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Preparation of Chromatin containing Ribosomal Deoxyribonucleic Acid from the Macronucleus of *Tetrahymena pyriformis*

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A method is described that enables a chromatin fraction containing ribosomal DNA (DNA containing sequences coding for rRNA) to be prepared from the macronuclei of growing or stationary cultures of *Tetrahymena pyriformis*. This material is obtained in yields of between 25 and 75% of the theoretical maximum. The DNA in this fraction was identified as ribosomal DNA on the basis of its density and molecular weight, and it appears not to be appreciably contaminated by other DNA. The method relies on the approximate assumption that ribosomal DNA is the smallest species of DNA in chromatin in the nucleus, and avoids the use of mechanical force, or enzyme action, to fractionate chromatin.

A major problem in the study of the structure and composition of chromatin containing actively transcribed DNA sequences is that these are almost invariably linked covalently to sequences not involved in transcription. Methods of separating active from inactive chromatin carry with them the risk of destroying the native structure of chromatin; for example the use of shearing forces to fragment chromatin has been shown to destroy its native periodic structure (Noll *et al.*, 1975). Further, chromatin fractions enriched in DNA sequences transcribed *in vivo* are almost certainly heterogeneous in their composition, containing a variety of unidentified genes. They may also include a proportion of chromatin that is not directly involved in transcription, or equally exclude chromatin that is intimately involved, though structurally different, from chromatin containing transcribed DNA sequences.

The chromatin containing the ribosomal DNA (rDNA) of the macronucleus of *Tetrahymena pyriformis* offers a solution to these and other problems. It has been shown that this DNA consists of multiple copies of an extrachromasomal molecule of mol.wt. 13×10^6 (Yao & Gorovsky, 1974; Gall, 1974; Engberg *et al.*, 1974). Each molecule is a giant palindrome (Karrer & Gall, 1976; Engberg *et al.*, 1976) and two copies of the gene(s) for rRNA occupy approx. 80% of its length (Engberg *et al.*, 1976). The rDNA accounts for 2% of the macronuclear DNA (Yao & Gorovsky, 1974), and is contained in nucleoli attached to the nuclear membrane, and only loosely associated with the rest of the macronuclear chromatin (Nilsson & Leick, 1970).

This rDNA is therefore probably the smallest species of DNA in the *Tetrahymena* macronucleus, excluding the likely presence of intermediates in replication, and it therefore seemed feasible to devise a method for the isolation of r-chromatin based on this physical property. Such a method is described in the present paper, and it provides a substantially homogeneous preparation of a single species of chromatin in sufficient quantity that, for example, analysis of its protein composition becomes possible (Jones, 1978).

Methods for the preparation of r-chromatin or r-chromatin-containing fractions have been described by Leer *et al.* (1976) and by Mathis & Gorovsky (1976). The method described in the present paper differs from these in several respects; for example I managed to avoid exposing chromatin to shearing forces, and, though chromatin is exposed in my method to non-ionic detergent, I have managed to avoid exposing it to either ionic detergent or, intentionally, to proteolytic enzymes.

Abbreviations used: rDNA, ribosomal DNA, the region of DNA coding for rRNA; r-chromatin, chromatin containing ribosomal DNA; CDTA, cyclohexanediaminetetra-acetic acid.

Experimental

Growth of organisms

T. pyriformis syngen 1 D/1 was grown at 28°C in a medium containing 0.5% (w/v) proteose peptone, 0.5% (w/v) tryptone and 0.1% (w/v) yeast extract (all from Oxoid, Basingstoke, Hants., U.K.). Stock cultures (5ml) were maintained in tubes, and used to inoculate 300ml of the same medium. The latter cultures were in turn used to inoculate 4 litres of medium that contained, in addition to the constituents above (per litre): 1g of sodium acetate;

1g of KH_2PO_4; 1g of glucose; 0.5mg of thiamin hydrochloride; 0.125g of streptomycin; 0.125g of benzylpenicillin; 100μl of poly(propylene glycol) 2025 as antifoaming agent. The complete medium was adjusted to pH7.5 by adding 2ml of 40% (w/v) NaOH. These cultures were vigorously aerated, and the organisms grew with a doubling time of about 4h. Cell numbers were measured with a Coulter cell counter.

Preparation of starved organisms

The method used was essentially that of Cameron & Jeter (1970). Organisms growing exponentially at cell densities between 1×10^5 and 1.5×10^5/ml in a 4-litre culture were harvested at $800g_{av.}$ in a MSE continuous-action rotor with a flow rate of 1 litre/min. The rotor was flushed, while still spinning, with 2 litres of 5mM-KH_2PO_4/0.5mM-$MgSO_4$, adjusted to pH7.5 with KOH. The pelleted organisms were resuspended in 4 litres of the latter solution, and incubated for 18h at 28°C with aeration. Cell numbers increased until 4h after starvation, and were then stationary until the organisms were harvested.

Radioactive labelling

DNA in growing organisms was radioactively labelled by the addition of either 12.5μCi of [*Me*-^{3}H]thymidine (sp. radioactivity 21 Ci/mmol)/litre or 2.5μCi of [*Me*-^{14}C]thymidine (sp. radioactivity 56mCi/mmol)/litre to cultures at least three generation times before harvesting. RNA was radioactively labelled by adding 12.5μCi of [5,6-^{3}H]uridine (sp. radioactivity 59Ci/mmol)/litre at 30min before a culture was harvested. Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Measurement of radioactivity

Radioactivities in cell homogenates, nuclei and chromatin fractions were measured after oxidizing portions in a Packard Tri-Carb 306 sample oxidizer. Radioactivities in fractions from glycerol gradients or CsCl gradients were measured after diluting samples to 1ml with water, and adding 10ml of a toluene/Synperonic NXP (Cargo Fleet Chemical Co., Eaglescliffe, Stockton, Cleveland, U.K.) scintillant (Wood *et al.*, 1975). Radioactive material on Whatman GF/C filters were measured after adding 3ml of a toluene-based scintillant containing 5g of PPO (2,5-diphenyloxazole) and 0.1g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre of toluene. This scintillant, with the addition of acetic acid (2ml/litre), was used for the measurement of radioactivity in agarose-gel slices.

Radioactivity was measured in a Packard Tri-Carb 3375 scintillation counter. When double-labelled samples were counted for radioactivity, or d.p.m. values required, a Packard Tri-Carb 3390 scintillation counter fitted with a model 544 absolute radioactivity analyser was used. Radioactivities of 100 c.p.m. or greater were counted to an accuracy of at least ±2.5% (S.D.).

Preparation of macronuclei

Nuclei were prepared by a modification of the method of Gorovsky (1970). Solutions used for the preparation of nuclei were: solution A [4% (w/v) acacia gum; 3% (w/v) sucrose; 10mM-Tris base; 1.5mM-$MgCl_2$; 5mM-spermidine trihydrochloride; 1mM-spermine tetrahydrochloride; adjusted to pH7.5 with HCl]; solution B [4% acacia gum; 3% sucrose; 10mM-Tris base; 2mM-EDTA; 1mM-CDTA; 2.5mM-spermidine trihydrochloride; 0.5mM-spermine tetrahydrochloride; adjusted to pH7.5 with HCl]; solution C was as for solution B with, in addition, 100mM-NaCl. All these solutions contained in addition 0.1mM-phenylmethanesulphonyl fluoride, added from a stock solution of 100mM-phenylmethanesulphonyl fluoride in dimethyl sulphoxide. All solutions were kept on ice during the preparation of macronuclei, and all the procedures described below were carried out on ice where possible, and otherwise at 3–5°C.

A 4-litre culture, grown to cell densities of between 1×10^5 and 1.5×10^5 organisms/ml, was poured on to 1.5 litres of ice. The cells were harvested at $1400g_{av.}$ in an MSE continuous-action rotor with a flow rate of 1 litre/min, and the rotor was flushed, while still spinning, with 2 litres of 10% (w/v) sucrose/10mM-Tris/1.5mM-$MgCl_2$, adjusted to pH7.5 with HCl. The pelleted cells were resuspended in the residue of this solution left in the rotor, and the suspension was centrifuged at $1250g_{av.}$ for 3min. Cells were then taken up in solution A, at a concentration of 8×10^6 cells/ml, and put in the 200ml-capacity cup of an MSE Ato-Mix homogenizer. Then 1ml of octan-1-ol/100ml of solution A was added, and the cells were homogenized at full speed for 30s. The resulting homogenate was made 8mM in EDTA, and homogenized at full speed for a further 10s. Nuclei were pelleted by centrifuging at $1250g_{av.}$ for 5min, and the supernatant and scum discarded. The pellet of nuclei was then washed twice in solution B, once in solution C, and finally in solution B, being centrifuged at $1250g_{av.}$ for 5min in each case.

Preparation of chromatin fractions

Two procedures were followed for the preparation of chromatin fractions.

(1) The pellet of nuclei, after the final wash of macronuclei in solution B, was resuspended in 2.05M-sucrose containing 0.5mM-CDTA (pH7.5), 0.1mM-phenylmethanesulphonyl fluoride and 0.1% dimethyl sulphoxide. These nuclei were pelleted by centrifuging at $196000g_{av.}$ in a Beckman SW41 rotor

for 30min. The resulting pellet was resuspended in 0.5mM-CDTA/0.1mM-phenylmethanesulphonyl fluoride/0.1% dimethyl sulphoxide/0.1% (v/v) Triton X-100, pH7.5, and layered on a 33ml 5–75% (v/v) linear glycerol gradient in the same solution. These gradients stood at 3°C for 2h before being spun at $3280g_{av.}$ in a Beckman SW27 rotor for 15min at 3°C.

(2) The second procedure was essentially the same, except that the 2.05M-sucrose solution contained in addition 0.05mM-spermidine trihydrochloride and 0.01mM-spermine tetrahydrochloride. After macronuclei had been centrifuged through this solution at $196000g_{av.}$ for 30min, the pellet was gently mixed to a homogeneous consistency, and the nuclei were resuspended in 5ml of water. Portions (2ml) of this suspension were gently pipetted into 1ml of a solution containing 30mM-Tris base, 0.3mM-CDTA, 0.3mM-phenylmethanesulphonyl fluoride, 0.3% dimethyl sulphoxide and 0.3% Triton X-100, adjusted to pH7.5 with HCl, and overlying a 33ml 5–40% (v/v) glycerol gradient containing 5mM-Tris base, 0.1mM-CDTA, 0.1mM-phenylmethanesulphonyl fluoride and 0.1% dimethyl sulphoxide, adjusted to pH7.5 with HCl. The suspension of macronuclei was gently stirred to ensure mixing with the overlay. The loaded gradients stood for 10min at 3°C before centrifuging at $52400g_{av.}$ for 30min in a Beckman SW27 rotor at 3°C.

Gradients resulting from either procedure were fractionated, in the cold (3°C), by pumping 1,1,1-trichloro-2,2,2-trifluoroethane (I.C.I., Macclesfield, Cheshire, U.K.) into the bottom of the gradients on an Isco 180 density-gradient fractionator. Fractions (approx. 2.0ml) were collected from the top of the gradient after passage through a flow cell attached to an Isco 222 u.v. analyser. The chromatin pellets remaining in the centrifuge tubes (bulk chromatin) were pooled and resuspended in 5ml of water by using a Polytron homogenizer at half-speed for 15s. Fractions containing r-chromatin were identified either from the extinction profile of the gradient or by measuring radioactivity in DNA in samples of gradient fractions. The appropriate fractions were pooled, and in some cases made 0.1mM and 0.02mM in spermidine trihydrochloride and spermine tetrahydrochloride respectively, and the r-chromatin was pelleted by spinning at $95400g_{av.}$ for 4h in a Beckman SW27 rotor at 3°C.

Preparation of macronuclei and chromatin fractions from starved organisms

The same procedure as for growing organisms was followed, with the following differences. Organisms were harvested at $2000g_{av.}$ in the continuous-action rotor, with a flow rate of 500ml/min. They were homogenized in solution A for 20s, and for a further 20s after the addition of EDTA. The CDTA concentration in the 2.05M-sucrose solution was increased to 1.25mM.

Preparation of DNA

DNA was prepared from chromatin or macronuclei by dissolving the material in 1M-NaCl/10mM-EDTA/1% (v/v) sarcosyl NL 35 (CIBA–Geigy U.K., Cambridge, U.K.). Ribonuclease (bovine pancreatic; BDH, Poole, Dorset, U.K.), previously heated at 100°C for 10min, was added to give a concentration of 100μg/ml, and the solution incubated for 4h at 37°C. Pronase (B grade; Calbiochem, Bishop's Stortford, Herts., U.K.), previously incubated at 37°C for 4h, was added at a concentration of 500μg/ml, and the solution incubated at 37°C overnight. After extraction with 3×2vol. of chloroform/3-methylbutan-1-ol (24:1, v/v), the solution of DNA was dialysed overnight against 1mM-Tris/HCl, 1mM-EDTA, pH7.5.

Analysis of DNA by density-gradient equilibrium ultracentrifugation

CsCl (BDH) was dissolved in solutions of DNA in 100mM-Tris base/1mM-EDTA, adjusted to pH8.0 with HCl, to give 12ml of a solution with a density of approx. 1.68g/ml. This was overlaid with liquid paraffin and centrifuged at $75000g_{av.}$ in a Beckman 60Ti rotor for 72h at 20°C (Flamm *et al.*, 1966). Fractions (0.5ml) were collected from the bottom of the tubes. The densities of these fractions were calculated from their refractive indices measured by using an Abbé refractometer. Samples (100μl) were diluted to 1ml with water for the measurement of radioactivity.

Analysis of DNA by agarose-gel electrophoresis

The buffer used in gels, reservoirs and DNA samples was 40mM-Tris base/5mM-sodium acetate/1mM-EDTA, adjusted to pH8.0 with acetic acid. Agarose was refluxed at 100°C in this buffer at a concentration of 0.75% (w/v) until dissolved, when ethidium (3,8-diamino-5-ethyl-6-phenylphenanthridinium) bromide was added to give a concentration of 0.4μg/ml, and the solution poured into 40cm×0.8cm (internal diameter) glass tubes. DNA, prepared as described above, was freeze-dried, dissolved in 40mM-Tris/HCl (pH8.0) buffer containing 10% (v/v) glycerol and subjected to electrophoresis at 120V for 16h.

DNA in these gels was detected by u.v. illumination. Gels containing radioactive DNA were cut into 0.5cm segments, and these were dried overnight at 37°C, before incubation in 0.5ml of NCS tissue solubilizer (Amersham/Searle Corp., High Wycombe, Herts, U.K.) for 4h at 50°C.

Chemical measurement of RNA, protein and DNA

Samples of chromatin were precipitated by adding 0.2 vol. of 100% (w/v) trichloroacetic acid, and filtered on 2.1 cm-diam. Whatman GF/C filters. The filters were washed with 10ml of 5% trichloroacetic acid followed by 10ml of ethanol, dried, then heated in 1ml of 5% trichloroacetic acid at 60°C for 60min. This extract was used for the measurement of RNA by the orcinol method (Munro & Fleck, 1966). Then 1ml of 0.2M-NaOH was added to the filter, which was heated at 60°C for 60min. Protein in this extract was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (Armour Pharmaceuticals, Eastbourne, Sussex, U.K.) as standard.

Radioactively labelled DNA was prepared from bulk chromatin as described above. Samples were taken for the measurement of radioactivity in DNA, and the concentration of DNA was calculated from the A_{260} of the solution. From this, the specific radioactivity of the DNA was calculated, and used to measure the quantity of DNA in various fractions of the same chromatin preparation.

Enzyme digestion of RNA or DNA in chromatin

Solutions of Tris/HCl, pH7.5, and $MgCl_2$ were added to samples of chromatin to give concentrations of 100mM and 1mM respectively. Ribonuclease (BDH) prepared as described above, or deoxyribonuclease I (ribonuclease-free; Cambrian Chemicals, Croydon CR9 6AG, Surrey, U.K.), was added to give concentrations of 100μg/ml, and the solutions were incubated at 37°C overnight. Samples lacking added enzyme were incubated in parallel. Then 0.1 vol. of 100% trichloroacetic acid was mixed with each sample at 0°C. Precipitated material was collected on Whatman GF/C filters, washed as described above, and the radioactivity in them measured.

Results

Preparation of macronuclei

In modifying the method of Gorovsky (1970) for the preparation of macronuclei from *T. pyriformis*, the most significant improvement was gained by the addition of EDTA to the suspension of organisms in solution A for part of the homogenization step. In the present work, in the absence of homogenization in the presence of EDTA an unacceptably large proportion of the radioactivity in DNA was lost in the scum that formed at the top of the tube after centrifugation of the homogenate, and as much as 50% of the resulting preparation of nuclei was lost when the nuclei were subsequently pelleted out of 2.05M-sucrose. The addition of EDTA during the homogenization step increased the yield of nuclei, measured after they were resuspended for a final wash in solution B, to between 60 and 90% of the radioactivity in the homogenate. Phase-contrast microscopy showed these macronuclei to be cleaner than those previously prepared. Less of the material was lost in the 2.05M-sucrose step, and 50–70% of the radioactivity in the homogenate was recovered in chromatin.

An additional benefit of this modification is that nuclei from starved organisms could be prepared in yields comparable with those from growing organisms. In the absence of EDTA during the homogenization step, the yields of macronuclei from starved organisms were poor (less than 25%), and these nuclei appeared to be heavily contaminated with non-nuclear material when examined by phase-contrast microscopy.

Preparation of chromatin fractions

In the first version of the method for the preparation of r-chromatin, analysis of the glycerol gradients in which chromatin was fractionated showed that between 0.5 and 1.5% of the radioactively labelled DNA in chromatin remained close to the top of the gradient in association with a peak in the A_{260} profile of the gradient (Fig. 1). The remaining radioactivity was in the gelatinous pellet.

^{3}H-labelled DNA prepared from this slowly sedimenting fraction was analysed on CsCl gradients together with [^{14}C]thymidine-labelled DNA prepared from whole macronuclei (Fig. 2). The [^{3}H]DNA was found to have a density greater than that of the DNA from whole nuclei, a property characteristic of the

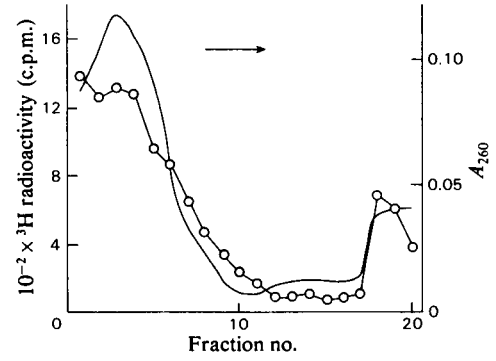

Fig. 1. *Fractionation of chromatin by method* 1 (*as described in the Experimental section*)
Macronuclei were prepared from growing organisms, radioactively labelled with [^{3}H]thymidine, and chromatin was fractionated in 5–75% glycerol gradients. The distribution of radioactivity (○) and the A_{260} profile (——) in one gradient are shown. The arrow indicates the direction of sedimentation.

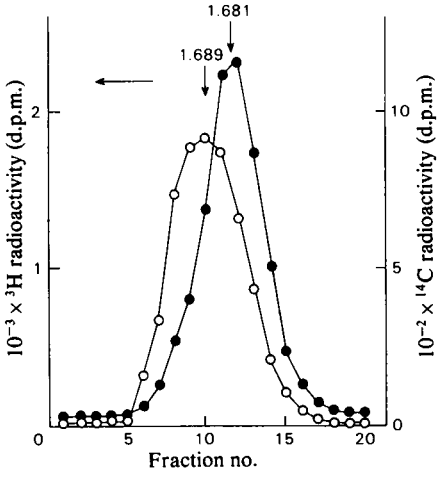

Fig. 2. *Buoyant density of DNA from the slowly sedimenting chromatin fraction*

[^{3}H]Thymidine-labelled DNA (○) from an r-chromatin fraction, extracted from growing organisms, together with [^{14}C]thymidine-labelled DNA (●) from whole macronuclei, was subjected to CsCl-density-gradient centrifugation. Gradients 12ml, overlaid with liquid paraffin, were centrifuged at 75000$g_{av.}$ in a Beckman 60 Ti rotor at 20°C for 72h. Fractions (0.5ml) were collected, and radioactivities in 0.1ml samples measured. The densities of gradient fractions were calculated from their refractive indices. The horizontal arrow indicates the direction of sedimentation.

rDNA from *T. pyriformis* (Engberg *et al.*, 1972; Gall, 1974). The symmetry of the peak containing rDNA suggests that the gradient fractions containing r-chromatin were not significantly contaminated by bulk DNA. Chromatin fractions were prepared from starved cells in the same way, and the slowly sedimenting fraction was found to contain DNA with the same properties as that from growing organisms.

This form of the method gave a fraction enriched in r-chromatin, but the A_{260} profile of the gradient suggested that the r-chromatin was not being separated from RNA released at the same time, and electron micrographs (not shown) of material from this fraction showed only large aggregates of what appeared to be pre-ribosomal particles. Centrifuging the gradients for longer times failed to achieve any separation of the r-chromatin-containing fraction from the u.v.-absorbing material.

Modification of this method included the addition of a low concentration of spermine and spermidine to the 2.05M-sucrose solution, and alterations to the ionic strength of solutions used in this and subsequent steps. These modifications were based on a rationale that permitted the regulation of the solubility of the chromatin at each step with considerable reproducibility. Bivalent cations were absent from the solutions in which nuclei and chromatin were prepared, and macronuclei were kept intact by the presence of polyamines (Gorovsky, 1970). Increasing the ionic strength of solutions caused the nuclei to swell, or become sticky, presumably by weakening the binding of polyamines to sites in the nuclei. Conversely, the nuclei could be kept intact at low concentrations of polyamines, provided that the ionic strength was sufficiently low.

The integrity of nuclei, in steps after their centrifugation through 2.05M-sucrose containing 0.05mM-spermidine trihydrochloride and 0.01m-M spermine tetrahydrochloride, was adjusted by varying the CDTA concentration in this solution, which presumably influenced the amount of polyamine remaining bound to the nuclei: 0.5mM-CDTA was used for nuclei from growing organisms, and 1.25mM-CDTA for nuclei from starved organisms. Resuspension of the resulting pellet in water maintained the nuclei intact, but attempts to resuspend the nuclei in 10mM-Tris/HCl, pH7.5, for example, often resulted in the production of an unmanageable aggregate. This had been a constant problem with the first form of the method. The suspension of nuclei in water could then be easily loaded on gradients where it was mixed with an overlay in which the nuclei were exposed to a higher ionic strength than at the same stage in the previous method. This resulted in the initially opaque suspension of nuclei clearing in the course of 10min, before the gradients were centrifuged at substantially higher g values than had been used before (52400$g_{av.}$ for 30min compared with 3280$g_{av.}$ for 15min previously).

Figs. 3(*a*) and 3(*b*) show gradients in which chromatin from growing and starved cells was fractionated. In both cases, the slowly sedimenting chromatin fraction was well separated from the chromatin pellet, and from the major peak in the A_{260} profile of the gradients. Regularly, between 0.5 and 1.5% of the radioactivity loaded on the gradient was recovered in this fraction, and the remaining radioactivity was in the gelationous chromatin pellet (bulk chromatin).

Agarose-gel electrophoresis of DNA from r-chromatin

DNA from the r-chromatin fraction extracted from growing organisms migrated on electrophoresis predominantly as a single band in 0.75% agarose gels (Fig. 4). Comparison of its mobility with those of bacteriophage-λ DNA and *Eco*R1-restriction-enzyme fragments of bacteriophage-λ DNA shows it to have a molecular weight of approx. 13×10^6, very similar to the values reported elsewhere for the rDNA from *T. pyriformis* (Gall, 1974; Engberg *et al.*, 1974). Electrophoresis of the DNA from the r-chromatin fraction of starved organisms gave the same result.

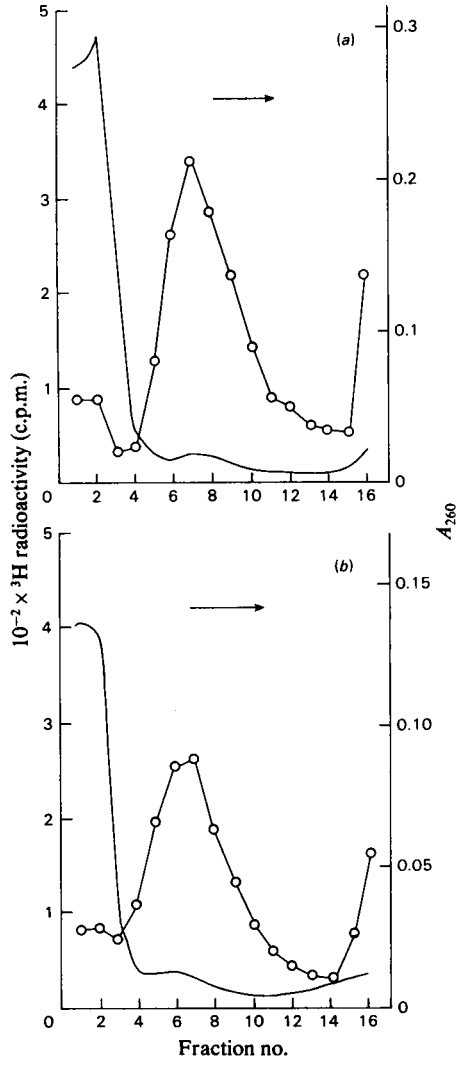

Fig. 3. *Fractionation of chromatin from growing (a) and starved (b) organisms by method 2 (as described in the Experimental section)*

Organisms were grown in the presence of [^{3}H]-thymidine. Growing organisms were starved as described in the Experimental section. The distributions of radioactivity (○) and the A_{260} profiles (——) in 5–40% (v/v) glycerol gradients are shown. In (*a*), the radioactivity in fractions 5–10 inclusive represents 0.9% of the radioactivity in the gradient and the pellet. In (*b*), fractions 4–9 inclusive contain 1.15% of the total radioactivity. The arrow indicates the direction of sedimentation.

Composition of fractions from preparative gradients

Table 1 shows, for two preparations, amounts of radioactivity in DNA in nuclei, in fractions from preparative gradients, and in the r-chromatin collected as a pellet by centrifuging the appropriate fractions from those gradients. The measurements described in the present paper of the amount of DNA in r-chromatin, as a percentage of the DNA in bulk chromatin, has depended on the assumption that all the radioactivity from radioactively labelled thymidine is only in DNA. That this is probably so is demonstrated by the observation that the incubation of r-chromatin with ribonuclease renders less than 7.5% of this radioactivity soluble in 5% trichloroacetic acid, whereas less than 5% remains precipitable after incubation with deoxyribonuclease I.

Table 1 also shows that a small amount of radioactivity remains at the top of preparative gradients. The amounts of this radioactivity, as a percentage of the total, are very variable, though usually less than half that in the r-chromatin fraction. This radioactivity is in DNA, as demonstrated by its digestion with deoxyribonuclease I.

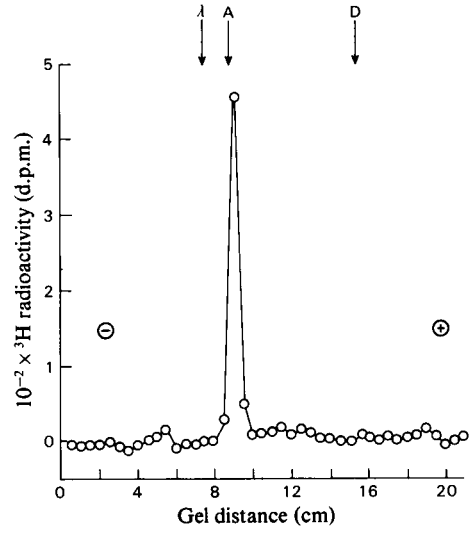

Fig. 4. *Agarose-gel electrophoresis of DNA from the slowly sedimenting chromatin fraction*

DNA was extracted from the slowly sedimenting chromatin fraction prepared from organisms grown in the presence of [^{3}H]thymidine. Samples of this DNA were electrophoresed in 40cm-long 0.75% (w/v) agarose gels at 120V for 16h. Samples of bacteriophage-λ DNA and of *Eco*R1 restriction-enzyme-digested bacteriophage-λ DNA (provided by Dr. A. F. Jones) were electrophoresed in parallel. DNA bands were detected by exposing the gels, which contained 0.4μg of ethidium bromide/ml, to u.v. light. Gels containing radioactive DNA were cut into 0.5cm segments, and the radioactivity in these was measured (○). Arrows indicate the positions of bacteriophage-λ DNA (mol.wt. 30.8×10^6) and restriction fragments A (mol.wt. 13.7×10^6) and D (mol.wt. 4.74×10^6) (Thomas & Davis, 1975).

Table 1. *Recoveries of radioactively labelled DNA at various steps in the preparation of chromatin fractions*

Results (in $10^{-3} \times$ c.p.m. of ^{14}C) are shown for two preparations (a and b). Cultures were grown in the presence of [^{14}C]thymidine. The yields of macronuclei, immediately before their final wash in solution B, are expressed as percentages (shown in parentheses) of the radioactivity in the cell homogenate. The radioactivities in the various parts of the preparative glycerol gradients are expressed as percentages (shown in parentheses) of the total radioactivity in the gradient. 'Residual fraction' refers to the top three fractions (6 ml) of the gradients. In these preparations, r-chromatin was pelleted by centrifugation in the absence of polyamines. Addition of spermine and spermidine at this stage increases the recovery of radioactivity in the r-chromatin pellet to about 90% of the radioactivity present in this fraction in the gradient, but makes the material more difficult to resuspend homogeneously. The recoveries of r-chromatin as a pellet are expressed as percentages (shown in parentheses) of the total radioactivity in the preparative gradient.

	$10^{-3} \times {}^{14}C$ radioactivity (c.p.m.)			
Preparation ...	a		b	
Preparation of nuclei				
Cell homogenate	1228		1098	
Nuclei	864	(70%)	910	(83%)
Fractions from 5–40% glycerol gradients				
Residual fraction	2.95	(0.47%)	2.8	(0.36%)
r-Chromatin	4.4	(0.7%)	10·8	(1·4%)
Pellet	625	(98.8%)	770	(98.3%)
r-Chromatin (95400*g* pellet)	3.6	(0.57%)	6.9	(0.88%)

The chemical composition of these fractions is shown in Table 2. As might be expected, the RNA/DNA ratio in the r-chromatin fraction is considerably greater than that in bulk chromatin. Most of the RNA from the macronuclei, however, remains either at the top or the gradient, or sediments with the bulk chromatin fraction.

When organisms were grown with both [^{14}C]-thymidine and [^{3}H]uridine, before preparation of chromatin fractions, analysis of preparative gradients (results not shown) showed that the distribution of radioactivity in RNA coincided closely with the A_{260} profile of the gradient. Since RNA is the major nucleic acid in the r-chromatin fraction, the small peak in the A_{260} profile that is associated with the r-chromatin fraction (Figs. 3*a* and 3*b*) probably represents a subfraction of macronuclear RNA that is intimately associated with r-chromatin.

Table 2. *Composition of fractions from preparative gradients*

The two preparations (a and b) described in Table 1 were used for the analysis of the chemical composition of the two chromatin fractions, and the residual fraction, from preparative gradients. RNA was measured by the orcinol method, protein by the method of Lowry *et al.* (1951) and the amounts of DNA in the fractions were measured indirectly as described in the Experimental section.

		Content (μg)			DNA/RNA/protein proportions
		DNA	RNA	Protein	
Residual fraction	a	10.7	659	1840	1:62:172
	b	4.9	675	1980	1:147:430
r-Chromatin (95400*g* pellet)	a	16.0	46	83	1:2.9:5.2
	b	18.0	87	144	1:4.8:8.0
Pellet (bulk chromatin)	a	2730	461	4000	1:0.17:1.46
	b	2030	649	3125	1:0.32:1.53

Sedimentation of r-chromatin in preparative gradients

For any particular set of conditions applied during the preparation of the r-chromatin fraction, the distance through which it sediments in preparative gradients varies very little. However, changes in some of these conditions leads to changes in its rate of sedimentation. For example, Fig. 5 shows two gradients that were centrifuged either 10 min or 45 min after nuclei had been loaded on them. The amount of radioactivity in the r-chromatin peak differed very little between the two gradients, but the r-chromatin from nuclei left for 45 min sedimented more slowly than that in the gradient left for 10 min.

The distance sedimented by the r-chromatin fraction can be altered in other ways. Lowering the CDTA concentration in the 2.05 M-sucrose from the usual 0.5 mM to 0.1 mM results in the r-chromatin sedimenting more rapidly, whereas the addition of spermidine and spermine, at concentrations of 20 μM and 4 μM respectively, immediately before these were loaded on gradients, increased the distance sedimented by 20%. These differences in sedimentation rate may reflect differences in the composition or the conformation of r-chromatin, possibly depending on the amount of spermine and spermidine remaining bound in this fraction.

Fig. 5 also shows that, when loaded preparative gradients were left for longer before centrifugation, a greater amount of radioactivity accumulated at the top of the gradient. This may represent the product of enzyme action on macronuclear DNA, or the release of low-molecular-weight DNA. Very occasionally the radioactivity in this part of the gradient amounts to a quantity greater than that in the r-chromatin fraction, but it appears always as a distinct fraction. This DNA appears not to contribute to the radioactivity in the r-chromatin

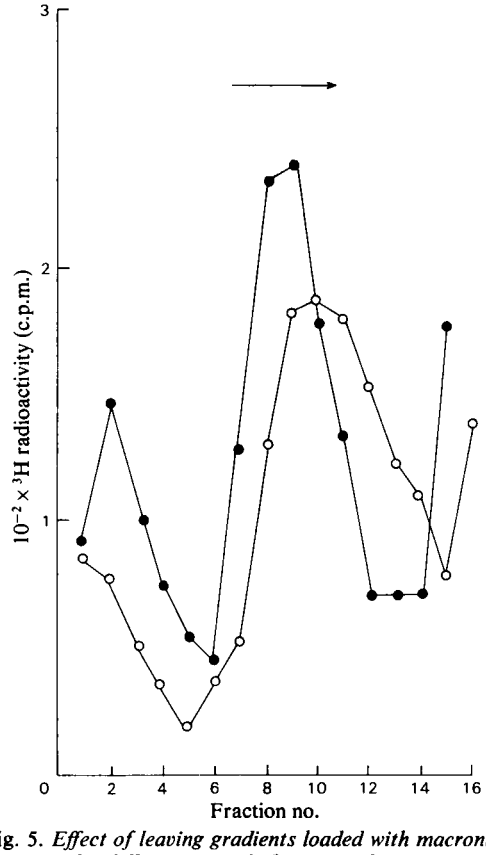

Fig. 5. *Effect of leaving gradients loaded with macronuclei for different times before centrifugation*
For this, 5–40% (v/v) glycerol gradients were loaded with equal amounts of macronuclei, prepared from organisms grown in the presence of [^{3}H]thymidine. The gradients were left for either 10min (○) or 40min (●) before centrifugation. In the gradient centrifuged after 10min fractions 9–14 inclusive contain 0.8% of the radioactivity in the gradient and the chromatin pellet, and in the gradient centrifuged 40min after loading fractions 7–12 inclusive contain 0.85% of the total radioactivity. The arrow indicates the direction of sedimentation.

fraction, which itself never amounted to more than about 75% of the theoretical maximum. Had the quantity of radioactivity in the r-chromatin fraction ever amounted to more than the theoretical maximum it would clearly be necessary to account for this by the presence of non-ribosomal DNA.

Discussion

The procedure described in the present paper provides a rapid method for the preparation of a r-chromatin fraction from the macronucleus of *T. pyriformis*. The DNA in this fraction has been identified as rDNA on the basis of its buoyant density (Engberg *et al.*, 1972) measured by equilibrium density-gradient centrifugation, and by its size (Gall, 1974) measured by agarose-gel electrophoresis. These data also suggest that the rDNA in this fraction has not been appreciably degraded, and that it is substantially free of contaminating non-ribosomal DNA. Elsewhere (Jones, 1978) I have shown that the histones present in both chromatin fractions show little evidence of proteolytic breakdown, with the exception of histone H1, which is the most sensitive of the histones to proteolysis. In some preparations this histone is present in only small amounts.

If the observation that the rDNA accounts for 2% of the DNA in the macronucleus of *T. pyriformis* (Yao & Gorovsky, 1974) applies to the strain of the organism used here, then my yields of r-chromatin, 0.5–1.5% of the total macronuclear DNA, represent a yield of between 25 and 75% of the macronuclear r-chromatin. It is important that this yield is achieved without exposing chromatin to shearing forces, though it may indirectly be exposed to these because of the necessity to homogenize organisms during the preparation of macronuclei. Intentional exposure to enzyme action has also been avoided. However, since *T. pyriformis* is equipped to digest micro-organisms, it is probably inevitable that chromatin will be exposed during its preparation to a range of enzyme activities originating in the organism itself. I have attempted to limit this by the exclusion of bivalent metal ions from most of the solutions used in the preparation of chromatin, by the inclusion of phenylmethanesulphonyl fluoride as a proteolytic-enzyme inhibitor, by keeping all solutions as cold as possible and by making the method as rapid as possible. The procedure, from the time when organisms are harvested to the fractionation of gradients, takes 3½h. However, no specific steps to limit ribonuclease activity during the preparation of these chromatin fractions have been taken.

Leer *et al.* (1976) have described a method for the preparation of a r-chromatin fraction similar in composition to that described in the present paper. They found it necessary, however, to expose chromatin to either proteolytic enzymes or ionic detergent at low concentrations. It is possible that endogenous enzyme activity fulfils a similar role in the method described in the present paper, but apart from the steps taken to combat this the reproducibility of the method argues against its dependence on the uncontrolled action of enzymes originating from the organism itself. However, it has been observed that the inclusion of Triton X-100 in the solution in which the nuclei are made to swell is essential for the liberation of r-chromatin from macronuclei. This is probably to be expected, since it appears that nucleoli in intact macronuclei are attached to the nuclear membrane (Nilsson & Leick, 1970).

The method has been applied to the preparation of a r-chromatin fraction from both growing and starved organisms. However, although nuclei have been prepared from starved organisms in yields comparable with those from growing organisms, consistently good yields of r-chromatin have not been obtained from these nuclei. The reason for this variability is not known, but it may be either that sufficiently clean nuclei are not obtained from starved organisms, or, as shown by the requirement for a higher CDTA concentration in the 2.05M-sucrose step, these nuclei may be in a physical state different from that of nuclei prepared from growing cells (Engberg *et al.*, 1972).

In its first form, the method used in the present paper produced a fraction from macronuclei that, although containing r-DNA, clearly included a large amount of RNA, and these two components were not separated by centrifuging preparative gradients for longer times. Modification of this procedure consisted of manipulations designed to displace spermine and spermidine residually bound to macronuclei, and resulted in the separation of an r-chromatin fraction from the bulk of RNA released coincidentally from the macronucleus. This r-chromatin fraction sedimented much more slowly than that originally prepared, and its rate of sedimentation could be altered by changing variables that possibly affect the amount of polyamine bound to it. These results suggest that the method could be modified by altering the concentration of spermine and spermidine and the ionic strength in the solutions used for the preparation of chromatin fractions, so that products ranging from those with a composition similar to nucleoli to those described in the present paper could be prepared.

The removal of polyamines from r-chromatin may affect its conformation, as well as its composition. Electron microscopy on the structure of material from the r-chromatin fraction (R. W. Jones, unpublished work) reveals a range of compact structures that appear to differ from each other in the degree of their compaction. The addition of spermine and spermidine to this material before electron microscopy resulted in the structures taking on a uniformly and highly compact appearance. It is therefore possible that the breadth of the r-chromatin peak in preparative gradients may be explained in part at least by the presence of structures exhibiting a range of conformations, and that alterations that result in different amounts of polyamine remaining associated with r-chromatin may influence its rate of sedimentation through effects on its conformation as much as on its composition.

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