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The Purification of DNA from the Genomes of *Paramecium aurelia* and *Tetrahymena pyriformis*[1]

SALLY LYMAN ALLEN and IAN GIBSON

Depts. of Botany and Zoology, Univ. of Michigan, Ann Arbor, Mich. 48104, U.S.A. and School of Biological Sciences, Univ. of East Anglia, Norwich, England

SYNOPSIS. Methods are described for the recovery of highly purified DNA from *Paramecium aurelia* and *Tetrahymena pyriformis* in high yields. Our DNA is only slightly contaminated with RNA and carbohydrate, and little or no protein can be detected. We could not reduce the RNA (orcinol-positive material) by further treatment (sephadex or hydroxyapatite chromatography and preparative CsCl gradients). At the extreme our DNA is contaminated with 15-20% RNA but the real value is most likely considerably lower than this. The DNA we have prepared from *Paramecium* and *Tetrahymena* shows all the properties of double-stranded, high molecular weight DNA when characterized by temperature melting, CsCl density gradient centrifugation and hydroxyapatite and sephadex chromatography. When denatured, it absorbs to nitrocellulose filters. The 2 major results of importance from our work reported here are: (1) There is similarity in base composition of DNA from different syngens of *Paramecium* (28% G+C for syngens 1, 2, 4, 5, and 9 and 29-30% G+C for syngen 8) while there is variation between the syngens of *Tetrahymena* (24-31% G+C for syngens 1, 4, 7, 10, 11, and 12); (2) the density of any *Paramecium* DNA varies depending upon whether the cells are grown in the presence of bacteria or in axenic medium. Our results are compatible with observations previously reported for *Tetrahymena* but contradict those made for *Paramecium*. The earlier reports of differences in base composition between syngens of *Paramecium* are probably due in large part to the use of stocks grown on bacteria.

WE have been investigating the DNA of the ciliated protozoa *Paramecium aurelia* and *Tetrahymena pyriformis*, with the ultimate aim of characterizing the DNA of the micronucleus and macronucleus of these cells in qualitative terms. We have presented elsewhere our detailed reasons for comparing the organization of the DNA molecules of these nuclei (1, 2). We hope to correlate genetic studies (1) which have shown that the macronuclear genotype causes cells within a clone to have different phenotypes with differences in the populations of DNA molecules present in macronuclei.

In order to isolate DNA or specific fractions of DNA and to characterize the molecules in terms of their renaturation properties following denaturation, it is essential to prepare DNA which is uncontaminated by RNA, protein, carbohydrates, lipids, etc. *It is also important to be certain that this DNA is derived from only the protozoan genome and not contaminated with DNA from bacterial sources. We believe that some of the earlier studies did not sufficiently eliminate such contamination nor indeed did they record the recovery of DNA from the cells. In such studies it is thus possible that only selected fractions of DNA were being examined and that contaminant molecules interfered in some of the qualitative and quantitative work. In the case of* Paramecium *DNA a potential source of contamination is the presence of DNA contributed from the food which in most cases is a single type of bacterium. This problem can be eliminated if* Paramecium, *like* Tetrahymena, *is grown under axenic conditions.*

In this report we will describe our methods for the preparation of DNA from *Paramecium* and *Tetrahymena* grown under axenic conditions. This DNA will then be characterized in terms of its purity, size, secondary structure, and base composition. We will also show the effects of bacterial contamination on the properties of *Paramecium* DNA.

MATERIALS AND METHODS

Strains: DNA was prepared from 14 stocks in syngens 1, 2, 4, 5, 8, and 9 of *Paramecium aurelia* and from 14 strains in syngens 1, 4, 7, 10, 11, and 12 of *Tetrahymena pyriformis* (Table 1). None of the *Paramecium* stocks possessed killer particles.

Growth of Cells: Paramecia were grown in axenic medium (23) or in bacterized grass medium in 1 liter Roux bottles for 5-12 days at 27 C. Usually 3-4 liters of paramecia were cultured in axenic medium yielding a wet weight pellet of 3-8 g depending on the stock used. To achieve the same yield with bacterized cultures 50-60 liters of culture medium were needed.

Tetrahymena were grown in 1% proteose-peptone (w/v) in 5 liter Povitsky bottles without aeration or shaking for 5-7 days at 23 C, except where indicated. The yield of packed cells from different strains varied from 0.3 to 1.0 ml per liter of culture.

Harvesting of Cells: Cells were harvested in a DeLaval (or Alpha-Laval) cream separator, suspended in Dryl's salt solution (10), and spun at 1,000 × *g* in pear-shaped tubes in an oil-testing centrifuge. The pellet of cells was then used for preparation of DNA.

Preparation of DNA:

Method 1: The packed cells were diluted to 10% concentration (v/v) in cold saline-EDTA (0.15 M NaCl, 0.1 M ethylenedinitrotrilotetraacetic acid, pH 8.0) and lysed by addition of 10% sodium lauryl sulfate (final concentration 0.5%). Within 1 min an equal volume of 90% phenol, adjusted to pH 9.0 with 1 M NaOH and at room temperature, was added, and the mixture was swirled on a shaker for 15 min at room temperature, then centrifuged for 5 min at 5000 rpm at 4 C in a Sorvall refrigerated centrifuge. The aqueous layer was pipetted off and again treated with phenol. A total of 3 consecutive phenol treatments was carried out. The aqueous layer was treated with 2 volumes of cold absolute ethanol and the precipitated DNA was spooled onto a glass rod; then the DNA was dissolved in 0.5 of the supernatant volume in 0.1 × SSC (where 1 × SSC = 0.15 M NaCl + 0.015 M sodium citrate). The salt concentration was adjusted to 1 × SSC by adding 10 × SSC and the DNA treated twice with α-amylase (Worthington Biochemical Corp., from hog

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TABLE 1. *Sources of DNA.*[1]

Syngen	Stock	Geographic Origin
		Paramecium aurelia
1	33	Maryland
	60	Virginia
	90	Pennsylvania
	513	France
	540	Mexico
	551	California
2	7	North Carolina
	114	Indiana
	562	Italy
4	51	Indiana
5	210	Ohio
8	138	Florida
	299	Panama
9	He-1	England
		Tetrahymena pyriformis
Syngen	Strain	Geographic Origin
1	7	Inbred, U.S.A. (A/C×C*)[2]
	8	Inbred, U.S.A. (A/C×C*)
	D	Inbred, U.S.A. (ALP−4×B)
	D/Congenic-1	Inbred, U.S.A. (C2×D)D[12]×C*
	C*	Inbred, U.S.A. (UM−226×B)
4	IN-3	India
7	UC-651	California
	UM-1215	North Carolina
	UM-1216	North Carolina
10	EN-131	England
11	AU-50-1	Australia
	AU-94-10	Australia
12	AU-F,2	Australia
	AU-115-3	Australia

[1] The syngen and strain is abbreviated in subsequent tables, in the figure and in the text in the following manner: syngen/stock or strain. For example, *T. pyriformis* 1/7 refers to syngen 1/strain 7.

[2] For details of origins of inbred strains, see reference 1.

pancreas; final concentration 50 μg/ml), once with a mixture of pancreatic and T1 ribonuclease (Sigma Chemical Co.; final concentrations 125 μg/ml and 20 "old" units/ml, respectively) and once with pronase B (Calbiochem; final concentration 500 μg/ml), each for 1 hr at 37 C. Between each enzyme treatment the DNA was deproteinized with phenol, precipitated with ethanol, spooled, and dissolved in 0.1 × SSC. After enzyme treatments the DNA was thrice precipitated with isopropanol following the procedure outlined by Marmur (16). The precipitate was then washed with a series of increasing ethanol concentrations, dissolved in 0.1 × SSC, and dialyzed overnight against 0.1 × SSC at 4 C. After 2 final precipitations in ethanol, the spooled DNA was dissolved in 0.1 × SSC and stored over chloroform at 4 C at a final concentration of 500-1000 μg/ml.

Method 2: The cells were frozen in liquid nitrogen, ground for 10 min with a mortar and pestle, suspended in 15-40 ml of culture fluid at approximately 20% concentration (w/v), lysed in 6% sodium 4-aminosalicylate, 1% NaCl and then shaken for 20 min with an equal volume of the following phenol mixture: 50 g phenol, 5.5 ml distilled water, 7 ml *m*-cresol, and 50 mg 8-hydroxyquinoline (14). The phases were separated by centrifuging at 7000 × *g* for 30 min at 4 C in a Sorvall refrigerated centrifuge, and the top aqueous layer was removed with a wide-mouthed pipette. The DNA was precipitated by adding 2 volumes of cold absolute ethanol, spooled onto a glass rod, and dissolved in 0.5 of the supernatant volume in 2 × SSC. The DNA was treated once with pancreatic ribonuclease (Sigma Chemical Co.; final concentration 50 μg/ml) for 1 hr at 37 C. The DNA was deproteinized with the phenol mixture, precipitated with ethanol, spooled, and dissolved in 0.1 × SSC. The DNA was precipitated once with isopropanol following the procedure outlined by Marmur (16). The precipitate was washed with a series of increasing ethanol concentrations, dissolved in 2 × SSC, dialyzed overnight against 2 × SSC at 4 C, again precipitated in ethanol, spooled, and dissolved in 2 × SSC, and stored over chloroform at 4 C at a final concentration of 500-1000 μg/ml.

In this method no α-amylase or pronase B were used.

Radioactive DNA: Thymidine-methyl-H^3 was added to test tubes containing 5 ml of culture at a concentration of 1 mCi/tube and the contents dispensed into a bottle. The final concentration of label was 1 μCi/ml of culture. The cells were grown and harvested, and the DNA was prepared as above. Specific activities 10-15,000 CPM/μg of DNA were obtained by this procedure.

Colorimetric Tests: DNA was assayed by the diphenylamine test of Burton (8), RNA by the orcinol test (9), protein by the Lowry method (15), and carbohydrate by the method outlined by Dubois et al. (11). Standard curves were constructed for each of these tests using salmon sperm DNA (Calbiochem), yeast RNA (Sigma Chemical Co.), serum albumin (Armour Pharmaceuticals), and anhydrous dextrose (Nutritional Biochemicals Corp.).

Tests for Purity of DNA:

a. *Alkali lability*: DNA samples containing 50 μg/ml in 0.1 × SSC were adjusted to 0.3 M KOH and incubated for 16 hr at 37 C. The treated DNA was then dialyzed against 1 × SSC overnight and tested for DNA and RNA by the diphenylamine and orcinol tests. Commercial yeast RNA was included as a control.

b. *Deoxyribonuclease digestion*: DNA samples containing 50 μg/ml in buffer (0.1 M sodium acetate, 0.1 M NaCl, 0.005 M $MgCl_2$) were incubated with deoxyribonuclease (Worthington; final concentration 200 μg/ml) for 16 hr at 37 C. The treated DNA was then dialyzed against 1 × SSC overnight and tested for DNA and RNA by the diphenylamine and orcinol tests. Commercial salmon sperm DNA was included as a control.

c. *Sephadex Chromatography*: Both high molecular weight and sheared DNA were used at a concentration of 70 μg/ml. Sheared DNA was prepared by sonication for 1 min at 1.5 amp in an MSE sonicator (Measuring & Scientific Equipment, Ltd.) using a probe 1 cm in diameter. We find that this treatment results in piece sizes of approximately 500 nucleotide pairs as determined by sucrose density gradient centrifugation and by direct measurement of electron microscope images. A total of 400 μg of DNA in 0.01 M sodium phosphate buffer, pH 6.8, was applied to either G-100 or G-200 sephadex columns, equilibrated in the same buffer. Elution was carried out at room temperature with 0.01 M phosphate buffer, pH 6.8, collecting 5 ml fractions, which were monitored by an ultraviolet recorder at 260 mμ. Peak fractions were assayed for DNA and RNA by the diphenylamine and orcinol tests.

d. *Hydroxyapatite Chromatography*: 200-1000 μg of sheared DNA (as described above) in 0.01 M sodium phosphate buffer, pH 6.8, were adsorbed to hydroxyapatite columns, equilibrated with 0.01 M phosphate buffer. Several elution schemes were used and elution was carried out in some experiments at room temperature and in others at 50 C. A flow rate of 1 ml/min was achieved by gentle air pressure. In one method step-wise elution was carried out at 50 C using columns 1 × 5 cm and 4 ml washes: 4 washes with 0.01 M phosphate buffer, 6 washes with 0.14 M phosphate buffer and 5 washes with 0.5 M phosphate buffer, all at pH 6.8. The eluates were assayed for optical density at 260 mμ and in some experiments tested for DNA and RNA by the colorimetric tests. To avoid interference with the color reaction in the diphenylamine test it was necessary to dialyze hydroxyapatite fractions against several changes of 1 × SSC for 24-48 hr. When radioactive DNA was used, the fractions

were precipitated with an equal volume of 10% TCA in the presence of methylated albumin (final concentration 25 μg/ml) and the precipitate collected on filters which were counted in a Tri-Carb liquid scintillation counter.

Denatured DNA samples (100 C for 10 min) were also adsorbed to columns and the elution scheme described above was carried out.

e. *Nitrocellulose filtration*: DNA samples in 2 × SSC were passed thru nitrocellulose filters and the filtrate examined for optical density at 260 mμ. Denatured DNA (in 0.1 × SSC, heated to 100 C for 10 min and fast-cooled by dilution with 9 parts of partially frozen 6 × SSC) was also passed thru filters and the filtrate and washes read for optical density. 12 mm circles were cut out from the filter, placed in 1 ml 0.1 M NaCl and the diphenylamine test carried out. The observed amount could be compared to the percentage of DNA retained on such a sized circle knowing the total amount retained on the whole filter. The latter value is calculated from the difference between the OD units in the original solution and in the filtrate.

Methods Used in the Characterization of DNA:

a. *CsCl density gradient centrifugation*: 1) *Analytical*: 2-5 μg of DNA in 0.6 ml 10 mM EDTA and 0.8 g CsCl (Harshaw Chemicals) were centrifuged in the Model E ultracentrifuge for 20 hr at 44,770 rpm at 25 C using the AnD rotor. At the end of the run UV-photographs were taken and tracings of the photographs made with a Joyce-Loebl integrating densitometer. As reference DNA's *E. coli* (1.710 g/cm^3) and poly d-AT (1.678 g/cm^3) were run simultaneously with experimental samples of DNA.

2) *Preparative*: *Tetrahymena*: At Michigan 600-800 μg of DNA in 1.8 ml of 0.1 × SSC were added to 6.3 ml CsCl solution in 0.03 M Tris, pH 7.48 ($\eta = 1.4181$; $\rho = 1.903$) and centrifuged in the preparative Spinco L2-50 centrifuge in the SW-50.1 rotor at 45,000 rpm for 46 hr at 22 C (The final density of the CsCl solution containing DNA was 1.711 g/cm^3). Samples (6 drops/tube) were collected at a flow rate of 0.5 ml/min and monitored by an ultraviolet recorder at 260 mμ. Fractions containing peak optical density were then pooled and tested for DNA, RNA, protein, and carbohydrate by the tests outlined above. These experiments were carried out with the help of Dr. Theo Staehelin (University of Michigan).

Paramecium: At the Univ. of East Anglia 100-500 μg of DNA in 0.5 ml of 2 × SSC were added to a tube containing 4.375 g of CsCl in 3.4 ml of 0.01 M Tris buffer, pH 8.2. The density of the CsCl solution was adjusted to 1.710-1.715 g/cm^3, and each tube was centrifuged at 33,000 rpm in the preparative Spinco L2 centrifuge in the SW-39 rotor for 60 hr at 25 C. Following centrifugation the contents of each tube were collected by siphoning at a rate of 0.5 ml/min (12 drops/tube). Tris buffer was then added to a final volume of 3 ml and the samples read at 260 mμ.

b. *Temperature Melting*: 20 μg DNA in either 0.1 × SSC or 1 × SSC were placed in cuvettes in either a Beckman or Unicam SP 800 spectrometer. The cell contents were heated by circulation of ethylene glycol with a Haake pump into the chamber around the cuvettes. The temperature of the cuvette containing SSC only ("blank") was monitored by a thermistor probe and the optical density at 260 mμ was read on all cuvettes. Pilot experiments determined the temperature at which the percentage increase in OD was 50% and readings were taken at 0.5-1.0 C intervals within a 5 C range on either side of this temperature. At other temperatures they were taken at 2-5 C intervals.

c. *Paper Chromatography of Base Composition*: The method of Wyatt (29) was followed in which 1 mg DNA was precipitated with ethanol, air-dried and hydrolyzed in 0.1 ml perchloric acid (72%) for 1 hr, cooled and diluted with 0.1 ml distilled water. The hydrolysate was applied to Whatman No. 1 paper strips (4 × 40 cm), dried and the bases separated by descending chromatography in a solvent containing 65 ml *iso*propanol, 17.7 ml concentrated HCl, 17.3 ml distilled water. After about 18 hr at 25 C the separated bases were observed by their ultraviolet absorbance and quantitated by determining the UV-absorbance at maximal absorption for each base of samples eluted from equivalent sections of the paper in 0.1 N HCl. As controls, salmon sperm DNA, *E. coli* DNA and equimolar mixtures of all 5 bases were run.

TABLE 2. *Recovery of DNA from different sources.*

Source	% Recovery	
	Method 1	Method 2
T. pyriformis		
1/7	50	—
1/8	50	—
7/UC-651	30	—
P. aurelia[1]		
1/540	50	65
8/138	52	70
8/299	45	68

[1] Grown axenically.

d. *Molecular Weight of DNA*: Determination of the molecular weight of DNA samples was carried out by sucrose density centrifugation using the relationship.

$$\frac{D_2}{D_1} = \left[\frac{M_2}{M_1}\right]^{0.35}$$

developed by Burgi and Hershey (7) where D_1 and D_2 are the distances sedimented by 2 samples and M_1 and M_2 are the molecular weights. Another method was also employed in which 1-2 μg of DNA were layered over 0.9 M NaCl, 0.1 M NaOH in a moving boundary cell and centrifuged at 44,700 rpm for several hours at 25 C in the Model E ultracentrifuge using the AnD rotor. Photographs were taken every 4 min. In these experiments *E. coli* DNA of known molecular weight 10^8 was used as a reference marker. For details of the method working out molecular weight for such alkaline gradients (see Studier, 25).

RESULTS

Recovery of DNA. In Table 2 we show the results of the technic used to prepare DNA in terms of the total recovery of DNA from the cell pellet. Only Method 1 was used for *Tetrahymena.* As shown in Table 3 for *T. pyriformis* 1/7 there was a gradual loss of DNA after each purification step. The final yield depended upon the strain and also on the age of the cells used (shown for *T. pyriformis* 1/7 in Table 4). The highest recovery (50%) was obtained from syngen 1 strains which were in the stationary phase. The recovery was lower from other syngens, 30% for UC-651 and considerably lower for UM-1215 and UM-1216, also from syngen 7. The cells of these strains seem to be more fragile and during harvesting there is a noticeable "fluffy" layer consisting of broken cells on top of the solid cell pack. Altho this layer was always discarded, it is possible that the loss of DNA may be attributed to high nuclease activity in the extract. In line with this reasoning the DNA from strains other than syngen 1 tended to be softer and more difficult to manage.

TABLE 3. *Purity of* T. pyriformis *1/7 DNA at various steps of purification (starting material 30 ml of packed cells).*

Step[1]	Treatment	Total Amounts (Mg) DNA	RNA	Carbohydrate	Protein
0	None	30	—	—	—
1	3 phenols	25	210	360	60
2	α-amylase	23	189	144	25
3	Ribonuclease	20	27	48	15
4	Pronase	19	10	23	11
5	Isopropanol	18.4	3.4	7.7	0
6	Dialysis	15.5	2.9	5.4	0

[1] Method 1.

Both Methods 1 and 2 were used in purifying DNA from *Paramecium*. The recovery of DNA was higher with Method 2 than with Method 1 (65% and 50%, respectively). Other methods were also tried, including that of Britten, Pavich and Smith (5), but none of these gave as high yields of pure DNA as the methods we describe.

Contamination of DNA Product. In Table 3 we show the degree of contamination of DNA samples by RNA, carbohydrate and protein, and how the level of contamination decreases with each purification step using Method 1. Table 4 compares the purity of DNA after the first phenol treatments (step 1) with the final product (step 6) from cells of different ages. Without enzyme treatments 90% of the nucleic acid is RNA in all types of cells. After the last step this level drops to 16-18%. In the phenol-treated samples there is a noticeable increase in contamination due to carbohydrate as the cell ages. The major source of this contamination is due to glycogen, which is known to increase as cells age (13, 18). In DNA not treated with α-amylase the glycogen appears as a discrete opalescent band in preparative and analytical CsCl gradients. With α-amylase treatment no band appears. Despite the enormous increase in carbohydrate in older cells, the level of contamination in the final product is not much higher than that found in logarithmic cells.

By these tests our final levels of contamination are 15-20% RNA (% nucleic acid), 23% carbohydrate (% total) and no detectable protein in DNA from *Tetrahymena*. In *Paramecium* these values are similar for Method 1 and perhaps slightly lower for Method 2.

Altho we made efforts to modify the purification procedure we could not lower the % contribution made by RNA, or more precisely the % appearing as orcinol-positive material. We questioned whether this was indeed RNA since the diphenylamine test gave an amount of DNA identical to that obtained by measuring optical density at 260 mμ. A number of other experiments and additional purification steps were carried out to determine the purity of the DNA and in particular to see if we could reduce the contribution made by material giving an orcinol-positive test. These experiments will be described in the next section.

TABLE 4. *Purity and yield of RNA from cell of different age* (T. pyriformis *1/7*).

Step 1[1]	1 Day 25 ml packed cells	3 Days 21 ml packed cells	5 Days 30 ml packed cells
DNA	19 mg	15 mg	25 mg
RNA	164	108	210
Carbohydrate	60	72	360
Protein	41	36	60
Step 6[1]			
DNA	5.4	6.1	15.5
RNA	1.0	1.3	2.9
Carbohydrate	1.4	2.3	5.4
Protein	0	0	0
% Recovery of DNA	22	29	53
% RNA/Nucleic Acid[2]	16	18	16
% Carbohydrate	14	22	23

[1] Method 1.
[2] % orcinol-positive material.

Tests for Purity

a. *Alkali lability.* Treatment with KOH should hydrolyze RNA specifically and not affect DNA. When yeast RNA was so treated, then there was a noticeable drop in the RNA content of the sample. There was no reduction in the orcinol-positive material of treated *Tetrahymena* DNA (Table 5). Similar results were also obtained with DNA from *Paramecium*.

TABLE 5. *Effect of different treatments on RNA content of* Tetrahymena *DNA.*

Sample	Purity	Treatment	μg DNA*	μg RNA*	% RNA[1]
1/7 DNA	Step 6	None	47	11	19
		+ KOH	45	10	18
		+ DNAase	4	<1	0?
Yeast RNA Commercial		None	2	50	96
		+ KOH	0	0	0
Salmon Sperm DNA Commercial		None	46	9	16
		+ DNAase	5	2	0?
1/7 (A)	Step 6	None	54	10	16
		Sephadex G100	45	9	17
		Sephadex G200	43	8	15
(B)	Step 6	None	51	9	15
		Sephadex G100	47	9	16
		Sephadex G200	41	7	15
1/7	Step 1	None	42	680	94
		CsCl Fraction 1	<1	33	100
		Fraction 2	24	40	62
	Step 6	None	37	9	20
		CsCl Fraction 2	37	10	21
1/7	Step 1	None	42	680	94
		HAP 0.01 M PB	0	110	100
		0.2 M PB	2	190	99
		0.3 M PB	45	20	31
	Step 6	None	37	9	20
		HAP 0.01 M PB	0	0	0
		0.2 M PB	0	0	0
		0.3 M PB	37	10	21

* Samples were diluted in 1 × SSC to give optical densities between 1.0 and 1.2, except for the first set of experiments where the dialysate was used directly. It was necessary to further dilute samples giving high readings with the orcinol test.
[1] % orcinol-positive material.

b. *Deoxyribonuclease digestion.* Prolonged treatment with a high concentration of deoxyribonuclease should result in the degradation of DNA. This was observed for salmon sperm DNA as well as for *Tetrahymena* DNA (Table 5). There was also a reduction in material giving an orcinol-positive test.

c. *Sephadex chromatography.* Purified DNA from *Tetrahymena* was sonicated and adsorbed to either Sephadex G-100 or G-200 columns. When eluted, only a single peak of optical density at 260 mμ was observed. The eluates were tested for their DNA and RNA content, and no reduction in orcinol-positive material was observed in the DNA eluted from the columns. The results from 2 different preparations of DNA are shown in Table 5.

d. *Density gradient centrifugation.* Preparative CsCl gradients of DNA from *Tetrahymena* and *Paramecium* purified by Method 1 only thru step 1 are characterized by an opalescent band which appears at a lesser density than the OD peak which contains DNA. This band contains glycogen since it is destroyed by α-amylase, is not destroyed by deoxyribonuclease, and does not incorporate H^3-thymidine. In addition to the glycogen band there are 2 peaks with optical density at 260 mμ: one at the bottom of the tube containing RNA and another midway up the tube containing DNA. When DNA is purified thru step 6 with Method 1, then only the DNA peak is present. Where radioactive DNA is used, only this peak is labeled. The results of colorimetric tests on fractions obtained from *Tetrahymena* DNA are shown in Table 5. Fraction 1 contains only RNA. Fraction 2 from DNA purified thru step 1 contains 62% RNA. There is less RNA (orcinol-positive material) in Fraction 2 from DNA purified thru step 6. However, it occurs in the same percentage as DNA *not* exposed to centrifugation.

Analytical centrifugation in CsCl showed a distinct DNA band in *Paramecium* samples with *E. coli* DNA as the marker. If ribonuclease was omitted in the preparation of DNA, then optically active material was seen as a smear on the gradients as well as the DNA band.

e. *Hydroxyapatite chromatography.* We have done extensive experiments with highly purified DNA from both *Paramecium* and *Tetrahymena* using the elution scheme outlined under methods. Some of the DNA was also radioactive. Native DNA which was not sonicated was irreversibly bound to the column. When sonicated, the DNA adsorbed to hydroxyapatite and was then completely removed in the 0.5 M wash. When denatured, the DNA was removed in the 0.14 M wash. Where radioactive DNA was used a complete correspondence was observed between the elution patterns of peak OD and peak radioactivity. These experiments indicate that we have double-stranded molecules in our DNA samples.

Another type of protocol was used to compare the behavior on hydroxyapatite of the same *Tetrahymena* DNA samples which were used in the preparative CsCl gradients above; that is, one purified thru step 1, Method 1, and the other thru step 6 (Table 5). The DNA samples were dialyzed extensively against 0.01 M phosphate buffer at pH 6.8, sonicated and then adsorbed on to hydroxyapatite columns at room temperature. Elution was then carried out with 3-5 column volumes of 0.01 M, 0.2 M and 0.3 M phosphate buffer, all at pH 6.8. With DNA purified thru step 1, RNA appeared in the 0.01 M and 0.2 M eluates, while almost all the DNA came off in the 0.3 M wash. This fraction contained 31% orcinol-positive material. With DNA purified thru step 6, no RNA appeared in either the 0.01 M or 0.2 M eluates, and all the DNA came off in the 0.3 M wash. This fraction contained 21% orcinol-positive material, an amount similar to that observed in the DNA sample originally put on the column.

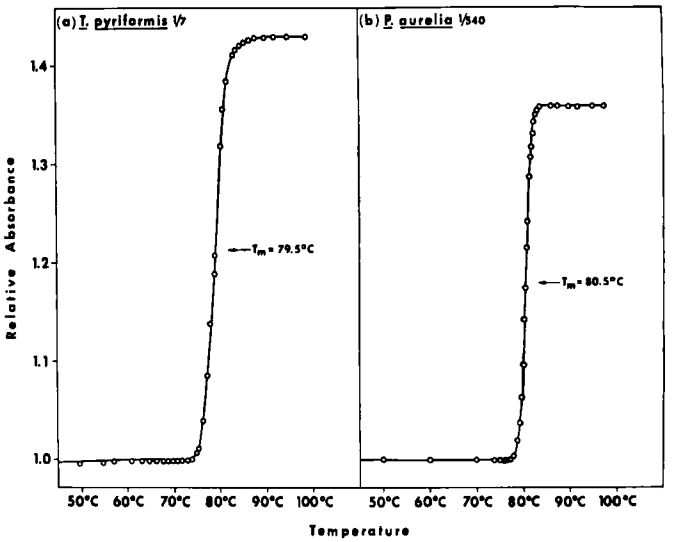

Fig. 1. Melting curves of DNA in 1 × SSC: (a) *T. pyriformis* 1/7; (b) *P. aurelia* 1/540.

f. *Temperature melting.* The shape of the melting profiles of purified DNA from both *Tetrahymena* and *Paramecium* is characteristic of DNA and unlike that of RNA (Fig. 1). They show a sharp melting range within an 8-10 C interval and a hyperchromicity of 43% (*Tetrahymena*: 1/7) and 35% (*Paramecium*: 1/540).

g. *Nitrocellulose filtration.* Native or sonicated double-stranded DNA did not remain complexed with the filters, nor did ribosomal RNA. Between 90-99% of the material passed thru. Denatured DNA did complex to the extent of 88-95%. This is a routine procedure in the hybridization technic involving nucleic acid molecules from many sources. This result indicates that we have at least 88-95% DNA molecules in our preparations.

To illustrate, in one experiment 90 optical density units (260 mμ) of highly purified *Tetrahymena* DNA were denatured and passed thru a large nitrocellulose filter. Eleven OD units were observed in the filtrate, none in the wash, which means that we would expect 79 OD units, or 88%, to have complexed to the filter. In terms of mg this means about 3.5 mg DNA. When 12 mm circles were cut from the filter, each could be shown by the diphenylamine test to contain 50 μg DNA, which is close to the 48 μg expected on a sample of the filter of this size. The DNA put on to the filter contained 20%

TABLE 6. *Base compositions of axenic and bacterized stocks of* P. aurelia.

Stock	Conditions of Growth	Buoyant density in CsCl g/cm³	Buoyant density in CsCl % G+C	T_m (1 × SSC) °C	T_m (1 × SSC) % G+C
1/33	Axenic	1.688 (1)[1]	28		
1/60	Axenic	1.688 (1)	28		
1/90	Axenic	1.688 (1)	28	81.0	28
1/513	Axenic	1.688 (1)	28		
	Bacterized, 6 days growth	1.692 (1)	33		
	Bacterized, 13 days growth	1.688 (1)	28		
1/540	Axenic	1.688 (8)	28	80.5-81.0	28
	Bacterized, 6 days growth	1.692 (2)	33	83.0	33
	Bacterized, 10 days growth	1.689 (1)	29		
	Bacterized, 14 days growth	1.689 (2)	29		
1/551	Axenic	1.688 (2)	28		
2/7	Axenic	1.688 (1)	28		
2/114	Axenic	1.688 (1)	28		
2/562	Axenic	1.688 (2)	28		
4/51	Axenic	1.689 (1)	29	81.0	28
5/210	Axenic	1.688 (1)	28		
	Bacterized, 12 days growth	1.691 (1)	32		
8/138	Axenic	1.690 (6)	30	81.5	30
8/299	Axenic	1.689 (3)	29	81.3	29
9/He-1	Axenic	1.688 (3)	28	81.0	28
	Bacteried, 9 days growth	1.690 (2)	30	81.5	30

[1] Number of different DNA samples examined in parentheses.

orcinol-positive material. Only 12% of the OD units failed to be recovered on the filter. This difference suggests that some of the orcinol-positive material is not RNA.

Characterization of DNA

a. *Molecular Weight.* The molecular weight of native *Tetrahymena* DNA (1/7) was determined by sucrose density gradients and that of native *Paramecium* DNA (1/540) by both sucrose density gradients and analytical centrifugation. *Tetrahymena* DNA had a molecular weight of 10^6. *Paramecium* samples ranged from 7×10^5 to 2×10^6 as measured by analytical centrifugation.

b. *Base Composition.* The base compositions of DNA from different strains and syngens of *Paramecium* and *Tetrahymena* are shown in Tables 6 and 7. The methods used to determine these are shown, i.e. T_m, CsCl density gradient centrifugation or direct chemical analysis.

An unexpected feature of the results with *Paramecium* is that the base composition of the DNA from a particular source varies depending upon whether it was grown on bacteria or in axenic medium. These DNAs were compared on preparative CsCl gradients but the few axenic

TABLE 7. *Base compositions of different strains of* T. pyriformis.

(a) Chromatography

Strain	% Guanine	% Adenine	% Cytosine	% Thymine	% G+C
1/7	11.8±0.4	36.8±0.5	12.2±0.3	39.2±0.5	24.0±0.5
1/8	13.1±0.2	36.0±0.6	12.7±0.2	38.2±0.7	25.8±0.4
7/UC-651	12.3±0.6	36.6±0.8	12.8±0.4	38.3±0.9	25.1±0.7

(b) Thermal denaturation

Strain	SSC	% Hyperchromicity	T_m (°C)	% G+C
1/7	1 × SSC	43	79.5	25
	0.1 × SSC	38	63.0	24
1/8	1 × SSC	40	80.0	26
	0.1 × SSC	36	63.5	25
1/D	0.1 × SSC	37	63.0	24
1/Congenic-1	0.1 × SSC	36	63.0	24
1/C*	0.1 × SSC	39	63.0	24
4/IN-3	0.1 × SSC	35	65.5	30
7/UC-651	1 × SSC	35	80.0	26
	0.1 × SSC	32	63.5	25
7/UM-1215	0.1 × SSC	27	63.5	25
7/UM-1216	0.1 × SSC	22	63.5	25
10/EN-131	0.1 × SSC	36	66.0	31
11/AU-50-1	0.1 × SSC	30	65.5	30
11/AU-94-10	0.1 × SSC	30	65.5	30
12/AU-F_12	0.1 × SSC	32	63.5	25
12/AU-115-3	0.1 × SSC	28	63.5	25

DNA preparations which were also run on the analytic ultracentrifuge gave the same DNA density. When paramecia are grown on *Aerobacter aerogenes,* it is surprising that the bacterial peak does not appear as a discrete entity. Control experiments show that *Aerobacter* has a different density (1.718 g/cm³). Since the cultures were starved we can only explain the observation in terms of differential extraction of *Paramecium* DNA. Why then does the latter have a different density from axenic DNA? We suggest that the bacterial DNA may become complexed with *Paramecium* DNA, thereby altering its secondary structure, and that this results in the observed density shift. The density of DNA extracted from paramecia which had been starved for a long time (15 days) resembled much more the density of the DNA from the same strain growing in axenic medium.

In *Tetrahymena* the base composition of the DNA from different syngens may vary (see Table 20, 1) while this does not seem to be true for *Paramecium* syngens grown in axenic medium. This result holds whether density measurements are used to determine base composition or temperature melting data (Table 6). In fact there is a strong correlation between the 2 methods for estimating base composition.

The base composition of the DNA from the strains of *Tetrahymena* examined in the present study varies from 24 to 31% (Table 7). Where temperature melting and chromatography were applied to the DNA from the same

strain, the base compositions estimated from both methods show close agreement. There is also agreement between the data from syngens 1, 4 and 7 shown in Table 7 with published data using buoyant densities in CsCl (26, 27) except that our values for syngens 4 and 7 are slightly lower. The only new information is that reported on syngens 11 and 12 from Australia. Their base compositions fall within the range of base compositions previously reported for the other 10 syngens (See Table 20, 1).

The results with *Tetrahymena* conform to those of other investigators but there is variation between our data and also between those of different investigators for the same stocks of *Paramecium*. We suggest that this may be due to different growth conditions and to differences in the effect of bacteria upon *Paramecium* DNA.

DISCUSSION

By the various criteria outlined in the methods section we feel we have developed technics which lead to the recovery of highly purified DNA from *Paramecium* and *Tetrahymena* in high yields. This compares favorably with methods previously published, some of which we have also tried (5, 21, 27). Our DNA is only slightly contaminated with RNA and carbohydrate, and little or no protein can be detected. All attempts to reduce the RNA, or more precisely, the orcinol-positive material, by further treatment (Sephadex or hydroxyapatite chromatography and preparative CsCl gradients) have met with failure. Either the RNA is too tightly complexed to the DNA to be removed or the orcinol test is giving us a false positive value. The latter is suggested by the experiments using alkali and deoxyribonuclease. Nitrocellulose filtration of denatured DNA also shows that more OD_{260} units complex to filters than would be expected on the basis of the orcinol test. Thus, at the extreme our DNA is contaminated with 15-20% RNA but the real value is most likely considerably lower than this.

The DNAs from both *Paramecium* and *Tetrahymena* show all the properties of double-stranded, high molecular weight DNA molecules when characterized by temperature melting, CsCl density gradient centrifugation, and hydroxyapatite and sephadex chromatography. When denatured they adsorb to nitrocellulose filters.

The temperature melting experiments and density gradients show no evidence for heterogeneity of the DNA molecules. By other technics, however, we have been able to separate fractions of DNA from both *Paramecium* and *Tetrahymena*. These results will be presented elsewhere (2).

The 2 major results of importance from our work reported here are:

1) There is a similarity in base composition of DNA molecules from different syngens of *Paramecium* while there is variation between the syngens of *Tetrahymena*.

2) The density of any *Paramecium* DNA varies depending on whether the cells are grown in the presence of bacteria or in axenic medium.

Estimates of base composition have been used as a means of classifying bacteria and fungi (19, 24). Previous studies on *Tetrahymena* have shown that different syngens may vary in base composition of their DNA molecules altho strain differences have not been disclosed (summarized in Table 20, 1). We assume that it is macronuclear DNA that is being examined here since it comprises a minimum of 89% of the cellular DNA. A small percentage is also contributed from the mitochondria. A satellite which could represent mitochondrial DNA has recently been seen by Chance (personal communication) in axenic stocks 540 and 299 (ρ = 1.699 g/cm^3) using analytical CsCl gradients of the DNA extracted from whole cells. Similar results were obtained by Suyama and Preer (28) using bacterized paramecia. In *Tetrahymena* the position with respect to satellites and mitochondrial DNA is ambiguous. Several investigators have claimed to have seen satellites representative of mitochondrial DNA in different strains of *Tetrahymena* (12, 27, 28). However, Brunk and Hanawalt (6) found that mitochondrial DNA had the same base composition as nuclear DNA.

Our work here has shown differences between some of the syngens of *Tetrahymena*, and we have extended this analysis by examining the base composition by different technics and by obtaining data from 2 new syngens. The results for a given strain by these different methods were remarkably similar in view of the differences which have recently come to light when DNA molecules are examined by different technics (4, 20, 30). However, even low G-C type DNA which both *Tetrahymena* and *Paramecium* possess behaves uniformly by the technics employed and conforms with the relationships developed between base composition and density and temperature melting which were worked out on organisms with high G-C content (17, 19).

The results with the syngens of *Paramecium* grown in axenic medium show there is homogeneity of base composition for syngens 1, 2, 4, 5, and 9 (28% G+C). The 2 stocks of syngen 8 seem to have a slightly higher value (29-30% G+C). Our finding of similarity in base composition between syngens is in contradiction to the observations of other workers (3, 21, 28; Pollack, unpublished). Our value for stock 299 of syngen 8 is similar to another estimate with DNA from cells grown in axenic medium (22). We have shown that the density of the DNA from the same strain varies depending upon whether bacteria are present in the growth medium. The effects are less marked if the cells are severely starved before they are harvested. Axenic cultures yield DNA which gives reliable and repeatable density values, and these are similar to those of DNA from starved cultures. DNA from axenic cultures of different syngens gives similar densities and similar melting curves with the same T_m. Despite the fact that the majority of our results were achieved with preparative CsCl gradients we have corroborated these wherever we have used analytic centrifugation.

The difference in our density values from those of other workers using the same stocks can be explained in 2 ways:

1) By the effect of bacterial DNA on the density of *Paramecium* DNA. We are not clear as to the process of association between *Paramecium* DNA and bacterial DNA but no bacterial DNA band is present in the preparative or analytic gradients with starved cultures, and there is a shift in the density of the *Paramecium* DNA on the gradients.

2) Only when marker DNA samples which equilibrate on either side of the unknown are run can accurate density values be obtained. Chance (personal communication) using analytic centrifugation carried out such experiments and finds slightly lower density values (1.686) for some of the *Paramecium* stocks (540, 551, 114, 562, 51, 138, and 299).

Despite the overall similarities in the base compositions of the DNAs of the syngens of both protozoa, we will show elsewhere that by nucleic acid hybridization studies there are low degrees of homology between the nucleotide sequences even where the base composition of the DNA molecules is the same. For such studies it is essential that the DNAs compared be pure. We believe that the efforts we have expended in developing the methods reported here have achieved this goal.

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