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BIOCHEMICAL CHARACTERIZATIONS OF THE DNA'S OF SEVERAL STRAINS OF THE DINOFLAGELLATE *CRYPTHECODINIUM COHNII*

by

GAIL C. KAPLAN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1978

Gail C. Kaplan

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

December 15, 1978
Date

Marion Himes
Chairman of Examining Committee
Professor Marion Himes

January 2, 1979
Date

Louis G. Moriber
Executive Officer
Professor Louis G. Moriber

R. G. Eckhardt
Prof. R. Eckhardt — Brooklyn College, Institution

Prof. J. Nishiura — Brooklyn College, Institution

Emerson S. Hawley
Prof. E. Hawley — Medgar Evers College, Institution

Regina O'Brien
Prof. R. O'Brien — Marymount College, Institution

Jon D. Cohen
Prof. J. Cohen — Albert Einstein College of Medicine, Institution

_____ — _____, Institution

The City University of New York

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INTRODUCTION

BIOLOGY OF C. cohnii

The dinoflagellates belong to a division of algae known as the Pyrrophyta. Most members are unicellular, biflagellated and some contain a definite cell wall. There are marine as well as fresh-water forms. Dinoflagellates are found throughout the world. Some are photosynthetic, while others are heterotrophic, parasitic, symbiotic, saprophytic or phagotrophic. Dinoflagellates, according to Dodge (1966), are intermediates between the prokaryotes and eukaryotes. They have characteristics of both groups. Prokaryotic features include condensed chromosomes throughout the cell cycle (Kubai and Ris, 1969), apparent lack of histones associated with the DNA (Rizzo and Noodén, 1974 a, b) possible chromosomal attachment to the nuclear membrane (when present, Kubai and Ris, 1969), the persistence of the nuclear membrane and nucleolus during mitosis and the absence of a typical mitotic spindle (Kubai and Ris, 1969). Eukaryotic characteristics include the presence of a nuclear membrane and nucleolus, large amounts of DNA per nucleus, as well as mitochondria, vacuoles, endoplasmic reticulum and golgi in the cytoplasm.

C. cohnii was first described as Glenodinium cohnii in 1885 by Seligo from Germany; later, Kuster in 1908 and Jollos in 1910 called the organism Gymnodinium fucorum. In 1914, Greissman found this organism in France and Naples. Later,

Kofoid and Swezy in 1921 referred to this organism as Gyrodinium and in 1933, Schiller called it Gyrodinium cohnii (Seligo). Biecheler in 1938 described C. (Gyrodinium) cohnii's plate formation as visualized by silver impregnation. She made an elaborate counting and description of the plates on the exterior of the organism. Such a description has never been exactly duplicated. These strains are no longer in existence.

Much later, Pavel Javornicky (1962) described the organism as Crypthecodinium biecheler (cohnii), isolated by L. Provasoli from rotting Fucus (a brown algae) from Woods Hole, Massachusetts and from Puerto Rico (McLaughlin). Javornicky treated C. cohnii with silver stain and saw plates similar to, but not identical with, those depicted by Biecheler. Javornicky suggested classifying cohnii in the following way: Class-Dinophyceae, Order-Dinococcales, and Family-Crypthecodiniaceae.

C. cohnii cells are divided into more-or-less equal halves - the epicone, or upper portion, and the hypocone, or lower portion. The cell is bisected by a "girdle" (cingulum) which forms a left-handed downward spiral into the sulcus. There is a transverse flagellum connected to the sulcus and a longitudinal flagellum attached to the cingulum. These and the lack of distinct thecal plates are the morphological features used to categorize an isolate as Crypthecodinium cohnii. They are also clearly heterotrophic and lack chloroplasts.

Beam and Himes (1977) obtained nine new isolates of

C. cohnii, found from Maine to Florida on the eastern seaboard, from Washington, Oregon and California on the western seaboard, and in Puerto Rico, Honduras, Taiwan and Hong Kong. These new isolates were found associated with diverse algal hosts. Following purification, the new strains have been characterized by Feulgen staining to determine chromosome number and relative amounts of DNA, life cycles, relative sensitivity to drugs such as ethidium bromide, colchicine and caffeine, growth curves, and inactivation by UV- and X-ray irradiation. Following mutagenesis and screening for motility mutants, mating was analyzed to determine sexual compatibilities.

All strains of C. cohnii from Maine to the western end of Long Island were found to be interfertile and compatible with the Puget Sound strain. In addition, isolates from Santa Monica, California, Yehlieu, Taiwan, Miami Beach, Florida and from Dead Horse Bay, Brooklyn, New York, were found to be interfertile and thus form another breeding group (Himes and Beam, 1978).

C. cohnii has been the subject of nutritional studies (Provasoli and Gold, 1961; Provasoli and Gold, 1962; Gold and Baren, 1966) and consequently can now be grown with relative ease in the laboratory. C. cohnii can be maintained on an agar medium. The organisms lose their flagella and become immotile; they form colonies through vegetative reproduction. They can be transferred to liquid media and thereupon develop flagella and become motile.

BIOCHEMICAL STUDIES OF *C. cohnii*

Colin K. Franker *et al.* (1973) described the morphotypic transition of cultures of *C. cohnii* synchronized by the method of Kubai and Ris (1969) who showed that division cysts adhere to modified polystyrene surfaces. The generation time for *C. cohnii*, under the particular growth conditions used by Franker *et al.*, was found to be ten hours. The swarmer phase (biflagellated zoospores) is approximately five hours, after which time there is an increase in predivision cysts (cysts without definite numbers of daughter cells). This phase lasts between 2.75 and 3.25 hours. As the number of predivision cysts, in turn, decreases, one then sees an increase in cysts containing two or four (occasionally eight) daughter cells. This period lasts about two hours. An increase in number of swarmer cells occurs again shortly afterward.

Franker *et al.* (1974) studied the timing of DNA synthesis in *C. cohnii*. The major period of DNA synthesis was determined by measuring ^{32}P incorporation into alkali-insoluble nucleic acid fractions taken from synchronously growing cells. Significant incorporation of ^{32}P into DNA begins at 5.5 hours and continues linearly until the eighth hour, an interval which coincides with the initiation of encystment and ends as one sees well-defined daughter cells within parent cysts.

Thermal elution profiles from hydroxylapatite columns of ^{32}P-labelled DNA obtained at various intervals during

synchronous growth suggest that in the first and middle two-thirds of the S phase the synthesis of "low melt" components is indistinguishable from that called a "high melt" region. After eight hours of labelling, four prominent fractions are resolved. Thus, there seems to be an "orderliness" to the sequence in which the DNA is replicated. This is reminiscent of the specific timing of heterochromatin and euchromatin in higher eukaryotes, according to these workers.

In 1974, Beam and Himes gave evidence for sexual fusion and recombination in C. cohnii; heretofore, C. cohnii was thought only to have a vegetative phase (Javornicky, 1962). Beam and Himes showed that C. cohnii is homothallic, possessing "gametes" and "zygotes". Complementation within zygotes following fusion of two nonallelic motility mutants was observed. Products of meiotic division included parentals or non-parentals but never tetratypes (1975). Details of these studies are found in the appendix.

The modified base, 5-hydroxymethyluracil, which partially replaces thymine was shown by Rae (1973) to be a component of dinoflagellate DNA. Rae found that DNA from C. cohnii has an average buoyant density of 1.715 g/cm^3, suggesting a G+C content of 56.1%. The DNA was also thermally denatured in 0.1 X SSC and shows a broad integral profile, suggesting a heterogeneity in base distribution within the DNA; the derivative profile indicated, according to the author, four melting components. The T_m is 68.5 °C, which represents a G+C content of 35.6%. Such

a discrepancy of percent G+C between CsCl buoyant density analysis and T_m analysis suggests the presence of a modified base.

Nucleotide analysis included digestion of ^{32}P-labeled DNA with deoxyribonuclease I and snake venom phosphodiesterase, and two-dimensional chromatography on unmodified cellulose plates using the solvent system of Randerath and Randerath (1967). Base analysis included hydrolysis of DNA in 88% formic acid according to Wyatt and Cohen (1953), followed by chromatography on plates or on Whatman #1 paper (Wyatt, 1955).

The percent G+C determined by nucleotide analysis was 41.3 and 5-hydroxymethyluracil (5HMU) content was 11.1% of the total nucleotides. The percent substitution of 5HMU for thymine was calculated to be 40.1.

In addition, the uniformity of the 5HMU distribution in C. cohnii DNA was investigated by determining the 5HMU:T ratio in various buoyant density fractions taken from DNA run in CsCl and in Ag^+-Cs_2SO_4 gradients. His findings were that different populations exist with respect to 5HMU to thymine ratios and that the different ratios are not related to the overall base composition of the fractions. Very rich 5HMU regions were not found.

Lastly, a linear relationship was observed between the amount of substitution and the increase in buoyant density over that expected from the G+C content. The following equation was derived:

$$\frac{\rho \text{ observed}}{\rho \text{ expected}} = 0.0236 \frac{5HMU}{5HMU + T} + 1$$

ρ expected was calculated from the equation $\rho = 0.098$ (G+C) + 1.660 (Mandel et al., 1968).

To ascertain as to whether the presence of a modified base was indicative of the Pyrrophyta in general or a phenomenon peculiar to C. cohnii, Rae extended his analyses to five different genera of dinoflagellates as well as to other algal groups (1976). Discrepancies in the percent G+C's calculated from the T_m's and from buoyant density analyses were seen only in the dinoflagellate group. Nucleotide data indicate the presence of 5HMU in all of the five dinoflagellate genera studied, but the percent of 5HMU varies considerably, ranging from 3.8% in Exuviella cassubica to 18.9% in Amphidinium carterae. In addition, 2.9% of 5-methylcytosine was found in E. cassubica, while less than 0.75% was found in C. cohnii.

Unlike Rae who did not compare strains of the same species or species within one genus, nine different strains of Crypthecodinium cohnii were chosen for the present study. These included two representatives of each of two widespread breeding groups and five genetically isolated strains, each representing a different breeding group. All the strains are morphologically and physiologically identical except for one strain, McLaughlin (MC), which is smaller. As shown by Beam and Himes (1977), all the strains, except for MC, have a doubling time of eight

hours, a minimal number of 50 chromosomes and similar DNA content. MC has a doubling time of 24 hours, a minimal number of 15 chromosomes and less than 1/10 the amount of DNA per nucleus relative to the other strains.

The question which we posed was: Are there biochemical differences in the DNA among these nine strains of C. cohnii-like dinoflagellates, and if so, can they be correlated with sexual compatibility? Does the DNA of the MC strain differ from that of others, paralleling morphological and physiological ones?

To answer these questions, a number of techniques were used:

1.) Thermal denaturation. Mandel and Marmur (1968) showed that the degree of stability to denaturing agents of DNA in the native, double-stranded state is proportional to the content of guanine plus cytosine base pairs of DNA; thermal stability of DNA increases linearly with G+C content (between 30 and 70% G+C). In addition, thermal denaturation is accompanied by an easily measurable hyperchromicity at the absorption maximum of DNA (260 nm.) The change of the native to the denatured configuration usually occurs over a small temperature range, with a total increase in absorbance of approximately 40%. From the T_m, or temperature at which 50% of the base pairs of the DNA double helix have separated, one can calculate the percent G+C using the appropriate formula. The shape of

the thermal denaturation curve indicates the distribution of base pairs. The T_m is dependent upon the ionic strength of the solvent. For most of the thermal denaturation studies 0.1 X SSC was used for convenience, as denaturation in 0.1 X SSC occurs at a lower temperature than using for example, 1.0 X SSC, as an increase in salt concentration increases the thermal stability of the DNA double helix against denaturation. Thermal denaturation also checks for the quality of the DNA preparation; a poor preparation which is denatured or contaminated with ultraviolet-absorbing material will not reach the 35-40% hyperchromic increase.

2.) Analytical CsCl buoyant density centrifugation. Additional information about DNA composition can be obtained with this technique: a) percent G+C calculated from the buoyant density of the DNA, b) DNA base distribution, c) DNA size distribution, as well as the presence of satellite sequences.

3.) Nucleotide and base analyses. Discrepancies in percent G+C calculated from the thermal denaturation and buoyant density gradient techniques were in fact found, and therefore led to measurements of the amounts of the four bases and of the modified base, 5-hydroxymethyluracil (5HMU) in the nine strains of C. cohnii. For this determination thin layer chromatography of 5'mononucleotides and of bases was employed.

It was hoped that characterization of the DNA's of representatives of the same and of different sibling species

might offer insights as to mechanisms of sexual isolation and diversification within Crypthecodinium cohnii-like dinoflagellates.

MATERIALS AND METHODS

Table I lists the strains of C. cohnii used in this study, where they were found and the algal "hosts" with which they were associated. All but three of these strains were isolated by Carl Beam and Marion Himes. They have ordered them into interbreeding groups using complementation tests between motility mutants. They have also characterized some of these organisms with respect to the parameters of drug sensitivity (e.g. ethidium bromide, caffeine), ultraviolet light and X-ray irradiation sensitivities and resistance to osmotic shock (Beam and Himes, 1977).

CULTURE CONDITIONS

The sea-water based medium of Gold and Baren (1966) was used to culture all dinoflagellate strains. Large quantities of cells were obtained as follows: 10^4 cells were inoculated into 100 ml of sterile media. When the cultures reached log phase growth, they were tested for contamination by the following method: a 0.1 ml sample was plated onto agar (1.5% agar in media plus 0.005% yeast extract and 0.005% hydrolyzed casein). The plates were incubated for two to four days and then microscopically examined for fungal and/or bacterial growth. If only

dinoflagellate colonies were observed, the 100 ml sample was added to 1500 ml of sterile media. All transfers were done under sterile conditions with continuous UV illumination. Neither temperature nor light were regulated during incubation.

It took approximately five to seven days for cultures of eight strains to reach log phase. MC were grown in 500 ml of media for about 14 days until the turbidity indicated that log phase was reached.

Before harvesting cells, the cultures were tested a second time for contamination as described. In addition, a drop of the cultures was examined microscopically to check for healthy growth and absence of contamination.

Cells in log phase were chosen for two reasons: 1) log phase cells contain about twice the DNA/cell as do stationary phase cells (Allen et al., 1975); 2) stationary phase cells encyst and become more resistant to osmotic shock.

RATIONALE FOR USE OF MOTILITY MUTANTS

To ensure that the eight similar wild type strains were identifiable as to breeding groups (Himes and Beam, 1978), motility mutants were also used for DNA analyses. MC has no stable mutants, but is morphologically recognizable. The mutant strains could be tested for complementation.

GENERAL METHOD OF DNA ISOLATION

The general method adopted for isolating DNA from all strains except for MC consisted of the following steps:

Cells were collected by centrifugation. To osmotically shock the cells, a volume of distilled water equal to that of the pellet was added. The viscous mixture was dispersed with broken-tip Pasteur pipettes. One-third volume of a 15% stock solution of SDS (sodium dodecyl sulfate) was added, and EDTA (ethylene diamene tetraacetic acid) was added to a final concentration of 0.05 M, pH 7.8-7.9. Tris-HCL (tris [hydroxymethyl] aminomethane), to a final concentration of 0.05 M, pH 7.8-7.9 and NaCl to a final concentration of 0.05 M were also added. An equal volume of phenol-chloroform (1:1), pH 8.0, was added, and the mixture shaken for 45 minutes to one hour (Marmur, 1961; Kirby, 1957). It was then centrifuged for 10 minutes, at 12,100 X g. The upper aqueous phase was removed with a large tip Pasteur pipette and shaken with the phenol-chloroform mixture as above. The interface was gently recovered with a spatula and redissolved in 0.01 M NaCl, 0.05 M EDTA, pH 7.8-7.9. The concentration of EDTA in the buffer for the interface material was later increased to approximately 0.20 M because DNA degradation occurred at the lower EDTA concentration.

The phenol-chloroform extraction was repeated for "interface DNA" as well as for aqueous phase DNA until

the subsequent interfaces had no visible material. The aqueous phase was adjusted to 0.15 M NaCl and the DNA precipitated by the addition of two volumes of 95% ethanol. The DNA was collected on a glass rod or pelleted by centrifugation for ten minutes, at 4,080 X g.

Depending upon the amount of DNA obtained, it was redissolved in three to five ml of 0.01 M NaCl, 0.05 M EDTA, pH 7.8-7.9. Ribonuclease A, previously dissolved in distilled water at 0.5 mg/ml and heated 15-20 minutes at 100 °C to inactivate contaminating deoxyribonucleases (Marmur, 1961), was added to a final concentration of 100 ug/ml. The solution was incubated for two hours at room temperature. Earlier, experiments were done with 50 ug/ml of ribonuclease. However, this was insufficient to remove most of the RNA associated with the DNA. After RNase digestion, the DNA was re-extracted with phenol-chloroform. To remove residual SDS, the DNA was either precipitated three to four times or dialyzed for 24-48 hours at room temperature against 0.01 M NaCl, 0.05 M EDTA, pH 7.8-7.9, with three to four changes, each of 500 ml, per two ml of DNA sample. The dialysis tubing was prepared by immersion in boiling water containing sodium bicarbonate for ten minutes and then thoroughly rinsed with distilled water. To remove residual phenol and chloroform, the DNA was then dialyzed against 0.1 X SSC or 1.0 X SSC (1.0 X SSC=0.15 M NaCl, 0.015 M Na citrate) in the cold for 24-48 hours, with four changes

each of 500 ml, per two ml DNA sample and then frozen or placed in the refrigerator with chloroform to prevent bacterial and fungal growth (Mandel, 1962).

The purity of the DNA samples was determined by the ratio of absorbance at 260:280 nm. This ratio varied from 1.8 to 2.0. The yield ranged from 1-13% of the total amount of DNA in the cells extracted. The amount of "interface DNA" was 50% of the aqueous phase DNA. To determine the yield of DNA, the total amount of DNA in the sample was determined by multiplying the number of cells in the sample by the average amount of DNA/cell, 6.9 pg (Rizzo and Nooden, 1973). The amount of DNA extracted was determined by spectrophotometric measurement of the purified DNA samples (Mandel and Marmur, 1968).

METHOD FOR MC DNA ISOLATION

The MC strain was resistant to osmotic shock. The cells were pelleted, frozen with dry ice and lysed in the Eaton press at 1500 lb. per square inch in a solution of 0.05 M EDTA, pH 7.8-7.9. Subsequent steps were as those in the general isolation procedure, except for the following changes: The DNA had to be precipitated with 0.15 M NaCl plus 2 volumes of 95% ethanol and stored at -20°C overnight. The DNA was pelleted by centrifugation at 4,080 X g, for ten minutes. In addition, before ribonuclease treatment, the DNA was dialyzed against NaCl-EDTA as described above to eliminate residual SDS.

Osmotic shock did not lyse all cells of any of the strains. To determine if lysed cells contained DNA different from that of unlysed cells, some strains were lysed with the Eaton press as described for MC's. Alternatively, pelleted cells were suspended in distilled water and then repelleted by centrifugation. Non-precipitable quantities of DNA were found in the supernatant. The pellets were lysed with the Eaton press as described above.

Bacterial DNA was extracted from frozen cells of strain E. coli B. 23.7 g of cells were suspended in 50 ml of 0.05 M Tris-HCL, pH 7.8-7.9, 0.05 M NaCl, and 0.05 M EDTA, pH 7.8-7.9; 16 ml of 15% SDS solution were added and 33 mg of self-digested pronase (stock solution at a concentration of 10 mg/ml in Tris-HCL-NaCl-EDTA); incubation proceeded for 21 hours at 37 °C. Another 50 ml of Tris-HCL-NaCl-EDTA were added with an equal volume of phenol-chloroform (Kirby, 1957). Further processing was the same as that for dinoflagellate DNA.

SP8 DNA was a gift from Jules Marmur.

THERMAL DENATURATION PROFILES

A Beckman Acta CIII spectrophotometer with a Haake temperature regulator was used to follow absorbance changes during thermal denaturation of DNA. Ethylene glycol was circulated around the cuvette chamber for temperature

regulation. Temperature increments were of the order of one degree per minute. Absorbance changes were followed on a recorder chart from 40 °C up to 100 °C at 260 nm. DNA samples were pre-equilibrated with solvent (1.0 X SSC or 0.1 X SSC). Optical density units ranged from approximately 0.600 to 0.800 or from 30 to 40 ug per sample. Three DNA samples were melted simultaneously, one always being E. coli DNA as a standard and all T_m values were corrected to E. coli DNA. The T_m of E. coli DNA is 74.0 °C in 0.1 X SSC (Mandel and Marmur, 1968; Marmur and Doty, 1962) and 90.5 °C in 1.0 X SSC (Marmur and Doty, 1962). DNA concentrations were adjusted such that the three samples were within about 0.1 optical density unit of each other. Samples were denatured in 2 ml quartz cuvettes with a 1 cm light path.

CSCL BUOYANT DENSITY GRADIENTS

CsCl buoyant density gradients were prepared by weighing out appropriate amounts of a saturated CsCl (optical grade) solution buffered with 0.01 M Tris-HCL, pH 7.9, and 1.5-2 ug of DNA sample dissolved in 0.1 X SSC. The amounts of each solution were chosen such that DNA's of buoyant densities 1.700 to 1.740 g/cm^3 would be measured in the same gradient.

SP8 DNA was used as a marker. It had a density of 1.7400 g/cm^3 as determined using E. coli DNA with a buoyant density of 1.710 g/cm^3 and Micrococcus

lysodeikticus DNA with a buoyant density of 1.731 g/cm^3 as reference markers (Schildkraut et al., 1962).

Samples were run in the Beckman Model E Analytical Ultracentrifuge in the ANG rotor, using double sector cells for approximately 20 hours at 44,000 rpm, 25 °C. MC DNA was centrifuged for 40 hours.

NUCLEOTIDE ANALYSIS

Purified DNA was precipitated with 0.15 M NaCl and two volumes of 95% ethanol, washed with 95% ethanol and then with ether and finally air dried. One mg/ml DNA samples (or commercial 5'mononucleotides from Sigma Biochem. Corp.) were dissolved in digestion buffer (0.01 M Tris-HCL, pH 7.6 and 5 mM $MgCl_2$), and then digested with bovine pancreatic deoxyribonuclease I, at a final concentration of 200 ug/ml for five hours at 37 °C. The pH was then adjusted to 8.5 with NaOH or with 1.0 M Tris and snake venom phosphodiesterase was added to a final concentration of 100 ug/ml and incubated for five hours at 37 °C (Ray and Hanawalt, 1964; Rae, 1973).

Twenty ul of digested DNA were spotted on 20 x 20 cm plates of MN CEL 300, 250 u thick (unmodified cellulose plates from Fisher Scientific), one ul at a time with drying in between each application. Two-dimensional ascending chromatography was performed in glass tanks. The first dimension solvent consisted of isobutyric acid:distilled water:ammonium hydroxide in the ratio of 66:20:1 (Dawid et al., 1970), and the second dimension solvent consisted

of saturated ammonium sulfate:1 M sodium acetate: isopropanol in the ratio of 80:18:2 (Markham and Smith, 1951).

Upon completion of the second dimension, the plates were dried for 1 hour. The 5'mononucleotides were located using shortwave ultraviolet illumination. The mononucleotides were eluted with 0.1 N HCL onto prewashed (in distilled water for at least 24 hours) Whatman #1 filter paper strips or the spots were scraped into test tubes. One ml of 0.1 N HCL was added to each nucleotide (if eluted onto paper, the paper was cut into small pieces and put into test tubes) and elution continued for 16-24 hours; the samples were rotary shaken during the elution period. The samples were then centrifuged at 2,000 rpm for ten minutes. Usually, the supernatants were pipetted out and centrifuged a second time. The optical densities of these samples were determined at 5 nm intervals from 230 to 310 nm (Randerath and Randerath, 1967).

Nucleotide concentrations were determined using the following information:

5'MONONUCLEOTIDES	λ_{max}*	$E_{max} \times 10^{-3}$*	MOLECULAR WEIGHT
d'AMP (pH 2)	258	14.3	330.1
d'CMP (pH 2)	280	13.5	306.1
d'GMP (pH 1)	255	11.8	346.2
d'TMP (pH 2)	267	10.2	321.1
HMUMP	264	10.2	337.1

*(References: Wyatt, 1955; Bendich, 1972; Beaven et al., 1955; Calbiochem-Properties of the Nucleic Acid Derivatives, 1964).

PEI-CELLULOSE (polyethyleneimine) CHROMATOGRAPHY

For preparation of PEI-cellulose plates (to determine degradation products, if any, of digested DNA's): Cellulose (MN 100) was suspended in 1500 ml of 0.1 N HCL, with three changes of acid, for 24 hours. At this time no further UV absorbing material eluted into HCL. The cellulose was then filtered and twice rinsed with 1800 ml of distilled water. The pH of the final filtrate was the same as that of distilled water. The cellulose was dried thoroughly and stored at room temperature until needed. Twenty grams of 50% polyethyleneimine were diluted with 700 ml of distilled water, adjusted to pH 6 with concentrated HCL and then made up to a final volume of one liter with distilled water. Twenty-three grams of cellulose were added to 145 ml of a 1% solution of PEI. Twenty x twenty cm glass plates, 250 u thick, were cleaned with soap and water and then with acetone. The cellulose in PEI mixture was then spread onto the glass plates. After air drying at room temperature the plates were prewashed by ascending chromatography with paper wicks attached to the tops of the plates with 10% NaCl and then with distilled water. Nucleotides were spotted onto these plates and chromatographed by one-dimensional ascending chromatography with 1 N acetic acid: 3 M LiCl in a 9:1 ratio (Randerath and Randerath, 1967).

BASE ANALYSIS

Purified DNA was precipitated and dried in a vacuum dessicator overnight. DNA, (or bases from a commercial source, Sigma Biochem. Corp.) 0.7 to 1.0 mg dissolved in 0.5 ml of 88% formic acid, was hydrolyzed in glass bombs at 175 °C, for 35 minutes. A great deal of pressure built up in the sealed glass bombs. Following hydrolysis, therefore, the tips of the bombs were flamed with a propane torch to burst the tips and release the pressure (Wyatt and Cohen, 1953). A file was used to cut off the neck of the bomb and the formic acid eliminated by pipetting a jet of air into the bomb. Once most of the formic acid was eliminated, the bombs were placed into a vacuum dessicator overnight.

The bases were redissolved in 1 N HCL at a concentration of 2.8 to 4 mg/ml. Alternatively, the volume was adjusted to yield approximately 2.8 ug of hydrolyzed DNA per ul of 1 N HCL. Fifty to 85 ul of sample were spotted, two ul at a time with drying in between, onto Whatman #1 cellulose strips, 50-60 cm long, 2.54-3.81 cm wide and 0.16 mm thick. The filter paper strips were prewashed with distilled water. Triplicate to quadruplicate samples were run, together with one to two blank pieces of paper as controls. A chromatography chamber, approximately 64.8 cm by 53.3 cm by 63.5 cm, was used. Descending one-dimensional chromatography was performed for 17-18 hours or until the solvent front had migrated about 35-38 cm

down the paper. The solvent consisted of isopropanol: HCL: H_2O in the ratio of 85:20.5:19.5. The chamber was kept saturated with solvent at all times using trays in the bottom of the chamber filled with solvent. Following chromatography, the cellulose strips were air dried at room temperature and the bases detected by UV illumination. The chromatography paper containing the bases was cut into small pieces and then placed into test tubes, into which were added 4 ml of 0.1 N HCL. The tubes were put onto a rotary shaker for 19-24 hours to elute the bases from the paper. After elution, samples were centrifuged at 2,000 rpm for ten minutes. Samples were read in 2 ml quartz cuvettes with a 1 cm light path in the Gilford 240 spectrophotometer. Optical densities were recorded at 5 nm intervals from 230 to 310 nm (Wyatt, 1951).

Base concentrations were determined using the following information:

BASE	λ max*	$E_{max} \times 10^{-3}$*	MOLECULAR WEIGHT*
A	260	13.0	135.1
G	250	11.0	151.2
T	265	7.95	126.1
C	275	10.5	111.1
HU	261	8.0	142.1

*(References: See page 27)

RESULTS

THERMAL DENATURATION ANALYSIS

Methodological

REPRODUCIBILITY

While whole cell DNA was used for all experiments, the contribution of mitochondrial DNA was so small as to be negligible in all results to be described.

The following set of experiments is concerned with the accuracy and precision of the thermal denaturation methods. Are the findings reproducible? Are the findings representative only of particular DNA isolations, or of DNA's isolated from particular times in the life cycle of C. cohnii? Do results depend on the solvent system used or are they due to the nature of the DNA's themselves? Figure 1 illustrates the reproducibility of the melting profiles of DNA from two strains, WH and SM. Three different samples of DNA from each strain were thermally denatured in 0.1 X SSC. The thermal denaturations of DNA's from these two strains represent two to five different DNA isolations for each strain and DNA isolated from stationary and log phase cells. The same was done for DNA's from the other strains, but the data are not shown here. The T_m of WH DNA varied within 0.5 °C (67.75-68.25) and the percent hyperchromic shift was within 2.1 (33.3-35.3%). The SM DNA T_m varied 0.75 °C and the percent hyperchromic shift within 2.2. The triplicate integral profiles are superimposable for each

strain. The first derivative plots indicate two to three different melting components for the DNA's of each strain; the different components show a nonrandom distribution of base pairs. E. coli DNA is included as a standard and all T_m values are corrected to E. coli DNA using a T_m of 74.00 °C (Mandel & Marmur, 1968; Marmur & Doty, 1962). Figure 1 thus indicates that the thermal denaturation method used here is highly reproducible and that DNA's isolated from stationary and log phase cells are identical according to this method.

As a further check on the fidelity of these thermal denaturation profiles, two different DNA's, from G and SM, were melted in 1.0 X SSC. This was done to make certain that the results were not due to the particular solvent used but rather to the characteristics of the DNA's themselves. The T_m of a given DNA changes in linear fashion with the logarithm of the salt concentration. Thus, values obtained at one ionic strength can be extrapolated to another ionic strength. The integral curve is a cumulative plot of denaturation of segments of DNA's which may differ in their average percent of G+C. AT-rich areas denature before GC-rich areas. The T_m's of the sequences with lowest average G+C and of highest average G+C should also shift with changes in ionic strength. By comparing thermal denaturation profiles in 0.1 X SSC and in 1.0 X SSC, artifactual changes in the slope of thermal denaturation profiles can be observed. (Mandel and Marmur, 1968).

Figure 2 compares melting profiles in 1.0 X SSC and in 0.1 X SSC, again including E. coli DNA as a standard. The 1.0 X SSC profiles were corrected and adjusted, using appropriate formulas: In 1.0 X SSC, G+C= $(T_m-69.3)2.44$; in 0.1 X SSC, G+C=$(T_m-53.9)2.44$ (Marmur and Doty, 1962; Mandel and Marmur, 1968). As can be seen, the integral and derivative plots are identical between the 1.0 X SSC and 0.1 X SSC solvent systems. Using the appropriate formulas and calculating the percent G+C from the T_m's, G DNA has a percent G+C in 0.1 X SSC of 33.8 and in 1.0 X SSC, 34.6; SM DNA in 0.1 X SSC has a 36.8% GC and in 1.0 X SSC, 37.1% GC. The percent hyperchromic shifts are also close for both solvents; G DNA in 0.1 X SSC has a 35.5% hyperchromic shift and in 1.0 X SSC, 38.9, while SM DNA in 0.1 X SSC shows a 36.5% increase in absorbance and a 37.4% increase in 1.0 X SSC.

In summary, the solvent system does not influence the reproducibility nor the characteristics measured of the DNA's.

NONPREFERENTIAL DNA ISOLATION

Several questions had to be answered concerning the methods of DNA isolation. Was there selection for particular DNA sequences; for example, did the two cell lysis procedures lyse only cysts or nonencysted cells? Examined microscopically, osmotic shock did not lyse all cells of any strain.

To answer the question of preferential cell lysis, cells were lysed by osmotic shock or by the Eaton press. The rationale is that the Eaton press would lyse all cell types, encysted and nonencysted, whereas osmotic shock would lyse only nonencysted cells. The DNA's were thermally denatured in 0.1 X SSC. The 260/280 nm ratio for all these DNA's was between 1.8 and 2.0. Since the DNA samples hadn't started to melt until 50.00 °C, no RNA or protein was in the samples.

Figure 3 represents thermal denaturation profiles of DNA's isolated from osmotically shocked and from Eaton press lysed cells of the SM strain. SM DNA from osmotically shocked cells has a T_m of 68.50 °C and a 34.2% hyperchromic shift. DNA from Eaton press lysed cells shows a T_m of 68.00 °C with a 29.7% hyperchromic shift. The profiles are identical. The lowered hyperchromic shift of DNA from Eaton press treated cells is expected since such harsh treatment of cells does cause some hydrogen and covalent bond breakage of DNA molecules (Davison, 1959). The difference in T_m's between DNA's of cells lysed by the two methods is within experimental error.

It appears that cysts and nonencysted cells contain the same type of DNA. If the DNA of cysts differs from nonencysted cells, the DNA's are either too low in quantity or the differences are not detectable under these experimental conditions. Table II-A summarizes the results.

Under certain extraction conditions, it is known that AT-rich DNA is found in the aqueous-phenol interface

(Kit, 1961). It was thought, therefore, that some DNA may have been lost at the interface. Consequently, DNA was recovered and isolated from the interface, and thermally denatured. Figure 4 illustrates the results obtained. PS DNA from the aqueous phase has a T_m of 66.50 °C, with a 33.5% hyperchromic shift, while interface DNA has a T_m of 67.00 °C and a 33.0% hyperchromic shift. These differences are insignificant. The integral and derivative plots are superimposable.

We can conclude, then, that according to these findings, aqueous phase DNA and interface DNA are identical. It appears that in using our procedures for DNA extraction, we are not preferentially selecting for particular DNA sequences. Table II-B summarizes these findings.

Biological Variation

SPECIFIC COMPARISONS BETWEEN NINE STRAINS OF C. cohnii

The following experiments were done to characterize the different strains with respect to T_m and thermal denaturation profiles of their DNA's.

Figure 5 shows integral profiles of thermally denatured DNA's from each of the nine strains of C. cohnii. An integral profile plots the increase in absorbance at 260 nm relative to increasing temperature (Mandel and Marmur, 1968). WH DNA is plotted together with each of the strains for easy comparison; E. coli DNA is included with the DNA of Y for a standard. None of the melting profiles is cor-

rected for thermal expansion (Mandel and Marmur, 1969) since every melt is corrected to the T_m of E. coli DNA. Distinct differences can be observed in the shapes of the integral profiles between the DNA's of the various strains. Two melting components are seen for the DNA's from each of the strains, with the possible exception of G DNA and MC DNA which may contain three melting components. The T_m's range from 66.50 °C for PS DNA to 75.25 °C for MC DNA. The hyperchromic shifts range from 33.5% for PS DNA and I DNA, to 37.0% for SM DNA. This is typical for double stranded DNA (Mandel and Marmur, 1968). MC DNA has a low hyperchromic shift of 18.8%, which may be due in part to the use of the Eaton press for cell lysis. The %GC's calculated from T_m's range from 30.7 to 52.1 (Mandel and Marmur, 1968).

If one compares the shapes of the integral plots of the thermal denaturation of DNA's of compatible strains, basic similarities are seen. The melting profiles of WH and PS DNA's are nearly parallel to each other, as are those of the DNA's from the SM and Y strains. The DNA's from these compatible strains differ significantly, however, in their T_m's. WH DNA has a T_m of 68.00 °C $\pm$ 0.20 °C, while that of its compatible strain, PS, has a T_m of 66.50 $\pm$ 0.41 °C; Y DNA has a T_m of 69.50 $\pm$ 0.35 °C, while that of its compatible strain, SM, has a T_m of 68.70 $\pm$ 0.31 °C. In contrast, the shapes of the integral profiles of DNA's of two incompatible strains, Y and To, differ. On the other hand, their T_m's are the same (69.50 $\pm$ 0.35 °C).

Figure 6 presents derivative plots of the melting curves of the DNA's of the nine strains. A derivative plot represents the change in absorbance per degree increment of temperature. It indicates the changes in the slope of the line from the integral plots. These changes illustrate various components within the DNA that denature at different temperatures, and these may vary in their base composition (Bernardi et al., 1970). WH DNA has been included in each derivative plot for comparison, and E. coli DNA is included with Y DNA as a standard. DNA's from all the strains except G and MC show two melting components; these exceptions may have up to three components. If the plots are superimposed after compensating for T_m differences, the shapes are similar for WH and PS DNA's and for those of SM and Y DNA's. The breadths of the curves for DNA's of members of the same breeding group are also very similar, especially when compared with the plots of To DNA and WH DNA, where distinct differences in shape are clearly observable.

Table III summarizes the T_m's and calculated percent GC's for the DNA's of the dinoflagellate strains, and includes standard deviations for both. PS DNA has the lowest T_m, 66.50 °C, with a corresponding calculated 30.7% GC; MC DNA has the highest T_m, 74.90 °C, with a calculated percent of GC of 51.2. The DNA's of the nine strains span a T_m range of 8.40 °C, with a 20.5% range in calculated GC.

The nonrandom base pair distribution is also indicated by bi- or triphasic thermal denaturation profiles (Mandel and Marmur, 1968). If the base pair distribution were random, one would obtain integral and derivative profiles like those for E. coli DNA, which have a constant slope. Each component of a multiphasic curve can represent a fraction of DNA which has a unique percent G+C. This can be determined from the T_m of that component. Table IV summarizes these calculations. For Ro DNA, the T_m's of individual melting components are 60.00 °C and 70.25 °C, with corresponding percent GC's of 14.9 and 39.9. Component 1 comprises 20.0% and component 2, 80.0%, of the total DNA. MC DNA has three melting components: the lowest, with a T_m of 67.25 °C and a percent GC of 32.6, comprises 26.0% of the total melt, while the highest, with a T_m of 77.50°C and a percent GC of 57.6, comprises 38.0% of the total melt. Ro and MC DNA's represent the most extreme examples of base heterogeneity and differences in T_m.

MUTANT VS. WILD TYPE STRAINS

Motility mutants were used for DNA analyses to ensure that the eight similar strains were identifiable as to breeding groups (see Materials and Methods). MC has no stable mutants, but is easily distinguishable morphologically from the other strains. DNA was isolated from the motility mutants as described, and thermally denatured in 0.1 X SSC.

Figure 7 compares these profiles with those of their wild type counterparts. In most cases, the profiles are identical. From figure 7a, DNA's from WH and WH_a show nearly identical integral and first derivative profiles; the T_m's are within 0.5°C of each other and the percent hyperchromic shifts within 1.1% of each other. Similar findings were obtained for DNA's from I and I_a, and for DNA's from To and To_b, as seen in figures 7b and 7c, respectively.

In some cases, the T_m's and percent hyperchromic shifts are lower in DNA's from the mutant strains when compared with those from their respective wild type strains (see Table V). For example, the profiles for PS and PS_p DNA's are nearly identical; however, the T_m of the DNA from the mutant is 1.25 °C lower than for DNA from the wild type, while the percent hyperchromic shift is only 1.7% lower in the DNA from the mutant (figure 7d). Figure 7e shows that DNA's from Ro and Ro_b have similar profiles, but Ro_b DNA has a T_m 2.25 °C lower and a 4.9% lower hyperchromic shift when compared with that from Ro. Figure 7f indicates similar findings for G and G_c DNA's.

In a few cases, the thermal denaturation profiles and hyperchromic shifts of DNA's from mutants differ greatly from those of the wild type. The DNA's from SM_a and Y_a strains were very sensitive to degradation as it took several attempts to extract DNA from these cells; several batches of cells yielded totally degraded DNA (the DNA disappeared after one or two purification steps). Figures

7g and 7h indicate that DNA's from SM and Y mutants, respectively, show the greatest disparities compared with DNA's from the wild type strains. The integral profiles look nearly identical. SM_a DNA has a 3.0 degree lowered T_m and a 17.3% lowered hyperchromic shift than SM DNA; Y_a DNA has a T_m 2.5 °C lower and a 20.4% lowered hyperchromic shift than Y DNA. The derivative plots are the same despite the lowered T_m's and percent hyperchromic shifts.

Three major conclusions were drawn from this experiment: 1. DNA's from some mutant strains are the same as those from wild type strains; 2. DNA's from other mutant strains are a little different from DNA's from wild type strains; 3. DNA's from two mutant strains are very different from those from wild type strains. There are only three strains in which the DNA's from the wild type have identical T_m's and thermal denaturation profiles, namely, WH, To and I. The possibility that these represent members of the same strain can be precluded by the fact that the DNA's of mutants of these three strains have the same thermal denaturation properties as do the wild types.

CSCL BUOYANT DENSITY GRADIENTS

A second, common method for DNA analysis, and one which requires little DNA (1.5-2. 0 ug), is that of buoyant density gradients. Schildkraut *et al*. (1962) demonstrated that a large number of DNA's from many different organisms showed that the buoyant density of DNA in CsCl is directly

proportional to its guanine plus cytosine content. Buoyant density determinations can be done with relative ease and reproducibility and can provide information on the degree of homogeneity of the DNA samples. When used in conjunction with UV-absorbance profiles, unusual bases can be detected, namely, in discrepancies in calculated percent GC's from T_m's and from buoyant densities.

Methodological

REPRODUCIBILITY

To determine the precision and reliability of buoyant density analyses, as well as reproducibility of findings, the following experiments were done. Figure 8a represents triplicate centrifugations in CsCl for DNA's from two different strains, PS and Y. These DNA's also represent DNA's taken from several different isolations (two to five) and from cells harvested at two different times in the life cycle, stationary and log phases. The DNA profiles for the individual strains are identical, as are the buoyant densities.

In summary, the buoyant density determinations are highly reproducible and DNA's isolated from stationary and log phases are identical according to this method.

NONPREFERENTIAL DNA ISOLATION

For reasons already given under the thermal denaturation studies, buoyant density analyses were performed on

DNA's isolated from osmotic shock and from Eaton press lysed cells of particular strains. No significant differences were found in these DNA's from thermal denaturation studies, so it was thought, though unlikely, that perhaps some differences would be discerned using buoyant density gradients. Figure 8b represents examples of buoyant density profiles of DNA's obtained from osmotically shocked cells, as well as from cells treated with the Eaton press. SM DNA from Eaton press lysed cells shows a greater span in buoyant density, indicating such a lysis procedure results in lower molecular weight DNA than osmotic shock treatment (Davison, 1959). The DNA's from the two lysis procedures have similar profiles and have the same average buoyant densities. SM DNA from osmotically lysed cells has a buoyant density of 1.7184 g/cm^3, and SM DNA from Eaton press lysed cells has a buoyant density of 1.7199 g/cm^3, or a percent G+C of 1.5 between the two DNA's. Table VI-A summarizes the results. One may conclude that the two DNA's are identical.

For the same reason discussed in the thermal denaturation section, DNA's isolated from the aqueous phase and from the interface were compared, also. Figure 8c represents profiles of PS aqueous phase and interface DNA's. The shapes of the profiles are essentially identical, as are buoyant densities. PS aqueous DNA has a buoyant density of 1.7171 g/cm^3, and interface DNA, a buoyant density of 1.7172 g/cm^3. Table VI-B summarizes the results.

The conclusion is that DNA's isolated from the aqueous phase and from the interface are identical and that particular DNA types are not being selected for with our methods for DNA isolation.

Biological Variation

SPECIFIC COMPARISONS BETWEEN NINE STRAINS OF *C. cohnii*

Figure 8d shows representative examples of buoyant density profiles of DNA's from each of the nine different dinoflagellate strains. One can quickly observe more pronounced differences in the buoyant density profiles than one saw from the thermal denaturation curves. DNA's from Y, SM and To have very pronounced heavy shoulders, together with wide spreads in buoyant densities. The three DNA's share similar profiles. WH and PS DNA's have less evident heavy shoulders, and share similar profiles.

I, G and Ro DNA's have light shoulders and have similar profiles.

MC DNA again stands out as the most distinguishable from the other DNA's; the buoyant density profile is much more heterogeneous than those of the other DNA's. This adds evidence to MC being in a group outside of the other strains.

Two compatible strains, WH and PS, have DNA's with identical profiles. DNA's from two strains of another breeding group, Y and SM, also have identical profiles.

Table VII summarizes these findings. The buoyant

densities range from 1.7171 in PS DNA to 1.7266 g/cm^3 in MC DNA, with a percent G+C calculated from these buoyant densities using the formula, $G+C=\frac{\rho-1.660\ g/cm^3}{0.098}$ (Schildkraut, Marmur and Doty, 1962), of 58.3 to 68.0. The buoyant density range is 0.0095 g/cm^3, with a corresponding percent G+C range of 9.7. Included in the table are buoyant densities calculated for heavy and light shoulders observed in profiles in every DNA except for that of MC, whose profile is too heterodisperse to show distinct regions. For this latter strain, DNA buoyant density spans were determined. Percent G+C's are also indicated, corresponding to respective buoyant densities. These measurements were done to give some idea of the extent of base distribution varieties within the DNA from each strain. Y DNA, for example, has a heavy shoulder, 0.0074 g/cm^3 heavier than its peak value, or 8.1% richer in GC (and/or HMU). On the other hand, Ro DNA displays a light shoulder 0.0071 g/cm^3 lighter than its peak value, corresponding to a percent GC of 7.3 (with, perhaps, an HMU contribution).

MC DNA displays a spread in buoyant densities ranging from 1.7184 g/cm^3 (=59.6% GC and probably some HMU) to 1.7333 g/cm^3 (=74.8% GC plus HMU). This heterodispersity could be due, in part, to the use of the Eaton press for cell lysis, as well as to a very nonrandom base pair distribution within the DNA itself.

There is no correspondence between the shoulders observed here and the various melting components observed in the thermal denaturation profiles. What is emphasized,

however, is the appearance of preferential base pair distribution observed in non-Guassian buoyant density profiles and in multiphasic thermal denaturation profiles of the DNA's of the nine strains.

MUTANT VS. WILD TYPE STRAINS

Figure 8e shows buoyant density profiles of DNA's from each of the nine dinoflagellate strains, together with DNA profiles of their respective motility mutants. These profiles were taken for reasons discussed in the thermal denaturation studies, namely, for identification of the interbreeding groups. I and I_a DNA's show nearly identical profiles; the I_a DNA profile appears a little more symmetrical without a definite light shoulder seen for I DNA; the buoyant densities are within 0.0007 g/cm^3 of each other. To_b DNA indicates the heavy shoulder observed for To DNA; however, its profile appears more symmetrical than that found for the wild type DNA. The two buoyant densities are within 0.0004 g/cm^3 of each other. WH_a DNA shows a peculiarity. Its profile shows a slightly light shoulder when compared with that from the wild type, which has a heavy shoulder. This observation is difficult to assess; the overall profiles are very similar but the buoyant densities differ by 0.0016 g/cm^3, a larger difference when compared to the DNA's from I or To. Ro and Ro_b DNA's also show similar profiles, but Ro_b DNA has a broader profile than that for the wild type DNA. The buoyant densities of the two differ by 0.003 g/cm^3. Buoyant

density profiles for DNA's from G and G_c, from SM and SM_a, from PS and PS_p, and from Y and Y_a are all identical; buoyant densities differ between DNA's of mutant and wild type by as little as 0.0001 g/cm^3 (SM and SM_a) and by as much as 0.0017 g/cm^3 (G and G_c). Table VIII summarizes the results. In general, the wild type and mutant DNA buoyant density profiles correspond.

NUCLEOTIDE ANALYSIS

The purpose of these studies was severalfold. Firstly, Peter Rae (1973) showed the presence of the modified base, 5-hydroxymethyluracil, in the DNA of C. cohnii (strain WH). Secondly, for all nine strains studied here, the percent G+C's determined from thermal denaturation data were different from those determined by buoyant density analysis. The actual percent G+C and percent hydroxymethyluracil (HMU) of the DNA's from each of these strains had to be determined by direct measurement. Thus the nucleotide composition of the DNA's of the various strains, using the technique of Randerath and Randerath (1967) was used. This technique is relatively easy to use and little DNA is needed. For nucleotide chromatography, DNase I and snake venom phosphodiesterse were used. DNase I is an endonuclease and creates a nick in the phosphodiester backbone of the DNA molecule, between the 3'pyrimidine nucleoside phosphoryl group and the 5'hydroxyl of the adjacent purine or pyrimidine nucleotide group. Snake venom phosphodiesterase is an exonuclease which hydrolyzes polynucleotides,

liberating primarily 5'mononucleotides (Schmidt, 1955). The products of digestion are separated with aqueous electrolyte solutions serving as the solvents. With two-dimensional chromatography, between 0.2 and 2 millimicromoles of individual mononucleotides can be detected under a shortwave UV light in a dark room (Randerath and Randerath, 1967). The solvent used in this study was chosen for its high resolving power.

Other chromatographic procedures, for example, ion exchange chromatography, require more material for analyses, about 0.5 mg for each base, and were therefore not used (Cohn, 1955).

To establish the identities of the individual 5'mononucleotides, various characteristics were measured. Each nucleotide was identified by its position on unmodified cellulose plates according to Rae (1973). UV spectra of the 5'nucleotides were taken and compared with 5'nucleotides obtained from commercial sources. Figures 9a-9c illustrate the results. The shapes are nearly identical. Some variations do exist but are expected given the low optical densities of some of the nucleotides, for example, HMU, T or G. The wavelength at which the nucleotides absorb maximally and the 250:260 and 280:260 nm ratios were also measured for each nucleotide. These numbers were compared with published data and with nucleotides from commercial sources. Table IX summarizes the results. The wavelengths of maximum absorption for individual mono-

nucleotides are nearly identical for the two sets of measurements. The ratios are, however, very different in a number of cases between the two sets of data. Migration and UV spectra were the primary parameters used to characterize the individual mononucleotides. They were consistent from experiment to experiment.

Table X' includes optical densities determined at the wavelengths of maximum absorption for the 5'mononucleotides, and Table X", percentages of each nucleotide within each DNA sample. Uncorrected values are data taken before correction with controls. To serve as controls, areas of the cellulose plates not containing any visible UV absorbing material but adjacent to the nucleotides were chosen. These control areas were treated in the same way as the actual nucleotide samples. UV spectra for the control areas are plotted in figure 9c-2. They all have the same basic shape, but differ in amount of UV absorbing material. For example, the control for d'AMP and d'TMP has a higher absorbance than that for HMUMP, and the control for d'CMP absorbs almost twice as much as that of the control for d'AMP and d'TMP. Due to such variations among the control values, mere subtraction of background would not give consistent and accurate results. To standardize the controls relative to each other and then with respect to their respective nucleotides, the following rationale was used: The absorption curve of a nucleotide eluted after chromatography is actually that of the nucleotide alone and that of non-nucleotide material. The absorption spectra of

the nucleotides dissolved in buffer show that they absorb very little at 230 nm. The material eluting from the control spots have maximal absorbance at 230 nm. Thus the absorption at 230 nm of nucleotides eluted after chromatography is actually that due to contaminating material. Thus the absorbances of nucleotides eluted after chromatography can be corrected by using the following formula:

$$A_m NT - \frac{A_{230} NT}{A_{230} c} \; A_m c$$

where Am^c and $A_{230}c$ are determined from the absorption curve of the control spots (figure 9c-2). Am^c is the absorbance of the nucleotide control at the wavelength of maximum absorption of the nucleotide in question. $A_{230}c$ is the absorbance of the nucleotide control at 230 nm.

$A_{230}{}^{NT}$ is the absorbance at 230 nm of the nucleotide eluted after chromatography.

Am^{NT} is the value obtained at the wavelength of maximal absorbance of the nucleotide in question.

Consistent results were thereby obtained, meaning d'AMP=d'TMP + HMUMP and d'GMP=d'CMP.

Reliability of the nucleotide technique was further tested by calculating recoveries of the material spotted onto the cellulose plates. The following formula was used: $C=\frac{O.D.}{Ex10^3}$ where C=molar concentration, E-molar extinction coefficient at the wavelength of maximum absorbance, and O.D.=optical density unit at the wavelength of maximum absorbance of the solution X total volume (Bendich, 1957).

This formula was used to calculate actual recoveries of individual nucleotides. The theoretical yields were calculated knowing how much material was spotted onto the cellulose plates and the percents of d'GMP, d'CMP, d'AMP, d'TMP and HMUMP.

Examples of percent yields are the following, from Tolo DNA:

d'AMP: 53.6

d'GMP: 58.2

d'TMP: 60.4

d'CMP: 42.9

HMUMP: 49.9

The recoveries do not differ drastically. These differences would slightly influence the results, but no correction was made in the final analysis.

DNA's from only six dinoflagellate strains are represented in Tables X' and X". Calf thymus DNA is included as a DNA with known base composition and lacking modified bases. A difficulty arose which precluded further use of the nucleotide chromatography technique. Rather consistently, only 50% of d'GMP relative to d'CMP was recovered when the DNA from the seventh strain was analyzed. This finding occurred at the time a newly purchased snake venom phosphodiesterase was used. It is known that frequently this enzyme is contaminated with a 5'nucleotidase. The nucleotidase has been shown to hydrolyze 5'd'AMP most rapidly, with 5'd'GMP hydrolyzed less rapidly (Sulkowski et al., 1963).

Several experiments were then carried out to find the source of the loss of d'GMP. Is the phosphodiesterase contaminated with an enzyme that is preferentially degrading d'GMP, or could the source of contamination involve the other enzyme used in our study, namely, deoxyribonuclease I? To determine if the enzymes used were respon-

for the lower recovery of d'GMP, the following experiment was done:

Commercial d'GMP, at a concentration of 1 mg/ml, was treated four ways:

1) dissolved in digestion buffer only
2) treated with DNase I only
3) treated with newly purchased snake venom phosphodiesterase only
4) treated with both DNase I and snake venom phosphodiesterase.

One would expect that if the phosphodiesterase were the source of the problem, treatments 3 and 4 should indicate 50% recovery of d'GMP relative to treatments 1 and 2.

Twenty micrograms of each of the four samples were spotted on unmodified cellulose plates, and two-dimensional chromatography performed as previously described. Treatments 1 and 2 showed approximately 60% recovery. This is in agreement with earlier results. Treatments 3 and 4 showed 28.5 and 36.0% recoveries, respectively. In other words, those samples treated with snake venom phosphodiesterase were recovered about 50% less than those not treated with the enzyme.

To determine if only d'GMP was affected by the phosphodiesterase, a similar experiment was done using commercial d'AMP. Deoxyadenosine was included in the chromatography and was dissolved in digestion buffer only. This was done to test whether d'AMP was being degraded to deoxyadenosine. The yields were unlike those for d'GMP. The d'AMP recoveries for the four treatments were within 10.0% of each other. In addition, there was only a 2 to 17% loss in material after chromatography. These findings are in disagreement with those cited in the beginning of this section, where a 53.6% recovery of d'AMP was noted. It is difficult to reconcile the two results, except to suggest, although it seems improbable, that the nucleotides are affected differently by the phosphodiesterase when they are in a polymerized state as compared to when they are individual nucleotides. In addition, none of the material in the treated d'AMP samples migrated on the plates like deoxyadenosine, indicating that d'AMP was not being converted to deoxyadenosine.

To identify the products of enzyme digestion by Rf values $\left(Rf=\frac{\text{distance migrated by the sample, in cm}}{\text{distance migrated by the solvent front, in cm}}\right)$ (Consden and Martin, 1944), the above five samples were chromatographed on PEI-cellulose plates prepared as described in the Materials and Methods section. 1 N acetic acid was the first solvent and once the solvent migrated 2 cm above the origin, the plates were put into a solvent consisting of 1 N acetic acid: 3 M LiCl in a 9:1 ratio.

This solvent was allowed to migrate 15-16 cm above the origin (Randerath and Randerath, 1967). The d'AMP treated in the four ways chromatographed with identical Rf values, 0.31-0.32. Deoxyadenosine in digestion buffer only migrated with an Rf value of 0.58, so it could easily be distinguished from d'AMP. Thus, it was shown again that d'AMP was not being dephosphorylated to deoxyadenosine or to any other detectable products following enzyme treatment.

The next experiment was similar to the previous one, except d'GMP was used, again to test whether enzyme treatments degraded the nucleotide into detectable products. Digested calf thymus DNA was also included to see if any differences could be noted with the effects of enzyme treatment on previously polymerized nucleotides vs. commercial nucleotides.

SAMPLE (each at 1 mg/ml)	TREATMENT
1. Guanine	1. Dissolved in digestive buffer.
2. Deoxyguanosine	2. Dissolved in digestion buffer.
3. d'GMP	3. Dissolved in digestion buffer.
4. d'GMP	4. DNase I treated
5. d'GMP	5. Snake venom phosphodiesterase treated.
6. d'GMP	6. DNase I and phosphodiesterase treated
7. d'AMP+deoxyadenosine	7. Dissolved in digestion buffer.

8. Calf thymus DNA

8. DNase I and phosphodiesterase treated.

The first four samples were spotted, 20 ug each, on one PEI-cellulose plate, and the last four, 20 ug each, on a second PEI-cellulose plate, as described. Guanine, deoxyguanosine and d'GMP had different Rf values. Thus, any d'GMP dephosphorylated by nucleotidases to deoxyguanosine could be separated from d'GMP. The d'GMP enzyme treated samples had the same Rf as d'GMP. Deoxyadenosine had a different Rf value than d'AMP. DNase I and snake venom phosphodiesterase treated d'GMP was not converted to deoxyguanosine. Calf thymus d'GMP migrated as expected, with no material with an Rf identical to guanine or to deoxyguanosine. The conclusion, then, is that d'GMP was not being dephosphorylated to other compounds.

Finally, to test whether various conditions used in the chromatographic procedure were responsible for lack of proper recovery of d'GMP, the unmodified cellulose plates were washed in a number of different ways. These included washing in 1 N HCL or in first dimension buffer. The time of elution of the nucleotides from the cellulose, as well as the normality of HCL used for elution, were also varied. None of these parameters significantly influenced d'GMP recover.

In summary, from the experimental procedures used, d'GMP was not found to be dephosphorylated into other compounds, nor to preferentially adhere to the cellulose plates. The question then, remains: What happened to 50%

of the d'GMP?

Looking at Table X", the average value GC for two runs of calf thymus DNA is 41.3%, which is within 0.7% of the published value of 42.0% (Belozersky and Spirin, 1960). For WH DNA, for which three chromatograms were run, percents for G+C varied by about 8.0 , and the percents for HMUMP varied by about 7.0. The values reported in Table X" must be taken as tentative values, since most of the DNA's cited were used for only one chromatographic run because of the problem already discussed.

BASE ANALYSIS

Due to the difficulties in the nucleotide chromatography experiments described in the previous section, a second approach was tried: that of base chromatography (Wyatt, 1955; Wyatt and Cohen, 1953). With base chromatography, there is no need for enzymes which seemed to alter results in the previous set of data. Large amounts of DNA are not needed for these analyses, either. In addition, the base data would be a double check on findings obtained from nucleotide studies.

Various characteristics were measured to establish the identities of individual bases. The bases isolated by hydrolysis of DNA and subsequently chromatographed, as well as those from commercial sources, were identified by Rf values on Whatman #1 filter paper, according to Wyatt and Cohen (1953). UV spectra were taken of the bases and compared with standards. Figures 10a-10c illustrate the

results of these measurements. The absorption profiles of the bases isolated from hydrolyzed DNA are the same as those for the standards. Again, like the 5'mononucleotides, hydroxymethyluracil (HMU) curves are slightly different due to the low optical densities. The wavelengths of maximum absorption, and the ratios at 250:260 nm and at 280:260 nm were also calculated for each base and the data compared with previously published values and with bases from commercial sources, together with Rf values. Table XI summarizes the results.

The wavelengths of maximum absorption are identical for the two sets of data. The 250:260 nm and 280:260 nm ratios are very close, while the Rf values are the same. All these parameters served to identify the bases. In Tables XII' and XII" are the results obtained when DNA's of the various strains were hydrolyzed and chromatographed for base analysis. Uncorrected values are shown only, for several reasons. Problems arose regarding the background, the background being areas not containing UV absorbing material on the chromatography paper. Such blanks were processed like those with sample. Following distilled water washing and prior to chromatography, no UV absorbing material eluted from the paper. Apparently, the paper gained UV absorbing material during the chromatographic run. Possibly, residue from the formic acid or from the running buffer condensing on the chamber walls interacted with the paper. In addition, the background varies between blank areas within one run and between different runs. For example, in two different representative runs, the following optical

densities at 230 nm were obtained:

BLANK FOR GUANINE:	0.060; 0.055
BLANK FOR ADENINE	0.031; 0.079
BLANK FOR CYTOSINE	0.025; 0.091
BLANK FOR HMU	0.031; 0.072
BLANK FOR THYMINE	0.055; 0.061

The blanks for guanine and adenine differ twofold (i.e. 0.060 vs. 0.031), and the blanks for adenine, between two different runs, differ by more than twofold. No way was found to control for such variabilities in background values.

From Table XII', it can be seen that in most cases recoveries are linear with increasing concentrations of material spotted on the paper. To determine consistency and reproducibility of the chromatographic technique used, percent yields were calculated for individual bases based on the formula described in the nucleotide chromatography section. Y DNA was used and three sets of determinations were made, representing three different runs:

BASE	PERCENT YIELDS
G	32.9, 32.8, 31.1
C	26.3, 24.4, 25.3
A	25.7, 23.4, 23.6
T	36.3, 37.0, 35.5
HMU	28.4, 31.4, 33.9

The values are consistent between different chromatographic runs. While the variations between different percent recoveries are not as great as those that were found for the

nucleotide data, there still exist some variations. This may influence the determinations of percents of G+C and of HMU.

Tables XII' and XII" include two DNA's with known base composition which served as standards to check for accuracy in results. The G+C value for calf thymus DNA is within 2.4% of published values (Belozersky and Spirin, 1960). The percent G+C for E. coli DNA is within 3.1% of the published value of 50% G+C (Belozersky and Spirin, 1960). Taking these two examples, then, it appears, everything else being equal, that data obtained from this chromatographic procedure contain at least a 3.0% error to begin with.

Without correcting for background, the data from Tables XII' and XII" are consistent. The accuracy, however, bears questioning. The amount of HMU is very low in DNA from the I, WH, To and Ro strains when compared with that found from the nucleotide analysis, about half in some cases. The percent G+C's determined by the two techniques are closer, with WH values varying the most. The WH values presented by Peter Rae (1973) match very closely with those derived from this nucleotide data. That was the primary reason for feeling that our HMU values from the base data were "abnormally" low. In addition, the high background obtained from our chromatographic runs and the finding that little HMU is present relative to the other bases all served to suggest that not all of the HMU was recovered after chromatography and elution. The yields of the bases derived from acid hydrolysis and chromatography of WH DNA were then calculated

using two different sets of data. These calculations assumed that DNA has 45.9% G+C and 6.1% HMU. These values are averages from three different chromatographies. The amount of any one base spotted on a chromatogram was determined by multiplying the amount of DNA spotted times the fraction that base represented of the total DNA. The following yields from two different chromatographies were obtained:

BASE	PERCENT YIELD
G	42.2; 50.2
C	29.5; 29.2
A	37.4; 37.7
T	36.7; 38.0
HMU	40.9; 44.0

If one determines the fractional amount of any base from the nucleotide data, the following results are obtained:

BASE	PERCENT YIELD
G	44.8
C	31.3
A	35.6
T	43.9
HMU	22.7

From the first set of calculations, HMU recovery is close to values for the other bases; from the second set, however, HMU is about 50% less when compared to guanine and thymine, and considerably less than cytosine and adenine. This 50% loss coincides with our findings of low HMU amounts recovered from the DNA's after acid hydrolysis, chromatography and elution.

One other possible source of loss of HMU, although unlikely, is based on the observation that following formic acid hydrolysis, a blackish residue appeared. Upon adding 1 N HCL, not all of the residue went into solution. Perhaps more of the HMU is insoluble compared to the other bases. There is no basis for this suggestion, however.

To ascertain whether incomplete elution of HMU from the chromatography paper was occurring, the time of elution and normality of HCL used for elution were varied. No difference in recovery of HMU was found. The paper was also examined under UV illumination and no detectable material was observed. If HMU was degraded to another UV absorbing compound, it was not visualized.

From Table XII", the percents of G+C range from 40.6 in PS to 48.8 in I; percents for HMU range from 4.8 in I to 13.6 in MC, with substitution of thymine for HMU of 18.8% for the I strain and 33.5% for the MC strain.

A method of correction was found which gave percents of HMU corresponding more closely to the findings derived from the nucleotide data. This was the curve correction calculations described in the previous section, using the same rationale and then, setting HMU equal to A=T+HMU. The average values then become:

STRAIN	%G+C	%HMU	%HMU FOR THYMINE
WH	43.2	10.8	38.0
PS	42.7	13.4	46.7
SM	48.7	12.2	47.5
Tc	44.5	13.6	48.9

STRAIN	%G+C	%HMU	%HMU FOR THYMINE
Ro	44.3	13.4	48.0
I	42.7	10.6	36.9

The data from base analyses have obvious weaknesses in certain values; the HMU percents are the most questionable. Further study is strongly warranted to ascertain more accurate values for the bases. One possible alternative to our technique is to label the DNA's with ^{32}P; this might eliminate the background problem. This, too, poses problems for not all of the strains of C. cohnii will grow in the medium (Provasoli and Gold, 1962) used for radioactive labeling. In addition, label such as ^{3}H-adenine does not enter the cells to any extent, if at all, according to some authors (Kaplan and Nishiura, unpublished findings). The problem, then, remains.

Unlike the other biochemical parameters studied, MC does not stand out as having abnormally high or low amounts of any base relative to the rest of the strains. This suggests that the heterogeneity observed in the thermal denaturation studies and in buoyant density profiles for the MC strain is due to heterogeneous base distribution.

Regarding the interbreeding groups, unlike findings in the thermal denaturation or buoyant density studies, no correlations are seen in percents of G+C, of HMU or of substitutions of thymine for HMU in terms of similarities between members of particular breeding groups. This again suggests that the similarities (and differences) found for members of the same breeding group are due primarily to the base

pair distribution within the DNA.

DISCUSSION

Using the techniques of thermal denaturation (Mandel and Marmur, 1968) and CsCl buoyant density gradient centrifugation (Schildkraut et al., 1962), comparisons were made between DNA's from nine different strains of Crypthecodinium cohnii. Integral and first derivative profiles were found to be very similar for DNA's of members of Crypthecodinium cohnii within the breeding groups and significantly different for DNA's of strains of different breeding groups of Crypthecodinium cohnii. MC DNA showed the most distinctive profile, and had a T_m almost 9.0 °C higher than that found for the strain with the lowest T_m (PS). Excluding MC DNA, the highest T_m was only 3.0 °C higher than the lowest T_m.

Using E. coli DNA as a standard, the asymmetric distribution of base pairs in C. cohnii DNA was demonstrated. DNA's from all strains showed two or three melting components, indicating a nonrandom distribution of GC and/or HMU bases, while E. coli DNA showed one melting component, indicating a random distribution of GC base pairs.

CsCl buoyant density gradient analyses revealed more outstanding differences among the DNA's from the various dinoflagellate strains. Heavy shoulders were found for DNA's of Y, SM, To, WH and PS, and light shoulders for DNA's from I, G and Ro. The heavy or light shoulders indicate different asymmetries in base distribution. The DNA's of the compatible strains, WH and PS, shared identical buoyant density profiles. DNA's from the two other genetically

compatible strains, Y and SM, also shared similar profiles that differed from those of PS and WH. However, incompatible strains also shared similarities in buoyant density profiles, for example I resembles Ro.

MC DNA again stood out from the other strains. It showed a very heterogeneous profile, with no distinct shoulders. However, its peak buoyant density did not differ from that of the other strains as much as its T_m. It had a buoyant density of 1.7266 g/cm^3, while the DNA of another strain, G, had a buoyant density of 1.7261 g/cm^3.

To ensure that the eight similar strains were identifiable as to breeding groups, DNA's from motility mutants of the eight strains were thermally denatured in 0.1 X SSC. MC has no stable mutants, but is morphologically distinguishable from the other C. cohnii strains. For all strains, except for SM_a and Y_a, thermal denaturation profiles were identical for mutant and wild type. T_m's and percent hyperchromic shifts were lower in the DNA's from the mutant strains, presumably due to degradation of these DNA's, as indicated by lowered percent hyperchromic shifts. One possible explanation for this degradation in mutant organisms may be correlated with the observation that it takes about twice as long for the mutants to reach log phase as their wild type counterparts. The cause for this is not known, but if cell division time is increased, there may be more

nucleases relative to wild type strains which are, in turn, activated during DNA extraction and isolation. SM_a and Y_a DNA's which exhibited the greatest differences compared with their wild type strains showed only about a 3.0 °C lowered T_m but a 20% lowered hyperchromic shift compared with the wild type DNA's; however, derivative plots were identical for both DNA types, given the differences cited above. Buoyant density profiles for the mutant and wild type DNA's were identical, except for those of WH and WH_a, the former having a slightly heavy shoulder and that of the mutant showing a slightly light shoulder. The significance of this observation is uncertain. In general, then, both thermal denaturation and CsCl gradient centrifugation verify the identities of DNA's extracted from mutant and from wild type strains. The differences observed between the DNA's of the mutant and parental wild types would, however, be interesting to pursue.

Chemical determinations of the DNA's from the nine strains were undertaken, using the techniques of nucleotide and base analyses. The results showed no correlation between percent G+C or 5HMU and sexual compatibility. MC DNA showed no outstanding characteristics in these parameters such as it did in the others.

The results obtained were different from those reported by Rae (1973):

STRAIN	T_m °C	$\rho g/cm^3$	%G+C	%5HMU	%5HMU for T
WH-Rae	68.5	1.7150	41.3	11.1	38.0
WH-our data	68.0	1.7182	41.5	14.3	52.5

The T_m's, percent G+C's and percent HMU's are essentially identical. The buoyant densities do differ, as do the percent substitutions of thymine for HMU. Since we used peak buoyant densities to obtain values, we thought that this could be the source of difference for the buoyant density differences. We therefore took average buoyant density measurements, but found the same values as those for the peak buoyant densities. In addition, about a 3% higher HMU was obtained which is reflected in a 14.5% difference for percent substitution of thymine for HMU. Such a difference is not unexpected given the sensitivity of the methods used here.

The media used or the conditions of growth might conceivably also affect the base composition of the DNA. Rae's growth medium was different from ours, yet his results and ours are quite similar. We sent several of our other strains to Rae who performed thermal denaturation studies on their DNA's and obtained results which, in some cases, varied by several degrees from ours (personal communication). We recently received from Rae some DNA from the Icaco strain (as well as his E. coli DNA standard), a strain from which he measured a T_m several degrees higher than ours. We melted Rae's Icaco DNA along with his E. coli DNA standard and obtained results which reproduced our earlier Icaco T_m data.

Our findings lead us to the question of correlating biochemical data with the genetic and physiological differences observed by Beam and Himes (1977) among the inter-

breeding groups. According to Nanney and McCoy (1976), "breeding tests are the foundations of taxonomic procedures in Paramecium: biochemical, geographical, or cytometric differences are acceptable criteria if correlated with breeding tests."

By the criteria of thermal denaturation and buoyant density analysis, DNA's from strains in the same breeding group are similar. However, the data also show that by these criteria, DNA's from strains in different breeding groups may also be similar. It is clear that sexual isolation may occur in the Caribbean species Ro and I without gross changes in DNA. Gold, also from the Caribbean, shows definite similarity with I and Ro as far as buoyant density profile, but differs in thermal denaturation parameters from the other two Caribbean strains.

The range of values observed in this study is the same order of magnitude that one sees between different families of dinoflagellates and between different algal groups. This is illustrated by the data of Rae (1976):

ORGANISM	T_m °C (%G+C)	$\rho g/cm^3$ (%G+C)
C. cohnii (WH)	68.5 (35.6)	1.7150 (56.1)
Amphidinium carterae	67.2 (32.5)	1.7230 (64.3)
Chlamydomonas reinhardii	80.2 (64.2)	1.7230 (64.3)

From our data:

STRAIN	T_m °C (%G+C)	$\rho g/cm^3$ (%G+C)
PS	66.5 (30.7)	1.7171 (58.3)
MC	75.25(52.1)	1.7266 (68.0)

The MC strain does not mate with any of the other dinoflagellate strains. Its morphological and physiological characteristics are also very distinctive. The thermal denaturation and buoyant density profiles show that the DNA from this strain is the most distinct of the C. cohnii group. It would, perhaps, be safe to put it into at least a different species group, as was pointed out earlier by Beam and Himes (1977).

The following table further emphasizes the diversity within C. cohnii, in terms of biochemical characteristics measured, when compared with other organisms:

ORGANISM	RANGE IN %G+C	RANGE IN T_m °C	RANGE IN ρ_g/cm^3
Tetrahymena pyriformis	9		
Paramecium aurelia	1		
Vertebrates	4		
Bacteria	50		
Gymnosperms			0.001
D. melanogaster vs. D. simulans		0.5	0
D. melanogaster vs. D. funebris		1.5	0.004
C. cohnii	8.2	3.0	0.009
MC	8.2	8.4	0.009

The different syngens (sibling species) of Tetrahymena pyriformis have G+C's differing by 9% (Allen and Li, 1974).

Paramecium aurelia varieties have G+C's within 1% of each other (Allen and Gibson, 1972). Bacterial DNA's range from 25 to 75%, and vertebrates differ by 4% G+C (Sueoka, 1961). D. melanogaster and D. simulans, sibling species, have nuclear mainband DNA's with the same buoyant density, and T_m's within 0.5 °C of each other. D. funebris, a more distantly related species, has nuclear mainband DNA differing by 0.004 g/cm^3 and a T_m about 1.5 °C lower than the former two species (Laird and McCarthy, 1968). Regarding the gymnosperms, species differ in buoyant densities by 0.001 g/cm^3 (Miksche and Hotta, 1973).

The ranges in the biochemical characteristics of the DNA's from the various strains of C. cohnii observed in this study are even greater than other related groups except for T. pyriformis. If one includes MC in this group, the differences are as much as 8.4 °C. Excluding MC, the T_m's differ by 3.0 °C. Including MC, buoyant densities differ by 0.009 g/cm^3; excluding MC, they differ by 0.009 g/cm^3. Using uncorrected base data or corrected base data, MC DNA values for G+C and HMU percents do not lie within the extremes in terms of highest or lowest percents G+C or HMU. Thus, for uncorrected base data, percent G+C's differ by up to 8.2, percents for HMU, 8.8 , and percents of substitution of thymine for HMU, 17.0; using corrected base data, percent G+C's differ by up to 6.1, percent for HMU, 3.0, and percent substitutions of thymine for HMU, 16.3. These ranges are at least as great, and more often, greater, than differences reported for the above groups of organisms

which include different genera and orders. All of these findings serve to emphasize the great heterogeneity present in organisms that have been placed within the same taxonomic species, Crypthecodinium cohnii.

As to the association of HMU with dinoflagellate DNA, a number of possible roles for modified bases in general have been suggested. Briefly (for further discussion, see appendix), Tosi et al. (1972) suggest base methylation may trigger heritable changes in the DNA with consequent developmental changes in an organism. Other workers (Sager and Kitchin, 1975; Riggs, 1975) suggest a role of DNA methylation in turning off particular genes or chromosomes (for example, heterochromatization of sets of chromosomes and of one of the two X chromosomes in the female). Rae and Steele suggest that the presence of 5HMU in dinoflagellates is a vestigial remnant from a primitive restriction system.

The nonrandom distribution of G+C and of HMU differs in the C. cohnii strains that are not compatible, i.e. different sibling species. Possibly, this evolutionary divergence occurred following sexual isolation. In terms of the nonrandom distribution of bases found in compatible strains, geographical isolation may have occurred first which then allowed for divergence in DNA. Since the WH and PS, and Y and SM do not mate in nature but do mate in the laboratory, one can rule out DNA differences of the order of magnitude found in this work as being responsible for sexual isolation.

Another question is what prevents mating between incompatible strains? As yet, we do not know at what step in the mating process successful mating is prevented. It could occur at the time of cell fusion, or cell attraction may not occur; the cells might fuse but the subsequent meiotic steps could be interfered with. Examples of all these and other types of sexual failure might occur within C. cohnii.

Studies of renaturation kinetics (Allen and Gibson, 1974) and isozyme patterns (Allen and Weremiuk, 1971) like the ones done for T. pyriformis might be helpful in answering the question of relevant differences between incompatible strains, and help decide what is a species as well as what are the causes of sexual incompatibility.

TABLE I-List of the Nine *C. cohnii* Strains Used

STRAINS	(ABBREV.)	COMPLEMENTATION	ORIGIN	ALGAL HOST
Woods Hole	(WH)	complement	Woods Hole, Mass.	Fucus
Puget Sound	(PS)	complement	Puget Sound, Wash.	Fucus
Yelieu	(Y)	complement	Yelieu, Taiwan	Sargassum
Santa Monica	(SM)	complement	Santa Monica, Calif.	Macrocystis
Tolo	(To)	no complementation with any other isolate	Tolo Harbor, Hong Kong	Mixture
Roatan	(Ro)	no complementation with any other isolate	Roatan Island, Honduras	Dictyota
Icaco	(I)	no complementation with any other isolate	Icaco Island, P.R.	Mixture
Gold	(G)	no complementation with any other isolate	La Paguera, P.R.	Unknown
McLaughlin	(MC)	no complementation with any other isolate	Phosphorescent Bay, P.R.	Unknown

TABLE II-A

T_m °C's and Percent Hyperchromic Shifts for SM DNA
Isolated from Osmotically and from Eaton Press Lysed Cells.

STRAINS	T_m °C	% HYPERCHROMIC SHIFT
$SM_{osmotic\ shock}$	68.50	34.2
$SM_{Eaton\ press}$	68.00	29.7

TABLE II-B

T_m °C's and Percent Hyperchromic Shifts for PS DNA
Isolated from Aqueous Layer and from Interface Phase.

STRAINS	T_m °C	% HYPERCHROMIC SHIFT
$PS_{aqueous\ phase}$	66.50	33.5
$PS_{interface}$	67.00	33.0

TABLE III

Summary of T_m °C's and Calculated Percent G+C's for the Nine Strains of *C. cohnii*

STRAINS	AVERAGE T_m °C (# times done)	%G+C
	*	*
WH	68.00(3)±0.20	34.4±0.49
PS	66.50(3)±0.41	30.7±1.00
Y	69.50(3)±0.35	38.1±0.86
SM	68.70(3)±0.31	36.1±).79
To	69.50(3)±0.35	38.1±0.86
Ro	69.40(2)±0.13	37.8±0.31
I	69.00(3)±0.18	36.8±0.79
G	67.40(3)±0.68	32.9±0.76
MC	74.90(3)±0.31	51.2±0.76

* = Standard Deviation

TABLE IV

Summary of Melting Components, Percent G+C's and Percents of Each Component from Thermal Denaturation Profiles of DNA's for the Nine Strains of C. cohnii.

STRAINS	COMPONENT #	T_m °C	%G+C	% OF EACH COMPONENT
WH	1	64.50	25.9	25.0
	2	74.00	49.0	75.0
PS	1	62.00	19.8	42.0
	2	71.25	42.3	58.0
Y	1	63.25	22.8	38.0
	2	72.50	45.4	62.0
SM	1	64.00	24.6	32.0
	2	72.75	46.0	68.0
To	1	65.50	28.3	48.0
	2	73.25	47.2	52.0
Ro	1	60.00	14.9	20.0
	2	70.25	39.9	80.0
I	1	63.25	22.8	33.0
	2	71.75	43.6	67.0
G	1	62.50	21.0	35.0
	2	70.00	39.3	46.5
	3	74.50	50.3	18.5
MC	1	67.25	32.6	26.0
	2	73.50	47.8	36.0
	3	77.50	57.6	38.0

TABLE V

T_m °C's and Percent Hyperchromic Shifts of DNA's from Wild Type and Respective Mutant Strains of C. cohnii.

STRAINS	T_m °C	PERCENT HYPERCHROMIC SHIFT
WH	68.00	34.0
WH_a*	67.50	35.1
PS	66.00	33.7
PS_p	64.75	32.0
Y	69.25	34.3
Y_a	66.75	13.9
SM	69.00	37.0
SM_a	66.00	19.7
To	69.75	36.5
To_b	69.75	34.8
Ro	69.50	31.0
Ro_b	67.25	26.1
I	68.80	33.5
I_a	69.25	33.3
G	67.75	36.5
G_c	67.00	28.0

* Small letters indicate mutant strains

TABLE VI-A

Buoyant Densities and Calculated Percent G+C's of SM DNA Isolated From Osmotically and From Eaton Press Lysed Cells

STRAINS	AVERAGE (PEAK VALUES) ρ g/cm^3	%GC
$SM_{osmotic\ shock}$	1.7184	59.6
$SM_{Eaton\ press}$	1.7199	61.1

TABLE VI-B

Buoyant Densities and Calculated Percent G+C's of PS DNA from Aqueous Layer and From Interface Phase

STRAINS	AVERAGE (PEAK VALUES) ρ g/cm^3	%GC
$PS_{aqueous}$	1.7171	58.3
$PS_{interface}$	1.7172	58.4

TABLE VII

Buoyant Densities and Calculated Percent G+C's for DNA's of Nine Strains of C. cohnii

STRAINS	AVERAGE* ρ + (#samples)±	AVERAGE %GC$^{\Delta}$	HEAVY SHLD %GC	LIGHT SHLD %GC	SPAN %GC
WH	1.7182 (3)	59.4±0.14$^{\square}$	1.7233 64.6		
PS	1.7171 (7)	58.3±0.31	1.7255 66.8		
Y	1.7173 (3)	58.5±0.45	1.7247 66.0		
SM	1.7184 (2)	59.6±0.071	1.7266 68.0		
To	1.7188 (2)	60.0±0.40	1.7243 65.6		
Ro	1.7230 (2)	64.3±0		1.7159 57.0	
I	1.7237 (2)	65.0±0.058		1.7147 55.8	
G	1.7261 (3)	67.4±0.43		1.7164 57.6	
MC	1.7266 (2)	68.0±0.35			1.7184=59.6 1.7333=74.8

* Represent peak values

\+ In g/cm^3

± Standard deviations for all buoyant densities were 0.

Δ Calculated from buoyant densities

$\square$ Standard deviations

TABLE VIII

Buoyant Densities and Calculated Percent G+C's of DNA's From Wild Type and Respective Mutant Strains of *C. cohnii*

STRAIN	AVERAGE (PEAK VALUES) @ g/cm3	%G+C
WH	1.7182	59.4
WH_a*	1.7198	61.0
PS	1.7171	58.3
PS_p	1.7174	58.6
Y	1.7173	58.5
Y_a	1.7188	60.0
SM	1.7184	59.6
SM_a	1.7183	59.5
To	1.7188	60.0
To_b	1.7192	60.4
Ro	1.7230	64.3
Ro_b	1.7260	67.3
I	1.7237	65.0
I_a	1.7230	64.3
G	1.7261	67.4
G_c	1.7278	69.2

* Small Letter Designates Mutant Strain

TABLE IX

Parameters Measured to Characterize 5' Mononucleotides

5'MONONUCLEOTIDE	λ max*	λ max+	250:260 nm*'	250:260 nm+'	280:260 nm*"	280:260 nm+"
d'GMP	255	245-250(4)	-	-	-	-
d'AMP	258	255-260(4)	-	-	-	-
d'CMP	280	275-280(4)	0.46	0.71(5)	2.12	1.55(5)
HMUMP	264	260-265(2)	0.70	0.88(6)	0.57	0.72(6)
d'TMP	267	260-270(4)	0.64	0.86(6)	0.74	0.78(6)

* = published values - Kallen et al., 1962; Takahashi and Marmur, 1963; Beaven et al., 1955; Wyatt, 1955.

+ = present data

Numbers in parentheses indicate # determinations made

*' = published values

+' = present data

*" = published values

+" = present data

TABLE X'

Summary of Nucleotide Chromatography; Optical Densities at λ max of Nucleotides of DNA's for Strains of C. cohnii

STRAINS	UG SPOTTED	d'GMP	d'CMP	d'AMP	d'TMP	HMUMP	
WH	15	0.144	0.274	0.460	0.145	0.213	Uncorrected*
		0.110	0.266	0.342	0.134	0.142	Corrected+
	15	0.343	0.467	0.507	0.284	0.262	Uncorrected
		0.155	0.269	0.339	0.076	0.115	Corrected
	15	0.331	0.364	0.582	0.220	0.198	Uncorrected
		0.205	0.328	0.440	0.120	0.101	Corrected
PS	15	0.426	0.278	0.615	0.273	0.232	Uncorrected
		0.205	0.179	0.384	0.087	0.126	Corrected
To	18	0.209	0.176	0.208	0.166	0.210	Uncorrected
		0.075	0.064	0.114	0.048	0.037	Corrected
Ro	11	0.611	0.293	0.674	0.180	0.190	Uncorrected
		0.193	0.196	0.282	0.107	0.094	Corrected
G	20	0.260	0.298	0.373	0.149	0.222	Uncorrected
		0.115	0.154	0.202	0.139	0.065	Corrected
CT	23	0.187	0.211	0.222	0.187	0.000	Uncorrected
		0.062	0.121	0.153	0.102	0.000	Corrected
	23	0.188	0.219	0.222	0.195	0.000	Uncorrected
		0.047	0.110	0.112	0.093	0.000	Corrected

*, + - Discussed in Results

TABLE X"

Summary of Nucleotide Chromatography; Percents of Nucleotides of DNA's for Strains of *C. cohnii*

STRAINS	%d'GMP	%d'CMP	%d'AMP	%d'TMP	%HMUMP	%GC	%HMU for T*	
WH	12.2	20.3	32.3	14.2	20.9	32.5	59.5	Uncorrected+
	11.6	24.7	29.9	16.4	17.4	36.3	51.5	Corrected±
	19.0	22.7	23.3	18.2	16.8	41.7	48.0	Uncorrected
	17.4	26.4	31.4	9.9	15.0	43.8	60.2	Corrected
	20.5	19.7	29.8	15.8	14.2	40.2	47.3	Uncorrected
	18.5	25.8	32.7	12.5	10.5	44.3	45.7	Corrected
					14.3	41.5	52.5	Average(corrected)
PS	24.2	13.8	28.8	18.0	15.2	38.0	45.8	Uncorrected
	22.0	16.8	34.1	11.4	15.7	38.8	57.8	Corrected
TO	21.7	15.8	17.6	19.8	25.1	37.5	55.9	Uncorrected
	23.4	17.2	29.2	17.2	13.1	40.6	43.2	Corrected
Ro	331.	14.9	29.9	11.2	11.8	49.0	51.3	Uncorrected
	23.5	23.7	27.9	14.9	13.0	47.2	46.6	Corrected
G	20.6	20.7	24.5	13.7	20.5	41.3	59.9	Uncorrected
	17.6	20.7	25.5	24.6	11.6	38.3	32.0	Corrected
CT	24.2	23.9	23.8	28.1	00.0	48.1	00.0	Uncorrected
	15.1	25.7	30.6	28.6	00.0	40.8	00.0	Corrected
	23.8	24.4	23.2	28.6	00.0	48.2	00.0	Uncorrected

TABLE X" cont.

STRAINS	%d'GMP	%d'CMP	%d'AMP	%d'TMP	%HMUMP	%GC	%HMU for T*	
CT	13.7	28.0	26.9	31.4	00.0	41.7	00.0	Corrected
						41.3	00.0	Average(corrected)

*-Calculated (Rae, 1973) from equation, $\frac{5HMU}{5HMU+T} + 1 \times 100$

+, $\pm$ -Discussed in Results

TABLE XI

Parameters Measured to Characterize Bases

BASE	λ max*	λ max+	250:260 nm*'	250:260 nm (aver.)+'	280:260 nm*"	280:260 nm (aver.)+"
Guanine	250	250(3)	1.37	1.20(4)	0.82	0.70(4)
Adenine	260	260-265(3)	0.78	0.77(4)	0.37	0.39(4)
Cytosine	275	275(3)	0.48	0.51(4)	1.50;1.53	1.20(4)
HMU	261	260(3)	0.75	0.53(2)	0.37	0.58(2)
Thymine	265	260-265(3)	0.67;0.69	0.64(4)	0.55;0.53	0.61(4)

Cont.

BASE	Rf Values (aver.)*"'	Rf Values (aver.)+"'
Guanine	0.24	0.24(6)
Adenine	0.34	0.33(6)
Cytosine	0.47	0.46(6)
HMU	0.62	0.61(6)
Thymine	0.78	0.74(6)

* Published values - See Table IX
\+ Present data
Parentheses indicate # determinations made

*' Published values
+' Present data
*" Published values

+" Present data
*"' Published values
+"' Present data

TABLE XII'

Summary of Base Chromatography; Optical Densities at λmax of Bases of DNA's for Strains of C. cohnii

STRAINS	EXPER.#	UG SPOTTED	G	C	A	T	HMU
WH	1	313	0.551	0.500	0.761	0.381	0.108
	2	226	0.474	0.358	0.555	0.285	0.084
	3	226	0.455	0.352	0.562	0.285	0.085
	4	226	0.464	0.369	0.582	0.279	0.067
PS	1	141	0.311	0.287	0.443	0.261	0.124
	2	168	0.314	0.264	0.438	0.247	0.104
Y	1	113	0.144	0.150	0.200	0.114	0.050
	2	151	0.192	0.180	0.244	0.155	0.074
	3	187	0.225	0.238	0.305	0.184	0.099
SM	1	141	0.380	0.324	0.521	0.262	0.101
	2	109	0.288	0.277	0.398	0.210	0.111
To	1	70	0.595	0.460	0.622	0.333	0.113
	2	199	0.335	0.265	0.369	0.203	0.062
	3	199	0.320	0.243	0.382	0.184	0.060
Ro	1	214	0.547	1.043	0.794	0.586	0.185
I	1	182	0.863	0.760	1.079	0.482	0.125
	2	238	1.153	0.967	1.276	0.584	0.133
	3	570	0.994	1.400	1.531	0.905	0.201

TABLE XII' cont.

STRAINS	EXPER.#	UG SPOTTED	G	C	A	T	HMU
G	1	212	0.330	0.315	0.399	0.216	0.134
	2	212	0.365	0.323	0.404	0.222	0.114
	3	212	0.379	0.339	0.406	0.231	0.126
MC	1	61	0.200	0.156	0.179	0.174	0.089
	2	198	0.103	0.111	0.067	0.089	0.044
CT*	1	58	0.093	0.119	0.146	0.152	0.000
E. coli	1	48	0.100	0.101	0.109	0.102	0.000

*CT = Calf Thymus

TABLE XII"

Summary of Base Chromatography; Percents of Bases of DNA's for Strains of *C. cohnii*

STRAINS	EXPER.#	%GC	%C	%A	%T	%HMU	%GC	%HMU for T*
WH	1	23.0	21.9	26.9	22.0	6.4	44.9	22.4
	2	25.9	20.5	25.7	21.5	6.6	46.4	23.5
	3	24.8	20.6	26.1	21.8	6.7	45.4	23.4
	4	25.5	21.2	27.3	21.1	4.8	46.7	18.5
	AVERAGE	24.8	21.1	26.5	21.6	6.1	45.9	22.1
PS	1	20.5	19.8	24.7	23.8	11.2	40.3	32.0
	2	21.7	19.1	25.6	23.7	9.9	40.8	29.5
	AVERAGE	21.1	19.5	25.2	23.8	10.6	40.6	30.8
Y	1	20.7	22.6	24.3	22.6	9.9	43.3	30.5
	2	21.3	20.8	22.9	23.7	11.3	42.1	32.3
	3	20.1	22.2	23.0	22.6	12.1	42.3	24.9
	AVERAGE	20.7	21.9	23.4	23.0	11.1	42.6	32.6
SM	1	22.8	20.5	26.5	21.8	8.3	43.3	27.6
	2	21.2	21.4	24.8	21.4	11.3	42.6	34.6
	AVERAGE	22.0	21.0	25.7	21.6	9.8	43.0	31.1
To	1	26.8	21.7	23.7	20.8	7.0	48.5	25.2
	2	26.0	21.5	24.2	21.7	6.6	47.5	23.3
	3	25.9	20.6	26.2	20.6	6.7	46.5	24.5
	AVERAGE	26.2	21.3	24.7	21.0	6.8	47.5	24.3
Ro	1	16.2	32.4	19.9	24.0	7.5	48.6	23.8

*Calculated (Rae, 1973) from Equation $\frac{5HMU}{5HMU+T} + 1 \times 100$

TABLE XII" cont.

STRAINS	EXPER.#	%GC	%C	%A	%T	%HMU	%GC	%HMU for T*
I	1	25.3	23.3	26.8	19.7	5.2	48.6	20.9
	2	27.3	24.0	25.6	19.1	4.1	51.3	17.7
	3	18.8	27.8	24.5	23.7	5.2	46.6	18.0
	AVERAGE	23.8	25.0	25.6	20.8	4.8	48.8	18.8
G	1	22.3	22.3	22.8	20.2	12.5	44.6	38.2
	2	24.2	22.4	22.7	20.3	10.4	46.6	33.9
	3	24.1	22.6	21.8	20.4	11.1	46.7	35.2
	AVERAGE	23.5	22.4	22.4	20.3	11.3	46.0	35.8
MC	1	22.8	18.6	17.3	27.4	13.9	41.4	33.7
	2	22.5	25.4	12.4	26.6	13.2	47.9	33.2
	AVERAGE	22.7	22.0	14.9	27.0	13.6	44.7	33.4
CT+	1	17.0	22.6	22.4	38.1	00.0	39.6	00.0
E. coli	1	22.8	24.1	21.1	32.1	00.0	46.9	00.0

+CT=Calf thymus

Figure 1. Integral and Derivative Profiles of Triplicate Thermal Denaturation Experiments for Two DNA's, WH and SM. For details, see text.

a) WH DNA with a T_m of 67.75 °C and a 33.2% hyperchromic shift(△—△); WH DNA with a T_m of 68.25 °C and a 35.3% hyperchromic shift(O—O); WH DNA with a T_m of 68.00 °C and a 34.0% hyperchromic shift(X—X).

b) Derivative profiles; see a) for symbol representations.

c) SM DNA with a T_m of 69.00 °C and a 37.0% hyperchromic shift(△—△); SM DNA with a T_m of 68.25 °C and a 35.5% hyperchromic shift(O—O); SM DNA with a T_m of 68.75 °C and a 37.6% hyperchromic shift(X—X).

d) Derivative profiles; see c) for symbol representations.

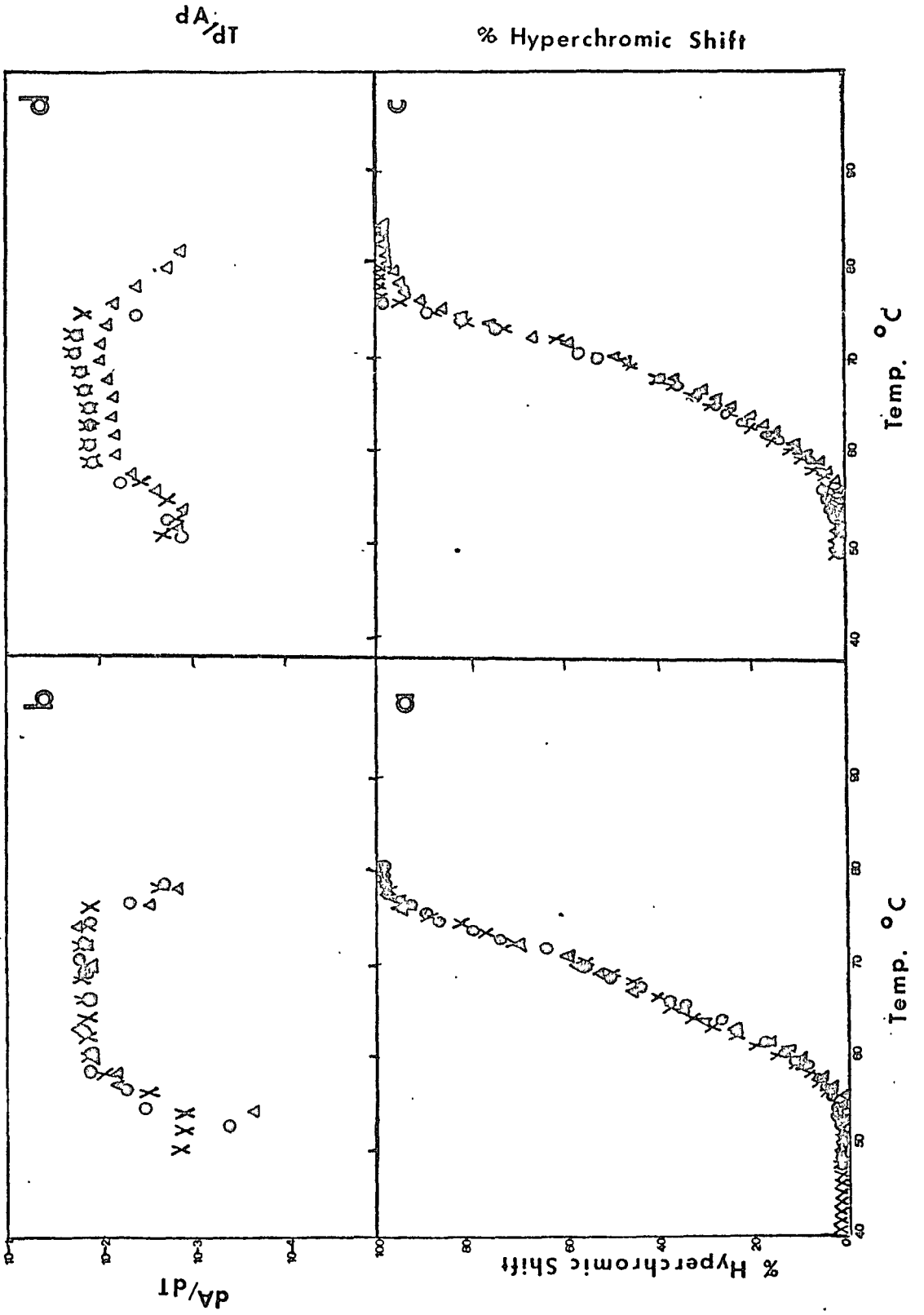

dA/dT
% Hyperchromic Shift
d
c
b
a
% Hyperchromic Shift
Temp. °C
Temp. °C
dA/dT

Figure 2. DNA's Extracted from Two Strains, G and SM, and Thermally Denatured in 0.1 X and in 1.0 X SSC. See text for details. E. coli DNA has been included in 2a and in 2b as a standard. T_m values in both solvents are corrected to E. coli DNA. The T_m values for DNA's thermally denatured in 1.0 X SSC have been corrected to values in 0.1 X SSC.

a) Integral profiles of G DNA thermally denatured in 0.1 X SSC (○—○) and in 1.0 X SSC(△—△); E. coli DNA (×—×).

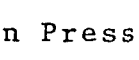
b) Derivative profiles of G DNA; symbols as in a).

c) Integral profiles of SM DNA thermally denatured in 0.1 X SSC(○—○)and in 1.0 X SSC (△—△).

d) Derivative profiles of SM DNA; symbols as in c).

dA/dT
% Hyperchromic Shift
d
c
b
a
Temp. °C
Temp. °C
% Hyperchromic Shift
dA/dT

Figure 3. Thermal Denaturation Profiles of SM DNA Extracted from Cells Lysed by Osmotic Shock or with the Eaton Press. 0.1 X SSC was the solvent used for thermal denaturation, as described in Materials and Methods.

a) Integral profiles of thermally denatures SM DNA's. DNA from osmotically shocked cells (O–O); DNA from Eaton Press lysed cells ().

b) Derivative profiles of thermally denatured SM DNA's. Symbols as in a).

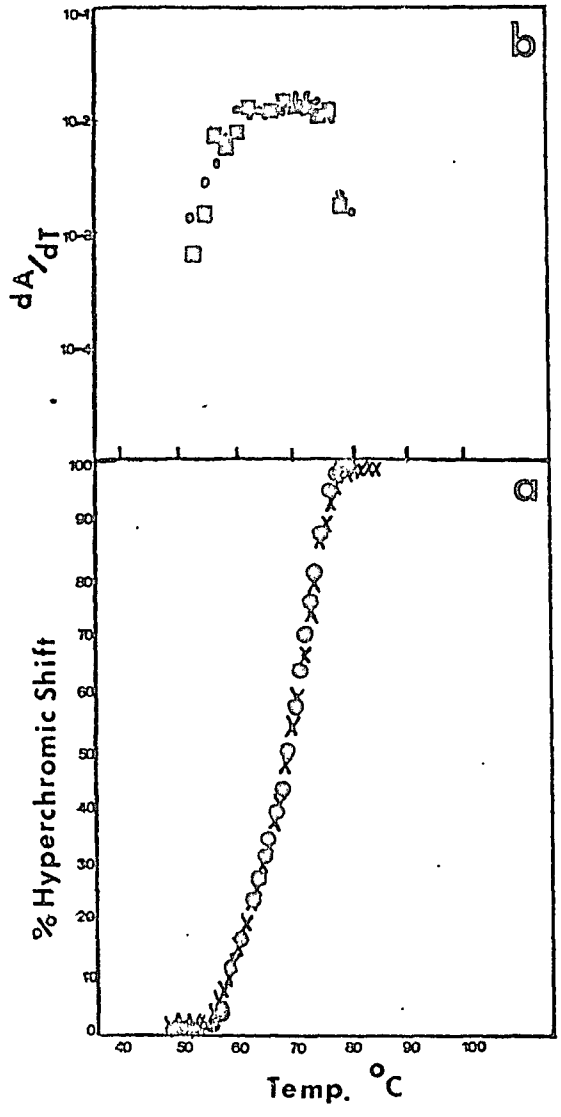

b
a
dA/dT
% Hyperchromic Shift
Temp. °C

Figure 4. Thermal Denaturation Profiles of PS DNA in 0.1 X SSC Isolated from the Aqueous Layer and from the Interface. Details in Materials and Methods.

a) Integral profiles of thermally denatured PS DNA's. DNA from aqueous layer (O–O); DNA from interface (Δ–Δ).

b) Derivative profiles of thermally denatured PS DNA's. Symbols as in a).

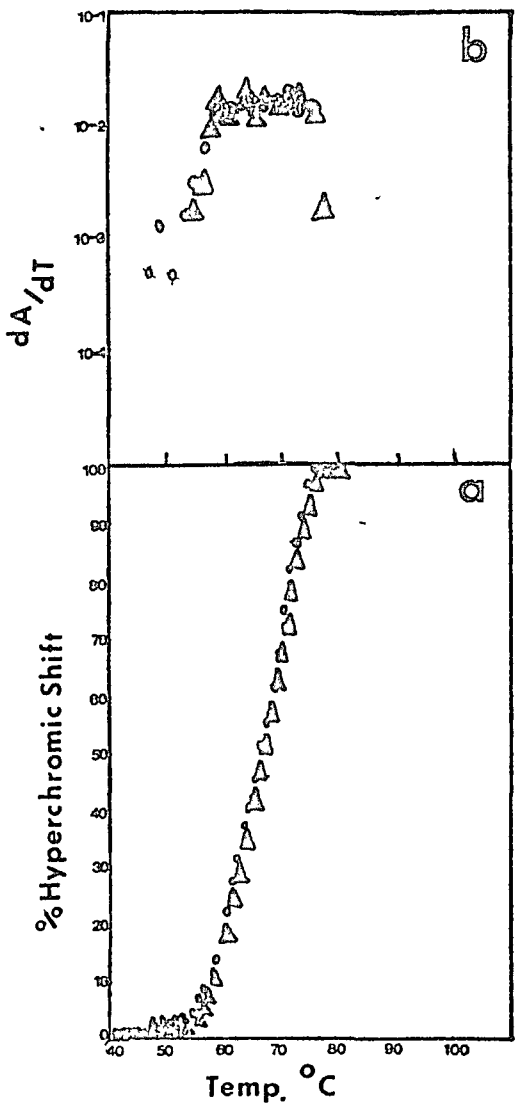

b
a
dA/dT
% Hyperchromic Shift
Temp. °C
40
50
60
70
80
90
100

Figure 5. Integral Profiles of Thermally Denatured DNA's in 0.1 X SSC from Each of Nine Strains of C. cohnii. DNA (from aqueous layer) was extracted from osmotically lysed cells of all strains, except for MC, which was lysed with the Eaton press, dialyzed 24-48 hours against 0.1 X SSC and thermally denatured. WH DNA has been included with each profile for comparison, while E. coli DNA is included with Y DNA as a standard. All T_m's are corrected to that of E. coli DNA, as discussed in Results. The WH DNA used here has a T_m of 68.00 °C, with a hyperchromic shift of 34.0%; E. coli has a 32.0% hyperchromic shift.

a) Y DNA(O–O), T_m of 70.00 °C, 34.0% hyperchromic shift; WH DNA(✕–✕); E. coli DNA(Δ–Δ).

b) SM DNA, T_m of 69.00 °C, 37.0% hyperchromic shift

c) To DNA, Tm of 69.75 °C, 36.5% hyperchromic shift.

d) PS DNA, T_m of 66.50 °C, 33.5% hyperchromic shift.

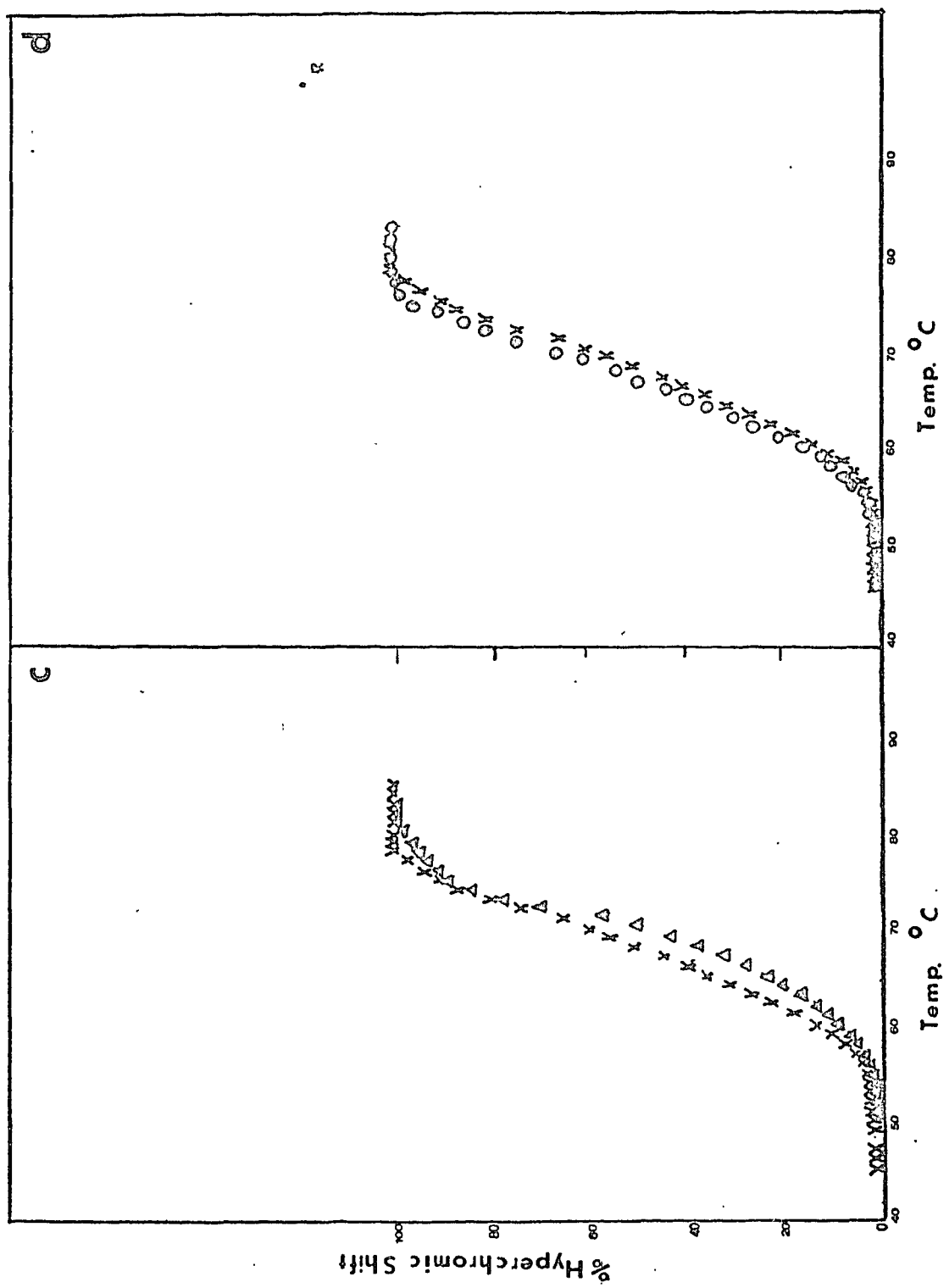
e) G DNA, T_m of 67.75 °C, 36.5% hyperchromic shift.

f) I DNA, T_m of 68.80 °C, 33.3% hyperchromic shift.

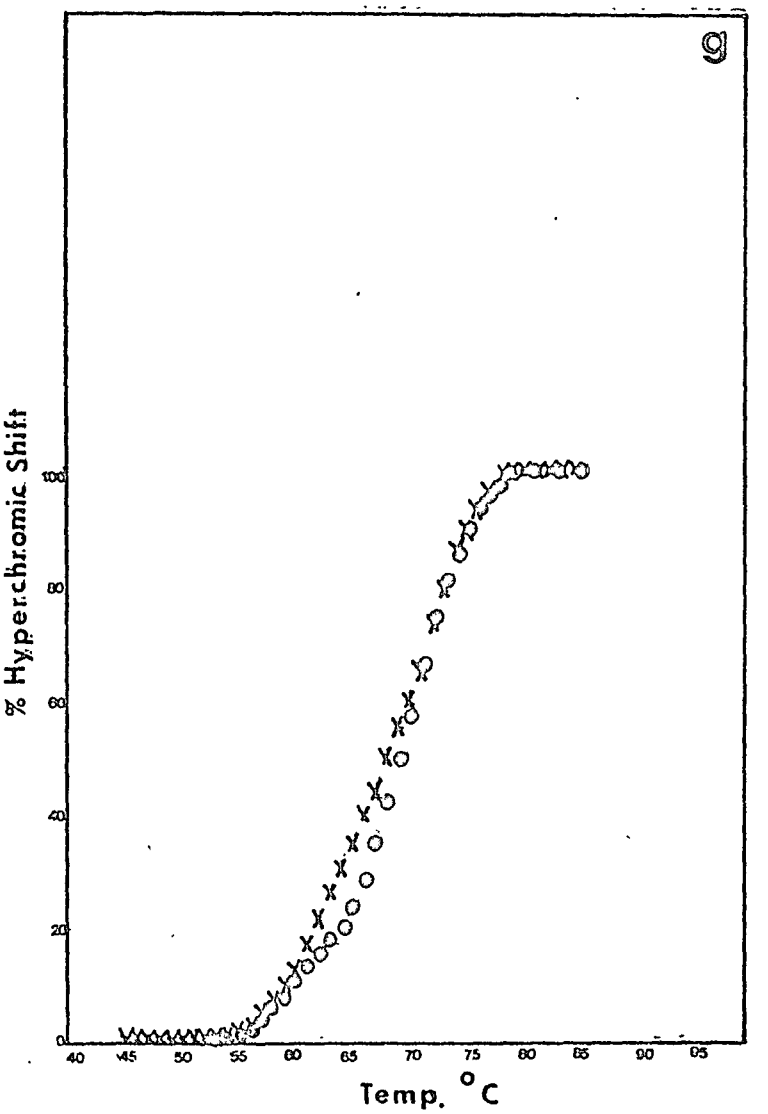
g) Ro DNA, T_m of 69.25 °C, 35.7% hyperchromic shift.

h) MC DNA, T_m of 75.25 °C, 18.8% hyperchromic shift.

% Hyperchromic Shift
a
b
Temp. °C
Temp. °C
% Hyperchromic Shift


% Hyperchromic Shift
d
c
Temp. °C
Temp. °C
% Hyperchromic Shift

% Hyperchromic Shift
Temp. °C
Temp. °C
% Hyperchromic Shift


g
% Hyperchromic Shift
0
20
40
60
80
100
40
45
50
55
60
65
70
75
80
85
90
95
Temp. °C

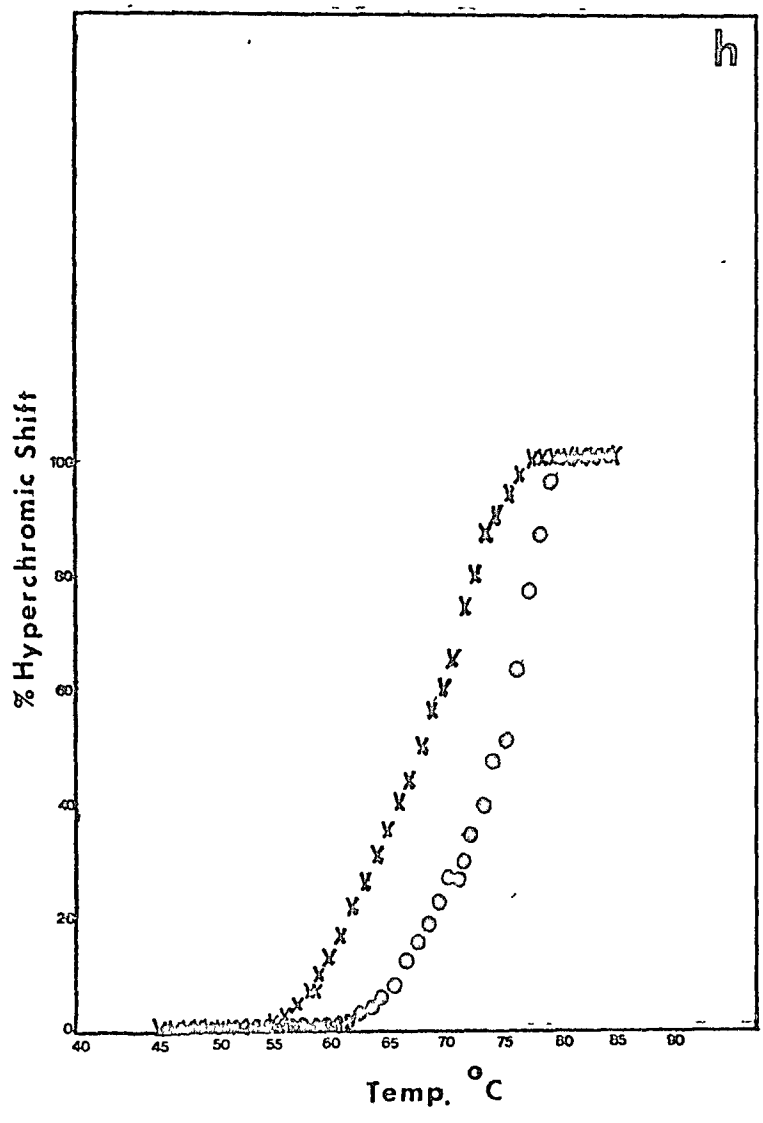

h
% Hyperchromic Shift
0
20
40
60
80
100
40
45
50
55
60
65
70
75
80
85
90
Temp. °C

Figure 6. Derivative Plots of Thermally Denatured DNA's in 0.1 X SSC from each of Nine Strains of C. cohnii. For procedure and symbols, see legend to figure 5.

a) Y DNA.

b) SM DNA.

c) To DNA

d) PS DNA.

e) G DNA.

f) I DNA.

g) Ro DNA.

h) MC DNA.

dA/dT
dA/dT
b
c
a
d
Temp. °C
Temp. °C

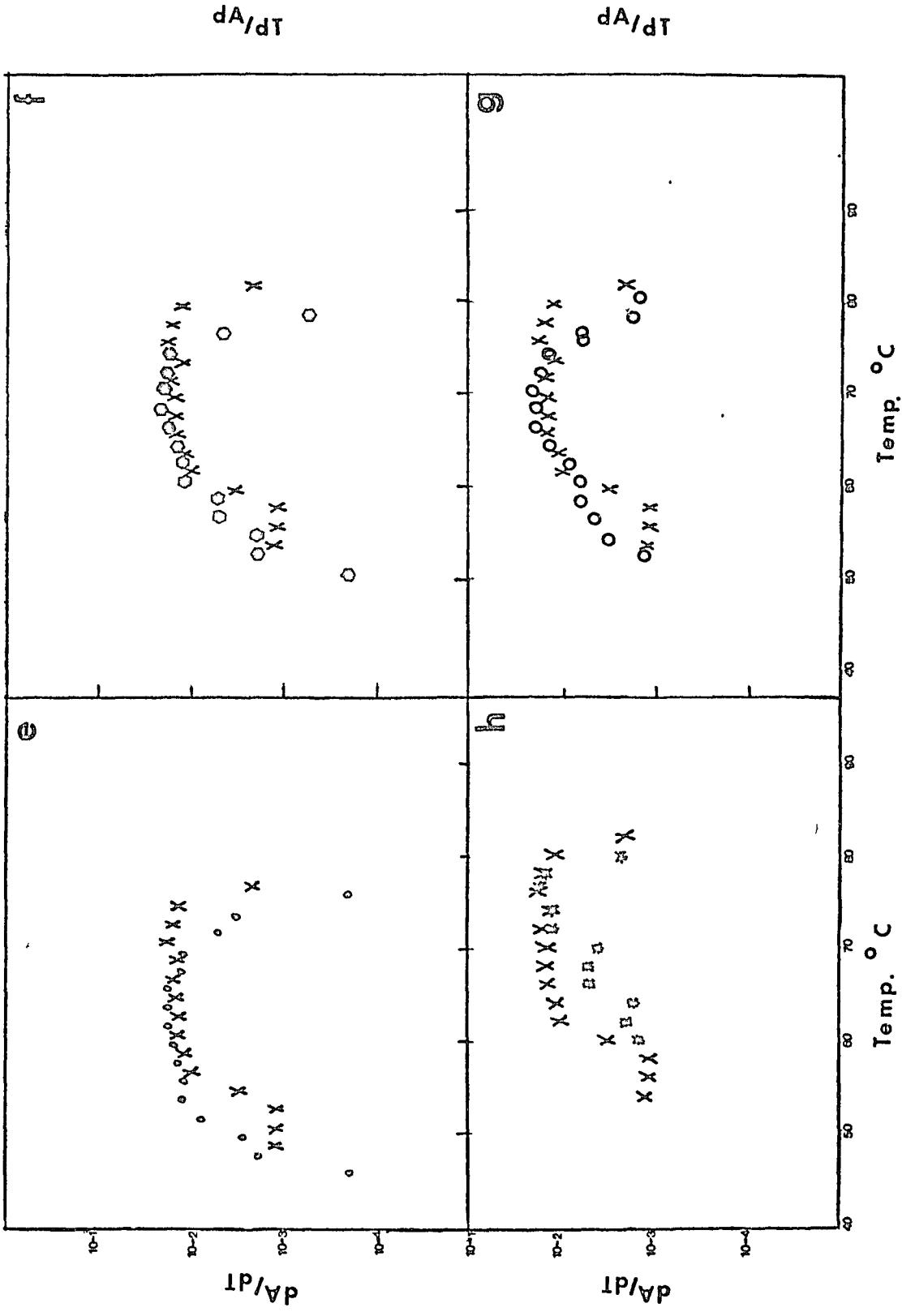

dA/dT
Temp. °C
dA/dT
Temp. °C
dA/dT
dA/dT

Figure 7. Thermal Denaturation Profiles of DNA's Isolated from Wild Type and Mutant Strains. DNA's were thermally denatured in 0.1 X SSC as described in Results.

a) Integral profiles of WH(✕—✕)and WH_a(o—o)DNA's.

a') Derivative profiles of WH and WH_a DNA's.

b) Integral profiles of I(△—△)and I_a(▽—▽)DNA's.

b') Derivative profiles of I and I_a DNA's.

c) Integral profiles of To(△—△)and To_b(o—o)DNA's.

c') Derivative profiles of To and To_b DNA's.

d) Integral profiles of PS(□—□)and PS_p(△—△)DNA's.

d') Derivative profiles of PS and PS_p DNA's.

e) Integral profiles of Ro(✕—✕)and Ro_b(o—o)DNA's.

e') Derivative profiles of Ro and Ro_b DNA's.

f) Integral profiles of G(✕—✕)and G_c(⊡—□)DNA's.

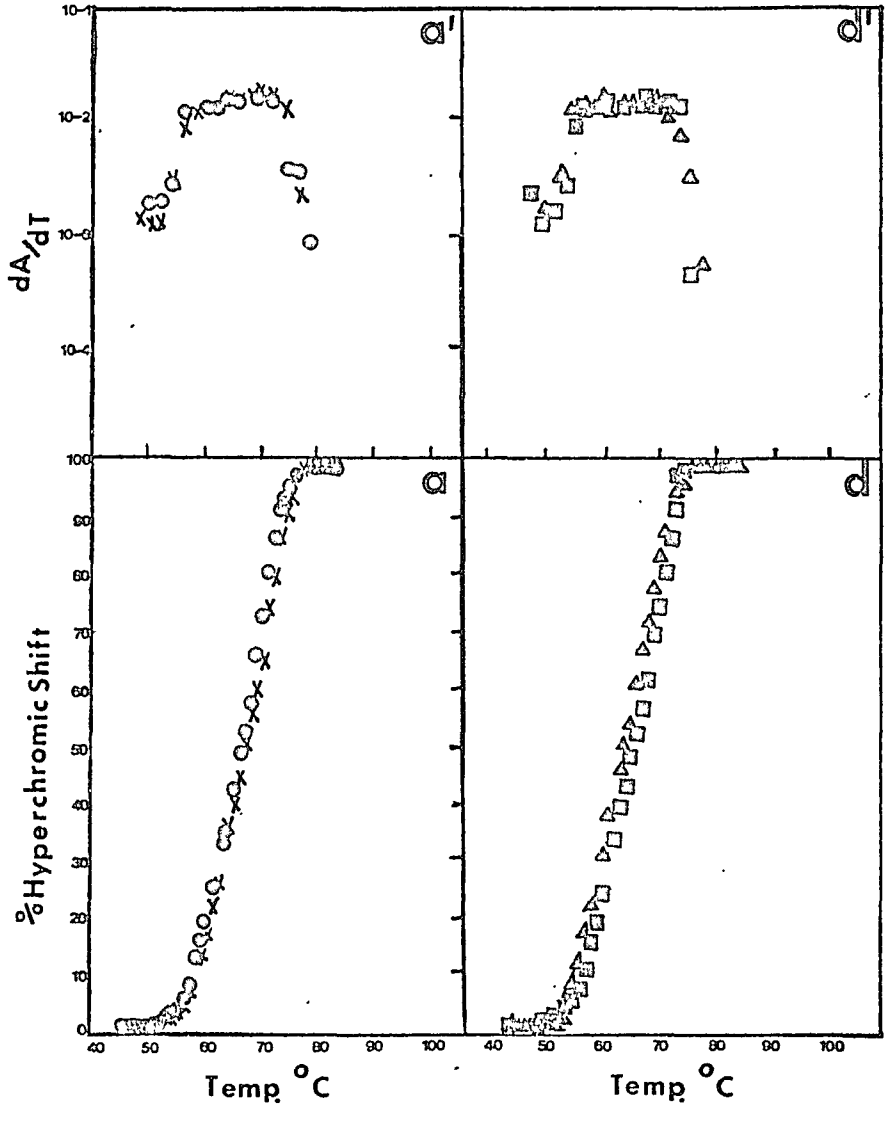
f') Derivative profiles of G and G_c DNA's.

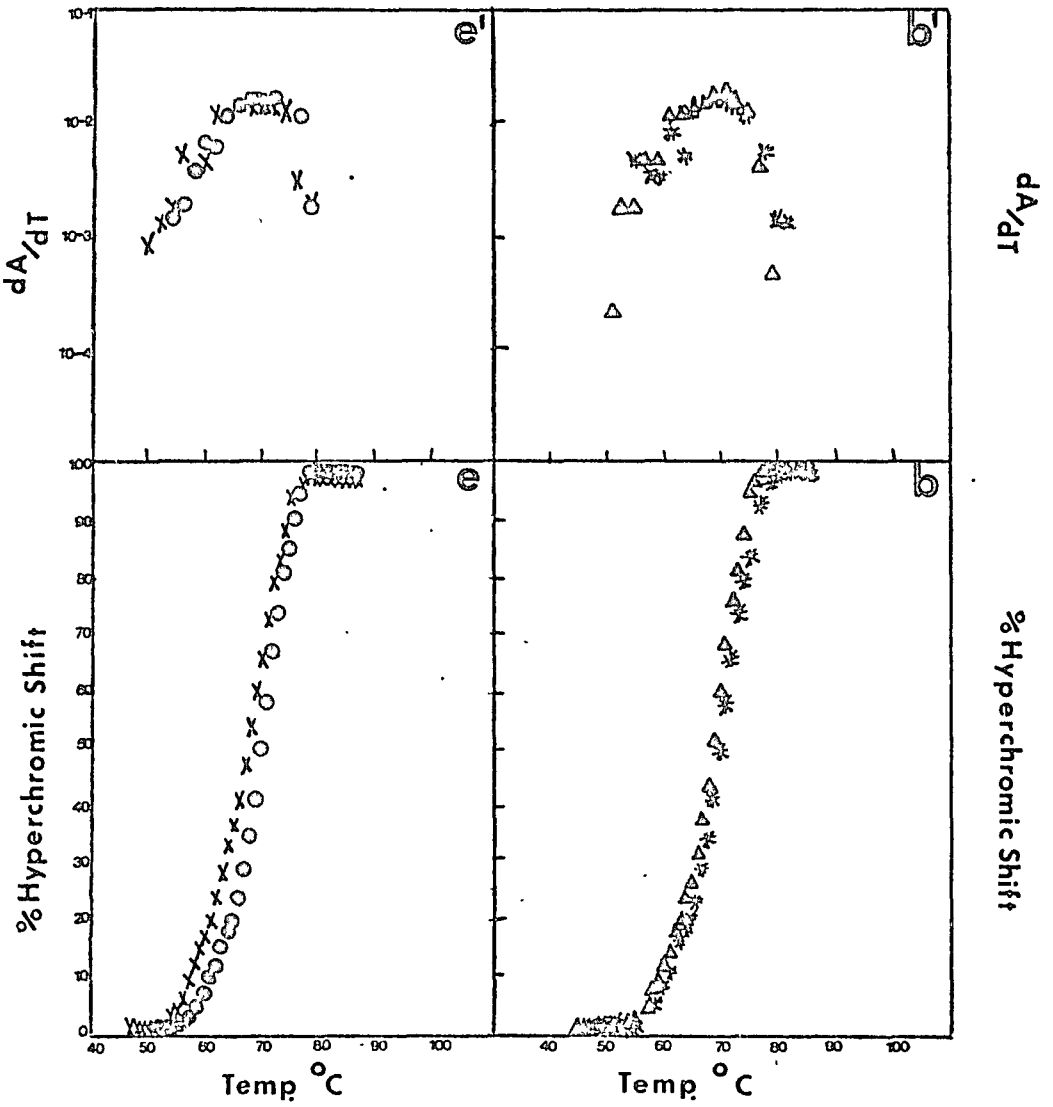
g) Integral profiles of SM(o—o)and SM_a DNA's.

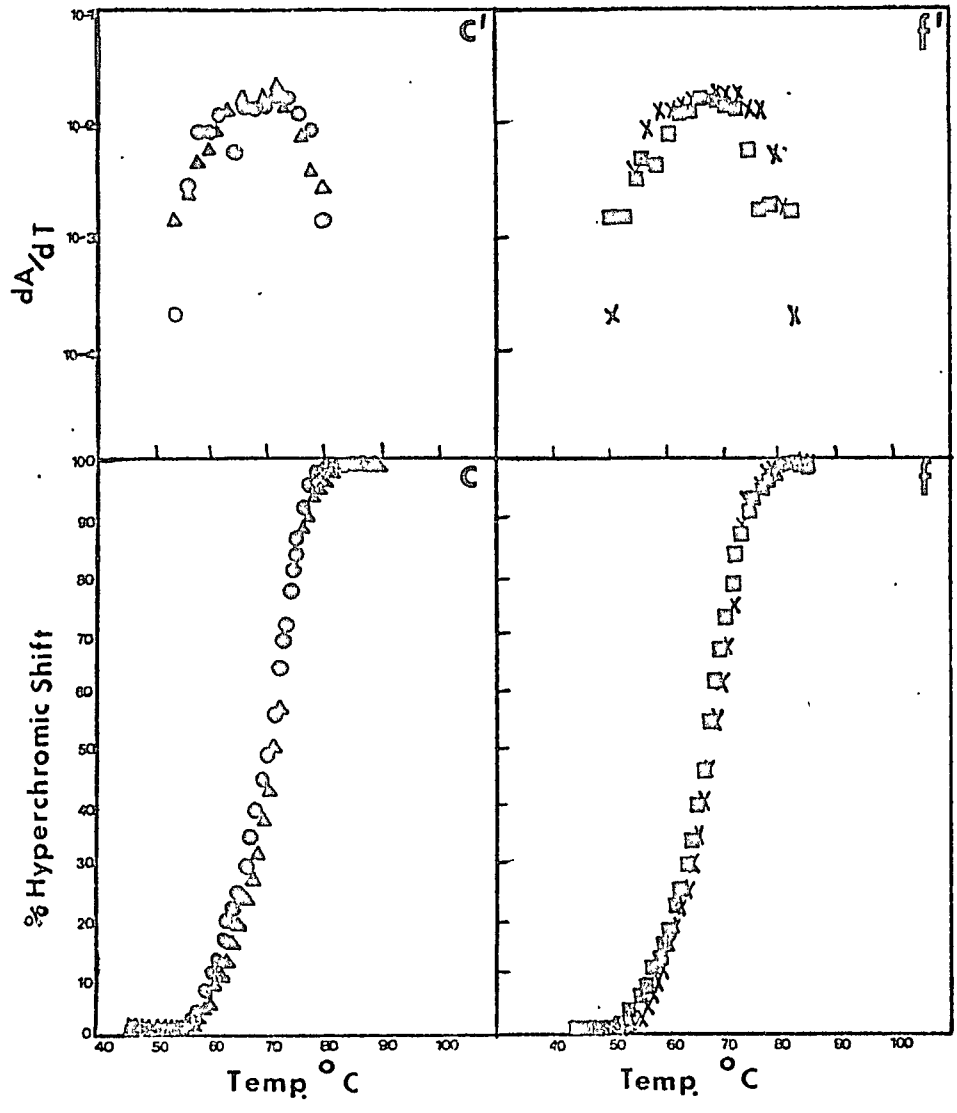
g') Derivative profiles of SM and SM_a DNA's.

h) Integral profiles of Y(✕—✕)and Y_a(⊡—□)DNA's.

h') Derivative profiles of Y and Y_a DNA's.


a'
a''
a
a
dA/dT
dA/dT
%Hyperchromic Shift
%Hyperchromic Shift
Temp °C
Temp °C


a'
b'
a
b
dA/dT
% Hyperchromic Shift
Temp °C
40
50
60
70
80
90
100


C'
f'
dA/dT
C
f
% Hyperchromic Shift
Temp. °C
40
50
60
70
80
90
100
0
10
20
30
40
50
60
70
80
90
100

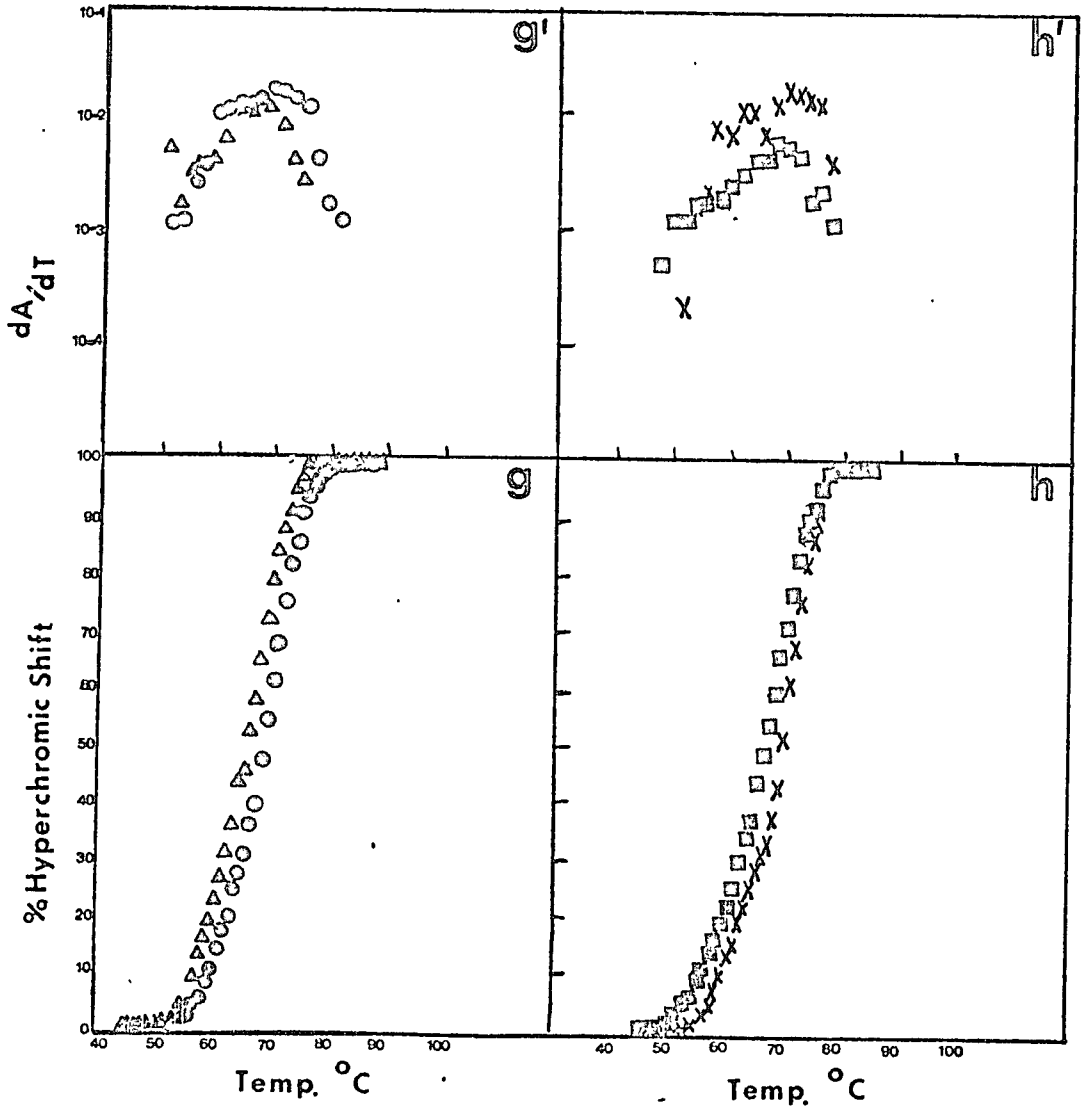

g'
h'
g
h
dA/dT
% Hyperchromic Shift
Temp. °C
40
50
60
70
80
90
100

Figure 8A. Triplicate CsCl Buoyant Density Profiles of PS and Y DNA's. Triplicate CsCl centrifugations of PS and Y DNA's were performed to illustrate reproducibility of buoyant density profiles and buoyant densities. See text for further details. 1,2,3: Buoyant density profiles of three different runs of PS DNA. 4,5,6: Buoyant density profiles of three different runs of Y DNA.

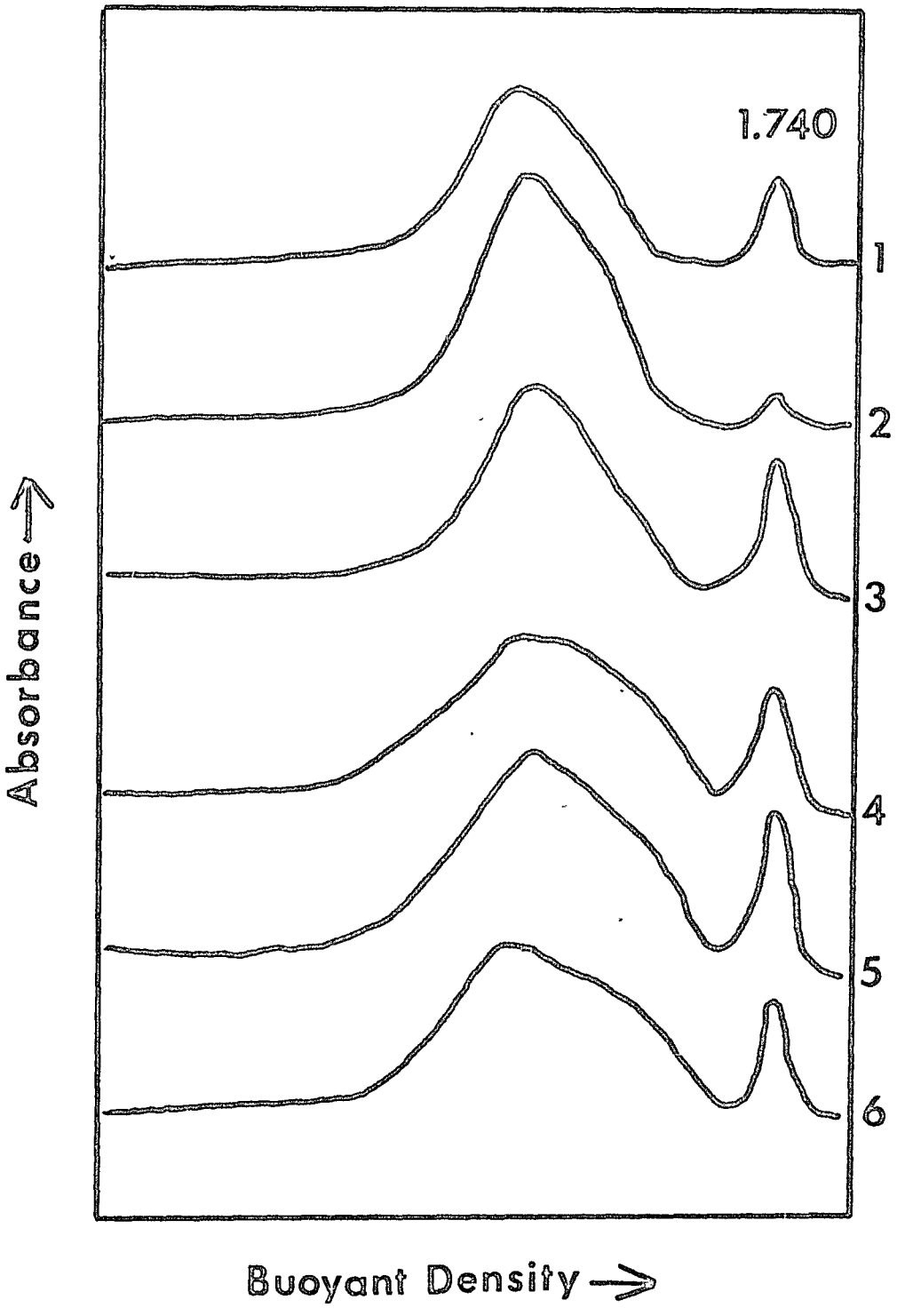

1.740
1
2
3
4
5
6
Absorbance
Buoyant Density

Figure 8B. CsCl Buoyant Density Profiles of SM DNA Extracted from Osmotically Lysed and from Eaton Press Lysed Cells. a': Buoyant density profile of SM DNA from Eaton press lysed cells. b': Buoyant density profile of SM DNA from osmotically lysed cells.

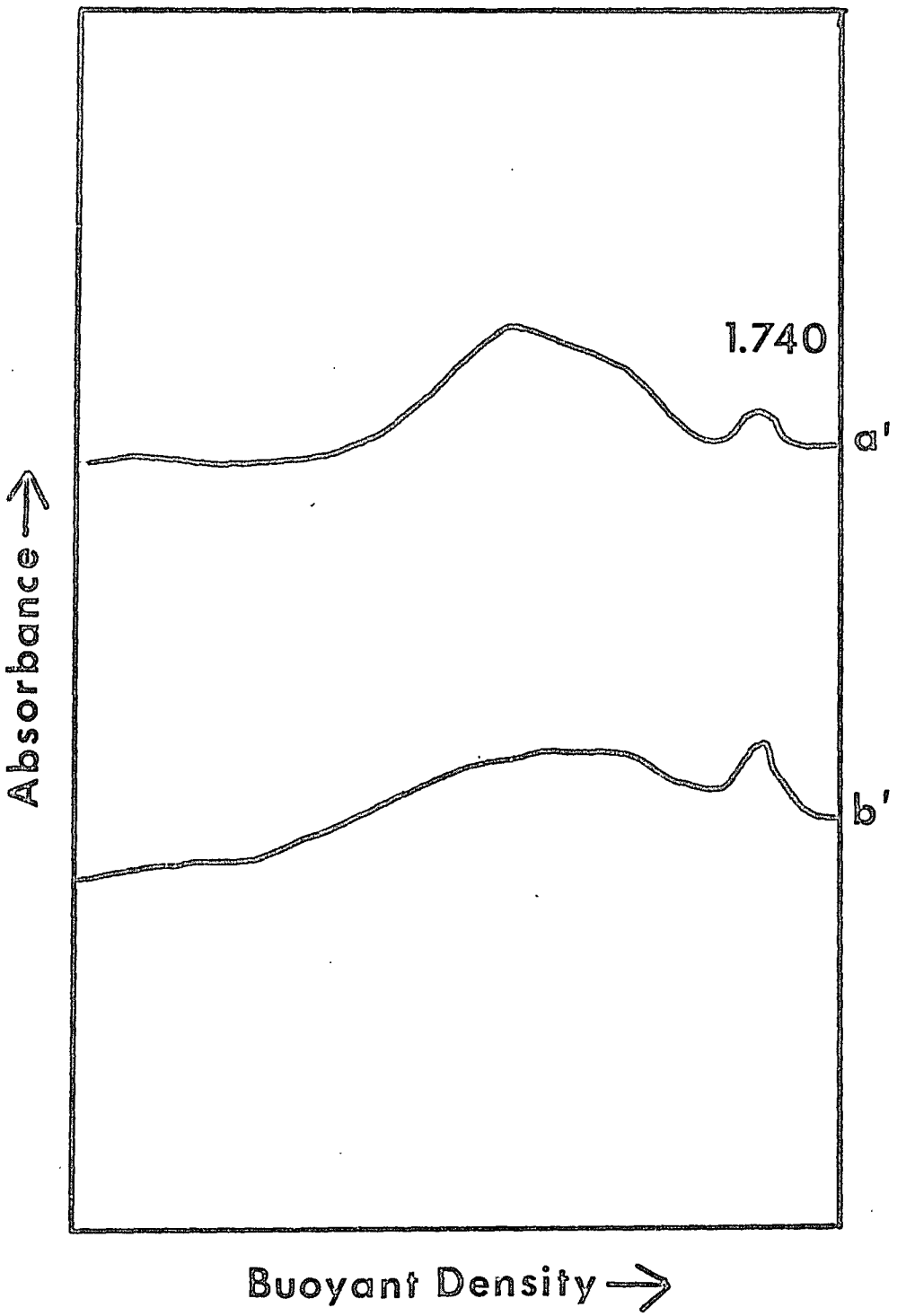

1.740
a'
b'
Absorbance
Buoyant Density

Figure 8C. Buoyant Density Profiles of PS DNA Extracted from the Aqueous Phase and from the Interface. PS DNA was extracted from the aqueous phase and from the interface as described in Materials and Methods and centrifuged in CsCl buoyant density gradients. a': Buoyant density profile of PS DNA extracted from the aqueous layer. b': Buoyant density profile of PS DNA extracted from the interface.

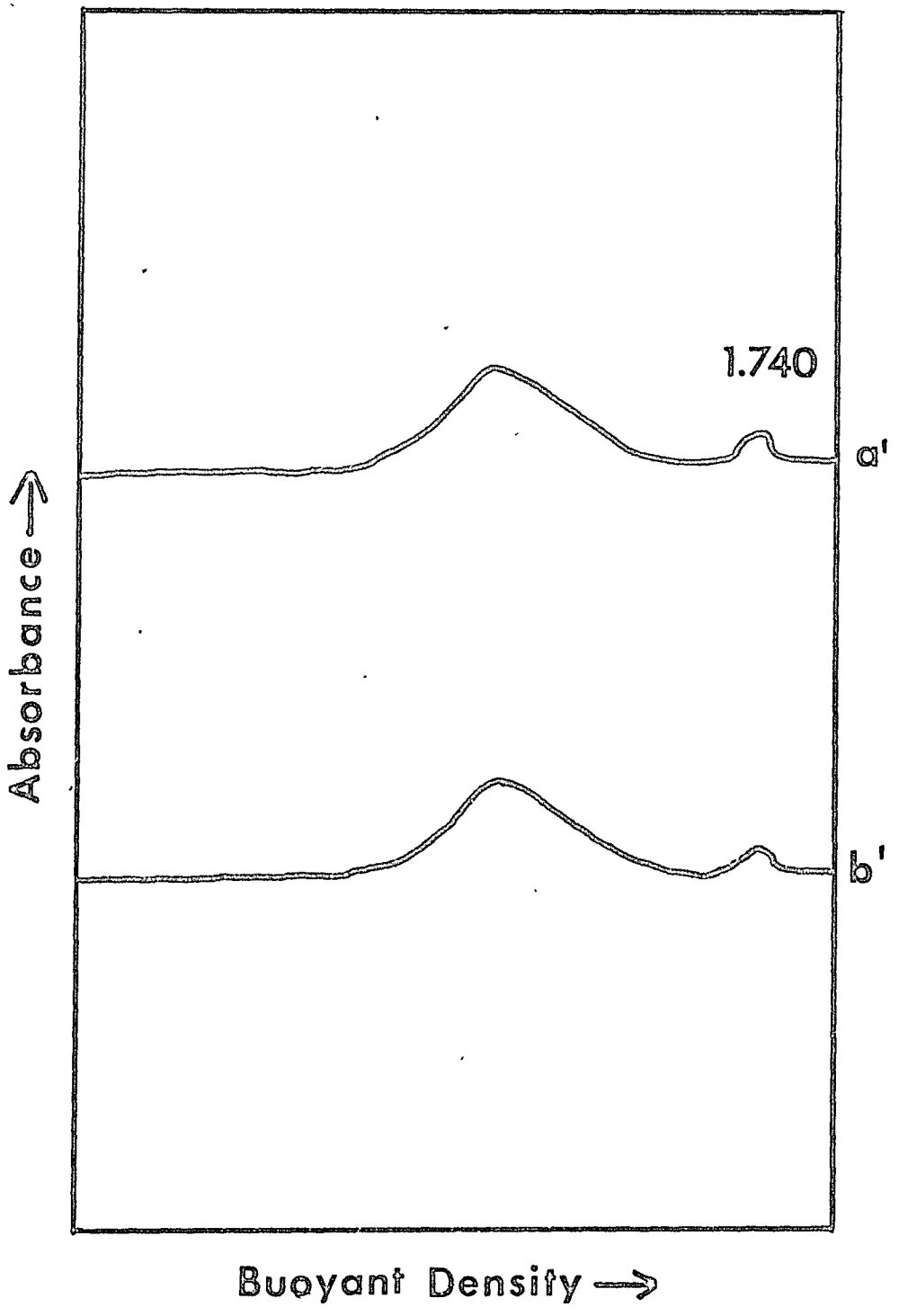
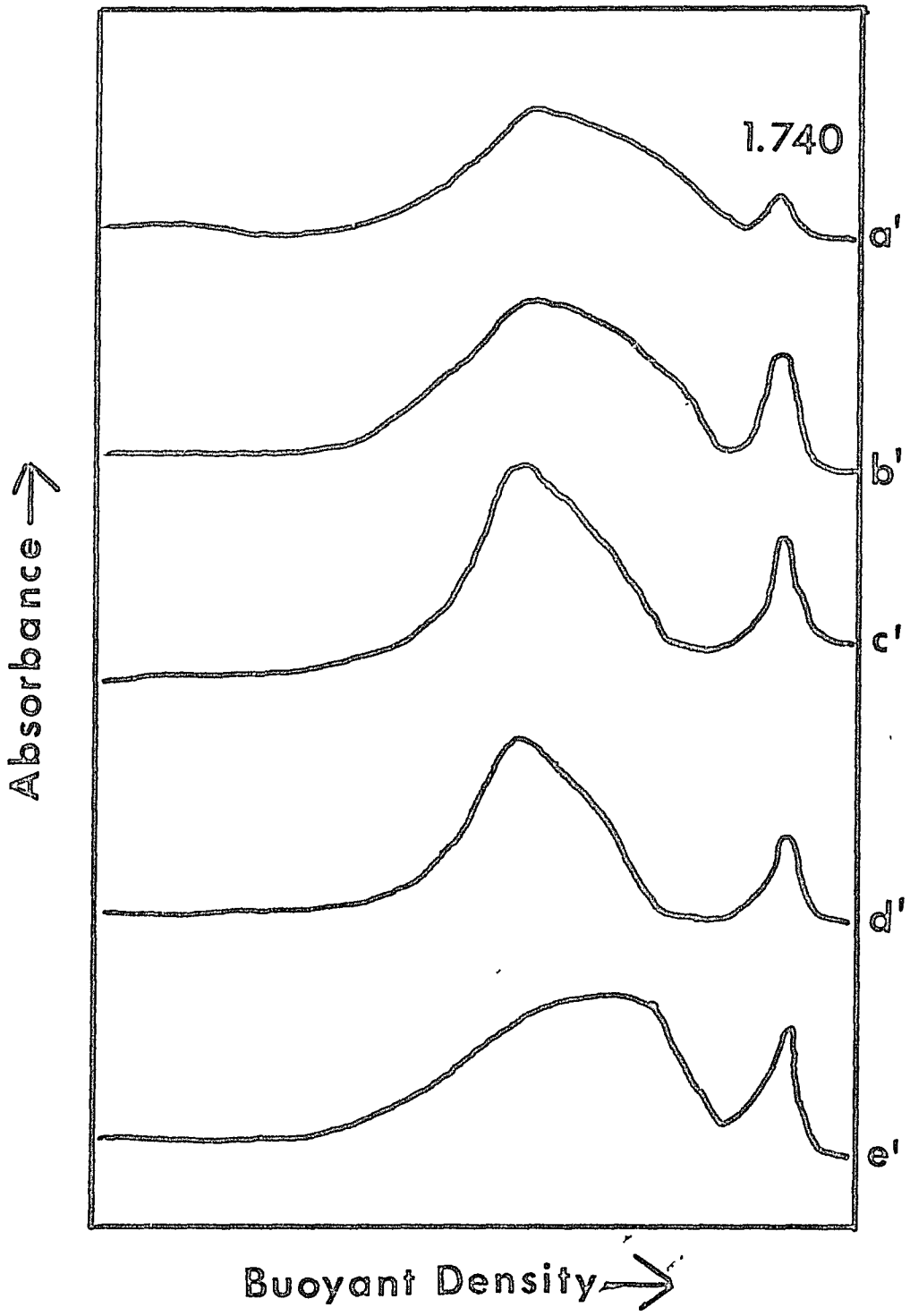

1.740
a'
b'
Absorbance
Buoyant Density

Figure 8D. CsCl Buoyant Density Profiles of DNA's from Each of the Nine Strains of C. cohnii. See Results for procedure details. Sp8 DNA and E. coli DNA, with densities of 1.740 and 1.710 g/cm^3, respectively, are included as buoyant density markers.

a') SM DNA; b') Y DNA; c') WH DNA; d') PS DNA; e') I DNA; f') Ro DNA; g') To DNA; h') G DNA; i') MC DNA; j') E. coli and Sp8 DNA's.


1.740
a'
b'
c'
d'
e'
Absorbance
Buoyant Density

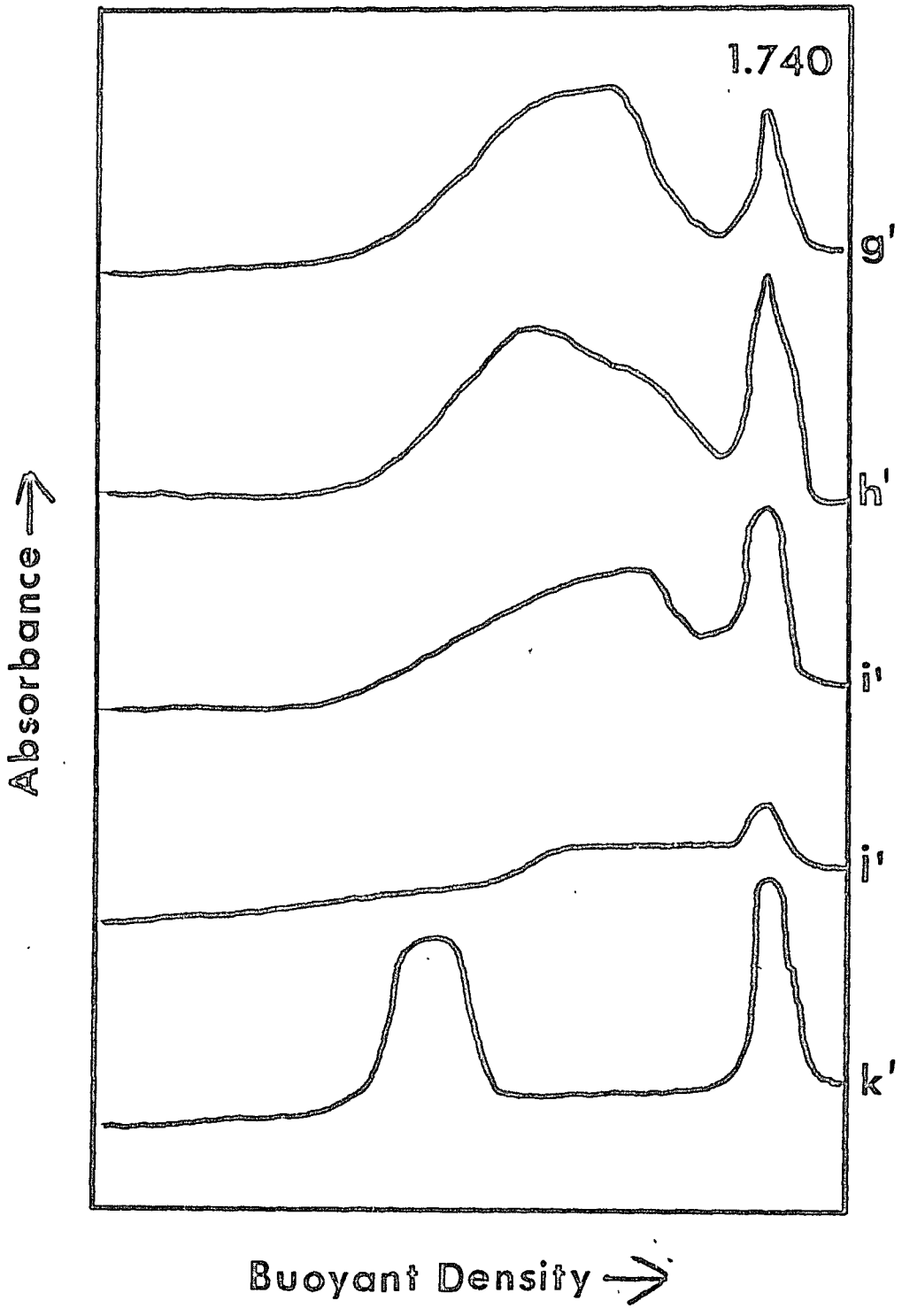

1.740
g'
h'
i'
i'
k'
Absorbance
Buoyant Density

Figure 8E. CsCl Buoyant Density Profiles of DNA's Isolated from Wild Type and Mutant Strains. See text for details.

a') SM DNA.

b') SM_a DNA.

c') I DNA.

d') I_a DNA.

e') Ro DNA.

f') Ro_b DNA.

g') WH DNA.

h') WH_a DNA.

i') To DNA.

j') To_b DNA.

k') Y DNA.

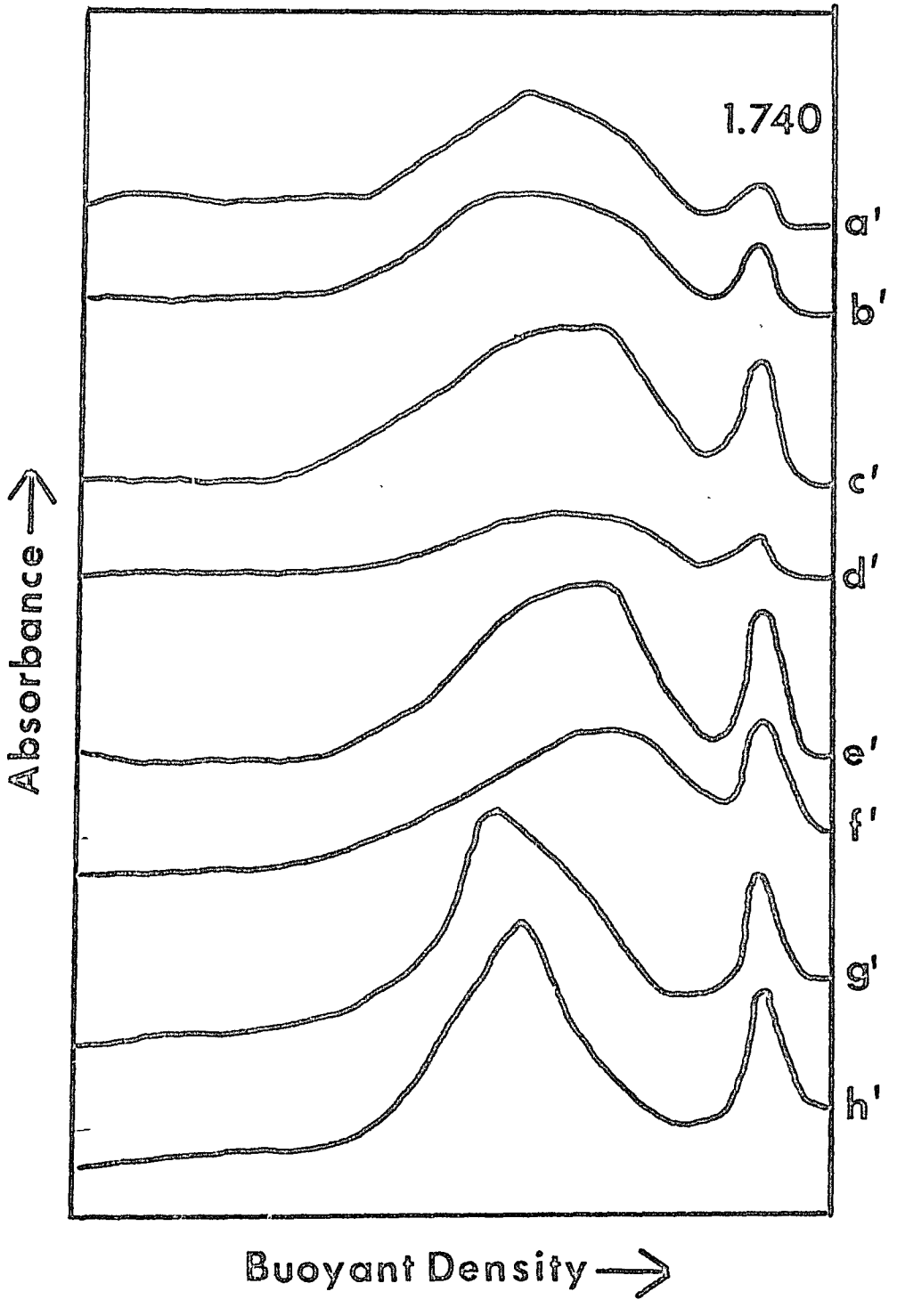
l') Y_a DNA.

m') PS DNA

n') PS_p DNA.

o') G DNA

p') G_c DNA.


1.740
a'
b'
c'
d'
e'
f'
g'
h'
Absorbance
Buoyant Density

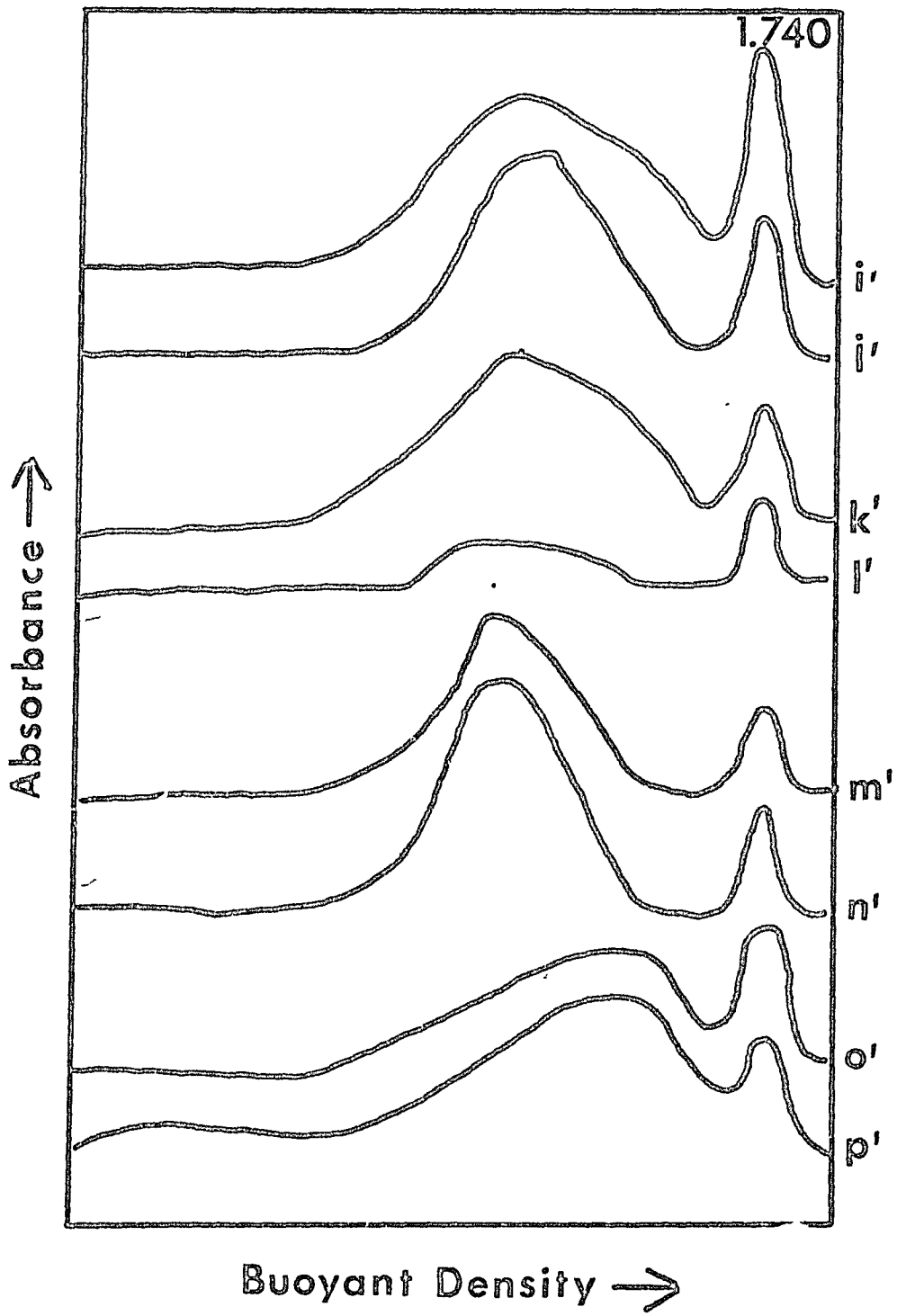

1.740
i'
i'
k'
l'
m'
n'
o'
p'
Absorbance
Buoyant Density

Figure 9. UV Spectra of 5'Mononucleotides. Details in Materials and Methods.

a-1) Commercial d'TMP (not hydrolyzed)( ); d'TMP from WH DNA( );d'TMP from Ro DNA ( ); d'TMP from calf thymus DNA ( ).

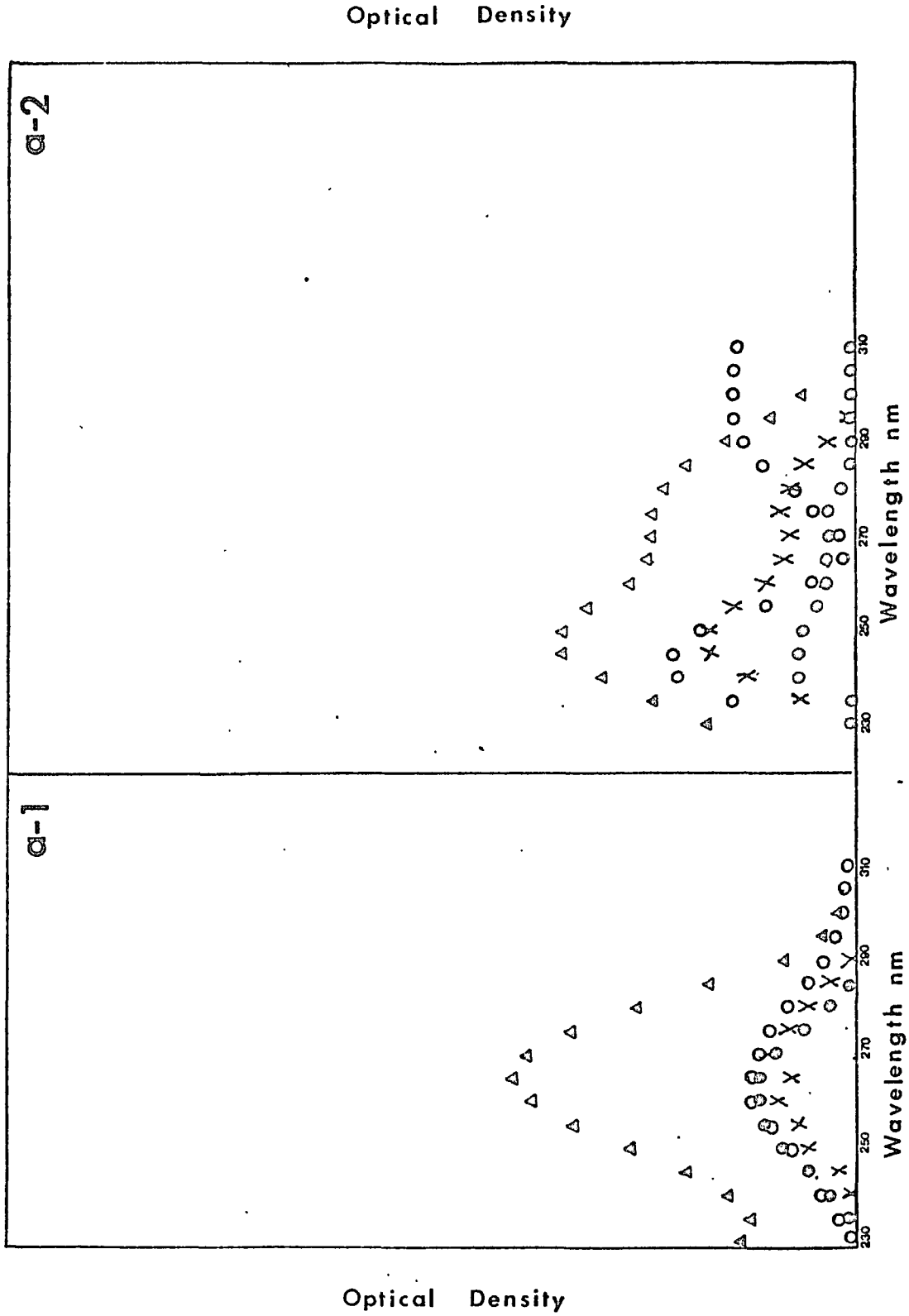
a-2) Commercial d'GMP (not hydrolyzed)( ); d'GMP from WH DNA( );d'GMP from Ro DNA ( ); d'GMP from calf thymus DNA ( ).

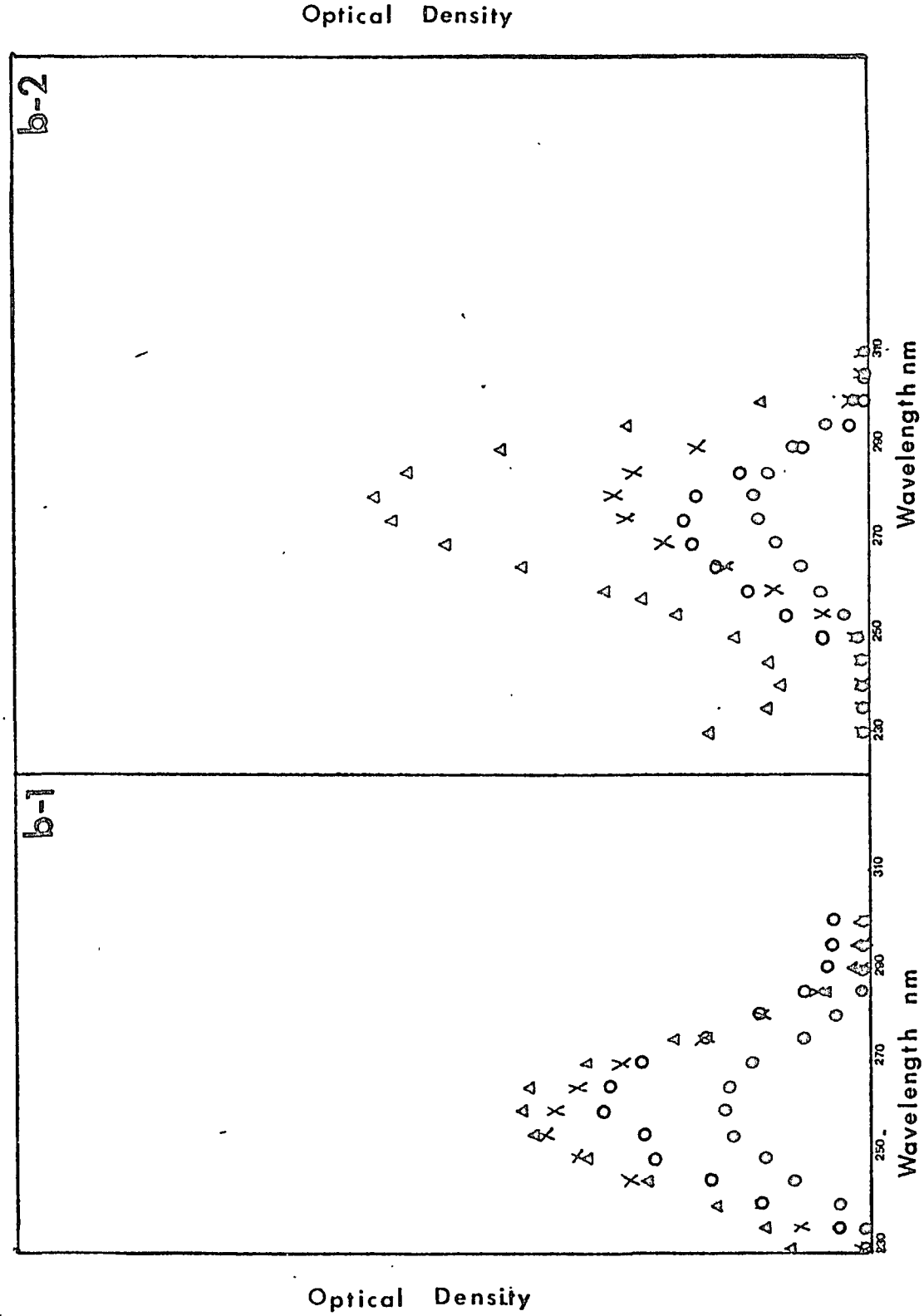
b-1) Commercial d'AMP (not hydrolyzed)( ); d'AMP from WH DNA( );d'AMP from Ro DNA ( ); d'AMP from calf thymus DNA ( ).

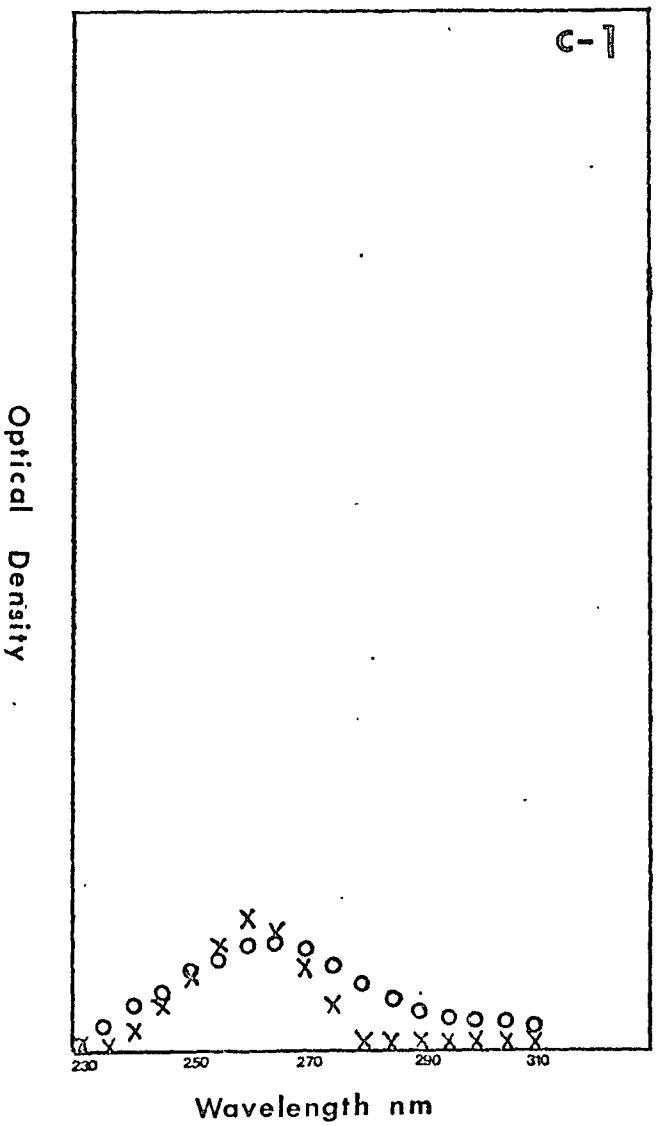
b-2) Commercial d'CMP (not hydrolyzed)( ); d'CMP from WH DNA( );d'CMP from Ro DNA ( ); d'CMP from calf thymus DNA ( ).

c-1) HMUMP from WH DNA( );HMUMP from Ro DNA( ).

c-2) Controls taken from WH DNA: d'CMP( ); d'AMP, d'TMP( ); d'GMP( ); d'HMUMP( ).


Optical Density
a-2
a-1
Wavelength nm
Wavelength nm
Optical Density


Optical Density
b-2
Wavelength nm
b-1
Wavelength nm
Optical Density


C-1
Optical Density
230
250
270
290
310
Wavelength nm

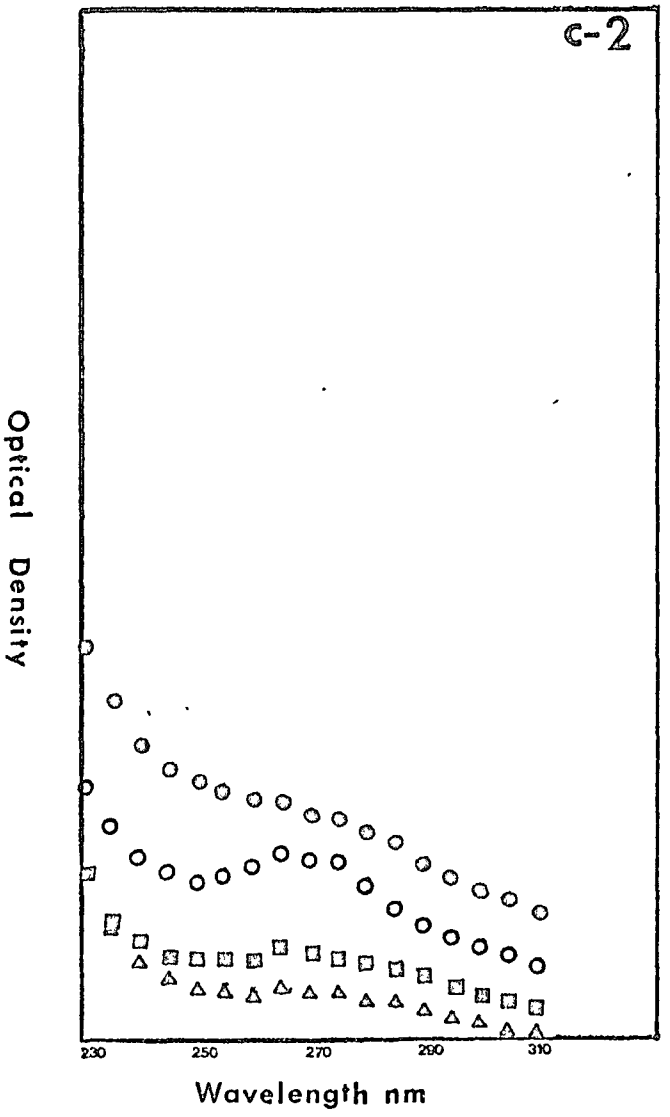

C-2
Optical Density
230
250
270
290
310
Wavelength nm

Figure 10. UV Spectra of Bases. Details in Materials and Methods.

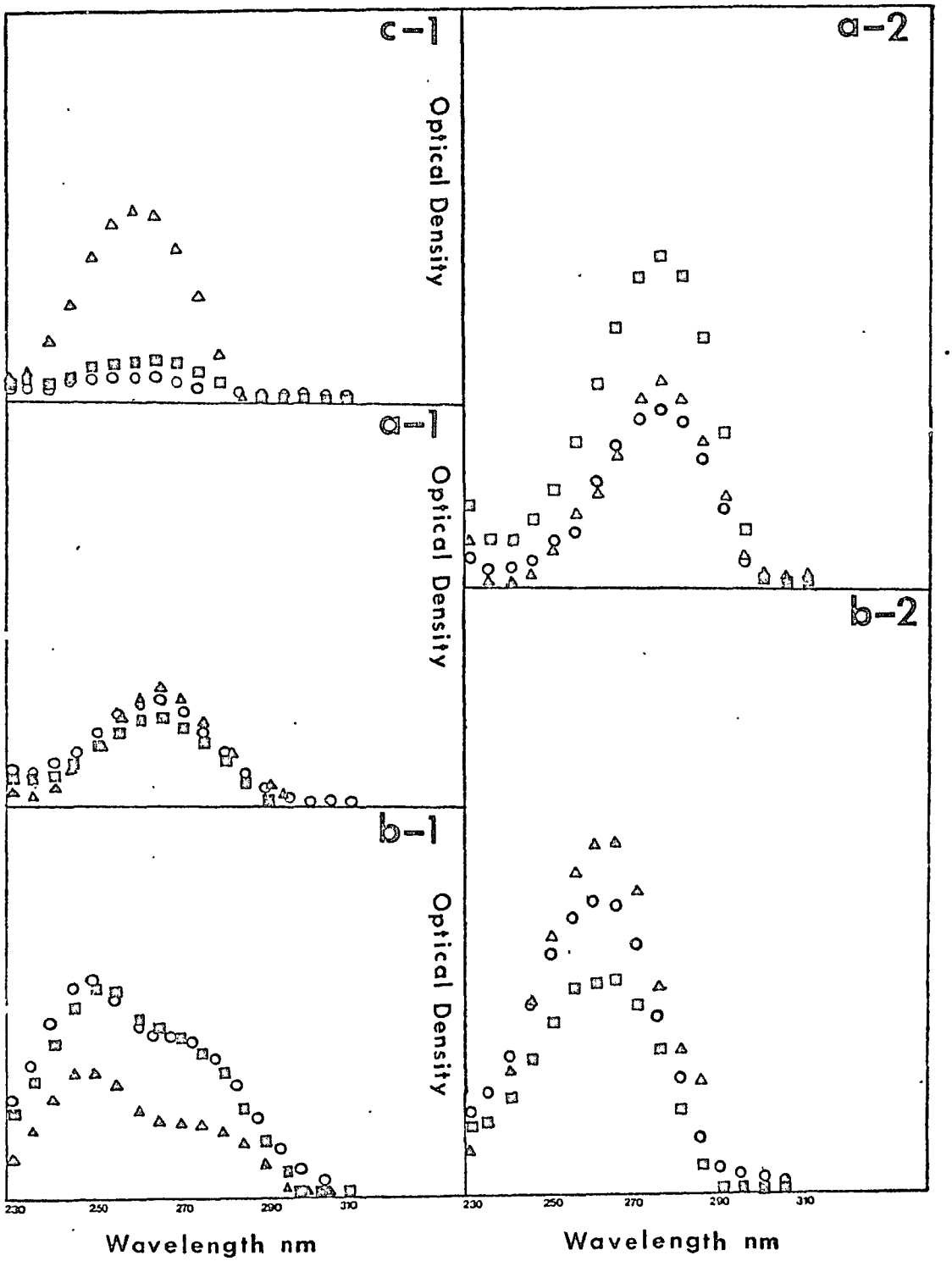
a-1) Commercial thymine, not hydrolyzed(Δ–Δ); commercial thymine, hydrolyzed(□–□); thymine from SM DNA ◎–◎ .

a-2) Commercial cytosine, not hydrolyzed(Δ–Δ); commercial cytosine, hydrolyzed(□–□); cytosine from SM DNA ◎–◎ .

b-1) Commercial guanine, not hydrolyzed (Δ–Δ); commercial guanine, hydrolyzed(□–□); guanine from SM DNA ◎–◎ .

b-2) Commercial adenine, not hydrolyzed (Δ–Δ); commercial adenine, hydrolyzed(□–□ adenine from SM DNA ◎–◎ .

c-1) Commercial HMU, not hydrolyzed Δ–Δ ; commercial HMU, hydrolyzed(□–□); HMU from SM DNA (◎–◎).


c-1
a-2
a-1
b-2
b-1
Optical Density
Optical Density
Optical Density
Optical Density
230
250
270
290
310
230
250
270
290
310
Wavelength nm
Wavelength nm

APPENDIX

BIOLOGY OF DINOFLAGELLATES

The Pyrrophyta include three classes- the Cryptophyceae, the Desmokontae and the Dinophyceae. The dinoflagellates belong to this latter class.

Dinoflagellates are encircled by a transverse groove (girdle). The two flagella are inserted in or near the girdle; one encircles the cell transversely while the other extends vertically backward.

Dinoflagellates usually have two pusules; these unique structures have a distinct membrane, are non-contractile and consist of a vacuole connected to the exterior of the cell by a canal opening into a flagellar pore of the cell wall. They are concerned with the intake of fluids (Smith, 1938).

Thecate structure is a feature of the dinoflagellates that serves to classify them into various genera. The basic structure of the theca consists of one outer membrane, beneath which lies one layer of flattened vesicles that may or may not contain wall plates or plate-like structures. Occasionally, there may be another continuous membrane beneath the vesicles. There are usually subthecal microtubules present below the theca. The number of thecal vesicles varies from several hundred in some species of Gyrodinium and Gymnodinium to two in members of the Prorocentrales (e.g. Prorocentrum and Exuviella). The vesicles may contain nothing, may show thin, dark-staining

structures or even polygonal plates. Such plates are composed of microfibrils perhaps of cellulose. The thecal plates are perforated by pores through which trichocysts are discharged.

The cell wall of nonmotile dinoflagellates (e.g. Pyrocystis) is bounded on the outside by a layer of resistant cuticular material, beneath which are twenty-four layers of cellulose microfibrils with alternating layers of microfibrils arranged at right angles to each other. The inner wall consists of randomly arranged microfibrils.

Eyespots are also present in the dinoflagellates. The most simple one is found in Woloszynskia coronata and it consists of globules (osmiophilic) without any visible membrane. In Woloszynskia tenuissima and in Peridinium westii, there are carotenoid lipid globules in and to one side of the chloroplast. In Glenodinium foliaceum, there are two layers of globules separated by a granular space and surrounded by a three-membraned envelope. Adjacent to the eyespot is a lamellar body consisting of a stack of up to fifty flattened sacs, each 16 nm thick and up to 750 nm wide. The lamellae are connected to the endoplasmic reticulum. Lastly, a highly developed eyespot is seen in the family Warnowiaceae, which is actually a large ocellus consisting of a dark, cup-shaped portion and a larger refractive structure together with a retinoid (Dodge, 1971).

Reproduction of motile genera is usually by vegetative division. Motile genera may also produce aplanospores

(cysts). Sexual reproduction occurs in some dinoflagellate groups also, for example, C. cohnii and Ceratium cornutum. The gametes are generally smaller than the vegetative cells and are motile. Meiosis in some species occurs while the zygote is motile, and in others occurs in the nonmotile cell-wall covered zygote (Loeblich, 1976, review).

NUCLEAR STRUCTURE AND MITOSIS

A considerable amount of work has been done on nuclear structure and on mitosis in the dinoflagellates. Since the first studies by Dodge and Grasse (1963; 1965) which show the coiled fibrils in condensed chromosomes in interphase, R. G. Zingmark (1970), after studying seven different species of dinoflagellates with the electron microscope, found two kinds of nuclei: (1) the first he called a "typical dinoflagellate nucleus"; for example, the nucleus seen in Gymnodinium, Gyrodinium dorsum, Peridinium and Prorocentrum micans, to name just a few. One observes distinct fibrillar chromosomes, and a double nuclear membrane with pores around the periphery. Occasionally, the outer of the two membranes extends and is confluent with the endoplasmic reticulum or with the Golgi apparatus; (2) the second type is observed in the vegetative cells of Noctiluca miliaris. The nucleus is different from the first type, the nuclear membrane being double but lined with annulated vesicles. The nucleolus of both types is the same and is composed of two different-sized granules; the outer layer of large granules surrounds and mingles

with the smaller ones.

L. Chunosoff and H. I. Hirschfield (1967) studied the nuclear structure and mitosis in Gonyaulax monilata, a marine, chainforming, photosynthetic and luminescent dinoflagellate. The chromosomes remain condensed throughout interphase. During metaphase (as in most dinoflagellates), the chromosomes are arranged at right angles to the plane of division and sister chromatids move apart with their ends pointing toward the division poles. During interphase, a structure called the "central body" is observed to be attached to the chromosomes at their ends. Using the proteolytic enzyme trypsin, the central body disappears and the chromatin becomes an amorphous mass.

In late prophase, the chromosomes form an entangled ball within which is the "central body". In metaphase, the chromosomes line up in parallel fashion. The "central body" divides into two and each end becomes situated at a division pole; the ends move and the chromatids separate. The "central body" remains attached to the chromosomes throughout mitosis.

Mitosis was also studied in Blastodinium, a parasitic dinoflagellate associated with pelagic copepods (Soyer, 1971). It is a binucleate organism, having one nucleus typical of a dinoflagellate (i.e. the chromosomes are always condensed) and the second similar to Noctiluca. Mitosis was studied in the dinoflagellate-type nucleus. The nuclear envelope plays a role in the orientation and

segregation of the chromosomes. Cytoplasmic invaginations containing many microtubules perforate the nucleus. Nuclear division is simple and no centrioles are observed. There is a "central body" which divides before the nucleus and the movements of the two central bodies with those of the chromosomes are interconnected.

In a paper by Kubai and Ris (1969), division in C. cohnii was elaborately described. During the division process, a complex process of invagination of the intact nuclear envelope occurs on one side of the nucleus which results in the formation of parallel, cylindrical cytoplasmic channels through the nucleus. These channels contain microtubules. Nuclear constriction occurs perpendicular to these channels. No associations are seen between the chromosomes and cytoplasmic microtubules. In dividing cells, however, chromosomes become V-shaped and their apices contact the membrane surrounding the cytoplasmic channels, which, in turn, may be involved in daughter chromosome separation.

The nucleus of Noctiluca scintillans is significant in terms of a possible structure illustrating nucleocytoplasmic exchanges (Afzelius, 1963). The nucleus is round and approximately 30 u in diameter. There is a layer of vesicles inside the nuclear membrane called annulated vesicles. The nuclear membrane is 150 °A wide and is composed of two parallel lamellae almost devoid of annuli. The double membranes of the annulated vesicles have tightly packed annuli and some of the vesicles are

continuous with the nuclear membrane and protrude into the cytoplasm. There are about 8,000 annulated vesicles per Noctiluca nucleus and about 80 annuli per vesicle. The author suggests a connection between the annulated vesicles with the cytoplasmic vesicles lying outside of the nuclear membrane which may serve as some sort of nucleocytoplasmic exchange structure.

CHROMOSOME MODELS

A number of models for the arrangement of chromosomal fibrils based on electron microscopy has been suggested. Grassé et al. (1965) envision a chromosome composed of many fibrils, each one in the form of a helix.

Y. Bouligand et al. (1968) (Livolant and Bouligand, 1978) observed that all chromosomes studied in four different species of dinoflagellates display longitudinal periodicity and that they are composed of filaments between 25 and 75 Å wide which, in turn, are grouped together to form segments with a diameter of approximately 200 Å. These workers attempt to ascertain the filament alignment within the chromosome. They suggested a model which consists of superimposed discs, equidistant from each other, forming a cylinder. If one makes an oblique section, one observes fibrillar arrays such as those seen in electron micrographs. There is also a longitudinal orientation in the chromosomes. The filaments are perpendicular in this direction and parallel to each other in a transverse plane.

O. K. Haapala and M. O. Soyer (1973), using electron microscopy of wholemount preparations of Prorocentrum micans chromosomes, suggested that chromatids of these polytenic chromosomes must be circular. The width of the so-called elementary chromosome fibril is about 30 Å and it, therefore, contains only one DNA double helix. Fibrils are seen with widths ranging from 60 to 200 Å , formed by several elementary fibrils. Their model resembles that of Grasse et al. (1965). When the chromosomes are spread, the periodicity observed is in the form of a linear array of "circular balls" and when stretched, give rise to a series of figure eights, each of which is due to the opening of two successive clusters. The length occupied by two balls is identical to one turn of the psiral chromatid coil. They suggested a degree of polytenization of about 1,000 fold. The figure eight model is similar to the Worcel-Burgi model of the bacterial nucleoid (Worcel and Burgi, 1972).

To extend the above study, Soyer and Haapala (1974) treated the chromosomes of Prorocentrum micans and Gyrodinium (Crypthecodinium) cohnii with pronase and with ribonuclease to further elucidate the structure of the chromosome. They concentrated on the extrachromosomal loops seen in spread and in thin-sectioned chromosomes using electron microscopy. Similar results were obtained with both dinoflagellates. Electron microscopy of untreated thin sections reveals two stages with regard to the extrachromosomal fibers. When there are no loops, nonfibrillar

material is seen primarily in the chromosomes; the diameter of the chromosomes is also greater than in the second stage when extrachromosomal loops and material around them are present.

Pronase treatment removes the nonfibrillar material of the chromosomes, as well as the material covering the extrachromosomal fibers. The structure of the fibrillar chromatin remains unchanged. One distinguishing effect of pronase on C. cohnii chromosomes, however, is in the removal of nonfibrillar chromosomal material which, in turn, results in the appearance of chromosomes resembling the figure eight configuration previously mentioned.

The conclusion drawn by the authors is that protein is associated with the chromosomes but removal with pronase does not affect (presumably) the structural integrity of the chromosomes.

When C. cohnii cells were treated with ribonuclease, the chromosomes were not as condensed as in untreated cells. Chromosomes fixed and sectioned showed that the packaging of chromatid fibrils was loosened and the chromosomes became extended, increasing about three times in length.

Ribonuclease treated P. micans cells showed chromosomes increasing in length five times. The figure eight configuration disappeared. Thus the two main effects of enzyme treatments on the dinoflagellate chromosomes, according to these authors, are: 1) removal of dense material from chromosomes by pronase and exposure, thereupon, of extrachromosomal loops, and 2) the loosening of the packaging of chromosomal

DNA by ribonuclease. They feel that extrachromosomal looped fibrils are an indication of genetic activity, resembling the lampbrush loops of newt diplotene chromosomes (Callan, 1967). Another interesting suggestion made is that uncoiling may be coarsely controlled by RNA which is responsible for tight supercoiling. In addition, the nonrandom distribution of the modified base, 5-hydroxymethyluracil, found in dinoflagellates (Rae, 1973)(discussed more fully later) may be scattered to aid in the overall supercoiling, or could also be located in areas binding structural RNA (Holmes et al., 1972).

There is, however, a potential problem in this study, in that no adequate controls were included. Cells were suspended in 0.5 M Na_2EDTA, 0.015 M Tris, pH 7.0 for enzyme digestion so that it is difficult to say whether such a high concentration of EDTA in itself affects the chromosomes in some way without the enzymes themselves.

This discussion concerning chromosome structure shows why Dodge (1966) called dinoflagellates, "mesocaryotes". Dinoflagellates, according to Dodge, are intermediates between the prokaryotes and eukaryotes. They have characteristics of both groups, namely, condensed chromosomes throughout the cell cycle (Kubai and Ris, 1969), apparent lack of histones associated with the DNA (Rizzo and Nooden, 1974 a, b), possible chromosomal attachment to the nuclear membrane (when present, Kubai and Ris, 1969), the persistence of the nuclear membrane and nucleolus during mitosis and the absence of a typical mitotic spindle

(Kubai and Ris, 1969); these characteristics are indicative of prokaryotes. The presence of large amounts of DNA per nucleus (Rizzo and Nooden, 1973), a nuclear membrane and nucleolus as well as cytoplasmic organelles, are typical of eukaryotes.

Glenodinim foliaceum illustrates a possible origin of chloroplasts in dinoflagellates (Dodge, 1971). Using both light and electron microscopy, the mesokaryotic nucleus is seen to contain numerous chromosomes with typical compact arrangement of fibrils; a nucleolus is also evident. During mitosis, the nucleus is penetrated by cytoplamic tunnels containing microtubules, as has been observed for other dinoflagellates (Kubai and Ris, 1969). With the light microscope, the "eukaryotic" nucleus is very varied in form; it contains much DNA as seen by Feulgen staining. With the electron microscope, the nucleus is bounded by a double membrane with typical nuclear pores. The main difference between the two nuclei lies in the finding that the mesokaryotic nucleus contains chromosomes condensed throughout the cell cycle while the "eukaryotic" chromosomes are dispersed during interphase.

One recent study on dinoflagellate chromosomes by Hamkalo and Rattner (1977) needs mentioning as these people tried to find eukaryotic nuclear characteristics within the mesokaryotic nucleus, namely, the presence of nucleosomes (nucleosomes are DNA-histone complexes forming regularly-spaced repeating fundamental units in chromatin (Olins and Olins, 1974). Hamkalo and Rattner used the microcentrifuga-

tion technique developed by Miller and coworkers (1970) to investigate the chromosomes of the free-living dinoflagellate, Prorocentrum micans. They found no indication of a globular repeat structure on the chromosome fibers, but did find structural information consistent with that found by Soyer and Haapala (1973, 1974b).

The mesokaryotic idea, as well as other work to be mentioned prompted the present author to undertake a study, biochemical in nature, on Crypthecodinium (Gyrodinium) cohnii.

Himes and Beam (1975; Beam and Himes, 1974) reported a detailed genetic and tetrad analysis on C. cohnii. Motility mutants were isolated from UV- or nitrosoguanidine-treated cells; some spontaneously arising mutants were also discovered. Their findings, in summary, indicate that "genes" for motility segregate as nuclear Mendelian units and that all genes observed (six in this study) show independent assortment with the possible exception of two. A peculiarity noticed was the absence of tetratypes, which the authors suggest may be attributable to one-division meiosis in C. cohnii. In addition, zygotic cysts were seen to contain 2, 4 or 8 daughter cells, the latter arising by one or two mitotic divisions, respectively, of the meiotic products.

An even more detailed genetic analysis of C. cohnii was published in 1977 by Beam et al. who isolated several new C. cohnii-like organisms, as well as additional motility mutants. As previously found, essentially all motility

genes (16) showed independent assortment, i.e. a 1:1 ratio of parental to nonparental ditypes. Epistatic interaction was also seen. From 30 different crosses, including five different strains, and representing 850 segregations, only two tetratypes were found. However, another finding and one certainly to contribute to the problem of the definition of the "species" was that Woods Hole, a strain found by L. Provasoli from Woods Hole, Massachusetts, and a second strain found by Beam and Himes from Puget Sound, Washington, were observed to be sexually compatible.

BIOCHEMICAL STUDIES OF DINOFLAGELLATES

A number of biochemical studies have been done on dinoflagellates. John Dodge (1964), working with *Prorocentrum micans* and using methyl green pyronin for staining RNA and DNA, and alkaline fast green for basic protein, found no RNA associated with the chromosomes nor in the cytoplasm, and found no basic proteins with the chromosomes, but did see positively stained material between and around the chromosomes.

In 1974 and 1977 Roberts *et al.* provided two lines of evidence for the dinoflagellate chromosome being haploid and uninemic. Using N-methyl-N' nitro-N-nitrosoguanidine, nutritional and carotenoid deficient mutants of *C. cohnii* were obtained at a frequency similar to that found by Adelberg *et al.* (1965) for *E. coli* K_{12}. Only a haploid nuclear condition is consistent with these results.

The second line of evidence was from the finding of Rizzo and Nooden (1973) that log phase cells contain 6.9 pg of DNA; using hydroxylapatite chromatography (Roberts et al., 1974), two kinetic classes of DNA were found-one class, comprising 60% of the total DNA reassociated as moderately repetitive sequences, while 40% renatured as essentially unique sequences. The C_0t values (=moles of nucleotide/liter X time) do not support the diploid-polyteny model for dinoflagellate chromosomes proposed by Haapala and Soyer (1973).

Allen et al. (1975) extended the study of Roberts et al. (1974) to include renaturation kinetics data on C. cohnii as well as on two other freeliving dinoflagellates, Gyrodinium resplendens and Gymnodinium sp.

C. cohnii DNA was sheared to fragment lengths of 500 to 600 base pairs, denatured and then renaturation followed with hydroxylapatite chromatography. The same two kinetic classes were found as in the previous study, namely, a repeated class comprising 55-60% and a highly complex class comprising 40-45% of the DNA. The repeated, renatured class (renatured to C_0t of 100) was melted and monitored optically. A 10% mismatch was calculated; the repeats are not perfect copies of each other. DNA was then sheared to longer fragment lengths (3000 base pairs) and renaturation monitored. It was found that an increase from 56% to 76% occurred on hydroxylapatite (when renatured to C_0t 20). According to these authors, the increase in binding at longer fragment lengths indicates

that repeated sequences are probably interspersed with unique DNA, forming large concatemers at 3000 base pairs when renatured.

G. resplendens, which has approximately ten times more DNA than C. cohnii, also shows the presence of a considerable amount of repeated DNA. Gymnodinium sp, besides having repeated classes, has 20 to 40% of the DNA renaturing too fast to measure (C_0t 2 X 10^{-3}). Thus, these findings add another eukaryotic characteristic to the dinoflagellates- the presence of interspersed, repeated sequences.

Work on ribosomal RNA processing in C. cohnii has also been done. Peter Rae (1970) isolated and characterized rRNA using sucrose gradient centrifugation and found two species, 16S and 25S subunits which are similar to those found in plants and other unicellular eukaryotes, such as Tetrahymena (Loening, 1968). Using radioactive labeling, Rae performed some kinetics of incorporation. By 2.5 minutes of labeling, a labeled RNA peak was found at 38 S; after 5 minutes of labeling, the 38S peak was very prominent but a peak of 27 S also appeared. By 10 minutes of labeling, an increase in radioactivity was seen at 16 S and at 27 S with a concomitant decrease in the 38 S region, and an indication of a 25 S peak appearing was observed. The conclusion drawn was that rRNA processing in C. cohnii follows that found in higher organisms.

In a later paper on ribosomal RNA in a dinoflagellate, Gressel and coworkers (1975) found somewhat different re-

sults than those of Rae, using polyacrylamide gel electrophoresis of rRNA subunits from the freshwater organism, Peridinium cinctum fa. westii. Apparently, this technique is more sensitive than sucrose gradient centrifugation. The findings indicated a molecular weight of 0.7×10^6 for the light rRNA subunit and 1.23×10^6 for the heavy rRNA subunit. The light rRNA subunit is typical of a eukaryotic organism, while the heavy rRNA subunit has a lower molecular weight than higher eukaryotes. the light rRNA subunit of prokaryotes is 0.56×10^6; fungi, green plants and some protozoa have heavy rRNA subunits with molecular weights of 1.3×10^6, with mammals having molecular weights of 1.75×10^6 (Loening, 1968). Thus, the dinoflagellates are intermediates with regard to this characteristic, also.

Several papers by Rizzo and Noodén describe isolation of dinoflagellate nuclei, together with the characterization of proteins associated with the nuclear DNA. In one paper (1973), four different species of dinoflagellates were used, C. cohnii included. The major difficulty in finding a proper procedure for nuclear isolation was in developing a procedure sufficiently effective to break the cell wall without breaking the nuclear membrane. This was accomplished by sonicating the cell pellet and running the material on a sucrose gradient containing Dextran and either Triton X-100, Nonidet P040 or Ficoll. Dextran increases the sucrose density and viscosity so nuclei and cell wall fragments sediment through the 2.4 M sucrose

more easily. From the light mocrographs, the nuclei appear to be pure but some cell wall fragments and starch grains are present. The basic findings, then, were an RNA:DNA ratio ranging from 0.21 in C. cohnii to 0.32 in Gymnodinium nelsoni; an acid soluble protein:DNA ratio of 0.08 in Peridinium trochoideum to 0.13 in G. nelsoni and an acid insoluble protein:DNA ratio of 0.99 in G. nelsoni to 1.22 in P. trochoideum, with a total protein:DNA ratio of 1.12 in G. nelsoni to 1.3 in P. trochoideum. The amounts of acid soluble and acid insoluble proteins are much lower than that found in typical eukaryotic nuclei (Johns, 1969).

In the next study (Rizzo and Nooden, 1974a), chromatin was isolated from C. cohnii and from P. trochoideum, from log and stationary phase cells. Two methods for chromatin isolation from purified nuclei were used. One method was a modification of that used by Towill and Nooden (1973) and the second method was similar to that of Hnilica (1967). In the first method, nuclei were washed in a buffer containing 0.14 M NaCl, 5 mM $MgCl_2$ and 10 mM Tris (pH 7.6). Heavy debris was centrifuged out. The nuclear pellet was resuspended in 10 mM Tris, 1 mM EDTA (pH 8.0) and sonicated. The chromatin was precipitated by adding solid $CaCl_2$ to a final concentration of 10 mM. In the second method, chromatin was extracted from washed nuclei by stirring overnight in 2 M NaCl and then precipitated with 20% trichloroaectic acid.

With both methods, chromatin isolated from log phase cells has more RNA, acid-soluble and acid insoluble protein relative to DNA than chromatin isolated from stationary

phase cells. Protein and RNA contents are higher for chromatin prepared by the second method. The ratio of acid-soluble protein:DNA is from 0.02 to 0.08 in the dinoflagellates as compared with a ratio of 1.0 in eukaryotes (Johns, 1969) and, in fact, resembles the ratio found in bacteria (Kadoya et al., 1964). Similar results were obtained with another dinoflagellate, P. trochoideum. Reported values in 0.1 X SSC (1.0 X SSC=0.15 M NaCl, 0.015 M Na citrate) were 67, 64 and 66 °C for C. cohnii DNA, whole chromatin and acid-extracted chromatin, respectively, and the melting profiles (not shown) were said to be indistinguishable from each other; in other words, DNA within chromatin is not more stabilized (e.g. against heat) than DNA itself.

In the accompanying paper, Rizzo and Noodén (1974b) partially characterized the "histones" and nonhistone chormosomal proteins of C. cohnii and P. trochoideum, using polyacrylamide gel electrophoresis and amino acid analysis. Two different chromatin isolation techniques were used (and mentioned in the previous paper) for comparative purposes. Both techniques gave essentially similar results, as did the protein isolated from the two different dinoflagellates.

Looking at the electrophoretic pattern of acid-insoluble proteins and comparing it to that of the corn plant, the pattern is less heterogeneous in terms of band number; also, the dinoflagellate acid-insoluble proteins are limited to a higher molecular weight range than those of corn, i.e. greater than 43,000. Acid-soluble proteins

extracted from log phase dinoflagellate cells include one major component which runs a little more slowly than histone IV (the fastest moving histone under the conditions used here). The molecular weight was determined to be approximately 16,000. The protein is not very basic; unlike typical eukaryote histones, it has a significant amount of tyrosine and phenylalanine (1.1% and 1.6%, respectively), 14.8% lysine and only 2.2% arginine, as well as one cystine residue. This basic protein is absent from stationary phase cells; a very thin band, however, is seen near the top of the gel which is absent from log phase cells.

Different patterns of nonhistones from chromatin of log and stationary phase cells were seen but the differences were not as striking as those seen with the acid-soluble protein.

To investigate the dinoflagellate containing two nuclei, Rizzo and Cox (1977) characterized the basic proteins of the binucleate dinoflagellate, Peridinium balticum. One of the two nuclei resembles a typical dinoflagellate nucleus and the second, a eukaryotic nucleus. The eukaryotic nucleus was separated from the dinoflagellate one and chromatin prepared basically using the $CaCl_2$ precipitation method previously described. Acid-soluble proteins isolated from the eukaryotic-type nucleus were electrophoresed on urea polyacrylamide gels and using calf thymus histones as markers, four bands ran with mobilities like those of H1, H2B, H2A and H4. Since they could not separate the dinokaryotic nucleus and found the same pattern for total

nuclear chromatin, they concluded that the source of the "histones" is from the eukaryotic nucleus. The conclusion was also based on the negative result obtained using alkaline fast green (Alfert and Geschwind, 1953) for the dinoflagellate nucleus and a positive test for the eukaryotic nucleus of P. balticum, as well as the finding here of histones from the eukaryotic nucleus free of the dinoflagellate nucleus.

MODIFIED BASES

A brief discussion is warranted here regarding the presence of modified bases in DNA to perhaps give us a clue as to the significance of their presence.

The only organisms outside of the dinoflagellates to contain 5HMU are the bacteriophages infecting Bacillus subtilis (Hemphill and Whitely, 1975; Kallen et al., 1962; Takahashi and Marmur, 1963). Wyatt and Cohen (1953) showed the presence of 5-hydroxymethylcytosine in lieu of cytosine in the DNA's of T2, T4, and T6 bacteriphages which infect E. coli using paper chromatography of acid hydrolyzed DNA.

Gorovsky et al. (1973) showed the presence of [^{6}N methyl]-adenine in the macronucleus of the eukaryote, Tetrahymena pyriformis. The amounts of macronuclear MeAde differed slightly between different strains, ranging between 0.65 to 0.80% of the adenine bases being methylated. The amount of MeAde in micronuclear DNA was at least tenfold lower than that found in the macronuclear DNA. In

addition, the amount of MeAde did not vary in different stages of culture growth or during starvation of cells in preparation for conjugation. This finding is interesting in light of the functions of the two different nuclei. The macronucleus is known to provide RNA for the cell, while the micronucleus functions in conjugation of the organism (Alfert and Das, 1959; Gorovsky and Goodard, 1969).

In a similar kind of study, Cummings et al. (1974) analyzed the DNA from five different syngens of Paramecium aurelia. They found, within the macronuclear DNA of the five syngens, from 2.1 to 2.5% [^{6}N methyl]-adenine replacing adenine. However, in contrast to the findings of Gorovsky et al. (1973) for T. pyriformis, micronuclear DNA from one P. aurelia syngen contained an amount of MeAde nearly equal to that found in macronuclear DNA (3.3% MeAde in the micronucleus). A second species, P. caudatum, showed a macronuclear DNA containing 4.7% of its adenine as MeAde.

[^{6}N methyl]-adenine has also been found in bacteria (Dunn and Smith, 1958).

5-methylcytosine is another modified base found in a large number of higher eukaryotes. For example, Dawid, Brown and Reeder (1970) studied the composition and structure of chromosomal and amplified ribosomal DNA's of the African clawed toad, Xenopus laevis. The rDNA from nuclear DNA of somatic cells of X. laevis contains ribosomal cistrons repeated 450 times. In oocytes, the rDNA is amplified approximately 1,000 times and these copies are

located extrachromosomally (Brown and Dawid, 1968). The two rDNA's were separated and nucleotide analyses performed. Chromosomal rDNA was found to contain 4.5% of its residues as 5MeC, whereas amplified rDNA contained no detectable 5MeC. The 5MeC residues were found to be located on both strands of chromosomal rDNA, including the gene regions that are transcribed *in vivo*. The overall percent G+C of the two rDNA's is the same, about 67%. The chromosomal rDNA showed a higher thermal stability compared to that of amplified rDNA, about 1.5 degrees centigrade. The two rDNA's, furthermore, could not be distinguished in terms of the complementary RNA's transcribed by *E. coli* RNA polymerase with respect to their hybridization to both rDNA's and the stability of these hybrids.

Nass (1973) compared 5MeC levels in viral transformed and nontransformed cultured mammalian cells, as well as levels in nuclear and mitochondrial DNA fractions. With growing baby hamster kidney (BHK) cells, the percent replacement of cytosine with 5MeC was 1.1 for nuclear DNA; in two transformed BHK lines, the percent replacements were 1.4 and 3.8. The 5MeC levels for the mitochondrial fractions remained fairly close, the nontransformed mitochondrial DNA having a 0.4% 5MeC replacement for cytosine and the two transformed cell lines having 0.5 and 0.6% replacements. Another finding was that the level of 5MeC varied with the stage of growth. For example, in L-cells (mouse fibroblasts) which were not growing, the mitochondrial DNA had a 0.2% and the nuclear DNA a 0.1%

replacement with 5MeC, while a growing culture had mitochondrial DNA with a 0.2% replacement but nuclear DNA with a 2.7% replacement.

Nass looked at *in vitro* methylation of the DNA's and found that mitochondrial DNA was a better substrate for nuclear methylase (five times better) than is nuclear DNA (i.e. mitochondrial DNA is undermethylated). Also, a mitochondrial DNA methylase was isolated.

Brown *et al*. (1977) examined the deoxyribonucleotide sequence specificity of a highly purified DNA methylase (400 fold purified) preparation obtained from Krebs II mouse ascites cells. Single stranded *E. coli* and native calf thymus DNA were methylated *in vitro* with methylase plus S-adenosyl-L-[^{3}H-Methyl]-methionine as the donor of methyl groups. The pattern of cytosine methylation resembles that predicted if methylation occurred in the sequences R-Yn-C-R (Y=pyrimidine, R=purine) and the sequences containing the newly methylated cytosines are very similar to those vertebrate DNA sequences found to be methylated *in vivo*.

Evans and Evans (1970) looked at the methylation of DNA in *Physarum polycephalum* during different times of the the mitotic cycle. They found that methylation of mainband nuclear DNA occurs during S and G_2 (there is no G_1) and that the rate of DNA methylation during G_2 is about 50% of the rate observed during S. Methylation was studied in two other DNA's- nuclear satellite and mitochondrial DNA. Methylation was seen in these, also.

One question arising with regard to base modification is when does such modification occur? Burdon and Adams (1969) demonstrated DNA methylation in cultured mammalian cells using synchronized cultures of mouse fibroblasts. Methylation was found to occur soon after the synthesis of the basic DNA structure. DNA synthesis was depressed by 94% using hydroxyurea, but DNA methylation continued up to one-third its normal level. With synchronized cultures, methylation occurred within an hour of the synthesis of the basic DNA polymer. Once made, the 5MeC was stable as no demethylation or deamination of this base was observed.

Guseinov et al. (1975) studied the distribution of 5MeC in the DNA of leaves of cotton plants, including healthy as well as wilt-infected ones. The DNA was first fractionated into highly repetitive, intermediate repetitive and into unique sequences. From healthy plants, it was shown that the highly repetitive and unique sequences are rich in G+C and the former is also rich in 5MeC; the percent 5MeC in the former is 5.8% and in the latter, 0.51%. The other fractions contain 5MeC in intermediate percentages. In wilt-infected plants, a twofold reduction is observed in the percent 5MeC in the highly repetitive fraction, the amount being about 2% and a similar reduction is present in the intermediate fractions also (and would, therefore, include the ribosomal cistrons). The amount of 5MeC in the unique fraction remains about the same. The amounts of the various DNA fractions and overall percent G+C's, however, remain nearly the same as those for the healthy

plants.

The authors mention unpublished findings that "super-methylation" of highly repeated sequences was found for animal DNA (which animal, though, is not mentioned).

A number of studies regarding modified bases in DNA has been done to ascertain the distribution of such bases. Rae's study (1973) has already been mentioned.

Harbers et al. (1975) looked at the 5MeC distribution in mouse L-cell satellite and mainband DNA's. DNA was labeled with [^{3}H-methyl]-methione and base analysis performed using two-dimensional thin-layer chromatography. The distribution of 5MeC was done on pyrimidine oligonucleotides using electrophoresis on cellulose acetate strips and homochromatography on DEAE-cellulose thin-layer plates. Mouse satellite DNA was found to contain approximately 3.5% 5MeC, roughly four times more than that of mainband DNA. 40% of the 5MeC was present in the sequence purine-5MeC-purine (Harbers et al., 1974); in pyrimidine oligonucleotides, ones such as CT_3 and C_2T_5 were preferentially methylated even though these oligonucleotides are not among the most common ones occurring in satellite DNA. In mouse mainband DNA, most of the 5MeC was also present in the sequence purine-5MeC-purine but methylation of the various pyrimidine oligonucleotides was very different than those found in satellite DNA, for example, C_2T.

Doskocil and Sorm (1962) looked for the distribution of 5MeC residues in the DNA of calf thymus, rat spleen, mouse leukemic liver and wheat germ. Calf thymus DNA

contained 1.5% 5MeC, rat spleen 1.3%, mouse leukemic liver 1.3% and wheat germ 6.7% 5MeC. The percent G+C's were 41.6, 41.5, 42.6 and 38.4, respectively. In all these DNA's, the highest degree of replacement of cytosine with 5MeC occurred in the sequence C-purine. In addition, wheat germ DNA has 5MeC in C-T and C-C.

5MeC distribution has also been visualized using the technique of immunofluorescence. Miller et al. (1974) made antisera to 5MeC which, in turn, reacted with the 5MeC residues in single-stranded DNA within fixed metaphase chromosomes and the residues then located by immunofluorescence. Mouse and human chromosomes were thus treated, and binding was observed in areas containing centromeric heterochromatin, i.e. in areas containing highly repeated sequences (Buhler et al., 1975).

In summary, then, several general trends emerge regarding the phenomenon of modified bases. More modification is seen in nuclear DNA's compared with organellar DNA's. Growing cells have more modification than non-growing cells. Transformed cell lines have more modified bases than non-transformed ones. Highly repetitive DNA's (excluding the amplified ribosomal cistrons of Xenopus laevis oocytes) show localization of modified bases.

POSSIBLE ROLES OF MODIFIED BASES

The presence of HMU, and its presence in such large amounts in C. cohnii, warrant some discussion of the phenomenon of modified bases with emphasis on possible

roles for these bases. An interesting idea was put forward by Tosi, Granieri and Scarano (1972) who studied 5-methylcytosine synthesis in developing sea urchin embryos. According to these authors, animal cells contain genes arranged into units of developmental information, called synchrons. A synchron is composed of a DP-gene, which codes for a DNA-modifying enzyme, and a number of gene clusters called DG-clusters, which are located in different locations on one or several chromosomes. A DG-cluster contains a specific prepromoter which is an area of DNA that specifically binds the product of the DP-gene, a promoter and the dependent structural gene. Two types of synchrons exist - the intermediate and the terminal. An intermediate synchron has among its structural genes one or more DP-genes. A terminal synchron is at the end of a cell differentiation pathway and has no DP-gene. Structural genes of the synchron are transcribed only if the prepromoter has a specific conformation which allows the promoter to bind RNA polymerase. A sequential synthesis during embryogenesis of DNA-modifying enzymes explains heritable turning on and off of specific synchrons according to a scheduled program. Specific DNA-cytosine-methylases and DNA-5MeC-aminohydrolases would bring about DNA modifications in prepromoters of the synchrons. The heritable changes would be GC to AT base transitions which occur at the times new cell lines arise.

Holliday and Pugh (1975) offer a similar kind of explanation to describe the differentiated state and the stability of the differentiated state. Extending Tosi et

al.'s model (1972), they feel that certain base modifications can lead to heritable changes in base sequences which, in turn, could control activity of adjacent structural genes. Holliday and Pugh explore these suggestions and add that such changes could operate developmental clocks which turn genes on or off after a specific number of cell divisions. They also propose that the same ordered control of the transcription of genes could be achieved by methylation of bases without changes in sequence. In addition, various methylating enzymes, during particular times of development, could methylate controlling areas such as promoters and operators, changing the conformation and thereby allowing or preventing RNA polymerase binding or repressor binding, respectively.

From another viewpoint, Sager and Kitchin (1975) propose that phenomena involving selective silencing by inactivation or elimination of specific chromosomes or of DNA molecules are all regulated by the same mechanism, namely, modification and restriction of DNA by enzymes with specificity for particular recogniztion sites. This proposal has a firm basis in the restriction-modification system of bacteria. Modifying enzymes may methylate particular DNA sites in the host cell; when a foreign DNA enters the host, unless it too is modified, it will be restricted, i.e. degraded by nucleases.

Riggs (1975) explains X-inactivation in mammals also in terms of DNA methylation. The author proposes that the inactivation center of one X chromosome is methylated

which, in turn, causes it to remain active. The model is based largely upon the restriction-modification system of bacteria. According to Riggs, in the zygote, before X chromosome differentiation, the primary inactivation center is unmethylated. The initial methylation is a slow process (requiring hours, otherwise both X chromosomes would be methylated). The chromosome then becomes activated, resulting then in the synthesis of two proteins. One protein changes the methylating enzyme so methylation of an unmethylated inactivation center (on the second X chromosome) is prevented. The altered methylase can, however, quickly methylate the half-methylated inactivation center. The second protein inactivates the X chromosome containing the unmethylated inactivation center. Thus, differentiation of the X chromosomes will be maintained through successive cell divisions and DNA replication.

Rae and Steele (1978) comprehensively review modified bases in eukaryotes, emphasizing the presence of 5HMU in dinoflagellates. They suggest a number of possibilities concerning the role of this modified base in dinoflagellates, favoring the view that 5HMU represents a neutral vestige of a mechanism that operated in earlier times. There existed in the earliest dinoflagellates or in their ancestors, a prokaryotic type of restriction-modification system. When this group of organisms began to diverge, the need for this protective restriction activity (considered to serve initially a purpose similar to that in bacterial systems today) was no longer needed with the advent of

other cellular characteristics, for example, the development of selective permeability. Without selective pressure to keep the restriction-modification system, restriction activity would need to be rapidly eliminated because of deleterious effects of relaxation of site specificity. The modification activity, however, could have become uncoupled with the restriction activity and need not have been eliminated if modification was functionally neutral. These proposals account for both the high levels of HMU in dinoflagellate DNA and the moderate degree of sequence specificity found for this base (Steele's unpublished findings are that almost twice as much HMU is bracketed by purines than thymine, i.e. 45% vs. 27%, and the 3'nearest neighbor of HMU is adenine about 50% of the time, with guanine or cytosine accounting for most of the remainder).

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