METHODS IN MICROBIOLOGY

Volume 18

Edited by

GERHARD GOTTSCHALK

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Institute for Microbiology
University of Göttingen
Göttingen, Federal Republic of Germany

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CONTRIBUTORS

Arthur G. Andrewes Chemistry Department, Saginaw Valley State College, University Center, Michigan 48710, U.S.A.

August Böck Institut für Genetik und Mikrobiologie, Universität München, 8000 Munich 19, Federal Republic of Germany

M. D. Collins Department of Microbiology, National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, England

G. E. Fox Department of Biochemical and Biophysical Sciences, University of Houston, University Park, Houston, Texas 77004, U.S.A.

John L. Johnson Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, U.S.A.

C. W. Jones Department of Biochemistry, University of Leicester, Leicester LE1 7RH, England

Synnøve Liaaen-Jensen Organic Chemistry Laboratories, Norwegian Institute of Technology, University of Trondheim, N-7034 Trondheim-NTH, Norway

W. Ludwig Institut Botanik und Mikrobiologie, Technische Universität München, D-8000 Munich, Federal Republic of Germany

H. Mayer Max-Planck-Institut für Immunbiologie, Freiburg i. Br., Federal Republic of Germany

J. Oelze Institut für Biologie II, Mikrobiologie, der Universität, Freiburg i. Br., D-7800 Freiburg, Federal Republic of Germany

R. K. Poole Department of Microbiology, Queen Elizabeth College, University of London, London W8 7AH, England

Karl Heinz Schleifer Technische Universität München, D-8000 Munich 2, Federal Republic of Germany

E. Stackebrandt[1] Institut für Botanik und Mikrobiologie, Technische Universität München, D-8000 Munich, Federal Republic of Germany

R. N. Tharanathan[2] Discipline of Biochemistry, C. F. T. R. I., Mysore 570013, India

Thomas G. Tornabene School of Applied Biology, Georgia Institute of Technology, Atlanta, Georgia 30332, U.S.A.

J. Weckesser Institut für Biologie II, Mikrobiologie, der Universität, Freiburg i. Br., D-7800 Freiburg, Federal Republic of Germany

[1] Present address: Institut für Allgemeine Mikrobiologie, Universität Kiel, D-23 Kiel, Federal Republic of Germany.

[2] Present address: Max-Planck-Institut für Immunbiologie, Freiburg i. Br., Federal Republic of Germany.

PREFACE

Volume 18 of "Methods in Microbiology" is concerned with components of bacterial cells that are of taxonomic interest. The techniques used for the analysis of some of these components are already widely applied and belong to the repertoire of many microbiological laboratories. This holds for the determination of the G + C content of bacterial DNA and of DNA–RNA homologies. Nevertheless, an up-to-date presentation of the appropriate methods seemed necessary. It is included in this volume together with procedures for the analysis of cellular components that are applied mainly in specialist laboratories. These methods deal with the analysis of 16 S ribosomal RNA, murein, lipopolysaccharides, ribosomal proteins, lipids, cytochromes, quinones, carotenoids, and bacteriochlorophylls. It is hoped that this volume will contribute to a wider application of these procedures and to a wider consideration of the taxonomic relevance of the components analyzed.

The editor wishes to express his gratitude first of all to the authors, who did an excellent job and who delivered their manuscripts, more or less, on time. Thanks are also due to the staff of Academic Press Inc. in London and to the Advisory Board Chairman, John R. Norris, who did everything to assure the rapid publication of this volume.

Gerhard Gottschalk

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1

Determination of DNA Base Composition

JOHN L. JOHNSON

Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA

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I. Introduction

The deoxyribonucleic acid (DNA) of most organisms contains the purine bases adenine (A) and guanine (G) and the pyrimidine bases thymine (T) and cytosine (C). Occasionally an organism may contain modified bases such as 5-methylcytosine or hydroxymethylcytosine. In most cases the modified base substitutes for only a small fraction of the parent base. The DNA in prokaryotic and eukaryotic organisms and in many of the viruses consists of double strands with specific base pairs (A + T) or (G + C) located at each position along the complementary strands. Since the recognition that the ratio of A + T to G + C base pairs may differ from one organism to the next and that the ratio in a given organism, usually expressed as mole percentage G + C (mol% G + C), is relatively constant, the mol% G + C values have been used for the comparative characterisation of organisms.

The chemical and physical properties of DNA are such that the base composition can be measured in several ways. Initially DNA preparations were hydrolysed and the bases separated by paper chromatography and then eluted from the paper and measured spectrophotometrically (Chargaff, 1955). Since then several physical and spectrophotometric properties of the DNA or DNA components have been correlated with the chromatographic data. This has resulted in several indirect methods for

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estimating mol% G + C values. It is the purpose of this chapter to review the methods presently available and provide protocols that incorporate recent advances in nucleic acid chemistry.

II. Methodology

The determination of the mol% G + C values of DNA can be separated into two parts, the isolation of the DNA and the determination of base composition.

A. DNA isolation

The isolation of DNA is the most time-consuming part of the mol% G + C analysis, and is the part most fraught with technical problems. Some of the procedures, such as CsCl buoyant density centrifugation, work best with high molecular weight DNA, and others, for example, base or nucleotide chromatography and spectrophotometric analysis, require DNA free from ribonucleic acid (RNA). In many instances, the mol% G + C analyses are done in conjunction with DNA homology studies (Chapter 2); therefore, most of the DNA isolation procedures will be covered in that chapter. Several procedures have been described for the rapid isolation of DNA from cells from medium-size culture volumes (Britten *et al.*, 1970; Cashion *et al.*, 1977; Gibson and Ogden, 1979; and Zadrazil *et al.*, 1973). Presented in this chapter is a rapid isolation procedure for the isolation of small amounts of RNA-free DNA (50–100 μg) suitable for an analysis.

1. Buffers and reagents

Sodium chloride–EDTA, pH 8.0: 0.15 *M* NaCl, 0.01 *M* ethylenediaminetetraacetic acid (EDTA).

Sodium dodecylsulphate (SDS): 20% (w/v) solution.

Sodium perchlorate: 5 *M* solution.

Phenol–chloroform: 1 : 1 (v/v) mixture of chromatography-grade liquid phenol equilibrated against the NaCl–EDTA salt solution and chloroform–isopentanol (24 : 1 v/v) to which is added 0.1% 8-hydroxyquinoline.

Tris–EDTA (TE) buffer, pH 7.2: 10 m*M* tris(hydroxymethyl)aminomethane (Tris), 1 m*M* EDTA.

TE-0.5 M NaCl: 0.5 *M* NaCl in TE buffer.

TE-1.0 M NaCl: 1.0 *M* NaCl in TE buffer.
TE-2.0 M NaCl: 2.0 *M* NaCl in TE buffer.
80% ethanol: 95% ethanol–water (24 : 1 v/v).
Proteinase K: prepare a 5-mg ml^{-1} solution in TE buffer just prior to use.
Ribonuclease (RNase): bovine pancreatic RNase, 0.5 mg ml^{-1} in 0.15 *M* NaCl, pH 5.0. Heat the solution at 80°C for 10 min to inactivate any traces of deoxyribonucleases (DNase). Store at −20°C. Just prior to use add T_1 RNase to the pancreatic RNase preparation to a concentration of 500 units ml^{-1}.
NACS-52: This is a gravity flow chromatography matrix which fractionates primarily by ion-exchange mechanisms. Bound nucleic acids are eluted from columns predominantly in order of increasing molecular weight. Prepare for use according the manufacturer's manual (Bethesda Research Laboratories, Inc.).

2. *Protocol*

1. Grow the cells in 20–40 ml of a suitable medium to the late log or early stationary phase of growth. Centrifuge the cells and resuspend them in 5.0 ml NaCl–EDTA salt solution. Using a French pressure cell, disrupt the cells into a flask containing another 5.0 ml of the salt solution, 0.5 ml of 20% SDS, and 2.5 ml of 5 *M* perchlorate. Add 0.15 ml of proteinase K solution and incubate for 1 h at 60°C.

2. Add 5.0 ml of phenol–chloroform and shake the flask for 20 min on a wrist-action shaker. Centrifuge the emulsion at 17,000 *g* for 10 min. Remove the aqueous supernatant to a second tube and add 2 volumes of cold 95% ethanol, mix, store in a −20°C freezer for 1 h, and again centrifuge as above. Pour off the ethanol, allow the tube to drain inverted on a paper towel for a few minutes, and then carefully add 10 ml of 80% ethanol (−20°C) so as not to disrupt the nucleic acid pellet. Rotate the tube so that the 80% ethanol will make contact with all of the surfaces of the tube. Place the tube in the −20°C freezer for 0.5–1 h and recentrifuge with the tube being placed in the same orientation as during the first centrifugation so that the pellet will not be dislodged. Pour off the ethanol and allow the tube to drain well and partially air dry.

3. Dissolve the nucleic acids in 2.0 ml of TE buffer and add 0.3 ml of the RNase mixture, mix and incubate at 37°C for 0.5 h. Add 0.5 ml of chloroform–isopentanol. Briefly mix two or three times on a vortex mixer and centrifuge at 17,000 *g* for 100 min.

4. Transfer the aqueous supernatant to another tube, add 0.2 ml of 3

M sodium acetate, mix, add 2 volumes of 95% ethanol, and mix again. Place the tube in the −20°C freezer for 1 h, centrifuge at 17,000 *g* for 10 min, and wash the pellet with 80% ethanol as outlined above. Air dry the pellet; a 37°C incubator works well.

5. Dissolve the pellet in 1.0 ml of TE. After the DNA is in solution, add 1.0 ml of TE-1.0 *M* NaCl.

6. Prepare a NACS-52 column, 0.5–0.75 ml bed volume in a Pasteur pipette or other small column, as recommended by the manufacturer (Bethesda Research Laboratories, Inc.). The NACS-52 used to pack the column must first be hydrated with TE-2.0 *M* NaCl buffer. Wash the column with several column equivalents of TE-0.5 *M* NaCl. Load the sample by gravity flow and pass it through a couple of times to ensure maximum binding of the DNA. The RNA fragments should not bind and will be eluted by running an additional 5.0–6.0 ml of the TE-0.5 *M* NaCl buffer through the column. The last of the buffer is removed from the column by gentle air pressure (a small rubber bulb works well for this) and the DNA eluted by sequentially adding 3 0.2-ml volumes of TE-1.0 *M* NaCl buffer. Force the buffer out between each addition with gentle air pressure.

7. Add 2 volumes of 95% ethanol to the eluate, mix, then place in the −20°C freezer and proceed with the centrifugation and 80% ethanol wash as outlined above. Dissolve the pellet in 0.5 ml of distilled water, and 50 μl of 3.0 *M* sodium acetate, mix, and add 1.0 ml of 95% ethanol for a final precipitation and pellet wash. After air drying the pellet, dissolve the DNA in 0.5 ml of distilled water. DNA isolated in this manner is ready for use in the optical and chromatographic methods of mol% G + C analysis.

3. Comments

1. This procedure works well for bacteria that are readily disrupted by passage through a French pressure cell; it may not work as well for Gram-positive cocci or small rods. Because of the small fragment size of DNA prepared in this manner, it will not work very well for buoyant density centrifugation.

2. The NACS-52 column step probably would not be needed for the T_m method.

3. Many of the procedures described below require DNA preparation free of RNA. The presence of RNA contamination can be demonstrated and an estimation of the amount obtained by electrophoresing DNA preparations in polyacrylamide gels (partially degraded RNA will migrate near the dye front), and then staining the gel with a silver stain (Beidler *et al.*, 1982; Igloi, 1983).

B. Methods for measuring DNA base compositions

Unique physical, chemical, and optical properties of DNA enable one to determine mol% G + C values in several different ways. Initially the bases were released from the DNA by acid hydrolysis, separated by paper chromatography, quantitatively eluted from the paper, and measured spectrophotometrically (Chargaff, 1955; Wyatt, 1955; Werner *et al.*, 1966). Although seldom, if ever, used at the present time, it is interesting to note that most other procedures for estimating mol% G + C values are based upon correlations with results obtained by quantitative paper chromatography.

1. Buoyant density centrifugation

The density of DNA in CsCl increases linearly with the mol% G + C. Schildkraut *et al.* (1962) were the first to publish an equation correlating the density of DNA preparations with mol% G + C values determined chemically. Subsequently, other investigators have carried out similar investigations (De Ley, 1970; Gasser and Mandel, 1968). Shown in Fig. 1 are the regression lines from the various studies. The slopes of the lines

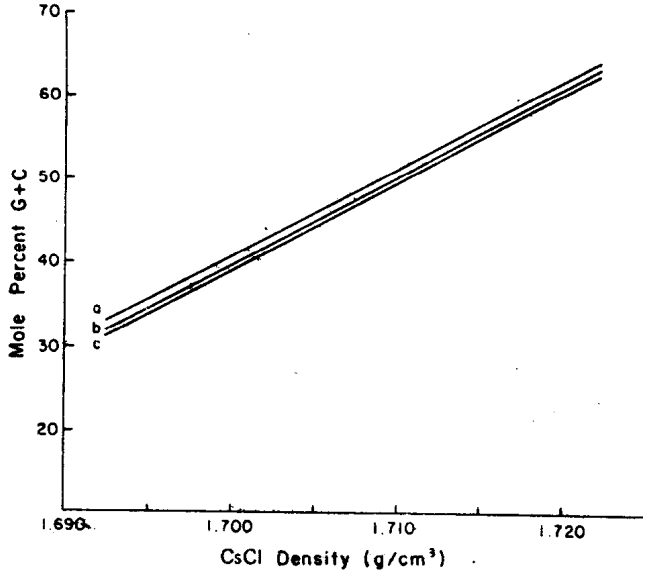

Fig. 1. Regression lines for published buoyant density equations: (a) Schildkraut *et al.* (1962); (b) De Ley (1970); and (c) Gasser and Mandel (1968).

are essentially the same although the constants do vary a little, such that the differences in mol% G + C using the different equations range from 1 to 2%.

The buoyant densities are best measured using high-molecular-weight DNA, since the width of the bands is a function of the fragment length. Advantages of the procedure are that only small amounts (a few micrograms) of DNA are required and it is not overly sensitive to RNA and protein contamination, although tenacious binding of pigments to DNA has caused density alterations (Enquist and Bradley, 1971). Disadvantages of the procedure are that an analytical ultracentrifuge is required and the typical centrifugation run is 44 h.

For a detailed description of the procedure the reader is referred to Mandel *et al.* (1968).

2. *Thermal melting profile (T_m)*

When a preparation of DNA is heated to high temperature, the bonding between the base pairs collapses and the two DNA strands separate (denature). This denaturation phenomenon can be measured readily with a UV spectrophotometer. As the DNA goes from the native state to the denatured, the absorbance at 260 nm will increase by about 40%. Although the melting curves appear as smooth transitions when the rate of temperature increase is in the range of 0.5–1.0°C min^{-1}, when high-resolution melting curves (temperature increase in the range of 0.05°C min^{-1}) are analysed as the derivative curves, the melting profile appears as a collection of subtransitions (thermalites) which have an average length of about 900 bp (Ansevin *et al.*, 1976; Vizard and Ansevin, 1976). This chapter will be limited to melting profiles obtained by rates of temperature increase in the 0.5–1.0°C range.

The midpoint temperatures of the thermal melting profiles (T_m) of DNA increase with increases in the mol% G + C. These values were first correlated with the chromatographically determined base composition of DNA by Marmur and Doty (1962). Although the A + T and G + C polymers did not fit on the linear regression line, there was a linear correlation for DNA preparations ranging from about 25 to 75 mol% G + C. De Ley (1970) and Mandel *et al.* (1970) have re-examined this correlation and have also correlated the T_m values with the CsCl buoyant density values. Regression lines for equations are shown in Fig. 2. Also included in Fig. 2 is the regression line for the equation by Owen *et al.* (1969) which includes the Na^+ molarity of the salt solution. The equations of De Ley (1970) and Marmur and Doty (1962) are nearly identical as is the slope of

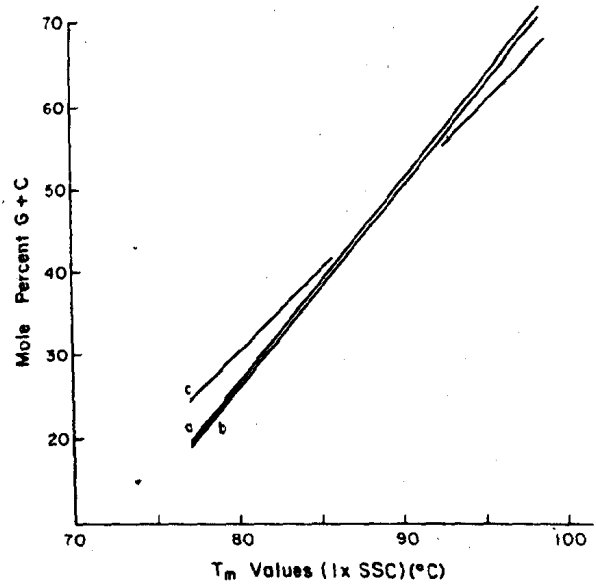

Fig. 2. Regression lines for published thermal melting point (T_m) equations: (a) Marmur and Doty (1962) and De Ley (1970); (b) Owen *et al.* (1969); and (c) Mandel *et al.* (1970).

the Owen *et al.* (1969) equation. The Mandel *et al.* (1970) equation has less of a slope, and as a result, when using this equation, mol% G + C values will be higher than with the other equations for mol% G + C DNA preparations that are less than 51 and lower for DNA preparations greater than 51 (when using *Escherichia coli* DNA as the standard).

The midpoint of the hyperchromic shift has been established by several different methods, including a graphic determination (Marmur and Doty, 1962), and the use of normal probability paper (Knittle *et al.*, 1968). Ferragut and Leclerc (1976) compared four methods and concluded that the graphic determination of T_m was the method of choice for routine analysis. This method is illustrated in Fig. 3.

The melting profile curve is generated by gradually heating a DNA sample in the cuvette chamber of an ultraviolet spectrophotometer and continuously measuring the absorbance of the sample(s) and the temperature within the cuvettes or cuvette chamber. Historically, the temperature of one of the cuvettes was measured using a mercury thermometer and the temperature was increased stepwise using an external temperature bath. Many of the newer spectrophotometers have electronically heated cuvette holders with thermistors embedded within them and are either microprocessor or computer controlled. When performing melting profiles, the two major variables about which an investigator must be concerned

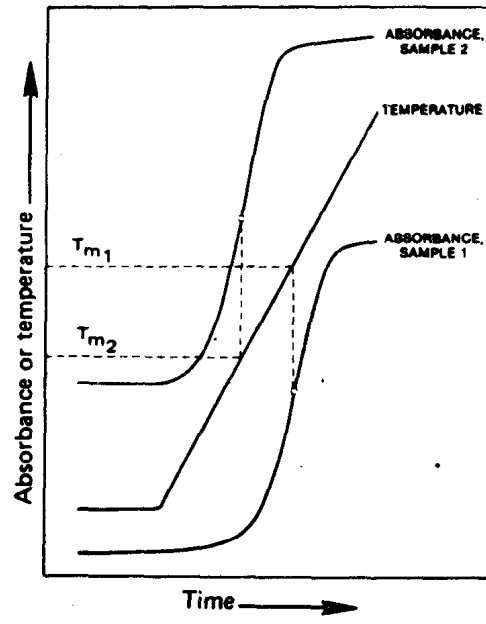

Fig. 3. Graphic representation of a thermal melting profile. The midpoint of the profile (T_m) is used for comparison with other T_m values. From Johnson (1981).

are the ionic strength of the buffer and the accuracy of the temperature. The ionic strength of the buffer has a large effect on the T_m of a DNA preparation (Schildkraut and Lifson, 1965). The ionic strength of the buffer can be standardised for the experiment by dialysing all of the DNA samples against the same batch of buffer, and the temperature is best standardised by including a DNA standard in each spectrophotometer run (*E. coli* b or K-12 is usually used).

(*a*) *Buffers and materials.* 1. *0.5× standard saline citrate (SSC):* Standard saline citrate is 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0, and other concentrations of it are indicated by a number such as 20× (20-fold concentration) or 0.1× (one-tenth the concentration). For most DNA preparations, the T_m values in 0.5× SSC are low enough to ensure that the hyperchromic shift is complete at a temperature of less than 100°C.

2. Wash pieces of dialysis tubing (½ × 51 in.) by placing them into a 1–2% solution of Na_2CO_3 and boiling for about 5 min. Rinse the tubing extensively with tap water and then place into a beaker of distilled water.

(*b*) *Protocol.* 1. Prepare 2–4 litres of 0.5× SSC, saving some of it for diluting the DNA preparations and for rinsing out cuvettes just prior to

putting the DNA samples into them. Divide the rest so the buffer can be changed once during dialysis.

2. Prepare 2- to 5-ml samples (depending on the size of the cuvettes to be used) of DNA at 50 μg ml^{-1}, using 0.5× SSC to dilute the stock DNA preparations. Prepare a 10- to 20-ml volume of the standard DNA (e.g., the DNA of *E. coli* b, which has a G + C content of 51 mol% and a T_m of 90.5°C in SSC), because it will be used in each instrument run.

3. Dialyse all of the preparations overnight in the 0.5× SSC at 4°C. Change the buffer once after the first few hours of dialysis. After dialysis, return the DNA preparations to screw-cap tubes.

4. Determine the melting profiles with an automatic recording spectrophotometer having a sample chamber that is heated by a circulating bath containing ethylene glycol, or preferably one that has an electronically heated cuvette holder. A thermistor should be located in the cuvette chamber, the cuvette holder, or in the cuvette. Having the thermistor within the cuvette is theoretically the best, but it is very convenient when it is located in the cuvette holder and there appears to be very little difference in accuracy. The temperature variations within an electronically heated cuvette holder can be determined by placing samples of the same DNA preparation into each cuvette position and comparing the resultant T_m measurements. Even when the thermistor is located in the chamber, and the chamber is heated by passing ethylene glycol through the chamber walls, cuvettes holding about 1.0 ml of sample may be heated linearly at rates greater than 1.0°C min^{-1}, although the actual temperatures do lag behind those of the circulating fluid. It is important to make two or three measurements on each DNA preparation for improved accuracy (De Ley, 1970). Neutralise any temperature variations that there may be from one cuvette position to the next by running the samples at different cuvette positions.

5. Start at 60°C and determine the T_m values as shown in Fig. 3.

6. Calculate the mol% G + C of each sample DNA by using the following Marmur and Doty equation (1962), which has been modified for normalising the T_m values in 0.5× SSC to T_m values in SSC. This general procedure can also be used for other ionic strength buffers:

$$\text{Mol\% G + C} = \frac{[(A - B) + C] - 69.3}{0.41}$$

where A = T_m of the DNA standard in SSC (for *E. coli*, 90.5); B = T_m of the DNA standard in 0.5× SSC; and C = T_m of the test DNA in 0.5× SSC.

The $A - B$ value is the difference between the T_m of the DNA standard in SSC and 0.5× SSC. Adding this value to the T_m of the test DNA normalises it to a T_m in SSC. The rest of the values are from the Marmur

equation. Extrapolating to 100 mol%, A + T intercepts the temperature axis at 69.3°C and the slope of the line is 0.41.

(*c*) *Comments.* These analyses are usually determined using cuvettes that are from 0.3 to 3.0 ml in capacity. These cuvettes are satisfactory when plenty of DNA is available; however, for DNAs that are difficult to isolate in large quantities, such as plasmid, phage, or a recombinant DNA fragment, there would be an advantage in using smaller cuvettes. To this end, microcuvettes have been constructed so that less than 1 μg of DNA is needed for an analysis (Ansevin and Vizard, 1979; Karsten *et al.*, 1980).

This procedure is not particularly sensitive to RNA contamination. If there is still secondary structure in the RNA fragments, the base line will gradually go up before the DNA hyperchromic shift begins. The DNA fragment size does affect the T_m. The T_m of high-molecular-weight DNA, e.g., isolated by the Marmur procedure (1961), is 1–2°C higher than the same DNA preparation after it has been passed through a French pressure cell. Therefore, all of the DNA preparations being analysed, including the standard, should be prepared in the same manner. Also it should be noted, that the fragment sizes of several DNA preparations may not be of similar lengths, even if they are prepared in the same way. Nuclease activity during DNA isolation can cause this type of problem. We have found this to be most pronounced among low mol% G + C organisms, for example, clostridia and fusobacteria (Selin *et al.*, 1983).

3. High-performance liquid chromatography (HPLC)

As previously noted, all of our present indirect methods of estimating G + C content are based on the acid hydrolysis of DNA and quantitative paper chromatography of the bases. However, because of the extensive work involved, few of these chromatographic analyses have been done since the early 1960s. Recent advances in HPLC instrumentation and columns now enable investigators to make direct determinations of the nucleic acid bases, deoxyribonucleosides, and deoxyribonucleotides rapidly and easily. A schematic representation of HPLC instrumentation is shown in Fig. 4. The components include (1) a solvent reservoir for the mobile phase; (2) a pump to move the solvent at high pressure through the column; (3) a pressure gauge to measure pump pressure; (4) an injection device, usually a sample loop; (5) a column; (6) a detector (for nucleic acid components, a UV photometer); and (7) a potentiometric recorder for producing a written record of the analysis, the chromatogram. The instrument can be microprocessor controlled to generate solvent gradients automatically, to

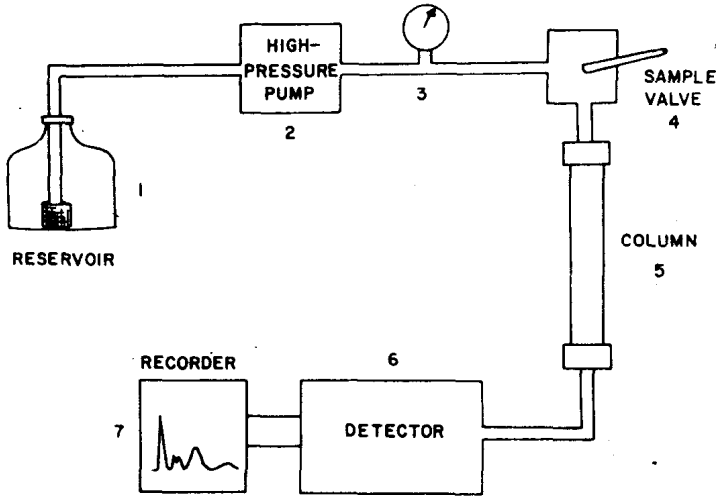

Fig. 4. Major components of a high-performance liquid chromatography system (HPLC). Reprinted from McNair (1980). © 1980 International Scientific Communications, Inc.

inject samples, and to quantitate the components by integration of the chromatograph peaks (McNair, 1980).

The UV detectors or monitors may be of a fixed wavelength (usually 254 nm), dual wavelength (254 and 280 nm), or variable wavelength (from about 190 to 300 nm). A photodiode array detector has recently been described which consists of 211 diodes covering a spectral range from 190 to 600 nm. This detector is able to take a spectrum in 10 ms (Elgass *et al.*, 1983). In addition to providing accurate analysis, it will allow for determining peak purity and peak identification. Other types of detectors that have been employed include fluorescence, conductivity, mass spectrometer, IR absorbance, refractive index (Wheals, 1982), voltametric, and polarographic (Štulík and Pacáková, 1983).

The three major types of HPLC columns used for the analysis of bases, nucleosides, and nucleotides are reversed phase, ion pairing, and ion exchange, either cation or anion (Zakaria and Brown, 1981). In reversed-phase chromatography (Caronia *et al.*, 1983; Ehrlich *et al.*, 1982; Gehrke *et al.*, 1978; Gehrke *et al.*, 1980; Hartwick *et al.*, 1979; Kraak *et al.*, 1981; Kuo *et al.*, 1980; Whitehouse and Greenstock, 1982; and Zumwalt *et al.*, 1982), which is used to separate both polar and non-polar compounds, hydrophobic interactions determine the extent of retention. A hydrophobic portion of the sample partitions into the hydrophobic surface of a chemically bonded material. The more polar or ionic solutes favour the