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PRODUCTION OF THYMINE DIMER IN DNA FROM MESOPHILIC AND THERMOPHILIC BACTERIA BY UV IRRADIATION

by

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PRODUCTION OF THYMINE DIMER IN DNA FROM MESOPHILIC AND THERMOPHILIC BACTERIA BY UV IRRADIATION

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Western Michigan University, 1987

The in vitro production of thymine dimer (TT) in DNA, isolated from the mesophile (B. licheniformis) and the thermophile (B. stearothermophilus), was carried out by UV irradiation (254 nm) at three different temperatures (35, 45, and 55°C). The production of TT from the thermophilic DNA was significantly higher than that from the mesophilic DNA. This was the case at all three irradiation temperatures. The increase in TT production per 10°C increase in irradiation temperature was four times higher for the thermophile than that for the mesophile. It was concluded that the DNA from the thermophile was photochemically less stable than that from the mesophile, even though the DNA of the thermophile was more heat stable than that of the mesophile.

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Fredrick Shu-Chung Yein

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CHAPTER I

INTRODUCTION

According to Watson and Crick's model (1), deoxyribonucleic acid (DNA) consists of two helical strands which form a double alpha-helix (Figure 1a). Each strand of the helix is a polynucleotide chain, and each nucleotide consists of a sugar (2-deoxyribose), a phosphate group, and a nitrogenous base (pyrimidine or purine). The common pyrimidines are thymine (T) and cytosine (C), and the common purines are adenine (A) and guanine (G). The bases are attached at the C-1 position of the sugar while the nucleotides are connected by phosphodiester linkages between C-3 of one sugar and C-5 of an adjacent sugar (Figure 1b). The backbone of DNA consists of alternating deoxyribose and phosphoric acid residues, and the bases project from the backbone of the strands toward the interior of the DNA molecule. The bases in one strand are complementary to those in the other strand; every A is paired to every T by two hydrogen bonds while every C is paired to every G by three hydrogen bonds (Figure 1c). The base-pairs thus formed are stacked, one above the other, in the interior of the double helix and are held together by hydrophobic interactions. The two helices are right handed and one strand cannot be unwound without unwinding the other strand. The pitch of the B form DNA helix is 34 angstroms, and there are 10 base pairs per turn of the helix. The molecular weight of the DNA varies widely depending on the isolation procedure; it is generally in the order of several million daltons.

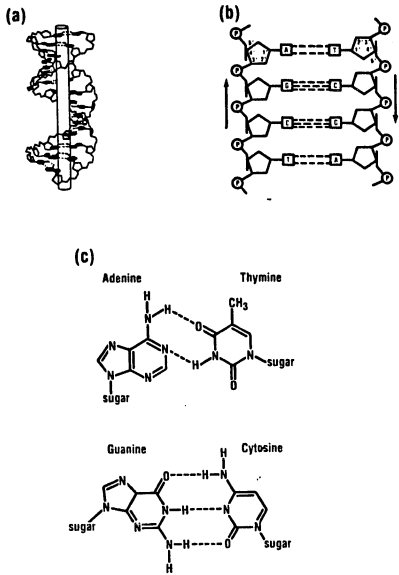
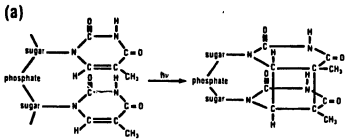


Figure 1. The Structure of DNA.

It has long been known that ultraviolet radiation (UV) can damage living cells. One effect of UV is the formation of pyrimidine dimers between two adjacent pyrimidine nucleotides in DNA (2,3). For example, two adjacent thymine bases on a strand of DNA may be fused by saturation of the C5-C6 double bond to form a cyclobutane ring between the bases (Figure 2a); four stereoisomers (Figure 2b) of the cyclobutyl dimer may exist (4).

Of the three possible types of pyrimidine dimers, the thymine dimer (TT) is the predominant photoproduct, while the cytosine-thymine dimer (CT) and the cytosine dimer (CC) are formed in relatively small amounts (5). Such a dimer, if not removed or repaired, poses a block to DNA replication and thus, may be lethal to living cells (3). To attenuate the catastrophic effects of this lesion, cells have developed mechanisms to repair such damage (6,7).

In dilute aqueous solution, thymine is virtually resistant to dimerization by irradiation with small doses of UV light (8). However, TT is formed readily at a high yield by UV irradiation of frozen thymine solutions. During freezing of a thymine solution, water becomes excluded, and the thymine molecules come close together to form aggregates. Formation of such aggregates has been demonstrated spectroscopically (9,10). These observations suggest that the close positioning of thymine molecules, and their proper orientation relative to each other are important factors in TT formation. These facts relating to frozen thymine solutions may explain the relatively easier TT formation by UV irradiation in DNA (11,12) as compared to that in aqueous thymine solutions. In double helical DNA, two adjacent thymine molecules are fixed in very close proximity; they are 3.4 angstroms apart on the DNA



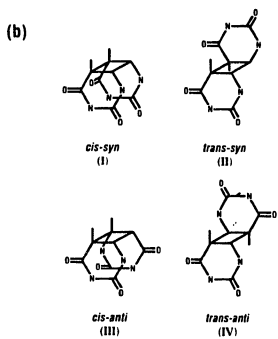


Figure 2. Thymine Dimer Formation.

strand. When two such thymine molecules are properly oriented relative to each other, cyclobutyl TT formation may readily take place upon UV irradiation. When aqueous solutions of DNA are irradiated, the TT stereoisomer of type I (Figure 2b) is the chief photoproduct; type II is formed in relatively small amounts, while type III and IV are scarcely present (13,14). During UV irradiation of an aqueous solution of thymine, a photohydrate (6-hydroxy-5,6-dihydro-thymine) is also formed. However, this photohydrate is highly unstable, and after the end of irradiation, it converts to the original thymine within 1.0 - 2.0 minutes. This photohydrate of thymine, possibly due to its instability, has never been isolated from irradiated DNA solutions (14).

Thermophiles are bacteria which grow at much higher temperatures (about 55 - 80^{o}C) than mesophiles which grow at moderate temperatures (about 20 - 45^{o}C). One of the most plausible theories of thermophily, the macromolecular theory, ascribes thermophily to physical-chemical differences of important macromolecules, resulting in the macromolecules of thermophiles being more heat stable than those of mesophiles (15). Among the studies supporting this theory are those of Stenesh et al. (16,17) on nucleic acids from mesophiles and thermophiles. It was found that DNA and RNA from thermophiles had higher G+C content and higher melting-out temperatures than those from mesophiles. This thermal stability of the nucleic acids could be correlated with the optimal growth temperatures of the organisms.

In an attempt to explain thermophily on the basis of the macromolecular theory, most studies so far have attempted to find a positive correlation between the optimal growth temperature and the thermal stability of specific macromolecules (16,17). On the other

hand, a study by Shaikh (18) showed that, in terms of the UV energy required to kill one cell, a thermophile (Bacillus stearothermophilus) showed an overall greater sensitivity to UV irradiation compared to a mesophile (B. licheniformis). These data suggest that there may be a difference between the thermal stability and the photochemical stability of such organisms in relation to the macromolecular theory of thermophily.

The purpose of this study was to further test the applicability of the macromolecular theory of thermophily by comparing the in vitro photochemical stability of DNA from a mesophile (B. licheniformis) to that from a thermophile (B. stearothermophilus). Photochemical stability is taken to be inversely proportional to the production of TT in the UV irradiated DNA.

DNA and its nitrogenous bases absorb strongly in the UV region (200 - 300 nm) with the maximum absorption around 260 nm. According to the Grothus-Draper law (19), photochemical changes take place only by the action of light absorbed by the system. Therefore, UV light at 254 nm was used as the light source for the irradiation throughout the entire study in order to produce the maximum photochemical effect on DNA. Since TT is the major UV photoproduct of DNA, only TT formation was investigated in this study. No attempt was made to quantitate each of the TT stereoisomers. The production of TT as a whole was measured by high performance liquid chromatography (HPLC) and the effect of irradiation temperature on TT production was determined.

CHAPTER II

MATERIALS AND METHODS

Synthesis of Thymine Dimer

For synthesis of pure thymine dimer (TT), the method described by Gunther and Prusoff (4) was followed. Thymine (800 mg, Calbiochem) was dissolved in 250 ml of distilled water. An aliquot of 10 ml of the thymine solution was transferred to a glass petri dish having a diameter of 95 mm. The dish was then placed in a dry-ice/ethanol bath to a depth of about 0.5 cm and gently swirled in order to freeze the solution into a solid layer with uniform thickness; there was only a thin layer of ethanol underneath the petri dish. The frozen thymine solution in the open dish was then irradiated with shortwave ultraviolet radiation (254 nm) provided by an ultraviolet lamp (Mineralight Model SL 2537, UVP Inc.). The irradiation was performed by placing the dish in a -20°C freezer, at a distance of 20 mm from the lamp, and irradiating for 30 min with a flux of 12 J sec^{-1} m^{-2}. The ultraviolet intensity was measured with a Black-Ray, shortwave ultraviolet meter (Model J225, UVP Inc.). At the end of the irradiation, the dish was removed from the freezer and the solid frozen solution was carefully chipped off with a spatula and transferred into a round bottom flask. The solution was then thawed at room temperature, and evaporated under reduced pressure at 60°C to about one-third of the original volume by using a rotatory evaporator with a thermostated water bath.

The concentrated solution was heated to boiling, filtered through a sintered glass funnel having medium pore size, and kept at 4°C for 48 hours for complete crystallization. The crystals were collected on an identical sintered glass funnel and then resuspended in absolute ethanol in a glass test tube. The crystals in the suspension were collected by centrifugation at 2000 x g at 4°C for 10 min and then washed five times with absolute ethanol by resuspension and centrifugation in order to remove residual thymine. After the last wash, the crystals were redissolved in 3 ml of boiling water and recrystallized at 4°C as described above. The crystals from the second crystallization were collected and dried in a vacuum desiccator at room temperature.

For the synthesis of tritiated thymine dimer, 1.0 mCi of thymine (methyl-^{3}H, specific activity 55 Ci/mmole, New England Nuclear, Inc.) was mixed with 120 mg of nonradioactive thymine in a total volume of 40 ml, and then thymine dimer synthesis was carried out by identical procedures to that described above.

Isolation of DNA

A mesophile, Bacillus licheniformis (NRS 243) and a thermophile, Bacillus stearothermophilus (B-10) were used for this study. The mesophile and the thermophile were grown, respectively, at 37°C and 55°C according to the procedures developed in this laboratory (20). The cells were grown aerobically in a 9.0 liter jar, using 4.0 liter of a medium consisting of 1% Trypticase (BBL) and 0.2% yeast extract (Difco). Additionally, 0.04 ml of antifoam (SAG 471, Union Carbide Corp.) were added for every liter of medium. Cell growth was followed by absorbance measurement at 540 nm, and the cells were harvested during their late

logarithmic phase (absorbance approximately 0.9 - 1.2). The culture was quickly chilled by plunging the jar into ice and the cells were collected by centrifugation at 16,000 x g at 4°C for 20 min, using a Sorvall centrifuge (Model RC5C, Sorvall Instruments, GSA rotor). The cells were washed and the DNA was isolated from the two organisms according to the procedure of Marmur (21). The purified DNA was dialyzed against water for 24 hours at 4°C, lyophilized and stored in an amber bottle at -20°C.

Analysis of Thymine Dimer

To prepare the TT solution for spectrophotometric analysis, 2.0 mg of TT, prepared as described above, were dissolved in 1.0 ml of 2 M NaOH and then 40 ml of sodium phosphate buffer solution (0.1 M, pH 7.0) was added. The pH of this solution was adjusted to 7.0 with 0.5 M HCl. The volume of the solution was then made up to 50 ml with the above sodium phosphate buffer. This TT solution was the working stock solution; it was stored in an amber vial at 4°C, and further diluted with the sodium phosphate buffer for spectrophotometric analysis.

Spectrophotometric measurements were made with a double beam analytical Hitachi spectrophotometer (Model 100-80A), using two quartz cuvettes (0.6 ml) having a 1.0 cm light path and scanning the absorption spectrum between 220 to 400 nm. The spectrum of a TT solution was compared with that of a thymine solution prepared in the same buffer.

In order to determine the photoreversibility of TT formation in solution, 7.0 ml of the TT solution (0.15 M) in a glass dish (60 x 15 mm; diameter x height) were irradiated, with gentle stirring, using UV light (254 nm) at a dose of 8 J sec^{-1} m^{-2}. At different irradiation intervals, 0.5 ml of the solution was removed and transferred into the

cuvette for spectrophotometric scanning.

Labeled thymine dimer (^{3}H-TT) was also prepared as described above and then dissolved to a concentration of 0.1 mg/ml in ammonium phosphate buffer (0.05 M, pH 3.5). To a 0.1 ml aliquot of the ^{3}H-TT solution were added 15 ml of ACS scintillation fluid (Amersham Corp.) and the solution was counted in a Packard Scintillation Counter (Model 3330). The counting efficiency was estimated by the external standard method (22). The specific radioactivity of ^{3}H-TT was calculated by using a molecular weight of 262 daltons.

Elemental analysis of TT was performed using a Perkin-Elmer Elemental Analyzer (Model 240B).

High Performance Liquid Chromatography (HPLC)

Standard solutions of cytosine, uracil, adenine, guanine (P-L Biochemical, Inc.) and thymine (Calbiochem), were prepared by dissolving 1.0 mg of each base in 2.0 ml of ammonium phosphate buffer (0.05 M, pH 3.5). Standard TT solutions were prepared by first dissolving 1.0 mg of either radioactive or non-radioactive TT in 0.5 ml of 2.0 M NaOH. The solution was then mixed with 0.5 ml of 0.1 M $NH_4H_2PO_4$ and finally carefully titrated to pH 3.5 with 0.5 M HCl. This solution was then made up to 2.0 ml by the addition of 0.05 M ammonium phosphate buffer (pH 3.5). In order to determine the retention time for each compound, a 0.02 ml aliquot of each of the properly diluted standard solutions (see below) was injected onto the chromatographic column under the following HPLC conditions:

HPLC: Kontron 720LC System with LC Pump 410

Column: uBondapak C18 Reverse Phase (Waters Assoc.), 300 mm x

3.9 mm I.D., 10 micron particle size.

Detector: Kontron 760, variable wavelength UV detector.

Detector wavelength: 230 nm and 260 nm.

Injector: Rheodyne Injector with 0.02 ml injection loop.

Solvent: 0.1 M ammonium phosphate buffer, pH 3.5, containing 0.1% acetonitrile.

Flow rate: 1.0 ml/min.

Recorder: Kontron 21, variable dynamic range and chart speed.

Concentration of Standards: Bases (stock solution diluted 1:250); TT or ^{3}H-TT (stock solution diluted 1:200).

In order to evaluate the chromatographic resolution of the six standards, 0.02 ml of a mixture of the standard solutions prepared above, was injected onto the column. The mixture contained about 0.002 mg of each standard per ml.

Purity of the unlabeled TT standard solution was determined by injecting 0.02 ml of solution onto the chromatographic column. Purity of the labeled TT standard solution was determined by adding 0.1 ml of radioactive TT solution, containing 1.2 x 10^{6} cpm, to 0.5 ml of unlabeled TT solution (1.0 mg/2.0 ml). An aliquot of 0.02 ml of this mixture was then injected onto the column for HPLC analysis. Fractions of column effluent were collected at 10 sec intervals and placed in scintillation vials containing 15 ml of ACS scintillation fluid. The radioactivity was then counted as described above.

Standard Curve for Thymine Dimer

Standard solutins of TT were prepared as described in the previous section (final concentration 0.1 mg/ml). This solution was then further

diluted with the ammonium phosphate buffer to prepare six more diluted standards. Aliquots of 0.02 ml of each standard were then analyzed by HPLC. The height of the TT peak was measured, and a standard curve was constructed by plotting peak height as a function of concentration.

Thermal Denaturation Profiles and Spectral Ratios of DNA

The DNA, isolated as described in the previous section, was dissolved in sodium phosphate buffer (1.0 mM, pH 7.0) to a concentration of about 0.04 mg/ml. The absorbance at 260 nm was measured in a Perkin-Elmer Lambda 5 Spectrophotometer equipped with a thermoelectric single cell holder (Model C550-0555) and temperature programmer (Model C570-0710). The temperature was programmed to rise from 25 to 75°C at a rate of 1.0°C/min. The melting out temperature, Tm, was defined as the temperature corresponding to the midpoint of the maximum change in absorbance as calculated from the thermal denaturation profile recorded on a chart recorder. Determinations of Tm were performed in duplicate and the Tm values were reproducible to within 0.3°C.

Spectral ratios of DNA were obtained from absorbance measurements at 230, 260, and 280 nm. For these measurements, the DNA was dissolved in saline citrate buffer (0.15 M NaCl, 0.015 M Na-citrate, pH 7.0), as described by Marmur (21).

Agarose Gel Electrophoresis of DNA

Horizontal slab gel electrophoresis was performed in a Mini-Sub DNA Electrophoresis System (BIO-RAD). The method of Maniatis et al. (23) was followed. The gel was prepared by dissolving agarose (BIO-RAD), to a concentration of 0.6%, in electrophoresis buffer (0.04 M Tris-acetate

buffer, pH 8.0, containing 0.002 M EDTA). The gel was cast into a slab on a removable glass plate and had dimensions of 50 x 70 x 5 mm (width x length x thickness). About 0.001 - 0.003 ml of either DNA solution (200 - 600 ng of DNA) or a marker solution was introduced into a well in the gel. Isolated DNA and DNA markers (Bethesda Research Lab.) with known number of kilo-base pairs (kb) were dissolved in loading buffer (electrophoresis buffer containing also 7% sucrose, and 0.025% bromophenol blue) before being applied to the gel. Electrophoresis was carried out at 22 - 24°C for 1.0 - 1.5 hours using a constant electric potential of 80 V. After electrophoresis, the DNA was visualized by immersing the gel in ethidium bromide solution (500 ng/ml water) for 45 min at room temperature, followed by destaining of the gel in 1.0 mM magnesium sulfate.

Molecular Weight of DNA

According to Maniatis et al. (23), the distance of DNA migration on agarose gel is inversely proportional to $\log_{10}$ of the number of its base pairs. Therefore, the number of total base pairs (Tbp) of DNA can be estimated from the migration distance of DNA in agarose gel electrophoresis and reference to the mobilities of DNA markers on the same gel. Moreover, Stenesh et al. (17) had reported that the base composition of B. licheniformis DNA was 47% (G+C) and 53% (A+T) while that of B. stearothermophilus DNA was 53% (G+C) and 47% (A+T). The molecular weight of a G-C or A-T nucleotide pair in DNA is 652.23 or 651.16 daltons, respectively. Taking all these data into consideration, the molecular weight (M.Wt.) of DNA can be calculated according to the following equation:

$$M.Wt. = (Tbp)[(\% G+C)(652.23) + (\% A+T)(651.16)]$$

UV Irradiation of DNA

For UV irradiation, DNA was dissolved in water to a concentration of 0.5 mg/ml, and 7.0 ml of this solution were then transferred to a glass dish (5.8 cm I.D. x 1.5 cm height). Irradiation was carried out in an incubator (Precision Scientific Co.; catalog # 31322). These DNA solutions had a concentration of 0.5 mg/ml and an absorbance of 12.1 when measured with a 1.0 cm light path at 254 nm. For irradiation, 7.0 ml of DNA solution were placed in a glass dish (5.8 cm I.D.) and the solution had a thickness of 0.265 cm. According to the Beer-Lambert Law, the absorbance of a solution is proportional to the length of the light path through the solution or the thickness of the solution, while the concentration of the solution is kept constant. Therefore, the DNA solution in the dish, by calculation, must have had an absorbance of 3.206 at 254 nm. Since absorbance = $\log_{10}$ (transmittance)$^{-1}$, the DNA solution thus had a transmittance of 6.215 x 10^{-4}. These calculation indicate that 99.937 percent of the UV irradiation was absorbed.

The temperature of the incubator could be adjusted within the range of 25 - 70°C, and the air in the incubator was moisturized by placing a pan of water in the chamber overnight. After setting the temperature of the incubator to the desired irradiation temperature, the incubator was allowed to equilibrate for 1.0 hour. To assure that temperature equilibrium prevailed throughout the incubator, the temperature of the water in the pan was measured by means of a Tele-thermometer (Model 43, Yellow Spring Instruments) having a small thermistor probe (YSI 423, Yellow Spring Instruments). Prior to irradiation, the DNA solution in the glass dish was placed in the incubator and allowed to equilibrate to

the irradiation temperature. That equilibrium temperature had been attained was verified by measuring directly the temperature of the DNA solution using the Tele-thermometer. The DNA solution was then irradiated with UV light (254 nm) using an intensity of 12 J sec^{-1} m^{-2} and an exposure time of 20 min. During irradiation, the solution was gently stirred by means of a micromagnetic stirrer. At the end of the irradiation, the glass dish was removed from the incubation chamber. A 0.01 ml aliquot of the DNA solution was saved for determination of the thermal denaturation profile and the rest of the solution was transferred to a Pyrex centrifuge tube (Corning 8122, VWR Scientific Co.). The glass dish was washed twice with 2.0 ml of water and the washes were added to the solution in the centrifuge tube. The DNA solution in the centrifuge tube was rapidly frozen in a dry-ice/acetone bath and freeze-dried by centrifugation at -60°C under vacuum in a Savant Speed Vac Evaporator System (Savant Instrument, Inc.). The freeze-dried DNA, packed at the bottom of the conical centrifuge tube, was then immediately subjected to hydrolysis.

Isolation of Thymine Dimer

Acid Hydrolysis of DNA

The freeze-dried DNA, prepared as described in the previous section, was hydrolyzed by the addition of 0.07 ml of 70% perchloric acid (Mallinckrodt, Inc.) and heating for 1.0 hour at 100°C in an oil bath according to the method of Bendich (24). During the hydrolysis, the heavy duty centrifuge tube was tightly capped by means of a plastic cap having a pressure fit liner in order to prevent evaporation of the

hydrolysis solution. As a result, some pressure built up in the tube during the reaction. After hydrolysis, the contents in the tube was cooled by placing the tube in ice, in order to relieve the pressure in the tube. After cooling, 0.35 ml of water was added to the tube to dilute the acid and to resuspend the black particles. After centrifuging down the particles, approximately 0.32 - 0.34 ml of the supernatant was carefully saved. The black solid was washed twice with 0.35 ml of 4% perchloric acid by resuspension and centrifugation, and the washes were combined with the supernatant.

Cation-Exchange Chromatography

The TT was extracted from the DNA hydrolysate (see previous section) by cation-exchange chromatography. This was performed on a disposable column, prepacked with AG 50W-X8(H^+) ion exchange resin and having a column bed of 0.8 x 4.0 cm in water (BIO-RAD). Before applying the DNA hydrolysate, the column was equilibrated first with 10 ml of 1.0 M HCl, and then with 10 ml of distilled, deionized water. The entire DNA hydrolysate (about 1.0 ml) was applied to the column. The tube containing the hydrolysate was rinsed thoroughly with 1.0 ml of water and the rinse was also applied to the column. Elution was carried out with six consecutive 1.0 ml portions water. The entire column effluent (total volume of 8.0 ml) was collected in a glass centrifuge tube. In order to completely remove the perchloric acid which may be harmful to the HPLC column, the effluent was titrated to pH 10 with 5 M KOH. The potassium perchlorate thus formed (a white precipitate) was collected by vacuum filtration, using a glass funnel having a fritted disc of medium porosity. The precipitate was washed once with 3.0 ml of water. The

filtrate was collected directly into a centrifuge tube which was placed under the funnel inside the vacuum flask. The filtrate was rapidly frozen in a dry-ice/acetone bath and lyophilized in a Savant Speed Vac Evaporator System.

Sample Preparation for HPLC Injection

The lyophilized residue obtained by cation-exchange chromatography was dissolved in 0.5 ml of water. The pH of this solution was carefully adjusted to 3.5 using concentrated phosphoric acid (Mallinckrodt, Inc.) and 0.5 M NaOH. Ammonium phosphate buffer (0.01 M, pH 3.5) was then added to bring the solution to a final volume of 1.0 ml. An aliquot of 0.02 ml was injected on the HPLC column for TT quantitation.

Recovery of Thymine Dimer

The recovery of TT, isolated as described above was assessed by adding 4.64×10^5 dpm of ^{3}H-TT (1.5 mCi/mmole) to 7.0 ml of DNA solution (isolated DNA; 0.5 mg/ml) and then proceeding to isolate TT from the DNA solution. At each major step, 0.05 - 0.1 ml of the solution was counted in 15 ml of ACS scintillation fluid. Quenching, resulting from the different compositions of the various solutions, was corrected for by the external standard method (22). The total radioactivity (dpm) was then calculated for the entire solution obtained at each step.

Yield of Thymine Dimer

The amount of TT obtained was calculated from the height of the TT peak of the HPLC chromatogram. The peak height was measured, and the

amount (nmoles) of TT in the applied 1.0 ml sample was obtained by interpolation from the TT standard curve. Since the 1.0 ml sample, applied to the HPLC column, was obtained from 3.5 mg of DNA, and since the molecular weight (M.Wt.) of the DNA can be estimated as described in the previous section, the yield of TT can be expressed in terms of moles of TT per kb of DNA. This is given by the following equation:

Yield of TT (moles TT / kb of DNA) =

(nmole TT/3.5x10^{-3}g)(mole/10^{9}nmole)(g.M.Wt./mole DNA)(mole/kb)

CHAPTER III

RESULTS AND DISCUSSION

Properties of the Isolated DNA

The DNA, isolated from the mesophile, B. licheniformis, and the thermophile, B. stearothermophilus, had spectral ratios of 0.48 : 1.0 : 0.54 at 230 : 260 : 280 nm, and there were no significant differences in spectral ratios between the DNA isolated from these two organisms. These spectral ratios are very similar to the values reported by Stenesh et al. (17) which were 0.45 : 1.0 : 0.55 at 230 : 260 : 280 nm. Thermal denaturation profiles of the DNA, carried out in 1.0 mM sodium phosphate buffer (pH 7.0), yielded Tm values of 67.7°C and 62.3°C for the DNA from the thermophile and the mesophile, respectively. This difference in Tm values again agrees with that reported by Stenesh et al. (17) which amounted to 5.0°C. Based on the electrophoretic pattern obtained with agarose gel (insert of Figure 3), the DNA isolated from both organisms was essentially homogeneous. By extrapolation of the standard curve (Figure 3), the DNA from the thermophile was found to contain 26 kb while that from the mesophile was found to contain 31 kb. Using the equation given above for the calculation of molecular weight (M. Wt.), the DNA isolated from the mesophile and thermophile was found to have a M. Wt. of 20 x 10^6 and 17 x 10^6 daltons, respectively. These values are somewhat higher than those estimated by Stenesh et al. (17) from viscosity measurements. Those authors arrived at a value of 10 x 10^6

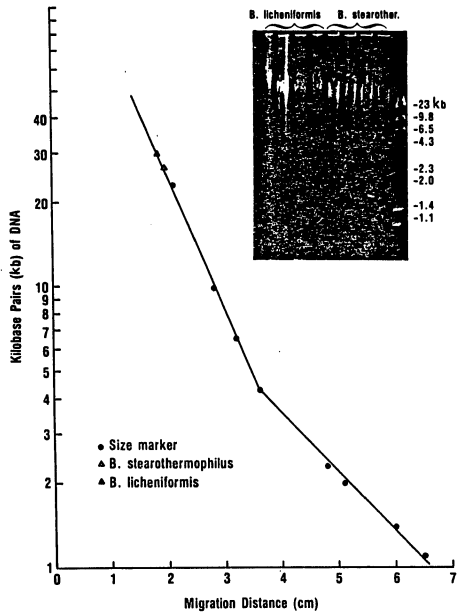


Figure 3. Agarose Gel Electrophoresis of DNA.

daltons for the molecular weight of the DNA.

Characterization of the Thymine Dimer Standard

It was pointed out previously that thymine in aqueous solution, has a maximum absorption at about 264 nm as can be seen from Figure 4. The TT, prepared by the method of Gunther and Prusoff (4), on the other hand, does not show this characteristic absorption peak of thymine. This is due to the fact that, while the dimer is formed by UV irradiation (254 nm) of frozen thymine solution, the C5-C6 double bonds are lost as the cyclobutyl TT structure is produced. Besides cyclobutyl TT, other non-cyclobutyl dimers are formed in minute quantity by UV irradiation of frozen thymine solutions (25). These dimers (referred to as photoadducts) also lack the absorption peak at 264 nm. They do, however, have an absorption peak at 316 nm where the cyclobutyl TT possesses no absorption.

It can been seen from Figure 4 that the prepared TT standard (0.15 mM) in sodium phosphate buffer (0.1 M, pH 7.0) showed no absorption peak at 264 nm while thymine (0.04 mM) in the same buffer showed a typical absorption maximum. Moreover, the TT had no absorption peak at 316 nm either. These absorption characteristics indicated that the prepared TT was a cyclobutyl dimer. The elemental analysis of the dimer (C 47.50%; H 4.78%; M 22.25%) agreed with that of thymine (C 47.62%; H 4.80%; M 22.21%). This indicates that the isolated TT was a dimer of thymine but not of thymine derivatives.

A very distinctive property of the cyclobutyl TT is its photoreversibility. The cyclobutyl TT, but not the other photoadducts, can be reverted to the thymine monomer by irradiating an aqueous dimer

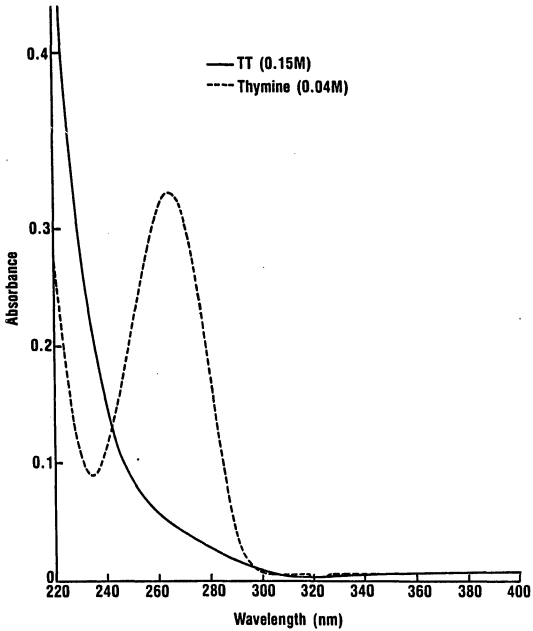


Figure 4. UV Spectra of Thymine and Thymine Dimer.

solution with UV light of the same wavelength (254 nm)(26). To check this, the prepared TT (0.15 mM) in sodium phosphate buffer (0.1 M, pH 7.4) was irradiated at room temperature for varying time intervals. The resulting spectral curves are shown in Figure 5, with the numbers indicating irradiation times in seconds. It can be seen that the UV spectrum of the TT solution changed continuously toward that of thymine, with an increasing absorption peak at 264 nm. This observation further confirms that the TT prepared in this study was indeed a cyclobutyl dimer of thymine.

The yield of TT, synthesized from T, was approximately 63% on the basis of the original amount of thymine. The specific activity of ^{3}H-TT was approximately 1.51 mCi/mmole.

Based on the UV spectra, shown in Figure 4, a wavelength of 230 nm was chosen as the detection wavelength for TT. At that wavelength, the absorption of TT was relatively strong while that of the four major bases present in DNA was minimal (27). At 230 nm, the molar extinction coefficients for T and TT in sodium phosphate buffer (0.1 M, pH 7.4) and with a 1.0 cm light path were determined to be 2144 and 1737, respectively.

Separation of Standard Thymine Dimer and Standard Bases by HPLC

High-performance liquid chromatography (HPLC) has been widely applied for the separation of nucleic acid bases. Recently, reverse-phase HPLC (RP-HPLC) has proven to be particularly effective for this purpose (28,29). However, none of the published methods involved attempts to separate TT and the nucleic acid bases simultaneously.

For the present study, it was desirable to be able to resolve the

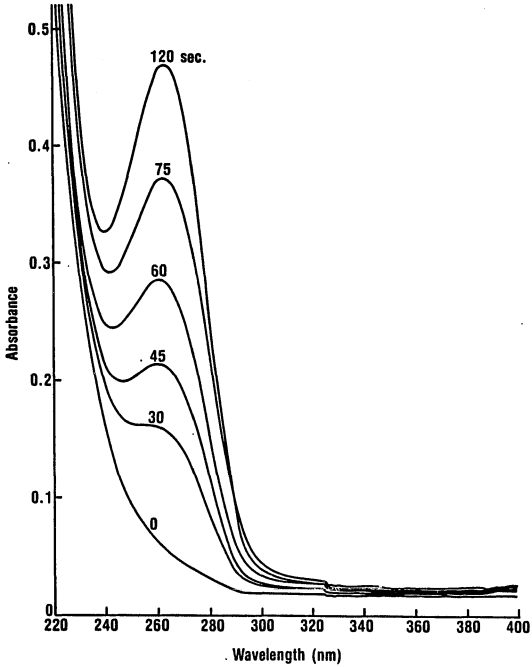


Figure 5. Photoreversibility of Thymine Dimer Formation.

five nucleic acid bases and TT under a single set of HPLC conditions so that the TT in the DNA hydrolysate could be determined directly without further extraction. In an attempt to achieve this, the RP-HPLC method of Miller (28) was first used without any modifications. The results were unsatisfactory. Following this, an HPLC system was developed in which the mobile phase consisted of 0.1% acetonitrile in ammonium phosphate buffer (0.1 M, pH 3.5). Using the HPLC conditions, as specified in Material and Methods, all six standards (five bases and the TT) could be totally resolved. These results are shown in Figure 6.

When a wavelength of 230 nm was used for detection of TT and the other bases, all six standards were detected. The order of elution, in terms of increasing retention time, was cytosine, uracil, guanine, adenine, TT, and thymine. Since TT has no significant absorption at 260 nm while the nucleic acid bases absorb maximumally around that wavelength, it was expected that changing the detection wavelength to 260 nm would significantly enhance the peaks of bases but diminish the TT peak. That that was the case can be seen from the chromatograms in Figure 6. This differential spectral detection method was helpful in identifying the TT peak and distinguishing it from the peaks of the other bases.

In order to assess the reproducibility of the HPLC system with respect to the retention time of each peak, a solution containing 0.001 - 0.002 mg/ml of each of the six standards was injected into the HPLC column in triplicate per day for three consecutive days. The retention times for the peaks were measured and are recorded in Table 1. It can been seen the retention times for all of the peaks were very reproducible with a maximum standard deviation of 6.6 seconds.

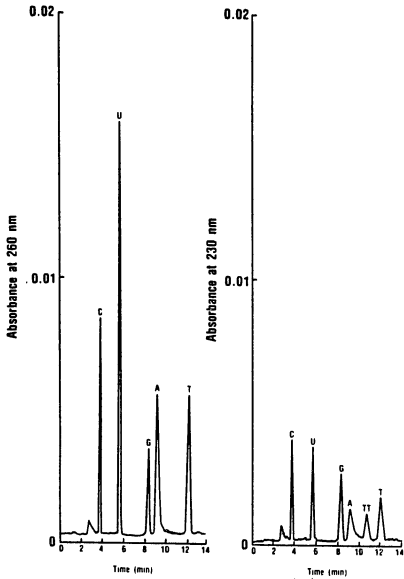


Figure 6. HPLC Chromatograms for TT and Standard Bases.

Table 1

Retention Times of Standards in HPLC

Standard	Retention Time (min) Average[a]	SD[b]	C.V.(%)[c]
Cytosine	3.40	0.02	0.6
Uracil	5.38	0.07	1.3
Guanine	8.06	0.06	0.7
Adenine	8.84	0.11	1.2
TT	10.44	0.05	0.5
Thymine	11.90	0.05	0.4

a. Average of nine measurements; triplicates per day for 3 days
b. Standard deviation
c. Coefficient of variation

Purity and Standard Curve of Thymine Dimer Standard

Since the molar absorptivities at 230 nm for TT and thymine are different, the purity of TT cannot be estimated by directly comparing the size of the TT peak to that of the thymine peak in an HPLC chromatogram. Hence, one must either construct an HPLC standard curve for T or use a different approach. In this study, a radioactive method was used for estimating the purity of TT. By using radioactively labeled TT (^{3}H-TT), synthesized as described under Materials and Methods, one can determine the purity of TT by measuring the counts in that peak and in the thymine peak. Since both the unlabeled and labeled TT were synthesized by an identical procedure, it was safe to assume that the

contamination with ^{3}H-T found for ^{3}H-TT would also apply for contamination of unlabeled T for unlabeled TT.

The results of such an experiment are shown in Figure 7. It can be seen that there are two peaks on the HPLC chromatogram. These correspond to TT and Thymine, respectively, as identified by the retention times. In the single experiment, described by this chromatogram, there was a total of 36.6 x 10^3 cpm in the two peaks; the TT and thymine peaks contained 35.2 x 10^3 and 1.05 x 10^3 cpm, respectively. Hence, a purity of 96.2% can be assigned to the TT prepared in this study.

Taking the purity of TT into account, solutions of TT were prepared as described under Materials and Methods. All told, six standard solutions were prepared, ranging in concentration from 7.5 to 133 nmole/ml. These six standard solutions were then run on HPLC, and a standard curve was constructed by plotting peak height (at 230 nm) against the corresponding concentration of TT. This is shown in Figure 8. Each point represents the average of triplicate determinations. Based on linear regression analysis (30), the line had a correlation coefficient (r) of 0.999, a slope of 0.098 cm/nmole/ml, and an intercept of 0.11 cm. The measurements of peak height had coefficients of variation from 0.9 to 4.7% throughout the whole range of the tested concentrations.

Quantitation of Thymine Dimer Isolated From DNA

An attempt was made initially to quantitate TT in the DNA hydrolysate by subjecting the latter directly to HPLC without any further extraction. The DNA hydrolysate was prepared for HPLC, as described under Materials and Methods. Briefly, the supernatant from the $HClO_4$

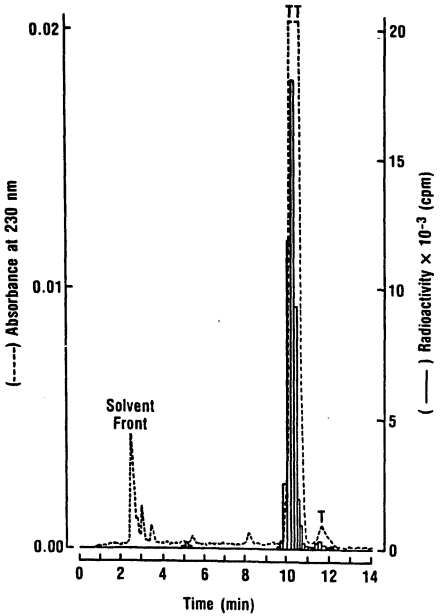


Figure 7. Determination of Thymine Dimer Purity by HPLC.

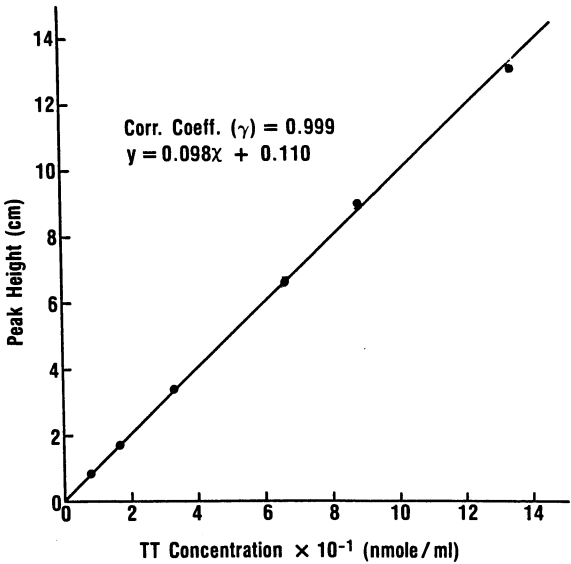


Figure 8. Standard Curve of Thymine Dimer.

hydrolysis of 3.5 mg of DNA was titrated to pH 10 with 5.0 M KOH in order to precipitate the perchloric acid in the form of potassium perchlorate. The supernatant was then lyophilized and redissolved in 1.0 ml of ammonium phosphate buffer (0.1 M, pH 3.5). A 0.02 ml aliquot of this solution was injected onto the HPLC column for analysis.

Two typical chromatograms for DNA hydrolysates from B. stearothermophilus are shown in Figure 9. One chromatogram is for DNA irradiated by UV at 55°C (see Materials and Methods). The other chromatogram is for a control in which the DNA was treated in an identical fashion to the irradiated DNA except that treatment was in the dark, in the absence of any irradiation. It can be seen from the data of Figure 9 that a TT peak, identified by its retention time of about 10.5 min, was present in the chromatogram of the irradiated DNA but not in the chromatogram of the control.

These results suggested that UV irradiation did indeed lead to the formation of TT in DNA, and that the TT in the DNA hydrolysate could be monitored by HPLC. However, formation of the TT peak was obscured, and baseline resolution was impeded, by the high concentration of adenine in the hydrolysate. In fact, the TT peak was almost entirely overlaid by the tail peak of adenine when the TT concentration was low. Hence, it became clear that it was difficult, if not impossible, to accurately quantitate the TT in the hydrolysate of irradiated DNA by such direct HPLC. Therefore, a cation-exchange chromatography step (see Materials and Methods) was introduced after the perchloric acid hydrolysis of DNA. The purpose of this chromatographic extraction was to remove the adenine from the hydrolysate so that the TT peak on the HPLC chromatogram would not be obscured. This should then allow an accurate quantitation of the

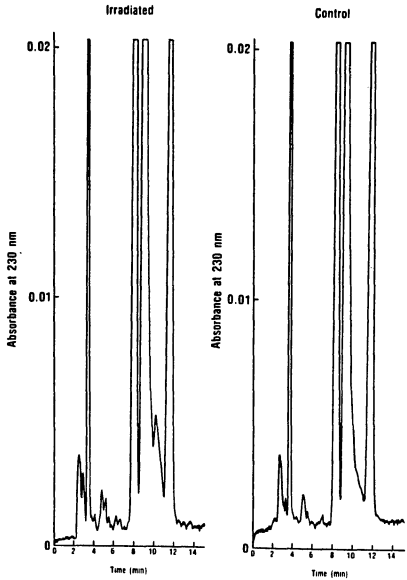


Figure 9. HPLC Chromatograms of DNA Hydrolysates.

low TT concentration in the DNA hydrolysate.

Of the four major DNA bases, three (adenine, cytosine, and guanine) exist as cations in solutions having a pH less than 3.0 (25,31). At such pH values, these bases are adsorbed onto a cation exchanger while thymine and TT are not adsorbed. The latter two could then be eluted with water. That this is the case is indicated by the chromatograms shown in Figure 10 for DNA from B. stearothermophilus, irradiated at 55°C. As can be seen from Figure 10, the use of cation-exchange chromatography resulted in disappearance of the adenine, cytosine, and guanine peaks, but the thymine and TT peaks remained. Most importantly, the TT peak was clearly resolved and easily identified; the TT peak was present when the effluent was scanned at 230 nm but was absent when the effluent was scanned at 260 nm.

These data indicated that, while our initial attempt to measure TT directly in the DNA hydrolysate was not feasible, a simple cation-exchange chromatographic extraction permitted the low TT concentration in the UV-irradiated DNA to be accurately quantitated by HPLC. Moreover, even if the cation-exchange extraction were incomplete, the presence of residual amounts of bases would not interfere with the accurate measurement of TT. This is the case since small amounts of bases lead to small, distinctive peaks, and all of the peaks, including that of TT, were well resolved by the HPLC technique. Lastly, addition of the cation-exchange extraction step did not affect the recovery of TT. Recovery of TT, beginning with the original DNA sample, and progressing toward preparation of the sample applied to the HPLC column, was essentially 100 percent at each step. These data (a single

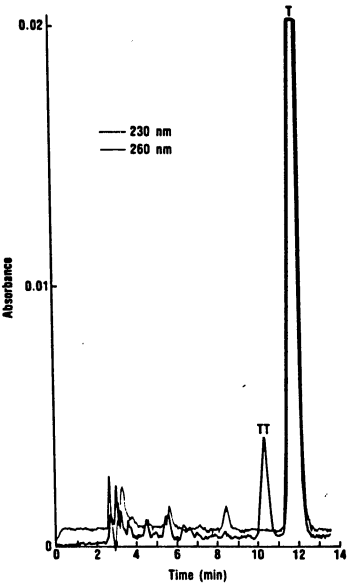


Figure 10. HPLC Chromatograms of a DNA Hydrolysate Subjected to Cation-Exchange Chromatography.

experiment) are shown in Table 2. It should be noted that, unlike thymine, the TT is very stable to acid hydrolysis (4).

Table 2

Recovery of ^{3}H-Thymine Dimer

Preparation Step	Total Radioactivity (dpm)	Recovery (%)
Original DNA sample	4.64×10^5	100
Acid hydrolysate	4.54×10^5	97.8
Cation-exchange eluate	4.49×10^5	96.8
Sample used for HPLC	4.45×10^5	95.9

In order to confirm that the TT peak of the chromatogram was indeed due to cyclobutyl TT and not due to noncyclobutyl TT, CT, or CC, the fractions containing the TT peak were pooled and the solution was scanned between 200 and 400 nm. A typical UV spectrum of TT, as shown in Figure 4, was obtained. The solution was then subjected to the photoreversibility test by irradiating with UV light (254 nm) for 2 min and then scanning between 200 and 400 nm as before; the typical UV spectrum of thymine, with an absorption maximum at 264 nm, was obtained. These observations confirmed that the TT peak of the chromatogram was, in fact, due to cyclobutyl TT.

Effect of Irradiation Temperature on Thymine Dimer Production

DNA in aqueous solution, was irradiated by a constant UV flux of 12

J sec^{-1} m^{-2} for 20 min at three different temperatures: 35°C, 45°C, and 55°C. At each temperature, a DNA control was processed. The control was treated in an identical fashion as the irradiated DNA except that it was treated in the dark, in the absence of any irradiation. After UV irradiation or dark treatment, a small aliquot of sample or control was used for Tm determination and the bulk of the material was used for isolation and quantitation of TT.

Results for the isolation and quantitation of TT (based on the standard curve of Figure 8) are shown in Table 3 and Figure 11. As can be seen, the production of TT, expressed as mole of TT per kb of DNA, was significantly higher in the thermophile than in the mesophile. This was true at all three irradiation temperatures. There was no detectable production of TT in any of the corresponding controls. Although the production of TT in the DNA from both strains increased with increasing irradiation temperature, there was a much greater temperature effect in the thermophile than in the mesophile. In other words, the TT production per 10°C increase in irradiation temperature was greater in the thermophile than in the mesophile. Thus, there was an average increase of TT production of 1.7 mole/kb per 10°C for the thermophile and 0.40 mole/kb per 10°C for the mesophile.

It has been reported by Stenesh et al. (17), that the mesophilic and thermophilic DNA contains 53 and 47 mole% (A+T), respectively. Therefore, it can be calculated that there are 265 moles of T/kb of mesophilic DNA and 235 moles of T/kb of thermophilic DNA. If all of the T's were present as adjacent T sequences (that is, as dinucleotides, T-T) and if all of these dinucleotides were able to be converted to thymine dimers (TT) by UV irradiation, then the maximum yield of TT which

Table 3

Effect of Irradiation Temperature on Thymine Dimer Production in DNA

Irradiation Temperature (°C)	Peak Height (cm)	TT Production (mole/kb)	Average[a]	SD[b]
B. licheniformis				
35	1.80	3.11		
	1.93	3.35	3.24	0.18
	1.88	3.26		
45	2.19	3.83		
	2.02	3.52	3.70	0.13
	2.15	3.76		
55	2.45	4.31		
	2.25	3.94	4.14	0.15
	2.37	4.16		

a. Average of triplicates
b. Standard deviation

Table 3--Continued

Irradiation Temperature (°C)	Peak Height (cm)	TT Production (mole/kb)	Average[a]	SD[b]
B. stearothermophilus				
35	2.40	4.34		
	2.22	4.00	4.16	0.14
	2.30	4.15		
45	3.06	5.60		
	2.75	5.01	5.26	0.24
	2.84	5.18		
55	4.22	7.80		
	4.15	7.66	7.64	0.14
	4.05	7.47		

a. Average of triplicates
b. Standard deviation

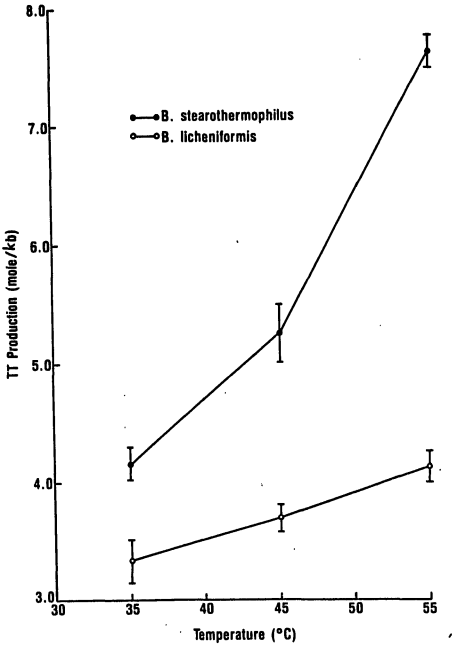


Figure 11. Effect of Irradiation Temperature on Thymine Dimer Production

could be expected for the mesophilic and thermophilic DNA would be 132.5 and 117.5 moles of TT/kb, respectively. Using these calculations and the data of Table 3, it follows that the formation of TT for the mesophilic DNA increased from 2.4 to 3.1% of the possible maximum value as the irradiation temperature was increased from 35 to 55°C. Under the same conditions, the formation of TT for the thermophilic DNA increased from 3.5 to 6.5% of the possible maximum value.

It has been reported (11,12,32) that local regions of denaturation are formed in double-stranded DNA upon irradiation. These regions of denaturation are due to the formation of TT and lead to a decrease in the Tm of the DNA. As can been seen from the data in Table 4, such denaturation appeared to have taken place in the thermophile so that the irradiated DNA had a lower Tm value than the control.

Table 4

Tm Values of Irradiated DNA

	Tm (°C)			
Irradiation	B. stearothermophilus		B. licheniformis	
Temperature (°C)	Control	Irradiated	Control	Irradiated
35	67.8	65.1	62.1	62.4
45	67.6	64.5	61.7	62.0
55	67.9	63.1	62.3	61.1

Moreover, the Tm value was lowered progressively as the irradiation

temperature was increased. In the mesophile, the Tm of the irradiated DNA and its control were essentially the same, and the Tm value decreased only slightly, if at all, as the irradiation temperature was increased. The lack of such changes may be due to the fact that TT formation was too low in the mesophile and hence did not affect the Tm value. It appears, therefore, that a decrease in Tm cannot be used as a sensitive measurement of TT production in UV irradiated DNA.

The data in Table 4 also suggest that the superior thermal stability of the DNA from the thermophile was greatly reduced by UV irradiation. The accumulation of a sufficient number of TT, with an impaired ability for complementary hydrogen bonding, resulted in a DNA that had less thermal stability. Thus, while the difference in Tm values between the mesophile and the thermophile was approximately $5^{o}C$ for the control DNA's, the difference was approximately $2^{o}C$ for the irradiated DNA's.

The thermal denaturation profiles of the control DNA's for the $55^{o}C$ experiments are shown in Figure 12. As can be seen, neither the thermophilic nor the mesophilic DNA started melting until the temperature was $56.5^{o}C$ or higher. Moreover, the Tm values and the denaturation profiles of these control DNA's were the same as those of DNA's that had not been exposed in the dark for 20 min at $55^{o}C$. These data suggest that the 20 min dark exposure at $55^{o}C$ did not begin to denature the DNA's so that they were in their native forms prior to UV irradiation. Therefore, the observed difference in TT production between the thermophilic and the mesophilic DNA must reflect a true difference in susceptibility to UV irradiation.

It must be remembered that a number of factors contribute to the thermal stability or instability of the DNA. For one, a DNA that is

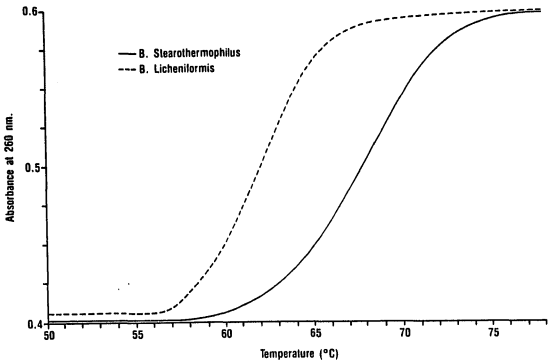


Figure 12. Thermal Denaturation Profiles of DNA Controls.

rich in G-C base pairs, which are linked by means of three hydrogen bonds, is expected to be more heat stable than a DNA rich in A-T base pairs, which are only linked by means of two hydrogen bonds. Additionally, however, the sequence of the bases is important. For example, a long stretch of A-T base pairs would tend to weaken (decrease the thermal stability) of the DNA much more than if these A-T base pairs were distributed randomly across the DNA. Thirdly, heterogeneity of a DNA preparation is likely to broaden the width of the thermal transition and may lower or raise the Tm accordingly.

It has been shown (17,32) that the thermal stability of DNA is proportional to its G+C content, and that the DNA of the thermophile, B. stearothermophilus, with a G+C content of 53 percent, was more heat stable than the DNA of the mesophile, B. licheniformis, with a lower G+C content of 47 percent. Moreover, It is reasonable to assume (but not necessarily true) that the A+T rich DNA of the mesophile would have a higher frequency of adjacent T sequences (T-T) than the G+C rich DNA of the thermophile. Since TT is formed between two adjacent thymines on the same strand of DNA, the A+T rich DNA from the mesophile would be expected to lead to higher production of TT than the G+C rich DNA from the thermophile. However, TT production in DNA is controlled not only by the frequency of T-T sequences, but also by the orientation and reactivity of the two adjacent thymines on the strand of the double helix. In solution, these vary (33,34) and are a function of the neighboring electronic clouds. For example, in order to be able to form cis-syn cyclobutyl TT which is the chief photoproduct, the C5-C6 double bonds of the pyrimidine rings of the two adjacent thymines must be in very close proximity, and the two thymines must be oriented in such a

manner that their pyrimidine rings are stacked in a reactive configuration with respect to the cyclobutane ring to be formed.

Thus, although the A+T rich DNA from the mesophile may have a higher frequency of T-T sequences, the production of TT by UV irradiation would be low if the two adjacent thymines at the T-T sites were not of proper orientation and reactivity to favor TT formation. Conversely, the G+C rich DNA from the thermophile may have a lower frequency of T-T sequences, but if the two adjacent thymines at the T-T sites were of proper orientation and reactivity, then a significant production of TT might be expected.

Such considerations about the reactivity of T relative to its location in a DNA strand and the effects of neighboring groups are illustrated by the studies of Haseltine et al.(33). These authors determined the distribution of dimer formation within a defined sequence in DNA fragments from pLj3 plasmid. They showed that no TT formation occurred between two remote T's, and, furthermore, that all of the adjacent T's had different reactivities toward TT formation following UV irradiation. The differences in reactivities were influenced by the nature of the nucleotides that flanked the adjacent T's. These data indicated that TT production in DNA is controlled not only by the frequency of T-T sequences, but also by the photochemical reactivity at the T-T sites on the strand of double helix. The photochemical reactivity thus was base sequence dependent.

To understand this, one must realize that the properties of aqueous DNA are quite different from those of crystalline DNA. When DNA is dissolved in solution, it is no longer subject to the influence of crystal lattice forces. Because of that, the base pairs of the DNA are

no longer centered on the helix axis and are not exactly perpendicular to the helix axis. Nevertheless, the base pairs are not coplanar but are arranged in a propeller-twist fashion, resulting in non-parallel base stacking.

The stability and orientation of two, non-parallel, stacked bases depends on hydrophobic interactions, electrostatic interactions, dipole-dipole and dipole-induced dipole interactions between the two heterocyclic rings. These interactions, in turns, are a function of the arrangements of the electron clouds in the confined space between two bases. Variations in the electron clouds, therefore, affect base-base interactions and, hence, stability and orientation of the bases. Consequently, in solution, the double helical structure of DNA displays a sequence dependent modulation (1,17,34).

Morever, in photochemical reactions, the excitation of a molecule by irradiation changes its electron cloud structure, thus changing its chemical reactivity. Any factor that influences the redistribution of electron density at the reactive site of the molecule must, therefore, affect its photochemical reactivity (14,34).

It is clear, therefore, that the reactivity of a given TT site depends on the base sequence where it is located and is bound to be affected by neighboring groups.

It appears that such considerations apply to the formation of TT in the mesophile and the thermophile of the present study, resulting in the finding that TT production was higher for the thermophile than for the mesophile. Thus, while the thermophilic DNA has greater thermal stability, it appears that it has lower photochemical stability than the DNA from the mesophile. Moreover, one can conclude that the orientation

and reactivity of adjacent T's for formation of TT was more favorable in the thermophile than in the mesophile.

CHAPTER IV

SUMMARY

The purpose of this study was to evaluate the in vitro photochemical stability of DNA from a mesophile (B. licheniformis) and a thermophile (B. stearothermophilus). The photochemical stability is assessed by measuring the in vitro production of TT in the DNA upon irradiation with UV light (254 nm).

In order to quantitate the TT formed, the irradiated DNA was hydrolysed and the hydrolysate was then subjected to cation-exchange chromatography. The sample was then analyzed by HPLC, using isocratic elution (0.1% acetonitrile in 0.1 M ammonium phosphate buffer, pH 3.5). This led to a complete separation of TT and the five major nucleic acid bases. These combined steps permitted the accurate quantitation of the small amounts of TT formed in the DNA upon irradiation. A standard of cyclobutyl TT was synthesized and shown to be 96.2% pure.

The results showed that the production of TT in the DNA from the thermophile was significantly higher than that in the DNA from the mesophile. This was true at all three irradiation temperatures (35°C, 45°C, and 55°C). Moreover, TT production in the thermophilic DNA increased by 1.7 mole/kb for every 10°C increase in irradiation temperature while TT production increased by only 0.40 mole/kb for every 10°C increase in temperature for the mesophilic DNA. The large amount of TT formed in the thermophilic DNA resulted in a 5°C drop in the Tm while no significant drop in the Tm was observed for the mesophilic DNA.

The DNA from the thermophile was more heat stable, but it was more vulnerable to UV irradiation at 254 nm, as compared to the DNA from the mesophile. It appears that photochemical stability and thermal stability of DNA do not vary in parallel fashion when evaluated in relation to thermophily.

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