ISOLATION AND CHARACTERISTICS OF A BACTERIOPHAGE FOR BACILLUS STEAROTHERMOPHILUS

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This thesis is affectionately dedicated to my wife Janice and to my children Dean, Marcie and Parris.

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ABSTRACT

A bacteriophage (TØ3) which infects the thermophilic bacterium Bacillus stearothermophilus ATCC 8005 was isolated and characterized. Infection of the bacterium by the bacteriophage was carried out at 60°C, the optimum growth temperature of the host. At 60°C the phage has a latent period of 18 minutes and a burst size of about 200. The phage is comparatively thermostable in broth. The half life of the phage is 400 minutes at 60°C, 120 minutes at 65°C, 40 minutes at 70°C and 12 minutes at 75°C. The activation energy for the heat inactivation of TØ3 is 56,000 cal. The buoyant density of TØ3 in a cesium chloride density gradient is 1.526.

Electron micrographs of TØ3 indicate that the phage has a regular hexagonal shaped head 57 mμ long. The morphology of the head is compatible with icosahedral symmetry. Each edge of the head is 29 mμ long, and there are 6 or 7 subunits along each edge. The tail of TØ3 is 125 mμ long and 10 mμ wide. There are about 30 cross striations that are spaced at 3.9 mμ intervals along the tail.

The DNA of phage TØ3 has a melting temperature of 88.5°C. Heat denatured TØ3 DNA can be extensively annealed in a high ionic strength environment. The buoyant density of TØ3 DNA in a cesium chloride density gradient is 1.695. TØ3 DNA contains: 42.7% guanine plus cytosine, as determined from the melting temperature; 43% guanine plus cytosine, as determined from the buoyant density; and 40.2% guanine plus cytosine, as determined by chromatographic separation and spectrophotometric estimation of the bases. The molecular weight of TØ3 DNA is 16.7×10^6 as determined from the band width of the TØ3 DNA

concentration distribution in a cesium chloride density gradient. Electron microscopy of TØ3 DNA revealed a single linear molecule that is 11.7 μ long. This corresponds to a molecular weight of 22.5×10^6.

Heat denatured TØ3 DNA forms two bands in a cesium chloride density gradient, one at a density of 1.707 and the other at a density of 1.715. After the separated bands are mixed and annealed in the centrifuge cell, the renatured TØ3 DNA forms a single band at a density of 1.699. These results indicate that the two complementary strands of TØ3 DNA have different buoyant densities in cesium chloride, presumably because they have different base compositions.

The characteristics of TØ3 are compared with those of other phages. A hypothesis is presented for a relationship between the base composition of one strand of TØ3 DNA and the amino acid composition of the proteins of TØ3.

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INTRODUCTION

Thermophilic bacteria.

The ability of certain organisms to grow at elevated temperatures has been of interest for many years (1). Organisms that have been found living at temperatures of 60° to 98°C include fishes, molluscs, arthropods, worms, algae and bacteria (1). The thermophilic microorganisms have been reviewed by Gaughran (1) and the thermophilic aerobic spore-forming bacteria by Allen (2). A book on the thermophilic fungi has appeared (3).

The thermophilic species of Bacillus are classified as those organisms whose optimum growth temperature is 55°C or above and which show slight if any growth at 37°C (4). This classification is somewhat arbitrary in that there is much overlapping, e.g. Bacillus subtilus may grow at 55°C but its optimum is 30 to 37°C (5). Most strains of Bacillus stearothermophilus have an optimum of 60°C; however, several have lower optima and will grow at temperatures as low as 33°C (5).

Thermostability of proteins of thermophilic bacteria.

Recent biochemical work with thermophilic bacteria has been concerned with the thermostability of proteins isolated from these organisms. Koffler, Mallett and Adye (6) isolated flagella from various mesophilic and thermophilic bacteria and studied the viscosity of solutions of these flagella under various conditions. They correlated a drop in the viscosity of solutions of flagella with the dissociation of the flagella into subunits. They found that the viscosity of solutions of flagella from thermophilic bacteria remained high at

temperatures up to 80°C. On the other hand, the viscosity of solutions of flagella from mesophilic bacteria decreased at about 55°C. They also found the flagella of thermophiles to be stable and the flagella of mesophiles to be unstable to the action of 6 M urea, 10 M acetamide or 0.0035 M sodium dodecyl sulfate. They concluded that the structure of the flagella of thermophilic bacteria is stabilized by more effective hydrogen and hydrophobic bonds than is the structure of the flagella of mesophilic bacteria. Koffler (7) has written a review of this and other work up to 1957 in which he supports the thermostable protein hypothesis of thermophily.

Campbell and his co-workers (8, 9, 10, 11) have isolated and investigated the properties of a thermostable α-amylase from Bacillus stearothermophilus. This enzyme, recrystallized 8 times, was active at temperatures up to 85°C and lost only 29% of its activity when incubated for 20 hours at 85°C (8). The enzyme preparation was homogeneous by sedimentation criteria (9). The sedimentation coefficient was found to be 0.762S and the molecular weight, as determined by sedimentation-diffusion, was 15,600. Neither the specific rotation nor the enzymatic activity was affected by treatment with 8 M urea or 4 M guanidine hydrochloride, both powerful hydrogen bond disruptors. They concluded that this enzyme "in the native state exists as a semi-random- or random-coiled, well hydrated molecule, with any secondary [tertiary] structure due to the presence of disulfide bonds." The amino acid composition was determined (10) and the enzyme was found to contain 145 amino acids, twenty two of which were glutamic acid, twenty two proline, eleven aspartic acid, eleven valine and nine glycine. The enzyme contains no tryptophan but

is very rich in the acidic amino acids and in proline. The molecular weight, as determined by the amino acid analysis is 15,600. There are two moles of amino-terminal phenylalanine per mole of enzyme (11). Proline and alanine were determined to be the carboxyl-terminal amino acids with one mole of each per mole of enzyme. Therefore, this α-amylase consists of two polypeptide chains. There were 4 cysteine residues found in the amino acid analysis (10), thus the two polypeptide chains could be held together by one or two disulfide bonds.

Thermostability of the deoxyribonucleic acid of Bacillus stearothermophilus.

Marmur (12) and Marmur and Doty (13) reported the thermal denaturation temperature (T_m) of the deoxyribonucleic acid isolated from Bacillus stearothermophilus strain 194 to be 87.5 and 88°C, corresponding to a base composition for this DNA of 46% guanine plus cytosine. Welker and Campbell (14) confirmed this determination but found that strain 194 is not a typical strain of this bacterium. They determined the T_m of 13 authentic strains of Bacillus stearothermophilus and found it to correspond to a base composition of 49 to 52% guanine plus cytosine. Their base compositions correspond to melting temperatures of 89 to 91°C. This is in the same range as the T_m found for many mesophiles (13), and in fact the DNA from many mesophiles has a T_m much higher than this (for example the DNA of Micrococcus lysodeikticus has a T_m of 99.5°C).

Since proteins isolated from this thermophilic organism are relatively thermostable whereas the deoxyribonucleic acid is not, the proteins are apparently responsible for the thermophilic nature of

this organism.

Bacteriophage for thermophilic bacteria.

Although there were early reports (15, 16, 17) of bacteriophage isolated for thermophilic bacteria, the bacteria involved were not true thermophiles. Later reports on bacteriophage include a few for thermophilic bacteria. White, Georgi and Militizer (18, 19) described a bacteriophage for Bacillus stearothermophilus. This phage produced plaques optimally at 65°C. It was stable in broth at 70°C. At 75°C the titer decreased by a factor of about 30 in 30 minutes. At 80°C the titer decreased by a factor of 100 in 30 minutes. The rate of inactivation was not constant but decreased with time. A temperature of 100°C for 30 minutes did not totally inactivate a lysate (18). The phage was less stable in phosphate buffer than in broth (19). In the broth used, about 75% of the phage adsorbed to the bacteria in 30 minutes. A low concentration of calcium chloride enhanced the adsorption.

Hirano (20) isolated a bacteriophage for an unspecified thermophilic bacterium. This phage exhibited a half-life in broth of 40 minutes at 65°C and 8 minutes at 72°C. Onodera (21) reported a phage that survived 2 hours at 100°C with no loss in activity. It was also reported that this phage contained 42% DNA and 32% RNA. Shafia and Thompson (22) investigated a phage for Bacillus stearothermophilus. They reported this phage to be very small, only 10 mμ in diameter and to have a half-life of 2 hours at 75°.

Welker and Campbell (14) have induced a temperate phage from Bacillus stearothermophilus. They reported a phage that has a 65 mμ diameter head, a flexuous tail 240 mμ long and 12 mμ wide. Phage

production was optimal at 55°C. The phage was stable at 55° and had a half-life of 30 minutes at 65°. The DNA of the phage was reported to have a sedimentation coefficient of 24.1S, a molecular weight of 12.1 X 10^6 and to contain 42% guanine plus cytosine. Calcium ion was required for adsorption of the phage to the bacteria.

Saunders and Campbell (23) have reported the investigation of the DNA of a phage for Bacillus stearothermophilus. This phage has a DNA that is 13.9 μ long, has a molecular weight of 26.7 X 10^6, and a sedimentation coefficient of 30.0S. It has a density of 1.705 and a base composition of 42% guanine plus cytosine. Upon heat denaturation or treatment with alkali the two complementary strands of the DNA can be separated.

Purpose of the present investigation.

The ordered tertiary structure of many proteins is not stable at temperatures above 40°C. Exposure to high temperatures denatures these proteins. Scheraga (77) indicates that the disruption of intramolecular hydrogen bonds is the primary cause of the thermal denaturation of proteins. The protein denaturing action of urea, acetamide and guanidine hydrochloride is also attributed to the efficiency with which they disrupt the hydrogen bonds involved in stabilizing the tertiary structure of proteins. The quaternary structure of the structural protein of the flagella (6) and the tertiary structure of an enzyme, α-amylase (8, 9, 10, 11), of the thermophilic bacterium, Bacillus stearothermophilus, are stabilized by bonds that are not heat labile and are not disrupted by hydrogen bond disrupting agents. The high proline content of the α-amylase of Bacillus stearothermophilus

also indicates that hydrogen bonds are relatively unimportant in stabilizing the tertiary structure of this enzyme. If heat stability and stability in the presence of hydrogen bond disrupting agents is a general characteristic of the proteins of Bacillus stearothermophilus, then the study of the structure of these proteins would augment our knowledge of the relationship between the primary amino acid sequence and the tertiary structure of proteins in general.

Our present understanding of the translation of information contained in the genetic material, deoxyribonucleic acid, to that contained in protein is that the nucleotide sequence in the DNA determines the amino acid sequence in the protein. The position of each amino acid in a protein is determined by the position of a corresponding nucleotide triplet in one strand of a DNA molecule. Each triplet codes for one and only one amino acid but each amino acid may be coded for by more than one triplet. The sequence of nucleotides in some of the codewords (triplets) has been determined (75). The codewords for proline are CCC, CCU, CCA and CCG. If the high proline content of the α-amylase of Bacillus stearothermophilus is a reflection of a general characteristic of the proteins of this organism, then it would be expected that one strand of the DNA of B. stearothermophilus would be rich in cytosine. This is of particular significance because methods are presently available for the determination of pyrimidine sequences in DNA (78). The CCC and CC sequences in one strand of B. stearothermophilus DNA should correlate directly to the proline content in the proteins of this organism.

The major problem with this type of approach is that a bacterium

is too complex, contains too much DNA and too many proteins. A much better organism to work with would be a small bacteriophage for a thermophilic organism. At the time this investigation was begun several phage for thermophilic bacteria had been described (18, 19, 20, 21, 24). None of these phage had been described in detail with regard to the physical and chemical characteristics of their protein and nucleic acids. The purpose of the present investigation was to isolate and characterize in detail the chemical and physical properties of the protein and nucleic acid of a bacteriophage for Bacillus stearothermophilus. It was hoped that such a study would lead to a further understanding of the relationship between the structure and composition of nucleic acids and proteins.

MATERIALS AND METHODS

Bacteria.

The host bacterium was selected as a single step mutant of Bacillus stearothermophilus ATCC 8005 resistant to 1 mg/ml streptomycin sulfate that was included in agar plates. This host is designated Bacillus stearothermophilus ATCC 8005 S^R.

Media.

The TYNGC broth used for liquid bacterial and phage cultures and for dilution of phage suspension for assay purposes contained 10 g of Bacto-Tryptone (Difco), 5 g of Bacto Yeast Extract (Difco), 10 g of NaCl and 1000 ml of distilled water. Glucose (1 g) and $CaCl_2$ to 2 X 10^{-3} M were added aseptically after autoclaving. The TYNGCM broth was the same as TYNGC except that $MnCl_2$ to 10^{-5} M was added aseptically after autoclaving. Agar plates contained 20 to 30 ml of either TYNGC or TYNGCM plus 2.5% Bacto-Agar (Difco). Top agar consisted of either TYNGC or TYNGCM plus 0.8% Bacto-Agar (Difco). TYNGCM medium was used in the isolation procedure. Subsequent investigation indicated that Mn^{2+} was not required in the media. All experiments subsequent to the isolation utilized TYNGC medium.

Buffers.

The buffers used in this investigation are as follows:

Phage buffer - 0.2 M NaCl, 0.01 M Tris (Tris(hydroxymethyl)-aminomethane), pH 7.15 at 25°C.

DNA buffer - 0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA (Ethylene-diaminetetraacetic acid, disodium salt), pH 7.5 at 25°C.

SSC - 0.15 M NaCl, 0.015 M trisodium citrate, adjusted to pH 7.1 at 25°C with HCl.

0.1 X SSC - SSC diluted by a factor of 10.

2.0 X SSC - a stock solution of 1.5 M NaCl, 0.15 M trisodium citrate, adjusted to pH 7.1 at 25°C with HCl, diluted by a factor of 5.

Phage assay.

The phage were assayed using the agar layer technique of Adams (25). Unless otherwise noted 0.1 ml of phage suspension was plated using 0.5 ml of a log phase culture of Bacillus stearothermophilus ATCC 8005 S^R (5 X 10^7 cells/ml) as a lawn in 1.5 ml of top agar. The concentration of infective phage particles in a phage suspension, as determined by this method, is reported as phage/ml.

Incubation.

Broth cultures were incubated as either 20 to 30 ml in a 125 ml screw cap flask or 300 ml in a 1000 ml screw cap flask in a New Brunswick Gyrotory water bath shaker. The incubation temperature was 60°C unless otherwise noted. Under these conditions the division time of the host was 20 min. Bacterial concentrations were determined by measuring the optical density at a wavelength of 600 mμ (OD_{600}). An optical density of 1.0 represented 5 X 10^7 cells/ml for Bacillus stearothermophilus ATCC 8005 S^R.

Agar plates for phage assay were incubated 5 to 6 hours at 60°C. The atmosphere in the incubator was humidified to prevent drying of the plates.

Phage isolation.

The isolation procedure used was essentially that used by Romig and Brodetsky (26) for the isolation of bacteriophages for Bacillus

subtilus. Their procedure was modified for use with Bacillus stearothermophilus.

Five grams of soil, obtained from various areas around the campus of the California Institute of Technology were suspended in 15 ml of tap water and stored at room temperature for two days. The soil was then resuspended by shaking and allowed to settle for 30 minutes. Five ml of the clearer top layer were then added to 5 ml of TYNGCM broth and then incubated with shaking for 4 hours at 60°C. Five ml of a log phase culture of Bacillus stearothermophilus ATCC 8005 S^R (5×10^{-7} cells/ml) along with streptomycin sulfate to 50 μg/ml were added, and the incubation was continued for 6 more hours at 60°C. This final culture was plated in 10 X serial dilutions using 0.2 ml of a log phase culture of Bacillus stearothermophilus ATCC 8005 S^R as a lawn. Isolated plaques were usually obtained by the 100 X dilution. Phage from individual plaques were picked up with a sterile needle, placed in 1 ml TYNGCM broth and again plated in 10 X serial dilutions. This single plaque isolation procedure was repeated three times to insure homogeneity of the phage stocks. After three successive single plaque isolations the final 1 ml of phage suspension was stored at 4°C for later use.

One of three phages that were obtained from a single soil sample was selected for further study and was designated TØ3 (Thermophilic Phage 3).

Preparation of phage stocks.

Phage stocks were prepared by infecting a 20 ml culture of the host organism, that contained 5×10^6 cells/ml, with 5×10^6 phage/ml. Incubation at 60°C was then continued until the OD_{600} dropped to less

than 0.5. The lysate was centrifuged at 10,000 X g for 10 minutes. The supernatant was removed with a pipette and filtered through a sterile HA Millipore filter. The pellet was discarded. Stocks prepared in this manner had a titer of 2 to 3 X 10^{10} phage/ml and were stable when stored at 4°C.

Purification.

Large quantities of phage were grown in 5 gallon carboys containing 15 liters of TYNGC broth per carboy. A temperature of 60°C was maintained in the medium by placing two carboys in a hot water bath maintained at 63°C. The medium was aerated vigorously with heated filtered air. Each 15 liter carboy was inoculated with 300 ml of a log phase bacterial culture, that had been grown the previous day and had been stored overnight at 4°C. When the optical density at 600 mμ reached 0.1 (5 X 10^6 cells/ml), the culture was inoculated with phage to a concentration of 10^7 phage/ml. Lysis of the culture began about 180 minutes after infection, and incubation was continued 150 minutes longer. Lysate titers were usually about 2 X 10^{10} phage/ml.

The cell debris was removed from the lysate by centrifugation in the KSB-R Servall Continuous Flow System, for the Servall RC-2 centrifuge, at 16,500 rpm and at a flow rate of 300 ml/min. The phage were then precipitated by the addition of 350 g of ammonium sulfate per liter of lysate (55% saturation). The precipitate was allowed to settle at 4°C for at least 2 days. Most of the clearer top layer was then siphoned off and discarded. The sediment was packed by centrifugation for 45 min at 14,600 X g. The supernatant was discarded, and the pellet was resuspended in 300 ml of a 55% saturated solution of $(NH_4)_2SO_4$. This

was then dialyzed against five 6000 ml changes of 0.2 M NaCl. The suspension was then removed from the dialysis tubing and treated with 10 μg/ml of deoxyribonuclease (Sigma Chemical Company), 10 μg/ml of ribonuclease (Sigma Chemical Company) and 0.02 M $MgCl_2$ at 37°C for 1 hour. The suspension was then centrifuged at 10,000 X g for 10 min. The sediment was washed with a small volume of 0.2 M NaCl and centrifuged at 10,000 X g for 10 min. The supernatants were pooled, and the sediment was discarded. The phage were then pelleted by centrifugation in the number 30 rotor in a Spinco model L ultracentrifuge at 78,000 X g for 2 hours. The pellets were removed, pooled and resuspended in 3 ml of phage buffer by stirring with a magnetic stirrer for 2 hours at 4°C.

The phage were then separated from contaminating bacterial protein by sedimenting them through a discontinuous CsCl gradient (27). Before centrifugation, the Spinco SW39 centrifuge tubes contained the following: a bottom layer of 1.5 ml of a CsCl solution with a density of 1.6, a middle layer of 2.0 ml of a CsCl solution with a density of 1.3 and a top layer of 1.0 to 1.5 ml of a crude phage suspension. This system was centrifuged at 36,000 rpm for 3 hours in the SW39 rotor. The tubes were removed and the visible phage band that occurred between the $\rho = 1.3$ and $\rho = 1.6$ layers was collected by the drop collecting technique of Weigle, Meselson and Paigen (28). The drops containing the visible phage band were collected in a separate container. The large brown band of contaminants at the top of the tube was separated from the phage band by several centimeters. Several bands collected in this manner were pooled. A volume of CsCl solution, with a density

of 1.5, was added to bring the total volume up to 10 ml. The phage were then banded in a continuous CsCl gradient (29) by centrifugation at 36,000 rpm for 20 hours in the SW39 rotor. After the phage band had been collected by the drop collecting procedure it was dialyzed against 3 changes of phage buffer. Any large debris remaining was removed by centrifugation at 10,000 X g for 10 minutes. The purified phage suspension was stored at $4^{o}C$ until used.

When smaller quantities of purified phage were needed in a shorter period of time, an alternate purification procedure was used. The bacteria were grown to the same concentration as above, but as 300 ml in a one liter flask in the New Brunswick Gyrotory shaker. Phage were added at the same concentration and the incubation was continued in the same manner. The cell debris was removed by centrifuging at 10,000 X g for 10 minutes. The supernatant was treated with deoxyribonuclease and ribonuclease as above. The phage were then pelleted by centrifuging for 2 hours at 78,000 X g. The procedure was identical from this point on. This procedure eliminated the time consuming $(NH_4)_2SO_4$ precipitation. The phage obtained were identical in all respects with those obtained by the ammonium sulfate procedure.

The cesium chloride used in these studies was obtained from two sources. The first, produced by Penn Rare Metals Inc., Revere, Pennsylvania, was designated 99.9% CsCl and was obtained from the Kawecki Chemical Co. Solutions of this product developed a slight precipitate upon standing. This was filtered out before use. A filtered stock solution with a density of 1.51 had an optical density at a wavelength of 260 mμ (OD_{260}) of 0.022. Filtered solutions of this CsCl were used

in the discontinuous gradients and for the phage buoyant density experiments described in the section on the Determination of the buoyant density of TØ3. Optical quality CsCl, obtained from the Harshaw Chemical Co. (Harshaw lots 16 and 17), was used in all other experiments. A stock solution of this CsCl at a density of 1.85 had an OD_{260} of 0.011.

Electron micrographs.

A diluted purified phage suspension was dialyzed against 3 changes of distilled water and then mixed (1:1) with either 4% phosphotungstic acid (30), pH 7.0, in 0.4% sucrose solution or a saturated solution of uranyl acetate (31, 32). The mixture was then placed on a standard electron microscope grid that was coated with a collodion film reinforced with a thin layer of evaporated carbon. The excess solution was removed with the edge of a piece of paper. The preparation was then air dried before examination in the Phillips EM200 electron microscope.

Determination of the spectrum of the phage.

To determine the ultraviolet absorption spectrum of TØ3, a purified phage suspension in phage buffer was diluted to about 100 μg/ml, and the spectrum was taken on the Cary model 15 spectrophotometer (Applied Physics Corp.). A sample of phage buffer was used as the blank reference.

Determination of the buoyant density of TØ3.

The buoyant density of TØ3 in a cesium chloride density gradient (29) was determined as follows. A purified suspension of phage TØ3

and a purified suspension of phage λ^+ were diluted and mixed to a final concentration of 3 X 10^6 phage TØ3/ml and 6 X 10^4 phage λ^+/ml with a CsCl solution whose density was 1.51. The final density of the solution was 1.50. Centrifuge tubes for the SW39 rotor of the Spinco model L ultracentrifuge were filled with 3.3 ml of mixed phage suspension and this suspension was overlayed with paraffin oil. The mixed phage suspension was then centrifuged in the SW39 rotor for 20 hours at 37,000 rpm at 4°C in the Spinco model L ultracentrifuge. The rotor was allowed to slow to a stop without a brake. The tubes were carefully removed and the contents were fractionated by the drop collecting technique (28). The individual drops were collected into 2.0 ml of sterile TYNGC broth except that every 10th drop was collected in a screw cap vial, for density determination. The screw cap vials were capped immediately after a drop was collected. The refractive index of each fraction in the screw capped vials was measured with a Zeiss refractometer. The density of these fractions was determined from the following relationship:

For a solution of CsCl in water,

$$\rho^{25} = 10.8601\ n_D^{25} - 13.4974 \quad (33),$$

where ρ^{25} is the density in gm/ml at 25°C

and n_D^{25} is the refractive index at 25°C.

The slope of the graph of ρ^{25} vs. fraction number was then determined by the method of least squares using density data from every tenth fraction from fraction number 20 to fraction number 80. The actual density gradient was adjusted so that the density at the λ^+ peak (see below) was 1.508 (34).

The position of the TØ3 phage band was determined by assaying each fraction for TØ3. The position of the λ^+ band was determined by assaying each fraction for λ^+ using Escherichia coli C600 as a lawn. Plates for the λ^+ assay were incubated overnight at 35°C. The λ^+ phage and Escherichia coli C600 bacteria were the gift of Mr. Elton T. Young of the California Institute of Technology.

Determination of the latent period of TØ3.

The latent period of phage TØ3 was determined by following the one step growth curve of the phage (35). The host organism was incubated in TYNGC broth at 60°C until the OD_{600} reached 1.0 (5 X 10^7 cells/ml). Phage to a multiplicity of infection of about 1.4 (7 X 10^7 phage/ml) were added, and the incubation was continued at 60°. Samples were taken every five minutes and their titers were determined.

Determination of the burst size of TØ3.

Due to poor adsorption of the phage to the bacterial cell wall the one step growth curve does not give an estimate of the burst size of TØ3. The single burst procedure of Ellis and Delbruck (35) was modified for use with this phage. The host bacteria were grown to a density of 2.5 X 10^7 cells/ml in TYNGC. Phage were added to a concentration of 1.5 X 10^8 phage/ml. The phage were allowed to adsorb for 10.0 min at 60°C. The culture was then cooled in an ice bath and centrifuged at 10,000 X g for 5 minutes. The pellet was washed twice with cold TYNGC broth to remove unadsorbed phage. A portion of the resuspended pellet was then diluted by a factor of 2.5 X 10^6 and 0.2 ml samples of this diluted culture were placed in each of 60 sterile test tubes.

Incubation was then continued at 60° for 50 minutes. Soft agar (1.5 ml) and 0.5 ml log phase host were added to each tube. The contents of each tube were poured into petri dishes that had been prepared beforehand. The plates were incubated as described in the section on Incubation.

Adsorption of the phage to the host.

The relative number of phage adsorbed to the host in 10 minutes was determined by infecting a culture of the host at a concentration of 2.5×10^7 cells/ml with 4×10^4 phage/ml. This system was incubated at 60° for 10 minutes. The culture was cooled in an ice bath and centrifuged for 5 minutes at 10,000 g. The supernatant was poured off and the pellet was washed 4 times with cold TYNGC broth. The phage titer in each washing and resuspended pellet was determined.

Thermal stability of TØ3.

The stability of the phage at high temperatures was measured by placing 20 ml of phage suspension containing 2×10^3 to 4×10^3 phage/ml in a 125 ml screw cap flask in a water bath at the appropriate temperature. The temperature was allowed to equilibrate for 5 min. One ml samples were taken at appropriate times and cooled in an ice bath. The titer of each 1 ml sample was then determined. The natural logarithm of the ratio of the initial titer to the titer at time t was plotted against the time t. A straight line was fitted by least squares to the data at each temperature, and the time of one half survival ($t_{\frac{1}{2}}$) was determined from the slope.

Host range.

A stock of phage containing 2×10^9 phage/ml when assayed using

B. stearothermophilus ATCC 8005 S^R for the lawn was assayed using ATCC strain 7953, 7954, 10149 or 12016 or Escherichia coli strain C or C600 for the lawn. The conditions for each were the same as that for assaying phage using strain 8005 S^R except that the growth temperature in broth and on plates was 50°C for B. stearothermophilus ATCC 7954 and 35°C for E. coli C and C600.

Demonstration of a phage "lysozyme".

To demonstrate the activity of an enzyme that lysed the bacteria, a sample of phage stock containing 100 phage per ml was assayed. After the initial incubation of 5 hours at 60° the incubation was continued at various temperatures for an additional 8 hours. After the 8-hour incubation the halos surrounding the plaques on plates incubated at 60° were sampled with a sterile needle and the needle was washed with one ml sterile TYNGC broth. Each one ml sample was then titered for phage.

Extraction of TØ3 DNA.

TØ3 DNA was extracted from purified phage in one volume of phage buffer (0.5 mg of phage per ml) by gentle shaking for five minutes with an equal volume of redistilled phenol (saturated with phage buffer). The mixture was centrifuged at 3,000 g for 2 minutes to break up the emulsion. The aqueous layer was removed with a pipet that had an inside diameter of 5 mm. The extraction was repeated 2 more times. The phenol layers were serially extracted twice with 0.5 volume of phage buffer and the aqueous layers were combined. The phenol in the combined aqueous layers was removed by 5 extractions with 0.5 volume ether. Nitrogen was bubbled through the aqueous layers for 30 minutes

at room temperature to remove the ether. The DNA solution was then dialyzed against 3 changes of DNA buffer or SSC at 4°C. Any insoluble material was removed by centrifugation at 20,000 X g for 10 minutes. The DNA solution was then diluted to a concentration of about 75 μg/ml. The ultraviolet absorption spectrum of a solution of TØ3 DNA in SSC was determined with the Cary 15 spectrophotometer using an SSC buffer blank. DNA solutions were stored at 4°C. The molecular weight of the DNA (as determined from the band width in an analytical cesium chloride density gradient) of one such solution, did not change over a two-week period.

Thermal denaturation and renaturation of TØ3 DNA.

The thermal denaturation studies of the DNA of bacteriophage TØ3 were performed according to the method of Marmur and Doty (13). A sample of Escherichia coli DNA used for comparison was the gift of Mr. Roger J. Radloff of the California Institute of Technology. The renaturation studies were performed by allowing the heat denatured samples to cool while in the spectrophotometer. The temperature decrease in the renaturation studies was linear from 95°C to 60°C at 3.9°C per min, the decrease was then progressively slower. The solvents used were: SSC, 0.1 X SSC and 2.0 X SSC. These studies were performed using a Cary 15 spectrophotometer and a Gilford automatic melting curve apparatus.

Determination of the buoyant density of TØ3 DNA.

The buoyant density of TØ3 DNA in a cesium chloride density gradient (29) was determined according to the method of Vinograd and Hearst (38). Escherichia coli DNA and Micrococcus lysodeikticus DNA were used to provide density references. The buoyant density of E. coli

DNA in a CsCl solution was assumed to be 1.704, and the buoyant density of M. lysodeikticus DNA in a CsCl solution was assumed to be 1.726 (39). Both of these samples of DNA were obtained from Mr. Roger J. Radloff. The centrifugation was performed in a 12 mm Kel-F centerpiece in the Spinco model E analytical ultracentrifuge. The centrifuge was operated at 44,770 rpm and at a temperature of 25°C. Ultraviolet optics including a corex filter were used. A -1° radial wedge top window was used. A series of exposures of from 1 to 5 minutes was made using Kodak Commercial film. A record of DNA concentration vs. radius was obtained from the developed film with a Joyce-Loebl double beam microdensitometer.

Band molecular weight of TØ3 DNA.

The molecular weight of TØ3 DNA as determined from the density gradient band shape (29), was calculated according to the procedure used by Studier for the determination of the molecular weight of the DNA of bacteriophage T7 (37). This procedure was devised from that given in earlier papers by Hearst and Vinograd (40, 41) and Hearst, Ifft and Vinograd (42). The hydration and compression parameters used were those determined for T4 DNA (41, 42, 43). The details of this procedure are given in the Appendix. The centrifugation was performed as described in the section on the Determination of the buoyant density of TØ3 DNA.

The method for computing the half-band width σ is described in the Appendix. For each density gradient several exposures were analyzed, and an average σ was computed. The molecular weight was then computed from the σ obtained.

Electron microscopy of TØ3 DNA.

The DNA of bacteriophage TØ3 was prepared for electron microscopy

according to the method of Kleinschmidt et al (44), except that the phage were suspended in a solution of 4 M ammonium acetate that contained 0.01% cytochrome c, before being osmotically shocked. The Phillips EM200 electron microscope was used. After development the negatives were projected onto the ground glass screen of a Nikon model 6 shadowgraph and the DNA molecules were traced. The length of the tracings was then determined with a map measure. The molecular weight was determined assuming the average molecular weight of the Na^+ salt of a nucleotide pair to be 662 and assuming a separation between base pairs of 3.46 Å (B configuration). This leads to a molecular weight to length ratio of 1.92×10^6 molecular weight units per micron (45, 46, 47).

Chemical composition of TØ3 DNA.

A. Base analysis.

The base analysis of TØ3 DNA was performed utilizing either whole phage or isolated DNA. The hydrolysis procedure used was that of Wyatt and Cohen (48, 49). Whole phage (0.8 mg) or phage DNA (0.4 mg) was dissolved in 98% formic acid in a pyrex tube that had an inside diameter of 4 mm and was about 30 cm long. The air in the tube was replaced with nitrogen and the tube was sealed. Hydrolysis was carried out at 175°C for 60 minutes. The tube was allowed to cool and the contents were frozen in a dry ice-methyl cellosolve bath. The tube was opened with a small flame, and the contents were evaporated to dryness by placing the tubes in a 60°C water bath and blowing a stream of air over them. The residue was then dissolved in 25 µl of 1 N HCl and two 10 µl portions were taken for chromatography. The bases were separated by descending chromatography on Whatman number 1 filter paper

that had previously been washed. The solvent for both the washing and chromatography was the solvent I of Kirby (50) and contained methanol: concentrated HCl:H_2O (70:20:10) by volume. Chromatography was continued until the solvent front had moved about 40 cm.

The chromatograms were dried and the ultraviolet absorbing spots were located with a short wavelength ultraviolet lamp. The spots were cut out and eluted with 0.1 N HCl into weighed vials. The weight of the vial plus the contents was determined, and from this the volume of the contents was determined (assuming a density of 1.00 g/ml). Ultraviolet absorption spectra were taken and the concentration of each base was determined from the optical density at the peak of absorbance using the extinction coefficients given by Bendich (51). From these data the molar ratios of the bases were determined.

B. From the thermal denaturation temperature.

The thermal denaturation temperature (T_m) of TØ3 DNA and the base composition corresponding to this T_m were determined according to the method of Marmur and Doty (13). The T_m and composition of a sample of Escherichia coli DNA (T_m = 90.5°, GC = 50 % (13)) were used as temperature and composition references. The Escherichia coli DNA was described in the section on the Thermal denaturation and renaturation of TØ3 DNA. The solvent used was SSC as described in the section on Buffers.

C. From the buoyant density.

The buoyant density of TØ3 DNA in a CsCl solution and the base composition corresponding to this buoyant density were determined according to the method of Schildkraut, Marmur and Doty (52). The buoyant density of E. coli DNA (1.710) and the buoyant density of

M. lysodeikticus DNA (1.731) were used as density references (52).

Strand separation and annealing of TØ3 DNA.

Separation of the two strands of TØ3 DNA was demonstrated in the following manner. A small sample of TØ3 DNA in SSC was heated in a boiling water bath for 10 minutes. This sample was then quenched in an ice bath. A sample containing 0.8 μg of this heat denatured DNA and 0.8 μg of TØ3 native DNA, in a CsCl solution (pH 8.0 with 0.01 M Tris) whose density was 1.700, was placed in a 12 mm Kel-F centerpiece and centrifuged in the ultracentrifuge as described in the section on the Determination of the buoyant density of TØ3 DNA. After 24 hours photographs were taken. The centrifuge cell was removed and tightened to 115 inch lb. The contents of the cell were mixed by inverting the cell several times. The annealing was accomplished by placing the intact centrifuge cell in distilled water in a 60° C water bath (53). The temperature was maintained at 60° for 1 hour and was then slowly decreased to 30°C over a period of 3 to 5 hours. The cell was then removed from the water bath, dried and retightened to 115 inch lb. Centrifugation was then carried out as before.

In order to increase the height of the peaks, without increasing the concentration of the heat denatured material, a 30 mm Kel-F centerpiece was used. In this experiment 2 μg of heat denatured TØ3 DNA and 0.5 μg of M. lysodeikticus DNA, in CsCl at a density of 1.705 (pH 8.0 with 0.01 M Tris), were placed in a 30 mm cell. A -2° radial wedge window was used as the bottom window and a -1° radial wedge was used as the top window. The 1st equilibrium run, the annealing and the 2nd equilibrium run were the same as those for the 12 mm cell.

RESULTS

Isolation.

Phage were isolated from four of the eleven soil samples that were examined. One of these four soil samples contained three types of phage, as distinguished by the morphology of the plaques produced by them. One of the three phage from this sample produced large clear plaques and was capable of producing a high titer lysate. This phage was chosen for further study and was called TØ3 (Thermophilic Phage 3).

Plaques produced by TØ3 on TYNGC media are shown in Figure 1. The size of the plaques varies from pin points up to about 2 mm. This variation in plaque size is probably due to poor adsorption of the phage to the bacterial cell. It could be eliminated by increasing the initial concentration of bacteria in the lawn. When the bacterial concentration was increased by a factor of 10 (by centrifuging the bacterial culture at 10,000 X g for 10 min and then resuspending the bacteria in one tenth the original volume) the plaque size became uniform, and the apparent titer of the phage suspension being assayed increased by a factor of 1.6. Presumably, at the lower bacterial concentration some of the phage do not adsorb soon enough to give visible plaques.

Attempts to infect 4 other thermophilic bacterial strains (Bacillus stearothermophilus ATCC strains 7953, 7954, 10149 and 12016) and two mesophilic strains (Escherichia coli C and C600) with TØ3 were completely unsuccessful.

Phage morphology.

The morphology of TØ3 is illustrated in the electron micrographs

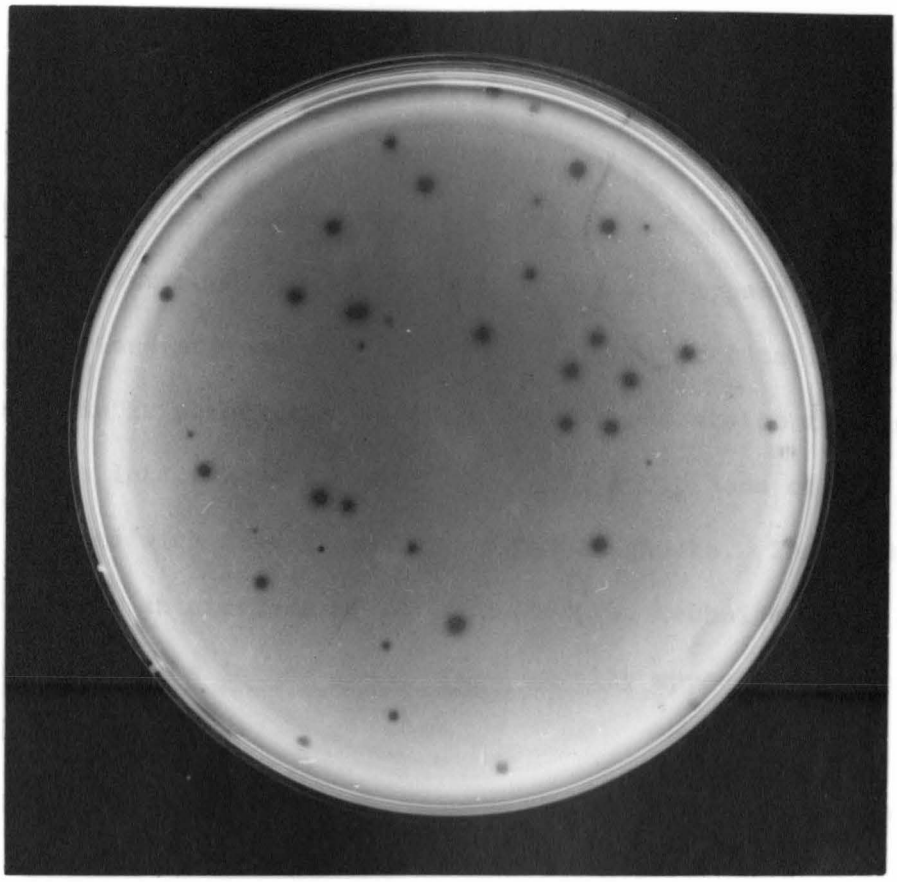

Figure 1

Plaques formed by bacteriophage TØ3. Five hours incubation at 60°C.

shown in Figure 2. Figure 2 A shows TØ3 and tobacco mosaic virus negatively stained with phosphotungstic acid (PTA). The length of the tail of the phage was found to be 125 mμ, assuming a length of 298 mμ (76) for tobacco mosaic virus. The length of the tail did not vary under the various staining conditions used. This length was used as a standard for magnification calibration when tobacco mosaic virus was not present.

Most of the phage particles are disrupted by the PTA negative staining technique. Apparently the PTA treatment removes the tail from the head and the nucleic acid contained inside the head is released into the surrounding medium. The empty heads appear as ghosts in the preparations stained with PTA. When uranyl acetate is used as a positive stain it penetrates the head protein and stains the nucleic acid inside the intact head as shown in Figure 2 D. None of the preparations stained with uranyl acetate contained ghosts. The ghosts must therefore be an artifact of the PTA staining technique. In addition to the ghosts the intact phage heads usually appeared swollen when a PTA stain was used.

A high magnification photograph of a PTA stained preparation is shown in Figure 2 B. The subunits of the head are resolved in this photograph, particularly along the edge of the phage head. There are 6 or 7 subunits to an edge. The length of the head, as determined from electron micrographs of preparations that were negatively stained with uranyl acetate, as is illustrated in Figure 2 C, is 57 mμ. The length of each edge of the regular hexagon-shaped head is 29 mμ. Assuming that there are 7 subunits per edge, each subunit has a diameter of approximately 4 mμ. Assuming that the subunits of the head are approximately

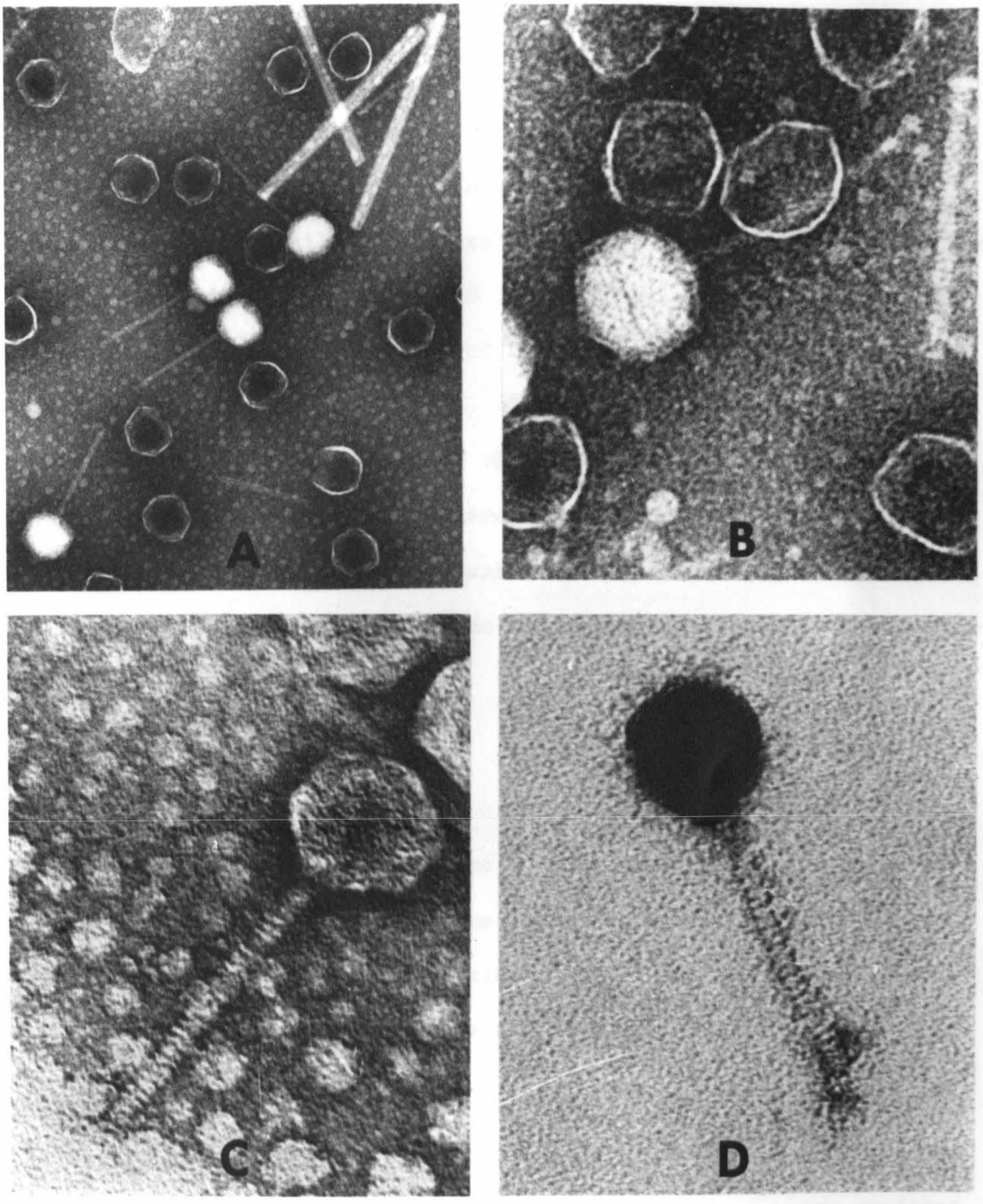

Figure 2

Electron micrographs of bacteriophage TØ3. A, phosphotungstic acid negative stain X 121,000; B, phosphotungstic acid negative stain X 332,000; C, uranyl acetate negative stain X 404,000; D, uranyl acetate positive stain X 368,000.

spherical, each subunit has a molecular weight of about 25,000. The dimensions and shape of the head are compatible with those of a regular icosahedron.

The details of the structure of the tail of TØ3 are also shown in Figure 2 C. The tail is 1250 mμ long and 10 mμ wide. The cross striation pattern of the tail is illustrated in this figure. There are about 30 cross striations that are spaced 3.9 mμ apart. The cross striation pattern changes near the junction of the tail and head, indicating that some type of collar may be present. The striations, the collar and possibly tail fibers at the end of the tail are illustrated in Figure 2 D. A careful inspection of preparations stained with PTA also indicates that tail fibers may be present.

An interpretation of the morphological characteristics of bacteriophage TØ3 is shown in Figure 3.

Spectrum of TØ3.

The ultraviolet absorption spectrum of a purified suspension of bacteriophage TØ3 is shown in Figure 4. The spectrum of the phage typically has 260:230:280 absorbance ratios of 1.00:0.75:0.62. The maximum absorbance occurs at a wavelength of 259 mμ and the minimum at 237 mμ. The ratio of absorbance at 259 to 237 is 0.62.

Buoyant density of TØ3.

The buoyant density of phage TØ3 was determined from the location of the infective phage particles in a cesium chloride density gradient (29). When bacteriophage TØ3 was banded in a cesium chloride density gradient a single sharp band of phage occurred at a density of 1.526. Three determinations all gave this value for the buoyant density of

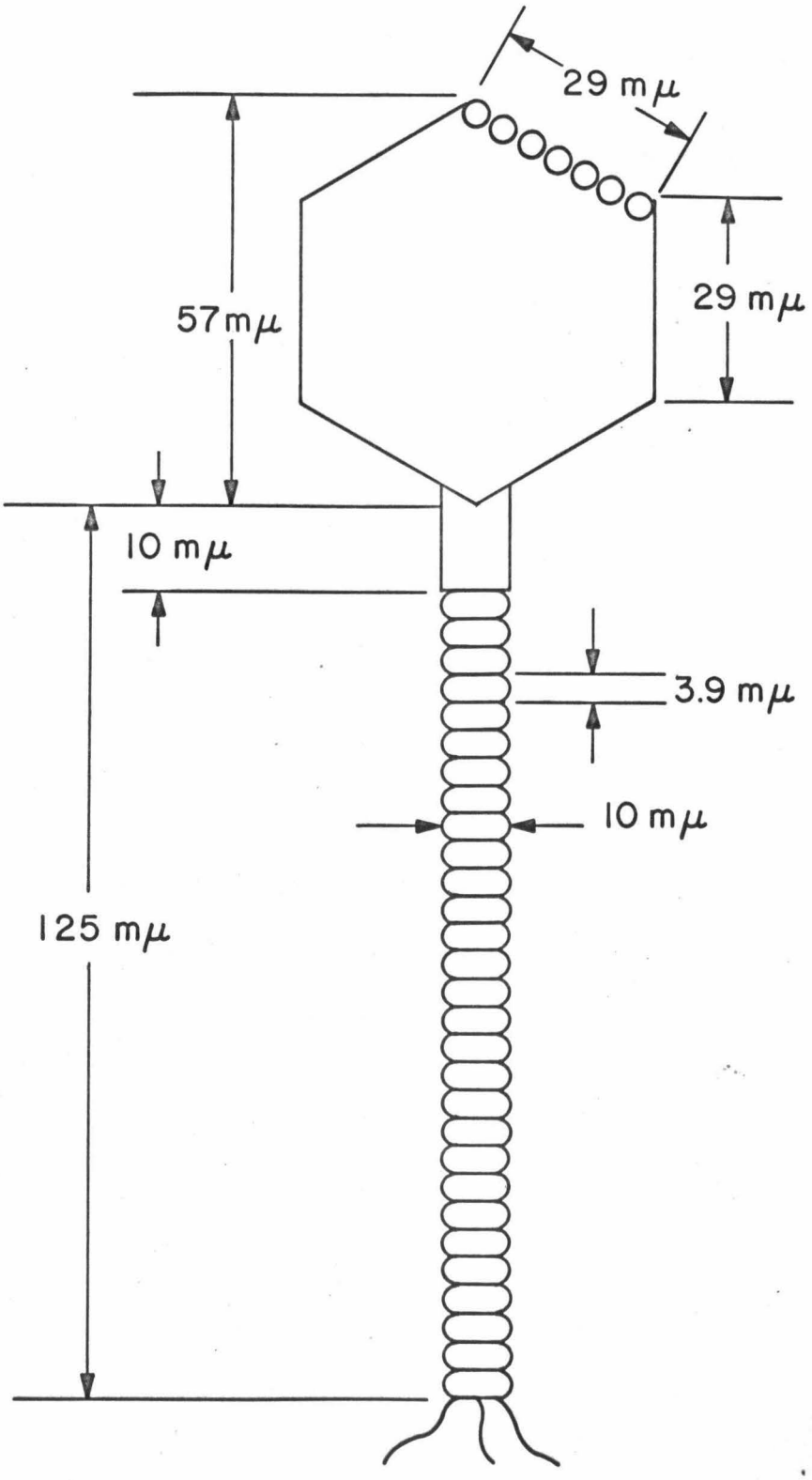

Figure 3

Bacteriophage TØ3.

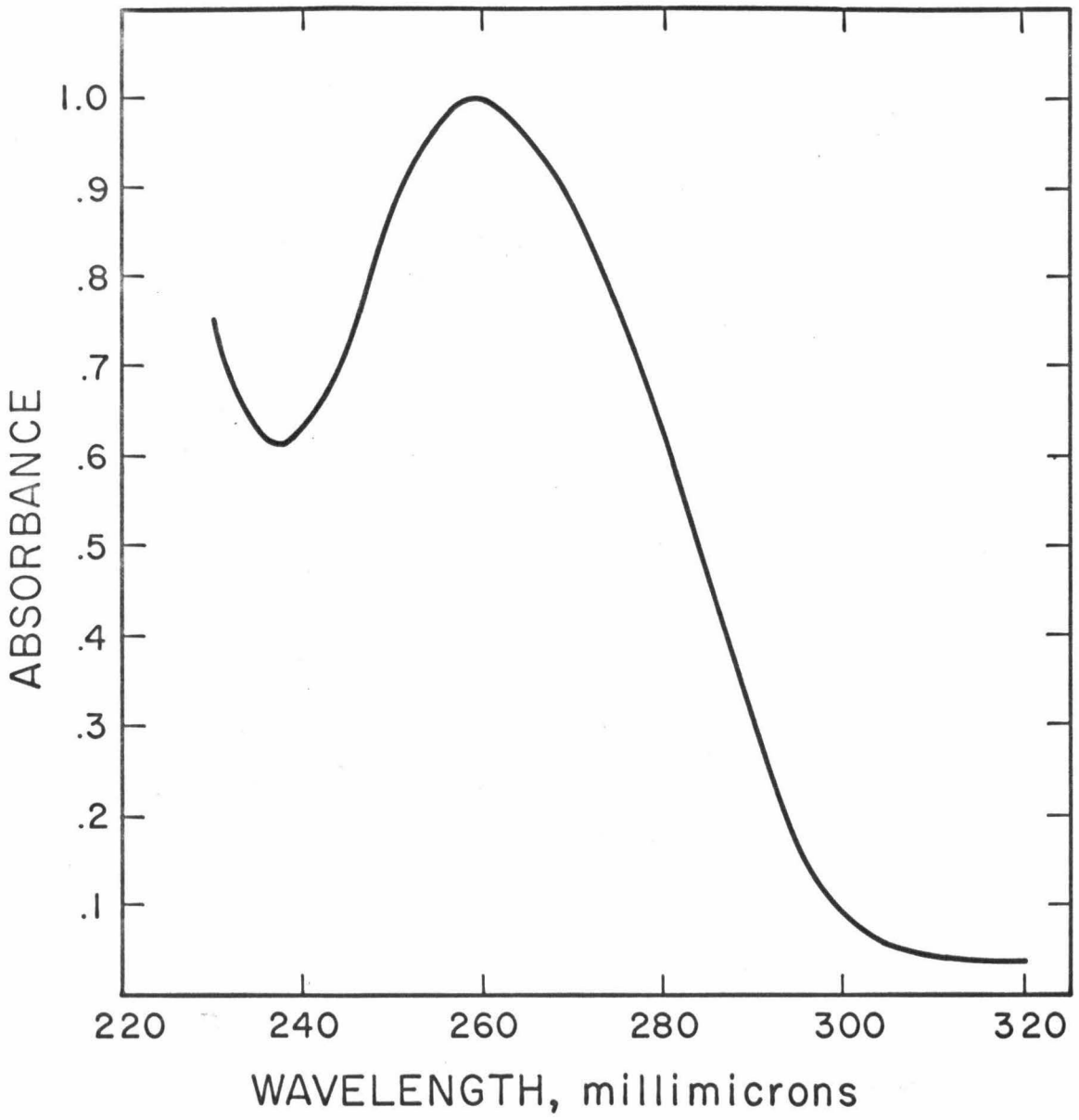

Figure 4

Spectrum of bacteriophage TØ3.

TØ3. The results of a density gradient experiment are illustrated in Figure 5. The TØ3 titer of each drop is indicated by a circle, squares indicate the phage λ^+ titer of each drop and triangles indicate the density of every tenth drop.

During the purification of the virus in which large quantities of phage were purified by the density gradient technique, a small second band sometimes occurred at a density of 1.48. The significance of this band has not been determined.

Latent period.

The latent period of TØ3 was determined from a one step growth curve as is illustrated in Figure 6. After infection of a bacterial culture with TØ3, the phage titer remained constant for 18 to 20 minutes. The titer then began to increase. The increase was rather slow and did not level off, as it should have done if all the bacteria had been infected at about the same time. Instead, the titer continued to increase, and the plateau was obscured by the asynchrony of the infection.

Burst size.

The burst size of TØ3 was determined from the results of a single burst experiment (35). In a single burst experiment samples containing, on the average, less than one infected bacterium are withdrawn from a bacterial culture infected with bacteriophage. The fraction P(r) of samples containing r infected bacteria is given by Poisson's formula,

$$P(r) = n^r e^{-n}/r! \quad ,$$

where n is the average number of infected bacteria per sample,

and e is the base of the natural logarithms.

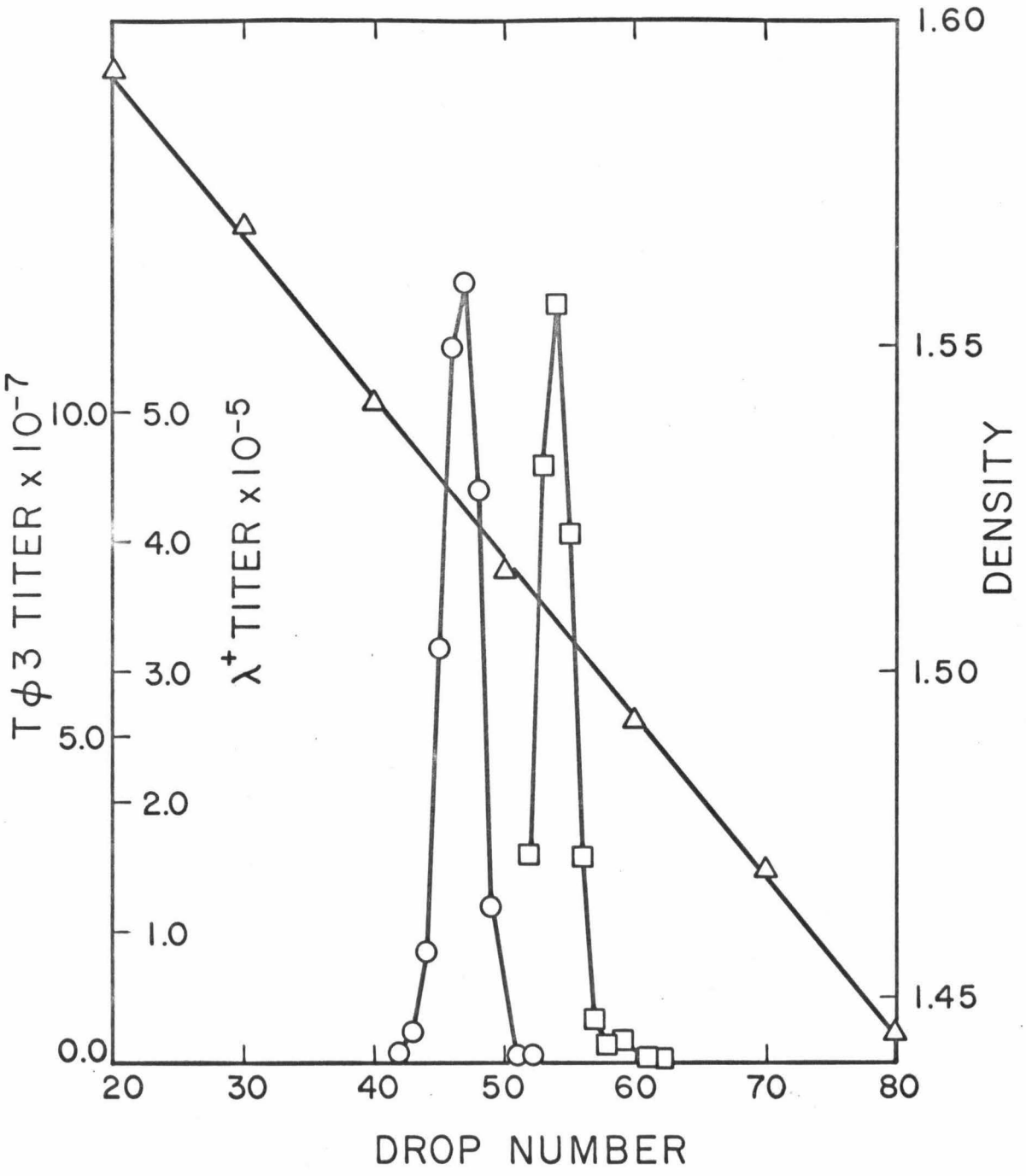

Figure 5

Buoyant density of bacteriophage TØ3 in a cesium chloride density gradient. Circles, TØ3 titer; squares, λ^+ titer; triangles, density.

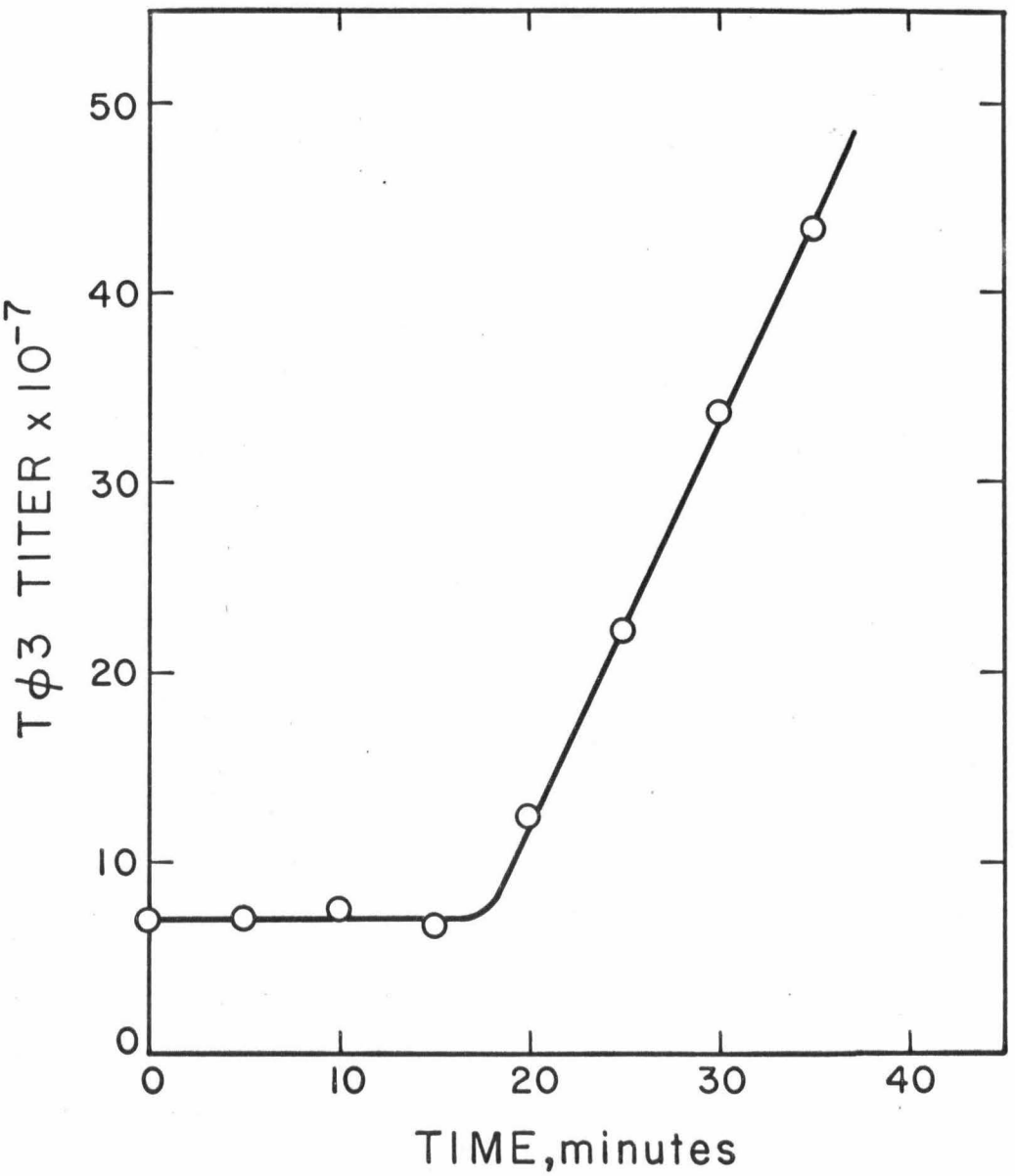

Figure 6

Bacteriophage TØ3 one step growth curve.

The average number of infected bacteria per sample, n, is determined from P(0),

$$P(0) = e^{-n} .$$

The results of a single burst experiment for phage TØ3 are given in Table 1. The fraction of plates containing 0 infected bacteria was 43/60 or 0.717.

$$P(0) = e^{-n},$$

$$0.717 = e^{-n}$$

and

$$n = 0.332 .$$

The average number of infected bacteria per sample was 0.332. The total number of infected bacteria in 60 samples was therefore 60 X 0.332 = 19.9. The total number of plaques was 4596 and the average burst size was 4596/20 = 230. The individual burst ranged from 54 to 783 with some of the counts being attributed to 2 or more infected bacteria in a single burst tube. The average burst size, obtained from two experiments, was 203.

Adsorption of the phage.

In a phage adsorption experiment in which the initial phage concentration was 2.4×10^4 phage/ml, about 2.9 percent of the phage were adsorbed in 10 minutes at 60^o at a bacterial concentration of 2.5×10^7 bacteria/ml. The percentage of phage adsorbed was also determined from the results of a single burst experiment. The initial phage concentration was 1.5×10^8 phage/ml. The concentration of infected bacteria after 10 minutes at 60^oC was 4.15×10^6 infected bacteria/ml. Therefore only $(4.15 \times 10^6/1.15 \times 10^8) \times 100 = 2.8$ percent of the phage had been adsorbed. The initial bacterial concentration was

TABLE 1

Variation of the burst size of TØ3

Number of plates	Number of plaques
32	0
8	1
2	2
1	5
17	54 to 783

Plaque count on plates containing more than 50 plaques.
54, 57, 125, 146, 161, 193, 198, 209, 221, 247,
258, 304, 358, 467, 679, 783.

A.	Total number of plates	60
B.	Number of plates containing 0 to 5 plaques	43
C.	Number of plates containing more than 50 plaques	17
D.	Total number of plaques in C	4596

2.5×10^7 bacteria/ml. These results indicate that the adsorption rate is independent of the phage concentration.

If calcium ion is not included in the media, the plaques formed by TØ3 are small, and the efficiency of plating is low. This is probably a reflection of a requirement for calcium ion for adsorption.

Thermal stability.

The thermal stability of phage TØ3 is illustrated in Figure 7. In broth the half life of TØ3 at 60°C is 400 minutes. At higher temperatures the phage is less stable, the half life at 65°C is 120 minutes, 40 minutes at 70°C and 12 minutes at 75°C.

The Arrhenius energy of activation for the heat inactivation reaction is given by:

$$\frac{d \ln k_n}{d T} = \frac{\Delta H_a}{RT^2}$$

where T is the absolute temperature

R is the gas constant (1.99 cal./deg.mole.),

ΔH_a is the activation energy in calories,

and k_n is the rate constant for inactivation.

The rate constant k_n is related to the half life by

$$k_n = \frac{\ln 2}{t_{\frac{1}{2}}} ,$$

where $t_{\frac{1}{2}}$ is the half life.

The activation energy, ΔH_a, for TØ3 in broth in the temperature range 60°C to 75°C is 56,000 cal.

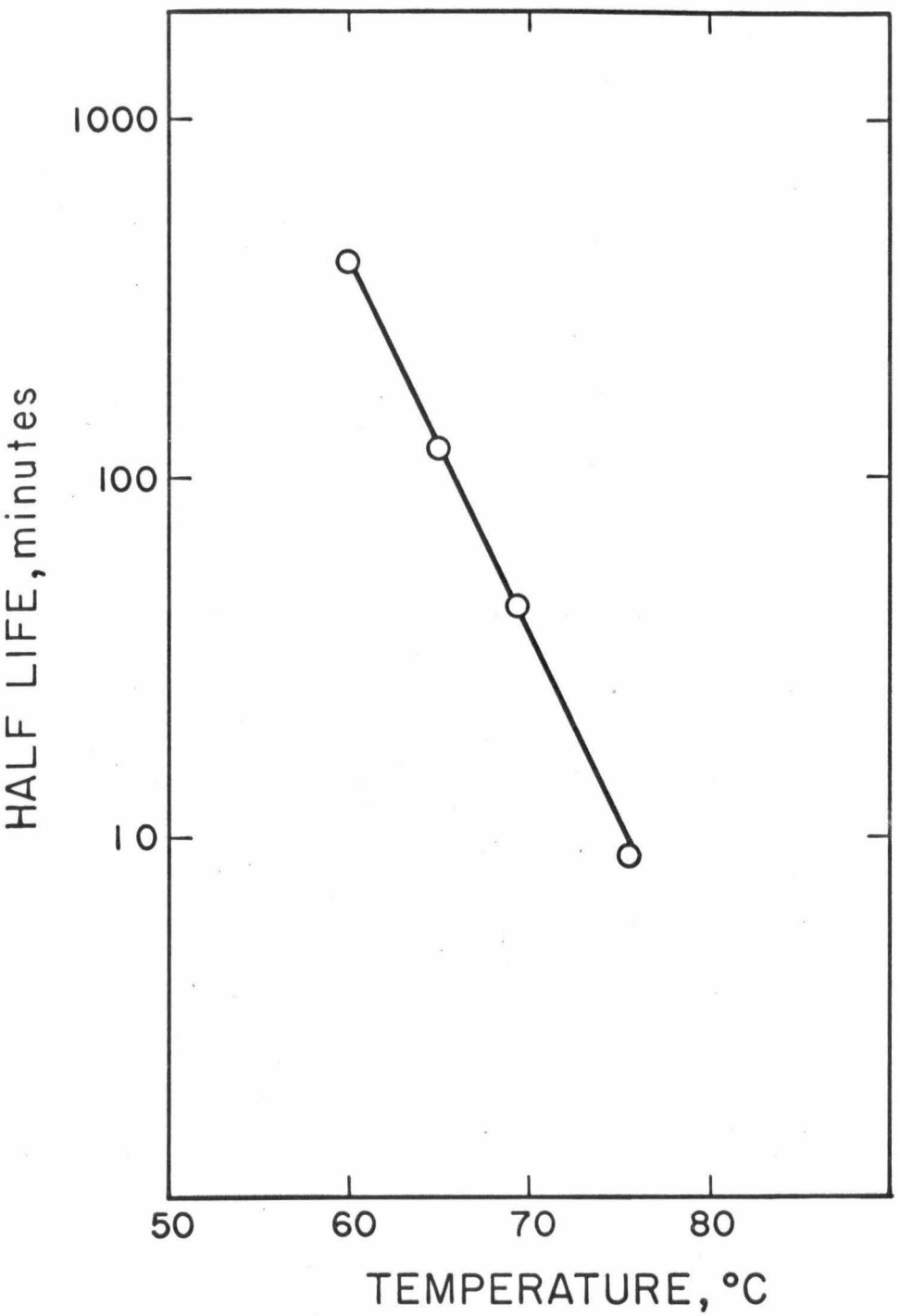

Figure 7

Thermal stability of bacteriophage TØ3 in broth.

Phage "lysozyme".

An enzyme that attacks and breaks down the bacterial cell wall can be demonstrated by continuing the incubation of plates containing phage TØ3 on a bacterial lawn for longer than 6 hours at 60°C. As illustrated in Figure 8, when this incubation is continued the plaque size continues to grow as a turbid halo forms around the clear central plaque. This halo is produced rapidly (4 hours) at 60°C, slowly (8 hours) at 48°C and even more slowly at 35°C and 25°C. At 4°C no halo is produced. There is essentially no further bacterial growth after the 6 hour initial incubation as judged by the turbidity of the areas of bacterial growth. Furthermore, there is practically no bacterial growth below 50°C. One phage was found in each of two samples, out of a total of 10 samples that were taken from the outer edges of 3 halos. The remaining eight samples contained no phage particles.

The interpretation of these results is that an enzyme that is active at temperatures of from 25°C to 60°C is produced in the central plaque during the initial incubation. During the second incubation the enzyme diffuses away from the plaque more rapidly than the phage. This enzyme attacks the cell walls of the bacteria that are present outside the plaque area. This leaves a turbid halo around the clear central plaque.

Spectrum of TØ3 deoxyribonucleic acid.

The ultraviolet absorption spectrum of the phage DNA is shown in Figure 9. The lower curve is the spectrum of the native TØ3 DNA, and the upper curve is the spectrum of heat denatured TØ3 DNA. Native TØ3

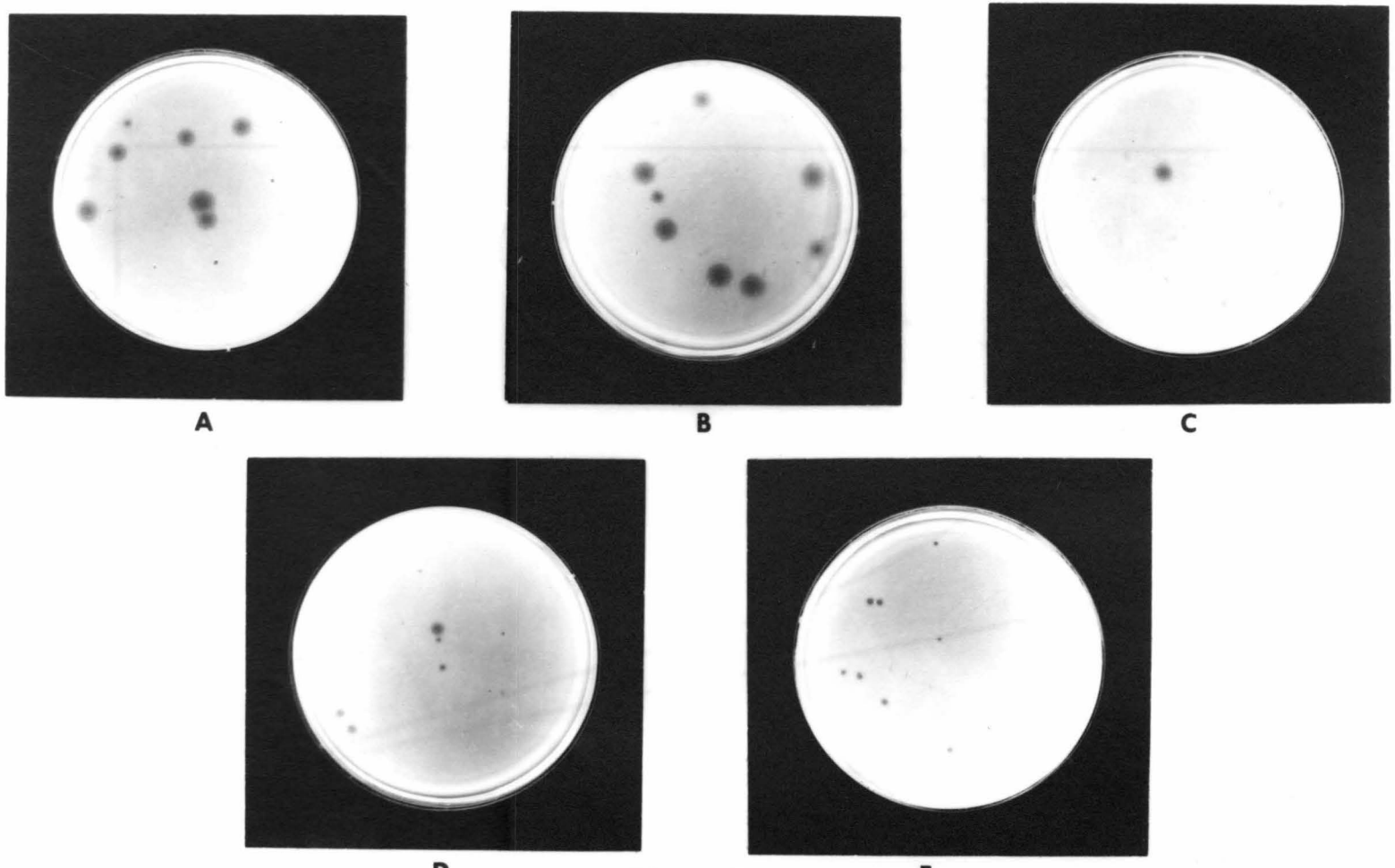

Figure 8

Photographs of plates indicating the action of bacteriophage TØ3 "lysozyme". Plates incubated 5 hours at 60°C and then incubated 8 hours at the following temperatures; A, 60°C; B, 48°C; C, 35°C; D, 25°C; E, 4°C.

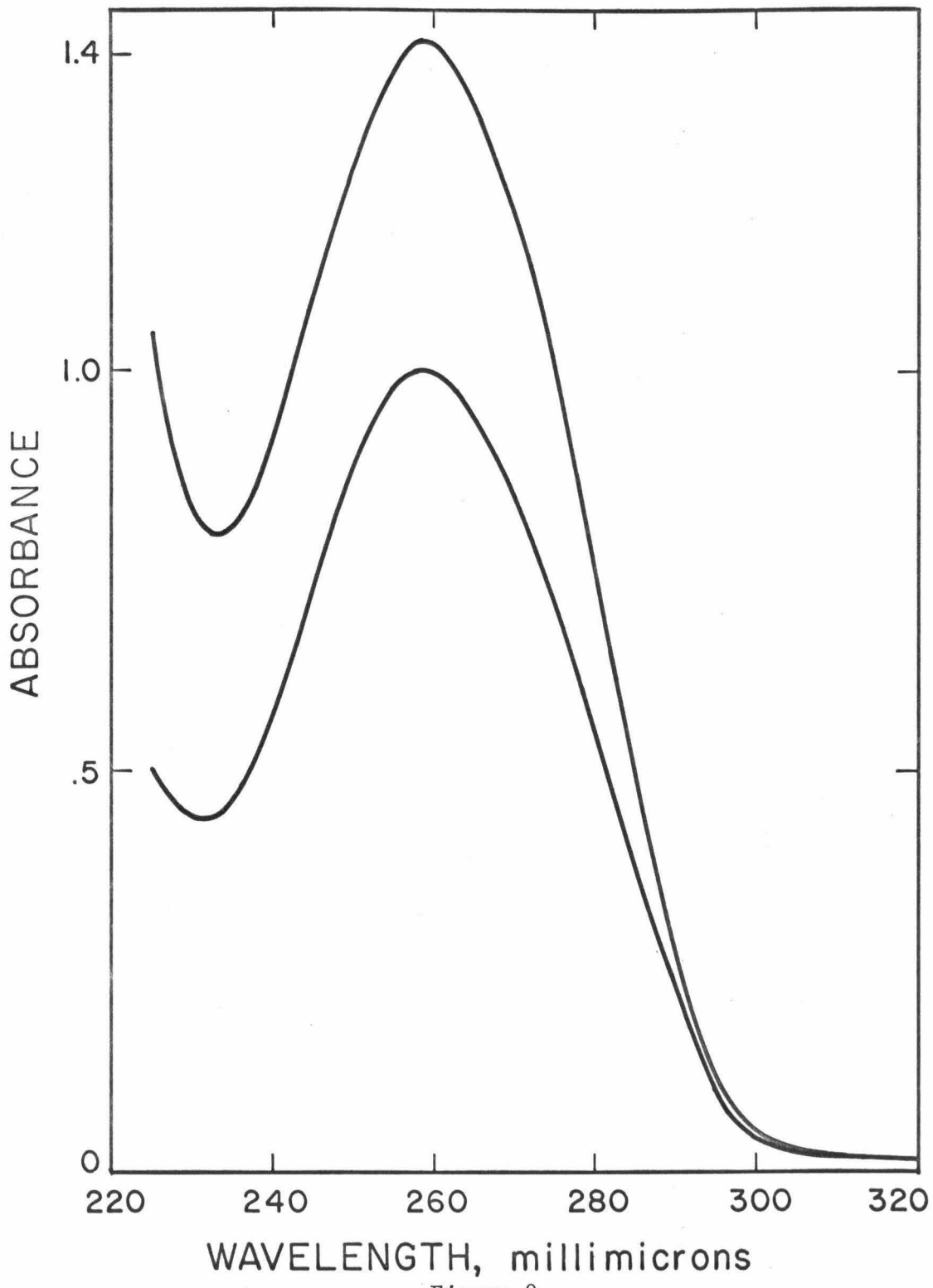

Figure 9

Spectrum of bacteriophage TØ3 DNA. Lower curvē, native DNA; upper curve, heat denatured DNA.

deoxyribonucleic acid typically has 260:230:280 ratios of 1.00:0.57: 0.56. The maximum absorbance occurs at a wavelength of 258 mμ and the minimum occurs at 233 mμ. Heat denatured TØ3 deoxyribonucleic acid has 260:230:280 ratios of 1.00:0.54:0.53. The maximum absorbance occurs at a wavelength of 259 mμ and the minimum occurs at 234 mμ.

Heat denaturation of TØ3 deoxyribonucleic acid.

When a solution of DNA is heated to a high temperature the double helical structure of the DNA is disrupted and the two polynucleotide chains separate (65). This separation results in a greater absorbance of ultraviolet light (hyperchromicity) by the DNA.

The heat denaturation of TØ3 DNA in three buffers with different ionic strengths is represented in Figure 10. As the temperature of a solution of TØ3 DNA increases, the absorbance at a wavelength of 260 mμ decreases from its value at room temperature to a minimum. The absorbance then rises rapidly and reaches a plateau. The midpoint of this rapid increase in absorbance is known as the melting temperature (T_m). The T_m of TØ3 DNA in 0.1 X SSC (circles) is 72.0°C, in SSC (squares) it is 88.5°C and in 2.0 X SSC (triangles) it is 92.0°C.

If the temperature of a solution of heat denatured DNA, at a high temperature, is allowed to decrease slowly, the DNA again assumes the native configuration with the two complementary strands specifically bonded together. This return to the native state is accompanied by a decrease in the absorbance of ultraviolet light (hypochromicity).

Figure 11 illustrates such a hypochromic effect for TØ3 DNA in various buffers. The samples that were heat denatured to obtain the data for Figure 10 were allowed to cool slowly. The annealing process

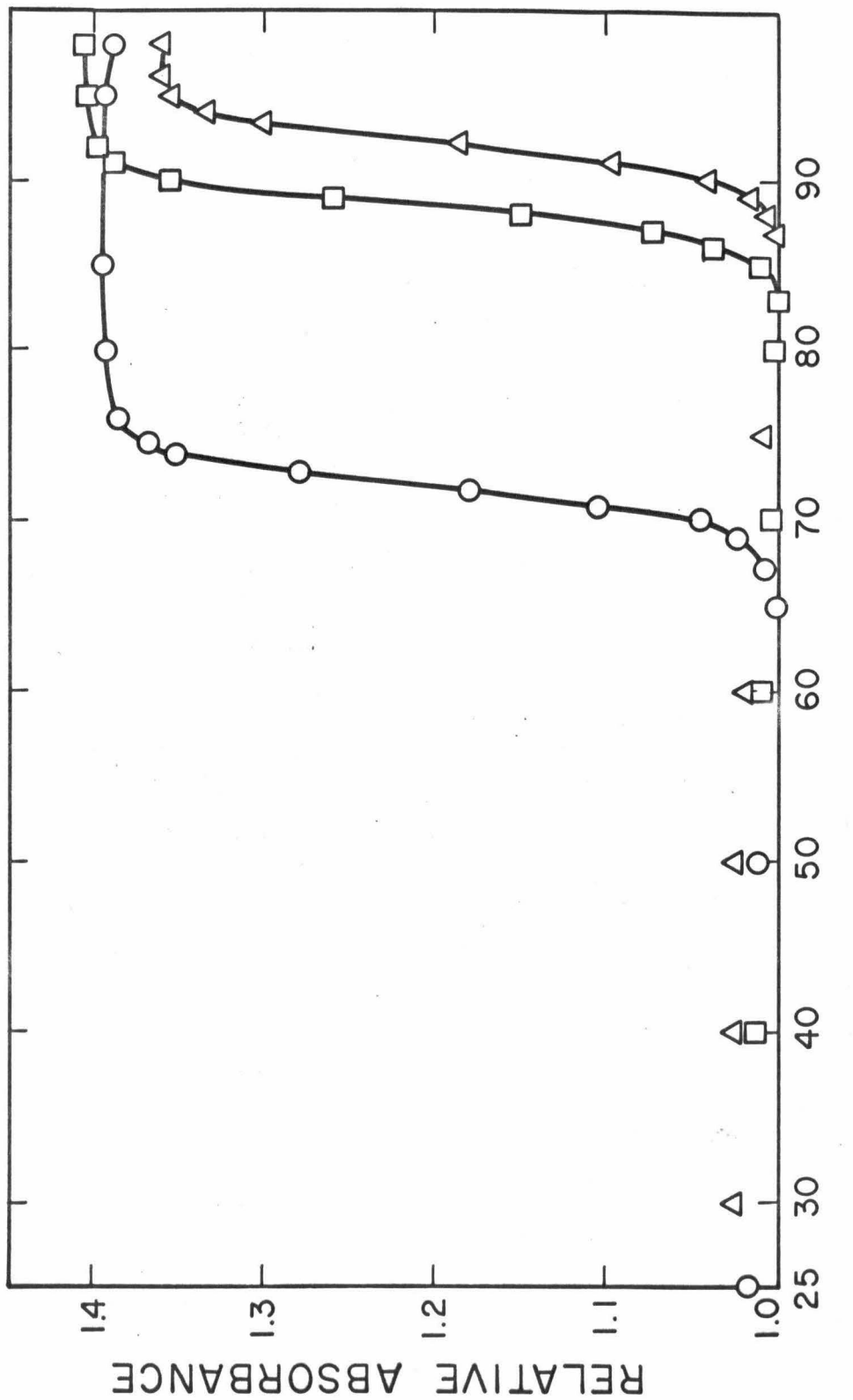

Figure 10

Thermal denaturation of bacteriophage TØ3 DNA in various buffers. Circles, 0.1 X SSC; squares, SSC; triangles, 2.0 X SSC.

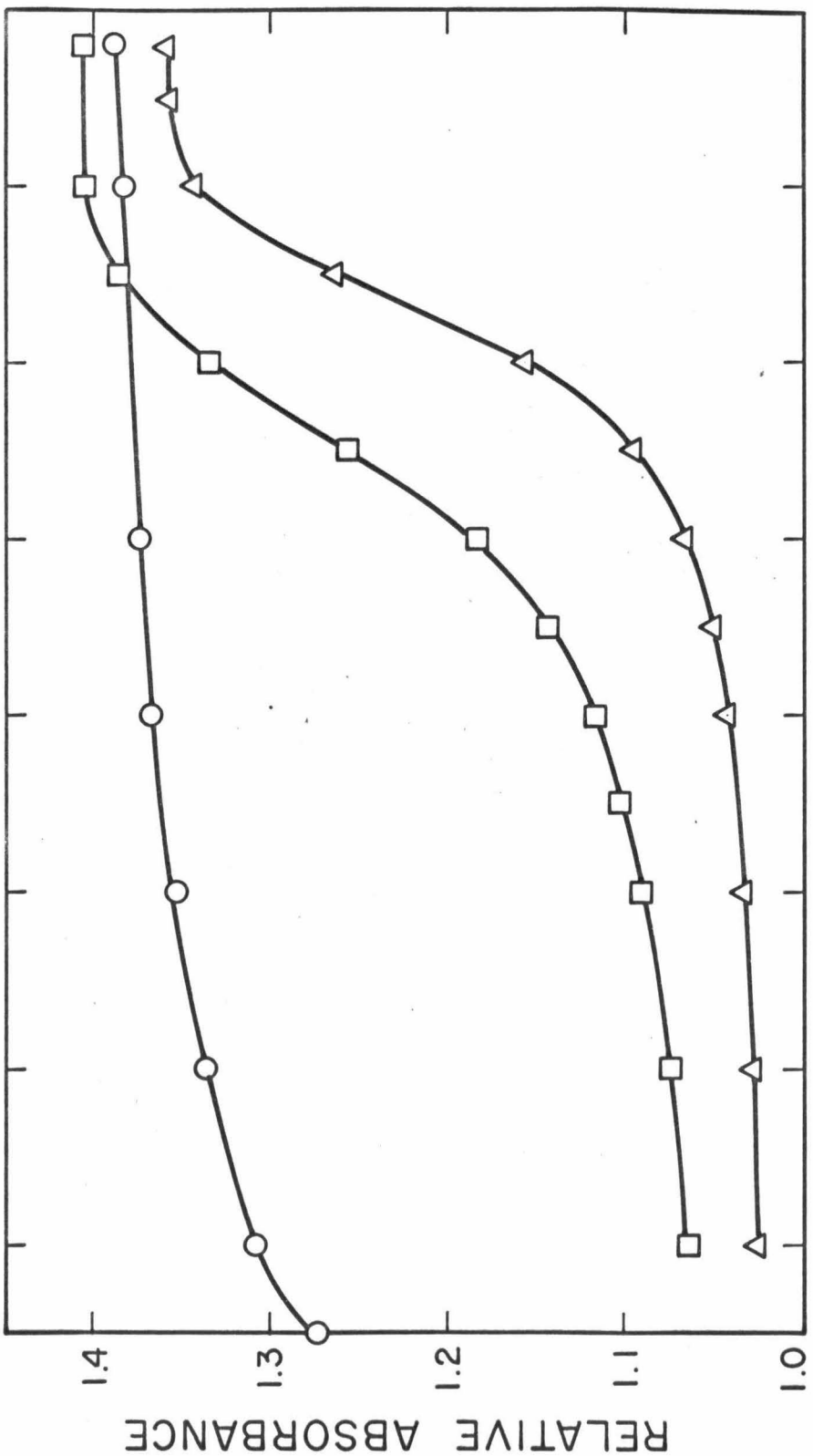

Figure 11

Thermal renaturation of bacteriophage TØ3 DNA in various buffers. Circles, 0.1 X SSC; squares, SSC; triangles, 2.0 X SSC.

was poor and incomplete in the low salt solvent (0.1 X SSC, circles) with 33% hypochromic effect. In SSC (squares) the annealing was more rapid and more complete with an 88% hypochromic effect. The annealing in the high salt solvent (2.0 X SSC, triangles) was the most rapid and most complete. The hypochromic effect was 100%; this means that the absorbance of the original native DNA solution was regained. This extensive renaturation in high salt solvents was used to great advantage in later experiments in which the physically separated strands of the TØ3 DNA double helix were annealed in a concentrated CsCl solution in an analytical ultracentrifuge cell.

Figure 12 is a representation of the hyperchromic effect when: A, a solution of TØ3 DNA (circles) in SSC was heated; B, a solution of a mixture of TØ3 DNA and Escherichia coli DNA (triangles) in SSC was heated; C, a solution of Escherichia coli DNA (squares) in SSC was heated. The T_m of the TØ3 DNA in SSC was 88.5°C. The solution of a mixture of TØ3 DNA and E. coli DNA had a melting curve that changed slope in the middle of the curve. This indicates that at the temperature at which the slope changes, the TØ3 DNA was almost completely denatured, and that the remainder of the hyperchromicity was due to the denaturation of the E. coli DNA. The T_m of E. coli DNA in SSC was 91.5°C. The melting profile of TØ3 DNA was much sharper than that of E. coli DNA. This is probably a reflection of the relative composition heterogeneity of the E. coli DNA fragments.

Buoyant density of TØ3 DNA.

The buoyant density of TØ3 DNA was determined from its position, relative to the position of DNA with a known density, in a cesium

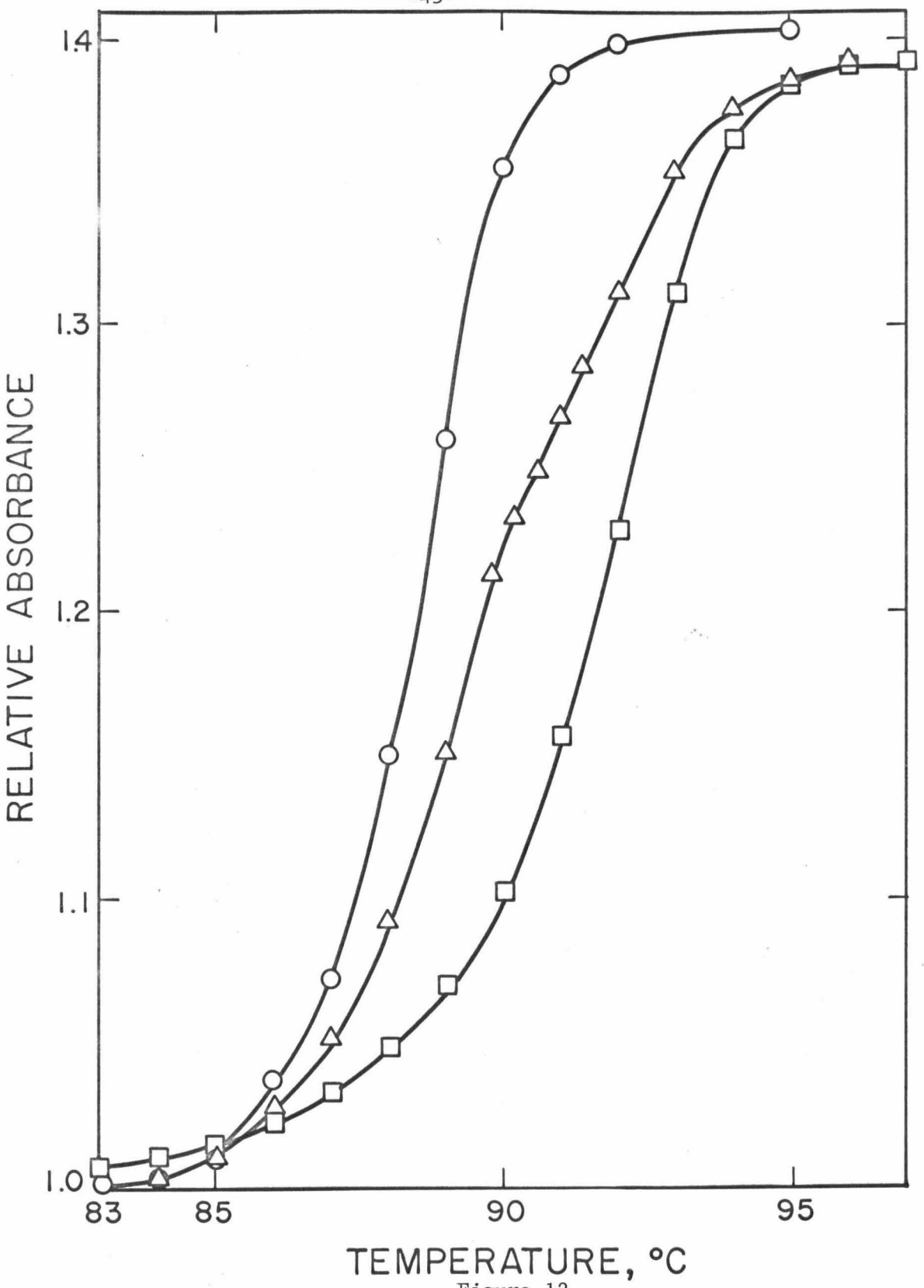

Figure 12

Thermal denaturation of DNA in SSC. Circles, TØ3 DNA; triangles, TØ3 DNA and Escherichia coli DNA mixture; squares, Escherichia coli DNA.

chloride density gradient (29). Figure 13 illustrates photographs that were taken utilizing the selective ultraviolet light absorbing properties of DNA. Figure 13 A represents a gradient that was formed from an initial solution that contained TØ3 DNA, Escherichia coli DNA and Micrococcus lysodeikticus DNA. TØ3 DNA formed a band at a density of 1.695. The gradient represented in Figure 13 B contained TØ3 DNA and Micrococcus lysodeikticus DNA. In the gradient represented in Figure 13 C only TØ3 DNA was present.

For accurate measurement of the relative position of the DNA bands, the original negative was analyzed by tracing the relative film exposure with a microdensitometer. This procedure gave a direct record of the light absorbed (and hence the DNA concentration) vs. radius from the rotor center. Densitometer tracings of the negatives used to produce Figure 13 are shown in Figure 14.

Molecular weight of TØ3 DNA.

The molecular weight of TØ3 DNA was determined from the band width of the concentration distribution that TØ3 DNA formed in a cesium chloride density gradient. The square of the half band width (σ) of the gaussian DNA concentration distribution in a density gradient is inversely proportional to the molecular weight of the DNA being studied if there is no density heterogeneity present in the DNA sample (29). Figure 15 illustrates a densitometer tracing of a TØ3 DNA band. The diagonal line is the record of exposure produced by an exponential aperture (66) included in the counterbalance to check the linearity of the film response. The half band width, σ, in this determination was 9.84×10^{-3} cm and the radius at band center, r_o, was 6.361 cm. For

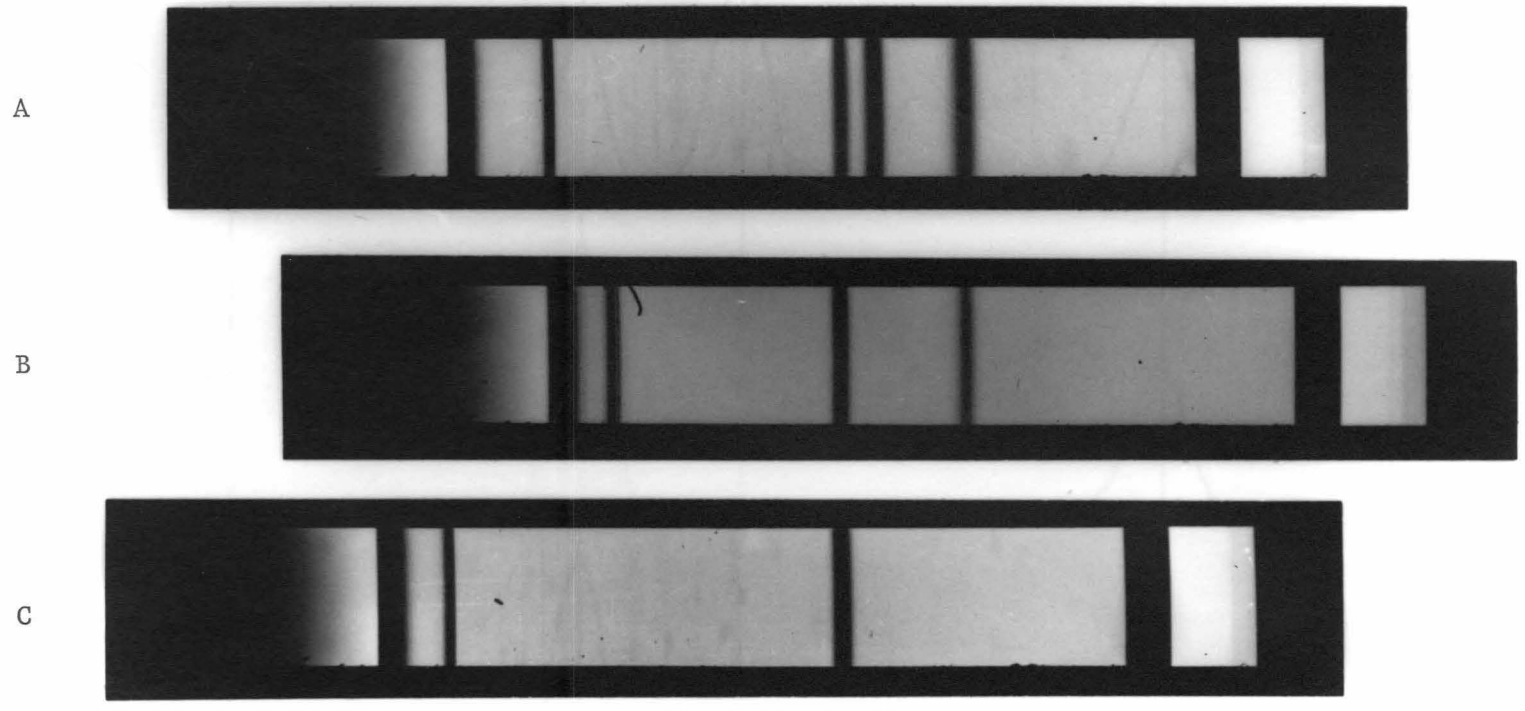

Figure 13

Photographs of bands formed by DNA in a cesium chloride density gradient at 44,770 rpm. A, TØ3 DNA, Escherichia coli DNA and Micrococcus lysodeikticus DNA; B, TØ3 DNA and Micrococcus lysodeikticus DNA; C, TØ3 DNA. The film record of the exponential aperture is at the left.

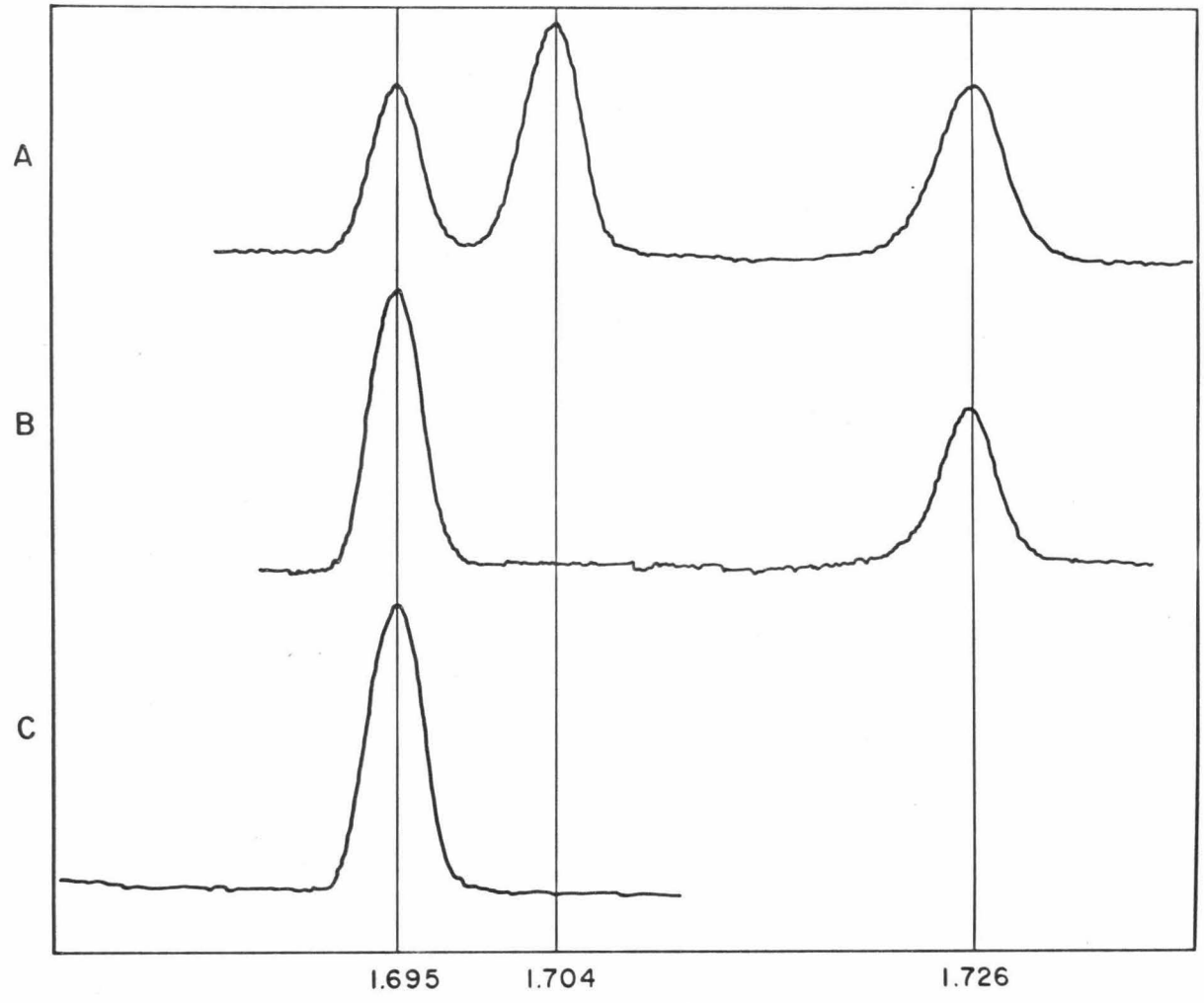

Figure 14

Densitometer tracings of photographs of bands formed by DNA in a cesium chloride density gradient at 44,770 rpm. A, TØ3 DNA, Escherichia coli DNA and Micrococcus lysodeikticus DNA; B, TØ3 DNA and Micrococcus lysodeikticus DNA; C, TØ3 DNA.

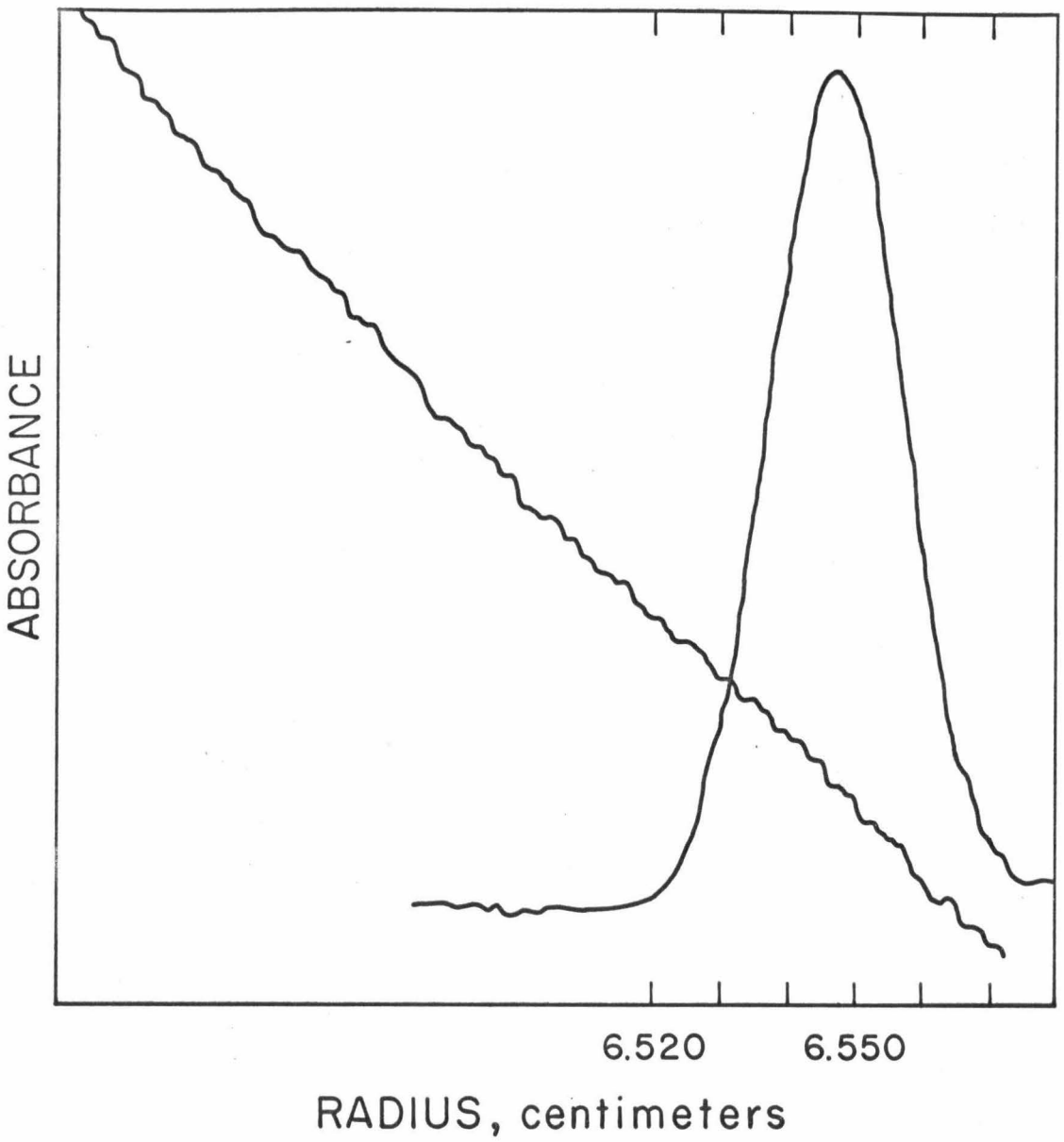

Figure 15

Densitometer tracing of a photograph of a TØ3 DNA band in a cesium chloride density gradient at 44,770 rpm. The diagonal line is the tracing of an exponential aperture included in the counterbalance to check the linearity of the film response.

TØ3 DNA (see Appendix):

$$M_{Na} = \frac{7.39 \times 10^4}{\sigma^2 r_o^2}$$

Thus the molecular weight of the sodium salt of TØ3 DNA observed in this experiment was 17.8×10^6. The average value of the molecular weight obtained from two determinations was 16.7×10^6.

Length and molecular weight of TØ3 DNA.

When particles of bacteriophage TØ3 in a high osmotic strength environment (4 M ammonium acetate) are removed from that environment and placed in a low osmotic strength environment (distilled water) some of the particles osmotically shock and burst, releasing their DNA into the surrounding medium. This DNA can be trapped in a cytochrome c film, shadowed and examined in the electron microscope (44). An electron micrograph of TØ3 DNA prepared in this manner is shown in Figure 16.

The average of 12 measurements of the length of different TØ3 DNA molecules was 11.7 ± 0.3 μ. The measured length of individual TØ3 DNA molecules ranged from 11.25 μ to 12.2 μ. Assuming 1.92×10^6 molecular weight units per μ, the molecular weight of the DNA of TØ3 is 22.5×10^6. The single DNA molecule of phage TØ3 has a mass of 3.73×10^{-11} μg and a volume of 3.7×10^{-5} μ^3.

The process of osmotically shocking the phage and trapping the DNA in a cytochrome c film yields three distinct configurations for the DNA molecule. The free random coil form, the compact form that crosses back over itself in a typical flower shape, and the form in which the DNA has not been completely released from the phage head. The first two forms are illustrated in Figure 16, and the 3rd form is illustrated

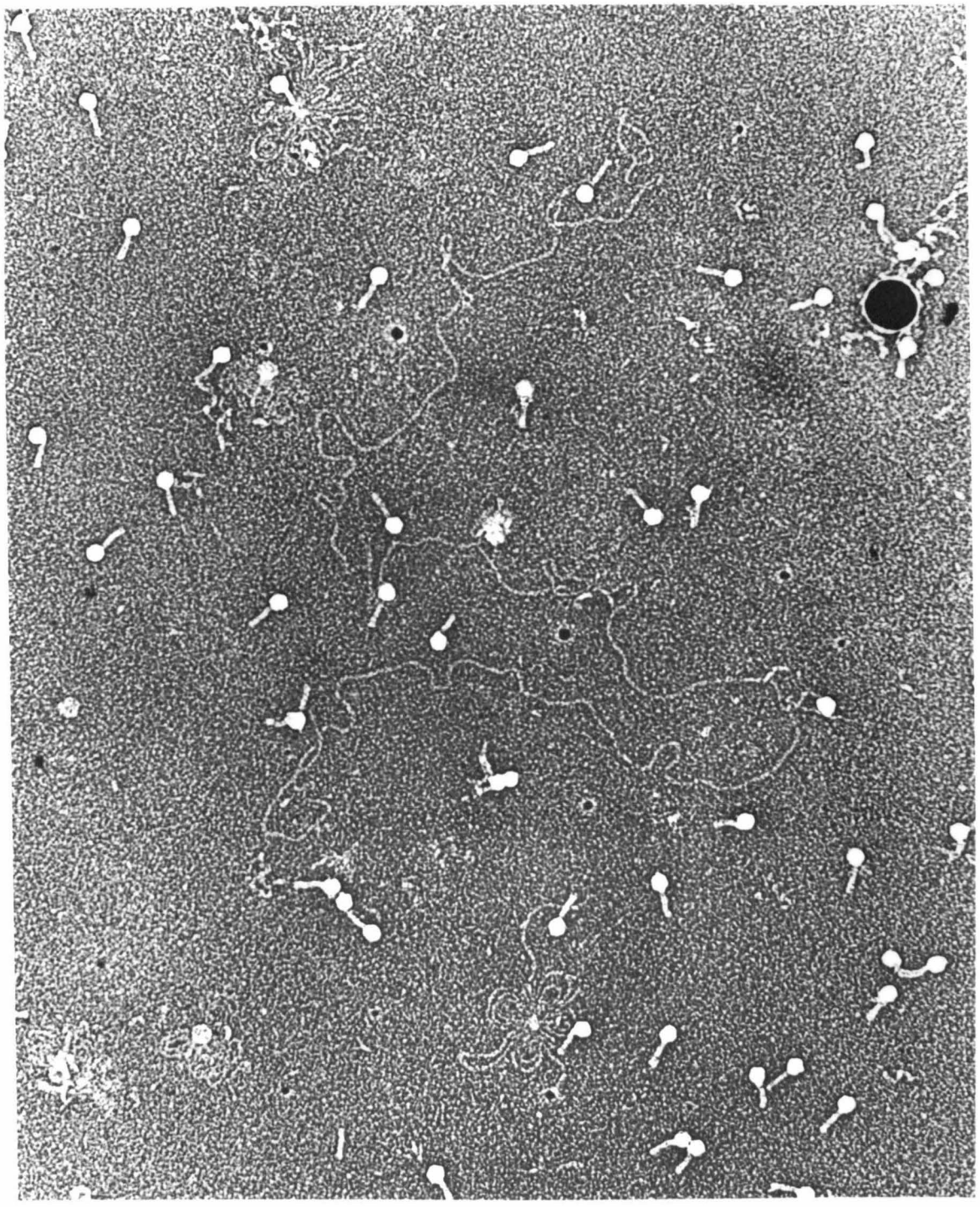

Figure 16

Electron micrograph of TØ3 and TØ3 DNA (treated with cytochrome c and shadowed with platinum), X 40,200.

in Figure 17. In some preparations it appeared that the DNA was being extruded through the tail while in others the tail was absent or it was attached to the end of the DNA molecule.

Base composition of TØ3 DNA.

A photograph of a paper chromatograph of formic acid hydrolyzed TØ3 phage is shown in Figure 18. The spots that absorbed ultraviolet light are outlined. The spots representing the bases present in the phage DNA are at the left. Control spots of adenine, guanine, thymine and cytosine are at the right. The two spots marked F are fluorescent spots that were present when whole phage was hydrolyzed but were not present when phage DNA was analyzed. The positions of the four bases present in the phage DNA match the positions of the control spots. The ultraviolet absorption spectra of the four bases present in TØ3 DNA matched the spectra of the corresponding control bases.

The results of the base analysis of TØ3 DNA are contained in Table 2. Based upon 3 determinations the DNA of TØ3 contains 29.0 $\pm$ 0.6% adenine, 19.4 $\pm$ 0.2% guanine, 30.8 $\pm$ 0.8% thymine and 20.8 $\pm$ 0.3% cytosine. The guanine plus cytosine content is 40.2%. The ratio of adenine to thymine is 0.94 and that of guanine to cytosine is 0.93.

The melting temperature of DNA has been found to be a function of its base composition (13). To utilize a marker DNA in the T_m determination a modified form of the equation of Marmur and Doty (13) was used. The difference in the T_m and the difference in the guanine plus cytosine content between two samples of DNA are related according to the equation:

$$\Delta GC = 2.44\ \Delta T_m,$$

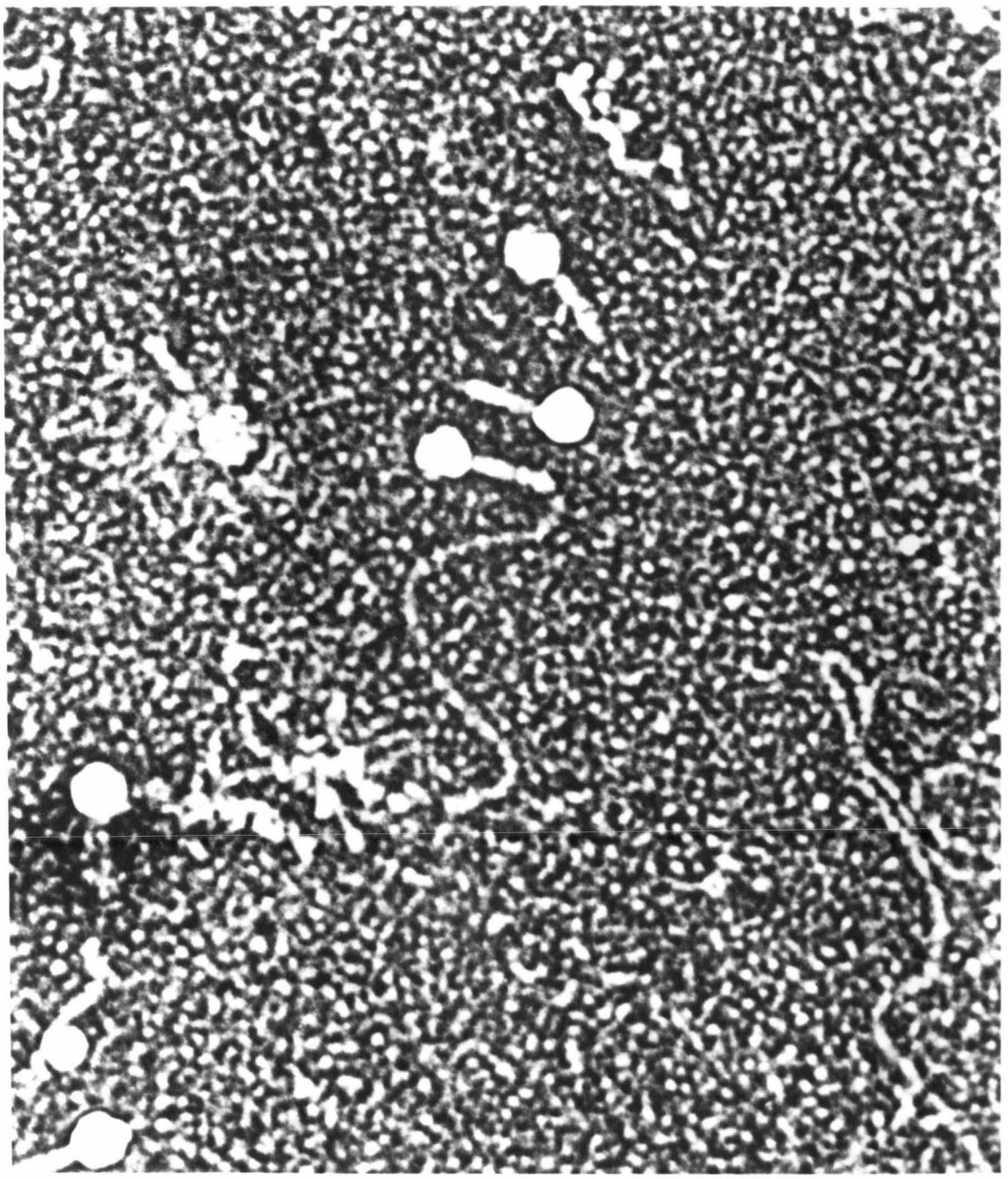

Figure 17

Electron micrograph of the DNA of bacteriophage TØ3 being extruded from the tail of the phage (treated with cytochrome c and shadowed with platinum), X 93,500.

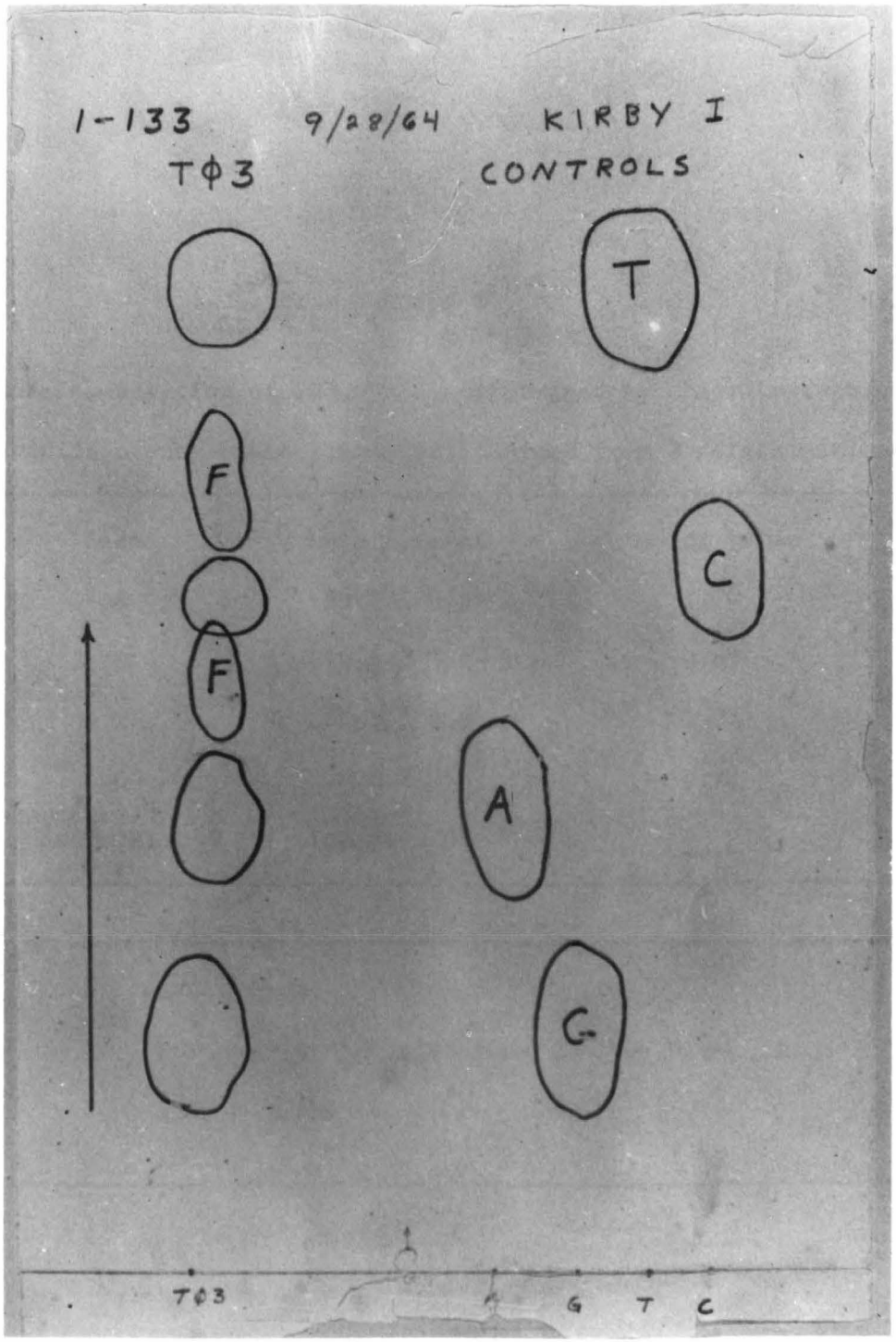

Figure 18

Photograph of a paper chromatogram of hydrolyzed bacteriophage TØ3. The spots that absorb ultraviolet light have been outlined. Spots representing the bases; A, adenine; G, guanine; T, thymine; C, cytosine are shown to the right and spots from TØ3 are on the left. The spots marked F are fluorescent.

TABLE 2

Base composition of TØ3 DNA as determined by chromatographic separation of the bases (averages obtained from 3 determinations).

Base	Mole percent	Molar ratio
A	29.0 ± 0.6	1.00
G	19.4 ± 0.2	0.69
T	30.8 ± 0.8	1.06
C	20.8 ± 0.3	0.72
Total	100.0	____

A/T = 0.94

G/C = 0.93

Purines (A+G)/Pyrimidines (T+C) = 0.94

(GC) = 40.2%

where ΔT_m is the difference of the melting temperature of the marker DNA and of the DNA of unknown composition,

and ΔGC is the percent difference of the guanine plus cytosine content of the marker DNA and of the DNA of unknown composition.

The T_m of Escherichia coli DNA was found to be 91.5°C and for TØ3 DNA it was 88.5°C. The ΔGC for these two samples of DNA is therefore:

$$\Delta GC = 2.44(3.0) = 7.3\%$$

Since E. coli DNA contains 50% G + C it follows that TØ3 DNA contains 42.7% G + C.

The buoyant density of DNA also depends upon its base composition in a known manner (52). If the density of TØ3 DNA is computed according to the methods of Schildkraut, Marmur and Doty, using their buoyant densities of 1.710 for Escherichia coli DNA and 1.731 for Micrococcus lysodeikticus DNA, a density of 1.702 is obtained for TØ3 DNA. The density of TØ3 DNA corresponds to a guanine plus cytosine content of 43%.

The base composition of TØ3 DNA obtained by three independent methods is presented in Table 3. Analysis by chromatographic separation of the bases gave 40.3% guanine plus cytosine. The melting temperature indicated a composition of 42.7% guanine plus cytosine. Finally, the buoyant density of TØ3 DNA corresponded to a guanine plus cytosine content of 43%.

Separation of the two complementary strands of TØ3 DNA.

According to the generally accepted Watson-Crick model for the

TABLE 3

Base composition of TØ3 DNA, summary.

Method	Mole percent G + C
Heat Denaturation, T_m	42.7
Buoyant Density	43
Chromatography	40.2

structure of deoxyribonucleic acid (67, 68), individual molecules of DNA are composed of two complementary polynucleotide chains. For every adenine on one chain there is a thymine on the other chain. For every cytosine there is a guanine on the other chain. This model dictates that the mole fraction of adenine must equal the mole fraction of thymine. The same relationship also must hold between guanine and cytosine. However, no such relationships between the bases are necessary for bases in a single polynucleotide chain. The usual base equivalence does not hold for the single stranded DNA of phage ØX174 (54). The two strands of the DNA of bacteriophage α have been shown to have different but complementary base compositions (55). The heat denatured DNA from phage α shows two bands in a cesium chloride density gradient (56), indicating that the two strands have different buoyant densities. The two strands of the DNA of the thermophilic bacteriophage TP-84 have different buoyant densities in cesium chloride. These two strands also have different but complementary base compositions (23).

As was illustrated in Figures 13 and 14, native DNA from phage TØ3 bands as a single peak at a density of 1.695 in a cesium chloride density gradient. Heat denatured TØ3 DNA forms two bands at densities of 1.707 and 1.715 as is indicated in the photographs in Figure 19, experiments 1 A and 2 A, and the densitometer tracings in Figure 20, experiments 1 A and 2 A. When the two separated strands are mixed together and annealed, the renatured DNA forms a single band at a density of 1.699. Photographs of the renatured band are shown in Figure 19, experiments 1 B and 2 B, and densitometer tracings are illustrated in Figure 20, experiments 1 B and 2 B. In experiment 1,

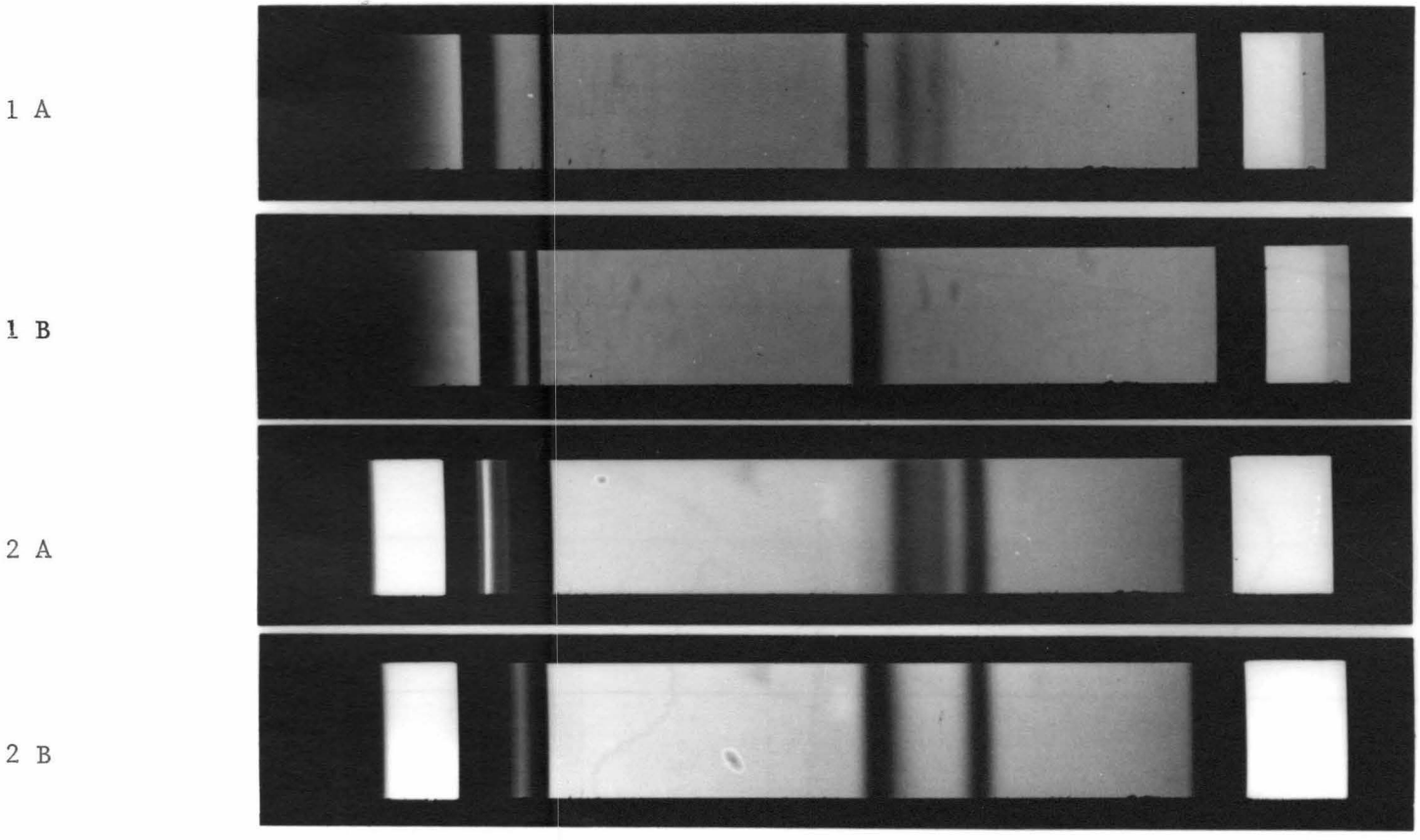

Figure 19

Photographs of bands formed by DNA in a cesium chloride density gradient at 44,770 rpm. 1 A, TØ3 DNA and TØ3 heat denatured DNA; 1 B, the material in 1 A, annealed and rebanded. 2 A, TØ3 heat denatured DNA and Micrococcus lysodeikticus DNA; 2 B, the material in 2 A, annealed and rebanded.

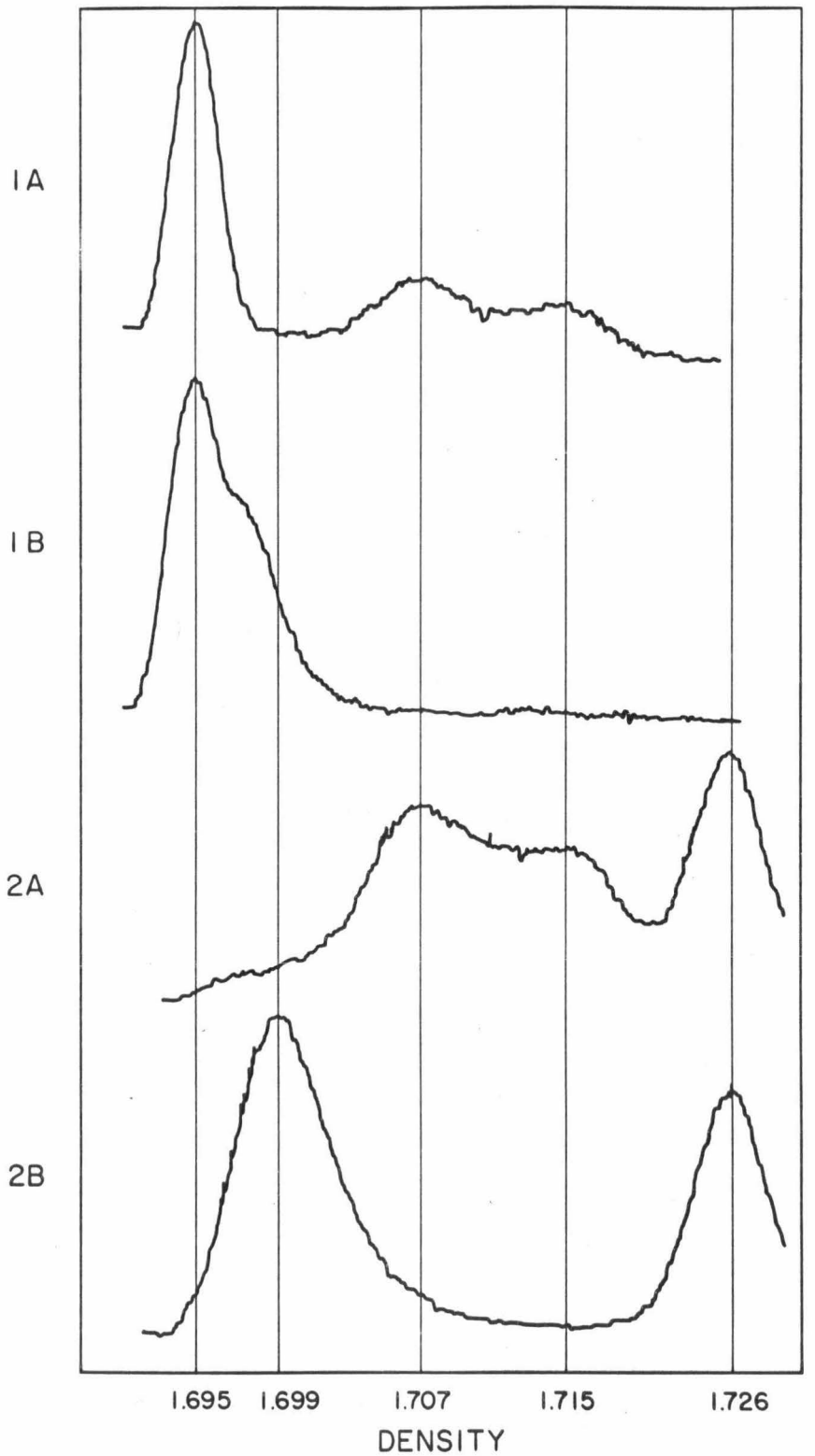

Figure 20

Densitometer tracings of photographs of bands formed by DNA in a cesium chloride density gradient at 44,770 rpm. 1 A, TØ3 DNA and TØ3 heat denatured DNA; 1 B, the material in 1 A, annealed and rebanded. 2 A, TØ3 heat denatured DNA and Micrococcus lysodeikticus DNA; 2 B, the material in 2 A, annealed and rebanded.

as illustrated in Figures 19 and 20, native TØ3 DNA was used to provide a density reference. The renatured DNA shows as a shoulder on the heavy side of the native peak in Figure 19 (1 B) and Figure 20 (1 B). In experiment 2, as illustrated in Figures 19 and 20, Micrococcus lysodeikticus DNA was used to provide a density reference. It is apparent that native TØ3 DNA is dissociated by heat denaturation into two subunits whose buoyant densities differ. These two subunits can be annealed together under conditions that are optimal for the renaturation of the separated complementary strands of the DNA double helix.

In the experiment from which Figures 19 (2 A) and 20 (2 A) were taken, a series of photographs taken after equilibrium was reached indicated that a shoulder was forming on the light side of the band of the light strand of TØ3 DNA. This shoulder appeared at the density of the renatured TØ3 DNA shown in Figures 19 (2 B) and 20 (2 B). In the region between the light and heavy peaks the concentrations of the two strands were high enough to promote some renaturation of the DNA. As the renaturation process was completed for an individual DNA molecule, the renatured DNA molecule would move in the gradient to a position corresponding to its new buoyant density. The renatured molecules thus migrated through the light strand peak region and increased the ultraviolet absorbance accordingly. The band representing the light strand is slightly darker than the band representing the heavy strand.

Several attempts were made to increase the height of the peaks, as seen in experiment 1 of Figures 19 and 20, by increasing the concentration of DNA. All attempts failed due to extensive renaturation of the DNA during the experiment. The use of the 30 mm centrifuge cell,

to increase the length of the light path through the cell, solved this problem by allowing the same concentration of heat denatured TØ3 DNA to be used as was used in experiment 1. In experiment 2 a 30 mm cell was used. The light path and hence the absorbance of ultraviolet light, by the DNA in the bands, increased by a factor of 2.5.

The separation of the strands was observed only when a fresh preparation of heat denatured DNA was used. One preparation that had been heat denatured and stored at $4^{o}C$ for 1 week showed extensive renaturation.

Additional observations.

During the course of this study several phenomena were observed that were not really pertinent to the problems being investigated at that time. They were not studied in detail and are thus listed here as observations that might be of interest.

The purification procedure for TØ3 involves centrifuging the lysate to remove the bacterial cell debris. When this cell debris was examined with a hand spectroscope, two dark absorption bands were observed, one at a wavelength of 570 mμ and the other at a wavelength of 533 mμ. These bands are a characteristic of a reduced cytochrome. Much lighter bands were present when uninfected bacteria from a control culture were examined. Apparently the cell debris from a phage lysate contains a higher concentration of cytochrome than the bacteria from an uninfected culture. It is not known if the presence of large amounts of cytochrome is the result of induction of a bacterial cytochrome by the phage infection, the synthesis of phage specific cytochrome or just the uncontrolled synthesis of cytochrome that was set off by the phage infection.

While performing some experiments to determine the effect that various ions and sugars have on bacterial growth and phage infection, it was observed that a medium in which sodium ion was replaced by potassium ion and glucose by sucrose, gave much better adsorption of TØ3 to its host than the usual TYNGC medium. However, no burst occurred during the 35 minutes of the experiment. In another experiment in which just the sodium ion was replaced by potassium a normal latent period was observed. Potassium or sucrose may enhance adsorption, but apparently sucrose either prevents or delays the burst.

It was found that a bacterial culture that had been grown overnight, well into the stationary phase of growth, gave uniform plaques when used as a lawn for titering phage preparations. The bacterial concentration in the lawn in these experiments was the same as in the usual phage assay. The apparent titer of the phage stock being assayed increased by 60% when stationary phase bacteria were used in the lawn. This increase in efficiency of plating was discovered too late to be of any use in the experiments reported here.

The optimum growth temperature of the host organism in TYNGC medium was found to be 60°C. The division times at various temperatures were: 50°C, 90 min; 60°C, 20 min; 65°C, 21 min; 70°C, 70 min.

DISCUSSION

Bacteriophage TØ3 is a virus that infects the thermophilic bacterium, Bacillus stearothermophilus, at a relatively high temperature of 60°C. The physical dimensions of phage TØ3 are similar to those of the E. coli phage λ (57). The size of TØ3 places it in a range between the larger phages of E. coli such as the T-even phages (57, 58) and the small phages of E. coli such as ØX174 (59). TØ3 is strikingly similar to phage α (60), a bacteriophage that infects Bacillus megaterium. The morphology of other bacteriophages for Bacillus stearothermophilus is not known in detail. Phage Øμ-4 is known to be very small with a reported diameter of 10 mμ (22), however, electron micrographs of this phage have not been published. Phage TP-1 has a head diameter of approximately 65 mμ and a tail 240 mμ long and 12 mμ wide (14). TØ3 is thus in a size range in between those two thermophilic phages.

The regular hexagonal appearance of the head of TØ3 probably indicates that the head has the form of an icosahedron, however, more detailed work is necessary to verify this. Although the head appears as a regular hexagon in all three staining procedures used (PTA negative, uranyl acetate positive and uranyl acetate negative) the actual size of the head varies considerably with the staining procedure. The length of the head when stained negatively with PTA was 65 mμ. The length when stained positively with uranyl acetate was 47 mμ. With a uranyl acetate negative stain it was 57 mμ long. The heads appeared swollen and there were many ghosts in the PTA preparations and the head outline was quite indefinite in the uranyl acetate positive stained preparations. Since the head outline was well defined and no ghosts

were present in the uranyl acetate negative stained preparations, measurements for the head are those taken from preparations stained in this manner.

The details of the structure of the tail of TØ3 were revealed by the uranyl acetate negative staining procedure. Further study is necessary to determine if the tail has a helical form as is probably the case of the E. coli phages, T2 (61) and T5 (62).

As would be expected TØ3 is more thermostable than are phage of mesophiles. In broth, for example, the half life of the E. coli phage T1 is about 12 minutes at 65°C (69), as compared to 120 minutes for TØ3. Another E. coli phage, T7, is less stable, having a half life of less than one minute at 60°C. The half life of TØ3 at 60°C is 400 minutes. At 65°C TØ3 is about 4 times as stable as the thermophilic phage TP-1 (14). The thermophilic phage isolated by Onodera was reported to be stable at 100°C (21). Phage TØ3 of course is not stable at this high temperature.

The rather normal melting temperature of the TØ3 DNA, taken together with the high ΔH_a for heat inactivation of the phage, indicate that the proteins of the TØ3 are primarily responsible for the thermostability characteristics (25) of TØ3. Many proteins of mesophilic organisms are denatured rapidly at temperatures above 40°C. The relative thermostability of the proteins of TØ3 is an indication that they have rather unique structural characteristics. These characteristics might consist of a unique amino acid composition, as is the case of the α-amylase of Bacillus stearothermophilus, or of a folding arrangement of the polypeptides that is particularly resistant to thermal denaturation.

Thus a further study of the proteins of TØ3 could yield some insight into the relationship between the primary amino acid sequence and the tertiary structure of proteins in general.

The study of the enzyme that is responsible for lysis of a TØ3 infected bacterium would also yield valuable information with regard to the relationship between structure and function of enzymes in general. Further study of the genetics and structure of TØ3 "lysozyme" would be even more meaningful in view of current research on the genetic control of the structure of T4 phage lysozyme (63, 64) and on the structural characteristics of egg white lysozyme (70, 71). However, TØ3 "lysozyme" may not be a lysozyme at all. In strict terms lysozyme is N-acetylmuramide glycanohydrolase and is known as muramidase. It has not been shown that TØ3 "lysozyme" is a muramidase. Until the enzymatic characteristics of TØ3 "lysozyme" are known, its relationship to T4 lysozyme should be taken as rather preliminary. This however in no way reduces the significance of this phage enzyme for it is of great interest in and of itself.

The poor adsorption of the TØ3 to the bacterial cell wall under all of the conditions studied may indicate that the present host is not the true host for this phage. On the other hand it may also mean that the proper environment for good adsorption has not been found. That the latter might be the case is strengthened by the fact that adsorption could be improved by utilizing various cation and sugar combinations in the media. This is also indicated by the finding that stationary phase bacteria serve as a better lawn than log phase bacteria. The effect that temperature has on adsorption could probably

be investigated to advantage once a system is found that allows good adsorption. Since no burst was found when potassium ion and sucrose were used in the media, their precise effect on the infected cell could be fruitfully investigated. This could presumably yield a good method for synchronizing the maturing process.

The deoxyribonucleic acid of TØ3 has proven to be extremely interesting. The determination of the molecular weight from the shape of the band in a cesium chloride density gradient indicates that this method can be used to determine DNA molecular weights to within 20%, provided the DNA is homogeneous with regards to density. This conclusion was also reached by Studier in his study on the molecular weight of the DNA of bacteriophage T7 (37). The 20% inaccuracy of this method also indicates that independent measurements of the molecular weight should be made in conjunction with any made by band width. Any density heterogeniety that was present in the sample of TØ3 DNA, that was used for the band molecular weight determination, would have broadened the band. The molecular weight that was determined from this broadened band would have been less than the actual molecular weight of native TØ3 DNA. Any degradation of the DNA in the sample would yield a similar result, a lower molecular weight than the molecular weight of native TØ3 DNA. The true molecular weight of TØ3 DNA must therefore be greater than or equal to the molecular weight that was determined from the band width.

The measurement of the length of the DNA molecule probably gives the best estimate of the actual molecular weight of a particular DNA molecule that can be made at the present time (45). The largest error

that is present in this type of determination is the calibration of the magnification; this amounts to about 10%. The length measurements using the map measure could be reproduced to within 3%.

The base composition of TØ3 DNA, as determined from the buoyant density and the T_m of TØ3 DNA, is probably more accurate than the composition that was determined in the spectrophotometric base analysis. The reason for this is the relative simplicity of the buoyant density and thermal denaturation experiments and the relative control that is possible when compared to the chromatographic isolation of the bases and the spectrophotometric determination of the base composition. This does not mean however, that these two methods should supplant the latter, for when a new system is being investigated, the isolation and identification of the bases is invaluable for the detection of bases other than the four that are usually present in DNA. The small inconsistancy that exists in the base composition, as determined by the T_m and buoyant density measurements on the one hand and the spectrophotometric base analysis on the other hand, could be an indication of the presence of small quantities of other bases in addition to the four that were detected. Any base, other than adenine, guanine, thymine and cytosine, that is present in TØ3 DNA would have to be present in small amounts because of the agreement between the base compositions that were determined from the T_m and the buoyant density. It should be mentioned that at the concentrations utilized in the chromatography, a base, other than adenine, guanine, thymine or cytosine, that was present in small amounts, would not have been recognized.

The increase in the melting temperature of TØ3 DNA with an increase

in the ionic strength of the solvent is analogous to the results that have been obtained with the DNA of other organisms (13). The enhancement of renaturation of DNA with increasing ionic strength of the solvent has also been observed for the DNA of other organisms (37, 79). The complete renaturation of heat denatured TØ3 DNA that was observed in a high ionic strength solvent (2.0 X SSC) is similar in extent to the renaturation of the DNA of other viruses (80). Bacterial DNA and mammalian DNA do not renature completely (80). That TØ3 DNA could actually be 100% annealed was shown in the strand separation and annealing experiments in which the bands representing denatured material were completely absent in the annealed samples. This is a good indication that the original sample of DNA was homogeneous and that the majority of the DNA molecules, obtained by the phenol extraction technique, contained few single strand breaks.

The separation of the two strands of the DNA of TØ3 yields additional evidence that the native DNA molecule consists of two subunits. The fact that the two separated strands can be reunited by annealing requires that the base compositions of the two separated strands be specifically complementary to each other. The finding that the two strands have different buoyant densities suggests that they also have different base compositions. The other alternative is that there is some molecule preferentially bound to one of the two strands that alters the density of that strand. This alternative would appear to be ruled out by the close agreement of the buoyant density, melting temperature and base analysis data. If the two bands do represent the two strands of the Watson-Crick double helix then their base compositions would

complement each other as is the case with phage α DNA (55).

Present evidence indicates that only one strand of the DNA double helix is used to supply information for protein synthesis (72, 73, 74). The amino acid analysis of the α-amylase of Bacillus stearothermophilus indicated that this protein is very rich in particular amino acids (10). Indeed more than 15% of the amino acid residues in this protein are proline. The messenger RNA coding triplets for proline are CCC, CCU, CCA and CCG (75). The DNA strand that codes for the messenger RNA of a protein that is rich in proline should be rich in the base complementary to cytosine. Thus the coding strand should contain more guanine than the non-coding strand. If this high proline content is a general characteristic of the proteins of thermophilic organisms then it would be expected that the proteins of TØ3 should also exhibit this feature. If an amino acid analysis of the proteins of TØ3 confirms this then it would be expected that one of the strands of TØ3 DNA would be rich in guanine. This particular strand should also be the one that would anneal to the messenger RNA of the infected bacterium.

The α-amylase of Bacillus stearothermophilus is also rich in the acidic amino acids, glutamic acid (15%) and aspartic acid (7.5%). The RNA coding triplets for glutamic acid are GAA and GAG, those for aspartic acid are GAU and GAC (75). If the RNA coding triplets of proline, glutamic acid and aspartic acid are considered together, they are conspicuously deficient in uracil. If the proteins of TØ3 are rich in these three amino acids then it would be expected that the coding strand of TØ3 DNA would be deficient in adenine, the complement of the uracil in the messenger RNA. Since the overall base composition of

TØ3 DNA does not show a deficiency in adenine, the non-coding strand should contain most of the adenine in TØ3 DNA. The coding strand of TØ3 should therefore be rich in thymine. One of the strands of the DNA of the thermophilic phage TP-84 is indeed richer in thymine than is the complementary strand (23).

If the proteins of TØ3 are rich in proline, glutamic acid and aspartic acid, the coding strand of TØ3 DNA should be rich in a) guanine, because the RNA codewords for proline are rich in cytosine, and b) thymine, because of the RNA codewords for proline, glutamic acid and aspartic acid are deficient in uracil. Preliminary evidence (81) suggests that one strand of TØ3 DNA is very rich in thymine and guanine.

Genetic experiments with either the isolated strands of TØ3 DNA or with heat denatured and annealed TØ3 DNA, in which one strand possesses one genetic marker and the other strand possesses another genetic marker, await the development of an infective DNA system utilizing TØ3 DNA. Once developed this whole system could provide support for the messenger hypothesis, the one strand transcription hypothesis, the MRNA-DNA hybridization experiments and the codeword determinations.

Another aspect of interest concerning the nucleic acids of thermophilic organisms is their transfer RNA. As has already been mentioned the α-amylase of Bacillus stearothermophilus is very rich in several amino acids. If this is a general characteristic of the proteins of this organism, Bacillus stearothermophilus could serve as a rich source of transfer RNA of specific amino acids, namely glutamic acid transfer RNA and proline transfer RNA.

APPENDIX

Calculation of the molecular weight of TØ3 DNA.

The molecular weight of a macromolecule is related to the width of the concentration distribution it forms in a density gradient by the equation:

$$M_{s,o} = \frac{RT}{\sigma^2 \bar{v}_{s,o} (d\rho/dr)_{eff}\, \omega^2 r_o} \qquad (40),$$

where $M_{s,o}$ is the solvated molecular weight of the macromolecule,

R is the gas constant (8.314×10^7 erg deg^{-1} $mole^{-1}$),

T is the absolute temperature (298^o K),

σ is the standard deviation of the concentration distribution at equilibrium,

$\bar{v}_{s,o}$ is the solvated partial specific volume of the macromolecule,

$(d\rho/dr)_{eff}$ is the effective density gradient,

ω is the angular velocity,

and r_o is the radius at the center of the concentration distribution.

The value of $1/\bar{v}_{s,o} = \rho_o = 1.695$ for TØ3 DNA, used in the calculations neglects pressure and introduces an error in $M_{s,o}$ of less than 1% (37). The effective density gradient, $(d\rho/dr)_{eff}$, is:

$$(d\rho/dr)_{eff} = \left(1/\beta^{\circ} + \psi\rho^{o^2}\right)(1-\alpha)\omega^2 r \qquad (40,41,42),$$

where $1/\beta^{\circ}$ is 8.40×10^{-10} (33),

ψ is 23.3×10^{-6} atm^{-1} (42)

or 2.3×10^{-11} cm^2 dyne^{-1},

ρ^{o} is the density at band center at atmospheric pressure, set equal to $\rho_o = 1.695$, the buoyant density of TØ3 DNA.

and α is 0.24 (41).

Thus $$(d\rho/dr)_{eff} = 6.89 \times 10^{-10}\omega^2 r,$$

and $$M_{s,o} = \frac{6.10 \times 10^{19}}{\sigma^2 (\omega^2 r_o)^2}.$$

The anhydrous molecular weight of the Cs salt of the DNA is:

$$M_{Cs} = M_{s,o}/(1 + \Gamma') \qquad (40),$$

where Γ' is the solvation of the DNA in grams of H_2O per gram Cs DNA.

However, $$1/\rho_o = (v_3 + \Gamma' v_1)/(1 + \Gamma') \qquad (40),$$

or $$\Gamma' = (1-\rho_o v_3)/(v_1\rho_o - 1),$$

where v_3 is the specific volume of anhydrous Cs DNA, taken to be 1/2.12 (41),

v_1 is the specific volume of solvated H_2O, taken to be 1.0.

Thus $$\Gamma' = 0.283,$$

and $$M_{Cs} = \frac{4.75 \times 10^{19}}{\sigma^2(\omega^2 r_o)^2}.$$

The molecular weight of the sodium salt of the DNA is found by

multiplying this by the ratio of the average molecular weight of a sodium deoxyribonucleotide to that of a cesium deoxyribonucleotide:

$$M_{Na} = M_{Cs} \ (331/441) \quad ,$$

$$= 0.751 \ M_{Cs} \quad ,$$

$$= \frac{3.57 \times 10^{19}}{\sigma^2 (\omega^2 r_o)^2} \quad ,$$

At 44,770 rpm, for TØ3 DNA,

$$M_{Na} \quad \frac{7.39 \times 10^4}{\sigma^2 \ r_o^2} \quad .$$

Determination of σ.

A graph of the equation for a gaussian distribution,

$$y = e^{-(r-r_o)^2/2\sigma^2} \quad ,$$

where the maximum value of y is 1, has a width of 2σ at $y = e^{-\frac{1}{2}}$, e being the base of the natural logarithms. A graph of y vs. r on gaussian graph paper yields a straight line. If y_2 is chosen at the center of the distribution ($y_2 = 1$) and y_1 at $e^{-\frac{1}{2}}$, then $r_2 - r_1 = \sigma$.

The concentration distribution of macromolecules obtained in a density gradient at equilibrium is gaussian (29) if the macromolecules are homogeneous with respect to density. A plot of the concentration of the macromolecule (normalized so that the maximum concentration is 1) vs. the radius, on gaussian graph paper should yield a straight line. The half band width σ can be determined from this plot. This procedure eliminates the necessity of an independent determination

of the radius at band center.

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