SATELLITE DNA IN HALOBACTERIUM SALINARIUM:
A PHYSICAL AND BIOCHEMICAL STUDY
SATELLITE DNA IN *HALOBACTERIUM SALINARIUM*:
A PHYSICAL AND BIOCHEMICAL STUDY

by

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree
Master of Science

McMaster University
June 1970.
The extremely halophilic bacterium, Halobacterium salinarium, contains a light density satellite DNA component which is 20% of the total DNA.

The purpose of this investigation was to study the physical characteristics of the satellite DNA by ultracentrifugation and electron microscopic methods in an attempt to answer the following questions:

(a) Does the amount of the satellite depend on DNA isolation conditions?
(b) What is the biological derivation of the satellite?
(c) What is the physical size(s) of the satellite?
(d) How many copies of the satellite occur in the cell?

The results of this investigation showed that the amount of the satellite is independent of isolation conditions, and that it exists in the form of closed circular DNA.
duplexes. Although the possibility that the satellite represents multiple forms of closed circular molecules could not be completely ruled out, the majority of the closed circles appeared to have lengths about 37 u, so that there might be eight copies of the satellite per bacterial genome.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. S.T. Bayley, for his patient guidance and invaluable advice throughout this research.

I also would like to thank Drs. I. Takahashi and J.J. Miller for permission to use their facilities in certain parts of this investigation, and to Mr. J.J. Darley for assistance in the fine-focusing of electron micrographs.

Thanks are also due to McMaster University Biology Department and the Province of Ontario Department of Education for financial assistance throughout this research.
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I. INTRODUCTION

In general, the DNA molecules isolated from any organism show a rather narrow, unimodal distribution upon isopycnic centrifugation in a cesium chloride density gradient. As the equilibrium banding position of the DNA is linearly related to its mole per cent G+C content (Schildkraut, Marmur & Doty, 1962), the unimodal distribution may be taken to indicate relative homogeneity in the overall base composition of the DNA.

In certain species of organisms, however, a bimodal distribution is observed, indicating the presence of a minor DNA component differing in density from the main band. By convention, the DNA components present in minor amounts are referred to as satellite DNA.

The first report of satellite DNA was in crabs. In several species, a light satellite comprising 10-30% of the total was found (Sueoka, 1961; Sueoka & Cheng, 1962; Smith, 1963, 1964). The unique property of this satellite is that it is composed of more than 90% alternating adenylate and thymidylate residues. Since these first reports, satellite DNA's have been found in a number of different eukaryotic organisms. In some instances these have been identified as cytoplasmic DNA, and are of mitochondrial
origin (Corneo, Moore, Sanadi, Grossman & Marmur, 1966; Rabinowitz, Sinclair, DeSalle, Haselkorn & Swift, 1965; Borst & Ruttenberg, 1966). Certain plants also possess satellite DNA which may consist of two bands, one associated with mitochondria and the other with chloroplasts (Edelman, Schiff & Epstein, 1965; Ray & Hanawalt, 1965; Green & Gorden, 1966; Iwamura & Kiwashima, 1969). On the other hand, the satellite component of pumpkin (Matsuda & Siegel, 1967), the toad *Xenopus laevis* (Brown & Dawid, 1968), and rat liver (Steele, 1968) have been found to be enriched in ribosomal cistrons, and may conceivably originate in the nucleolus (Matsuda & Siegel, 1967).

Nuclear satellite DNA also has been found in some mammals, such as the mouse, cow, and man (Corneo, Moore, Sanadi, Grossman & Marmur, 1966; Borst & Ruttenberg, 1966; Corneo, Ginelli & Polli, 1967; Corneo, Ginelli, Soave & Bernardi, 1968). Its contribution to the total DNA varies from 10% in mouse to a mere 1% in man. The function of this nuclear satellite is not known. The most extensively investigated satellite is that of the mouse. This contains twice as much 5-methylcytosine as the main band (Salomon & Kaye, 1969). It undergoes rapid thermal renaturation (Flamm, Bond, Burr & Bond, 1966), and this has been interpreted to imply that it is composed of 300 to 400 nucleotide pairs which are repeated a million times in the genome (Waring & Britten, 1966). Evidence on whether this satellite
is transcribed is conflicting. Harel, Hanania, Tapiero & Harel (1968) observed that one of the complementary strands was transcribed. Flamm, Walker & McCallum (1969), on the other hand, reported negative findings. Whatever explanation is offered for the function and evolution of the satellite, it must take into account Henning & Walker's (1970) recent finding that there are gross differences in the amount, density, and reassociation rate of satellite DNA between even closely related species of rodents.

Satellite DNA is also present in prokaryotic organisms. Minor secondary bands have been reported in cases of interspecies episome infection (Marmur, Rownd, Falkow, Baron, Schildkraut & Doty, 1961). Extrachromosomal elements have also been found in various bacterial cells, characterized, and in some cases examined in the electron microscope. These include the colicinogenic factor $E_1$ from *Proteus mirabilis* (DeWitt & Helinski, 1965; Roth & Helinski, 1967; Bazaral & Helinski, 1968a); the colicinogenic factors $E_1$, $E_2$, and $E_3$ from *E. coli* (Bazaral & Helinski, 1968b); $F'$ *lac* and $F$ sex factors from *E. coli* (Freifelder & Freifelder, 1968; Freifelder, 1968); circular DNA of uncertain function from *Shigella paradysenteriae* Y6R (Jansz, Zanderberg, Van der Pol & Van Bruggen, 1969), *Staphylococcus aureus*, and *Shigella dysenteriae* Y6R (Rush, Gordon, Novick & Warner, 1969). Without exception, intact DNA from these elements have been found to be in the closed
circular duplex conformation.

Joshi, Guild & Handler (1963) first reported that Halobacterium salinarium contains an unusually large proportion of a light density satellite which amounts to 20% of the total. The buoyant densities of the satellite and the main band were found to be 1.718 and 1.727 g/ml in cesium chloride, implying G+C contents of 58% and 67%, respectively. Chemical analysis of the DNA, however, failed to reveal the presence of unusual bases which might have accounted for the density heterogeneity. The possibility that the satellite represents a contaminating culture is ruled out by the results from microbiological purification (Joshi, Guild & Handler, 1963).

More recently, Moore & McCarthy (1969a, 1969b) have reported parallel work on the satellite of the extreme halophiles. This indicates that the presence of a satellite component seems to be characteristic of all non-photosynthetic strains of extreme halophiles, although its relative amount varies from 10% in H. cutirubrum to 36% in an unidentified species of Halobacterium referred to as "Isolate III". The same authors also tried to establish whether the halophilic satellite represents an episomal element by treating halophilic cultures with mutagenic agents such as acriflavin, acridine orange, and mitomycin. Their results were not conclusive, partly because not all episomal elements are eliminated by these agents, and partly because these agents
do not easily dissolve in the high-salt medium of the extreme halophiles. DNA-DNA renaturation rate studies, on the other hand, suggest that both the satellite and the main band renatured at the same rate. This is taken by the authors to mean that the satellite is not present in multiple copies. The same renaturation rate studies also established the genome size of the halophilic bacteria to be $4.1 \times 10^6$ nucleotide pairs long, which is approximately the size of that of *E. coli* ($4.5 \times 10^6$ nucleotide pairs, Cairns, 1963).

The present study is undertaken to study the physical properties of the satellite DNA in the hope that such information will shed light on its possible significance in the halophilic bacteria. As *H. salinarium* contains twice as much satellite as *H. cutirubrum*, it has become the organism of choice for the present study, the scope of which is two-fold:

(a) To find out whether the halophilic satellite represents an extraction artefact, and whether its presence depends on the method of DNA isolation. Both Joshi, Guild & Handler (1963) and Moore & McCarthy (1969a, 1969b) have used Marmur's method (1961) to isolate DNA. It is not known what effect isolation conditions will have on the presence of two species of DNA in *H. salinarium*. One is reminded of a peculiar phenomenon in the preparation of crab DNA: DNA prepared using chloroform as a protein denaturant shows the d(A-T)-rich satellite, whereas that
prepared using phenol does not (Skinner & Triplett, 1967). As the explanation for this difference is unknown, one needs to find out if this phenomenon occurs in the halo-philic DNA as well. Again, should the satellite represent an extraction artefact, its amount is unlikely to remain constant from preparation to preparation, especially if different isolation procedures are used.

(b) To find out the physical characteristics of the halophilic satellite. There can only be two alternatives to the biological derivation of the satellite. It may either represent specific pieces of chromosomes fragmented from the bacterial genome during DNA extraction, or it may represent an extrachromosomal element. In view of the rapidly growing list of such elements found to be closed circles, a direct approach to the latter alternative is to find out if it is in a closed circular conformation. On the other hand, if the satellite does not exist as closed circles, one can still characterize its size. In either event, knowing the size of the satellite will allow one to determine the number of copies of the satellite per cell.

In order to provide some answers to these questions, sucrose gradient sedimentation experiments have been performed on unfractionated DNA to see whether the satellite consists of a sufficient number of small identical molecules of molecular weights such that they may be detected as a single recognizable peak. Ethidium bromide-cesium chloride
isopycnic centrifugation experiments have been performed in order to detect closed circular DNA. The results from the latter experiments have been substantiated by examining the size and shape of the isolated satellite under the electron microscope.
II. MATERIALS AND METHODS

Sources of Chemicals

Unless specified, all organic and inorganic compounds were obtained from Fisher Scientific Co., Fairlane, New Jersey. Potassium tartrate was from J.T. Baker Chemical Co., Phillipsburg, New Jersey; optical grade cesium chloride was from Harshaw Chemical Co., Cleveland, Ohio; analytical grade cesium chloride and cesium sulphate were from American Potash & Chemical Corp., West Chicago, Illinois; analytical grade phenol was from Mallinckrodt Chemical Co., St. Louis, Missouri.

Bovine serum albumin, pancreatic ribonuclease type III, and cytochrome C type III were obtained from Sigma Chemical Co., St. Louis, Missouri; adenine, adenosine, and uridylic acid were from General Biochemical Co., Chagrin Falls, Ohio; casamino acids and yeast extract were from Difco Laboratories, Detroit, Michigan; polyethylene glycol ("Carbowax 6000") was from Union Carbide Chemical Co., N.Y.; dextran T500 (number average molecular weight 185,000) was from Pharmacia Ltd., Uppsala, Sweden; thymidine-methyl-³H (1 mC/ml, 6.7c/m-mole) was from New England Nuclear Corp., Boston, Mass.; ethidium bromide was from Calbiochemical Co., Los Angeles, California; and platinum-carbon pellet was from Ladd Research Industries, Burlington, Vermont.
Growth of Bacteria

*H. salinarium* strain 1 was obtained from the National Research Council in Ottawa. The composition of the growth medium was similar to that described by Sehgal & Gibbons (1960), except that 0.02% calcium chloride was added to obtain better growth (Dr. M.G. Gochnauer, personal communication). The composition of the complex medium is given in Table 1.

The inoculum consisted of a 24-hour culture grown in 200 ml complex medium which was added directly to four litres of complex medium in a New Brunswick fermentor. The culture was maintained at 37°C with an aeration rate of three litres of air per minute and an agitation rate of 300 r.p.m. Under such conditions the culture generally reached late log phase of growth 50 hours after inoculation. Figure 1 shows the growth curve of *H. salinarium* under conditions as described. Turbidity measurements were taken by withdrawing 3 ml samples from the fermentor at convenient times and reading them in a Bausch & Lomb SP 20 spectrophotometer at 660 mu.
Table 1. Composition of the Complex Medium for the Growth of Halophilic Bacteria

<table>
<thead>
<tr>
<th>Ingredient</th>
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<tr>
<td>Yeast extract (Difco)</td>
<td>10 grams</td>
</tr>
<tr>
<td>Casamino acid (Difco)</td>
<td>7.5 grams</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>2 grams</td>
</tr>
<tr>
<td>Magnesium sulphate .7H2O</td>
<td>20 grams</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>250 grams</td>
</tr>
<tr>
<td>Sodium citrate .2H2O</td>
<td>3 grams</td>
</tr>
<tr>
<td>Calcium chloride .2H2O</td>
<td>0.2 grams</td>
</tr>
<tr>
<td>Ferrous sulphate .7H2O</td>
<td>1 ml</td>
</tr>
<tr>
<td>(4.98% ferrous sulphate .7H2O</td>
<td></td>
</tr>
<tr>
<td>acidified with 1 ml N HCl per</td>
<td></td>
</tr>
<tr>
<td>100 ml</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre final volume</td>
</tr>
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About 800 ml of distilled water was added, and the pH adjusted to 7-8 with NaOH. The mixture was heated for 5-10 minutes and the precipitate filtered. The volume of the filtrate was brought to one litre, and the pH adjusted to 6.2 with HCl. The medium was autoclaved at 1.2 atmospheres for at least 20 minutes.
Fig. 1. Turbidity Curve of H. salinarium in New Brunswick Scientific Fermentor.

4 litres of culture in the complex medium of Sehgal & Gibbons (1960) were grown at 37°C at an aeration rate of 3 litres/min. and agitation at 300 r.p.m. Yield was about 4 grams of cell paste per litre of culture in late log phase.
Cells were harvested in late log phase of growth by centrifuging in a Sorvall RC-2 centrifuge at 8,000 x g for 10 minutes at 4°C. The cell paste was washed twice with about 200 ml of the NaCl wash medium of Bayley & Kushner (1964) consisting of 4.3M NaCl-0.3M KCl-0.08M MgSO₄. The yield in cell paste was about 16 grams from 4 litres of culture.

Analytical Methods

Estimation of protein was by the method of Lowry, Rosebrough, Farr & Randall (1951). 1 ml of DNA solution was mixed with 1 ml of a solution containing 1% potassium tartrate. After 5 minutes reaction time at room temperature, 0.5 ml of 1N Folin reagent was added, and the mixture allowed to stand for another 30 minutes. The intensity of the blue colour was read at 500 μm in a Beckman DU spectrophotometer. Bovine serum albumin was used as standard (figure 2).

Estimation of RNA was by the orcinol reaction of Schneider (1957). Orcinol reagent was prepared, immediately before use, by dissolving 0.1 gram of orcinol in 10 ml concentrated hydrochloric acid containing 0.5 gram of ferric chloride. The reaction consisted of heating 1.5 ml aliquots of the DNA preparation with equal volumes of the orcinol reagent in a boiling water bath for 20 minutes. The intensity of the green colour was read at 660 μm in a Beckman DU spectrophotometer, using 1-cm cells. Adenosine
was used as standard curve (figure 3).

Absorption spectrum of DNA was taken in a Unicam SP 800A spectrophotometer between 300 and 200 μm, using 1-cm cells.

**Analytical isopycnic centrifugation.** The density gradient consisted of 56% (w/w) optical grade cesium chloride in 0.02 M Tris, pH 8 buffer containing 0.02 O.D. *H. salinarum* DNA and 0.01 O.D. SB19E DNA to serve as reference. The latter DNA was provided by Dr. I. Takahashi, and has a buoyant density of 1.703 g/ml in cesium chloride. Centrifugation was performed at 44,770 r.p.m. for 18 hours at 25°C, after which period the distribution of the DNA bands was photographed by ultraviolet light.

Buoyant densities of the satellite and main band were calculated using the position of SB19E DNA as reference, according to the equation given by Sueoka (1961) in the density range from 1.65 to 1.75 g/ml at 25°C:

\[ p = p_o + 4.2w^2 (r^2 - r_o^2) \times 10^{-10} \text{ g/ml} \]

where \( p \) is the buoyant density of the unknown DNA band, \( p_o \) the buoyant density of the reference DNA, \( w \) the speed of rotation in radians/second, \( r \) the distance of the unknown band from the center of rotation, and \( r_o \) the distance of the reference band from the center of rotation. The centerpiece cavity of the Model E ultracentrifuge is known to have a length of 1.4 cm, and the centripetal end of the cell is 5.810 cm from the center of rotation (Mandel,
Fig. 2  Bovine Serum Albumin Standard Curve for the Estimation of Protein Content in DNA by the Folin Reaction of Lowry, Rosebrough, Farr & Randall (1951).

Intensity of the blue color was read at 500 mu in a Beckman DU spectrophotometer.
Fig. 3  Adenosine Standard Curve for the Estimation of RNA Content in DNA by the Orcinol Reaction of Schneider (1957).

Intensity of the green colour was read at 600 mu in a Beckman DU spectrophotometer, using 1-cm cells.
Preparative isopycnic centrifugation. Earlier centrifugation experiments to check the densities of purified DNA and the amount of the satellite were performed in density gradients composed of 5 ml of 53% (w/w) analytical grade cesium chloride in 0.02M Tris, pH 8 buffer containing 0.3 O.D. H. salinarium DNA. Centrifugation was performed in a Spinco SW 50 rotor at 33,000 r.p.m. for 60-70 hours at 25°C.

After centrifugation, 8 drop-fractions (corresponding to volumes of about 0.07 ml) were collected from the bottom of the tube through a 23-gauge needle. For refractive index measurement, every fifth fraction of the gradient was immediately sealed with parafilm wax as soon as its collection was complete. The remaining fractions were diluted with 1 ml aliquots of 0.15M NaCl-0.015M sodium citrate, pH 7 buffer (SSC buffer). Positions of the DNA peaks were determined by reading the absorbances of the diluted fractions at 260 μm in a Beckman DU spectrophotometer, using 1-cm cells.

Linearity of the density gradient was determined from plots of refractive index against fraction number. Refractive index was read by placing a droplet from the corresponding fraction onto the stage of a Bausch & Lomb refractometer. Densities of the DNA peaks were calculated from their refractive indices, using the relationship given
by Ifft, Voet & Vinograd (1961):

\[ p^{25} = 10.8601 \ n_D - 13.4974 \]

where \( p^{25} \) is the density of cesium chloride at 25°C, and \( n_D \) the refractive index at the same temperature.

In order to reduce the lengthy centrifugation time, we have tried various approaches to achieve isopycnic condition within the shortest possible period of time. Preforming a multiple-step density gradient by manually layering cesium chloride solutions of progressively decreasing densities over one another allowed a saving of ten or more hours of centrifugation time. On the other hand, the extreme care involved in handling the gradient made it somewhat impractical for routine application. A somewhat easier way, which gave similar density profiles, was to preform a linear, continuous density gradient by mixing two equal volumes of cesium chloride solutions of densities 1.8 and 1.5 g/ml by means of a Buchler gradient former, so that the average density was about 1.7 g/ml, the density expected of purified halophilic DNA.

We also sought to reduce the centrifugation time by initially centrifuging a uniformly mixed cesium chloride solution of density 1.7 g/ml at a high speed of 50,000 r.p.m. in a Spinco 65 fixed-angle rotor, so that isopycnic condition could be attained rapidly. After 24 hours, the speed was reduced to only 34,000 r.p.m. for 21 hours so that DNA bands could be better resolved in a shallower
density gradient. This approach was similar to that described six months later by Anet & Strayer (1969).

We found it most convenient, however, to combine the merit of all these methods by forming a step-gradient and centrifuging at a high speed to attain isopycnic condition, and then reducing the speed to produce a shallower density gradient. Step-gradient of cesium chloride, similar to that described recently by Brunk & Leick (1969), was prepared by gently layering 2.5 ml of a 49% (w/w) solution of cesium chloride in 0.02M Tris, pH 8 buffer over an equal volume of a 64% (w/w) solution containing 0.05 O.D. of H. salinarium DNA and 0.03 O.D. commercial salmon sperm DNA as reference. Centrifugation was performed in a Spinco SW 50 rotor at 42,000 r.p.m. for 14 hours at 25°C, after which period the speed was reduced to 30,000 r.p.m. for 24 hours. The density profile and the narrowness of the DNA bands obtained by this approach were at least as good as those obtained by previous methods.

**Ultraviolet Scanning of Density Gradient.** Distribution of the DNA bands was occasionally scanned by means of an ISCO Model 170 ultraviolet analyser. A saturated solution of cesium sulphate was used to displace the content of the gradient from the bottom of the centrifuge tube at a pump rate of 0.2 ml/minute into 1 cm x 7.5 pyrex tubes. Immediately after delivery of each 0.25 ml fraction, the corresponding tube was sealed with parafilm wax to prevent evaporation of
the contents. Refractive index measurement and subsequent determination of the buoyant densities of the DNA bands were as described earlier on.

In later centrifugation experiments, the density difference between the satellite and the main band was checked by determining the ratios of their relative positions from salmon sperm reference DNA (buoyant density = 1.703 g/ml in cesium chloride, Schildkraut, Marmur & Doty, 1962). The results were in good agreement with the calculated values, accurate to two decimal places. Although these give no indication of the absolute densities of the DNA peaks, one may, in the light of the fairly consistent density values obtained from previous refractive index measurements, assign values of 1.72 and 1.73 g/ml to the densities of the satellite and the main band, respectively (figures 9 and 16).
III. A NEW APPROACH TO THE ISOLATION OF DNA

Problems in DNA Isolation

Conventional methods of DNA isolation generally involve fairly extensive use of organic solvents as protein denaturants. A classical procedure using chloroform has been developed by Marmur (1961). As chloroform is a rather ineffective denaturation agent, the procedure tends to be tiresome as twenty or more extraction steps are generally required. A somewhat simpler procedure has been developed by Takahashi (1965).

Phenol, on the other hand, has been one of the most effective protein denaturants known. Isolation procedures based on phenol extraction offer greater economy of time and effort over that of Marmur. Kirby and co-workers, for instance, have studied its methodology extensively over the past decade, and have devised many useful techniques for its application (Kirby, 1957, 1958, 1959, 1961, 1962, 1964; Frearson & Kirby, 1964; Kirby, Fox-Carter & Guest, 1967).

Saito & Miura (1963), on the other hand, have developed a simpler procedure whereby DNA is phenol-deproteinized at pH 9 by one single extraction, such that little RNA is extracted with the DNA. Residual RNA is further removed by RNase treatment, as in the procedure of Marmur (1961). The yield of phenol-extracted DNA is found
to be better than that of chloroform-extracted: the former gives 3 mg of DNA per gram cell paste, whereas the latter gives only 2 gm of DNA. Phenol extraction also gives a higher molecular weight product over chloroform extraction, as estimated by their relative cotransfer indices which are indications of the proportion of joint transformation of closely linked markers (Saito & Miura, 1963). The level of protein contamination in the final DNA preparation is comparable between the two methods: both give less than 2% protein by the method of Lowry, Rosebrough, Farr & Randall (1951). RNA content, however, was not mentioned by the authors.

As these conventional purification procedures rely upon the use of organic solvents as protein denaturants, alcohol precipitation, and resuspension of DNA, changes in the structure of DNA is likely to be encountered. Noll & Stutz (1967), for example, found that phenol often causes selective losses of DNA by entrapment in precipitates of denatured protein, particularly at the interphase. They believe that in many cases the use of phenol has produced misleading artefacts, but they did not specify the latter. Instead, they advocated the use of sodium and lithium dodecyl sulphate as protein denaturants. Kirby (1967) also admits that some of his phenol procedures may cause some denaturation of DNA. Again, Skinner & Triplett (1967) have reported that the crab d(A-T)-rich satellite is not observed
in phenol-extracted DNA preparations, although the explanation for this phenomenon remains unknown.

In view of these limitations in conventional DNA isolation procedures, Albertsson has recently devised a polymer phase system for the fractionation of DNA which appears to offer considerable advantages over previous methods (Albertsson, 1960, 1962a, 1962b, 1965; Albertsson & Baird, 1962; Rudin & Albertsson, 1967). The phase system operates on the principle that DNA, protein, and RNA may be selectively separated without loss of biological activity in an aqueous solution containing 5% dextran and 4% polyethylene glycol, under favourable ionic conditions. The great advantage of this method, however, lies in the fact that denatured DNA may be quantitatively removed from native DNA in one extraction.

Other advantages of this system are:

(a) RNase treatment is unnecessary. Commercial RNase preparations may contain trace amounts of DNase which may not be completely inactivated even upon heating at 80°C before use, so that it may be safer to avoid using RNase as a means of purifying DNA. Also, RNase is stable towards denaturation, and is difficult to remove from the purified DNA. Investigation into the biological information content of the satellite may involve DNA-RNA hybridization experiments. Clearly, it is essential to free the DNA preparation from even trace amounts of RNase for
experiments of this sort.

(b) DNA is never precipitated throughout the phase extraction. This avoids lengthy overnight shaking periods usually necessary to resuspend precipitated DNA, during which time damage to the DNA may occur through shearing stress introduced by continuous shaking, or by contaminating nuclease activity which may not be completely removed at this stage. By avoiding several overnight shaking periods, isolation of DNA may be accomplished within the same day.

(c) The yield of DNA is high. Rudin & Albertsson (1967) estimated that over 90% of the total DNA from _E. coli_ is recovered.

Favre & Pettijohn (1967) have proposed a procedure which gives an even higher level of purity than that of Rudin & Albertsson (1967), with fewer extraction steps. Since the unique feature of the polymer phase system lies in the isolation of fully native DNA, and since one of the aims of the present study is to see if the presence of two species of DNA in _H. salinarium_ depends on extraction conditions, we have adopted the latter procedure to the routine preparation of halophilic DNA.

The effect of various extraction procedures on the presence and relative amounts of two species of DNA in _H. salinarium_ will now be examined, beginning with the chloroform-RNase method of Marmur (1961).
(a) Isolation of DNA by Chloroform-Alcohol and RNase

Although the methodology is essentially that of Marmur's (1961), a protocol obtained from Dr. Sol Spiegelman has been followed. The latter consists of slight modifications in both the amounts of reagents used and the sequence of chloroform extraction and RNase treatment.

Extraction Procedure

Preparation of lysate. After harvesting and washing the cells at 4°C in the NaCl wash medium of Bayley & Kushner (1964), the cell paste was suspended in minimum volume of the same wash medium. Lysis of cells was accomplished by taking advantage of the fact that the cell membrane of the extreme halophilic bacteria disintegrates in hypotonic solutions deficient in sodium ions (Abram & Gibbons, 1960, 1961; Stockenius & Rowen, 1967). A solution of 0.1M EDTA-0.15M NaCl, pH 8 was added to the cell suspension to a concentration of 10 ml/gram of paste while gently stirring by means of a magnetic bar. Lysis of the halophiles was instantaneous. The EDTA was added to inactivate DNase. The lysate was made 3% in sodium dodecyl sulphate by adding 18 ml of a 25% detergent solution in the same EDTA-NaCl buffer to the 128 ml of lysate.

Chloroform-alcohol extraction. The lysate was made 1M in sodium perchlorate. An equal volume of chloroform and 1/10 volume of isoamyl alcohol was added. Extrac-
tion of cellular proteins and lipids was accomplished by vigorously shaking the mixture in a volumetric flask on a mechanical reciprocal shaker in a 4° cold room for ten minutes. The resulting emulsion was separated into three layers by a ten minute centrifugation at 16,300 x g in a Sorvall RC-2 centrifuge at 4°C.

The viscous upper aqueous phase contained the DNA, and was carefully siphoned off. The interphase consisted of denatured protein, and the bottom phase of lipids and proteins in chloroform. The upper phase was again extracted with an equal volume of chloroform-alcohol, and the procedure repeated five times until a white protein precipitate was no longer visible at the interphase.

**Ethanol precipitation.** The DNA preparation was precipitated with twice its volume of 95% ethanol in the following manner: the DNA-containing aqueous phase was placed in a beaker in an ice-water bath. Ethanol was added in drops by means of a separatory funnel while the contents were being slowly stirred by a cork-screw shaped glass rod. DNA precipitated around the rod and was removed. Excess liquid was drained by pressing the rod against the sides of the beaker. The yield of DNA at this point was about 1.4 grams of DNA in wet weight from 16 grams of cell paste.

**Resuspension of DNA.** The DNA precipitate was generally very difficult to dissolve, and was best dissolved
by suspending it in 80 ml of a low ionic strength buffer containing 0.015M NaCl-0.0015M NaCitrate, pH 7 (i.e., in 1/10x "SSC" buffer) with overnight shaking. When solution was complete, it was made "standard" in salt concentration by adding 1/10 volume of a 10xSSc solution.

**RNase treatment.** 8.8 mg. pancreatic RNase, dissolved in 1 ml SSC buffer, was heated for 10 minutes at 80°C in a water bath in order to destroy possible contaminating DNase activity. After cooling, the solution was added to 88 ml of DNA solution to a final concentration of 100 μg/ml. Incubation was carried out at 37°C for two hours. RNase was then removed by making the solution 1M in sodium perchlorate and extracting it six times with chloroform-alcohol, followed by ethanol precipitation and resuspension in buffer as described.

**Isopropanol precipitation.** Finally, trace amounts of RNA and polysaccharides were removed by making the preparation 0.3M in sodium acetate by adding 1/10 volume of a 3M sodium acetate buffer. This provided a favourable ionic environment for subsequent isopropanol precipitation. Exactly 0.54 volume of isopropanol was added in drops in much the same manner ethanol precipitation was performed. The DNA precipitate at this stage appeared clear, rather than milky as it did just after ethanol precipitation. It was resuspended in 1/10 SSC with overnight shaking, and finally made standard in salt concentration as described.
Results

The yield of DNA from 16 grams of cell paste was 20 mg, estimated from its absorbance at 260 μm, on the basis that a one per cent solution of DNA gives an absorbance of 200 units in a 1-cm cell (i.e. $E_{\text{1cm}}^{1\%} = 200$).

The levels of protein and RNA contamination in the DNA preparation were less than 2%, as estimated by the phenol reaction of Lowry, Rosebrough, Farr & Randall (1951) and orcinol reaction of Schneider (1957). Figure 4 shows the absorption spectrum of the purified DNA preparation. The absorbance ratio at 280/260 was 0.53. The 260/230 ratio was 2.23.

The presence of two species of DNA in DNA so prepared is clearly shown in an ultraviolet photograph taken during isopycnic centrifugation in a Model E analytical ultracentrifuge. As shown in figure 5, the DNA bands, starting from right to left, are those of SB19E, the satellite, and the main band. The calculated values for the density of the satellite and the main band were 1.716 and 1.725 g/ml, respectively, by the relationship given by Sueoka (1961). Although both densities were 0.002 g/ml less dense than those reported by Joshi, Guild & Handler (1963), the density difference between the two species was consistent with their reported value. Moore & McCarthy (1969b), on the other hand, cited values of 1.718 and 1.725 g/ml, respectively. Our results therefore agree
within experimental error with those reported.

Figure 6 shows a density profile of chloroform-extracted DNA upon preparative centrifugation. The satellite DNA is seen as a prominent shoulder on the light density side of the gradient. Its proportion, estimated by weighing the areas under the curve, is about 19-20% of the total. This is in good agreement with that reported by Joshi, Guild & Handler (1963), and by Moore & McCarthy (1969b). In all three cases, DNA was prepared from late log phase of growth by the method of Marmur (1961). Thus it seems clear at this stage that the amount of satellite remains constant whenever DNA is extracted by chloroform.

(b) Isolation of DNA by Phenol-pH 9 and RNase

Isolation Procedure

Unless specified, the operational details of the procedure were similar to those described for chloroform extraction. The salient feature of the present method is that protein is denatured by a single phenol extraction.

Preparation of phenol-pH 9 buffer. Loose crystalline phenol (200 grams) was freshly redistilled immediately prior to use in order to remove trace amounts of oxidized forms of the compound which might otherwise affect effective extraction of DNA. Redistilled phenol, which boiled at 182°C, was saturated with an alkaline buffer containing 0.1M tris-HCl-1% sodium dodecyl sulphate-0.1M NaCl, pH 9 at 4°C. This corresponded to 80% (w/w) phenol.
Figure 4. Ultraviolet Absorption Spectrum of DNA purified by Chloroform and RNase

0.5 ml of DNA solution in 0.15M NaCl-0.015M NaCitrate, pH 7 buffer was diluted six times with the same buffer. The absorption spectrum was taken by a Unicam SP 800A spectrophotometer, using 1-cm cells.
Figure 5. **Ultraviolet Absorption Photograph of DNA Purified by Chloroform and RNase**

Picture was taken 18 hours after centrifugation in a Beckman Spinco Model E analytical centrifuge at 44,770 r.p.m. at 25°C. The DNA band at the far right was that of SB19E, and was used as reference (buoyant density = 1.703 g/ml).
Figure 6. Density Profile of DNA Purified by Chloroform and RNase.

The density gradient consisted of 5 ml of 53% (w/w) cesium chloride in 0.02M Tris, pH 8 buffer containing 0.3 O.D. halophilic DNA. Centrifugation was performed in a Spinco SW 50 rotor at 33,000 r.p.m. for 62 hours, 25°C.

8 drop-fractions were collected from the bottom of the centrifuge tube through a 23-gauge needle. The appropriate fractions were diluted with SSC buffer to 1-ml volumes, and the absorbance of each fraction read at 260 μm in a Beckman DU spectrophotometer, using 1-cm cells. Buoyant densities of the peaks were estimated from their refractive indices, using the relationship given by Ifft, Voet & Vinograd (1961).
Phenol extraction. Tris-SDS-pH 9 buffer (160 mls) was added to 16 grams of cell paste, harvested in late log phase of growth from four litres of culture, so that the lysate concentration was 10 ml of buffer per gram of cell paste. An equal volume of the saturated phenol-buffer was added to the lysate suspension; and the mixture shaken vigorously for 20 minutes in a mechanical reciprocal shaker in the cold. Conditions for centrifuging the emulsion and siphoning the aqueous DNA phase were described in the previous section.

RNase treatment. Although Saito & Miura (1963) advocated using a combination of pancreatic RNase and T1 RNase so that ribonucleotide sequences resistant to one type of RNase action would not be so to the other type, we have obtained less than 1% RNA content in some of our DNA preparation by pancreatic RNase activity alone. The RNase, following heating in SSC buffer to destroy possible DNase activity, was added to the DNA solution to a final concentration of 100 µg/ml under incubation conditions described in the previous section.

Results

Purified DNA was obtained in high yield. Up to 39 mg of DNA was recovered from 16 grams of cell paste. This is about twice the amount recovered in the previous procedure. The high recovery was undoubtedly due to fewer number of extraction necessary by phenol so that loss of
DNA was minimized.

The level of protein content in five preparations of DNA was less than 2%. RNA content was less than 4%, and was at 1% level in two of the preparations. The level of purity given by the phenol procedure is therefore comparable to that by the chloroform. Figure 7 shows an absorption spectrum of the purified DNA. The absorbance ratio at 280/260 was 0.56. The ratio at 260/230 was 2.28.

Figure 8 shows the density profile of phenol-extracted DNA upon preparative centrifugation. The presence of a satellite component is indicated by a shoulder on the light density side of the gradient. Its proportion is about 20% of the total. The calculated densities of the DNA peaks, however, are slightly higher than those of figures 5 and 6, although the intrinsic density difference between the two species (0.009 g/ml) is consistent with that in figure 5. The discrepancy probably represents experimental errors involved in refractive index measurement.

The presence of a satellite component is more clearly demonstrated in an enlarged ISCO tracing, showing the density profile of DNA peaks in a shallower density gradient (figure 9). The DNA peaks, from right to left, are those of salmon sperm reference, the satellite, and the main band. The satellite is again 19-20% of the total. The densities of the halophilic DNA are approximated by their
Figure 7. Ultraviolet Absorption Spectrum of DNA purified by Phenol and RNase.

0.5 ml of DNA solution in 0.15M NaCl-0.015 NaCitrate, pH 7 was diluted six times with the same buffer.
Figure 8. Density Profile of DNA Purified by Phenol and RNase.

Conditions of centrifugation and collection of the gradient were as described in figure 6.
Figure 9. Ultraviolet Tracing of DNA Purified by Phenol and RNase.

The gradient consisted of 2.5 ml of a 49% (w/w) solution of cesium chloride in an 0.02M Tris, pH 8 buffer over an equal volume of a 64% (w/w) solution of cesium chloride containing 0.05 O.D. halophilic DNA and 0.03 O.D. commercial salmon sperm DNA. Centrifugation was performed in a Spinco SW 50 rotor at 42,000 r.p.m. for 14 hours at 25°C, and then at 30,000 r.p.m. for 24 hours. The tracing shown is an enlargement of a smaller tracing given by an ISCO Model 170 ultraviolet analyzer at 254mu, at a scan speed of 0.2 ml/minute.
positions from salmon sperm DNA as described earlier on. In view of the constancy in the amount of the satellite, it seems evident that its presence in the halophilic bacteria is independent of phenol treatment, contrary to the case in crab d(A-T)-rich satellite (Skinner & Triplett, 1967).

(c) Isolation of DNA by Polyethylene Glycol-Dextran Phase Extraction.

General Description of the Polymer Phase System

Definition of partition coefficient (Berg, 1963). Liquid-liquid extraction is a partitioning process based on the selective distribution of a third component in two immiscible phases. The distribution of this third component between the two phases follows the distribution law when the system is at equilibrium:

\[
\frac{c_1}{c_2} = D = \text{distribution coefficient}
\]

where \(c_1\) and \(c_2\) represent the concentration of the third component in the upper and lower phases, respectively and \(D\) is a constant. Such an expression of the distribution law is valid only for ideal system. In practice, the distribution often deviates from this law owing to the association or dissociation of the solute molecules in one or both phases. The solute molecules may even undergo a change in structure or composition as they change phases.
An exact expression of the distribution law therefore states that

$$\frac{a_1}{a_2} = K = \text{partition coefficient}$$

where $a_1$ and $a_2$ represent the activity of the third component in the upper and lower phases, respectively, and $K$ is a constant.

Nonetheless, the partition coefficient for a dilute solution approaches the distribution coefficient, and therefore may be adequately expressed as a concentration ratio.

**Application of the Brönsted approximation to phase systems.**

Partition of a component in a phase system generally obeys the Brönsted approximation (1931a, 1931b):

$$\frac{c_1}{c_2} = K = e^{Ma/RT}$$

where $c_1$ and $c_2$ represent the concentration of the component in the upper and lower phases, respectively, $K$ the partition coefficient, $R$ the gas constant, $T$ the absolute temperature, $M$ the molecular weight of the partitioned component, and $\lambda$ is a constant which depends on the physical and chemical properties of the phases and the partitioned component. These properties include the ionic strength of the phases, the charged groups on the partitioned molecule, hydrophilic
and hydrophobic groups of the phase polymers and the partitioned molecule, their structure and molecular weight, and so on. The combined effect of these factors on the value of $\lambda$ is therefore difficult to predict. Nonetheless, one may deduce from the above formula that even with small changes in the value of $\lambda$, $K$ should change drastically if $M$ is sufficiently large, as with biological macromolecules. Thus, even with a small positive change in $\lambda$, $K$ can still be very large. On the other hand, with a small change in $\lambda$ to the negative sign, $K$ will become very small.

In short, the Brønsted equation states that a small change in the phase composition will cause macromolecules to be transferred effectively from one phase to the other. This is the theoretical basis underlying the polyethylene glycol-dextran phase system for the isolation of DNA.

**Extraction Procedure**

The operational details to be described represent a synthesis of various techniques described by Albertsson (1965, 1967) and Favre & Pettijohn (1967) so that the most effective procedure may be performed using relatively simple equipment. For example, the procedure of Favre & Pettijohn was adopted as the authors claimed that it gave a higher level of purity with less extraction steps than that of Rudin & Albertsson (1967). As this procedure called for use of a counter-current extraction apparatus for equilibration, the alternative approach of simply
mixing the contents by hand in a separatory funnel was adopted.

The complete procedure, in summary, consists of the following:

(1) One extraction of lysate with chloroform to denature and remove protein, lest naturally occurring protein-DNA complexes (e.g., polymerase-DNA complexes) be isolated instead of naked DNA (Favre & Pettijohn, 1967); (2) overnight dialysis of the DNA-containing aqueous phase to bring the ionic environment to a level favourable for phase partitioning; (3) one equilibration in a phase system containing 3mM Na₂HPO₄ and 7mM NaH₂PO₄ buffer. This step removes denatured DNA; (4) four equilibrations of the resultant upper phase with twice its volume of a fresh lower phase containing 5mM Na₂HPO₄ and 5mM NaH₂PO₄ to remove RNA and remnant protein not removed by chloroform; (5) four equilibrations in 5mM Na₂HPO₄-5mM NaH₂PO₄-20mM NaCl buffer, using an upper phase volume three times the volume of the lower phase. These steps concentrate the DNA in the lower phase and further remove protein and RNA; (6) one extraction in fresh 5mM Na₂HPO₄ and 5mM NaH₂PO₄ buffer at pH 8.3, using an equal volume of upper phase. This brings the purified DNA back to the upper phase. The total number of equilibrations, of course, may be modified, depending on the level of purity desired.
Lysate preparation *H. salinarium*, grown in four litres of complex medium (Table 1), was harvested in late log phase and washed twice with NaCl wash medium. As the ionic environment in the first phase system must be kept at 3mM Na$_2$HPO$_4$ and 7mM NaH$_2$PO$_4$ for effective partition, the cell paste, 16 grams in wet weight, was lysed directly in a sodium phosphate buffer of the same strength so that the desirable ionic environment could be approached in the shortest length of time upon dialysis. The concentration of the lysate suspension was 10 ml of buffer per gram of cell paste.

**Removal of protein.** The lysate preparation was extracted once with chloroform, so that DNA could partition independently of protein-DNA complexes.

**Dialysis of the lysate.** As trace amounts of chloride ions would cause DNA to partition in the bottom phase, it was necessary to remove these ions by dialysis before phase extraction. In order to allow for a two-fold dilution in ionic strength upon equilibration with an equal volume of phase system, the 160 ml lysate preparation was dialysed overnight against twice the ionic strength necessary for phase partition, i.e., against 6mM Na$_2$HPO$_4$ and 4mM NaH$_2$PO$_4$.

**Phase equilibration.** An equal volume of an aqueous mixture containing 8% (w/w) polyethylene glycol and 10% (w/w) dextran was equilibrated with 160 ml of the dialyzate in a 1-litre capacity separatory funnel by manually
inverting the vessel thirty times. After 5 minutes of centrifugation at 16,300 x g in a Sorvall RC-2 centrifuge at 4°C to hasten phase separation, the dextran-rich lower phase was discarded.

To the upper phase containing DNA was added twice its volume of a lower phase containing 5mM each of Na₂HPO₄ and NaH₂PO₄ which had been held in reserve. After another thirty times of equilibration, and 5 minutes of centrifugation, the lower phase was again discarded. Again, twice its volume of fresh lower phase was added, and the procedure was repeated to a total of four times.

Further purification was achieved by adding 0.187 grams of solid NaCl directly to the 160 ml phase system to a final molarity of 20mM in NaCl. This concentrated the DNA into the lower phase. The upper phase was in turn discarded, and three times its volume of fresh upper phase added to the lower phase. The vessel was inverted another thirty times and centrifuged as described. The procedure was repeated to a total of four times. Purification of DNA was now complete.

Removal of phase polymers. As polyethylene glycol was much easier than dextran to remove from the DNA solution, it was desirable to transfer the purified DNA from the lower phase into a blank upper phase. Rather than using a neutral buffer system described by Favre & Pettijohn (1967) which required up to four equilibrations to transfer
the DNA, we have applied Albertsson's (1965) observation that a high \( \text{HPO}_4^-/\text{H}_2\text{PO}_4^- \) ratio favoured partition of the DNA into the upper phase. The neutral buffer system of Favre & Pettijohn was therefore made alkaline by adding KOH to pH 8.3 to increase the fraction of \( \text{HPO}_4^- \). In three tests, over 90% of the DNA was recovered upon a single equilibration in this manner.

Polyethylene glycol could then be quantitatively removed from the DNA preparation by one extraction with chloroform (Rudin & Albertsson, 1967). Alternatively, if cesium chloride density gradient of the DNA is to follow, no extraction with chloroform is even necessary, as the polymer floats at the top of the density gradient (Alberts, 1967).

Results

The yield of DNA was 2.8 mg per gram cell paste, or 45 mg from 16 grams cell paste, estimated on the basis that \( \varepsilon_{\text{1cm}1} = 200 \). The recovery was therefore comparable to that obtained by phenol extraction.

Figure 10 shows the absorption spectrum of the purified DNA. The absorbance ratio at 260/236 was 2.26. The ratio at 280/260 was 0.52. The latter value was thus in good agreement with that of 0.53 cited by Rudin & Albertsson (1967) for purified \( E. \text{coli} \) DNA containing less than 1% protein. The protein content estimated by the colorimetric method was less than 1%. RNA content was
Figure 10. Ultraviolet Absorption Spectrum of DNA Purified by Polyethylene Glycol and Dextran

DNA solution in 5 mM sodium phosphate buffer was diluted three times with the same buffer.
less than 2%.

Figure 11 shows an enlarged ISCO ultraviolet tracing of the density profile of the purified DNA. The satellite, seen as a prominent shoulder on the light density side of the gradient, is about 18% of the total area under the curve. This is consistent with the 19-20% observed in DNA prepared by other methods. As denatured DNA is expected to be selectively removed by the present procedure, the persistence of two species of DNA in the final product strongly suggests that both DNA species are fully native. The partition coefficients of native, denatured, and transfer RNA from the halophilic bacteria will be examined in the following section.

(d) Partition Coefficients of DNA

Procedure

In order to define the phase system more precisely, it was necessary to monitor the partition coefficients of macromolecules during various stages of phase equilibration. As the ultraviolet absorbance of DNA is directly related to its concentration, its partition coefficient, i.e., its concentration ratio between the upper and lower phases, could be determined from its absorbance ratio between these two phases.

Clearly, the spectrophotometric approach would be valid only if there was no interference due to absorbancy of protein or RNA, or both. For this reason, a phenol-
Figure 11. Density Profile of DNA Purified by Polyethylene Glycol and Dextran

The preparation of the step-gradient, conditions of centrifugation, and ultraviolet scanning of the gradient were similar to those described in figure 9, except that 0.5 O.D. halophilic DNA was included, and that salmon sperm DNA was omitted in the gradient. 0.25 ml-fractions were collected while the gradient was being scanned by the ISCO ultraviolet analyzer, and the refractive indices of these fractions read.
purified DNA preparation with less than 2% protein and RNA content was used as test material. DNA (7ml) at a concentration of 600 ug/ml was dialysed overnight against phosphate buffer containing 6mM Na₂HPO₄ and 14mM NaH₂PO₄ in order to bring the ionic environment close to that required for effective partitioning.

**Thermal denaturation of DNA.** To test the partition coefficient of denatured DNA in polyethylene glycol-dextran, DNA was thermally denatured as follows:

1. Dilution of the DNA to a low concentration to reduce the probability of reannealing: 1 ml of the undialysed DNA solution at a concentration of 600 ug/ml in "SSC" buffer was diluted ten times with distilled water so that both the DNA concentration and the ionic strength of the solution were sufficiently low for denaturation.

2. Thermal denaturation in the presence of formaldehyde: 0.05 ml formaldehyde was added to the diluted DNA solution to a final concentration of 0.5%. The chemical is believed to prevent strand-reannealing by forming methylene linkages with the free amino groups of cysteine, guanine, and adenine made available after strand-separation (as cited by Mahler & Cordes, 1966). The DNA solution was then subdivided into three smaller volumes of roughly 3 ml aliquots in 5-ml capacity pyrex tubes so that thermal equilibration could be readily established during heating. The latter period was 20 minutes in a boiling water bath.
(3) Quick chilling of the contents was performed by immersing the pyrex tubes in an ice-brine bath for 10 minutes.

(4) Concentration of the DNA solution to an O.D. level suitable for spectrophotometric analysis: A convenient way of doing this was by means of negative pressure dialysis using polyethylene glycol. After chilling, the contents were pooled and transferred to a narrow diameter dialysis tubing, and the ends were tied off. The dialysis sac was placed in a large beaker and polyethylene glycol flakes were evenly sprinkled around the sac. Within an hour in the cold, the volume of the contents were reduced from 10 ml to about 2 ml.

The concentrated DNA preparation was dialysed overnight against phosphate buffers corresponding to the ionic strength at each stage of the phase system, so that appropriate ionic conditions could be obtained.

Preparation of tRNA. Phenol-purified tRNA from *H. cutirubrum*, an extreme halophile of close taxonomic relationship to *H. salinarium*, was provided by Mr. B.N. White.

Determination of partition coefficient. Upon phase separation, 0.5 ml samples were withdrawn from the upper and lower phases, diluted with 2.5 ml water, and the absorbance read at 260 μm in an Unicam SP 800A spectrophotometer, using 1-cm cells. Small amounts of absorbance
by the blank phase material were corrected from the sample readings by subtracting 0.02 O.D. and 0.06 O.D. units from the readings given by the upper and lower phases, respectively.

**Effect of DNA concentration on partition coefficient.**

To test the capacity of the phase system to accommodate different concentrations of DNA, 2 ml of the test material, after overnight dialysis against 3mM Na$_2$HPO$_4$ and 7mM NaH$_2$PO$_4$, was diluted with convenient volumes of the same buffer. The concentrations of the diluted DNA solution was estimated by their O.D. in an Unicam SP 800A spectrophotometer. 2 ml samples of the diluted solutions were inverted with equal volumes of a phase system containing 8% (w/w) polyethylene glycol and 10% (w/w) dextran in a manner described earlier on.

**Results**

Table 2 compares the partition coefficients of native DNA, denatured DNA, and tRNA from the halophiles at various ionic conditions to that cited by Favre & Pettijohn (1967) for the corresponding molecules from *E. coli*.

The pattern of partitioning of halophilic nucleic acids is quite similar to that of *E. coli*, with only small deviations in the absolute values. Native DNA from both organisms partition in favour of the upper phase in the first two ionic environments, and in the lower phase in the third.
Table 2a. Raw Data of Partition Coefficient of DNA and tRNA from *Halobacterium*

<table>
<thead>
<tr>
<th>Ionic Environment (mM)</th>
<th>Native DNA</th>
<th>Denatured DNA</th>
<th>tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>NaH₂PO₄</td>
<td>NaCl</td>
<td>Partition Coefficient = corrected O.D.₂₆₀ in upper phase / corrected O.D.₂₆₀ in lower phase</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>0</td>
<td>1.07 = 9</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0</td>
<td>1.15 = 115</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>20</td>
<td>0.02 = 0.019</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>10</td>
<td>pH8.3</td>
</tr>
</tbody>
</table>

* Correction made by subtracting 0.02 O.D. and 0.06 O.D. units from the readings given by the upper and lower phases, respectively.
<table>
<thead>
<tr>
<th>Ionic Environment (mM)</th>
<th>Partition Coefficients</th>
<th>Halobacterium</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Native DNA</td>
<td>Native DNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Denatured DNA</td>
<td>Denatured DNA</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>NaH₂PO₄</td>
<td>NaCl</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0</td>
<td>115</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>20</td>
<td>0.019</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>10</td>
<td>pH 8.3</td>
</tr>
</tbody>
</table>

Table 2. Comparison of Partition Coefficients of Nucleic Acids from Halobacterium and E. coli
Denatured DNA from both organisms, on the other hand, partition in the lower phase in all three environments. Transfer RNA, in comparison, partitions evenly among the two phases in the first two environments, so that its amount becomes reduced two-fold with each equilibration.

Figure 12 shows the partitioning tendency of native and denatured DNA during phase extraction. Figure 13 demonstrates the capacity of the phase system to accommodate different concentrations of DNA without altering the partition coefficient. This was attributed to the highly aqueous environment of the phase system.

Discussion

It should be clarified that extracting the lysate once with chloroform to separate naturally occurring protein-DNA complexes does not invalidate the many advantages the polymer phase system holds over conventional procedures, since (1) extensive treatment with organic solvent is avoided; (2) no precipitation of DNA is encountered, so that any danger of alcohol-induced conformational change in the DNA helix, similar to that observed by Lang (1970), is avoided; (3) no treatment with RNase is necessary; and (4) potential organic solvent-induced artefacts, such as those Noll & Stutz (1967) spoke of would be eliminated by the phase system, along with denatured DNA.

It is interesting to find that DNA from *H. salinarium* obeys the same partition pattern as *E. coli* DNA. As it
has been shown that partition coefficient depends closely on the conformation of the molecule undergoing partitioning, the similarity in partition coefficients may be taken to mean that, given the ionic environment, the conformation of halophilic DNA is no different from that of *E. coli*. This idea is also suggested by the similarity in the 280/260 absorbance ratios given by purified halophilic and *E. coli* DNA. It is possible, however, that DNA from the extremely halophilic bacteria *in vivo* is protected from precipitating by specific proteins.

The phenomenon of phase partition raises another intriguing question. Could a similar mechanism be operative *in vivo*? It is possible that transport of macromolecules from one location in the cell to another is brought about by changes in the local phase composition, analogous to the partition of DNA in the phase system. Changes in the ionic composition in this case may perhaps be initiated by changes in the permeability of the cell membrane.

Thus far, all three extraction procedures gave similar results: purified DNA invariably contained an 18-20% satellite; the differences in the densities of the two species of DNA were within experimental error; phenol had no effect on the presence of the satellite; and polyethylene glycol-dextran did not alter the density profile of the DNA. Taken together, the results indicate that the presence of the satellite DNA is unlikely to be due to a
Fig. 12 Partition Coefficients of Native and Denatured DNA as a Function of Ionic Environment.

The phase system consisted of 4% (w/w) polyethylene glycol and 5% (w/w) dextran at 4°C.

--- native DNA; -- thermal denatured DNA.
Fig. 13  **Partition Coefficient of Native DNA as a Function of DNA Concentration.**

The phase system consisted of 4% (w/w) polyethylene glycol and 5% (w/w) dextran in 3mM Na$_2$HPO$_4$ and 7mM NaH$_2$PO$_4$, at 4°C.
random extraction artefact.
IV. ON THE PHYSICAL NATURE OF THE SATELLITE DNA

The next aim of the present study is to physically characterize the satellite DNA, and to establish whether it originates from the bacterial chromosome during extraction, as a result of pre-existing "weak linkages" in the chromosome, or whether it represents DNA element(s) having its own physical integrity. It was hoped that the satellite might consist of a number of small identical molecules of molecular weights sufficiently different from that of the main band, such that they might be detected as a separate peak on a sucrose density gradient.

(a) Fractionation of DNA by Sucrose Density Gradient

Preparation of DNA. DNA was isolated by phenol extraction, under conditions described earlier.

Preparation of Sucrose Gradient. Linear gradients of 35 ml 5-20% (w/w) sucrose in 0.015M NaCl-0.0015M NaCitrate, pH 7 buffer were prepared by means of a Buchler gradient former at speed 5.5, low voltage of peristaltic pump action. 1.5 ml of DNA solution containing 8 O.D. was usually layered on top of the gradient by means of a pipette.

Linearity of the preformed gradient was checked by preparing a 5 ml gradient of 5-20% (w/w) sucrose under similar conditions. After 2.5 hours of centrifugation at 32,500
r.p.m. at 4°C in a Spinco SW 50L rotor (about the same g value as that at 27,000 r.p.m. in a SW 27 rotor), 5 drop-fractions (about 0.08 ml) were collected from the bottom of the centrifuge tube through a 23-gauge needle. The refractive index of every fifth fraction was read in a Bausch & Lomb refractometer (figure 14).

Sucrose gradient sedimentation. Sedimentation was performed in a Spinco SW 27 rotor from 7 to 14 hours at 27,000 r.p.m. at 4°C. 1-ml fractions were collected by puncturing the bottom of the centrifuge tube, and the absorbance at 260 μm was read in a Unicam SP 800A spectrophotometer. Fractions under each of the two peaks were pooled separately and dialysed overnight against 0.015M NaCl-NaCitrate pH 7 buffer to remove the sucrose for subsequent cesium chloride centrifugation.

Estimation of molecular weights from sedimentation values. The relationship given by Studier (1965) for native DNA was used:

\[ S_{20,w}^0 = 0.0882 M^{0.346} \]

where \( S_{20,w}^0 \) is the sedimentation coefficient of a given particle in a solvent with the density and viscosity of water at 20°C, and M is the molecular weight of the particle.

Results

Figure 15 shows the sedimentation profile of \( H. \) salinarium DNA after 8 hours of sedimentation on a sucrose
Figure 14. Calibration of the Linearity of Preformed Sucrose Gradients

5 ml of a 5-20% (w/w) sucrose in 0.015M NaCl-0.0015M NaCitrate, pH 7 buffer was prepared by mixing 2.5 ml of a 5% sucrose solution with an equal volume of a 20% solution by a Buchler gradient former at speed 5.5, low voltage of peristaltic pump action. After 2.5 hours of centrifugation at 32,000 r.p.m. in a SW 50L rotor, 5 drop-fractions were collected from the bottom of the tube through a 23-gauge needle. Refractive index of every fifth fraction was read in a Bausch & Lomb refractometer.
gradient. The profile consists of a broad peak, followed by a slower sedimenting peak near the top of the gradient. The latter peak varied in proportion, depending on the preparation. The faster sedimenting peak has a sedimentation value of 30S, and the slower sedimenting peak of 2S, as estimated from the position of reference *E. coli* ribosomes centrifuged in a separate tube. These values correspond to molecular weights of $2 \times 10^7$ and $9 \times 10^3$ daltons, respectively, as calculated from the relationship of Studier (1965). These values may not be absolute, as Smith, Schaller & Bonhoeffer (1970) calculated the molecular weight of a 30S particle to be $3 \times 10^7$ daltons by the relationship of Burgi & Hershey (1963).

Longer periods of sedimentation only caused the 30S peak to spread out more diffusely without revealing individual components within this peak. Samples pooled from this peak showed the normal proportion of the satellite upon isopycnic centrifugation in a cesium chloride gradient (figure 16a). Samples from the 2S peak, however, did not give a peak within the density range of the gradient (figure 16b), presumably because it represented low molecular weight nucleotide fragments generated during phenol extraction. A 1:1 O.D. mixture of the 30S and 2S peaks again gave only the picture of the 30S peak (figure 16c).

Sedimentation in an alkali gradient in the presence of 0.1N NaOH did not alter the overall profile of the peaks,
The gradient consisted of 8 O.D. of DNA layered on top of a linear 5-20% (w/w) sucrose in 0.015M NaCl-0.0015M NaCitrate, pH 7. Sedimentation in a Spinco SW 27 rotor was performed for 8 hours at 27,000 r.p.m. at 4°C. 1-ml fractions were collected from the bottom of the tube through a 20-gauge needle.
Figure 16. Density Profile of 30S and 28 DNA Peaks from Sucrose Gradients.

Three gradients, each consisting of 2.5 ml of 49% (w/w) cesium chloride in 0.02M Tris, pH 8 buffer layered over an equal volume of 64% (w/w) cesium chloride in the buffer, containing, respectively:

(a) 0.13 O.D. 30S DNA + 0.1 O.D. salmon sperm DNA;
(b) 0.13 O.D. 2S DNA + 0.1 O.D. salmon sperm DNA;
(c) 0.13 O.D. 30S DNA + 0.13 O.D. 2S DNA

Centrifugation was performed in a SW 50.1 rotor at 42,000 r.p.m. for 18 hours at 25°C, and then at 30,000 r.p.m. for 27 hours. The density profiles shown were reproduced from an ISCO ultraviolet tracing at a scan speed of 0.25 ml/minute. Buoyant densities of the halophilic DNA peaks were approximated by their positions from salmon sperm DNA as described.
even though the peaks sedimented at slower rates. The latter observation was in agreement with Studier's (1965) finding that at low ionic strengths, alkali denatured DNA has a more extended conformation than native DNA, hence a lower S value.

These sucrose gradient sedimentation experiments were not successful in separating the satellite from the bulk DNA. The results from figure 16 clearly shows that the satellite component sedimented together with the rest of the 30S component. The latter peak is skewed toward the denser side of the sucrose gradient, indicating a heterogenous distribution in the molecular weight in the preparation. Nonetheless, as a distinct peak corresponding to an 18-20% satellite is not observed, one must conclude that the molecular weights of both the satellite and the main band in the DNA preparation were sufficiently similar so that attempts to separate the two species of DNA on the basis of size differences is impractical. In other words, the results indicate that, whatever the in vivo molecular weight of the satellite might have been, the intrinsic heterogeneity in the molecular weights of a purified, and therefore inevitably sheared DNA preparation would have masked the presence of a discrete satellite peak in a sucrose density gradient.

Since reducing the molecular weight of bulk DNA to $2-3 \times 10^7$ daltons did not allow separation of the satellite
from the main band, this must mean that the minimum size of the satellite is at about the same value. Knowing the genome size of the halophilic bacteria \((2.7 \times 10^9\) daltons, Moore & McCarthy, 1969b), the maximum number of copies of the satellite must not be more than 18 to 27 per genome, depending on whether \(2-\) or \(3 \times 10^7\) daltons is chosen to represent the molecular weight of a 30S particle. The true figure probably will be less, since these estimates were based on the minimum size found in a sheared DNA preparation.

(b) Fractionation by Ethidium Bromide-Isopycnic Centrifugation

In order to further characterize the satellite DNA, it was necessary to isolate the component in its intact physical form, hopefully in the form of a closed circular duplex. A convenient method of detecting closed circular duplexes is to subject a DNA preparation suspected of containing such duplexes to isopycnic centrifugation in the presence of ethidium bromide. The interaction of the phenanthridine dye with DNA has been studied extensively (Le Pecq, 1965; Waring, 1965), and it has been known to bind between DNA base pairs by intercalation, a term introduced by Lerman (1961). On the basis of model building studies, Fuller & Waring (1964) concluded that the DNA helix unwinds by \(12^\circ\) for every molecule of dye bound.

Vinograd, Lebowitz, Radloff, Watson & Laipis (1965)
proposed that closed circular duplex DNA's contain superhelical turns which are maintained by chemical forces in the molecule, although the number of these superhelical turns has subsequently been shown to vary inversely with the ionic strength (Bode & MacHattie, 1968). As these superhelical turns introduce mechanical torsional stress into the molecule, three events occur when such a molecule is treated with ethidium bromide, depending on the dye concentration (figure 17, modified after Crawford & Waring, 1967):

(a) at low dye concentrations (less than 5.4 μg/ml), a superhelix readily unwinds upon intercalation;

(b) at a critical dye concentration, the superhelix completely unwinds, so that the molecule now assumes a circular but non-superhelical conformation;

(c) at high dye concentrations (greater than 100 μg/ml), further unwinding of the duplex introduces left-handed twists instead, so that it now possesses superhelical turns in the opposite chemical sense.

The creation of these new superhelical twists, however, re-introduces mechanical torsional stress, as well as a more ordered conformation, into the DNA, relative to event (b). These two effects result in an increase in the free energy of formation of the DNA-dye complex, and is energetically unfavourable. Hence, at high dye concentrations, dye absorption by a superhelical DNA is restricted. On the other hand, there is less restriction to the absorption of
The Watson-Crick double helix is represented as a single continuous line.

(a) represents an intact superhelix with an arbitrarily assigned number of superhelical turns;

(b) the number of superhelical turns decreases upon random dye intercalation. The latter is represented by bars perpendicular to the helix axis;

(c) at a critical point, the superhelix completely unwinds;

(d) and (e) represent formation of a left-handed helix due to further intercalation of the dye at saturating concentrations.
(a) → (b) → (c) → (d) → (e)
dye by a linear molecule, since the latter is free to rotate upon intercalation. As the absorption of the dye lowers the buoyant density of DNA, linear molecules become lighter in density than superhelical DNA. The density difference between a superhelical DNA and its nicked or linear derivative has been found to be 0.035 to 0.036 g/ml for polyoma DNA, at 100 μg/ml dye concentration (Radloff, Bauer & Vinograd, 1967).
(b.1) **Effect of Adenine and Deoxyadenosine on the Incorporation of Thymidine into DNA**

As ethidium bromide absorbs strongly in the ultraviolet region, it was necessary to use radioactively labelled DNA for preparative dye-isopycnic experiments so that the distribution of DNA peaks would not be masked by the dye concentration gradient. For this reason, thymidine-methyl-\(^3\)H has been used to label _H. salinarium_ DNA, since the uptake of thymidine has been found to be negligible in strains of _E. coli_ and _B. subtilis_ (Crawford, 1958; Rachmeler, Gerhart & Rosner, 1961). Even so, the uptake of thymidine is restricted as cultures of these organisms have been found to convert it rapidly to thymine, presumably via an inducible phosphorylase enzyme (Rachmeler, Gerhart & Rosner, 1961; Bodmer & Schildkraut, 1964).

According to Boyce & Setlow (1962), addition of deoxyadenosine at a concentration of 250 \(\mu g/ml\) to the growth medium of prototrophic strains of _E. coli_ enhances the uptake of exogenous thymidine by a factor of three over that of the control. The effect of this deoxynucleotide on the incorporation of \(^3\)H-thymidine into DNA of _H. salinarium_ was compared with that of adenine which is one of the ingredients in the synthetic medium (Table 3).

**Growth media** Halophiles were grown in the synthetic medium of Onishi, McCance & Gibbons (1964), containing either 100 \(\mu g/ml\) adenine or 250 \(\mu g/ml\) deoxyadenosine.
Growth conditions. Precultures for inoculating purposes were grown in 15 ml complex medium for 24 hours with two consecutive subculturings so as to obtain log phase cells. The cells were washed with sterile 25% NaCl wash medium, and resuspended in the same wash medium to a turbidity of 0.32 unit at 660 μm in a Bausch & Lomb SP 20 spectrophotometer. Aliquots of this cell suspension (0.75 ml) were added to 15 ml synthetic medium in 250 ml-capacity side arm flasks. The cultures were shaken at 37°C on a New Brunswick Scientific gyrotory shaker at minimum speed of rotation. Thymidine-methyl-³H (100 uC) was added to 15 ml of culture in early log phase of growth. A growth period of 21.5 hours was given so that a substantial amount of thymidine could be incorporated. As the generation time of H. salinarium is 7.5 hours in the synthetic medium, this corresponds to nearly three generations of growth.

Assay of incorporation of ³H-thymidine into DNA.
The incorporation of label into DNA was determined by extracting 0.1 ml of the culture with 0.9 ml 10% cold TCA, followed by filtration through Millipore filter (0.45 μm size). The level of input radioactivity was determined by diluting 0.1 ml of the culture with 0.9 ml water and directly applying 0.01 ml of the dilution onto Millipore filter in order to reduce quenching due to the presence of salt crystals from the growth medium on the filter. The filters were dried for twenty minutes in an oven at 70°C,
Table 3. Composition of Synthetic Medium for the Growth of Halophilic Bacteria (after Onishi, McCance & Gibbons, 1965).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>gram/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casamino acids'</td>
<td>7.5</td>
</tr>
<tr>
<td>Adenine&quot;</td>
<td>0.1</td>
</tr>
<tr>
<td>Uridylic acid</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>250</td>
</tr>
<tr>
<td>Magnesium sulphate.7H₂O</td>
<td>20</td>
</tr>
</tbody>
</table>

Stock solution of mineral salts containing, in mg per 100 ml,

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>100 ml/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>10</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>5</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>5</td>
</tr>
<tr>
<td>NaCitrate</td>
<td>50</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>0.03</td>
</tr>
<tr>
<td>CaCl₂.7H₂O</td>
<td>0.7</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>0.044</td>
</tr>
<tr>
<td>FeCl₂</td>
<td>0.23</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.005</td>
</tr>
</tbody>
</table>

The pH was adjusted to 6.2 with KOH before making up to volume. The medium was autoclaved at 1.2 atmospheres for at least twenty minutes.

' Replacing fifteen amino acids in the original medium

" Replacing adenylic acid in the original medium
and counted for ten minutes in a Beckman LS-250 scintillation counter.

Results

As shown in Table 4, deoxyadenosine not only did not give three-fold enhancement in the incorporation of thymidine, but consistently gave poorer incorporation than adenine.

In view of the fact that deoxyadenosine has no adverse effect on bacterial growth rate, it is difficult to say at this point whether or not the discrepancy was due to differences in the membrane permeability of the halophilic bacterium to the nucleoside, or whether different enzymes were induced in response to the substance. The level of thymidine uptake by the halophile (11-20%), nonetheless, compares favourably with that normally obtained for E. coli and B. subtilis (10-12%, Mahler, 1967).

As adenine was observed to give better incorporation of thymidine into DNA, growth medium containing 100 ug/ml of the pyrimidine was used to prepare tritium-labelled DNA for ethidium bromide-isopycnic centrifugation experiments for the detection of closed circular duplexes.
Table 4. Effect of Adenine and Deoxyadenosine on the Incorporation of Thymidine-methyl-³H into H. salinarium DNA

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Adenine (a)'</th>
<th>Deoxyadenosine (b)&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm present per 0.801 ml culture</td>
<td>cpm precipitated by TCA from 0.1 ml culture</td>
</tr>
<tr>
<td>I</td>
<td>802</td>
<td>14161</td>
</tr>
<tr>
<td>II</td>
<td>770</td>
<td>16612</td>
</tr>
<tr>
<td>III</td>
<td>583</td>
<td>10313</td>
</tr>
</tbody>
</table>

' Normally present in the synthetic medium at a concentration of 100 ug/ml.

" Replacing 100 ug/ml adenine, at a concentration of 250 ug/ml.
(b.2) **Ethidium Bromide-Isopycnic Centrifugation**

**Preparation of lysate.** Cells were labelled, harvested, and washed twice with 25% NaCl wash medium as described in previous sections. The method for preparing the sarkosyl lysate was adapted from that given by Bazaral & Helinski (1968) for *E. coli*. The pellet, containing about 60 mg of cells by wet weight, was resuspended in 1 ml of an hypotonic buffer containing 0.05M NaCl-0.5M Tris-0.005M EDTA, pH 8, and 500 ug/ml pancreatic RNase which had been heated for 10 minutes at 80°C in a water bath to destroy DNase activity. Lysis was instantaneous. 0.5 ml of a 2% sarkosyl solution in water was then added, and the mixture drawn in and out of a Pasteur pipette ten times to selectively shear the main chromosomal component. The assumption underlying this operation was that bacterial chromosome, having a molecular weight of about $10^9$ daltons (Moore & McCarthy, 1969b) would be sheared into smaller linear pieces. Closed circular DNA of smaller molecular weight, under identical conditions, would be less susceptible to shear damage. Upon isopycnic centrifugation in the presence of ethidium bromide, these linear pieces would absorb more dye and band in the light density side of the gradient; whereas closed circular DNA, on the other hand, would absorb less dye and band at a higher density, and could therefore be isolated. As we did not know the size of the satellite, we have chosen to pipette the lysate ten
times, instead of twenty times as Bazaral & Helinski (1968) have done, in order to avoid excessive shearing of the DNA. Finally, another ml of the RNase-buffer was added, and the mixture again drawn in and out of a Pasteur pipette three more times.

**Ethidium bromide-isopycnic centrifugation.** The sheared lysate (2 ml) was added to a solution of cesium chloride containing 175 ug/ml ethidium bromide in 0.1M sodium phosphate buffer, pH 7 in amounts described by Bazaral & Helinski (1968).

Centrifugation was performed in a Spinco 65 fixed-angle rotor for 44 hours at 44,000 r.p.m. at 20°C. Fractions of either 29 or 19 drops were collected from the bottom of the centrifuge tube through a 20 gauge needle. Each fraction was precipitated with 2 ml of 10% cold TCA in the presence of 0.1 ml bovine serum albumin as carrier, centrifuged for 5 minutes at low speed in an International centrifuge to collect the precipitate, and filtered through a Millipore filter (0.45 u pore size). Each filter was then washed with 5 ml portions of fresh TCA and dried in an oven at 70°C for fifteen minutes. Radioactivity of the samples was counted, 5 minutes each, in a Beckman LS-250 scintillation counter.

Refractive indices of the even numbered fractions of the first twelve fractions were read by means of a Bausch & Lomb refractometer. This was meant to avoid losing the
DNA peaks on the stage of the refractometer, as well as unnecessary contamination of the latter. Buoyant densities of the peaks were extrapolated from their refractive indices as described.

Cesium chloride-isopycnic centrifugation. In order to equate the dense, minor peak in ethidium bromide gradients with the light density satellite, conventional cesium chloride gradients were prepared by mixing 0.45 ml of the lysate with a solution composed of 11.44 g cesium chloride in 8.11 ml 0.1xSSC buffer, so that the final density of the mixture was 1.72 g/ml. Centrifugation was occasionally performed concurrently with gradients containing ethidium bromide under conditions just described. Fractions of 19 drops were collected, and assayed for radioactivity as described.

Results

A minor DNA band with a density higher than that of the main band was observed, although its relative proportion to the main band varied from a maximum of 17% to a minimum of 2-3%, depending on the particular lysate preparation. Figure 23 shows a dye-buoyant density profile of a DNA preparation containing an 8% dense, minor band.

Table 5 lists the density differences between the minor and the main band, and the relative proportion of the minor band to total. The average density difference between the two DNA bands was 0.026-0.027 g/ml. In the light of the
Figure 18. Ethidium Bromide-Isopycnic Centrifugation of Sarkosyl Lysate of *H. salinarium*

2 ml. of sarkosyl lysate was added directly to a solution of cesium chloride containing 175 ug/ml. ethidium bromide. Centrifugation was performed in a Spinco 65 fixed-angle rotor at 44,000 r.p.m. for 44 hours at 20°C. 0.38 ml. fractions were collected by puncturing the bottom of the centrifuge tube with a 20-gauge needle. Radioactivity was assayed by TCA precipitation. Buoyant density of the peaks were extrapolated from their refractive indices, using the relationship of Ifft, Voet & Vinograd (1961).

The dense, minor peak is 8% of the total.
reported value of 0.035-0.036 g/ml density difference between a closed circular DNA and its nicked derivative (Radloff, Bauer & Vinograd, 1967), it is significant to note that, if correction be made for the intrinsic density difference of 0.009 g/ml between the satellite and the main band, the theoretical value comes out to be (0.035/0.036-0.009) g/ml, or 0.026 to 0.027 g/ml, which is in agreement with the experimental values.

Again, the magnitude of the density difference between the two DNA bands suggests that the minor band did not arise from denatured DNA, as the latter is normally only 0.015 g/ml more dense than native DNA in a cesium chloride density gradient (Sueoka, Marmur & Doty, 1959).

As can be seen in Table 5, in three out of thirteen gradients, the main DNA was split into two peaks. The density differences between the two were 0.014, 0.015, and 0.021 g/ml, respectively (lines 1-3, Table 5). In the rest of the gradients, however, the main band was seen as one peak, even when the resolution was improved by collecting smaller fractions. Judging from their large amounts, these peaks were unlikely to have derived from the satellite component, and could have derived from insufficiently sheared fragments of different overall base composition. Although Bazaral & Helinski (1968) also observed similar variation in the number of peaks within the main band, the explanation for this phenomenon remains uncertain at present.
Table 5. Buoyant Density Differences Between the Dense, Minor and Major Peaks

<table>
<thead>
<tr>
<th>Dye-CsCl Gradient</th>
<th>Density Difference between Major and Minor Peaks (g/ml)</th>
<th>% Major Peak</th>
<th>% Minor Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.030</td>
<td>(58) 95</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>(37)</td>
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Values within brackets represent those of split major peaks.
The significant feature of these centrifugation experiments is that they strongly indicate the presence of an ethidium bromide-resistant closed circular DNA which may be equated with the light satellite. A possible explanation for the low proportion of the dense, minor peak, relative to the main band (Table 5) may be that a significant proportion of otherwise intact closed circles is broken during shearing of the sarkosyl lysate. Still, more direct evidence, such as that from electron microscopy, is called for.
(b.3) **Electron Microscopy of DNA**

Electron microscopic examination of DNA from the halophilic bacterium would reveal the size and form of the satellite, and would hopefully answer the following questions:

1. Whether the dense minor band in ethidium bromide was indeed composed of closed circular DNA, and not artefacts.

2. Whether the dense minor band could indeed be equated with the light satellite observed in conventional cesium chloride gradients. One needed to compare the sizes of the DNA isolated from the respective bands; and

3. Whether the satellite exists as multiple sizes of DNA having different molecular weights, or whether it exists as one discrete class of molecules. If the latter is true, one might then calculate the number of copies of the satellite in the cell. The answer to both questions must come from a statistical measurement of the lengths of these molecules.

The principle of the Kleinschmidt monolayer technique (Kleinschmidt & Zahn, 1959; Kleinschmidt, 1967) is to spread DNA in as nearly a two-dimensional form as possible over an aqueous solution (such as ammonium acetate buffer) with the help of a globular protein having sufficient surface-spreading activity. To do this, DNA is mixed, in solution, with a basic protein (such as cytochrome C) so that binding
of the DNA to protein occurs. The protein-DNA mixture is floated onto the ammonium acetate hypophase in a flat trough. Since the protein is surface-denatured, it forms a monolayer film, with the DNA absorbed to it by basic side groups of amino acid residues. The protein-DNA film is then transferred to a solid support (such as a film of parlodion over a copper grid) and dried. The preparation may be shadowed to enhance contrast, and is ready for electron microscopic examination.

Procedure

Preparation of DNA. DNA samples were pooled from the appropriate regions of the peaks in both the ethidium bromide-cesium chloride gradients (figure 19a) and conventional cesium chloride gradients (figure 19b), as indicated by the shadowed areas. The pooled fractions, about 0.3 ml in volume, were dialysed overnight against SSC buffer to remove ethidium bromide and cesium chloride for subsequent electron microscopy. The concentrations of the dialyzates were estimated by their absorbances at 260 μm, using a 0.3 ml-capacity microcell with 1-cm pathlength. The dialyzates were used for electron microscopy without delay.

Spreading of DNA-cytochrome film. 0.2 ug DNA in a volume of about 0.02 ml was mixed with 40 ug cytochrome C (1 mg/ml in 4M ammonium acetate buffer, pH 7) to a weight ratio of 1 part DNA to 200 parts cytochrome C. The droplet was floated via a glass slide onto a solution containing
0.5 ammonium acetate buffer, pH 7, filled to just overflowing in a 9.5 x 9.5 cm plastic trough. After allowing ten minutes for stabilization, a small area of the film was picked up by briefly touching a parlodion-carbon coated copper grid (200 or 300 mesh) horizontally to the surface of the film. The grid was then dried in 95% ethanol for 20 seconds.

**Shadowing.** The specimens were rotary shadowed at a rate of 60 r.p.m. at an angle of 60° from an evaporating platinum-carbon pellet weighing about 12 mg. The base plate containing the specimen was placed about 3 cm away and 0.3 cm above the pellet. Shadowing was performed under a vacuum of 10⁻⁵ torr, in an Edwards Model 4 vacuum unit.

**Electron microscopy.** The shadowed specimens were photographed in a Carl Zeiss EM 9 electron microscope, at magnifications of 5,600, 10,000 and 17,000 x.

**Measurement of contour length** of the DNA was done using a Keuffel & Esser number 11 map measurer. Photographs, printed at 3x magnification, were projected 4.3 times on a screen by an American Optical Delineascope so that measurement could be made with less than 10% deviation.

**Calculation of molecular weight** was as follows:

\[
\text{Molecular weight} = \frac{\text{Measured Length}(\text{nm})}{3.4(A)} \times \frac{1}{\text{Total magnification}} \times 553
\]

where 3.4 A is the internucleotide distance of double helical
Figure 19. Isolation of Sarkosyl-DNA from Ethidium Bromide-Cesium Chloride Density Gradient

(a) 2 ml sarkosyl lysate was added to a solution of cesium chloride containing 175 ug/ml ethidium bromide in 0.1M sodium phosphate buffer, pH 7, to a final density of 1.60 g/ml. Centrifugation was performed in a Spinco 65 fixed angle rotor at 44,000 r.p.m. for 44 hours, 20°C. 29 drop-fractions were collected from the bottom of the centrifuge tube, precipitated with TCA, and assayed for radioactivity. Buoyant densities of the DNA peaks were extrapolated from their refractive indices.

Figure shows the density profile of the DNA containing a 9% dense, minor band.

(b) 0.45 ml sarkosyl lysate was added to a solution of cesium chloride in 0.1 x SSC buffer, pH 7, to a final density of 1.72 g/ml. Centrifugation was performed concurrently with gradient (a). Figure shows a 20% light satellite DNA.

Fractions under shaded areas were pooled separately and dialyzed overnight against SSC buffer to remove cesium chloride and ethidium bromide.
DNA in the B configuration, and 653 the average molecular weight of a deoxynucleotide base pair.

Results

When examined under the electron microscope, the dense, minor DNA band isolated from ethidium bromide-cesium chloride gradients consisted mainly of closed circular molecules, thus confirming the physicochemical data from ethidium bromide gradients. The length distribution of forty molecules is given in figure 20(a), thirty-one (78%) of which were seen as closed circles. As estimated from the histogram, the average length of the molecules is about 37.5 \( u \). The statistical average of thirty-four molecules within the range of 25 to 55 \( u \) is calculated to be 37\( ^+ \)7\( u \).

A representative molecule with a length of 37.8 \( u \) is shown in figure 21. An example of one of two larger molecules, with a length of 52 \( u \), is shown in figure 22. At the other extreme, 15% of the total were considerably smaller in size, with a length of 14\( ^+ \)3 \( u \) (figure 23).

Main band DNA from the same ethidium bromide gradients, in contrast, appeared as a mixture of long, extended and randomly oriented fibers. No circular forms could be distinguished (figure 24). The lengths of these fibers were not measured.

Closed circular DNA was again revealed in fractions isolated from the satellite band in conventional cesium chloride gradients. The length distribution of thirty-
Figure 20. **Length Distribution of DNA Isolated from Ethidium Bromide-Cesium Chloride and from Cesium Chloride Gradients**

(a) represents the length distribution of DNA isolated from the dense, minor band in figure 19(a). The histogram is based on measurement of forty molecules, thirty-one (78%) of which were closed circles.

(b) represents that of DNA isolated from the light satellite band in figure 19(b). The histogram is based on measurement of thirty-three molecules, eleven (33%) of which were closed circles.

Shaded areas represent closed circular molecules.
Figure 21. Electron Micrograph of DNA from the Dense, Minor Band in Ethidium Bromide-Cesium Chloride Gradients

DNA was prepared for electron microscopy by the Kleinschmidt technique. The specimen was rotary shadowed at an angle of 6° by a platinum-carbon pellet. The length of the molecule is 37.8 u. Magnification 16,800 x.
Figure 22. Electron Micrograph of DNA from the Dense, Minor Band in Ethidium Bromide-Cesium Chloride Gradients

This is one of two larger molecules, having a length of about 52 u. Magnification 30,000 x.
Figure 23. Electron Micrograph of DNA from the Dense, Minor Band in Ethidium Bromide-Cesium Chloride Gradients

The molecule is 13.8 u long. Magnification 30,000 x.
Figure 24. Electron Micrograph of DNA from the Main Band in Ethidium Bromide-Cesium Chloride Gradients
Magnification 16,800 x.
three molecules, eleven of which were closed circles, is given in figure 20(b). From the histogram, the average length of ten closed circles is seen to be about 37.5 u. The statistical average of the latter molecules is calculated to be $37 \pm 3\text{u.}$ The close similarity in the length distribution of these closed molecules with those isolated from ethidium bromide gradients strongly suggests that the latter molecules may indeed be equated with the satellite.

Figure 25 shows a closed circle with a length of 37.5 u. A third of the linear molecules, on the other hand, were only about half as long as the closed circles, suggesting that these represented broken halves of intact circles. At the other extreme, one circular molecule was found to be 63 u long, and is shown in figure 26.

Fractions isolated from the main band in conventional cesium chloride gradients, in contrast, did not contain circular molecules. The general appearance of the DNA fibers was similar to those obtained for the DNA from the main band in ethidium bromide gradients, and is shown in figure 27.

Again, an additional line of evidence may be taken to indicate that the satellite is physically identical to the dense, minor band in ethidium bromide gradients. During lysate preparation, some damage to the physical integrity of the satellite might have occurred, so that only a third of the molecules remained closed circles in one test,
preparation (figure 20b). Therefore only a third of the 20% satellite, or 7% of the total DNA, ought to be restrictive to dye absorption. This has indeed been observed. The same lysate preparation gave rise to dense, minor bands which averaged to 7% of the total in ethidium bromide-cesium chloride gradients (Table 5, gradients 12 and 13; figure 19a).
V. Discussion

The foregoing results suggest that the presence of two species of DNA in *H. salinarium* is not due to random extraction artefacts, as the relative amount of these two bands is seen to be constant, regardless of the extraction conditions. The differences in the buoyant densities of these two bands are also consistent within experimental error. The presence of the satellite in its normal proportion after phenol extraction clearly indicates that the peculiar phenomenon in which the presence of the crab d(A-T)-rich satellite is observed only upon chloroform extraction, but not phenol, does not apply to the halophilic bacteria. Results from polyethylene glycol-dextran extractions suggest that both DNA species in the halophilic bacteria represent native forms of the molecule, as denatured DNA has been demonstrated to partition very differently from native forms in such a system. The constancy in the density and proportion of the satellite from preparation to preparation also tends to exclude the possibility that
Figure 25. Electron Micrograph of DNA from the Satellite Band in Cesium Chloride Gradients

The photograph shows a linear and a closed circular molecule. The latter is 37.5 u long. Magnification 16,800 x.
Figure 26. Electron Micrograph of Larger Circular DNA from the Satellite Band in Cesium Chloride Gradients.

This molecule is 63 u long. Magnification 16,800 x.
Figure 27. Electron Micrograph of DNA from the Main Band in Cesium Chloride Gradients

Magnification 16,800 x.
it represents glycogen material which may be mistaken for DNA in cesium chloride gradients, since the constancy of the latter substance depends critically on the preparatory condition (Brunk & Hanawalt, 1966). A stronger piece of evidence against this possibility comes from the observation that the satellite incorporates radioactive thymidine just as well as the main band.

The results from sedimentation in sucrose gradients indicate the minimum size of the satellite to be about 2-3 x 10^7 daltons, as it sedimented together with main band DNA sheared to similar sizes. Further characterization of the satellite in ethidium bromide-cesium chloride gradients suggests that it occurs as closed circles, which is an indication of its physical autonomy from the bacterial chromosome. Evidence that the dense, minor band in ethidium bromide gradients does indeed represent a dye-resistant closed circular DNA is provided by electron microscopic examination of these molecules. The latter examination also provides further evidence to equate these circular duplexes with the light satellite, in that the length distributions of the molecule from these two sources show close similarity to each other, with the peaks of the histograms coinciding at 37.5 u (figure 20). Two out of forty molecules in figure 20(a) appeared rather entangled (figure 22), which might have contained more than one molecule in each unit. This would account for their higher-than-average values. For example,
the complex illustrated in figure 22 might contain a linear piece of DNA together with a closed circle, but since its physical continuity could not be convincingly established, this complex has been plotted as a linear molecule in the histogram.

In view of the few smaller circles seen in figure 20(a), and one large circle from the satellite (figure 26), it is unclear whether there are multiple forms of these circular molecules, or whether these molecules are artefacts. A possible explanation for the origin of these smaller molecules is that they represent fragments derived from larger pieces of DNA which have managed to form artificial circles. Whatever the origin of these circles may have been, they are not seen in figure 20(b). If these circles turn out to be genuine, they may perhaps represent yet an unidentified class of circular DNA which may not be related to the satellite per se, but is nevertheless banded together with closed circular satellite in ethidium bromide-cesium chloride gradients.

The 65 u long circular molecule shown in figure 26 is rather puzzling. The size of this molecule suggests that it might be a dimer of two smaller molecules, although the possible points of overlap of the smaller molecules are not readily discernable from the electron micrograph. More determinations on the lengths of these molecules undoubtedly will help decide whether they are mono- or poly-disperse,
although present statistics seem to indicate that, with the exception of the one molecule, most of the closed circles isolated directly from the satellite have lengths about 37 u. If later statistics do indeed bear out a unimodal distribution for the size of the satellite DNA, a molecule of 37 u long is calculated to weigh $7.1 \times 10^7$ daltons, and is in the right order of magnitude as predicted from sucrose gradient sedimentation studies. The size of the satellite DNA may be compared to that of the bacterial genome which has been estimated to be $2.68 \times 10^9$ daltons (Moore & McCarthy, 1969b). This means that the majority of the satellite molecules are about 3/100 the size of the bacterial chromosome, so that there must be eight copies of the satellite DNA per genome to account for 20% of the total DNA. The question remains open as to whether all eight copies are identical, or whether they all have different nucleotide sequences.

Our finding that the satellite DNA from _H. salinarium_ exists as closed circular duplexes, with the majority of the molecules having a molecular weight close to $7.1 \times 10^7$ daltons such that there may be eight copies of the satellite per bacterial genome appears to contradict Moore & McCarthy's (1969b) interpretation that it is not an episome of a size about $10^7$ daltons, and that it is not present in multiple copies. These authors based their interpretation upon the observation that both the satellite and the main band DNA
renatured at the same rate. It is known, however, that the method they used is not sensitive enough to detect the presence of 2 to 10 copies of part of the genome (Britten & Kohne, 1965-1966), even if the satellite DNA is composed of eight identical molecules of $7.1 \times 10^7$ daltons each. It seems clear that the discrepancy between our finding and theirs is due only to their interpretation of their results, and not to mutually exclusive evidences.

One may also estimate the potential genetic content encoded in each satellite molecule, assuming a monodispersed averaged length of 37 u. An averaged-sized protein molecule contains about 350 amino acid residues (Watson, 1965). A DNA molecule of 37 u long contains $1.1 \times 10^5$ nucleotide pairs. On the basis of the triplet code, each strand of the DNA duplex is sufficient to code for 105 protein molecules. It would be interesting to find out whether the nucleotide sequence(s) in the satellite DNA is repetitious, such that each molecule codes for identical copies of protein molecules. Preliminary hybridization tests reported by Moore & McCarthy (1969b) seem to suggest that the satellite DNA is transcribed. If this is true, it would be interesting to find out the functional significance of the satellite DNA, since it seems to be present in all species of extreme, non-photosynthetic halophiles (Moore & McCarthy, 1969b). Although the genetics of the extreme halophiles has yet to be studied, the circularity of the
satellite DNA nonetheless establishes its physical autonomy from the bacterial chromosome. One may therefore identify the satellite DNA as an extrachromosomal element, in the operational sense that it represents a "stably inherited component of the cell genome when physically separate from the chromosome" (Novick, 1969).


Partition studies on nucleic acids. I. Influence of electrolytes, polymer concentration and nucleic acid conformation on the partition in the dextran-polyethylene glycol system.


136. Rapid equilibrium isopycnic cesium chloride gradients.


properties of F' lac and F DNA.


Replication of chloroplast DNA of tobacco.


Variations in the DNA from two rodent families (Cricetidae and Muridae).


the nature of bonds between deoxyribonucleic acid and protein.


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Steele, W.J. (1968). J. Biol. Chem. 243, 3333. Localization of deoxyribonucleic acid complementary to ribosomal ribonucleic acid and preribosomal ribonucleic acid in
the nucleolus of rat liver.
A morphological study of Halobacterium halobium and its lysis in media of low salt concentration.
II. Dependence of the density of deoxyribonucleic acids on guanine-cytosine content.
Twisted circular form of polyoma viral DNA.
Growth of *H. salinarium* in Complex and Synthetic Media

Radioactive labelling of halophilic DNA could not be performed in the complex medium devised by Sehgal & Gibbons (1960), as the medium contained unknown quantities of thymine derivatives from yeast extract, which would therefore compete with radioactive thymidine for incorporation into DNA. Hence we have found it necessary to establish the growth of *H. salinarium* in a chemically defined medium, such as that described by Onishi, McCance & Gibbons (1964).

We also wanted to study the growth of the halophilic bacteria in the synthetic medium for another reason. As far as we are aware, studies on the nutritional requirements and growth of these halophiles have been based exclusively on turbidity readings (Brown & Gibbons, 1955; Sehgal & Gibbons, 1960; Dundas, Srinivasan & Halvorson, 1963; Onishi, McCance & Gibbons, 1965). Just how well these turbidity readings relate to actual growth has not been described. Even so, the turbidity readings described by Onishi, McCance & Gibbons (1964) have been followed for only one passage after inoculation. The cells used for these experiments typically were grown in a more complete complex medium, harvested, washed free of the medium, and inoculated into respective test media. It has not been clear whether the resultant turbidity curves observed were a genuine reflection of bacterial growth in the test media. For these reasons, we have undertaken a more extensive study of
the relative growth of the halophiles—using in this instance \textit{H. salinarium}—in the complex and synthetic media. We have also compared turbidity readings with results from total and viable cell counts.

**Procedure**

**Growth media.** The composition and preparation of the complex medium is given in Table 1. A defined synthetic medium devised by Onishi, McCance & Gibbons (1964) was adopted, except that adenine was used in place of adenylic acid. The composition of the medium is given in Table 3.

**Growth conditions.** Precultures for inoculating purposes were grown in 15 ml complex medium for 24 hours with two consecutive subculturings so as to obtain log phase cells. The cells were washed with sterile 25% NaCl wash medium, and resuspended in the same wash medium to a turbidity of 0.32 unit at 660 \( \mu \text{m} \) in a Bausch & Lomb SP 20 spectrophotometer. 0.75 ml aliquots of this cell suspension were added to 15 ml complex, or synthetic medium (depending on the nature of the study) in 250 ml-capacity side arm flasks. The cultures were shaken at 37\(^{\circ}\)C on a New Brunswick Scientific gyrotory shaker at minimum speed of rotation.

**Growth Measurements.** Turbidity of cultures was read directly in a Bausch & Lomb SP 20 spectrophotometer against blank medium at 660 \( \mu \text{m} \).

In order to demonstrate that the turbidity curves obtained in the synthetic medium is a genuine reflection of
growth, and not due to nutrients carried over from the complex medium either inter- or intra-cellularly, cells were prepared from 15 ml of complex medium and inoculated into 15 ml synthetic medium under conditions just described. Turbidity readings were taken at various time intervals until the culture reached early stationary phase of growth. 0.75 ml was withdrawn from the culture and transferred into 15 ml fresh synthetic medium. This process of subculturing was repeated at 24-hour intervals to a total of eleven times before turbidity readings were again taken.

Total cell count was obtained by counting the number of cells, under phase contrast microscopy, in a Petroff-Hauser bacterial counter. The latter was calibrated such that twenty unit squares within the chamber corresponded to a volume of 1x10^{-6} ml. At a magnification of 1,300x, *H. salinarium* appeared as slender rods, approximately 0.5 x 2 u in size, and were easy to count. Cells from seven different areas were counted, and the figures averaged for plotting figures 20 and 21.

Viable cell counts were obtained by evenly distributing 0.1 ml aliquots of cell dilutions ranging from 10^{-6} to 10^{-8} x, onto 2% agar plates containing complex medium, six plates for each dilution. The plates were sealed with Saranwrap to keep moist, lest the high-salt medium crystallize. Colonies appeared after one week of storage at 37°C in a humid incubator, and the counts were averaged.
Results

Figure 28 shows the relative turbidity readings of *H. salinarium* in complex and synthetic media, under similar conditions of growth. The slight lag of the halophile upon inoculation in the synthetic medium is taken to represent an adaptation period from an enriched complex medium to a defined synthetic medium possessing all the essential nutrients but nevertheless less rich in growth elements. The generation time of *H. salinarium* under these conditions of growth was 6 hours in the complex medium, and 7.5 hours in the synthetic.

Figure 29 shows that the turbidity curves given by *H. salinarium* is indeed a genuine reflection of its growth characteristic in the synthetic medium, and not due to nutrients carried over from the complex medium which would make the synthetic medium appear more complete than it is. The small lag observed in the first turbidity curve may have been due to an adaptation period in the synthetic medium, following growth in an enriched complex medium. Once the cells commence to grow, however, they do so at a characteristic rate regardless of the number of times of subculturing.

Plots of turbidity, total cell count, and viable cell count for cells grown in complex and synthetic media are given in figures 30 and 31, respectively. It is evident that turbidity readings are not a reliable measurement of cell growth at high densities, since results from total and viable
cell counts indicate greater extent of growth. In fact, a
dilution curve of turbidity against total cell count
indicates that turbidity reading deviates from linearity at
values above 0.5 unit at 660 mu (figure 32). The discrepancy
between values given by total and viable cell counts is not
surprising, as the former method does not distinguish dead
cells from viable ones.

The results indicate that _H. salinarium_ does indeed grow
well in the synthetic medium, even after it has been sub-
cultured many times. The results also indicate that turbidity
reading is inadequate at high densities as a measurement of
growth, and must be supplemented by viable cell count if
more precise growth index is needed.
Fig. 28. Relative Turbidity of *H. salinarium* in Complex and Synthetic Media.

0.75 ml. of cells in log phase of growth was inoculated at a turbidity of 0.32 unit at 660 mu into 15 ml. complex (----) and synthetic media (x--x).
Fig. 29. Turbidity Curve of *H. salinarium* in Synthetic Medium.

0.75 ml cell suspension in 25% NaCl wash medium at 0.32 unit at 660 μm was inoculated into 15 ml synthetic medium (x—x). 0.75 ml of the culture was subcultured into another 15 ml synthetic medium, and the process repeated at 24-hour intervals for eleven cycles before turbidity readings were again taken (—).
Fig. 30 Comparison of Turbidity, Total Cell Count, and Viable Cell Count in Complex Medium.

Cells in log phase of growth were resuspended in NaCl wash medium to a turbidity of 0.32 unit at 660 μm. 5 ml. of the suspension were inoculated into 100 ml. of complex medium.

Growth was measured at various time intervals. Turbidity readings were taken directly in a Bausch & Lomb SP20 spectrophotometer at 660 μm. 0.1 ml. aliquots were withdrawn for Petroff-Hauser total cell count (Δ—Δ) and viable cell count (Δ—Δ).
Fig. 31  Comparison of Turbidity, Total Cell Count, and Viable Cell Count of *H. salinarium* in Synthetic Medium.

Conditions of inoculation and growth were as described in fig. 20.

---, turbidity measurements; △—△, total cell count; △—△, viable cell count.
Fig. 32 Correlation between Turbidity and Total Cell Count at Different Cell Dilutions.

Total Cell Count was taken of a culture of H. salinarium grown to a turbidity of 0.76 unit at 660 mu in synthetic medium. The culture was subsequently diluted with convenient volumes of the same medium and the total cell counts corresponding to each dilution were taken.
TURBIDITY 660

TOTAL CELL COUNT ($10^6$ CELLS/ML)