PART I

STUDIES ON MYCOPLASMA GALLISEPTICUM

PART II

AUTORADIOGRAPHY OF CHROMOSOMAL DNA FIBERS FROM CHINESE HAMSTER CELLS

Thesis by

Arthur D. Riggs

In Partial Fulfillment of the Requirements
For the Degree of
Doctor of Philosophy

California Institute of Technology

Pasadena, California

1966

(Submitted January 21, 1966)

This thesis is dedicated to my parents who, by their excellent example, taught me the value of creative thought and hard work.

ACKNOWLEDGMENTS

I am deeply grateful to Dr. Herschell K. Mitchell for his understanding, patient guidance, and excellent example of both personal and scientific conduct. I would also like to thank the many other people here at Caltech who have influenced my development as a scientist and as a person. I would particularly like to express my thanks for the friendship of my associates on the second floor of Alles and elsewhere at this institute.

I thank Dr. Allen J. Hodge and Dr. Jerome Vinograd for generously allowing me to use their equipment.

My sincere thanks also go to Mr. Joel A. Huberman, without whom the work reported in Part II of this thesis would not have been done. Working with Joel has been a pleasure.

I thank most of all my wife, Jane, and my children, Karen and Lynelle, for making these years of hard work and study actually enjoyable.

The financial support of the National Science Foundation, the United States Public Health Service, and the Arthur McCallum Foundation is gratefully acknowledged.

I am also grateful to Miss Robbi Hunt for making sacrifices to type my thesis.

ABSTRACT

Part I

The cell size, DNA content, and chromosome size and structure of Mycoplasma gallisepticum A5969 was studied. After calibrating a phase microscope with polystyrene latex spheres of known diameter, it was determined that the average cell volume in the living state is only $36 \pm 9 \times 10^{-15}$ cm^3, which is equivalent to the volume of a sphere 0.40 ± 0.04 μ in diameter.

By a DNA determination method based on analysis for thymine, the DNA content of M. gallisepticum was found to be $4.0 \pm 0.6\%$ of the dry weight. The DNA per colony forming unit was found to be 300×10^6 daltons. For these experiments, the cells were harvested by centrifugation. The results of later experiments, in which the cells were directly precipitated from a broth culture by the addition of trichloroacetic acid (TCA), suggested that there had been DNA loss during the centrifugation steps of the earlier experiments and that the DNA per clone forming unit is $1{,}200 \pm 200 \times 10^6$ daltons.

The Cairns autoradiographic technique was used to visualize the chromosome of M. gallisepticum. The DNA autoradiograms seen indicated that its chromosome is 630 ± 150 μ long. This corresponds to $1{,}200 \pm 300 \times 10^6$ daltons of DNA. The DNA autoradiograms often showed "replication forks" and were sometimes circular.

The Kleinschmidt technique was also used to provide evidence on the size and structure of the chromosome of M. gallisepticum. Tangles

of DNA were found that were very similar to those obtained from bacteria. The total length of DNA in the tangles indicated that the chromosome of M. gallisepticum is 500 $\pm$ 200 μ long, a value that supports the estimate obtained by autoradiography.

Part II

Linear DNA autoradiograms were found when the Cairns technique was applied to Chinese hamster cells. At least 6% of these autoradiograms are more than 0.8 mm long - roughly the size of the E. coli chromosome and considerably longer than previously reported DNA fibers from higher organisms. Some rare autoradiograms are as long as 1.6 - 1.8 mm. The implications of these results in terms of models of chromosome structure are discussed.

TABLE OF CONTENTS

Part I

Studies on *Mycoplasma gallisepticum*

ABREVIATIONS

CFU	colony forming units
EDTA	ethylenediaminetetraacetic acid
NADH	reduced diphosphopyridine nucleotide
$O.D._{260}$	optical density at 260 millimicrons
PPLO	pleuropneumonia-like organisms
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid

P A R T I

INTRODUCTION

Brief History of Pleuropneumonia-Like Organisms.

The disease contagious pleuropneumonia of cattle was, and in some parts of the world still is, one of the major cattle diseases, causing enormous economic loss. Louis Pasteur recognized that this disease was caused by an infective agent which was not visible in his microscopes and would not grow in ordinary nutrient broth (1). In 1898 Noccard and Roux (2) succeeded in growing this agent in cell-free media, but it was not until 1910 that Bordet (3) and Borrel et al. (4) described the distinctive pleomorphic morphology of this agent which they called Asterococcus mycoides. It was also shown to pass through filters that retained all known bacteria.

In 1923 Bridre and Donatien (5) showed that contagious agalactia of sheep was caused by an organism with characteristics similar to, but not identical with, those of the "pleuropneumonia organism". In 1934 another organism of this type was isolated from dogs (6). After this slow start, progress was more rapid, and by 1941 it was recognized that these "pleuropneumonia-like organisms" were widely dispersed in nature and had unique properties that distinguished them from bacteria, rickettsia, and viruses (7). It is now known that pleuropneumonia-like organisms, usually abreviated as PPLO, are almost ubiquitous among the domesticated mammals and birds; PPLO have been found in all the common laboratory animals except rabbits and hamsters and man himself can harbor at least six different species (8).

Nomenclature.

The term pleuropneumonia-like organism (PPLO) is still in common use for describing this group of organisms, but it is being replaced by the nomenclature suggested by the classification scheme of Edward and Freundt (9). According to this scheme, the PPLO are classified as follows in the seventh edition of Burgey's Manual (10):

Division I. Protophyta Sachs, 1874, amend. Krassilnikov

Class II. Schizomycetes von Naegeli, 1857

Order X. Mycoplasmateles Freundt, 1955

Family I. Mycoplasmataceae Freundt, 1955

Genus I. Mycoplasma, Nowak, 1929

Throughout the rest of this thesis, the term mycoplasma will be used in place of PPLO.

Many workers in the mycoplasma field would prefer setting up a separate class for the mycoplasma between the Schizomycetes (which include the eubacteria) and the Microtatobiotes (the rickettsia and viruses) (8), whereas some, led by Dienes (11), have suggested that mycoplasms are merely stabilized variant forms of eubacteria.

General Properties of the Mycoplasmas

The following are the main characteristic properties of the microorganisms belonging to the genus Mycoplasma:

1. They grow and multiply in cell-free media. In liquid media they produce only slight turbidity or granularity even at maximum growth (12).

2. They produce distinctive colonies on solid agar medium. These colonies may be as small as 10 to 20 μ in diameter and are usually less than 600 μ. As a rule, the colonies have a dark center and a more transparent peripheral zone; thus they somewhat resemble in appearance a fried egg (7, 12).

3. They lack cell walls (13, 14), and perhaps as a result they are highly pleomorphic. Most broth cultures contain spherical and rod shaped cells of various sizes and, in addition, have filaments, disk-like structures, "ring", club, and star forms (7, 10, 12).

4. They are filterable. All members of this group that have been tested produce small viable cells (usually called elementary bodies or minimal reproductive units) that can pass through filters that retain bacteria. Ultrafiltration studies have shown that these minimal reproductive units range from 125 to 250 mμ (7, 12, 15, 16), and structures of this size have been seen in the electron microscope (12, 13, 17). The minimal reproductive units are usually only a small fraction of the total viable cells, the majority of cells being between 0.3 and 1.0 μ in diameter (11, 19).

5. They have a distinctive mode of reproduction. Most workers are in agreement with Klieneberger-Nobel (12) who believes that the small, filterable minimal reproductive unit grows into a larger cell which, lacking a rigid cell wall, can assume a variety of forms, some of which may be filaments. This larger cell may divide by segmentation, but eventually a differentiation of the cytoplasm occurs and "condensed" areas are produced which then develop into spherical granules

that later become the minimal reproductive units. The "condensed" areas usually occur only at the periphery of the large cells, and the number of granules produced depends on the size of the cell but may be one, two, or more. The granules may either be shed or remain attached to the mother cell, and, if conditions are favorable, they grow again into large cells.

Those workers who do not accept the above mode of reproduction agree with Freundt (10, 18) that the minimal reproductive units give rise to a coenocytic mycelial structure, within which spherical "condensed" elements form. Later, constrictions develop between the spheres and a chain of coccoid elements is formed. These elements are thought to be released by fragmentation of the chain and become the new minimal reproductive units.

The main difference of opinion is over the relative importance of the mycelial structures. There is agreement that the minimal reproductive units are formed within the cytoplasm of the mother cell, and this mode of reproduction may distinguish the mycoplasma from all other microorganisms.

6. They are stable forms showing no history of conversion from, or reversion to, a bacterial parental form. Although there have been several reports suggesting the reversion of mycoplasmas to bacteria (20-23), none is convincing, and there seems to be general agreement that the mycoplasmas are stable. One report (23), in which the reversion of avian mycoplasmas to the bacteria H. gallinarum was demonstrated, can probably be completely discounted because of the recent

results of Rogul et al. (23). Using the DNA-DNA hybridization technique of McCarthy and Bolton (25), it was shown that the extent of hybridization of DNA from the avian mycoplasmas with DNA from H. gallinarum was not above background. This result indicates almost a complete lack of genetic relatedness between the organisms and seems to rule out conversion of one to the other.

L-forms of Bacteria

L-forms are variant forms of bacteria that have lost the ability to produce cell walls (12, 13). The in vitro conversion of bacteria to intermediate, frequently reverting L-forms and then to stable, non-reverting L-forms is now firmly established (8, 12, 26). The conversion can be induced by a variety of treatments, including high salt concentration and penicillin.

Stable L-forms show a remarkable resemblance to the mycoplasmas. Their colonies can be difficult to distinguish from mycoplasma colonies; their cells are highly pleomorphic and small, filterable "reproductive units" are even produced (12). L-forms differ from the mycoplasmas in that: a) They grow to greater turbidity in liquid media and form larger colonies on solid medium (12); b) they are nutritionally less exacting (27); c) the viable, filterable particles of the L-forms are about 300 mμ or larger (12, 28), whereas most mycoplasmas have viable particles as small as 150 mμ; and d) perhaps most importantly, L-forms have a history of conversion from, and reversion to, bacteria.

Although the differences are perhaps sufficient to distinguish the two groups of organisms, the similarities suggest that the mycoplasmas may well have evolved from bacteria.

Nutrition and Metabolism of the Mycoplasmas

The mycoplasmas are nutritionally quite exacting organisms, and most will grow only in complex, chemically undefined bacteriological media (tryptose, heart infusion, etc.) supplemented with serum or a serum fraction. Almost all strains require cholesterol and other lipids for growth (30), and these growth factors are usually supplied by the serum. According to Smith (27), other components of the serum, phospholipids and protein, are necessary only to permit incorporation of the required lipids into the cell and perhaps to act as general detoxifiers.

A completely defined medium has recently been developed by Tourtellotte et al. (31) that supports good growth of M. laidlawii B, a saprophytic mycoplasma not requiring cholesterol for growth. This medium consists of glucose, inorganic salts, twenty amino acids, eight vitamins, oleic acid, thymidine, adenosine, cytidine, guanosine, and one or more peptides obtained from a tryptic digest of RNase. The peptide requirement is nonspecific and two chemically defined hexapeptides could support optimum growth. A similar medium but containing 1% serum albumin had earlier been reported (32) to support good growth of another, perhaps related, saprophytic strain, M. laidlawii A. A partially defined medium based on a complex tissue

culture medium with 21 amino acids and 13 vitamins has recently been reported (33) to support the growth of the avian pathogen M. gallisepticum, strain 293, but no growth was obtained unless serum was added and growth was poor unless peptone was added.

The energy metabolism of the mycoplasmas is of two types -- fermentative and nonfermentative. The nonfermenters oxidize lactate, acetate, and other short chain fatty acids (27, 34), and some strains may also obtain energy from the degradation of arginine to ornithine (35, 36). The fermenters carry on either a homofermentative catabolism, producing lactic acid as an end product, or heterofermentative catabolism, producing lactate, acetic acid and CO_2 as the main end products (30, 37, 38). Many strains apparently lack Krebs cycle enzymes (37) and the cytochrome system (30, 34). Flavoproteins may be the enzymes responsible for electron transfer from NADH to oxygen (34,39). M. laidlawii has some of the enzymes of the hexose monophosphate shunt (38).

The biosynthetic capacities of the mycoplasmas are for the most part unknown, but their complex nutritional requirements suggest a rather limited array compared to most bacteria. Lipid biosynthesis has received some attention (27, 40), and protein synthesis in M. laidlawii is apparently normal, involving activating enzymes, t-RNA, and ribosomes (41).

Chemical Composition and Macromolecular Structure

In overall chemical composition, mycoplasma differ from bacteria only in that they lack cell wall constituents (43) and usually contain considerable amounts of nonsaponifiable lipid (42). Razin et al. (44) have analyzed 8 mycoplasma strains and found that on a dry weight basis they were 55-63% protein, 12-16% lipid, 3-8% carbohydrate, 8-17% RNA, and 4-7% DNA. Another study (46) has given similar results. Lagenfeld et al. (45) compared the total nucleic acid phosphorous content of 13 mycoplasma with that of 9 bacteria and found that on a dry weight basis they were very similar.

The base ratios of the DNA from three mycoplasma species have been determined by chemical means (46, 47, 48) and the G-C contents of eight species have been determined by buoyant density or thermal denaturation measurements (24, 46, 49, 50). The G-C content varies widely, from 24 to 47 mole %, and there may be a tendency for mycoplasma DNA to have a somewhat lower G-C content than most bacterial DNA. There are no data, however, to indicate that mycoplasma DNA is in any way "abnormal".

Recent electron microscopic studies (13, 51, 52, 53) have extended previous studies (12, 14) and confirmed that the basic ultrastructure of the mycoplasmas is similar to that of bacteria. Although they lacked cell walls, all 19 strains examined had a triple-layered cell membrane about 75 Å thick, a nuclear region, and ribosomes. Most of the strains studied had intracellular and extracellular electron dense, membrane bound bodies 130 to 210 mμ in diameter. These are presumably the minimal reproductive units. There were also intracellular condensations

100 to 200 mμ in diameter which had no limiting membrane but were located near protrusions of the cell membrane. These condensations are thought to be developing minimal reproductive units.

A recent study of the membrane structure of M. laidlawii may be of great general interest (54). Membranes obtained from this mycoplasma were disaggregated by detergent into fairly uniform subunits with an uncorrected sedimentation constant of 3.3 S. Upon removal of the detergent by prolonged dialysis in the presence of di- or multivalent cations, membrane-like structures reformed. Observation in the electron microscope indicated that the reformed membranes had a triple layered structure similar to the original membranes, and the reformed membranes retained at least one membrane bound enzymatic activity (NADH oxidase).

The Recent Surge of Interest in the Genus Mycoplasma

The mycoplasmas are no longer "forgotten" organisms. There were about 90 publications concerning them in 1964 alone. They are receiving, and will continue to receive, widespread attention for many reasons, perhaps foremost of which are:

1. Mycoplasmas are important pathogens. The economic loss due to mycoplasma infection of domestic animals is very large (12). Moreover, at least one serious human disease, primary atypical pneumonia, is caused by a mycoplasma (55) and another mycoplasma may play a role in respiratory-tract and genital-tract diseases (8). Mycoplasmas have even been implicated in leukemia, a variety of solid tumors, lupus erythematosis, and rheumatoid arthritis (8). The significance

of these latter findings, however, remains to be proven. It seems likely that the mycoplasmas were either picked up as contaminants or were just secondary infections.

2. Mycoplasmas are important tissue culture contaminants. Surveys (56-60) have shown that 50 to 75% of all continuous cell lines contained mycoplasma. Contamination was usually unnoticed because as many as 10^7 mycoplasma cells per ml may be present in tissue cultures without producing obvious cytopathic effects (60). The majority of tissue culture contaminants have been identified as M. hominis or M. orale, human mycoplasma species that are commonly found in the mouth and throat of healthy individuals (8). Fortunately, mycoplasmas can be eliminated from tissue cultures by the use of antibiotics, specific antiserum, or slightly elevated temperatures (8).

3. Mycoplasmas have been recognized as the smallest and possibly the simplest organisms capable of growth and multiplication in cell-free media. That as a group they have the smallest average cell size may be debatable, but their minimal reproductive units, which can be as small as 130 mμ (7, 12, 15, 16), are almost certainly the smallest free-living organisms. In one mycoplasma strain, H39, the majority of the viable cells may be less than 220 mμ because filtration of a H39 culture through a 220 mμ Millipore Filter has been reported not to reduce the viable count (16). To serve as a reference, it should perhaps be pointed out that vaccinia virus is 220 by 260 mμ (16), whereas E. coli is about 800 by 2000 mμ in the living state (61).

Purpose of the Present Investigation

It was the small cell size and the possible functional simplicity of the mycoplasmas that stimulated the present study. In particular, it had been reported by Morowitz et al. (46) that M. gallisepticum, strain A5969, had an average cell size of 250 mμ (about 125 times smaller in volume than a typical E. coli cell), and, moreover, the DNA content per cell was thought to be perhaps less than that of the bacteriophage T2 (130×10^6 daltons). Since such a small amount of DNA can code for only a limited number of enzymes and structural proteins, this organism promised to contain only those proteins most essential for life. Without question, further study of this organism was warranted.

In view of their critical importance, it was thought necessary to confirm and extend the results of Morowitz et al., and, therefore, the purpose of part of the present investigation was to provide conclusive evidence as to cell size and cellular DNA content of M. gallisepticum.

Also, since the chromosome of a microorganism with such a limited amount of DNA promised to be small and thus amenable to study, a major portion of the present investigation was devoted to experiments designed to give information on the structure and size of the chromosome of M. gallisepticum. The length of a microorganism's chromosome, of course, also gives a minimal estimate of the organism's DNA complement.

METHODS

Strain

Mycoplasma gallisepticum, strain A5969 was obtained as a gift from Dr. Mark E. Tourtellote and is the same strain studied by Morowitz, et al.(46).

Media and Propagation

Unless otherwise noted, the cells were grown in the tryptose broth medium described by Morowitz, et al. (46). This broth was prepared by adding 50 ml of concentrated SB solution (see section on Buffers) to 20 g of tryptose (Difco) in 950 ml of tap distilled water. To this broth was added one ml of 0.5% thiamine HCL, one ml of a 2% suspension of phenol red in water, and two ml of 6.2% thallium(ous) acetate. After autoclaving and cooling below 50°C, 50 ml of sterile 20% glucose and 10 ml of PPLO Serum Fraction (Difco) were added. The final pH was 8.0 at room temperature. This method of preparation was found to be very convenient. The concentrated SB solution and other solutions could be kept indefinitely. The broth could be stored for at least two months at 0-5°C without affecting its ability to support growth.

For routine propagation, one inch O.D. culture tubes containing 16 ml of medium were used, and the cultures were incubated at 36°C without aeration or shaking. To obtain larger quantities of cells, three liter culture flasks containing 1000 to 1500 ml of medium were used, and the incubation temperature was 35° C.

Some cultures were always kept frozen at -15° C. Growth could be obtained from a frozen culture after as long as seven months, but normally about every three months a frozen culture was thawed, sub-cultured once or twice, and then refrozen.

Dilute Growth Medium for Autoradiography Experiments

For labeling with tritiated thymidine for autoradiography, the cells were grown in a modification of the usual tryptose broth. The concentration of tryptose was reduced to only 4 g/L, and 10 μg/ml of aminopterin (Sigma, practical grade) and 200 units/ml of penicillin G (E. R. Squibb and Sons, buffered) were included.

Buffers

The following buffers were frequently used in the present investigation:

SSC - 0.15 M NaCl, 0.015 M trisodium citrate, adjusted to pH 7.0 at 25° C with HCl.

Tris-saline - 0.15 M NaCl, 0.04 M tris (hydroxymethyl) aminomethane, adjusted to pH 8.0 at 25° C with HCl.

SB solution - 100 g of NaCl, 100 g of tris (hydroxymethyl) aminomethane, tap distilled water to make 1000 ml of solution; adjusted to pH 8.3 at 25° C with HCl.

Growth Assays

Culture turbidity was measured using a Bausch and Lomb Spectronic 20 colorimeter fitted with an adapter to hold one inch test tubes. The optical density was read at 650 mμ, where phenol red and other constituents of the medium do not adsorb.

Colony forming units (CFU) were assayed by plating onto solid medium which consisted of tryptose broth containing 1% agar (Difco) and 2% PPLO serum fraction. (For many experiments 2% agar was used, but it was found that with some batches of agar, growth was better at the lower agar concentration.) One percent heart infusion broth, prepared according to Morowitz, et al. (16) was also added for many experiments without any apparent affect on the results. Twenty ml of agar medium was used per 100 mm petri dish, and the surface of the agar was dried by incubating the plates at 35° C for 12 hours. The plates can be kept for at least one week if care is taken to prevent further drying.

The culture to be assayed was serially diluted in tryptose broth without glucose or serum fraction. Then four drops, each about 0.02 ml but together accurately totaling 0.08 ml, were placed on the agar surface using a 0.1 ml pipette. The drops were allowed to sink into the agar at room temperature (up to three hours at room temperature did not affect colony counts), and then the plates were placed in moist refrigerator bags. The bags were sealed and incubated at 35° C for 4 to 7 days, after which the small, "fried egg" type colonies were easily observed and counted using a Nikon Model 6 Shadograph. At

10X magnification, a drop covered about two-thirds of the viewing screen. Up to 1000 colonies per drop could be readily counted if the observation screen was covered with Saran Wrap and each colony marked off as it was counted.

Characterization of the Organism

a. Agglutination of chicken red blood cells. The agglutination of chicken red blood cells -- which is a distinguishing characteristic of M. gallisepticum (12) -- was demonstrated by the procedure of Fahey and Crawley (62). One volume of a culture of M. gallisepticum (or various dilutions of the culture) was mixed with an equal volume of a 1% suspension of chicken red blood cells. A control was also done using just tryptose medium. When the red blood cells of the control had settled into a compact mass, scoring for agglutination was done. A positive result was a sediment that did not form a compact mass. The red blood cells were prepared in the following way. Blood was obtained from a white leghorn chicken by heart puncture and mixed with SSC; the blood cells were pelleted by low speed centrifugation, washed three times with saline-citrate, and then suspended in tris-saline to give a concentration of 1% (v/v).

b. Buoyant density of M. gallisepticum DNA. DNA was extracted from M. gallisepticum by the proceedure of Marmur (77), and its buoyant density in a CsCl density gradient was determined according to the method of Vinograd and Hearst (63). E. coli and Micrococcus lysodeikticus DNA, both obtained from Dr. Larre Egbert, were used as density references. Mycoplasma DNA was added, along with E. coli and M. lysodeikticus DNA, to a concentrated (ρ = 1.856 g/cm^3) solution of CsCl (Harshaw, Optical Grade) buffered at pH 8.0 with 0.01 M tris (hydroxymethyl) aminomethane. Glass distilled water was added to bring the final density to about 1.70 g/cm^3, and a sample of this solution, containing about 0.5 µg of each type of DNA, was centrifuged for 24 hours at 44,770 rpm in a Spinco model E ultracentrifuge. The temperature was regulated at 25° C. A 12 mm Kel-F centerpiece and a -1° radial wedge top window was used. Photographs were taken using ultraviolet optics that included a purple corex filter; the negatives were scanned with a Joyce-Loebl double beam recording microdensitometer.

Fixation of Cells for Electron Microscopy

Two methods of cell fixation were used. The first method was similar to the procedure used by Morowitz et al. (46) except that formaldehyde vapors were used instead of osmic acid vapors. Cells from a tryptose broth culture were pelleted by centrifugation at 6000 x g and resuspended in distilled water. A drop of the suspension was placed on a parlodion covered electron microscope support grid, and the cells were fixed by allowing the drop to stand in formaldehyde vapors

for 30 minutes to one hour at room temperature. The fixed cells were usually examined without shadowing, but in one experiment, they were shadowed with carbon at a 2:1 angle before examining.

The second method of fixation was a modification of the procedure of Klieneberger-Nobel (12). Cells from a tryptose broth culture were pelleted by centrifugation at 6000 x g for 10 minutes and resuspended in an equal volume of tris-saline buffer. A drop of this cell suspension was placed on a formvar film floating on a tris-saline buffer plus 10% formaldehyde, adjusted to pH 7.0 for most experiments. After 10 minutes for fixation, the film was transferred onto distilled water for one hour and then onto an electron microscope support grid.

The formvar film was made by dipping a glass slide into an 0.3% solution of formvar in chloroform and then allowing the slide to drain in an atmosphere partially saturated with chloroform. The edges of the slide were scrapped with a razor blade and then the formvar film was floated off onto water. The film was transferred from one solution to another by use of a fine wire screen.

Observation of Fixed Cells and Size Measurements Using the Electron Microscope

A Phillips E.M. 200 electron microscope was used to examine the fixed cells and to take electron micrographs. When size measurements were to be made, the microscope was carefully adjusted to give accurate magnifications just before use. The magnifications of the microscope were calibrated using as a reference the lines on a carbon replica of a diffraction grating (Ladd Research Industries).

Observation and Photography with the Phase Microscope

A drop of broth culture was placed on a clean slide and quickly covered with a cover glass. In some cases the slide was immediately examined using a Zeiss Photomicroscope with phase optics, but usually the edges of the cover glass were sealed with paraffin and the slide was allowed to stand at room temperature for one-half to two hours before examination in the phase microscope. During this time the cells stuck to the surface of the glass and were immobilized. Photographs of the immobilized cells were taken using the standard photographic procedure for the Zeiss Photomicroscope. Adox KB 14 film was used.

Cell Size by Phase Microscopy

Using a Zeiss Photomicroscope, photographs were taken at 400 power of cells that were immobilized on glass by the procedure described in the preceding section. The negatives were used to project the images of the cells onto a white sheet of paper and the outlines of the cells were traced onto the paper. Cells in at least ten different frames of the negative were traced, and cell size was determined from these tracings. (The projector had a film holder that held the position of the film constant; if one frame was in focus, they all were.)

The accuracy of the microscope magnification was checked using the rulings on a Petroff-Hauser Bacterial Counter as a reference. The magnification of the projection step was also measured, but the magnification of the projector was not critical because polystyrene latex spheres which were to serve as a known size reference were measured

at the same session as were the cells, without changing the focus or the position of the projector.

The Apparent Size of Latex Spheres in the Phase Microscope

Polystyrene latex spheres of two known sizes, 0.264 ± 0.006 μ and 0.365 ±0.008 μ (Dow Chemical Corp.), were used to determine the size distortion of objects that were near the limit of resolution of the phase microscope. A drop of a suspension of the spheres in water was spread on a slide and allowed to dry. After drying, which fixed the spheres to the glass, a drop of water was placed on the slide, followed by a cover glass. The apparent diameter of the latex spheres was then determined using the same microscope, the same magnification, and the same procedure as for the M. gallisepticum cells.

Growth and Harvesting Procedures for DNA Content Determinations

Cells were grown in tryptose broth without thallium acetate, but containing 200 units/ml of penicillin. One liter of this medium, prewarmed to 35° C, was inoculated with 16 ml of a stationary phase culture. After the appropriate growth period at 35° C, the culture was transferred to 250 ml plastic (Nalgene or polycarbonate) centrifuge bottles, and these were centrifuged at 10,000 x g for 30 minutes. The centrifuge was refrigerated, so the temperature of culture medium dropped to about 5° C during the centrifugation. Working in a 5° C cold room, the supernatant was decanted and the pellets were quantitatively taken up in 10 ml of tris-saline and transferred to a 50 ml

cellulose nitrate centrifuge tube. The bottles were rinsed twice more with 10 ml portions of tris-saline, and the washes were combined with the first cell suspension. The cells were pelleted by another centrifugation at 6,000 x g for 15 minutes, and resuspended in 10 ml of distilled water. Good suspension of the cells was obtained by sucking the pellet up and down in a pipette. An aliquot of this cell suspension was now taken for dry weight determination, and other aliquots were taken for DNA determination.

Dry Weight Determination

An aliquot of the above washed cell suspension sufficient to give 10 to 20 mg of dry weight was added to a beaker that had been dried to a constant weight under high vacuum over P_2O_5. This beaker was put inside another beaker which was then covered with tin foil to keep out any dust or other debris. The cell suspension was dried at first under aspirator vacuum over moist KOH until most of the water had been removed, and then under high vacuum over P_2O_5. The beaker was weighed daily and drying was continued until a constant weight was reached.

DNA Determination by Thymine Analysis

a) Hydrolysis of cells. Cells were hydrolyzed according to the method of Jervell (64). Using a long tipped Pasteur pipette, a one ml sample of the same cell suspension used for the dry weight determination was placed in a 7 mm O.D., 10 cm long pyrex tube that was sealed at one end. The suspension was evaporated to dryness under aspirator vacuum

over moist KOH, and then 1.0 ml of 98-100% formic acid was added. The tube was sealed and heated at 175° C for two hours. After cooling to room temperature, the pressure that built up inside the tube during hydrolysis was released by carefully melting just the tip of the sealed tube. (It was necessary to use long tubes because shorter tubes built up higher pressure and often exploded in the oven.) The tube was then opened and the formic acid was evaporated under aspirator vacuum over moist KOH. After evaporation was complete, 0.5 ml of 1.5 N HCl was added to the tube, the sides of the tube were carefully rinsed, and the tube was heated in a boiling water bath for 15 minutes to insure solubilization of the thymine and other cellular constituents.

b) Chromatography of cell hydrolysate. The column chromatographic method of Wall (65) was used to separate thymine from all other cellular constituents in the hydrolysate. An ion exchange column was prepared from Dowex 50-X8, 200-400 mesh resin (or Spinco Ion Exchange Resin type 50A, which is a sulphonated polystyrene resin like Dowex 50). Prior to preparation of the column, the resin had been cleaned using the following procedure; two washes with hot 6 N HCl, one wash with water, two washes with room temperature 1 N NaOH, one wash with water, and one wash with 1.5 N HCl. The washes were conveniently done on a sintered glass funnel. The cleaned resin was suspended in 1.5 N HCl and, after settling and decantation to remove the fines, it was poured as a slurry (40 g per 50 ml) into a glass chromatography tube to form a column 1.0 cm in diameter and 60 cm high. Just before use, the column was washed overnight with 1.5 N HCl to reduce the background

of ultraviolet absorbing material in the column effluent. This background could never be completely eliminated, but after sufficient washing it reached a constant low value. After each use with cell hydrolysates, the resin was cleaned and the column remade using the above procedure.

The chromatography was performed as follows. Cell hydrolysate, in a 0.5 ml volume of 1.5 N HCl, was carefully added to the top of the column. The tube that had contained the cell hydrolysate was washed with an additional 0.5 ml of 1.5 N HCl and this too was added to the column. After washing the cell hydrolysate onto the resin with another one ml of 1.5 N HCl, elution was started. The column was eluted with 1.5 N HCl using two pounds of nitrogen pressure to increase the flow rate. The flow rate was usually about 30 ml/hr. Slower flow rates did not improve separation. Either one or two ml fractions were collected, and the absorption of these fractions at 260 mμ ($O.D._{260}$) was measured.

For some experiments, the effluent was passed through a Beckman Liquid Micro-Aperature Flow Cell which allowed monitoring of the optical density at 260 mμ with a Cary model 15 spectrophotometer. To adapt this spectrophotometer for use with the Beckman flow through cell, it was only necessary to construct a special cuvette mask with appropriately positioned windows. Even with the special mask, changes in the hot spot of the hydrogen lamp caused a small periodic oscillation of the pen, but this was not a serious problem because of its reproducibility and relatively high frequency.

c) Calculation of thymine concentration and the resulting DNA estimate. The fractions containing thymine were identified by their location and by their characteristic ultraviolet absorption spectrum. The concentration of thymine in these fractions was calculated from their $O.D._{260}$ above background by using the extinction coefficient of thymine in 1.5 N HCl. This was determined by dissolving a known quantity of pure thymine in 1.5 N HCl. The volume of each fraction containing thymine was measured by determining the weight of liquid in each fraction, and the total amount of thymine recovered from the column was calculated and used as the basis for an estimate of the DNA present before hydrolysis.

To calculate the DNA equivalent to the measured amount of thymine, it was only necessary to know (in addition to the molecular weights of thymine and the nucleotides in DNA) the percentage of M. gallisepticum DNA that is thymine. This was reported by Morowitz et al. (46) to be 33 ± 2 mole %, on the basis of both chemical analysis and buoyant density measurements. The buoyant density of M. gallisepticum DNA and thus also its base ratio was confirmed by experiments reported in this thesis.

Thymine Analysis Controls

The qualitative and quantitative aspects of the chromatographic step were determined by chromatographing a known amount (usually 20 μg) of thymine (Sigma Chemical Co.), and the overall procedure -- formic acid hydrolysis followed by chromatography -- was first checked by

using thymidine (Calbiochem, A grade) and later by using calf-thymus DNA (Sigma Chemical Co., type II). The amount of DNA analyzed (about 400 μg) was calculated from the $O.D._{259}$ of the stock DNA solution in SSC. The ε(P) of calf-thymus DNA was taken to be 6650 at 259 mμ (66, 67); its mole fraction thymine was taken to be 0.26 (68). To check on the suitability of the procedure in the presence of protein, 12.5 mg of serum albumin (Bovine, Fraction V, Sigma Chemical Co.) was added to 400 μg of DNA before hydrolysis.

To determine the recovery of thymine in the presence of cellular constituents, the following experiment was performed. One microcurie of thymidine-methyl-H^3(6.7 C/mmole, New England Nuclear Corp.) was added to a quantity of cell residue similar to that usually used for DNA determinations. The residue was analyzed for thymine using the normal procedure, and then the thymine containing fractions were pooled and evaporated to dryness. The thymine was dissolved in one ml of hyamine hydroxide (Packard Instrument Co.) and this was added to 15 ml of scintillation fluid (2 g/l 2,5-diphenyloxazole, 0.05 g/l 2-ρ-phenylenebis (5-phenoxyazole)) and counted using a Nuclear-Chicago 770 series scintillation counter. The channels ratio method (69) was used to monitor quenching. The tube that had contained the thymine was washed with another one ml of hyamine hydroxide and this was counted separately to check on the complete solubilization of thymine by hyamine hydroxide.

To serve as a standard for complete recovery of label, 1 μc of tritiated thymidine was dried in a test tube and then taken up in hyamine hydroxide and counted by the method just described.

The Keck DNA Determination Method

This method, which makes use of the reaction of indole with the deoxyribose in hydrolyzed DNA, was developed by Keck (70). The procedure used was as follows. One-half ml of cell suspension containing about 15 μg of DNA was added to 0.5 ml of 10% TCA in a centrifuge tube. The tube was heated for 20 minutes at 90° C and then centrifuged at 10,000 x g for 10 minutes. The supernatant was quantitatively transferred to another small test tube and then 0.5 ml of an 0.06% indole solution and 0.5 ml of 2.5 N HCl were added. After mixing, the test solution was heated for 15 minutes at 90° C, cooled, and then extracted two times with one ml portions of n-pentyl acetate (Matheson, Coleman, and Bell). The extractions were best performed by inverting the test tube a given number of times, usually five times.

A calf-thymus DNA standard curve was done simultaneously with each determination of cellular DNA, using the same reagents and heating for the same length of time. The color due to DNA was slowly, but significantly, extracted with n-pentyl acetate, so the extractions of the standard curve and the unknown DNA solution were performed identically.

After extraction with n-pentyl acetate, the optical density of the aqueous phase was measured at 460 mμ using a Cary model 15 spectrophotometer.

DNA Content by Direct TCA Precipitation

A 14 ml aliquot of a culture of M. gallisepticum grown in tryptose broth without phenol red was cooled in an ice bath for 15 minutes, and then an equal volume of ice cold 10% TCA was added. After 30 minutes at 0° C, the resulting precipitate was collected by centrifugation at about 400.x g for 15 minutes, and resuspended in 20 ml of cold 5% TCA. After another 30 minutes at 0° C with occasional stirring, the suspension was again centrifuged at 400 x g for 15 minutes. The supernatant was discarded and then 1.0 ml of 5% TCA was added to the pellet. After resuspension, the TCA precipitate was heated for 20 minutes at 90° C, cooled, and repelleted by centrifugation at 400 x g for 30 minutes. The supernatant from this centrifugation was collected, its volume determined by weighing, and assayed for DNA by the Keck method just described.

As a necessary control, 14 ml of a sterile tryptose broth medium without phenol red was treated exactly as was the mycoplasma culture.

Thymidine Content of Tryptose Broth

Thymidine-Methyl-H^3 (New England Nuclear Corp., 6.7 curies/ mmole) was added to a logarithmically growing culture of M. gallisepticum to make 25 μc/ml. At zero time and at hourly intervals thereafter, a one milliliter sample of the culture was taken and added to one milliliter of 10% TCA at 0° C. After standing at 0° C for 30 minutes or more, the precipitate was collected on a HA Millipore Filter (Millipore Filter Corp.) and washed with five one milliliter portions of cold 5% TCA. After the first four samples had been taken, the culture was divided

in half, 20 μg/ml of nonradioactive thymidine was added to one portion, and sampling of both portions was continued. The Millipore filters from each sample were dried, suspended in scintillation fluid and counted as described in the section on Thymine Analysis Controls. The channels ratio method (69) was used to monitor quenching.

Total Thymine in Tryptose Broth

To determine the total thymine (thymine, thymidine, thymidylic acid, etc.) content of the growth medium, 0.625 g of tryptose and 0.3 ml of PPLO Serum Fraction were dissolved in 25 ml of 98-100% formic acid. Then, a one ml aliquot of this solution was hydrolyzed and analyzed for thymine according to the procedure described in the section on DNA Determination by Thymine Analysis.

Autoradiography Experiment

Ten milliliters of dilute growth medium, with no added thymidine, was inoculated with 0.1 ml of an early stationary phase culture of M. gallisepticum. An aliquot (0.025 ml) of this inoculated medium was added to an 0.5 ml conical test tube containing 4 μg of dry thymidine-methyl-H^3 (14 curies/mmole, New England Nuclear Corp.). After stirring to dissolve the thymidine, the tube was stoppered with cotton and placed inside a larger test tube with a water saturated atmosphere. To the rest of the inoculated medium, unlabeled thymidine was added to give a concentration of 16 μg/ml. This nonradioactive culture was assayed for clone forming units, and then both radioactive and nonradioactive cultures were incubated at 36° C for 24 hours. Sterile

technique was used throughout the preceding operations. After incubation, both cultures were assayed for clone forming units, and then the following procedure, recently developed by Cairns (71, 72), was applied. (Only the outline of this procedure was described by Cairns. Many of the details of the procedure are or may be somewhat different. These details could be important.) The radioactive culture was diluted 170 or 1,100 fold into lysis solution (1.5 M sucrose, 0.05 M NaCl, 5 μg/ml calf-thymus DNA (Sigma, Type I), 0.01 M EDTA, pH 8.0), and about one milliliter of this dilute cell suspension was placed in a dialysis chamber, as follows.

The dialysis chamber, which was constructed as depicted in Figure 1, was filled by inserting teflon or polyethylene tubing down through the hollow arm and carefully injecting about one milliliter of cell suspension. The chamber was not completely filled, so with a little care, no solution got into the arm. After filling, the plastic tubing was removed, and the mouth of the arm was sealed using a short piece of tubing closed at one end. It was essential to seal the arm, because otherwise osmotic effects caused solution to enter the chamber and partially fill the arm.

After filling, eight of the chambers were clamped in an upright position in 200 ml of lysis solution plus 0.5% sodium dodecyl sulfate (Duponol C, Dow Chemical Corp.) for one hour and then transferred to 200 ml of dialysis solution (0.05 M NaCl, 0.005 M EDTA, pH 8.0). The dialysis solution was changed about every hour for 10 hours. During the dialysis procedures, gentle convection stirring was effected by having only the

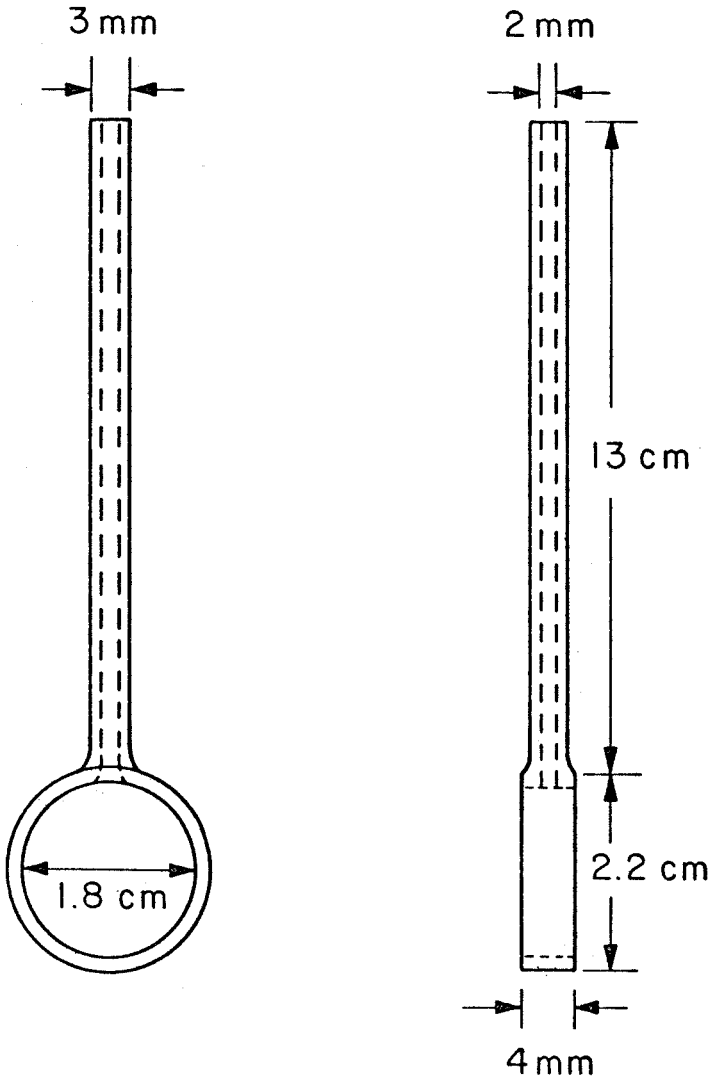

Figure 1. Dialysis Chamber. A 3 mm Pyrex tube was fused to a 2.2 cm Pyrex tube, and then the large tube was cut to form a thin cylinder with an arm for filling and handling. Using plastic dissolved in amyl acetate as a glue, a VM Millipore filter (50 mμ average pore size, Millipore Filter Corp., Bedford, Mass.) 25 mm in diameter was glued to each end of the glass cylinder.

bottom of the beaker containing the dialysis solutions in a 37° C water bath. The temperature of the dialysis solutions equilibrated at 35° C. After the dialysis period, the chambers were removed from the solution and fixed firmly in place by taping to a wooden block; then a small hole was punched with an insect pin in the bottom of one of the millipore filters bounding each chamber. A chamber usually emptied about two-thirds in 10 to 15 minutes, but complete drainage often took one-half to one hour. All during the dialysis procedure and until the filters were dry, great care was taken to avoid any shaking of the chambers, but some slight disturbance was unavoidable in handling.

When the filters were dry, each was cut out and glued to a slide, using plastic dissolved in amyl acetate. The filter was overlaid with Kodak AR 10 stripping film by dipping the slide into clean distilled water and bringing it up underneath a piece of film floating on the surface (73). The film covered slides were air dried and then stored in a CO_2 atmosphere over Drierite for two to five months at -15° C in a light tight box.

The slides were developed for 20 minutes in Kodak 19b developing solution at 20° C. (This solution was made up just prior to use according to the formula given on the pamphlet that comes with every box of stripping film; filtration was often necessary to remove a very fine precipitate that formed.) After development the slides were rinsed in distilled water for 30 seconds, fixed for 20 minutes in Atkinson Paper Fixer, and rinsed in distilled water for two to four hours. While the slides were under water, the stripping film was carefully peeled from

both slide and filter and placed on another clean slide. After air drying overnight in a dust free environment, the film was covered with Permount and a cover glass.

Observation, Photography, and Length Measurements

The autoradiograms of the DNA molecules were observed and photographed using a Zeiss photomicroscope. The autoradiograms were most easily discerned if dark field optics were used, but most photographs were taken with phase optics, which gave a cleaner background. Routinely, a 10 x objective was used and the total magnification on the photographic film was only 64 times. Prints were made, being careful to show the complete frame so that the enlargement of each frame could be accurately determined. The lengths of the DNA autoradiograms were determined by using a map measuring device (K & E co., D1741). The accuracy of this device was checked by measuring known distances set off by a ruler; the accuracy of the magnification steps involved was checked using for reference the rulings on a haemocytometer or Petroff-Hauser bacterial counter.

The Kleinschmidt Technique

To obtain information on the size and structure of the chromosome of M. gallisepticum, a technique developed by Kleinschmidt (74) was adapted for use with this organism. Cells from a ten ml aliquot of an early stationary phase culture were pelleted by centrifugation at 6,000 x g for 10 minutes at 5° C. For several experiments the pellet was resuspended

in one ml of distilled water, but for other experiments the pellet was resuspended in a saline-EDTA solution (0.85 % NaCl, 0.02 M EDTA, pH 8.5). After various incubation times and temperatures, which will be specified in RESULTS, 0.1 ml of the cell suspension was added to 0.9 ml of a 4 M ammonium acetate solution containing 0.01 % cytochrome c (and for some experiments 0.01 M EDTA). After five minutes at room temperature, 0.02 ml of this solution was allowed to run slowly down a specially cleaned glass slide onto the surface of an 0.0005 M EDTA solution, pH 7.5. (The glass slide had been cleaned with hot chromic acid cleaning solution and was thus hydrophilic.) The cytochrome c spread as a monolayer over the surface of the dilute EDTA solution, and the DNA which was released from the cells by osmotic shock was trapped in the monolayer. An electron microscope support grid covered with a film made from 2% parlodion was touched to the monolayer, dipped into 95% alcohol for 15 seconds, then dipped into isopentane for 10 seconds, and finally air dried. The grid was shadowed with one or two platinum-carbon pellets (Ladd Research Industries) at a distance of 6 cm and at a 10:1 angle, while being rotated at 41 revolutions per minute.

The shadowed grid was examined in a Phillips E. M. 200 electron microscope, and, after adjusting the microscope to give accurate magnifications, electron micrographs of the DNA fibers were taken. The magnifications of the microscope were checked using as reference the lines on a carbon replica of a diffraction grating (Ladd Research Industries).

Length measurements were made by projecting the electron micrograph negatives onto a white sheet of paper, tracing the images of the DNA fibers onto the paper, and running a map measuring device (K & E Co., D1741) over the tracings.

RESULTS

Characterization of the Organism

The cellular morphology, colony morphology, and growth characteristics of the M. gallisepticum stock culture used for the present investigation were the same as those of the original culture of M. gallisepticum A5969 obtained from Dr. Mark E. Tourtellote; in addition, these characteristics were the same as those of a second culture of A5969 obtained from Dr. Tourtellotte almost a year later. Since each of these characteristics is quite distinctive, they are, taken together, probably sufficient to prove that A5969 -- the organism reported by Morowitz, Tourtellotte, et al. (46) to have a small cell size and low DNA content -- was the organism being studied. However, to provide additional characterizing data, two additional types of experiments were performed.

First, the ability of the stock culture to agglutinate chicken red blood cells was checked. This is a characteristic of M. gallisepticum that distinguishes it from all other mycoplasma species (12, 55), with the possible exception of M. pneumoniae (75), which, however, is an organism that requires a special growth medium and would not grow in tryptose broth. It was found that M. gallisepticum cultures, and ten fold dilutions of cultures, strongly agglutinated chicken red blood cells, whereas tryptose broth was without effect. These results suggested that the stock culture was indeed M. gallisepticum.

As a second characterization procedure, the buoyant density of what was expected to be M. gallisepticum DNA was determined and compared with published values. A densitometer tracing of the bands formed when a mixture of M. gallisepticum, E. coli, and Micrococcus lysodeikticus DNA was centrifuged to equilibrium in a cesium chloride density gradient is shown in Figure 2. Knowledge of the initial density of the solution allowed the middle peak to be assigned to E. coli DNA and the peak at the higher density position to be assigned to M. lysodeikticus DNA; then, taking the buoyant density of E. coli DNA to be 1.704 (76), the densities of the other two peaks were calculated using the relationship $\Delta\rho = 0.020\ \bar{r}\ \Delta r$ (63). The calculated density of M. lysodeikticus DNA (1.727 gm/cm^3) was in good agreement with the expected value [1.726 gm/cm^3 (76)], a result that confirms the original peak assignments. Similar ultracentrifuge experiments performed by Dr. Larre Egbert (80) using the same CsCl and marker DNA solutions, showed that neither the E. coli nor the M. lysodeikticus DNA preparations contain material that bands at a position corresponding to the third peak. Therefore, the peak at a buoyant density of 1.687 gm/cm^3 must represent M. gallisepticum DNA. A repeat experiment also gave this same value.

Morowitz et al. (46) have reported that the buoyant density of M. gallisepticum A5969 DNA is 1.693 gm/cm^3. However, they took the buoyant density of E. coli DNA to be 1.709 gm/cm^3 and used the relationship $\Delta\rho = 4.15 \times 10^{-10}\omega^2(r^2 - r_o^2)$ to calculate buoyant density differences. When this formula was applied to the present data, M. gallisepticum DNA was found to be 0.016 gm/cm^3 lighter than E. coli, which

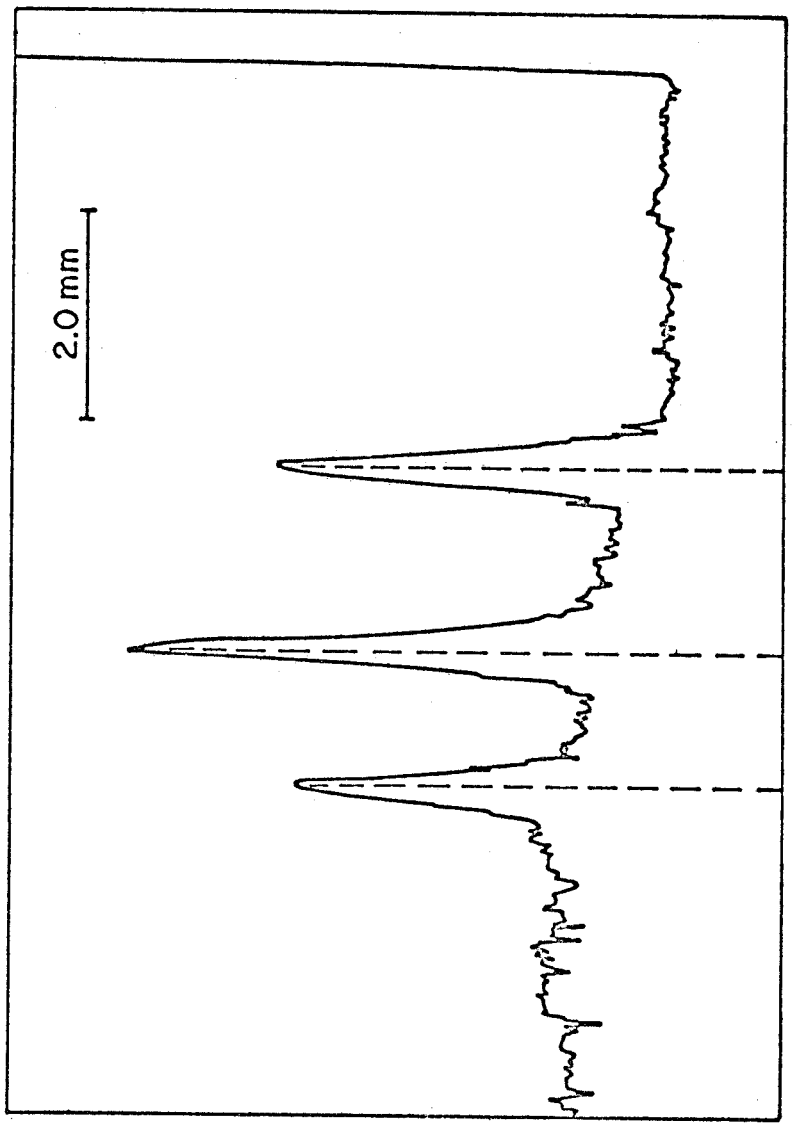

Figure 2. Densitometer tracing of bands from M. gallisepticum, E. coli, and M. lysodeikticus DNAs, centrifuged in CsCl for 24 hr at 44,770 rev/min and 25°C. The middle peak was 6.66 cm from the center of rotation, and the scale in the upper right gives the total magnification. See text for peak assignments and calculation of densities.

is exactly what Morowitz et al. (46) reported. This result suggests that the same organism was studied in the present investigation as was studied by Morowitz et al. (46).

It is also pertinent that the cell forms seen upon routine observation of a turbid broth culture in the phase microscope were found to fit very well with the following description of gram stained M. gallisepticum cells given by Nelson (81):

> . . . minute immobile cells which may be found as single or double units and in aggregates varying from a few cells up to large compact masses of innumerable units. In shape they are commonly spherical but may be slightly elongated, appearing as extremely short bacilli. In size they are generally under 0.5 μ but may be so minute as to be barely visible.

When this observation is considered along with the other observations and experimental results already discussed, there can be little doubt that M. gallisepticum A5969 was being studied in the present investigation.

Growth Curves

Typical growth curves of M. gallisepticum , strain A5969, growing in tryptose broth without aeration at 36° C are shown in Figure 3. Growth was monitored either by assaying for colony forming units (CFU) or by measuring optical density at 650 mμ. The optical density increases logarithmically for about 23 hours, and the maximum optical density (0.26 optical density units with a 2.2 cm path length) is reached at about 27 hours. The concentration of CFU also increases logarithmically at about the same rate as the optical density for the first 20 hours.

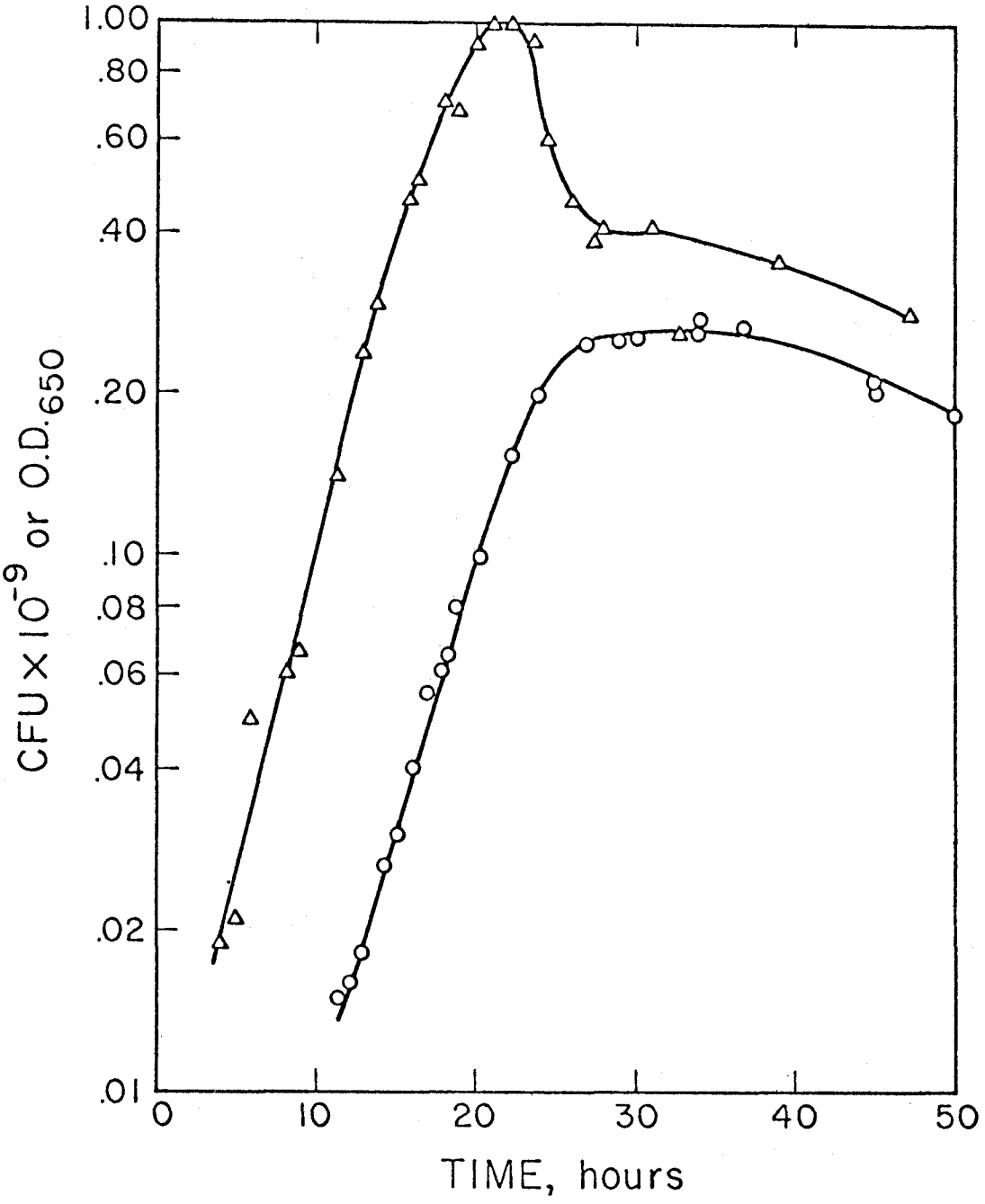

Figure 3. Typical growth curves of M. gallisepticum A5969. See text for details. Triangles, concentration of CFU; circles, optical density at 650 mμ.

By either growth monitoring procedure, the generation time is 2.8 ± 0.2 hours during this early period of growth.

The maximum concentration of CFU obtained is 1×10^9 per ml, and this is reached at 21 hours. After reaching a peak, the concentration of CFU decreases rapidly to about 4×10^8 per ml and then stays roughly constant until the culture is about 36 hours old. The optical density does not fall after reaching its peak but stays roughly constant during the stationary phase. The rapid decrease in the concentration of CFU was found to be closely correlated with the onset of clumping of the individual cells to form cell aggregates. Figure 4a is a micrograph taken with a phase microscope of cells from a 16.5 hour culture. There are no large clumps of cells. Cells from a 26 hour culture are shown in Figure 4b. In this age culture, clumps of cells are very common; most clumps contain only a few cells, but some clumps can be very large, containing perhaps as many as 50 cells.

As an alternative to optical density or CFU measurements, it was convenient and often sufficiently accurate to determine the physiological age of a culture by observing its color. This was possible because the pH of a culture varied with its age, and phenol red was included in the growth medium to serve as a pH indicator. A dark-red culture was still in the logarithmic phase; a reddish orange culture was in the stationary phase, and an orange or yellow culture was in the death phase.

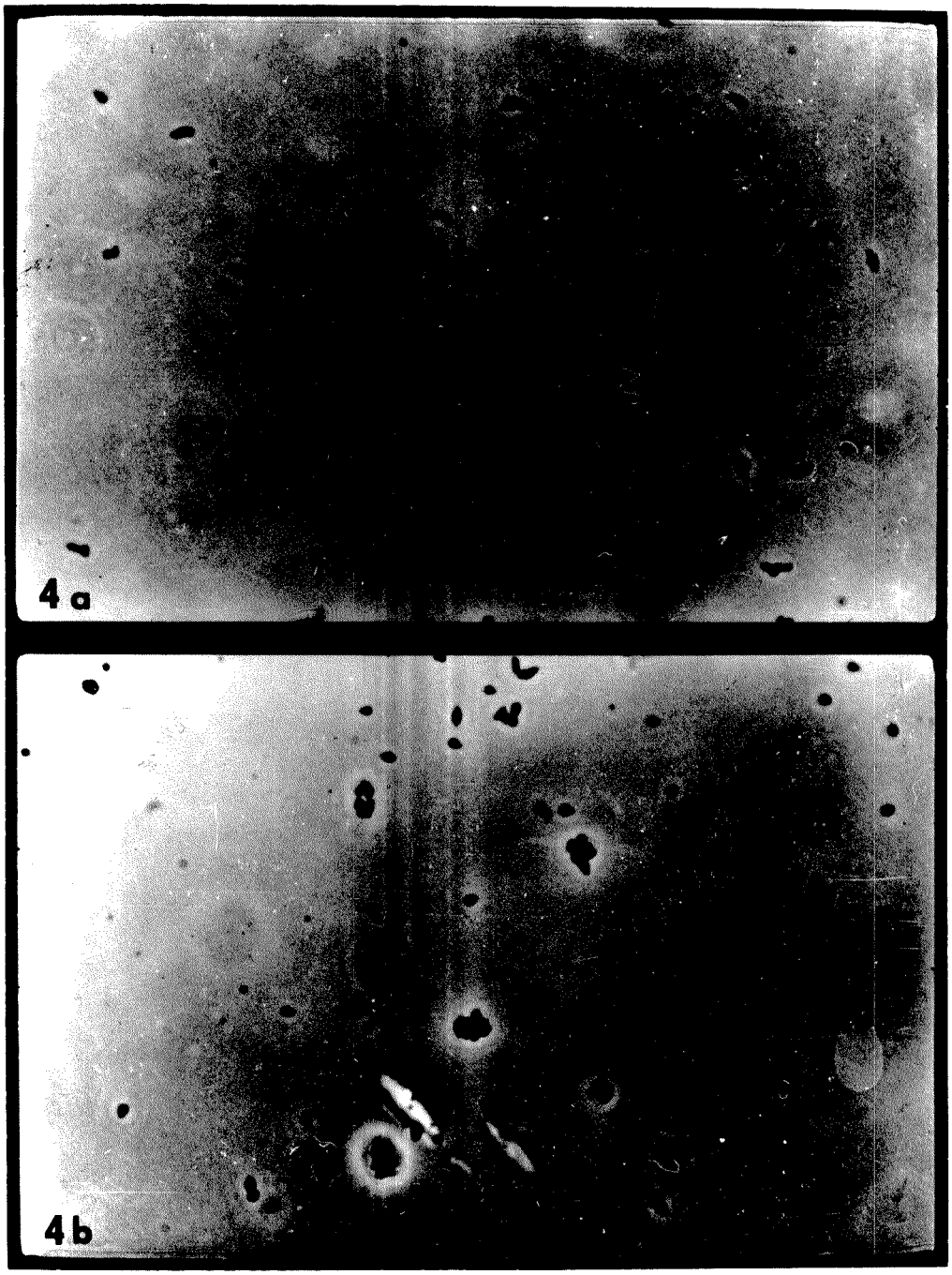

Fig. 4. Phase micrographs of M. gallisepticum cells immobilized on glass. (a) Cells from a logarithmic phase culture. (b) Cells from an early stationary phase culture. X 1450.

Cell Morphology by Electron Microscopy

When cells of M. gallisepticum were fixed according to either of the two methods employed in the present study and examined in the electron microscope, only round cells were observed. In one experiment where the fixed cells were shadowed with carbon at a known angle, the cells were found to be spherical. Depending on the preparation, the state of preservation of the cells varied but there was no definite indication of other than round cells. Fixation by floating on a formvar film over neutral 10% formalin was found to give the best preservation and some typical cells prepared by this fixation method are shown in Figure 5.

The observation of spherical cells agrees with reports on the cell morphology of M. gallisepticum A5969 made prior to 1965 (46, 52). In a recent report by Maniloff et al. (51), similar results, i.e., spherical cells, were obtained if harvesting by centrifugation preceded fixation. They found, however, that if fixation by glutaraldehyde preceded harvesting, then elongated and tear-drop shaped cells predominated. Using strains W and JA of M. gallisepticum, Domermuth et al. (53) similarly found nonspherical cells. The results of these electron microscope studies suggest that the spherical shape may be an artifact produced by harvesting before fixation. In the present study harvesting by centrifugation always preceded fixation, so spherical cells were to be expected.

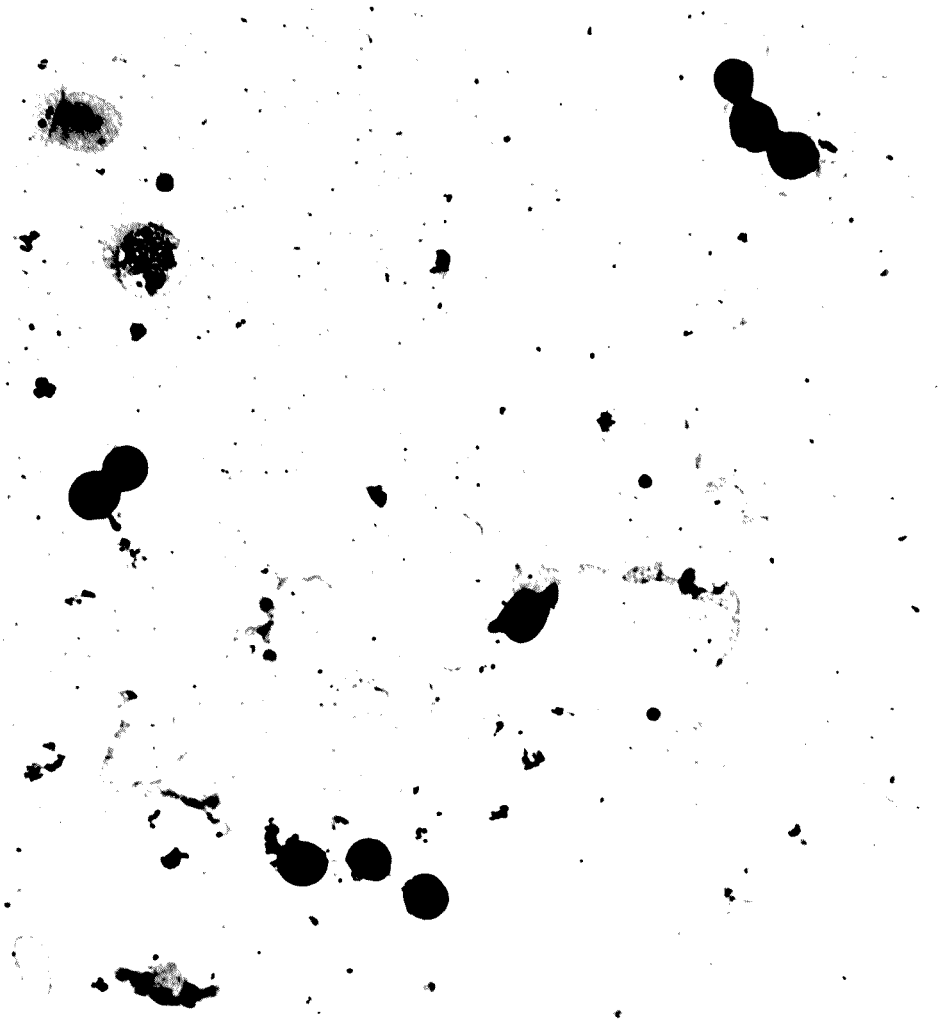

Fig. 5. Electron micrograph of M. gallisepticum cells from a stationary phase culture. Fixation was by floating on a formvar film over neutral formalin. The cells are the large, black objects; the other objects are probably debris from the medium, lysed cells, and formvar film. X 20,000.

Cell Morphology by Phase Microscopy

The observation of M. gallisepticum cells freely floating in broth culture is difficult because their small size and brownian motion makes accurate focusing almost impossible. However, it was found that when a drop of a broth culture was placed on a glass slide and covered with a cover slip, most of the cells in the drop stuck to the glass within one-half hour. Observation and photography of the cells stuck to glass were greatly facilitated by their immobility, and for this reason all the photographs taken in the present study were of cells immobilized on glass. The morphology of free-floating cells was not noticeably different from those stuck to glass.

A typical field of a late logarithmic phase culture is shown in Figure 4a, and a collection of the more common cell types seen in such a culture is shown in Figures 6-A through J. Most cells did not appear to be spheres although some spheres were present. Cells in the shape of tear-drops, short rods, or short "lumpy" filaments were common (Figures 6-A through 6-D). A three-lobed structure such as shown in Figures 6-E and 6-F was not infrequent nor was a structure such as that in Figure 6-H, which appears to be a sphere with two short protrusions on each side. Short filaments were somewhat more common in earlier logarithmic phase cultures, but they were always less common than the tear-drop and rod-like cells.

It should perhaps be pointed out that these cells are so small that two spherical cells adjacent to each other would not be resolved; they would appear as a short rod. The structure in Figure 6-I is

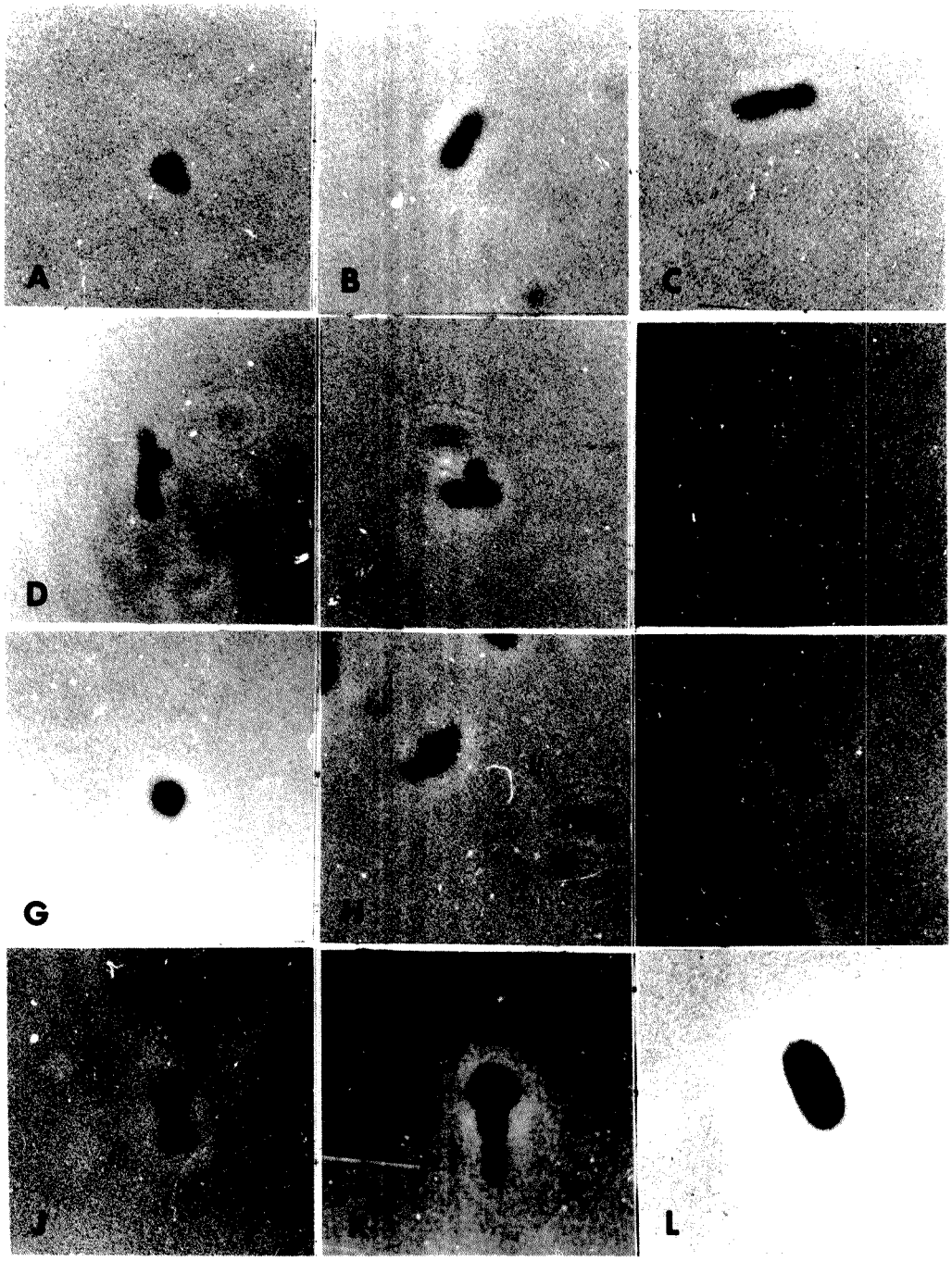

Fig. 6. Phase micrographs of cells immobilized on glass. (A) through (J), M. gallisepticum cells from a logarithmic phase culture. (K), a M. gallisepticum cell from a stationary phase culture. (L), an E. coli cell. All X 6300.

probably two contiguous spherical cells. In fact, most of the structures seen could be interpreted as a collection of spherical cells. This, however, is not the most reasonable interpretation in light of the electron microscope studies of Domermuth (53) and Maniloff (51).

A typical field of an early stationary phase culture is shown in Figure 4b. Although many cells are in clumps, making observation of their morphology difficult, those cells not in clumps are predominately tear-drop in shape. (If observed while floating freely in solution the tear-drop shaped cells tended to appear spherical because of the difficulty in keeping them in focus.) The rod-like and filamentous cells seen in a younger culture were still occasionally present but were much less frequent. Giant spherical cells were also seen occasionally in stationary phase cultures. A giant cell from which three smaller cells may have arisen is shown in Figure 6-K. In late stationary phase cultures, normal sized spherical cells predominated.

The cell forms seen in the present study may be of some significance, especially since there is somewhat of a controversy over the mode of replication and classification of M. gallisepticum. From a growth curve study, Kelton (78) proposed that M. gallisepticum (strains J and S-6) reproduces by binary fission. Domeruth et al. (53) have suggested, however, on the basis of their gross morphology and ultrastructural studies using the electron microscope, that M. gallisepticum (strains J and W) reproduces by the growth of "elementary bodies" which form in short protusions from the cell proper. The study of

Maniloff et al. (51) on strain A5969 also indicated the existence of short protrusions from the cell proper.

The present studies can not, of course, give conclusive evidence as to the mode of replication of A5969, but the cell forms seen suggest the following hypothesis. Usually a cell sends out a short protrusion forming a tear-drop cell such as that in Figure 6-A. The protrusion grows in size while still attached to the mother cell to form a rod-like cell with one end smaller than the other, such as that in Figure 6-B. Finally, a constriction is formed and two spherical cells are produced (Figure 6-I). Replication is then a type of budding process. Not infrequently a longer protrusion may be formed which divides into several cells (see Figure 6-K), or a cell may send out more than one protrusion. Whether or not the details of this hypothesis are true, it seems likely that multiplication is not predominately by simple binary fission.

In 1962 Klieneberger-Nobel suggested (12) that M. gallisepticum strains do not belong to the mycoplasma group but should be considered as separate "coccobacilliform bodies". Her argument was based to a large degree on data suggesting that M. gallisepticum had a regular coccoid morphology and apparently multiplied by binary fission. The results of the present investigation suggest that M. gallisepticum does not, in general, have a regular coccoid morphology and does not multiply by simple binary fission. Weibull (79) has recently examined cells of two "authentic" species of mycoplasma while they were immobilized in agar, and the cell forms seen were similar to those seen

in the present investigation. When the electron microscope studies of Domermuth (53) and Maniloff (51) are also considered, there seems to be no firm basis for separating M. gallisepticum from the other mycoplasmas.

Cell Size by Electron Microscopy

The diameter of the cells in micrographs such as Figure 5 were measured and the distribution of cell diameters obtained from one experiment are shown in Figure 7. There are occasional large cells up to 1.1 μ in diameter and a few as small as 0.13 μ in diameter, but most of the cell diameters are within 0.05 μ of the mean, which is 0.32 μ. Ninety seven per cent of the cells have diameters less than or equal to 0.40 μ. This distribution is typical for five out of the six experiments of this type that were performed. The method of fixation (formaldehyde vapors or floating on a formvar film over formalin) did not greatly affect the size distribution; the average cell diameter was always less than 0.4 μ. In one experiment in which a formvar film and an acid formaldehyde solution were used for fixation, the average cell diameter was only 0.24 μ. This diameter agrees with the value of 0.25 μ reported by Morowitz et al. (46) from a similar electron microscope study in which osmic acid vapors were used for fixation. In the present study the low value could not, however, be repeated and must be considered atypical. In fact, cells in different areas of the same film were "normal", suggesting that subtle variations in fixation conditions can alter the apparent size of the cells. This

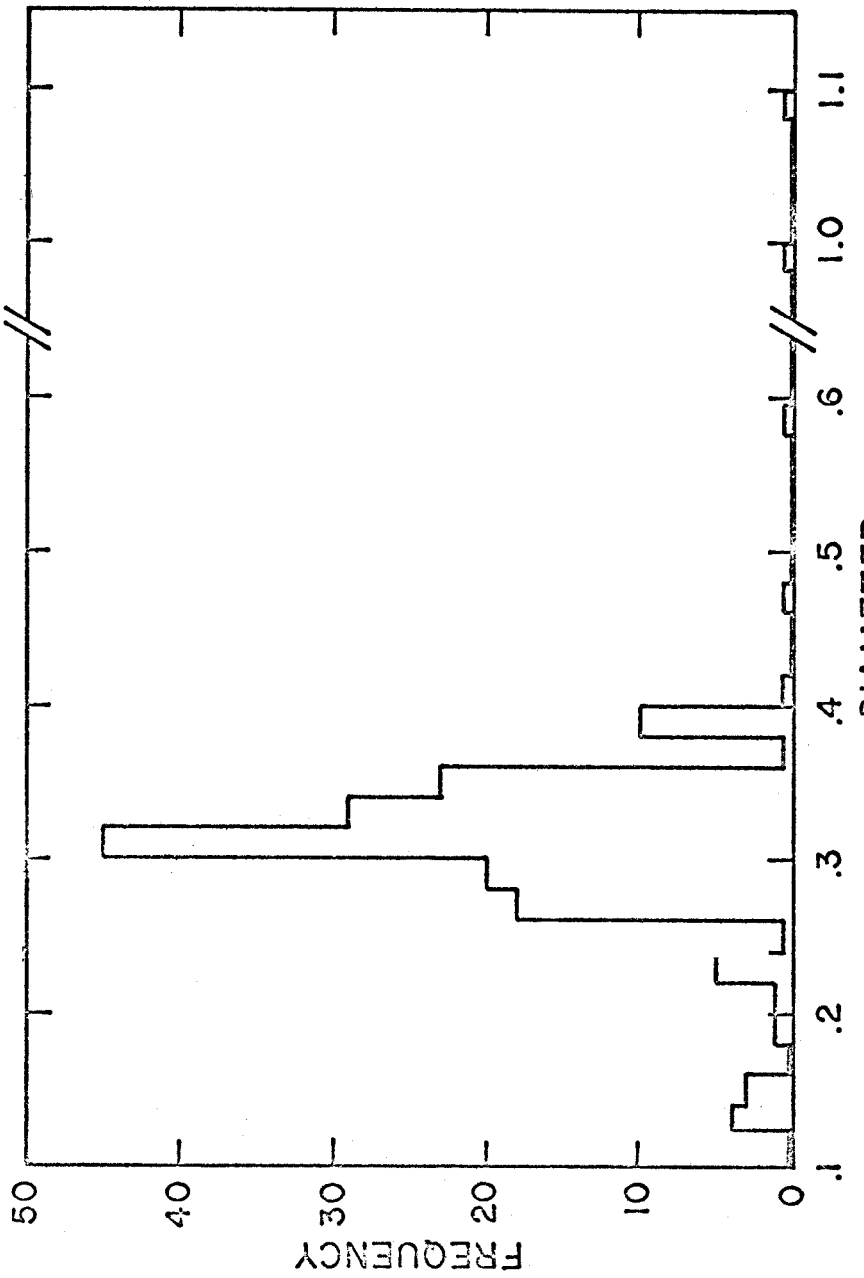

Figure 7. Distribution of cell diameters as determined by electron microscopy.

possibility seems even more likely in light of the recent studies of Maniloff et al. (52) in which cells of strain A5969 were harvested, fixed by either osmium tetroxide or glutaraldehyde, and thin sections examined in the electron microscope. They found round cells about 0.55 μ in diameter. If the apparent average cell size can vary from 0.25 μ to 0.55 μ depending on the method of fixation, it seems unlikely that electron microscope studies by themselves can give reliable data concerning cell size in the living state.

Cell Size by Phase Microscopy

To avoid the uncertainties introduced by the harvesting and fixation steps necessary before examination in the electron microscope, estimates of cell size were made using phase microscopy. In Figures 6-A through 6-J typical logarithmic phase *M. gallisepticum* cells are shown, and in Figure 6-L an *E. coli* cell is shown at the same magnification. The *E. coli* cell is from an overnight culture of strain W3110; it is 1.0 μ wide and 2.1 μ long and thus may be considered a typical *E. coli* cell (61). Most of the *M. gallisepticum* cells are obviously much smaller than the *E. coli* cell, but quantitative size measurements were not straightforward because the *M. gallisepticum* cells are so small that their size is distorted by diffraction effects in the light microscope.

However, meaningful size measurements were obtained after the microscope was calibrated by using polystyrene spheres of known diameters. When polystyrene latex spheres known to be 0.365 ± .008 μ in diameter

were measured with the phase microscope, they had an apparent size of 0.53 ± .03 μ; latex spheres known to be 0.264 ± .006 μ in diameter had an apparent size of 0.44 ± .03 μ. Thus the diameters of the latex spheres were exaggerated by 0.17 ± .03 μ, an amount roughly equal to twice the resolving power of the microscope.

The focus of the microscope at the time that the size measurement photographs were taken had some effect on the apparent size of the latex spheres, since there was as much as a 13% variation in the diameter of the spheres determined from different frames of the same negative. However, if ten or more frames were used to obtain the average diameter, the variation in sphere diameter determined from independent experiments was reduced to about 5%.

The contrast was considerably better for the latex spheres than it was for the cells, and, conceivably, the degree of contrast might affect the amount of distortion. It was thus necessary to show that the refractive index difference (which determines the contrast) between the spheres and the surrounding medium does not greatly affect the apparent size of the latex spheres. To show this, the apparent diameters of the latex spheres in sucrose solutions of varying refractive index were measured. It was found that varying the refractive index from 1.3330 (pure water) to 1.4588 (a saturated sucrose solution) had no significant effect on the apparent size of the latex spheres. When the latex spheres were in concentrated sucrose solutions, they had about the same contrast as the cells had in tryptose broth. The latex spheres should, then, make good size references, and the size

distortion calculated for them should apply to the cells, especially since, as will be shown later, the diameter of an average cell is close to that of the 0.364 μ latex spheres.

Because M. gallisepticum cell size is close to the limit of the resolving power of the phase microscope, there was often uncertainty as to whether a particle was a single large cell or a group of smaller cells. Therefore, it was informative to do the size measurements using two different procedures. In the first procedure (the "liberal" procedure), cells were considered separate if there was a slight constriction between them, that is, each "lump" in a particle was considered to be an individual cell. In the second procedure (the "conservative" procedure) cells were considered to be separate only if there was a very definite constriction between them. Thus many lumpy particles were counted as a single cell.

Regardless of whether the "liberal" or the "conservative" procedure was used, cell volume was calculated after: a) approximating each cell by one or more spheres or rods, whichever was appropriate and b) correcting the dimensions of these spheres or rods by subtracting the correction factor determined for the latex spheres. The distributions of cell volumes thus obtained for cells from an early stationary phase culture are shown in Figure 8. When the "liberal" procedure was used, the average cell volume is 2.7×10^{-14} cm^3, which is equivalent to the volume of a sphere 0.37 μ in diameter. When the "conservative" procedure was used, the average cell volume is $4.5 \times 10^{-14} cm^3$, which is equivalent to the volume of a sphere 0.44 μ in diameter. The true

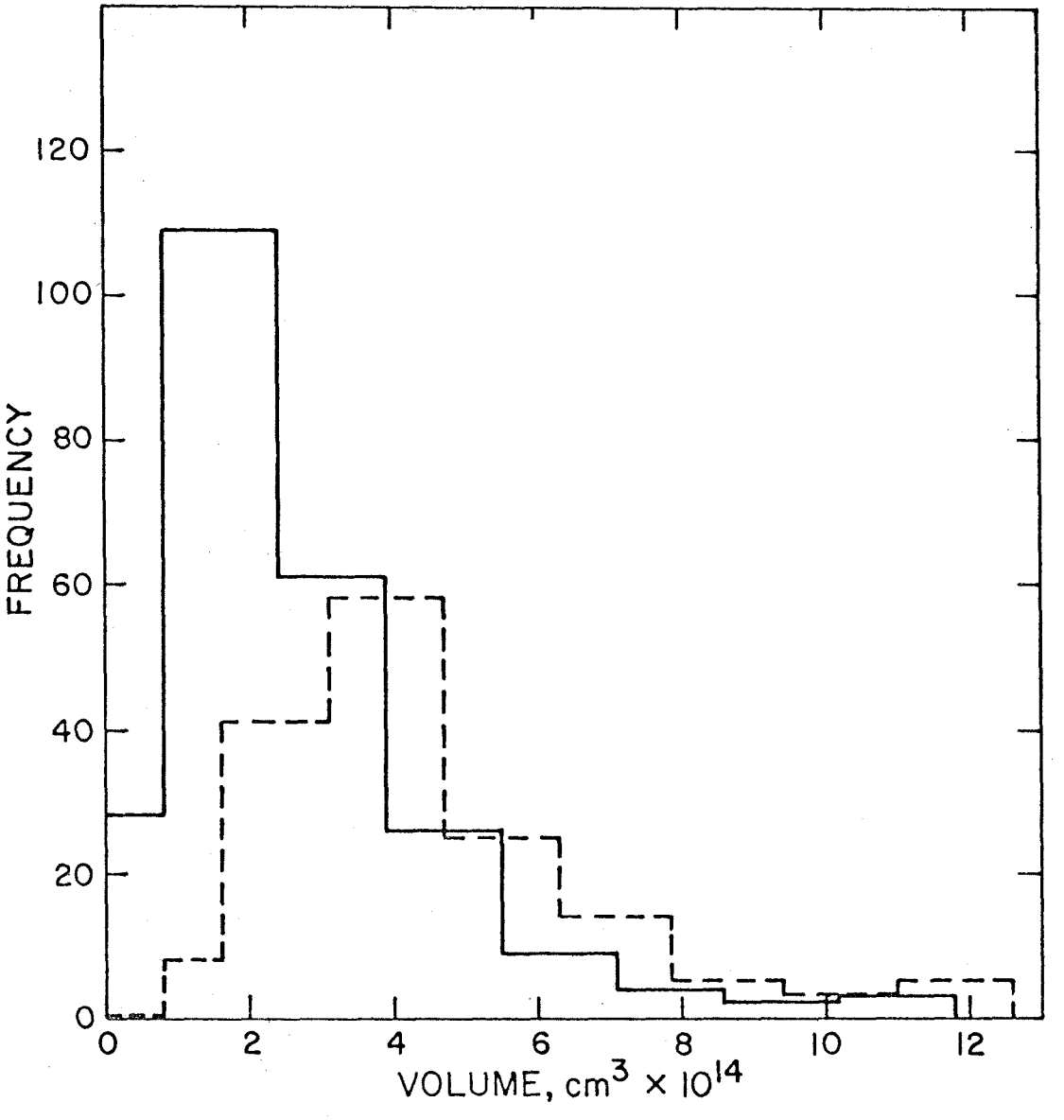

Figure 8. Distributions of cell volumes as determined by phase microscopy. Solid line, "liberal procedure"; dotted line, "conservative" procedure. (See text for explanation.)

distribution of cell volume, as determined by this method, is probably somewhere between the distributions shown in Figure 8. The "liberal" procedure probably counts many protrusions as individual cells; the "conservative" procedure probably counts many individual cells as part of a larger cell.

One of the main points to be made from this study is, however, that even with the "conservative" procedure, the cells of M. gallisepticum are very small. An average E. coli cell in the living state has a volume of about $1 \times 10^{-12} cm^3$ (61). Thus even the larger M. gallisepticum cells, which have a colume of about $1 \times 10^{-13} cm^3$ are ten times smaller than an average E. coli cell; the average M. gallisepticum cell ($4.5 \times 10^{-14} cm^3$) is at least 22 times smaller, and the smallest M. gallisepticum cells ($1.2 \times 10^{-14} cm^3$) are at least 80 times smaller.

The other point to be made from this study is that although the cells of M. gallisepticum are very small, the average cell volume is probably three to five times larger than that of a 0.25 μ sphere, which was the average cell size indicated by the electron microscope study of Morowitz et al. (46).

Introduction to the DNA Determination Method

Prior to the present investigation, the Keck method (70) and the salmine precipitation method (82) had been used by Morowitz et al. (46) to determine the DNA content of M. gallisepticum. The Keck method utilizes the color reaction of indole with deoxyribose in TCA precipitated and partically hydrolyzed DNA, whereas the salmine precipitation

method depends on the specific precipitation of DNA from an alkaline solution by salmine. The results obtained by these methods were in fair agreement -- 3.7 to 4.6% of the dry weight of M. gallisepticum cells was DNA. Morowitz et al. (46) used these values, along with their data on cell size (a sphere 0.25 μ in diameter) and water content, to calculate that M. gallisepticum may have as little as 50×10^6 daltons of DNA per cell. This is a very small amount of DNA; a T2 bacteriophage contains close to 130×10^6 daltons of DNA (84), and an E. coli cell contains at least $2{,}100 \times 10^6$ daltons of DNA (85).

Independent confirmation of the DNA content data pertaining to M. gallisepticum was certainly called for, but to rule out the possibility that the DNA of M. gallisepticum was in some way protected and thus resistant to TCA or salmine precipitation, it was thought advisable to use a DNA determination method based on different chemical and physical principles. Therefore, a DNA determination method based on analysis for thymine was developed.

Formic acid at elevated temperatures is commonly used to hydrolyze DNA (83), and Jervell has reported (64) that this method of hydrolysis quantitatively releases thymine from a nucleoprotein TCA precipitate. Wall (65) has reported that thymine can be separated from all other components of a cell hydrolysate by ion exchange chromatography. In the present investigation, the methods of Jervell and Wall were combined to form a method for determining the thymine content of dried, whole cells, and then the thymine content data were used to estimate the DNA content of the cells.

Characterization of the Chromatography Step of the DNA Determination Method

The curve in Figure 9 shows the separation and relative positions of most of the bases and nucleosides that elute from a Dowex 50 column with 1.5 N HCl. For a given size column the positions of the peaks were reproducible within three or four milliliters. The thymine containing peak (labeled T on the graph) could be easily distinguished from the uracil containing peak (U) when the complete ultraviolet absorption spectrums of the materials in the peaks were measured, since thymine has a distinctive absorption maximum at 264 mμ, whereas uracil has an absorption maximum at 259 mμ. Thymine is well separated from the other bases by this procedure.

DNA Determination Controls

Several experiments were done to ascertain the quantitative aspects of the DNA determination method. The results of these experiments are summarized in Table 1, and are discussed below.

a) Recovery of thymine and thymidine. The recovery of a known quantity of thymine added to the Dowex column was found to be 99%. Therefore, the chromatography step (at least in the absence of other cellular constituents) is probably more than adequate. Two separate experiments were also done in which known amounts of thymidine were subjected to formic acid hydrolysis and then chromatographed. The hydrolysis of thymidine to thymine was complete for both experiments, because of the elution patterns showed only thymine peaks. The recovery

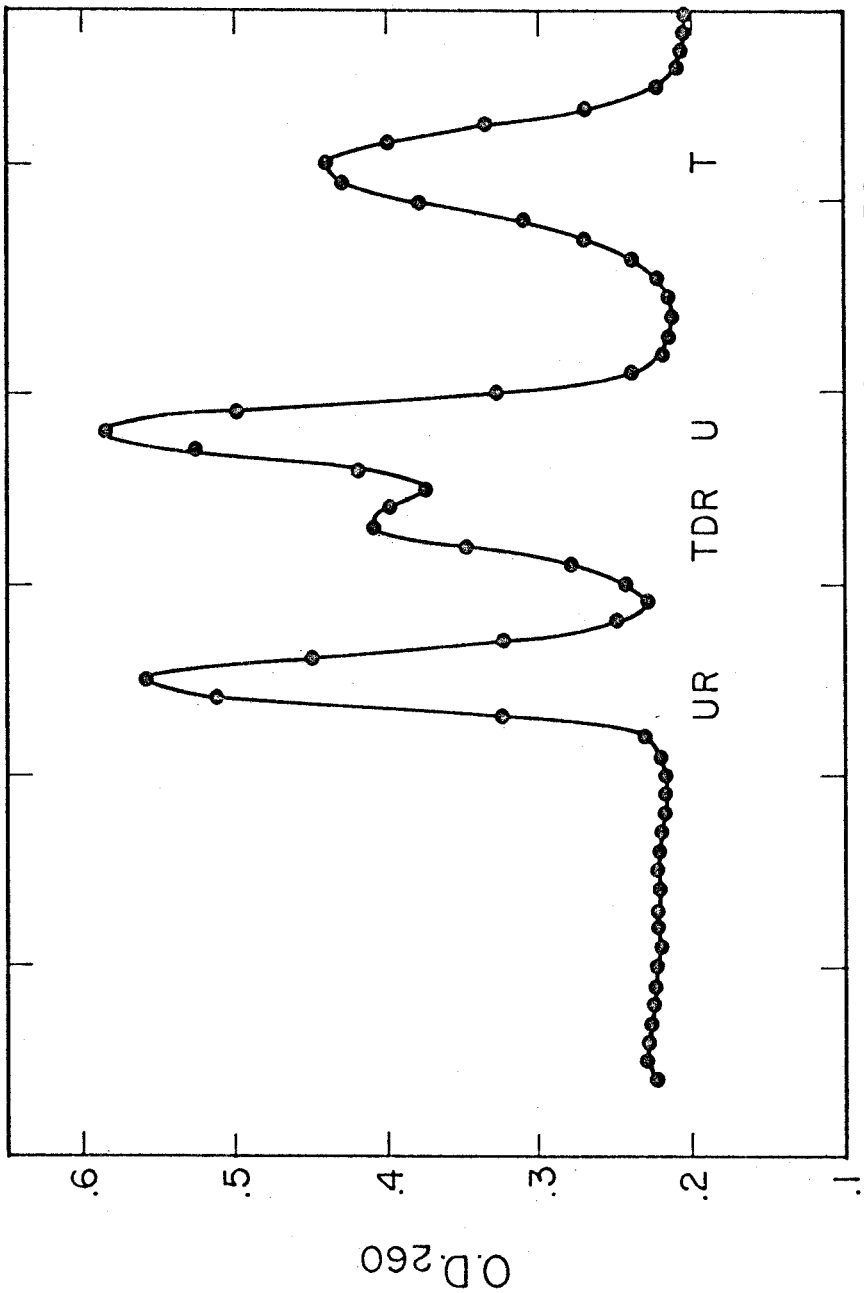

Figure 9. Elution pattern obtained when known nucleosides and bases were chromatographed on Dowex 50. A Beckman flow cell was used for this experiment. UR, uridine; TDR, thymidine; U, uracil; T, thymine.

TABLE 1.

DNA Determination Controls

Exp. #	Description	% Recovery of Thymine	µg Thymine Recovered
1	Thymine	99	42.6
2	Thymidine	87, 94*	55.1, 59.2
3	Calf Thymus DNA	> 90	---
	Calf Thymus DNA plus Albumin	98	15.6
4	Labelled Thymidine plus Cells	85	---
5	Calf Thymus DNA	106, 103	12.6, 12.2
	Cells		9.9, 9.7
	Predicted Sum		12.4 + 9.8 = 22.2
	Cells plus Calf Thymus DNA		22.8

*Each value represents a separate experiment.

of thymine was 87% of theoretical for the first experiment and 94% for the second experiment.

b) Recovery of thymine from DNA. The solid line in Figure 10 shows the pattern that was obtained when calf-thymus DNA was hydrolyzed and chromatographed. Besides the main thymine (T) peak, there are two small peaks corresponding to uracil (U) and to what may be uracil deoxyriboside (UDR). The later peaks probably arise from the deamination of deoxycytosine during the formic acid hydrolysis.

The dotted line in Figure 10 is the pattern obtained when calf-thymus DNA was hydrolyzed in the presence of a 25 fold excess of serum albumin. The flow rates during chromatography varied slightly and if these were corrected for, the curves would be almost identical. After flow rate correction, the areas under the thymine peaks were not significantly different (6.6 cm^2 vs 6.5 cm^2). Due to technical difficulties with the fraction collector, the recovery for the determination with DNA alone could not be calculated, but the recovery for the determination with DNA plus albumin was 98% of theoretical. Since the areas under the peaks were almost the same, the recovery for the determination with DNA alone must also have been over 90%.

c) Recovery of labeled thymidine hydrolyzed with cells. When a known amount of radioactive thymidine was added to the cell residue before hydrolysis, it was found that 85% of the label was recovered as thymine. Since extra steps were needed to determine the radioactivity in the thymine containing fractions, this value is probably an underestimate. Also, the thymidine was labeled with tritium in the methyl

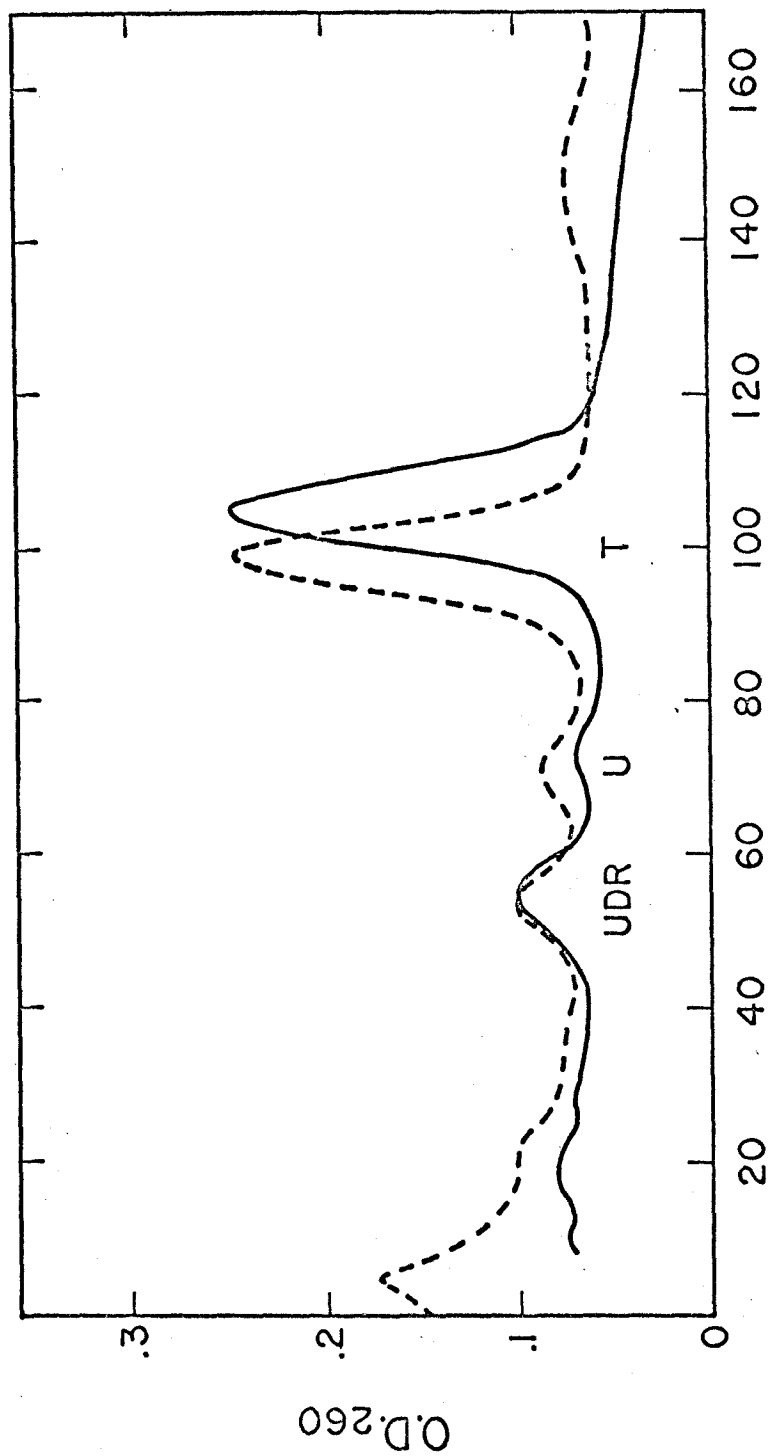

Figure 10. Elution pattern obtained when a formic acid hydrolysate of calf-thymus DNA was chromatographed on Dowex 50. A Beckman flow cell was used for this experiment. Solid line, pure calf-thymus DNA hydrolysate; dotted line, calf-thymus DNA plus serum albumin hydrolysate. UDR, uracil deoxyriboside; U, uracil; T, thymine.

group, and under the strongly acidic conditions and high temperature of the hydrolysis, there may have been some loss of tritium by hydrogen exchange. There was no detectable label in any peak other than that for thymine.

d) Additivity of calf-thymus DNA and cellular DNA. The last experiments were done to determine if DNA added to dried, whole M. gallisepticum cells could be quantitatively recovered. First calf-thymus DNA by itself was hydrolyzed and analyzed for thymine (essentially 100% recovery was obtained), and then M. gallisepticum cells by themselves were assayed for thymine. Finally, calf-thymus DNA was added to cells and the mixture was hydrolyzed and assayed for thymine. If significant destruction of the thymine in the added calf-thymus DNA did not occur, then the thymine recovered from the mixture should be the same as that predicted on the basis of the separate experiments. The data shown in Table 1, experiment 5 indicate that there was no significant destruction of thymine. This experiment also gives some idea about the reproducibility of the assay system, since most determinations were done in duplicate, and only a 3% variation was found.

These control experiments, taken together, indicate that the destruction or loss of thymine from DNA during the entire procedure is less than 10%, and the method should be satisfactory for its intended purpose, namely, the determination of the DNA content of M. gallisepticum cells.

Elution Pattern for Cell Hydrolysates

When M. gallisepticum cells that had been harvested and washed as in METHODS were hydrolyzed with formic acid and chromatographed on Dowex 50, an elution pattern was obtained such as that in Figure 11. Only three well separated peaks are significant. From their elution position and absorption spectrum, the first peak represents uridine (UR) and the second uracil (U). As expected from the work of Jervell (64), uridine is not completely hydrolyzed to uracil during the formic acid hydrolysis. Thymidine is more labile and is completely hydrolyzed to thymine, which elutes to give the third peak (T). All other U.V. absorbing compounds are retained on the column until well after the thymine peak.

Cellular DNA Content

The DNA present in the harvested and washed cells was calculated from the thymine recovered from the ion exchange column and these data were conbined with those on the dry weight of the cells analyzed to give the percentage of the dry cellular material of M. gallisepticum that was DNA. The data on percent DNA from six experiments are plotted in Figure 12 as a function of the age of the culture that was analyzed. A typical growth curve (a reproduction of Figure 2) is included in Figure 12 to show the physiological ages of the cultures that were analyzed. It is apparent that, as determined by this procedure, DNA is 4.0 ± 0.6 percent of the dry cellular material regardless of the age of the culture. This result agrees very well with the published values (46).

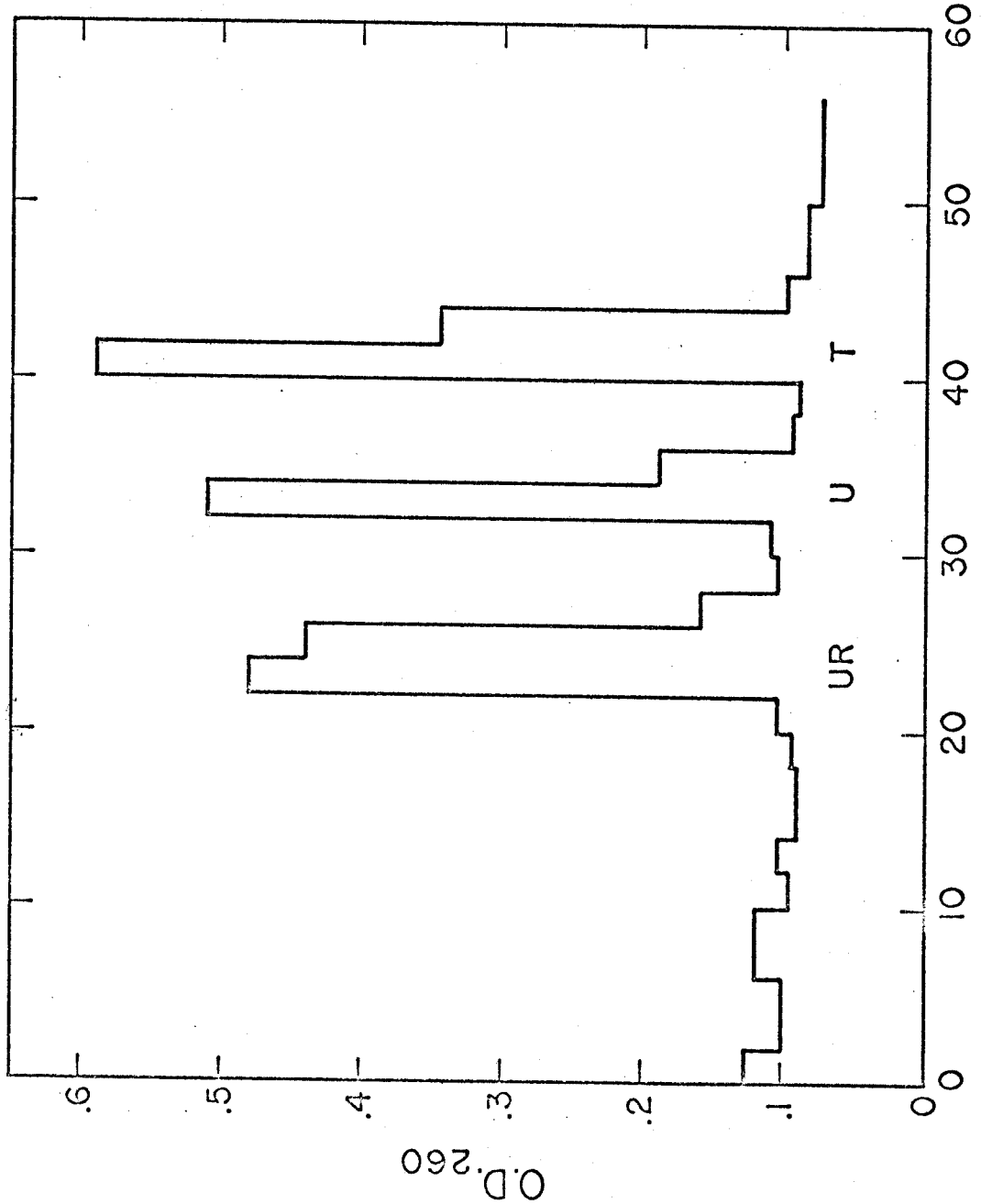

Figure 11. Elution pattern for cell hydrolysates. UR, uridine; U, uracil; T, thymine.

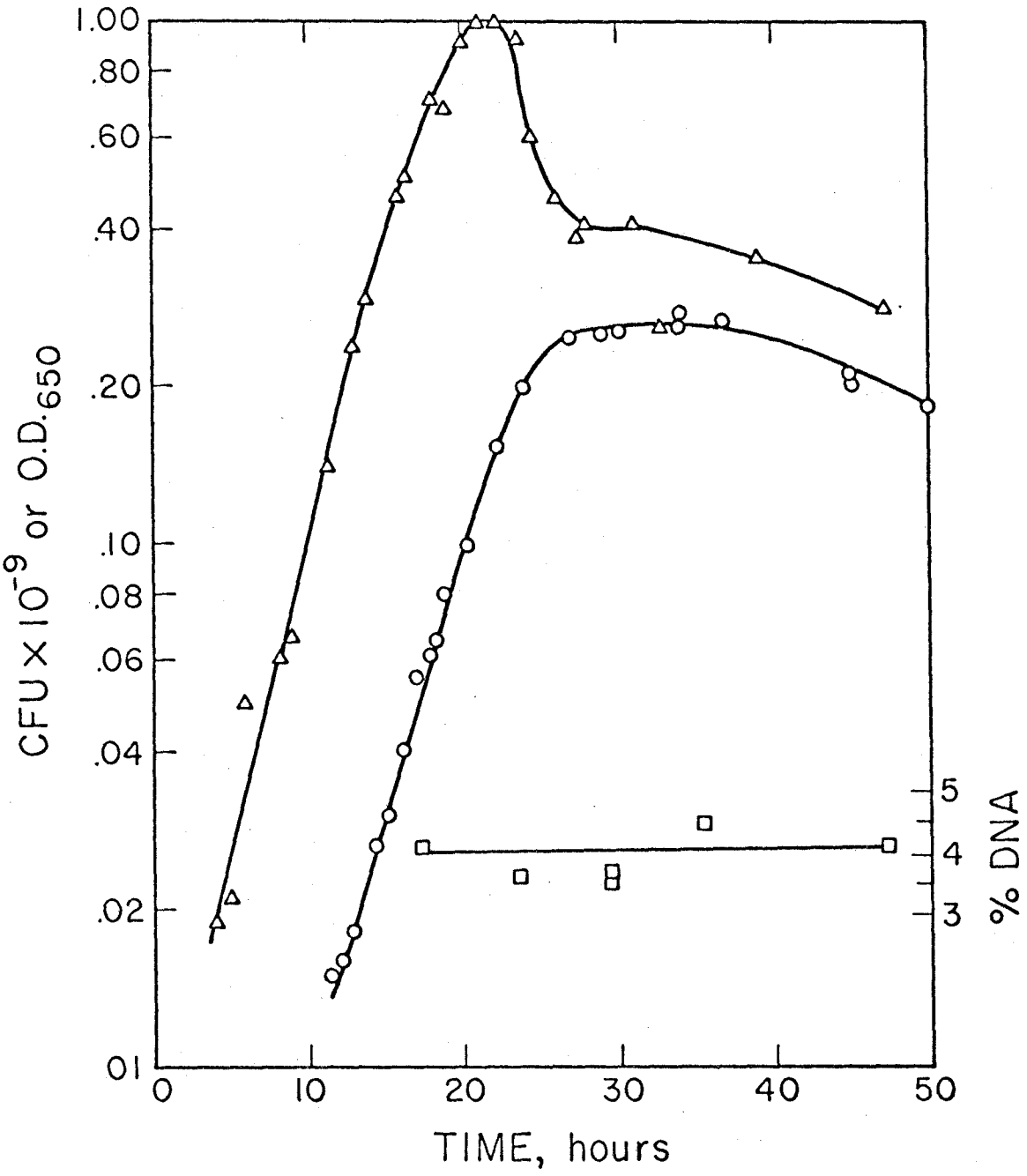

Figure 12. Percentage DNA as a function of culture age. Typical growth curves are also shown. Squares, percent DNA; triangles, concentration of CFU; circles, optical density at 650 mμ.

As was done by Morowitz et al. (46), the percent DNA data were combined with cell size and water content values to get an estimate of DNA content per cell. The data in the section on Cell Size by Phase Microscopy indicated that the volume of an average M. gallisepticum cell is $3.6 \pm 0.9 \times 10^{-14} cm^3$. If the cells are 75% water (Morowitz et al. (46) have published information suggesting this value) and have a density of 1.1 gm/cm^3, then they have $4 \pm 1 \times 10^{-14}$ gm of dry cellular material per cell. If 4% of the dry cellular material is DNA, then the cellular DNA content is $240 \pm 60 \times 10^6$ daltons. This value is considerably larger than the 50×10^6 daltons reported by Morowitz et al. (46), but is still very low for an organism capable of an autonomous existance.

Experiment with the 17.5 Hour Culture

The experiment on the DNA content of the 17.5 hour culture had five modifications and additions to the usual procedure and each needs to be considered in some detail:

1) Since extrapolation from previous experiments with the aid of the optical density growth curve indicated that only about five milligrams of dry cellular material could be expected from one liter of a 17.5 hour culture, it was especially important to ascertain that all sedimentable material obtained from a culture was indeed of cellular origin and not just debris from the medium. Therefore, the tryptose medium was filtered through a HA Millipore filter (0.45 μ average pore size), and all solutions and water used to wash and suspend the

cells were similarly filtered. All glassware and other containers used were carefully cleaned just prior to use.

2) The low expected dry weight also necessitated an additional wash, by suspension of the cells in distilled water and repelleting them by centrifugation, to remove the one or two milligrams of salt and buffer that could have been in the pellet.

3) The supernatants from each centrifugation were saved for later examination. This was thought to be especially important for the last wash with distilled water. Cellular constituents including DNA could have been lost in this step due to cell lysis by osmotic shock.

4) Again by extrapolation from previous experiments with the aid of the optical density growth curve, only 200 ± 50 μg of DNA were expected per liter of a 17.5 hour culture. With this low amount of DNA, the DNA assay method based on analysis for thymine would no longer have been sufficiently accurate without further modifications of the procedure. Therefore, this assay method was not used for this experiment, but instead the Keck method of DNA assay (70) was used.

5) The colony forming unit (CFU) assay does not yield meaningful data for cultures older than 21 hours because of extensive clumping of the cells. However, in a 17.5 hour culture clumping has not yet begun, so a CFU assay was included in this experiment, and this allowed calculation of perhaps significant DNA per CFU values.

Before presenting the results of the analysis of the cells, it is worth pointing out the following two sets of results and conclusions. First, examination of the supernatant from the initial centrifugation

in the phase microscope indicated that essentially all cells had been spun down, and the lack of turbidity of the supernatants of the second and third centrifugations indicated that over 90% of the cells had been spun down for each of these centrifugations. Therefore, DNA is not lost due to incomplete sedimentation of the cells. Secondly, the distilled water wash had no DNA detectable by the Keck method, and, moreover, its absorption in the U.V. was very low; no more than expected from the slight contamination with tryptose medium. There was, therefore, no significant loss of DNA or other cellular constituents from the cells during this step.

The data concerning the DNA content of the cells are shown in Table 2 along with the predicted values. The good agreement between the predicted and measured values suggests (but does not prove) that none of the variations made for this experiment greatly affected the results, The Keck method seems to give results comparable to those obtained with the thymine analysis method.

The value of 300×10^6 daltons of DNA per CFU is especially significant because a) it is very low and b) it at least partially confirms the data of Morowitz et al. (46) who reported values ranging from 70 to 400×10^6 daltons per CFU.

DNA Determination by Direct TCA Precipitation

To avoid any possible DNA loss from M. gallisepticum cells during the harvesting procedure employed during all previous DNA determinations, several experiments were performed in which cells were directly

TABLE 2.

Summary of Data From the Experiment With the 17.5 Hour Culture

	Measured	Predicted
mg dry wt./L	4.6	5
μg DNA/L	201 ± 22*	200 ± 50
% DNA	4.4	4
CFU/ml	4×10^8	6×10^8
daltons DNA/CFU	300×10^6	-

*Range of four determinations

precipitated from a tryptose broth culture by the addition of TCA to the culture. The TCA precipitate was then assayed for DNA using a procedure based on the Keck method (see METHODS for details).

Complete precipitation of what could be as little as 4 μg/ml of cellular material was assured by the fact that tryptose broth medium contains about 1 mg/ml of serum protein. Conceivably, this large excess of protein and co-precipitated tryptose broth constituents could have seriously interfered with the DNA assay. This was not the case, however, as the following discussion will indicate.

Curve A in Figure 13 is the spectrum obtained when the TCA precipitate from a 24 hour culture was analyzed for DNA by the Keck method. The spectrum is not noticeably different from that obtained with pure DNA. Curve B in Figure 13 is the spectrum obtained when the TCA precipitate from a tryptose medium control was analyzed by the same method. Although not negligible, the absorption produced by the control was low and fairly reproducible. This same background absorption was almost certainly present in the solution from the analysis of the culture because cellular material was always less than one percent of the total TCA precipitate. Therefore, the background absorption could be corrected for. When 45 μg of DNA was added to the control before TCA precipitation, analysis of the precipitate for DNA by the Keck method indicated that the precipitate contained 39 μg of DNA. Therefore, the direct TCA precipitation procedure for DNA analysis is probably reliable, giving, if anything, slight underestimates.

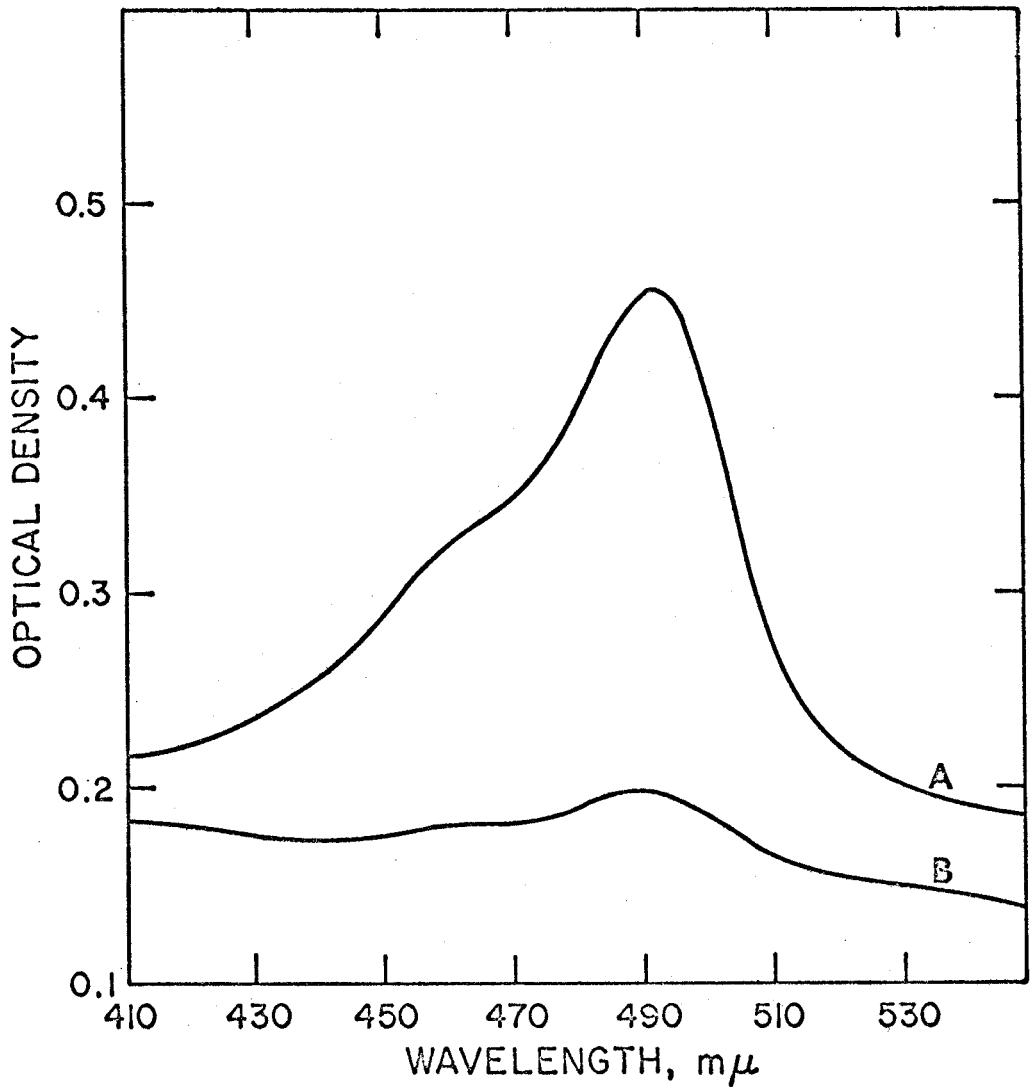

Figure 13. Visible spectrum of the colored product resulting from the Keck DNA determination method. Curve A, product obtained when a TCA precipitate from a 24 hour M. gallisepticum culture was analyzed. Curve B, product obtained when a tryptose medium control was analyzed.

The results obtained from three experiments using the direct TCA precipitation procedure for DNA analysis are presented in Table 3. CFU assays were also included in these experiments, and DNA per CFU values were calculated. The cultures that were analyzed were examined in the phase microscope and it was found that for each experiment, only a small minority of the cells were in clumps. The CFU values are also close to those expected on the basis of the ages of the cultures. Therefore, the DNA per CFU values are probably meaningful. These values ($1200 \pm 200 \times 10^6$ daltons of DNA per CFU) are much larger than those previously obtained from experiments in which harvested cells were analyzed.

A fourth experiment was done in which samples of a 24 hour culture were analyzed for DNA by the direct TCA precipitation procedure, while the cells from the main portion of the culture were harvested as usual and then analyzed for DNA by the Keck method. During the harvesting procedure even more care than usual was taken to keep the temperature low while washing the cells. The result of this experiment was that the direct TCA precipitation procedure gave DNA values that were 2 to 2.5 times higher than those of the procedure in which harvesting preceded analysis. These results suggest that there is DNA loss during the harvesting procedure.

DNA loss during the initial centrifugation is a reasonable hypothesis because of the way in which this centrifugation was done. About 200 ml of warm culture was placed in a 300 ml centrifuge bottle and the bottle was placed in a room temperature angle head centrifuge

TABLE 3.

Results Obtained Using the Direct TCA Precipitation Procedure for DNA Analysis

Exp. #	Age of culture in hours	µg DNA per ml of culture	CFU per ml x 10^{-8}	Daltons of DNA per CFU x 10^{-6}
1	20.3	2.1	10	1260
2	18.5	1.0	6	1000
3	20.5	2.1	9.5	1340

rotor, which was then placed in a refrigerated centrifuge and the centrifugation begun. During the first few minutes a great many of the cells in the still warm culture would be spun against the side of the centrifuge bottle and then rolled down the side to the bottom. This treatment may well damage the soft, perhaps "sticky" mycoplasma cells, and nucleases could be activated.

The literature contains at least two sets of observations that indirectly support the DNA loss hypothesis. The first are observations made by Jones and Walker (86) during their studies on Mycoplasma mycoides. They found that when cells were harvested by passing a culture through a refrigerated continuous-flow centrifuge, the culture had to be precooled to 0° C to prevent degradation of cellular DNA. In the present investigation the M. gallisepticum cultures were not precooled; so by analogy with M. mycoides, DNA degradation could have occurred. The second observations are those made by Maniloff et al. (51) while studying M. gallisepticum A5969. They found that when cells were first harvested and then fixed and observed in the electron microscope, different results were obtained than when cells were fixed before harvesting. The cells that had been harvested first were round rather than elongated and had apparently lost much of their "cytoplasmic ground substance"; their nuclear region also appeared abnormal, with threads of nuclear material radiating out from the main region. If the cells of M. gallisepticum are altered this drastically during centrifugation, then it seems reasonable to suggest that enzymatic degradation of the DNA may have been initiated in some or perhaps all cells.

Although additional direct TCA precipitation experiments are called for, the data obtained so far with this procedure certainly suggest DNA loss during the harvesting procedure and cast doubt on the significance of all previous DNA content values, including those published by Morowitz et al. (46, 87).

Introduction to the Autoradiography and Kleinschmidt Experiments

Since the studies based on chemical analyses for DNA have not yielded conclusive evidence as to the DNA content of M. gallisepticum, it is fortunate that two techniques have recently become available that are more direct and allow observation of a microorganism's chromosome; the length of which gives a minimum estimate of the organism's DNA complement.

The first technique is that of autoradiography. Cairns (71, 72, 84) has developed this technique so that autoradiograms of individual DNA molecules can be obtained. In outline, the procedure used in the present investigation was to release tritium labeled DNA from M. gallisepticum by lysis with detergent, trap this DNA on a Millipore filter, and subject it to autoradiography.

The second technique is that developed by Kleinschmidt et al. (88, 89) in which DNA released from a microorganism is trapped on a cytochrome c monolayer and then observed in the electron microscope.

Development of a Dilute Medium for Autoradiography

In order to see individual DNA molecules by the Cairns technique of autoradiography, tritium-labeled thymidine of very high specific activity (at least about 4 C/mole) must be incorporated into the DNA. However, as shown by the results of the following two preliminary experiments, the tryptose broth medium normally used for the growth of M. gallisepticum contains a relatively large amount of thymidine. The first preliminary experiment was one in which the incorporation of radioactive thymidine into a logarithmically growing culture of M. gallisepticum was measured. The results of this experiment are shown in Figure 14. Nonradioactive thymidine was added to a portion of the culture at the time indicated by the arrow. The change in the rate of incorporation of radioactive thymidine upon the addition of a known quantity of nonradioactive thymidine permitted the calculation of the initial concentration of thymidine in the medium. The data shown in Figure 14 lead to a value of 11 μg of thymidine per milliliter of tryptose broth. This estimate is not likely to be greatly in error, because a second experiment was done (see METHODS for details) in which an analysis of the constituents of the medium indicated 18 μg of thymine per milliliter of tryptose broth. Thymidine and most other thymine containing compounds would have been hydrolyzed to free thymine during the procedure.

This high thymidine content would have led to an unacceptable dilution of the added radioactive thymidine. It was, therefore, necessary to alter the growth medium. Fortunately, good growth could

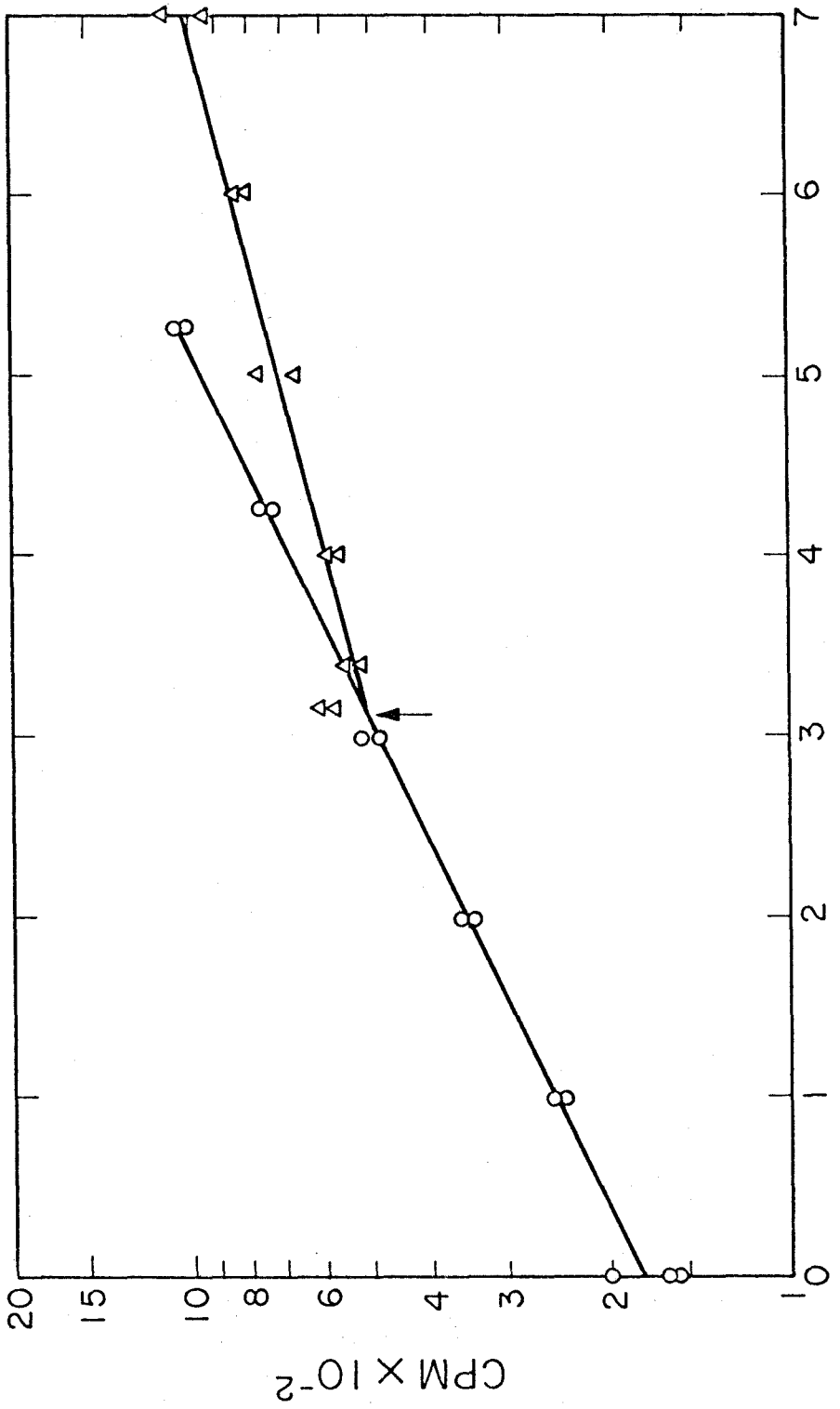

Figure 14. Incorporation of thymidine-methyl-H^3 into cold TCA insoluble material by a logarithmically growing culture of M. galliseptícum. At the time indicated by the arrow, a known quantity of nonradioactive thymidine was added to one portion of the culture. Circles, no nonradioactive thymidine added; triangles, nonradioactive thymidine added.

still be obtained when the concentration of tryptose in the medium was reduced to one fifth the normal amount, thereby decreasing the thymidine concentration to an acceptable level (about 2 μg/ml).

M. gallisepticum may be able to carry out an endogenous synthesis of thymidine (a single experiment, which was not repeated, indicated a two to three fold dilution of exogenous thymidine by intracellular processes). In an attempt to block this biosynthesis, aminopterin was included in the dilute growth medium. Up to a concentration of 10 μg/ml, aminopterin had little if any affect on the growth rate in the dilute medium containing added thymidine. Aminopterin has been reported to cause a requirement for thymidine in another strain, Mycoplasma laidlawii (90).

Growth of Cells for Autoradiography

For autoradiography, M. gallisepticum was grown on dilute medium containing 10 μg/ml of aminopterin and 16 μg/ml of added thymidine. Preliminary experiments indicated that the generation time was 3.6 hours in this medium.

Four separate autoradiography experiments were performed as described in METHODS. In each experiment there was a culture growing in the presence of radioactive thymidine (specific activity about 12 C/mmole) and a control culture growing in nonradioactive thymidine. CFU assays were made and the resulting data from the first experiment indicated that although the radioactivity markedly decreased the amount of growth, the number of viable cells increased about 16 times (from

1×10^7 to 2×10^8 CFU/ml) during the 24 hour growth period. None of the CFU assay plates of the second experiment had any colonies. This probably was due to the surface of the plates being too dry, since the same batch of plates had supported growth a week earlier. The autoradiography of both of these experiments was successful, and the results were not noticeably different. Therefore, growth probably occurred in the second experiment as in the first. The last two experiments were done using different batches of tritium-labeled thymidine and dilute growth medium. In these experiments no growth was indicated for the radioactive culture, and growth with cold thymidine was also poorer than expected on the basis of previous experiments. The autoradiography for these experiments was not successful because different cell lysis procedures were used (0.1% SDS or 1% Brij 58) and the DNA, although sufficiently labeled, was inadequately spread.

Size of DNA Autoradiograms

As shown in Figures 15a and 15b, linear DNA autoradiograms longer than 400 μ were found. Most of the long linear "molecules* had a tangle of DNA at one end, making an accurate length measurement impossible; the molecule in Figure 15a is a good example of this. Linear molecules about 250 μ long were also found that from their

*The word molecule will often be used to refer to DNA autoradiograms. There is, however, no proof that these autoradiograms represent single DNA molecules; several smaller molecules could be joined end to end by "linkers".

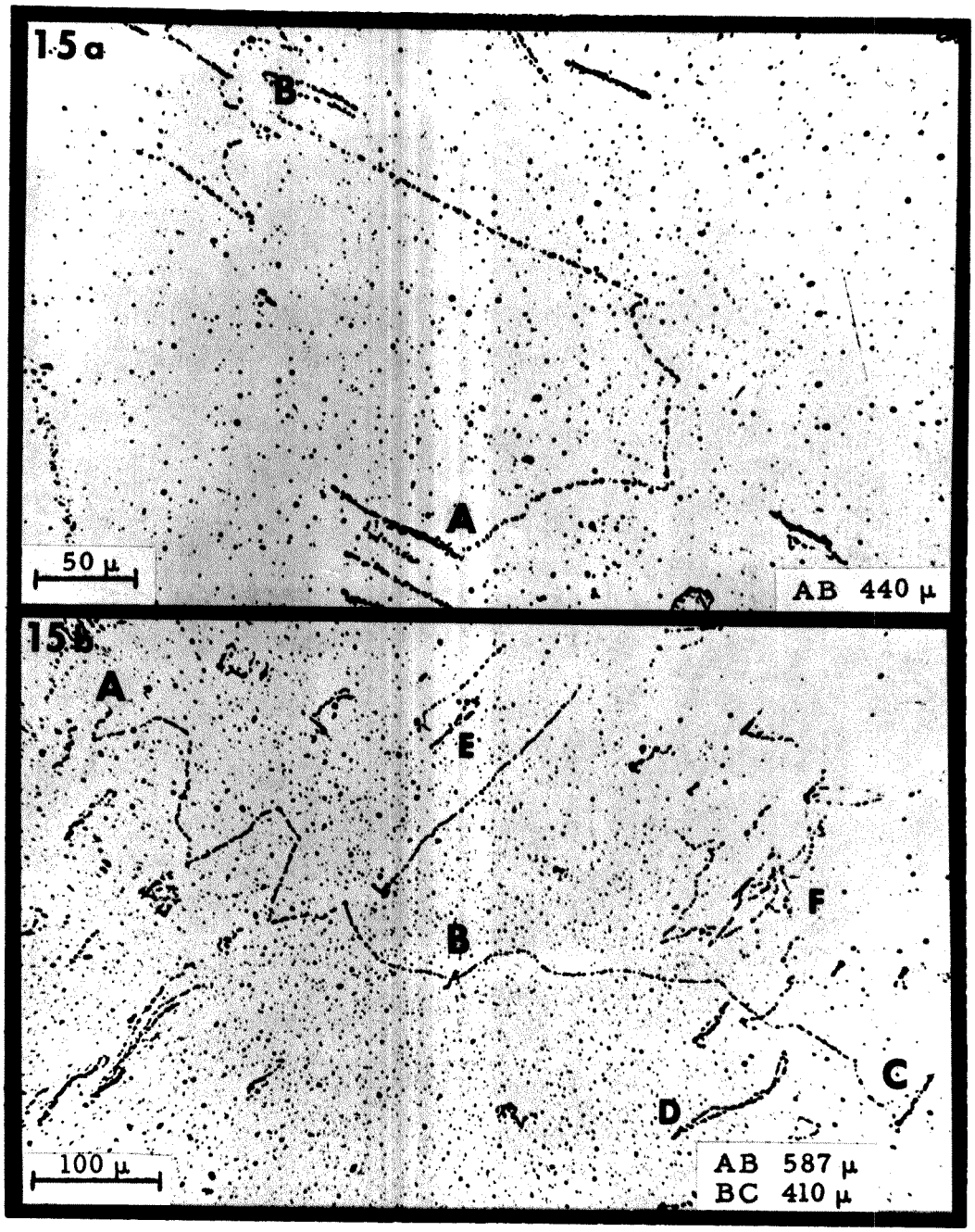

Fig. 15. Autoradiograms of M. gallisepticum DNA. Insets on right of both (a) and (b) give the length of DNA thread between the indicated points. The meanings of points D and E in (b) are explained in text. For both (a) and (b) the concentration of labeled DNA was about 13×10^{-4} μg/ml, and the exposure time was four months.

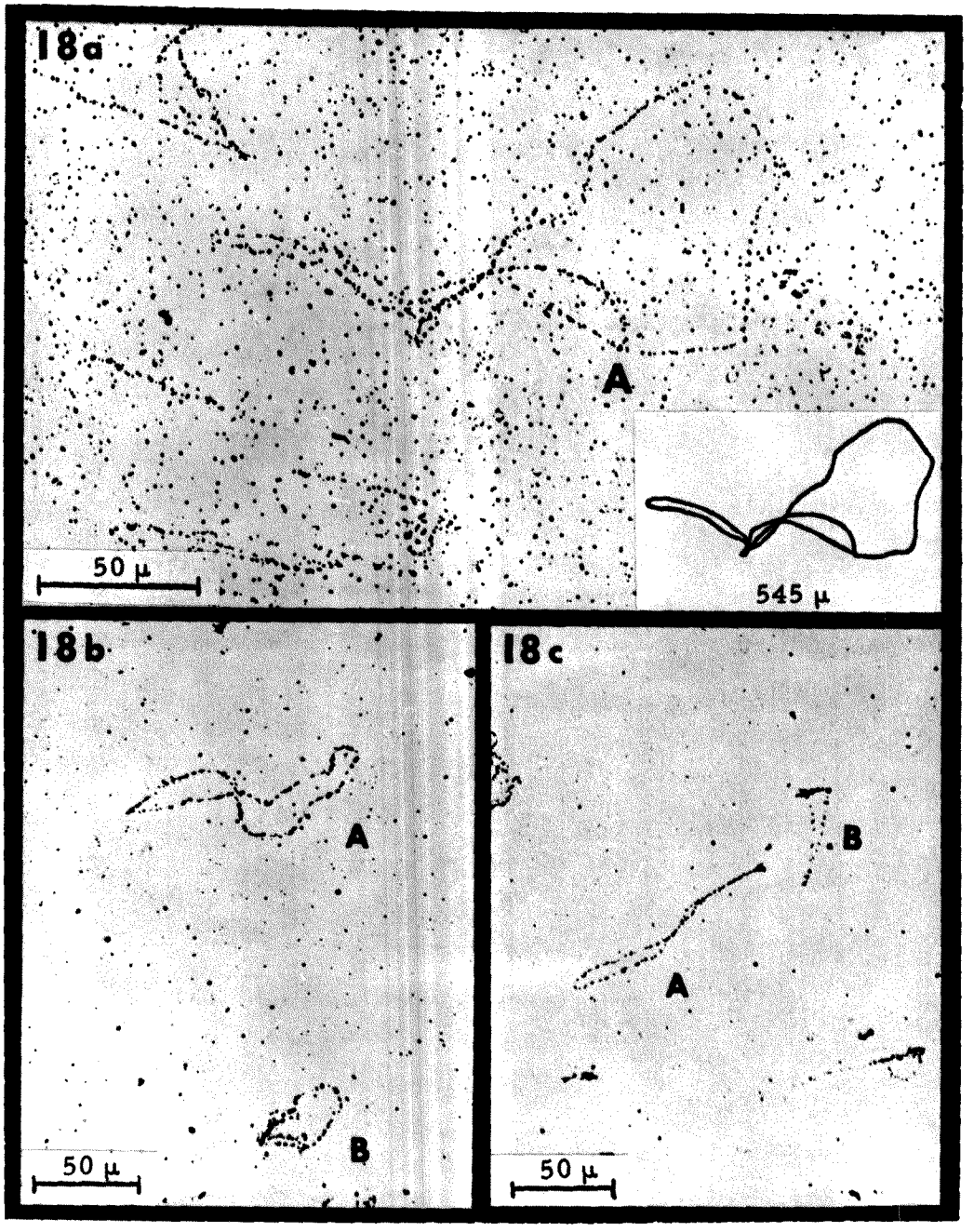

Fig. 18. Autoradiograms of M. gallisepticum DNA. Inset on right of (a) gives a possible interpretation and the resulting chromosome length estimate. The meanings of the capital letters in (a), (b), and (c) are explained in the text. For (a), (b), and (c) the concentration of labeled DNA was about 2 x 10^{-4} μg/ml, and the exposure time was 4 months.

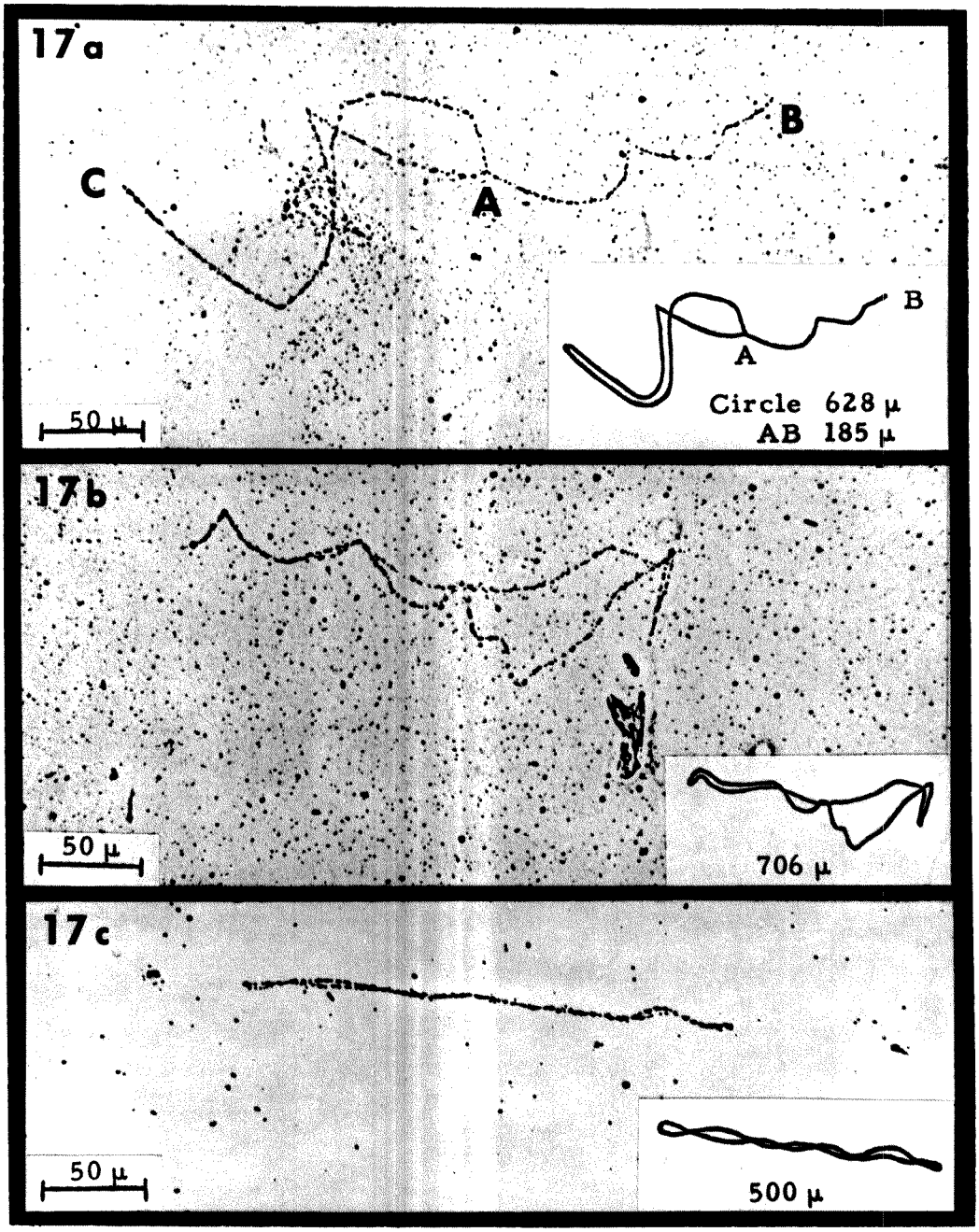

Fig. 17. Autoradiograms of M. gallisepticum DNA. Insets on right give possible interpretations and the resulting chromosome length estimates. The meanings of the capital letters are explained in the text. For (a), (b), and (c) the concentration of labeled DNA was about 2 x 10^{-4} μg/ml. (a), (b) Exposure time was 4 months. (c) Exposure time was 5 months.

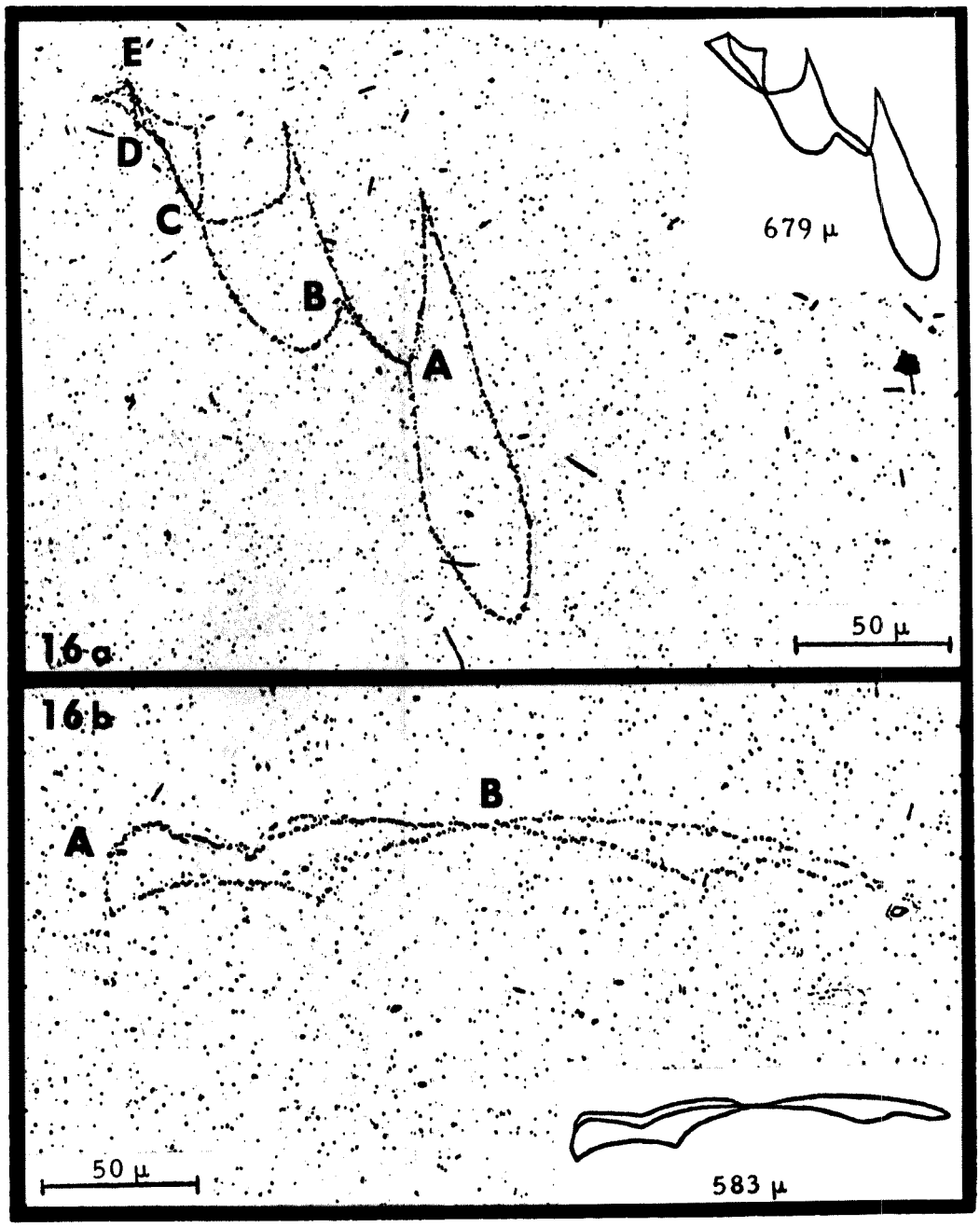

Fig. 16. Autoradiograms of M. gallisepticum DNA. Insets on right of both (a) and (b) give possible interpretations and the resulting chromosome length estimates. The meanings of the capital letters in both (a) and (b) are explained in the text. (a), (b) The concentration of labeled DNA was about 2 x 10^{-4} μg/ml. (a) Exposure time was 5 months. (b) Exposure time was 4 months.

grain density (grains per unit length) appeared to be double. Occasionally the threads of such a molecule were partially separated, confirming that it was indeed double (see Figure 17c).

Most molecules, such as the one displayed in Figure 16a, can best be interpreted with the aid of quantitative grain counts obtained from higher power micrographs. On the average, the molecule in Figure 16a has a grain density of about 0.5 grains per micron, but regions AB and CD have 0.9-1.1 grains per micron. These regions, therefore, consist of two adjacent threads of DNA, as is suggested visually for the region AB. There can be little doubt (especially when all molecules observed are considered) that the DNA to the right of C is one continuous thread 567 μ long; but this is not the complete molecule. Since the region CD is double, point C has 5 DNA threads emanating from it. It follows that one thread either begins or ends at this point; thus, this molecule was probably in the act of replicating. Point E was interpreted as having 3 strands converging to it. With the preceding interpretation of points C and E, the molecule must be very nearly as shown in the inset of Figure 16a. Allowing for the extra DNA due to replication, the complete molecule is 679 μ long. Variations in the interpretation of the region to the left of C have small effect on the size estimate.

Autoradiography can not be expected to give extremely accurate size estimates. Some stretching and undetected folding or coiling could and apparently does occur. Cairns (84, 91) found that molecules of T2 DNA with about the same number of total grains varied from 45

to 59 μ in length; molecules of λ DNA varied from 13 to 23 μ. For larger DNA molecules a similar variation was found, a value of 1100 to 1400 μ being reported by Cairns (85) for the chromosome of E. coli. Therefore the molecules shown in Figures 16b thru 18a all support the length estimate obtained from Figure 16a. The interpretation of each molecule is shown at the lower right of each Figure along with the resulting size estimate. In each case the interpretation was made with the aid of grain counts and an analysis similar to that given in detail for Figure 16a. For most molecules, alternative reasonable interpretations would not greatly affect the size estimate.

Note that if the chromosome before replication in Figure 17a is considered to run from C to B, then the chromosome would be 500 μ long, and this establishes a rather firm lower limit on the chromosome size estimate.

Since the molecules interpreted as circles suggest that the size of the chromosome is around 630 μ, the complete linear DNA thread shown in Figure 15b is too long to be considered one molecule. The excess length must be interpreted as due to replication or aggregation. It is probably not a coincidence that 587 μ from the end A there is a small tangle of DNA (Point B), making such a interpretation almost mandatory. (This was the only linear molecule found that could be interpreted as longer than 600 μ.)

Size Distribution of DNA Autoradiograms

In these experiments, most molecules were not spread in such a way that an unambiguous interpretation of structure was possible. However, in favorable areas, most were sufficiently spread that an estimate of total DNA content was possible, and the size distribution shown in Figure 19 was obtained. All linear and tangled molecules in an area covering about one-third of a millipore filter were photographed and measured. If there was doubt as to whether a tangle of DNA should be considered one molecule or two, it was considered to be two. Often tangles of DNA would have centers or folded areas with confluent grains. A great deal of DNA could be hidden in these centers. Therefore, the length estimates are probably underestimates.

Most molecules were small, 47% being less than or equal to 190 μ. The peak at 145 μ is probably not significant, especially since molecules shorter than 25 μ were difficult to distinguish from background and many could have been missed. At any rate, the molecules were not sharply clustered in a particular size range. A size distribution of this pattern may be characteristic of the method, because as will be shown in Part II of this thesis, a similar size distribution was obtained for autoradiograms of Chinese Hamster DNA. This type of distribution very likely arose from the degradation of larger molecules during the procedure. Degradation could result from one or a combination of several factors: 1. Shear breakage; 2. Radiation damage; 3. DNase activity during the Cairns procedure; 4. DNase activity in damaged or dead cells before the Cairns procedure; 5. Poor

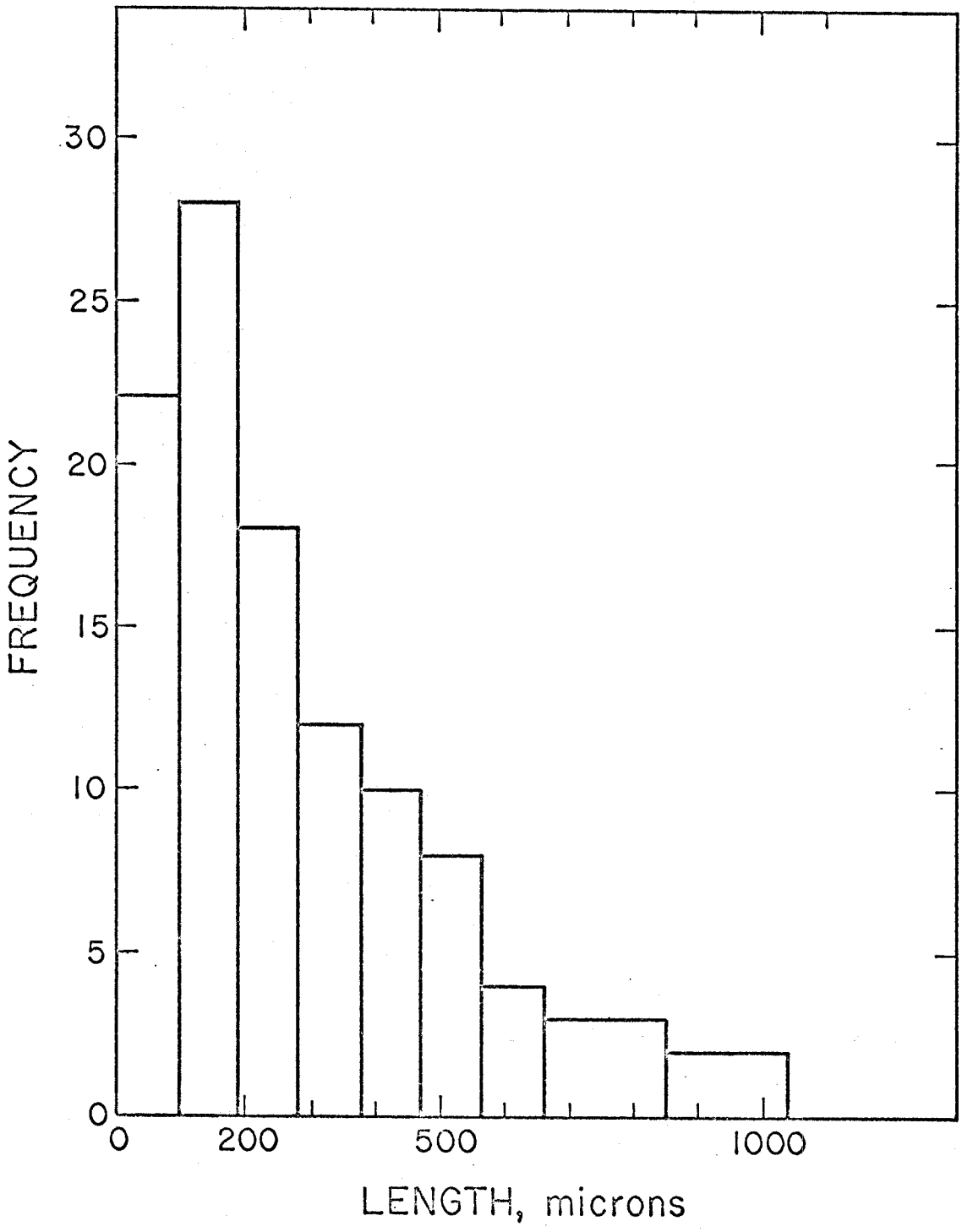

Figure 19. Size distribution of M. gallisepticum DNA autoradiograms. Concentration of labeled DNA was about 2×10^{-4} µg/ml.

spreading of DNA. Poor spreading was definitely a factor but its quantitative significance is unknown.

The most critical point to be made from the size distribution, however, is that molecules 500 μ or longer were certainly not rare, making up 20% of the total. Thus the size distribution does not dispute the value of 630 ± 150 μ arrived at by an analysis of the rare (1-3%) well spread circular and linear molecules. The "molecules" with greater than 750 μ of DNA are easily explained as resulting from DNA replication.

Replication Forks

Forks, such as those in Figure 17a, at point A and Figure 18a at point A, were seen quite frequently. About 10% of the molecules photographed for the size distribution were found to have a definite fork, but even more presumptive forks were present. The forks seen were almost all like the fork in Figure 17a in that all three arms of the fork were of equal grain density, and there was no detectable tangling of DNA at the center of the fork. Since the cells were probably growing logarithmically, these forks are likely to be "replication forks" such as seen by Cairns in E. coli (72, 85).

To confirm that these are indeed replication forks it would be desirable to observe [as Cairns (72) has done] pulse labeled molecules and molecules that were doubly labeled in one arm and singly labeled in the other arms. Experiments designed to produce such molecules were not done because under the present labeling conditions, DNA helices with only one strand labeled would be difficult to detect.

Even with both strands labeled, four month exposure was necessary to get adequate grain densities. This suggests that the radioactive thymidine was considerably diluted before its incorporation into DNA, despite the precautions taken to prevent such dilution.

Circles

As shown in Figures 16a thru 18a autoradiograms were obtained that could be interpreted as circles and circles replicating as circles. The autoradiogram in Figure 16b is perhaps the clearest example obtained in the present study of what may be an unbroken replicating circular chromosome. This, of course, agrees with the chromosome structure and mode of replication seen by Cairns (72, 85) for E. coli, and may be one of the most significant observations made in the present study. However, these circular molecules were found only after a very extensive search; thirty fairly clear circles of the appropriate size were found out of an estimated 3,000 to 10,000 molecules. These circles must, therefore, be somewhat suspect. Moreover, smaller circles, varying uniformly from about 100 μ to 450 μ in circumference, were present at about the same frequency as the larger ones. Figure 18b has an example of an almost perfect small circle at point A. These small circles usually had roughly the same grain density as the larger circles.

Cairns (72) also observed circles of varying sizes in E. coli and interpreted them as arising from breakage, at both the growing point and the initiation point, of a circular molecule replicating

as a circle. This is credible because both of these points are unique and might be weaker than the rest of the molecule. However, in the present study, it is also possible that these circles were artifacts of a different sort. Molecules were often folded and tangled so that circles were produced (see Figure 15b point F; Figure 18b point B; Figure 18c point B). Usually these circles were recognized as artifacts without difficulty, being just a loop of DNA apparently dragged out of a tangled center; but sometimes the center was less obvious or not present (see Figure 18b point A; Figure 18c point A; Figure 15b points D and E). The maximum path length of a β particle from a tritium decay is only about 2 μ in stripping film (91), and a single grain may develop to about one micron, so many centers of DNA could conceivably be hidden. Many small circles do seem to have small, barely detectable centers, for example, point A in Figure 18c. These centers could be the remnants of the original cells, which were less than 0.4 μ in diameter. If most of the DNA had escaped from the cell remnant, as would be the case for the large molecules seen, it would be especially easy for the "center" to go undetected. If this explanation were correct, then the autoradiographic data reported here can not give strong evidence as to circularity; the ends of apparently circular molecules could be separated by the entire width of the original cell.

Significance of the Autoradiographic Length Measurements

The autoradiography experiments were carefully scrutinized for sources of large error and none was found, as the following discussion will indicate.

For these experiments, the cells were grown, using sterile technique, in medium containing penicillin and thallium acetate, both of which are potent growth inhibitors for bacteria. Additionally, the radioactive culture was assayed for growth by plating on solid medium, and no colonies other than those characteristic of M. gallisepticum were found.* Two completely independent successful experiments were done, and the results of the first agreed with those of the second. Thus, contamination of the radioactive cultures can be ruled out.

The only possible sources of error in the Cairns autoradiographic procedure leading to large overestimates of length are:

1. Errors in magnification and in measurement of the autoradiograms.
2. Stretching of the DNA molecules.
3. Stretching of the stripping film.
4. Intermolecular aggregation of some sort.
5. Complete artifacts, e.g., scratches.

The first possible source of error can be eliminated because both the magnification and the measurement steps were carefully checked. The

* It should be pointed out that even in the experiment where no mycoplasma colonies were obtained, the lack of bacterial or fungal colonies is strong evidence against contamination.

combined error of the magnification steps was always less than 3%; the map measuring device used to measure the autoradiographs had less than 1% error when measuring reasonably linear molecules and even for highly tangled molecules the reproducibility was better than 5%.

The second and third possibilities combined are unlikely to lead to errors greater than about 20%, because: a) this procedure gives reasonable length estimates for λ, T2, and E. coli chromosomes (72, 84, 85, 91); b) the area of the stripping film that covered the Millipore filter could usually be distinguished after processing of the film was completed, and this area remained the same size and shape as the filter it had covered. Thus, no net stretching of the film occurred; c) the grain density of highly stretched molecules should be much less than the average grain density, and this was not the case for the great majority of the long molecules seen.

The fourth possibility, aggregation, is rendered unlikely by the fact that during lysis and dialysis, the concentration of radioactive DNA was very low; depending on the dilutions made prior to lysis, it was either about 2×10^{-4} or about 13×10^{-4} μg/ml. Aggregation should, then, be a rare event, and, moreover, should be strongly concentration dependent. Since about 20% of the DNA molecules were "long" regardless of the labeled DNA concentration, aggregation while in solution was probably not a significant factor. Furthermore, a different type of aggregation, overlap of molecules on the filter, can not be a significant factor, because most overlaps would be

recognized as such, and overlaps were not common since less than a thousand DNA molecules were present on most Millipore filters.

The fifth possibility, complete artifacts, was given careful consideration. Occasional scratches and non-DNA fibers were present, but with a little experience these could easily be distinguished from true DNA autoradiograms; the structure of DNA autoradiograms is quite distinctive. The best argument for the reality of what are considered to be DNA autoradiograms is that the concentration of DNA threads seen varied as expected with the concentration of labeled DNA.

It therefore seems that even a combination of the possible errors cannot be used to explain away the large molecules; as determined by this method, the chromosome of M. gallisepticum is almost certainly about 630 ± 150 μ long.

Morowitz et al.(46) have shown that on the basis of its base composition, buoyant density, and melting behavior, the DNA of M. gallisepticum exists as a typical Watson-Crick double helix. As will be shown in the section of this thesis titled Kleinschmidt Technique Results, the DNA also appears "normal" in the electron microscope. If during the exposure of the autoradiographic film, the DNA was in the B form i.e., 1.92×10^6 daltons per micron (92,94) then a DNA autoradiogram 630 ± 150 μ long corresponds to $1,200 \pm 300 \times 10^6$ daltons of DNA.

Kleinschmidt Technique Results

The length of the chromosome of M. gallisepticum was also estimated from measurements based on electron micrographs such as Figure 20, in which DNA threads are seen spreading out from a "center". DNA threads of this type spreading from a center are characteristic results of the Kleinschmidt technique when the DNA is released from a microorganism by osmotic shock during the spreading procedure (74, 89). The frequent pairing and what appears to be coiling of the DNA threads in Figure 20 and 21 may not be characteristic results of the technique per se and will be commented on later.

The cluster of DNA threads in Figure 20 is one of the smallest clusters that were seen. Obviously larger clusters were common, and these were thought to arise from the lysis of a clump of cells. This is reasonable because examination of the cell suspension before spreading it on a water surface showed that clumps of cells were present. However, single cells were also present, and it was therefore assumed that the smallest clusters of DNA were derived from single cells. Even these small clusters had a large amount of DNA, much of which was highly tangled or coiled. As a result, accurate length measurements were impossible, but the total length of DNA in the cluster in Figure 20 was estimated to be 508 μ; and the length estimates of three other small clusters were 497, 432 and 557 μ.

More extensive measurements were not done because; a) the large amount of DNA in the clusters made measurement very tedious; b) the tangling and frequent tight coiling of the DNA threads made it

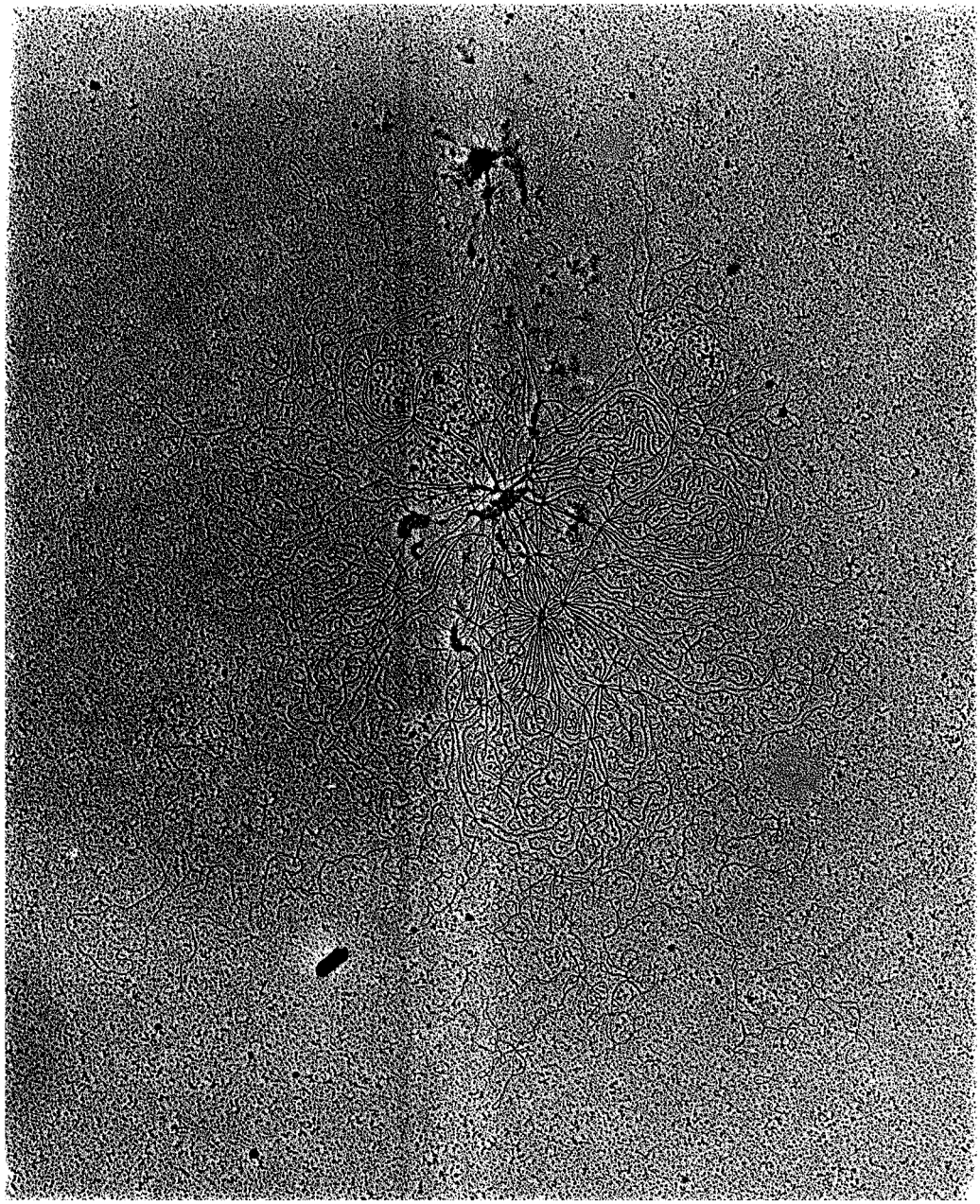

Fig. 20. Electron micrograph of DNA threads released from a M. gallisepticum cell by osmotic shock and trapped on a cytochrome c monolayer according to the method of Kleinschmidt et al. (89). X 19,600.

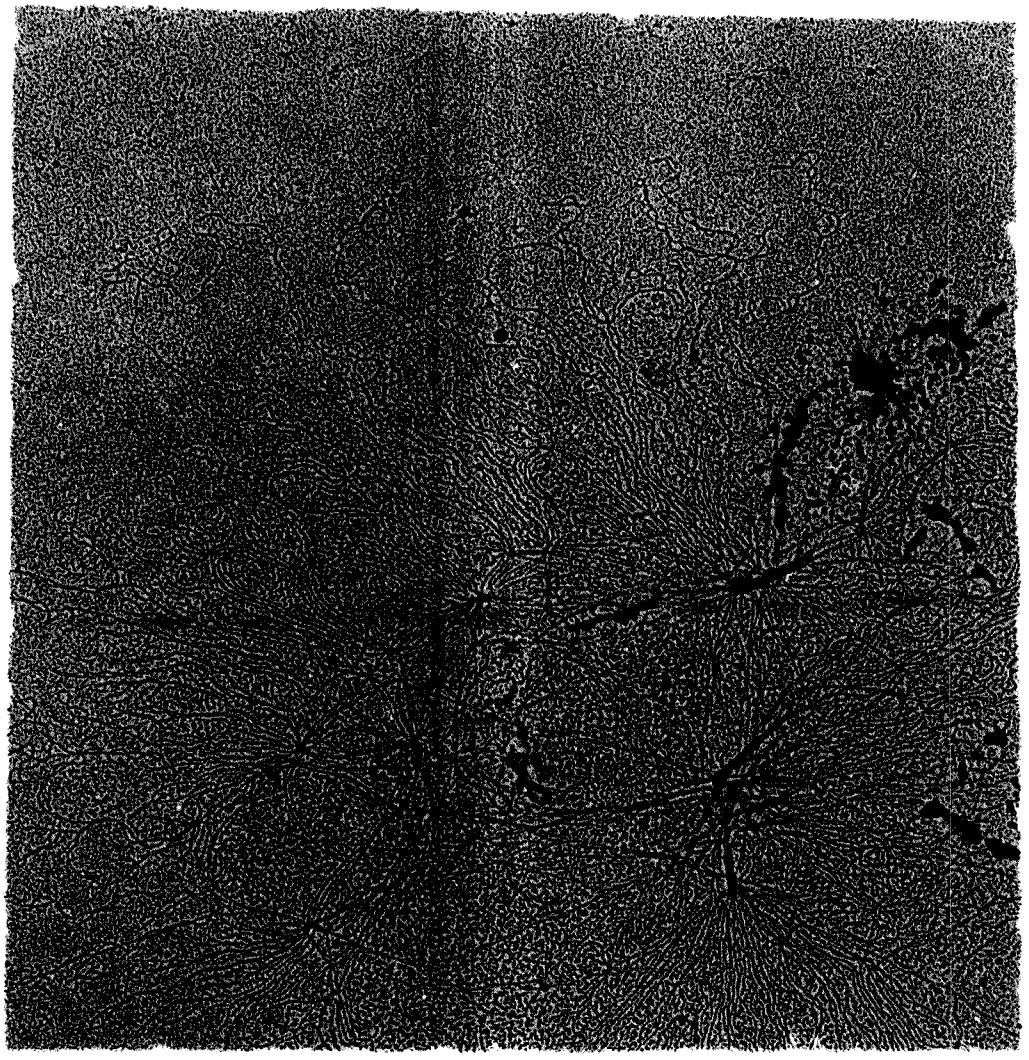

Fig. 21. Electron micrograph of DNA threads released from a M. gallisepticum cell by osmotic shock and trapped on a cytochrome c monolayer according to the method of Kleinschmidt et al. (89). X 31,600.

impossible to follow them closely with a map measurer, and it was often difficult to decide whether a thread was single or double; thus the error in the length measurements could be very large, perhaps as much as 40%; c) after a few measurements, comparisons by eye were probably sufficiently accurate.

Literally thousands of DNA clusters were scanned, and a class of smaller clusters was not found*. If the chromosome of M. gallisepticum had been, for example, 150 μ, then clusters of this size would easily have been distinguished from the larger clusters. Therefore, the following rather firm conclusion can be drawn from the Kleinschmidt studies: the total length of DNA in a M. gallisepticum cell must be "large"; it is almost certainly 500 ± 200 μ. Since only stationary phase cells were used for these studies, most cells may contain only a single complement of DNA (93), and if this is so, the length of the M. gallisepticum chromosome suggested by these studies agrees rather well with the length estimate obtained from the autoradiographic studies.

There is good evidence (see 92) that DNA trapped on a cytochrome c film and observed according to the Kleinschmidt procedure is in the B configuration and thus contains 1.92×10^6 daltons of DNA per micron. Therefore, as determined by this method, the DNA content of M. gallisepticum cells is at least $1{,}000 \pm 400 \times 10^6$ daltons.

---

* Some very short DNA threads as small as 5 μ were usually present but these were of variable size and thus are probably degradation products. It should also be pointed out that most clusters contained two or more "ends", but since there may have been some degradation, the significance of these ends cannot be ascertained.

Observations on Untangling of the DNA

A major problem with the Kleinschmidt technique experiments was to obtain sufficient untangling of the DNA threads so as to produce measurable clusters of DNA. In the early experiments, cells of M. gallisepticum were pelleted by centrifugation, resuspended in distilled water, and then mixed with an ammonium acetate-cytochrome c solution before spreading on a water surface. In all these experiments, the DNA threads were, as a rule, paired and apparently coiled around each other forming "supercoils" similar in appearance to those found for polyoma virus DNA by Vinograd et al. (97). In addition, a great deal of DNA was usually in the center of the cluster. Figure 21 has an example of this type of cluster and also many good examples of supercoils.

In later experiments, it was found that if cells were resuspended in saline plus 0.02 M EDTA at pH 8.5 and then allowed to incubate at 37° C for at least 10 minutes before adding the ammonium acetate solution and spreading, all supercoils and, in fact, almost all clusters disappeared. The DNA was actually spread too much for measurement of large molecules; several sequential electron micrographs were required to follow large molecules, and the molecules were eventually "lost".

If the incubation in the presence of EDTA was done at 0° C for as long as 30 minutes, tight clusters with many supercoils were still prevalent. Incubation at room temperature for 15 to 30 minutes produced some "loosening" of the clusters, although supercoils were still

common. The length measurements were obtained from such slightly "loosened" clusters.

The significance of the supercoils is difficult to evaluate, but the coiling probably does not occur after lysis of the cells. There are at least three reasons for making this conclusion:

1. Conditions at the time of lysis and spreading were kept the same, only the incubation conditions were varied.
2. The actual spreading of the film did not seem to affect the results. In one experiment, cytochrome c was replaced by trypsin, and although the spreading of the protein film was much better than usual, only tight clusters and supercoils were observed.
3. Under the conditions of spreading that were used, λ DNA did not form any supercoils.

If the supercoils did not form at the time of lysis, then they were present before lysis, and they may well be the result of the high degree of ordering that is required to keep the DNA compacted within a nuclear region smaller than the whole cell.

Kleinschmidt et al. (89) also observed "supercoils" when spheroplasts of M. lysodeikticus were formed and spread according to their standard procedure, in which the spheroplasts were formed by the action of lysozyme at pH 5.0. However, they found that if the pH was raised to pH 7.0 for the incubation with lysozyme, not only was the untangling of the DNA better but shorter DNA threads were also observed. They

attributed this result to the activation of a DNase at the higher pH. Some preliminary results obtained in the present investigation also suggest that the untangling of the DNA may be aided by DNase activity. It was found that if M. gallisepticum cells were incubated in 0.10 M EDTA, the untangling of the DNA threads seemed to be less than if the incubation was done in 0.02 M EDTA. The explanation for this result could be that 0.02 M EDTA kills the cell, activating a DNase; but does not completely inhibit DNase activity because of the presence of divalent cations from the growth medium. The higher concentration of EDTA may more fully inhibit DNase activity. Alternative explanations cannot, of course, be eliminated without further experimentation.

DISCUSSION

As stated in the introduction, the purpose of part of the present investigation was to confirm the cell size and DNA content of M. gallisepticum A5969 reported by Morowitz et al. (46). A perusal of Table 4, in which a summary of both the published results and the results reported here are presented, will show that the published data could be repeated, and thus they were correct within the limitations of the methods employed. However, the results of several additional experiments reported here suggest that the methods employed by Morowitz et al. did not give valid results. For reasons already mentioned, the value for the cellular DNA content based on the autoradiography experiments and supported by the Kleinschmidt experiments is probably a sound lower limit*, but this value is at least four times higher than any based on chemical analyses of harvested cells.

The direct TCA precipitation experiments, in which harvesting of the cells by centrifugation was avoided, were the last experiments performed and were done in an effort to find the explanation for the large discrepancy between the results based on chemical analyses and those based on the more direct physical techniques. At first glance, the DNA content values obtained using the direct TCA precipitation procedure seem to be in good agreement with those obtained using the autoradiography and Kleinschmidt procedures. The close agreement may

---

* The conversions from length units to molecular weight units were made using the value 1.92×10^6 daltons per micron. Cairns (85) has used a somewhat higher conversion factor of 2.54×10^6 daltons per micron.

TABLE 4.

Summary of the Results of the Present Investigation and also of Published Results

Description	Results Reported Here	Published Results (46)
1. Average cell volume		
a) by electron microscopy	about 17×10^{-15}, but one experiment suggested 7×10^{-15} cm^3	8×10^{-15} cm^3
b) by phase microscopy	$36 \pm 9 \times 10^{-15}$ cm^3	-
2. Percentage DNA of harvested cells	4.0 ± 0.6	4.0 ± 0.4
3. DNA content based on cell size and percentage DNA	$240 \pm 60 \times 10^6$ daltons per cell*	50×10^6 daltons per cell
4. DNA/CFU		
a) based on DNA analyses of harvested cells	300×10^6 daltons/CFU	$250 \pm 178 \times 10^6$ daltons/CFU
b) based on DNA analyses of unharvested cells	$1200 \pm 200 \times 10^6$ daltons/CFU	-
5. DNA content as determined by autoradiography	$\geq 1200 \pm 300 \times 10^6$ daltons/cell	-
6. DNA content as determined by the Kleinschmidt technique	$\geq 1000 \pm 400 \times 10^6$ daltons/cell	-

* The difference between this value and the published value is due to the use of a larger cell size in the calculations.

be more apparent than real, however, because first of all, these analyses were performed on logarithmically growing cells and their DNA content should be larger than the minimum value by a factor of 1/ln 2 (85). Secondly, the DNA content values obtained using this procedure are based on CFU assays, and, in light of their fastidiousness with regard to growth conditions, there is no compelling reason to believe that the "plating efficiency" of M. gallisepticum cells is 100%. Nevertheless, the results of the direct TCA precipitation experiments do suggest that DNA loss during harvesting by centrifugation is the explanation for a large part of the discrepancy.

The chromosome of M. gallisepticum seems to be similar in structure to that of bacteria. The displays of DNA threads observed by electron microscopy are not greatly different either from those observed by Kleinschmidt et al. (89) for Micrococcus lysodeikticus or from those observed by MacHattie et al. (94) for Hemophilus influenzae. The DNA autoradiograms obtained for M. gallisepticum were also similar in structure to those obtained by Cairns (71, 72, 85) for E. coli. The length of the chromosome of M. gallisepticum is, moreover, only about 50% smaller than that of E. coli; the chromosome of M. gallisepticum is almost certainly 630 ± 150 μ long, whereas that of E. coli has been reported by Cairns (85) to be 1250 ± 150 μ long. The chromosome of Hemophilus influenzae may even be smaller than that of M. gallisepticum. Berns and Thomas (95) found that the DNA complement of Hemophilus is probably $750 \pm 50 \times 10^6$ daltons,

which suggests that the chromosome is only about 400 μ long. Using the Kleinschmidt technique, MacHattie et al. (94) found about 800 μ of DNA per Hemophilus cell, but most cells were thought to have two nuclei. Since, in terms of chromosome size or DNA content, M. gallisepticum is not greatly smaller than some bacteria, it should also be comparable in functional complexity, despite its very small cell size.

It is of interest to note that Maniloff et al. (52) found that from an ultrastructural point of view, M. gallisepticum cells appear to be highly organized, and they reasoned:

> A small organism could be expected to function within its spatial limitations in one of two extreme ways: either it could be completely structureless with all cellular processes being carried on in a homogeneous phase, or it could be highly structured thereby imposing a high degree of organization on all its processes. The latter choice seems to have been made in M. gallisepticum . . . The replicative structures seem formidable for such a small cell . . .

A highly organized cell would presumably require a large amount of genetic information, such as could be supplied by the large chromosome and DNA complement reported here.

Other mycoplasma species may have smaller chromosomes than M. gallisepticum: as determined by autoradiography, M. laidlawii A has a chromosome about 400 μ long (96) and Kleinschmidt technique results have suggested that the mycoplasma H39 has a chromosome only about 200 μ long (93). If functional simplicity is desired, H39, not M. gallisepticum appears to be the best organism for further study.

REFERENCES

1. Edward, D.G., J. Gen. Microbiol. 10, p. 27 (1954).

2. Nocard, E., and E.R. Roux, Ann. Inst. Pasteur 12, p. 240 (1898).

3. Bordet, J., Ann. Inst. Pasteur 24, p. 161 (1910).

4. Borrel, A., E. Dujardin-Beaumetz, Jeantet and Jouan, Ann. Inst. Pasteur 24, p. 168 (1910).

5. Bridre, J., and A. Donatien, C. R. Acad. Sci., Paris 177, p. 841 (1923).

6. Shoetensack, H.M., Kitasato Arch. 11, p. 277 (1934).

7. Sabin, A.B., Bacteriol. Rev. 5, p. 1 (1941).

8. Hayflick, L., and R.M. Chanock, Bacteriol. Rev. 29, p. 185 (1965).

9. Edward, D.G. ff., and E.A. Freundt, J. Gen. Microbiol. 14, p. 197 (1956).

10. Breed, R.W., E.G.D. Murray, and N.R. Smith, Bergey's Manual of Determinative Bacteriology, 7th ed., The Williams & Wilkins Co., Baltimore (1957).

11. Dienes L., in Recent Progress in Microbiology VII, p. 511, Univ. of Toronto Press (1963).

12. Klieneberger-Nobel, E., Pleuropneumonia-like Organisms (PPLO): Mycoplasmataceae, Academic Press, Inc., New York (1962).

13. Domermuth, C.J., M.H. Nielson, E.A. Freundt, and A. Birch-Anderson, J. Bacteriol. 88, p. 727 (1964).

14. Van Iterson, W., and A.C. Ruys, J. Ultrastruct. Res. 3, p. 282 (1960).

15. Elford, W.J., Brit. J. Exp. Path. 10, p. 26 (1929).

16. Morowitz, J.H., M.E. Tourtellotte, and M.E. Pollack, J. Bacteriol. 85, p. 134 (1963).

17. Cuckow, R.W., and E. Klieneberger-Nobel, J. Gen. Microbiol. 13, p. 149 (1955).

18. Freundt, E.A., Ann.N.Y. Acad. Sci. 79, p. 312 (1960).

19. Weibull, C., and B.M. Ludin, J. Bacteriol. 84, p. 513 (1962).

20. Wittler, R.G., S.G. Cary, and R.B. Lindberg, J. Gen. Microbiol. 14, p. 763 (1956).

21. Kelton, W.H., R.F. Gentry, and E.H. Ludwig, Ann. N.Y. Acad. Sci. 79, p. 410 (1960).

22. Smith, P.F., D.M. Peoples, and H.E. Morton, Proc. Soc. Exp. Biol. N.Y. 96, p. 550 (1957).

23. McKay, K.A., and R.B. Truscott, Ann. N.Y. Acad. Sci. 79, p. 465 (1960).

24. Rogul, M., Z.A. McGee, R.G. Wittler, and S. Falkow, J. Bacteriol. 90, p. 1200 (1965).

25. McCarthy, B.J., and E.T. Bolton, Proc. Nat. Acad. Sci. U.S. 50, p. 156 (1963).

26. Dienes, L., and H.J. Weinberger, Bacteriol. Rev. 15, p. 245 (1951).

27. Smith, P.F., Bacteriol. Rev. 28, p. 97 (1964).

28. Weibull, C., Proc. Soc. Exp. Biol. N.Y. 113, p. 32 (1963).

29. Weibull, C., J. Bacteriol. 90, p. 1467 (1965).

30. Razin, S., in Pleuropneumonia-like Organisms (PPLO): Mycoplasmataceae (E. Klieneberger-Nobel, author), p. 91, Academic Press Inc., N.Y. (1962).

31. Tourtellotte, M.E., H.J. Morowitz, and P. Kasimer, J. Bacteriol. 88, p. 11 (1964).

32. Razin,S., and A. Cohen, J. Gen. Microbiol. 30, p. 141, (1963).

33. Fabricant, C.G., J. Fabricant, and P.J. van Demark, J. Gen. Microbiol. 35, p. 135 (1964).

34. Smith, S.L., P.J. van Demark, and J. Fabricant, J. Bacteriol. 86, p. 893 (1963).

35. Smith, P.F., Ann. N.Y. Acad. Sci. 79, p. 543 (1960).

36. Schimke, R.T., and Barile, M.F., J. Bacteriol.86, p. 195 (1963).

37. Tourtellotte, M.E., and R.E. Jacobs, Ann.N.Y. Acad. Sci. 79, p. 521 (1960).

38. Castrejon-Diez, J., T.N. Fisher, and E. Fisher, Jr., J. Bacteriol. 86, p. 627 (1963).

39. Somerson, N.L., and H.E. Morton, J. Bacteriol. 65, p. 245 (1953).

40. Tourtellotte, M.E., R.G. Jenson, G.W. Gander, and H.J. Morowitz, J. Bacteriol. 86, p. 370 (1963).

41. Tourtellotte, M.E., and H.J. Morowitz, Bacteriol. Proc., p. 112 (1963).

42. Smith, P.R., and G.H. Rothblat, J. Bacteriol. 83, p. 500 (1962).

43. Plackett, P. Biochem. Biophys. Acta 35, p. 260 (1959).

44. Razin, S., M. Argaman, and J. Avigan, J. Gen. Microbiol. 33, p. 477 (1963).

45. Langenfeld, M.G., and P.F. Smith, J. Bacteriol. 86, p. 1216 (1963).

46. Morowitz, H.J., M.E. Tourtellotte, W.R. Guild, E. Casto, and C. Woese, J. Mol. Biol. 4, p. 93 (1962).

47. Jones, A.S., and R.T. Walker, Nature 198, p. 588 (1963).

48. Lynn, R.J., and P.R. Smith, J. Bacteriol. 74, p. 811 (1957).

49. McGee, Z.A., N. Rogul, S. Falkow, and R.G. Wittler, Proc. Nat. Acad. Sci. U.S. 54, p. 457 (1965).

50. Neimark, H.C., and J.J. Pene, Proc. Soc. Exptl. Biol. Med. 118, p. 517 (1965).

51. Maniloff, J., J.H. Morowitz, and R.J. Barrnett, J. Bacteriol. 90, p. 193 (1965).

52. Maniloff, J., J.H. Morowitz, and R.J. Barrnett, J. Cell Biol. 25, p. 139 (1965).

53. Domermuth, C.J., and M. Nielson, E.A. Freundt, and A. Birch-Anderson, J. Bacteriol. 88, p. 1428 (1964).

54. Razin, S., H.J. Morowitz, and T.M. Terry, Proc. Nat. Acad. Sci. U.S. 54, p. 219 (1965).

55. Eaton, M.D., Ann. Rev. of Microbiol. 19, p. 379 (1965).

56. Pollock, M.E., G.E. Kenney, and J.T. Syverton, Proc. Soc. Exptl. Biol. Med. 105, p. 10 (1960).

57. Barile, M.F., W.F. Malizia, and D.B. Riggs, J. Bacteriol. 84, p. 130 (1962).

58. Macpherson, I.A., and K. Allner, Nature, 186, p. 992 (1960).

59. Rothblatt, G.H., and H.E. Morton, Proc. Soc. Exptl. Biol. Med. 100, p. 87 (1959).

60. Carski, T.R., and C.C. Shepard, J. Bacteriol. 81, p. 626 (1961).

61. Luria, S.E., in The Bacteria (I.C. Gunsalus and R.Y. Stanier, eds.) Vol. I, p. 1, Academic Press, N.Y. (1960).

62. Fahey, J.E., and J.F. Crawley, Canad. J. Comp. Med. 18, p. 264 (1954).

63. Vinograd, J., and J.E. Hearst, Fortschr. Chem. Org. Naturstoffe. 20, p. 372 (1962).

64. Jervell, C.R., Arch. Biochem. Biophys. 78, p. 157 (1958).

65. Wall, J.S., Anal. Chem. 25, p. 950 (1953).

66. Shikawa, K., Nature 207, p. 529 (1965).

67. Inman, R.B., and D.O. Jordon, Biochem. Biophys.Acta 43, p. 206 (1964).

68. Tamm, C., M.E. Hoden, and E. Chargaff, J. Biol. Chem. 195, p. 49 (1952).

69. Bush, E.T., Anal. Chem.35, p. 1024 (1963).

70. Keck, C., Arch. Biochem. Biophys. 63, p. 446 (1956).

71. Cairns, J., J. Mol. Biol. 4, p. 407 (1962).

72. Cairns, J., J. Mol. Biol. 6, p. 208 (1963).

73. Boyd, G.A., Autoradiography in Biology and Medicine, p. 213, Academic Press Inc., N.Y. (1955).

74. Kleinschmidt, A.K., D. Lang, D. Jacherts, and R.K. Zahn, Biochem. Biophys. Acta 61, p. 857 (1962).

75. Del Guidice, R.A., and R. Pavia, Bacteriol. Proc., p. 71 (1964).

76. Vinograd, J., J. Morris, N. Davidson, and W.F. Dove, Jr., Proc. Nat. Acad. Sci. U.S. 49, p. 12 (1963).

77. Marmur, J., J. Mol. Biol. 3, p. 208 (1961).

78. Kelton, W.H., J. Bacteriol. 83, p. 948 (1962).

79. Weibull, C., and B.M. Ludin, J. Bacteriol. 84, p. 513 (1962).

80. Egbert, L.N., Ph.D. Thesis, California Institute of Technology, Pasadena, California (1966).

81. Nelson, J.B., J. Exptl. Med. 63, p. 515 (1936).

82. McIntire, F.C., and M.F. Sproull, Proc. Soc. Exp. Biol., N.Y. 95, p. 458 (1957).

83. Wyatt, G.R., in The Nucleic Acids (E. Chargaff and J.N. Davidson, eds.) Vol. I, p. 259, Academic Press, New York (1955).

84. Cairns, J., J. Mol. Biol. 3, p. 756 (1961).

85. Cairns, J., Cold Spr. Harb. Symp. Quant. Biol. 28, p. 43 (1963).

86. Jones, A.S., and R.T. Walker, Nature 198, p. 588 (1963).

87. Morowitz, H.J., and R.C. Cleverdon, Biochem. Biophys. Acta 34, p. 578 (1959).

88. Kleinschmidt, A., and R.K. Zahn, Z. Naturf. 14B, p 770 (1959).

89. Kleinschmidt, A., D. Lang, and R.K. Zahn, Z. Naturf. 16B, p. 730 (1961).

90. Razin, S., J. Gen. Microbiol. 28, p. 243 (1962).

91. Cairns, J., Cold Spr. Harb. Symp. Quant. Biol. 27, p. 311 (1962).

92. Caro, L.G., Virology 25, p. 226 (1965).

93. Bode, H.R., and H.J. Morowitz, Biophys. Soc. Abst., p. 109 (1965).

94. MacHattie, L.A., K.I. Berns, and C.A. Thomas, J. Mol. Biol. 11, p. 648 (1965).

95. Berns, K.I., and C.A. Thomas, J. Mol. Biol. 11, p. 476 (1965).

96. Riggs, A.D., unpublished results.

97. Vinograd, J., J. Lebowitz, R. Radloff, R. Watson, and P. Laipis, Proc. Nat. Acad. Sci. U.S. 53, p. 1104 (1965).

P A R T II

INTRODUCTION TO PART II

AUTORADIOGRAPHY OF DNA FIBERS FROM CHINESE HAMSTER CELLS

Autoradiography of individual DNA "molecules" has yielded very valuable information about the size and structure of the chromosomes of bacteriophage (31), bacteria (2), and mycoplasma (see Part I of this thesis). All of these organisms are, according to the nomenclature of Ris (32), prokaryotes. It seemed obviously desirable to extend the technique of autoradiography to observe individual DNA "molecules" from a higher organism, i.e., a eukaryote. Joel A. Huberman independently arrived at this same conclusion and we therefore collaborated in performing several experiments, on Chinese hamster cells, that proved to be very fruitful. The results of these experiments are presented in Part II of this thesis as a manuscript that has been submitted for publication.*

The introduction to the manuscript is short, so a few additional words of introduction may be helpful to the reader. No attempt will be made to review the vast amount of literature pertaining either directly or indirectly to eukaryotic chromosome structure, but rather the reader is refered to recent reviews on the subject (33, 34, 35, 36). However, the results of the experiments of DuPraw (37) and of Wolfe (38) are probably the most important recent contributions

---

* The major burden of writing this manuscript was born by Joel A. Huberman, but I am responsible for any errors in fact or presentation that are present.

to our knowledge of chromosome structure and deserve to be discussed. Both DuPraw and Wolfe trapped eukaryotic metaphase chromosomes (and also interphase DNA fibers) on a protein monolayer, then dehydrated and dried them according to a critical point method. By this procedure, the metaphase chromosomes retained most of their gross structural features; and, in addition, more detailed ultrastructure could be discerned in the electron microscope. The basic structure was observed to be a fiber about 230 Å in diameter that seemed to be irregularly folded to form the metaphase chromosome seen by light microscopy. DuPraw (37) treated these fibers with trypsin and found a "core" that was apparently a single Watson-Crick DNA helix. The number of individual fibers that constitute a chromatid is not known, but DuPraw (33) has hypothesized that a single continuous fiber may be folded to form a metaphase chromatid. It should be pointed out, however, that a single fiber need not be a single DNA molecule.

In the following manuscript we do <u>not</u> use the word fiber to imply that 230 Å diameter fibers like those observed by DuPraw and Wolfe produced the autoradiograms that we observed, although we have not rigorously excluded this possibility.

AUTORADIOGRAPHY OF CHROMOSOMAL DNA FIBERS FROM CHINESE HAMSTER CELLS*

By Joel A. Huberman and Arthur D. Riggs

DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY

PASADENA, CALIFORNIA

Ignorance of the true length of the DNA molecules in the chromosomes of higher organisms has always been a major obstacle to understanding chromosome structure. Consequently attempts have been made, usually with the aid of electron microscopy, to estimate the size of DNA in higher organisms. Solari[1] has reported the longest such DNA measured before now - a DNA fiber from a sea urchin sperm at least 93 μ long.

The autoradiographic technique developed by Cairns[2] for visualizing DNA has allowed measurement of fibers much longer than 93 μ. It has been used successfully by Cairns[2] with bacterial DNA, and by Riggs and Mitchell[3] with DNA from PPLO. This paper presents the results we obtained by applying the Cairns technique to Chinese hamster cells grown in tissue culture.

Methods. - Incorporation of H^3-thymidine: Cells of Chinese hamster fibroblast strain B14FAF28 (a gift from Dr. T. C. Hsu) were grown as monolayer cultures on plastic Petri dishes in Eagle's medium supplemented with 10% calf serum. At a cell density of 10^5 cells/ml of medium, 5-fluorodeoxyuridine (FUDR, courtesy of Hoffmann LaRoche Laboratories, Inc.), an inhibitor of thymidine biosynthesis, was added to make 0.05 μg/ml. Uridine was added to 2.5 μg/ml at the same time. About 10 hr

later H^3-thymidine (14 curies/mmole, New England Nuclear Corp.) was added to 4 µg/ml. Incubation was continued for 35-40 hr. Then the cells were harvested by a 10-min treatment at 37°C with 0.05% trypsin in TD (0.137 M NaCl, 0.005 M KCl, 0.007 M NaH_2PO_4, 0.025 M tris, pH 7.4, containing 100 mg/l of streptomycin sulfate and 5×10^5 units/liter of penicillin G) and diluted to about 400 cells/ml in TD.

Lysis and spreading procedure: The method usually used was a modification by Riggs and Mitchell[3] of the procedure developed by Cairns.[2] The cells suspended in TD were diluted tenfold into "lysis medium" (1.0 M sucrose, 0.05 M NaCl, 0.01 M EDTA, pH 8.0). Usually calf thymus DNA was added at this point to 5 µg/ml. One ml of cell suspension (about 40 cells) was then introduced through a polyethylene tube into a dialysis chamber. Construction of dialysis chambers is outlined in Figure 1. The cells were lysed by dialysis for 3 hr at 34°C against 250 ml of 1% sodium dodecyl sulfate (SDS) in "lysis medium." Further dialysis (6 changes of 2 hr each) against "dialysis medium" (0.05 M NaCl, 0.005 M EDTA, pH 8.0) served to remove SDS and unincorporated thymidine. Finally, the dialysis chambers were removed from the "dialysis medium" and emptied, either by draining through a small hole pierced in one of the VM filters, or by siphoning through the glass inlet tube. In the process of emptying, some DNA was trapped on the VM filters and was spread out as the liquid meniscus moved past.

Single cell method: In some cases, cells were suspended in "lysis medium" at an average concentration of 0.5 cell/µl. Drops of 2 µl were placed (one drop per filter) on VM filters which had been coated with

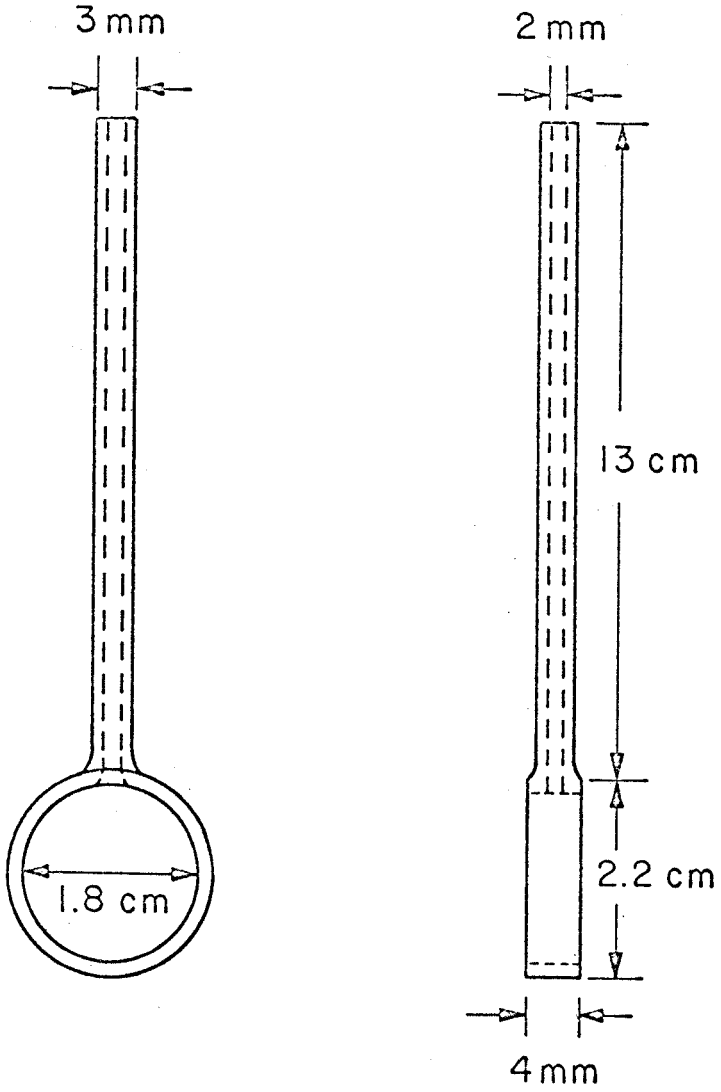

Figure 1. Dialysis Chamber. A 3 mm O.D. Pyrex tube was fused to a 2.2 cm O.D. Pyrex tube. The large tube was then cut to form a thin cylinder with an arm for filling and handling. Using plastic dissolved in amyl acetate as a glue, a VM Millipore filter (50 mμ average pore size, Millipore Filter Corp., Bedford, Mass.) 25 mm in diameter was glued to each end of the glass cylinder.

a thin film of silicone grease around the outer edge and soaked in "lysis medium." The drops were examined microscopically. Those drops containing single cells were diluted to 0.1 ml with "lysis medium." The drops, still on the filters, were dialyzed first against 1% SDS in "lysis medium" (3 hr) and then against "dialysis medium" (6 changes of 2 hr each) by floating the filters on the surface of the appropriate solutions. The liquid remaining on top of each filter was drained off through the filter by transferring the filter to a dry surface and then placing the point of a wedge-shaped piece of bibulous paper underneath its center. In this way all DNA was trapped on the filter.

Pronase digestion: In some cases, after the fifth change of dialysis medium, dialysis was continued for 14.5 hr against SSC-tris (0.15 M NaCl, 0.015 M trisodium citrate, .01 M tris, pH 8.0) containing pronase (Calbiochem, B grade) at a concentration of 50 µg/ml. Under these conditions, there was no detectable nuclease activity in this lot of pronase. Some of the pronase was probably adsorbed by the Millipore filters. Hence, after pronase treatment, the contents of the dialysis chambers were always collected by siphoning and then assayed for pronase activity. The assay medium contained SSC-tris and 5 mg/ml of casein. Incubation was carried out at 37°C for 0.5-14.5 hr, and then trichloroacetic acid was added to a final concentration of 5%. Precipitated material was removed by centrifugation and the optical density of the supernatant was determined at 280 mµ. An $O.D._{280}$ of 1.61 corresponded to the conversion of 5 mg/ml of casein to acid-soluble form.

Autoradiography: The filters were allowed to dry thoroughly, and were then cut from the dialysis chambers, glued to glass microslides, and covered with Kodak AR-10 Autoradiographic Stripping Film (Eastman Kodak Co.). The slides were placed in light-proof boxes along with some $CaSO_4$ drying agent and exposed for 1-4 months in a CO_2 atmosphere at -15°C. At the end of this period, the film was developed in Kodak D-19b at 20°C for 20 min. After development the stripping film was peeled from the slide to which the filter had been glued and mounted on a new, clean slide with Permount and a coverglass.

Observation: The slides were usually scanned with a microscope at 100X using dark-field optics to increase contrast. Most photographs were taken at 40X with dark field. The lengths of individual DNA autoradiograms were determined from photographic enlargements using a map measurer. For grain counts, photographs of the area of interest were taken at 400X with phase optics, enlargements were made, and divisions corresponding to 4 μ were marked off along an imaginary line interpolated through the grains of the autoradiogram being examined. All grains in each interval were counted if they fell within a distance corresponding to 1.5 μ of the imaginary line.

To obtain length distributions, the image produced by a microscope with dark-field optics was projected onto a ground glass screen. All DNA autoradiograms in each field of view were then traced onto tracing paper. Hundreds of autoradiograms, corresponding to areas of one third of a Millipore VM filter or more, could readily be traced in this way. Lengths were determined from the tracings with a map measurer.

Results. - Observed distribution of lengths: In describing our results we shall use the word "autoradiogram" to refer to any apparently continuous line of grains caused by decay of tritium incorporated into DNA. We shall also use the word "fiber" to refer to any single thread consisting of DNA and other substances associated with DNA. The DNA in a single fiber need not be a single molecule.

The basic experiment reported here has been repeated four times, and the results of all experiments are in agreement.

Figure 2 shows an autoradiographic field from one of the more concentrated areas on a filter. The longest DNA autoradiogram visible (arrows) is 1.1 mm long, and there are many shorter autoradiograms. The distribution of lengths observed on a filter chosen for the clarity and good spreading of its DNA autoradiograms is given in Figure 3. One can see that the most frequent autoradiograms are shorter than 0.1 mm. However, 6% of the autoradiograms are longer than 0.8 mm. Figure 4 shows the distribution obtained when the same data are weighted according to length. It is apparent that 50% of the total length of autoradiograms is accounted for by autoradiograms equal to or longer than 0.5 mm.

The longest autoradiograms we observe are of special interest. Figure 5 shows a very long autoradiogram which we have used for grain count studies, and two more long autoradiograms are shown in Figure 6.

The significance of these autoradiograms depends on the answers to four questions:

a) Were the autoradiograms produced by Chinese hamster cell DNA?

b) If so, was the DNA of chromosomal or extrachromosomal origin?

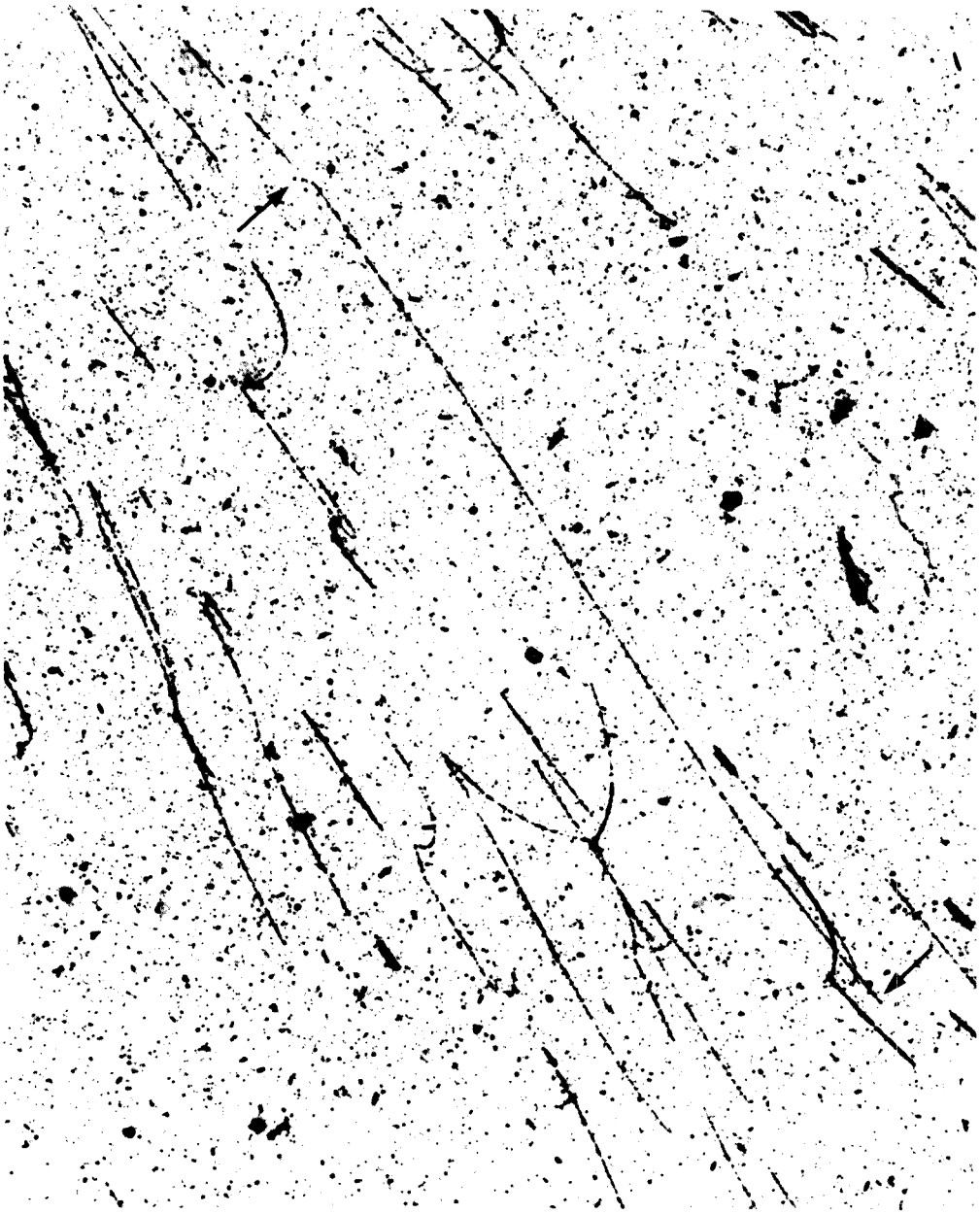

Figure 2. Typical DNA autoradiograms. Arrows indicate long autoradiogram. Exposure time was 3 months. X 125.

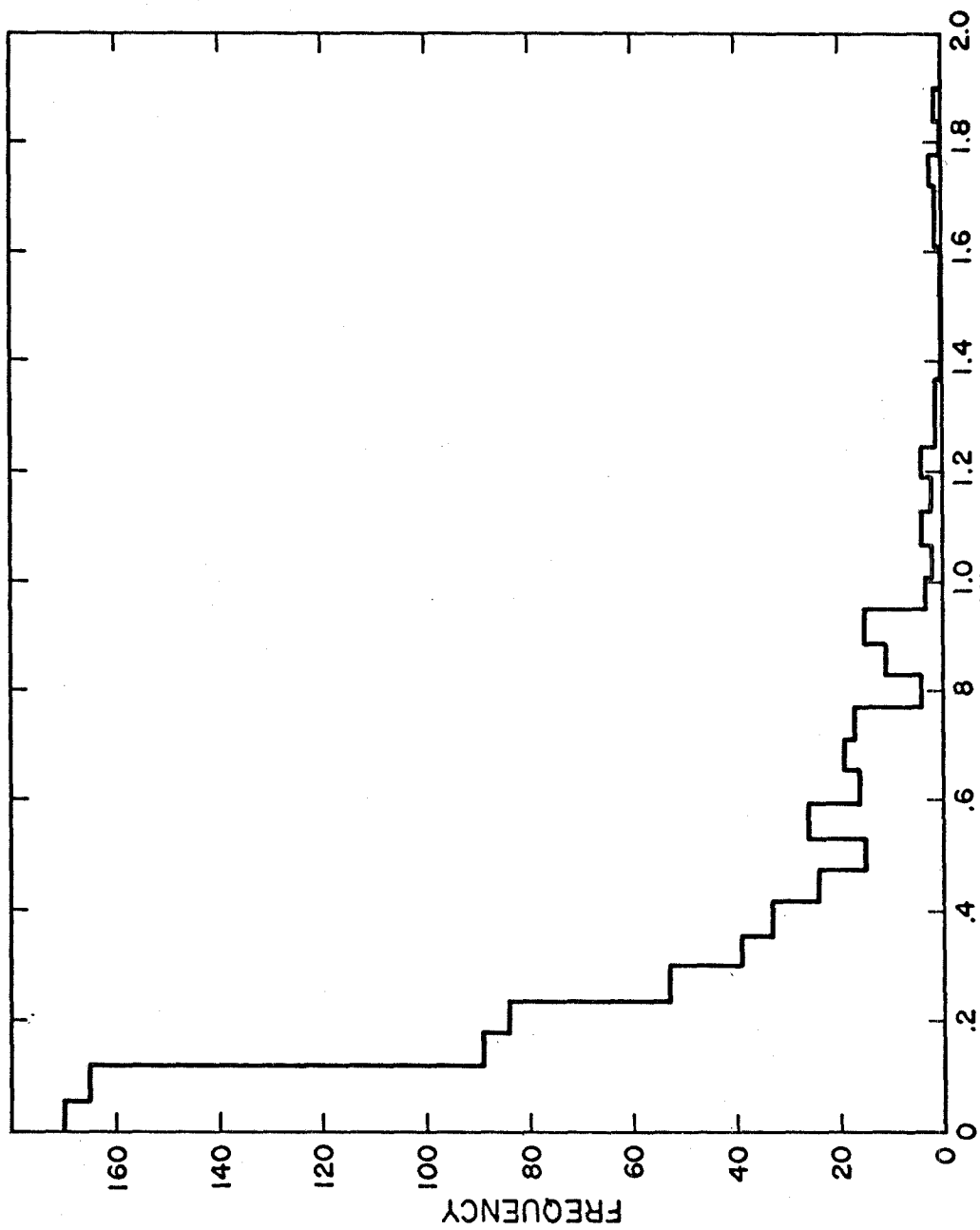

Figure 3. Frequency of DNA autoradiograms as a function of length.

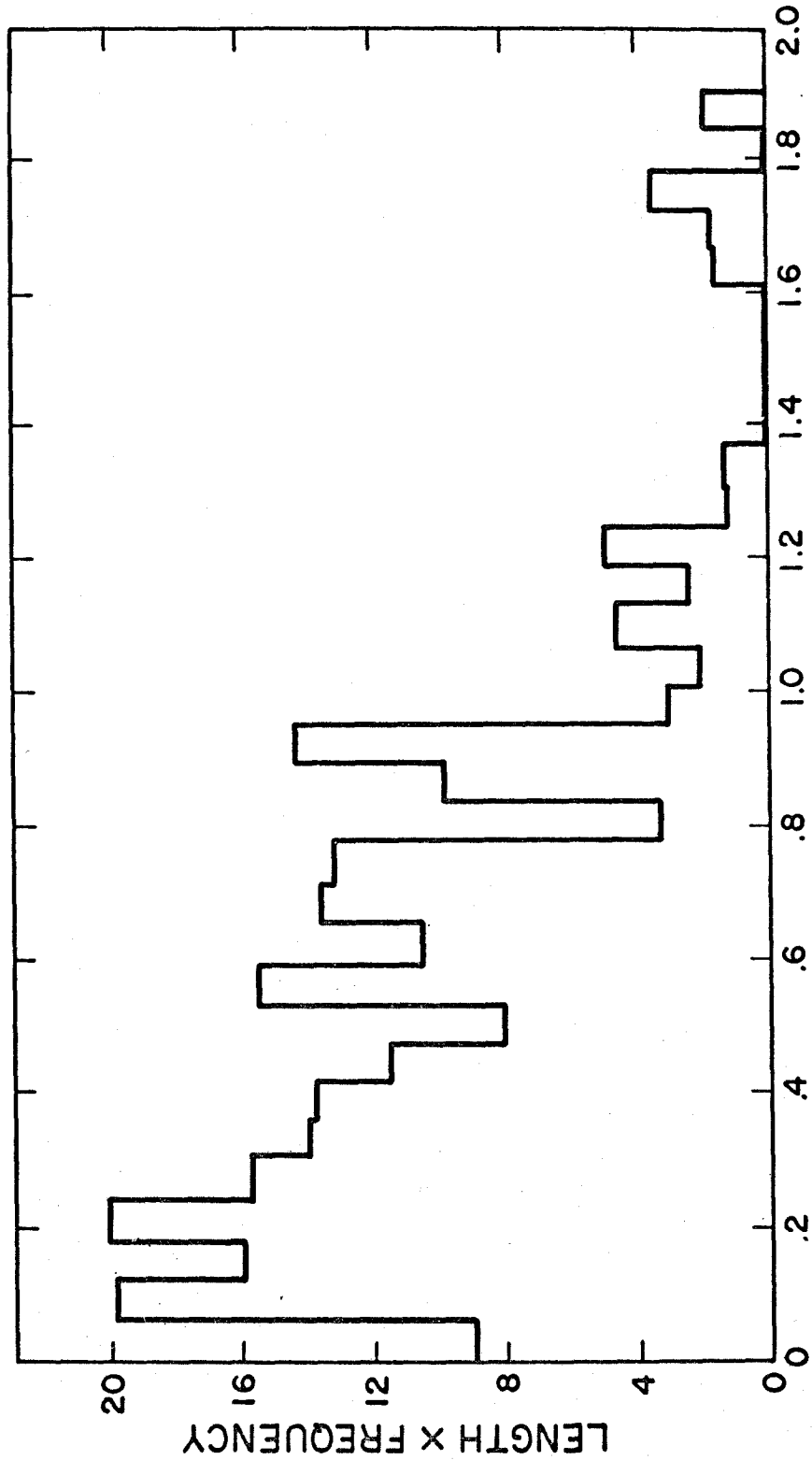

Figure 4. Frequency times length of DNA autoradiograms as a function of length. Data are the same as in Figure 3.

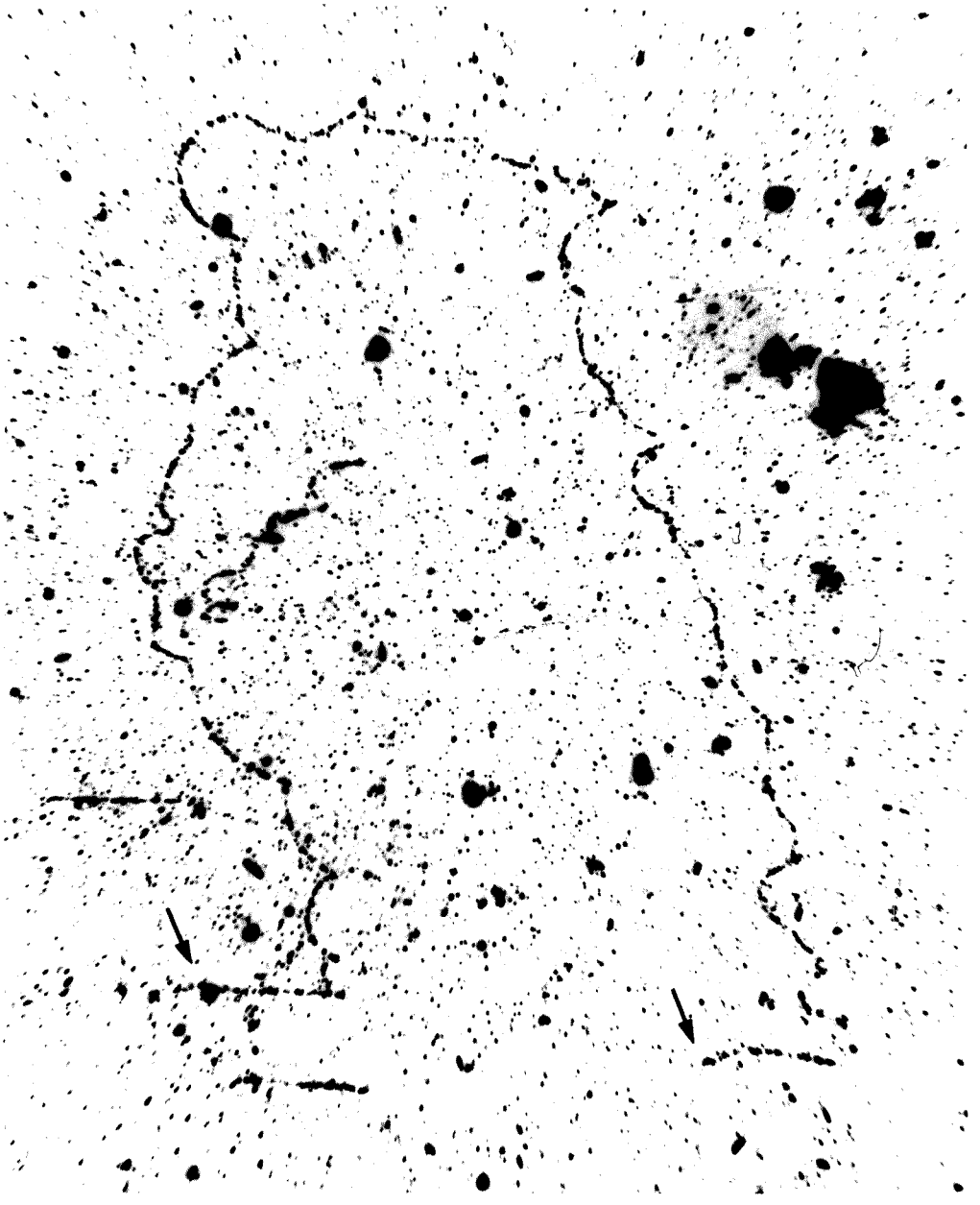

Figure 5. A DNA autoradiogram 1.6 mm long (between arrows). Exposure time was 3 months. X 248.

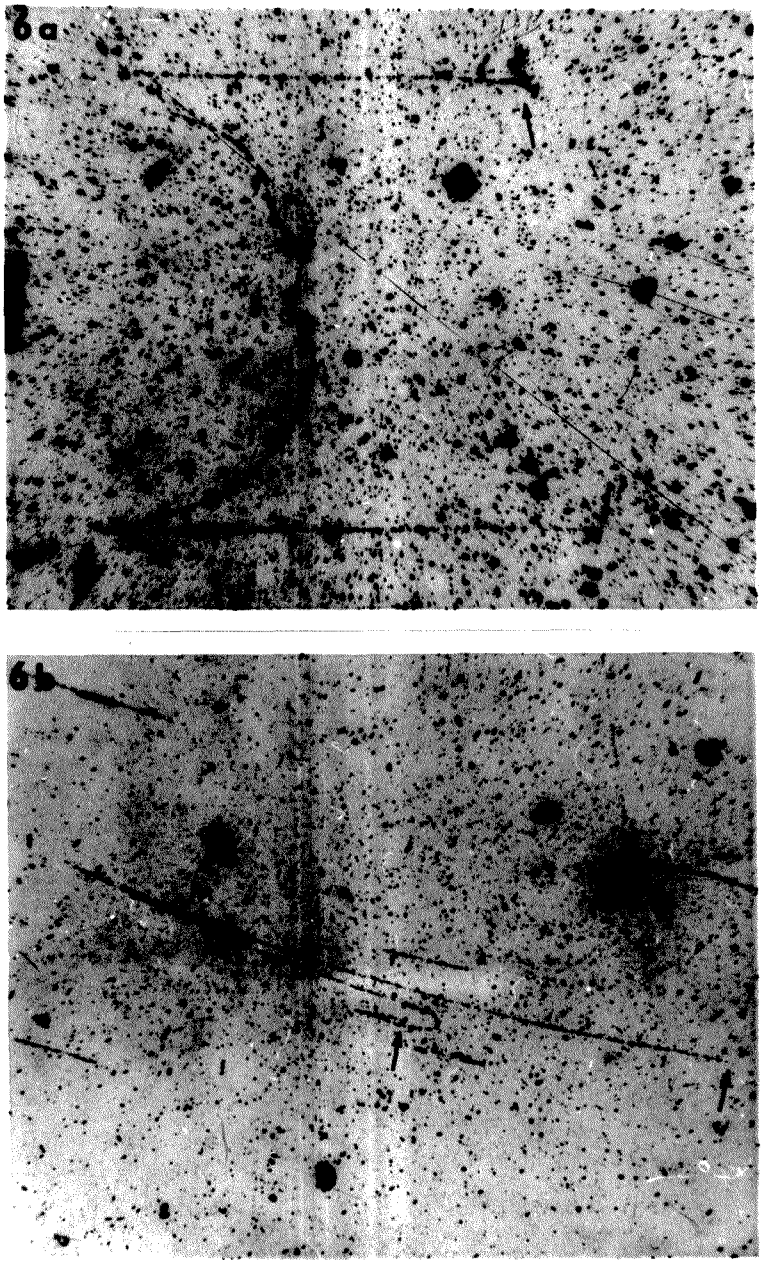

Figure 6. a) A DNA autoradiogram 1.4 mm long (between arrows). Exposure time was 3 months. X 148.

b) A DNA autoradiogram 1.6 mm long (between arrows). Exposure time was 3-1/2 months. X 99.

c) Are the autoradiograms the same length as the DNA producing them or are they distorted?

d) Were they produced by single DNA fibers or by unnatural aggregates of DNA?

That the autoradiograms were produced by DNA is suggested by the fact that no autoradiograms were found if the cells were not lysed. Consequently the autoradiograms must have been produced by some cell component; they could not be due to scratches on the stripping film (scratches were sometimes observed, but they could be distinguished from DNA autoradiograms). The only known cell component of such size into which thymidine is incorporated is DNA.

The DNA producing the autoradiograms must have been Chinese hamster cell DNA and not DNA from a contaminating microorganism for we were unable to detect any contamination. Microscopic examination of the cells used for the experiments showed no microorganisms, and tests of the stock culture for bacteria and PPLO were negative.[4]

We can not rule out the possibility that a small proportion of the autoradiograms might be due to DNA of extrachromosomal (e.g. mitochondrial) origin, but most of them were certainly produced by chromosomal DNA since at least 7% of the DNA in a single Chinese hamster cell is required to account for all the autoradiograms produced by such a cell. This estimate, which was made by our single cell technique (see Methods), is certainly too low because a great deal of the DNA from single cells was tangled and produced unmeasurable autoradiograms. Nevertheless, it is highly unlikely that extrachromosomal DNA could, by itself, account for even

7% of the DNA in a single cell. Furthermore, in our first experiment it was necessary to calculate the proper number of cells to put in the dialysis chambers. In making this calculation we assumed that all the DNA in the cells would (a) be released from the cells and (b) produce autoradiograms if trapped on a filter. Accordingly, we used a cell concentration (20 cells/ml) just high enough to give a DNA concentration inside the dialysis chambers (2×10^{-4} µg/ml) that had been found to be about optimum for the Cairns procedure when applied to PPLO.[3] The resulting frequency of autoradiograms was similar to that obtained with PPLO. This agreement suggests that our assumptions were correct.

The possible measuring errors which could lead to large overestimates of length are: (1) errors in magnification and in measurement of the autoradiograms; (2) overstretching of the DNA fibers; and (3) stretching of the stripping film. The first possible source of error can be eliminated because both the magnification and the measurement steps were carefully checked. The combined error of the magnification steps was always less than 3%. The map measurer we used had less than 1% error when measuring straight lines, and even for highly twisted lines the reproducibility was better than 5%.

The second and third possibilities combined are unlikely to lead to errors greater than about 25% because the same procedure gives values for the E. coli chromosome with a variation from the mean of less than 12%[2] and these values are in agreement with independent determinations.[2] In addition, the area of the stripping film that covered the Millipore filter could usually be distinguished after processing of the film was

completed, and this area remained the same size and shape as the filter it had covered. Thus, no net stretching of the film occurred. Also, the linear grain density produced by abnormally stretched fibers should be much less than the average linear grain density. This was not the case for the great majority of long autoradiograms seen.

Several lines of argument support the conclusion that most of the DNA which produced our autoradiograms consisted of single fibers rather than aggregates. We counted grains in 4 μ intervals over entire long autoradiograms (see Methods), and we found that the number of grains per interval followed a Poisson distribution. Table 1 shows the grain count data from the autoradiogram in Figure 5. Overlaps or discontinuities in the DNA fibers responsible for the autoradiograms would have produced deviations from the Poisson distribution provided that such abnormalities extended for a few microns or more. The low concentration of labeled DNA present in the dialysis chambers (usually 4.0×10^{-4} μg/ml) also is an argument against the possibility of aggregation of labeled DNA fibers. Furthermore, neither varying the concentration of labeled DNA from 2.0 to 20×10^{-4} μg/ml nor omitting the calf thymus DNA from the dialysis chambers had any significant effect on the autoradiogram lengths.

In summary, the autoradiograms are unexaggerated representations of single fibers of Chinese hamster DNA, mostly or entirely of chromosomal origin. Consequently, they are significant as representations of the longest apparently continuous DNA fibers yet reported for higher organisms. Indeed, our longest autoradiograms are more than fifteen times longer

TABLE 1

Grain Count Data

Number of grains per 4 μ interval	Observed Frequency	Expected Frequency
0	13	12.1
1	39	42.6
2	69	75.1
3	88	88.5
4	86	78.0
5	62	55.3
6	35	32.4
7	13	16.4
8	6	7.2
≥9	1	4.5

Data are taken from the autoradiogram shown in Figure 6b. The expected frequency is calculated using the Poisson formula and the mean number of grains per interval (3.53). $\chi^2 = 6.3$, which is well under the rejection limit of 13.4 at the 10% level for 8 degrees of freedom.

than the DNA fiber reported by Solari,[1] and they are even somewhat longer than the 1.1-1.4 mm reported by Cairns[2] for the E. coli chromosome.

Effect of pronase on autoradiogram lengths: We have performed one successful experiment in which the contents of some dialysis chambers were dialyzed against pronase in SSC-tris for 14.5 hr (see Methods) after the cells had been lysed by SDS. Controls were dialyzed against SSC-tris for the same length of time. The pronase activity inside the dialysis chambers at the end of dialysis was sufficient to solubilize 0.14 mg of casein per hour under the conditions of the assay. The longest autoradiograms from the pronase-treated filters were just as long as those from the controls. We conclude tentatively that the DNA fibers producing the autoradiograms do not contain linkers readily susceptible to pronase under the conditions we employed. To date, experiments with higher concentrations of pronase have failed because pronase attacks the stripping film.

Discussion. - We do not yet have enough information to establish definitely the relationship between the autoradiograms we observe and the DNA molecules in chromosomes. It is possible that the chromosomal DNA molecules are shorter than our autoradiograms. If so, the molecules must be joined tandemly (by linkers of another substance) to form fibers at least as long as our autoradiograms, and the linkers must be resistant to both SDS and pronase under the conditions employed.

On the other hand, the DNA molecules could be longer than the autoradiograms for many reasons. The cells may be incompletely lysed during preparation or the DNA fibers may not be completely untangled. Even

if the fibers are properly untangled, they may be incompletely stretched out or they may have contracted when drying. Portions of the fibers may be held away from the stripping film in the pores of the Millipore filter. The fibers may be partially degraded by nuclease action, by mechanical shear, or by tritium decay, and, finally, the fibers may not be completely labeled. Further experiments are required to test these possibilities.

It is not unlikely, however, that some of the autoradiograms we observe may be close to the true length of chromosomal DNA molecules. There is now considerable evidence that individual chromosomes of higher organisms contain many independent DNA replication points. The existence of multiple replication points can be explained most simply in terms of independently replicating DNA molecules, and several criteria suggest that these hypothetical DNA molecules should be about the size of our longer autoradiograms.

Evidence for multiple replication points comes from numerous experiments, with both animal[5-21] and plant[22,23] cells, which show that tritiated thymidine can be incorporated into many separate sites in single chromosomes after pulses which are short compared to the time required for complete DNA replication. Furthermore, the giant chromosomes of Drosophila are sufficiently extended so that the separate incorporation points can sometimes be counted. Plaut and Nash[24] find up to 50 incorporation points per Drosophila chromosome, but they consider the true number to be higher.

The tremendous total length of DNA in the chromosomes of higher organisms also suggests that chromosomes must contain many replication

points. Even at the fast bacterial rate of DNA synthesis (up to 100 μ per min[2]), fifteen hours would be required to replicate all the DNA of an average Chinese hamster chromosome if there were one replication point per chromosome.[25] Total DNA synthesis takes only about 6 hr in these cells.[6,20] Furthermore, the heterochromatic X chromosome of female Chinese hamster cells, one of the larger chromosomes, is known to replicate in about 1-1/2 hr.[20] Other animals, too, synthesize DNA more rapidly than would be expected on the basis of a single replication point per chromosome. Early cleavages in the embryos of many invertebrates occur at intervals of less than 30 min,[26] and for the first 10-12 divisions after fertilization in Drosophila the entire mitotic cycle takes less than 10 min.[27]

If the assumption is made that each DNA replication point in a chromosome corresponds to one DNA molecule, then it should be possible to estimate the average length of chromosomal DNA molecules. An estimate of the number of replication points per chromosome has been made only for Drosophila (Plaut and Nash[24]). If this estimate (50) is divided into the average Drosophila chromosomal DNA content of 1.5-7.5 cm,[28] a length of 0.3-1.5 mm for an average chromosomal DNA molecule is obtained.

If, in addition, the rate of DNA synthesis in Chinese hamster cells is assumed to be the same as that in E. coli, then the maximum possible length for DNA molecules in the heterochromatic X chromosome can be estimated directly as 9 mm. Since all the molecules of the heterochromatic X may not replicate at once, 9 mm is probably an overestimate.

These estimates of chromosomal DNA length in Chinese hamster cells and in Drosophila are of the same order of magnitude as our longer

autoradiogram lengths. This agreement suggests that it is possible that some of our autoradiograms represent whole chromosomal DNA molecules. If so, then the bonds holding the molecules together in the chromosome may be sensitive to SDS.

We also do not have enough information to establish the arrangement of DNA molecules in chromosomes. However, we can make some preliminary conclusions. Our knowledge of genetics suggests that, on a large scale, DNA molecules are arranged in linear sequence but that circularity of individual molecules is possible.[29] We find no evidence for circular molecules in our autoradiograms. Although we find no evidence against the possibilities that our autoradiograms either represent fragments of originally larger circular molecules or represent molecules once held in circular configuration by SDS-sensitive bonds, circles shorter than about 1 mm in circumference and without SDS-sensitive bonds probably could not have produced our autoradiograms. In particular, our results cannot be easily explained in terms of small circles of the type recently reported by Hotta and Bassel[30] for DNA from boar sperm.

Summary. - Linear DNA autoradiograms are found when the Cairns technique is applied to Chinese hamster cells. At least 6% of these autoradiograms are more than 0.8 mm long - roughly the size of the E. coli chromosome and considerably longer than previously reported DNA fibers from higher organisms. Some rare autoradiograms are as long as 1.6-1.8 mm. The implications of these results in terms of models of chromosome structure are discussed.

We gratefully acknowledge the advice and assistance of Dr. Giuseppe Attardi and of Dr. Herschel K. Mitchell, in whose laboratories this work was done.

*This work was supported by U.S. Public Health Service grants GM-11726 and 5-F1-GM-21,622 and by the Arthur McCallum Fund.

[1]Solari, A. J., these Proceedings, 53, 503 (1965).

[2]Cairns, J., in Synthesis and Structure of Macromolecules, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), p. 43.

[3]Riggs, A. D., and H. K. Mitchell, in preparation.

[4]Tests were performed by inoculating Petri plates prepared with either commercial PPLO agar or special PPLO agar [Randall, C. G., L. G. Gafford, G. A. Gentry, and L. A. Lawson, Science, 149, 1098 (1965)] with aliquots of stock cell culture, incubating the plates aerobically at 37°C for at least 7 days, and then examining the plates microscopically.

[5]Rudkin, G. T., and P. S. Woods, these Proceedings, 45, 997 (1959).

[6]Taylor, J. H., J. Biophys. Biochem. Cytol., 7, 455 (1960).

[7]Lima-de-Faria, A., Hereditas, 47, 674 (1961).

[8]Painter, R. B., J. Biophys. Biochem. Cytol., 11, 485 (1961).

[9]Stubblefield, E., and G. C. Mueller, Cancer Res., 22, 175 (1963).

[10]Morishima, A., M. M. Grumbach, and J. H. Taylor, these Proceedings, 48, 756 (1962).

[11]Gilbert, C. W., S. Muldal, L. G. Lajtha, and J. Rowley, Nature, 195, 869 (1962).

[12]Gay, H., Carnegie Inst. Wash. Year Book, 62, 503 (1963).

[13]Keyl, H. G., and C. Pelling, Chromosoma, 14, 347 (1963).

[14]Plaut, W., J. Mol. Biol., 7, 632 (1963).

[15]Schmid, W., Cytogenetics, 2, 175 (1963).

[16]Moorhead, P. S., and V. Defendi, J. Cell Biol., 16, 202 (1963).

[17]Swift, H., in The Nucleohistones, ed. J. Bonner and P. Ts'o (San Francisco: Holden-Day, Inc., 1964), p. 169.

[18]Gabrusewycz-Garcia, N., Chromosoma, 15, 312 (1964).

[19]German, J., J. Cell Biol., 20, 37 (1964).

[20]Hsu, T. C., J. Cell Biol., 23, 53 (1964).

[21]Petersen, A. J., J. Cell Biol., 23, 651 (1964).

[22]Taylor, J. H., Proc. Tenth Intern. Congr. Genet., 63 (1959).

[23]Wimber, D. E., Exptl. Cell Res., 23, 402 (1961).

[24]Plaut, W., and D. Nash, in The Role of Chromosomes in Development, ed. M. Locke (New York: Academic Press, 1964), p. 113.

[25]Using the diphenylamine method, we have determined the DNA content of the average log phase Chinese hamster cell to be 10 picograms. Correction for DNA synthesis gives about 7 picograms per diploid chromosome complement. The average cell in the strain we use contains 23 chromosomes. Consequently there are about 9 cm of DNA per average chromosome.

[26]Costello, D. P., M. E. Davidson, A. Eggers, M. H. Fox, and C. Henley, Methods for Obtaining and Handling Marine Eggs and Embryos, Marine Biological Laboratory, Woods Hole, 1957.

[27]Sonnenblick, B. P., in Biology of Drosophila, ed. M. Demerec (New York: John Wiley and Sons, Inc., 1950), p. 62.

[28]These figures are based on the estimate of 0.2-1.0 picogram DNA per haploid Drosophila genome [from Ritossa, F. M., and S. Spiegelman, these Proceedings, 53, 737 (1965)].

[29]Stahl, F., J. Chim. Phys., 58, 1072 (1961).

[30]Hotta, Y., and A. Bassel, these Proceedings, 53, 356 (1965).

[31]Cairns, J., Cold Spr. Harb. Symp. Quant. Biol.,27, 311 (1962)

[32]Ris, H., and B.L. Chandler, Cold Spr. Harb. Symp. Quant. Biol., 28, 1 (1963).

[33]DuPraw, E.J., Nature, 206, 338 (1965).

[34]Taylor, J.H., in Molecular Genetics, ed. J.H. Taylor (New York: Academic Press, 1963), p. 65.

[35]Kaufman, B.P., H. Gay, and M.R. McDonald, Int. Rev. Cytol, 9, 77 (1960).

[36]Steffensen, D., Int. Rev. Cytol., 12, 163 (1961).

[37]DuPraw, E.J., these Proceedings, 53, 161 (1965).

[38]Wolfe, S.L., and B. John, Chromosoma, 17, 85 (1965).