THE POSSIBILITY OF BRANCH CONFORMATION IN *Azotobacter vinelandii* CHROMOSOMAL DNA CARRYING MULTIPLE GENE COPIES AND ITS FOLDED STATE IN THE CELL

THESIS

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By

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Chromosomal DNA of *A. vinelandii* thought to carry multiple gene copies was examined in efforts to visualize its chromosomal structure using electron microscopy. The chromosomal DNA of *A. vinelandii* may have multiple circular genomic units carrying multiple copies of genes. There may be one or more genes per circular unit. The circular structures may be branched out from a larger circular chromosomal unit carrying single-copy gene groups including *nif,* hypoxanthine, adenine, methionine, and uracil auxotrophy genes by forming three-strand branch structures at many places. Each circular unit seems to be bound to the cell membrane at different sites.

Three possible branch construction schemes and their replication modes are postulated in this study.

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CHAPTER I

INTRODUCTION

It is known that *Azotobacter vinelandii,* an aerobic, gram-negative, nitrogen-fixing bacterium (46), has multiple genes (33, 47, 49). Studies of DNA renaturation with *A. vinelandii* and *Escherichia coli* in their mid-exponential growth phase showed that the two microorganisms had similar Cot values, which meant they had extensive lengths of similar DNA sequences (47, 49). The sedimentation coefficient of the released, folded chromosome of *A. vinelandii* is almost identical to that of *E. coli* in that they both settle equally in a centrifugal field at a rotor speed of 17,000 revolutions per minute (rpm) (49). Because they have similar characteristics of renaturation kinetics and sedimentation rates, it has been suggested that the two microorganisms may have DNA similarities. However, the DNA content per cell of *A. vinelandii* is much larger than that of *E. coli* (48, 76). *Escherichia coli* cells contain 3.5 $X10^{-15}$ g of chromosomal DNA per cell, whereas exponentially growing cells of *A. vinelandii* have a content of 1.5 X 10^{-13} g DNA per cell (48).

Sadoff (49) claims that each exponentially growing cell of *A. vinelandii* contains as many as 27 copies of its chromosomal material and that each unit is equivalent to the chromosome of *E. coli*. Robson (78) agrees with Sadoff in principle but sets the number of chromosomes at approximately 40 per cell.

The fact that it is difficult to isolate mutant cells of *A. vinelandii* supports the notion that *A. vinelandii* may have multiple copies of its genome. Mutations of *A. vinelandii* have been reported with varying degrees of success. These occur spontaneously (25) and also after mutagenesis with UV radiation (33, 65, 66), hydroxylamine (25), N-methyl-N-nitro-N-nitrosoguanidine (NTG) (4, 22), ethylmethane sulfonate (EMS) (42), and by the use of transposons (31). In spite of these positive results in obtaining mutations, difficulties in the isolation of certain types of mutants have been found. Isolation of mutants blocked in ß- hydroxybutyrate metabolism, or at specific morphological stages of development, and isolation of auxotrophs relating protein and nucleic acid metabolism are known to be more difficult (36, 41, 42, 49). Also, when enzyme or antibiotic resistance mutation rates of azotobacter were compared to those of *E. coli* , it was obvious that a different set of

rules applied.

Genetic redundancy in the *A. vinelandii* cell can explain the difficulty in obtaining mutants. That is, if there are many copies of each gene, the organism will not behave as a haploid organism as does *E. coli* but rather as a eukaryotic cell with allelic genes. In this view, in order to obtain a mutation in *A. vinelandii,* all copies of the same gene would have to mutate at the same place at the same time. The probability of this happening is quite large. If *A. vinelandii* had 500 genes and if it had 27 copies of each gene, the probability of all 27 alleles mutating during one generation would be $1 \div 500^{27}$. If the mutations accumulated from one generation to the next, the probability would be the same. If 1×10^6 gene mutations occur during a given time, it would take $1 \div 500^{27} \times 1 \times 10^6$ intervals of time to achieve a mutation that would affect phenotype.

Mutants in *A. vinelandii* sometimes tend to revert to wild type even after several subcultures (32, 40, 42, 65, 75). This kind of instability of mutant phenotypes in addition to the difficulty in isolating mutants must be considered as a separate mechanism since mutation indicates the presence of only one gene, i.e., haploid condition. No further comment will be made on mutant reversion.

It is known that some mutants of *nif* genes (4, 22, 42, 55, 60, 75) and respiratory genes (28, 29, 40) can be easily isolated in *A. vinelandii* . The kinetics of *nif* gene mutations are in effect identical to those of haploid genes. That is, they show a critical target number of one. Why are these mutants so easily found, while other mutants like amino acid auxotrophies are very hard to get in *A. vinelandii* ? It is obvious that the *nif* genes do not appear in multiple copies in *A. vinelandii* ? Medhora *et al.* (41) suggested that *A. vinelandii* might have a unique, heretofore undescribed, chromosome or a single-copy plasmid that contains the *nif* genes (41). From this idea, Maia *et al.* challenged Medhora's explanation that nitrogen-fixing genes could be present on single-copy plasmids. Maia *et al.* proposed that if *nif* genes existed in single-copy plasmids, then all cultures of azotobacter that can grow on nitrogen-free media should contain single-copy plasmids. These could be either single copy, free, double-stranded, circular DNA molecules or as a single copy plasmid integrated into the bacterial genome(39). Maia *et al.* examined thirty laboratory strains of *A. vinelandii* and two cultures isolated from water, which could grow on nitrogen-free media and found that only six of the thirty two, four laboratory

strains and two isolated from water, possessed double-stranded, covalently bonded, circular (plasmid) DNA. The six cultures were not affected in their ability to produce nitrogenase and nitrogenase reductase nor to fix nitrogen when they were cured of their plasmids (39). Also, the non-nitrogen-fixing mutant, UW-1, contained a plasmid but it too showed no differences in its ability to fix nitrogen between plasmid-bearing and cured cultures (39). Maia *et al.* concluded that there was no basis for Medhora's statement (41) regarding nitrogen fixation and plasmids.

Punita *et al.* (47) reported that *leu* B, *nif* H, *nif* D, and *nif* K genes appeared in at least 80 copies of each gene in stationary-phase cultures of *A. vinelandii.* They used Southern blotting of *A. vinelandii* chromosomal DNA probed with ^{32}P-labeled *nif* H, D, and K fragments, respectively, isolated from the cloned plasmid pMP2 carrying the *nif* HDK gene fragments of *A. vinelandii.* In these studies, the intensity of the autoradiograms of *A. vinelandii* chromosomes was similar to the control, standard pMP2 DNA, prepared as the equivalent of the 80-copy material (47).

The ß-Lactamase gene found on ampicillin resistant pMP2 or pMM113 plasmids (47) was inserted into the *A. vinelandii*

chromosome by single-point crossover recombination. It was found that 80 copies were produced per cell of *A. vinelandii* when they were cultured for many generations in the presence of ampicillin (47). With this genomic multiplicity, Punita *et al.* suggested that those multiple copies of the genes in *A. vinelandii* were not on a single large chromosome in the manner of tandem reiteration but were presented on as many copies of the *A. vinelandii* chromosome and that the ampicillin resistance gene existed in the form of one copy in each chromosome(47).

The foregoing proves that the unique genomic content of *A. vinelandii* is not simple as in other bacteria including *E. coli* . *Escherichia coli* contains one single supercoiled double-stranded circular DNA molecule which is considered normal for bacteria. The mutation behavior of *A. vinelandii* can not be explained on the same basis as is that of bacteria such as *E. coli.* The simplest explanation is one based on the assumption that *A. vinelandii* has multiple chromosomes each of which would be equivalent to that of *E. coli.* However, this is untenable in view of *nif,* hypoxanthine, adenine, methionine, and uracil auxotrophy mutations.

This beings so, how can the genomic irregularities in *A.*

vinelandii be explained in terms that can be reconciled with experimental observations? How can multi-copy genes, large amount of chromosomal DNA, and haploid genes such as *nif,* hypoxanthine, adenine, methionine, and uracil be organized in the *A. vinelandii* cell?

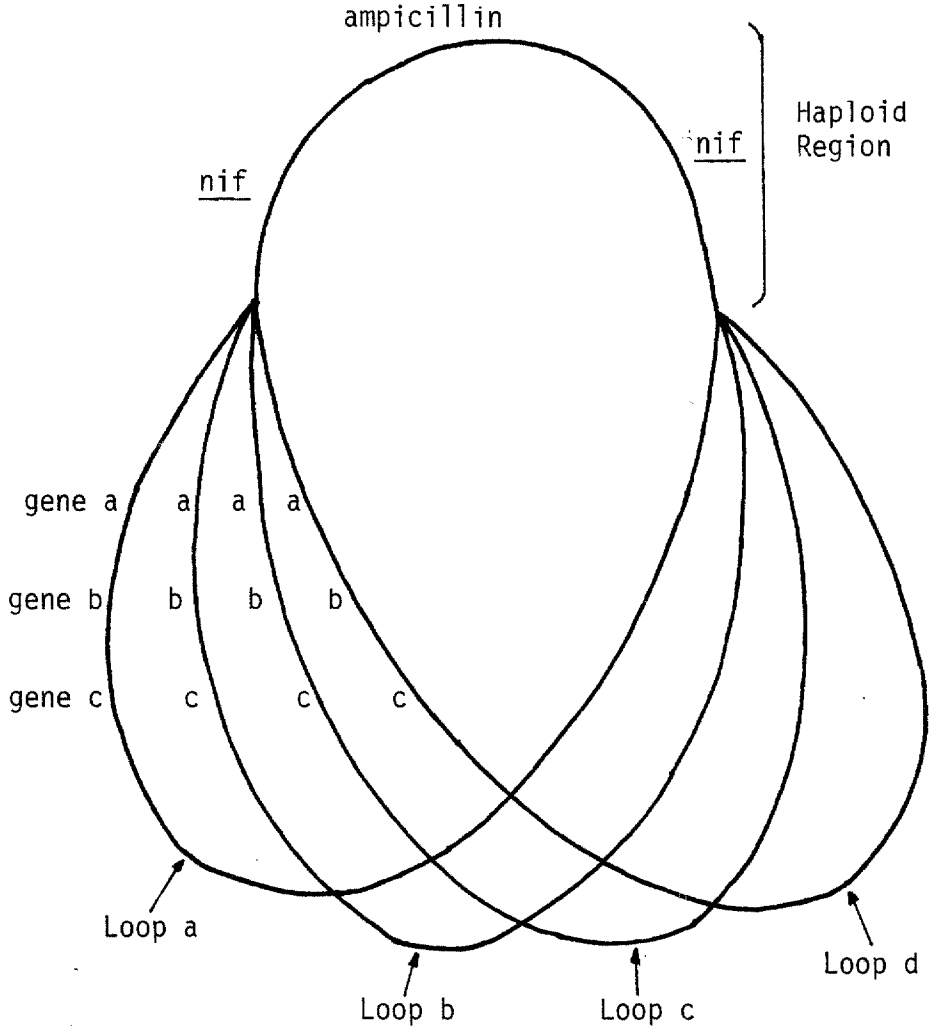
Vigorous studies on bacterial genomic structures have been pursued in *E. coli* by many researchers around the world. All evidence supports the concept of a circular, folded chromosome such as that of *E. coli.* Electron microscope studies (16, 17, 62, 71, 72) have confirmed these studies and provided visual evidence of chromosome structure. In *A. vinelandii,* however, few studies have been devoted to visualizing the structural configuration of the chromosomal DNA. Most of the studies attempted have been unsuccessful and at this time, there is no data which permits visualizing the physical appearance of the azotobacter chromosome.

This study was designed to show the configuration of the chromosome of *A. vinelandii* and also its attachment to the cell membrane. Attempts were made to visualize the coiled DNA, unfolded DNA, and DNA attached to the cell membrane. It was also decided to investigate fragments of the DNA in order to detect

structural details as seen in "weak" segments of DNA. The structure of azotobacter DNA has been examined previously in several published papers (33, 47, 49) but unambiguous results have not been reported nor has an explanation been tendered that would reconcile the possibility of a chromosome with both haploid and polyploid domains been proposed.

Three possibilities of chromosomal DNA conformation that would explain observed data were investigated. One model proposed is a single, very large, double-stranded circular DNA in which many genes appear in tandem but others in single copy. Another possibility is based on a hypothetical structure such as that shown in Figure 1. A third possibility was considered in which the whole single chromosome was composed of sub-circular members of DNA connected by branching segments in which one double-stranded DNA molecule branched out to give two or three double-stranded DNA molecules. The *nif* genes and any other easily mutated genes were located on a circular member as a single copy and the rest of the genes located on the other circular segments, one copy per each member DNA semi circular element. In this case, however, a significant problem in chromosomal DNA replication could be expected.

Figure 1. Hypothetical structure of *A. vinelandii* chromosome. The chromosome is composed of a haploid region containing *nif,* hypoxanthine, adenine, methionine, and uracil genes. Genes resistant to mutations are thought to exist on multiple circular units carrying one gene per one unit. Those units (loop a, loop b, loop c, and loop d in this figure) are branched out from the haploid chromosomal region and the number of the loops can be 27 loops (49), 40 loops (78), or 80 loops (47).


ampicillin
nif
nif
Haploid
Region
gene a
gene b
gene c
a
a
a
b
b
b
c
c
c
Loop a
Loop b
Loop c
Loop d

All attempts to reconcile the proposed structure with the process of DNA synthesis (3, 10, 18, 23, 50) as seen in *E. coli* and other bacteria were fruitless. If the proposed structure is correct, the synthesis of DNA in *A. vinelandii* must be different from that seen in other bacteria.

The experimental design for this study focused on finding the specific configurations of DNA during the replication processes of the proposed DNA structure. Preparations for visualizing the folded state of membrane-attached and released chromosomal DNA were examined by electron microscopy. Isolation of chromosomal DNA from cells of *A. vinelandii* was based on lysing the cells with non-ionic detergent in 1.0 M NaCl solution. This is the method proposed by Stonington and Pettijohn for the folded chromosomal DNA of *E. coli* (62). The method of Worcel and Burgi (71, 72) was modified and used for carrying out cell lysis and isolation of uncoiled DNA. Preparations of both forms of DNA (membrane-attached and released chromosomal DNA) were examined by electron microscopy using the cytochrome c monolayer method derived from the Zahn-Kleinschmidt technique (13). DNA fragments were also examined to see the details of DNA conformations occurred during the replication proces-

ses The aqueous and the formamide technique were used for spreading DNA on the hypophase solution (14).

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains, Media, and Culture Conditions

Bacterial cultures for this study were obtained from Dr. G. R. Vela's laboratory. *Azotobacter vinelandii* strain A and strain ATCC 478 preserved in 40% glycerol-water mixture at -20°C were used for all experiments. The cultures were grown at 30°C in modified Burk's nitrogen-free medium (63). *Escherichia coli* K12 grown at 37°C in LB medium (51) was used for comparison with *A. vinelandii* . One hundred milliliter cultures of *A. vinelandii* in 500 ml Erlenmeyer flasks were grown on the reciprocal shaker for 48 hours. The cells were harvested by centrifugation at 6,000 rpm for 8 minutes at 4°C prior to the end of exponential growth, and washed by resuspending, shaking, and centrifugating in 30 ml buffer [50 mM Tris–Cl {Tris(hydroxymethyl) aminomethane; Sigma Chemical Co., St Louis, MO} and 20 mM EDTA (disodium salt of ethylenediaminetetraacetic acid; Sigma Chemical Co.) adjusted to pH 8.0 with 1 M HCl (hydrochloric acid; Sigma Chemical Co.) using the Corning pH

meter (Model 240, Corning Science Products; Corning, NY). The cell pellet was resuspended, shaken, and centrifuged again in 10 ml buffer [10 mM Tris–Cl (pH 8.0) and 1 mM EDTA]. The final pellet was preserved at 0°C in an ice bath until needed.

Cell Lysis and Chromosomal DNA Isolation

The method used for *E. coli* K12 by Stonington and Pettijohn (62) was used to isolate chromosomal DNA from *A. vinelandii* and *E. coli.* Some modifications in the procedure were adopted from related papers (71, 72). The temperature during lysis was kept constant because the form of isolated DNA is determined by lysis temperatures. Lysis at 0 to 4°C results in membrane-attached, folded chromosomes while lysis at room temperature gives released, folded chromosomes. In these experiments, it was found that lysis at 10°C yielded both types of DNA. Membrane-attached, folded chromosomes were particularly easy to find in the prepared material. The procedures of Stonington and Pettijohn (62) for lysis were scaled up in volume for this work. Before lysis with non-ionic detergent (1% Brij-58, 0.4% deoxycholate), the cell wall was disrupted or partially removed by lysozyme treatment in hypertonic

environments of 20% (w/v) sucrose solution buffered with Tris-Cl and EDTA. Consequently, osmotically sensitive sphaeroplasts were produced. The high concentration of NaCl during lysis and purification stabilized the folded state of the chromosome by neutralizing the negatively charged DNA molecules. Each step in this work was immediately followed by the next step with no intervals of time elapsed during the entire procedure.

The *A.vinelandii* cell pellet obtained from two days culture in Burk's nitrogen-free medium was resuspended thoroughly in 1.0 ml of cold solution (called Solution A in this work) containing 10 mM Tris-Cl (pH 8.0, preserved at 4°C), 10 mM sodium azide (Sigma Chemical Co.), 20% (w/v) sucrose (Mallinckrodt, Paris, KY), and 0.10 M sodium chloride (Sigma Chemical Co.). This step was performed in an ice bath for the successful production of sphaeroplasts. Sphaeroplasts were produced by adding 0.25 ml of Solution B [50 mM EDTA, 4 mg/ml of egg white lysozyme (Muramidase; Mucopeptide, Sigma Chemical Co.), and 0.12 M Tris-Cl adjusted to pH 8.0] to the resuspended cells, and mixing carefully. The preparation was kept for exactly 30 seconds in an ice and water mixture bath. Worcel and Burgi (72) noted that longer times with lysozyme resulted in

premature lysis in the absence of NaCl resulting in viscous DNA, and that shorter times with lysozyme are insufficient for lysis of the cells giving low yields of DNA (72). After 30 seconds exposure of the cells to lysozyme, 1.25 ml of ice-cold Solution C [1% Brij-58 (polyoxyethylene 20 cetyl ether; Sigma Chemical Co.), 0.4% deoxycholate (Sigma Chemical Co.), 2.0 M NaCl, and 10 mM EDTA] was immediately added, and gently mixed. The cells were then transferred to a 10°C stationary water bath for several minutes until the cell lysate became clear but not sticky. The best results were obtained in 10 to 15 minutes, depending on the amount of cell material. Diethyl pyrocarbonate (Sigma Chemical Co.), a nuclease inhibitor, was added to a concentration of 0.1% just before adding Solution C. During lysis with Solution C, the lysate was slowly rotated a few times with care for conserving the folded state and preventing the breakage of DNA molecules. The relatively clean lysate was then centrifuged at 4,000 X G at 4°C for 5 minutes to remove cell debris in a Sorvall RC5C Centrifuge (DuPont Co., Wilmington, Del.). The supernatant was gently layered on 35 ml of 10 to 30% (w/v) continuous sucrose gradient (1) containing 10 mM Tris-Cl (pH 8.0), 1.0 M NaCl, 1 mM EDTA, and 1 mM ß-mercaptoethanol (2-

hydroxyethyl mercaptan; Sigma Chemical Co.) with extreme care. Sorvall OTD 75B Ultracentrifuge and Sorvall AH-629 Swinging Bucket Rotor (DuPont Co.) were used for this purpose. The gradient was then centrifuged at 17,000 rpm at 4°C for one half hour to separate the folded DNA molecules. Worcel and Burgi (71) reported that this method yielded folded chromosomes of *E. coli.* This method failed to give folded DNA from *A. vinelandii.* The centrifugation was extended to one hour and then up to six hours without acceptable results. The azotobacter DNA obtained from these procedure was broken as was expected due to the physical forces of centrifugation. The lysis method was slightly modified for isolation of released DNA. Fifteen minutes of treatment with Solution C followed by 10 minutes of standing at room temperature, and 12 minutes of centrifugation at 17,000 rpm at 4°C yield suitable results. In this work, T4 phage was used as a reference marker for sedimentation rate.

Fractions containing DNA molecules were collected from the bottom of the thin wall ultracentrifuge tube by puncturing with a 16 gauge hypodermic needle 1 1/2 inches long (Becton Dickinson & Co., Rutherford, NJ). The large end to the syringe was broken off and the

resulting hollow tube connected to a flexible silicon tubing of 1/16 inch inside diameter. The gradient solution was allowed to flow through the silicon tube and divided into aliquants of 1.5 ml each without dropping in order to reduce DNA breakages. Polypropylene micro-centrifuge tubes autoclaved before using were employed to collect the individual fractions. The optical density of each fraction was measured at 260 nm using the Beckman DU-40 Spectrophotometer. The amount of DNA in each fraction which showed high absorbance at 260 nm was calculated [increase of 1.0 in absorbance at 260 nm means increase of 50 μg/ml of DNA in that solution (51)] and DNA solutions prepared for electron microscopy within 30 minutes of separation.

Preparations of DNA for Electron Microscopy

All glassware including reagent bottles and glass slides for spreading DNA were washed with detergent, boiled for one hour in 5% Isoclean (Sigma Chemical Co.), rinsed thoroughly with distilled water followed by a rinse in 1 N HCl, ten times with distilled water, and, finally, with double distilled water. Cleaned glass slides were stored in double-distilled water filtered through 500 ml bottle

filter having 0.22 μm pore size membrane filter (Costar Corp., Cambridge, MA). All water for solutions, reagents, and final rinsing was double-distilled and passed through 0.22 μm membrane filters.

Basically, two types of preparation methods for spreading DNA were used. One was an aqueous method introduced by Coggins (13), and the other was a formamide technique carried out by Davis *et al.* (14). Various detailed techniques for electron microscopy of nucleic acids (21, 27, 35, 44, 59) were examined for this work and those procedures which gave the best results were employed.

Grid Preparation and Collodion Supporting Film

Copper wire grids of 200 mesh (Ted Pella, Inc., Redding, CA) were cleaned by soaking for one half to 2 hours in 10% acetic acid (glacial; Sigma Chemical Co.), washing three times in distilled water, rinsing twice with acetone (Sigma Chemical Co.); and stored in acetone. A stainless steel screen, which was bent equally on both sides to keep the screen from the bottom of a glass dish, was placed in a clean glass dish and filled with water to the top of the screen (Figure 2). The grids were placed on the screen just below the surface of the water and a drop of 3.5% collodion solution (Polys-

Figure 2. Experimental design for collodion coating on grids. Put a stainless steel screen, which is equally bent on all sides to keep the screen from the bottom, in a clean glass dish (dia. = about 15 cm). Fill the dish with water to 1 cm above the top of the screen. Place grids on the screen and fall a drop of 3.5% collodion solution in n-amyl acetate. Allowed it to form a thin film and to dry. Pick up the film with tweezers and discard it. Make another collodion film by repeating the above procedure. Lower the water level slowly by suction with a 50 ml polyproylene syringe from the bottom of the dish so that the film could gently fall onto the grid surfaces.

ciences, Inc., Warrington, PA) in n-amyl acetate (Polysciences, Inc.) was layered on the water surface and allowed to form a thin film. The film was allowed to dry for 1 to 2 minutes. This first film was lifted with tweezers and discarded because it contained dust particles that may have fallen on the water surface. A second film was immediately formed on the water surface by the same method. After the film was formed, water was removed slowly by suction with a 50 ml polypropylene syringe from the bottom of the dish so that the film would gently fall onto the grid surfaces. The screen with collodion coated grids was transferred to a Whatman No. 1 Filter Paper and allowed to dry in a 60°C oven for three hours. The coated grids were removed from the screen and stored in a desiccator. They were used within two weeks.

Spreading Chromosomes in Aqueous Condition

Special care was taken in handling all DNA preparations. Glassware and grids were protected from chemical contaminants and fingertip DNAse since this would result in degrading the isolated DNA. All solutions were filtered through 0.2 μm Nalgene disposable syringe filters. The autoclaved micropipet tips were always rinsed

with 0.2 μm filtered double distilled water just before picking up the solutions. The micropipet tips for handling DNA samples were cut to the point which could result in a 2 mm diameter opening. In addition, all polyproylene microcentrifuge tubes carrying DNA were rinsed as above and also autoclaved prior to use.

One hundred microliters of spreading solution containing 25 μl of 2 M ammonium acetate (Sigma Chemical Co.) and 4 mM EDTA (pH 7.5), 50 μl of DNA and water (5 to 10 μg DNA/ml), and 25 μl of horse heart cytochrome c (type VI; Sigma Chemical Co., Cat.# C-7752) with 0.4 mg/ml were prepared just before use. Freshly made hypophase solution (0.25 M ammonium acetate (pH 7.5)) was placed in a clean Millipore filter box (9 X 9 X 2 cm). The spreading solution was applied by running slowly and steadily down an acid-cleaned glass slide, which was immersed in the box by resting one end on the rim of the box at an angle. After approximately one minute, during which a monolayer of DNA and protein formed, the chromosomal DNA was picked up by pressing the collodion-coated grids onto the DNA-protein monolayer surface at the area within 1 - 2 cm from the slide-hypophase boundary. Liquid adhering to the grids was removed by blotting with Whatman No. 1 filter paper. Five or six grids were

prepared within five minutes from each sample of DNA prepared.

Spreading Chromosomes by Formamide Technique

The basic method is exactly the same as that with the aqueous method but different in that formamide solution is used instead of ammonium acetate. High purity formamide (99.9%) purchased from Electron Microscopy Sciences (Cat. #15745; Fort Waxhington, PA). In this method, the DNA spreading solution was composed of 25 µl of DNA and water (5 to 10 µg DNA/ml), 50 µl mixture of 60% (v/v) formamide and 40% 0.5 M Tris–Cl (pH 8.5)-50 mM EDTA, and 25 µl of horse heart cytochrome c (0.4 mg/ml). The hypophase solution (20% formamide, 10 mM Tris-Cl, and 1 mM EDTA) was made up five minutes or less prior to use and filtered through a 0.2 µm syringe filter.

Staining and Metal Shadowing for Contrast

The staining solution was a 1:100 dilution of uranyl acetate (50 mM uranyl acetate (J. J. Baker Chemical Co., Phillipsburg, NJ) and 50 mM HCl in 95% ethanol, stored at 4°C in the dark) in 85% aqueous ethanol, and used within 30 minutes. The staining solution was also filtered through 0.2 µm pore size filter membrane. The prepared

grids with deposited DNA were stained for 30 seconds in staining solution and rinsed with 85% ethanol for 15 seconds. They were allowed to air dried in a desiccator.

The stained grids were shadowed with platinum-palladium (Pt-Pd; 80:20) wire (0.008" dia.; Ted Pella, Inc., Redding, CA). Although visible contrast could be obtained with just uranyl acetate stain, viewing with the electron microscope was always better when the grids were shadowed with metal. The grids were placed on the rotating stage vertically about 10 cm below the metal source (3.5 cm of Pt-Pd wire twisted tightly around a 0.02" diameter tungsten wire) in the bell jar of a JEOL JEE-4X Vacuum Evaporator (JEOL Engineering Service Co., LTD., Tokyo, Japan). The stage was tilted at an angle of 8° from the metal source and rotated at about 40 rpm. The metal was evaporated under the 4 X 10^{-4} Pa vacuum by heating the source with a current of 16 to 17 amperes for 40 seconds.

The shadowed grids were thinly coated with carbon to give resistance to the layered collodion film on the grids under the electron beam because the collodion film was easily torn by the heat of electron beam. This work was also done in the bell jar of the vacuum evaporator. The grids were placed on the slowly rotating

stage vertically below the carbon source. Just one second evaporation time was sufficient to lay a thin carbon coat at 4 X 10^{-4} Pa vacuum and sufficiently high heating current.

Electron Microscopy

JEM-100CX II equipped with the EM-ASID4D (ultrahigh resolution scanning system) (JEOL, LTD., Tokyo, Japan) was used to visualize the prepared samples. Pictures were taken on Kodak Electron Microscope Film (Eastman Kodak Co., Rochester, NY). All photographic materials and chemicals used in this works were purchased from Eastman Kodak Company (Rochester, NY).

CHAPTER III

RESULTS

The isolation of intact, folded chromosomal DNA from *Escherichia coli* has been reported in several publications by Worcel and coworkers (16, 62, 71, 72). In their studies, two separate bands of DNA were obtained from sucrose gradients. One was the slow fraction of 1,300-2,200 Svedberg units (S) which contained free but folded chromosomes, and the other was the fast fraction containing particles of 3,000-4,000 S, which were mostly membrane-attached chromosomes. In their studies, these were examined by electron microscopy.

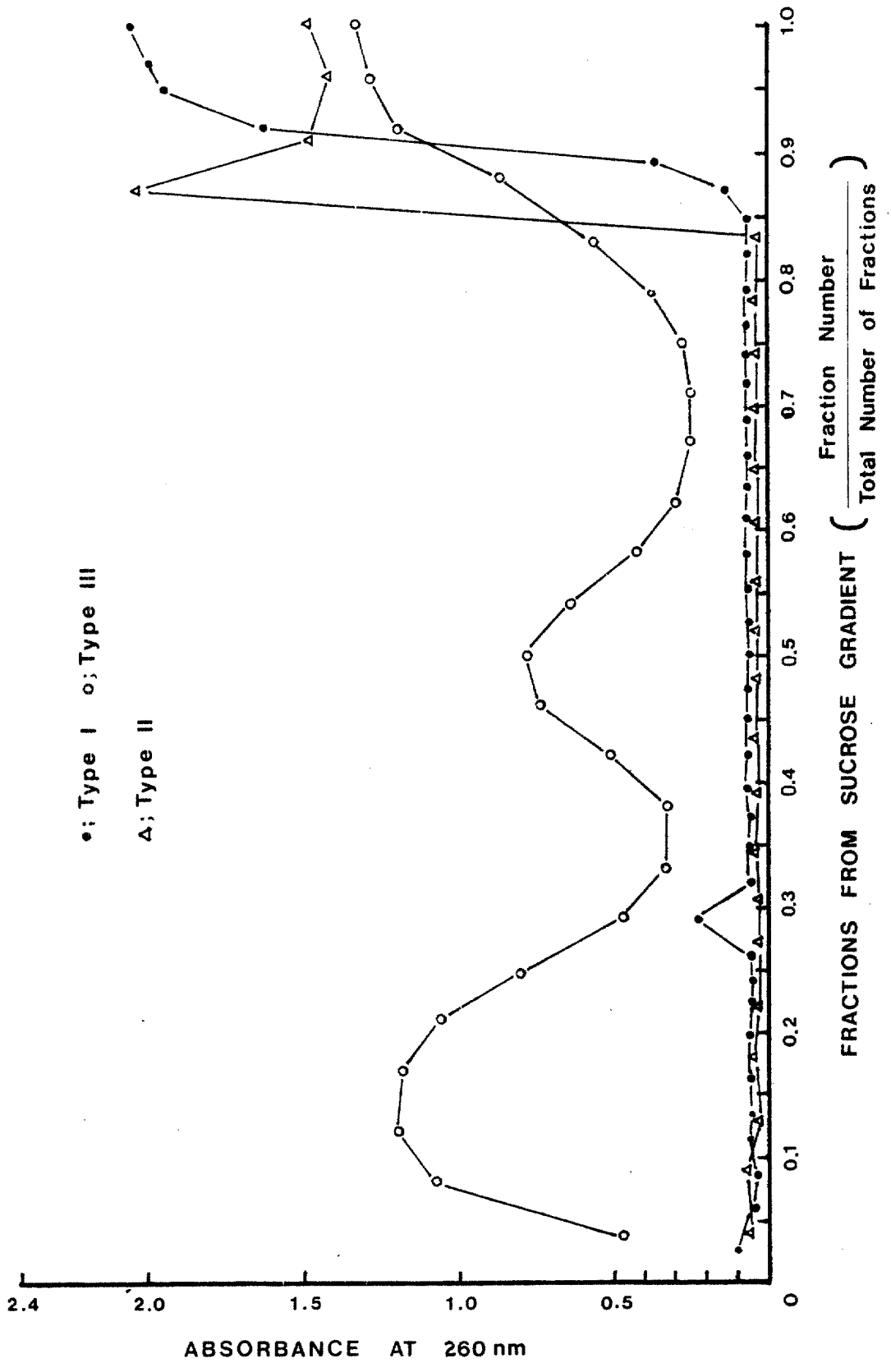
Folded chromosomal DNA from *A. vinelandii* could not be detected by the methods used for *E. coli.* Other researchers have failed to isolate the membrane-bound chromosomes of 3,200 S of azotobacter (33, 49). In this work, the clear separation of fast sedimentation DNA from slow sedimentation DNA was not accomplished, and the well-spread intact, folded chromosomes of *A. vinelandii* were not found by electron microscopy. Since we could

not isolate the folded membrane-bound DNA of *A. vinelandii,* we agree with Sadoff *et al.* (49) in saying that the chromosomal structure of azotobacter DNA is different from that of *E. coli.*

Spectrophotometry at 260 nm of fractions collected from the sucrose gradients are shown in Figure 3. After 30 minutes of centrifugation at 17,000 rpm, the sample DNA layered on the sucrose gradient remained on the top of the sucrose gradient with very little diffusion downward. A small increase in absorbance at 260 nm was detected in the fast sedimentation portion of the gradient (Type I in Figure 3). Generally, however, separation did not occur (Type II in Figure 3). Increase in centrifugation time gave a better separation of bands, but this resulted in breakage as well as unfolding of chromosomal DNA, probably by sheer forces in centrifugation (Type III in Figure 3). These facts imply that the chromosomal DNA of *A. vinelandii* does not behave the same structural characteristics as does that of other bacteria such as *E. coli.* This assertion supports the conclusion described immediately above and is also in agreement with the report of Sadoff *et al.* (49).

The gradient from the small absorption peak, Type I (Figure 3), was prepared for study by electron microscope. Electron

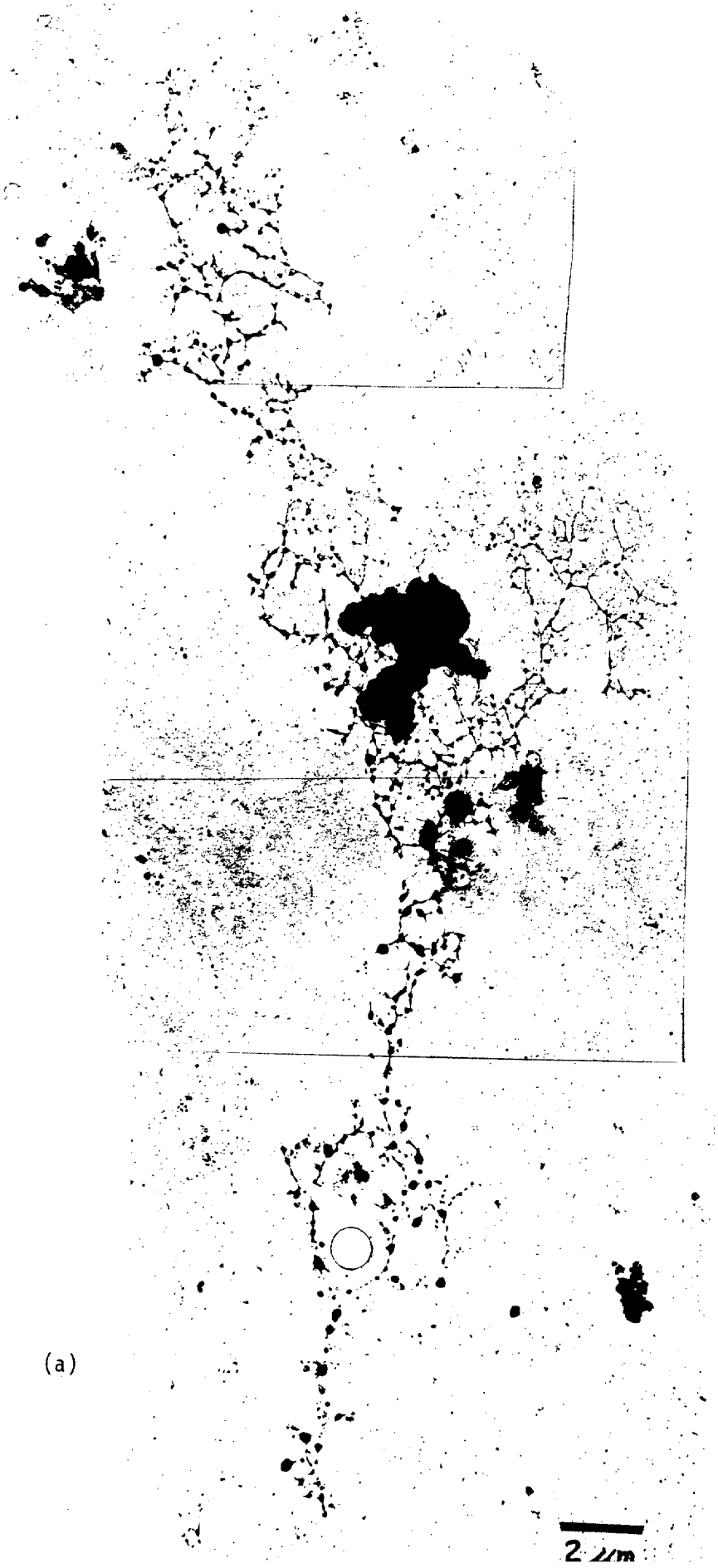
Figure 3. Typical absorbance at 260 nm of each fraction from the sucrose gradient. Symbols : • = Type I, fractions prepared with both 30 minutes of centrifugation at 17,000 rpm at 4°C, and by the released, folded chromosome method; Δ = Type II, fractions collected from a preparation after 30 minutes of centrifugation at 17,000 rpm at 4°C; o = Type III, fractions obtained after 6 hours of centrifugation at 17,000 rpm at 4°C. Type III shows that breaking of DNA occurred resulted in various sizes of DNA fragments after 6 hours of centrifugation. All types were prepared by the same lysis method for membrane-attached folded chromosomal DNA represented in the Materials and Methods.


•; Type I o; Type III
Δ; Type II
ABSORBANCE AT 260 nm
2.4
2.0
1.5
1.0
0.5
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
FRACTIONS FROM SUCROSE GRADIENT (Fraction Number / Total Number of Fractions)

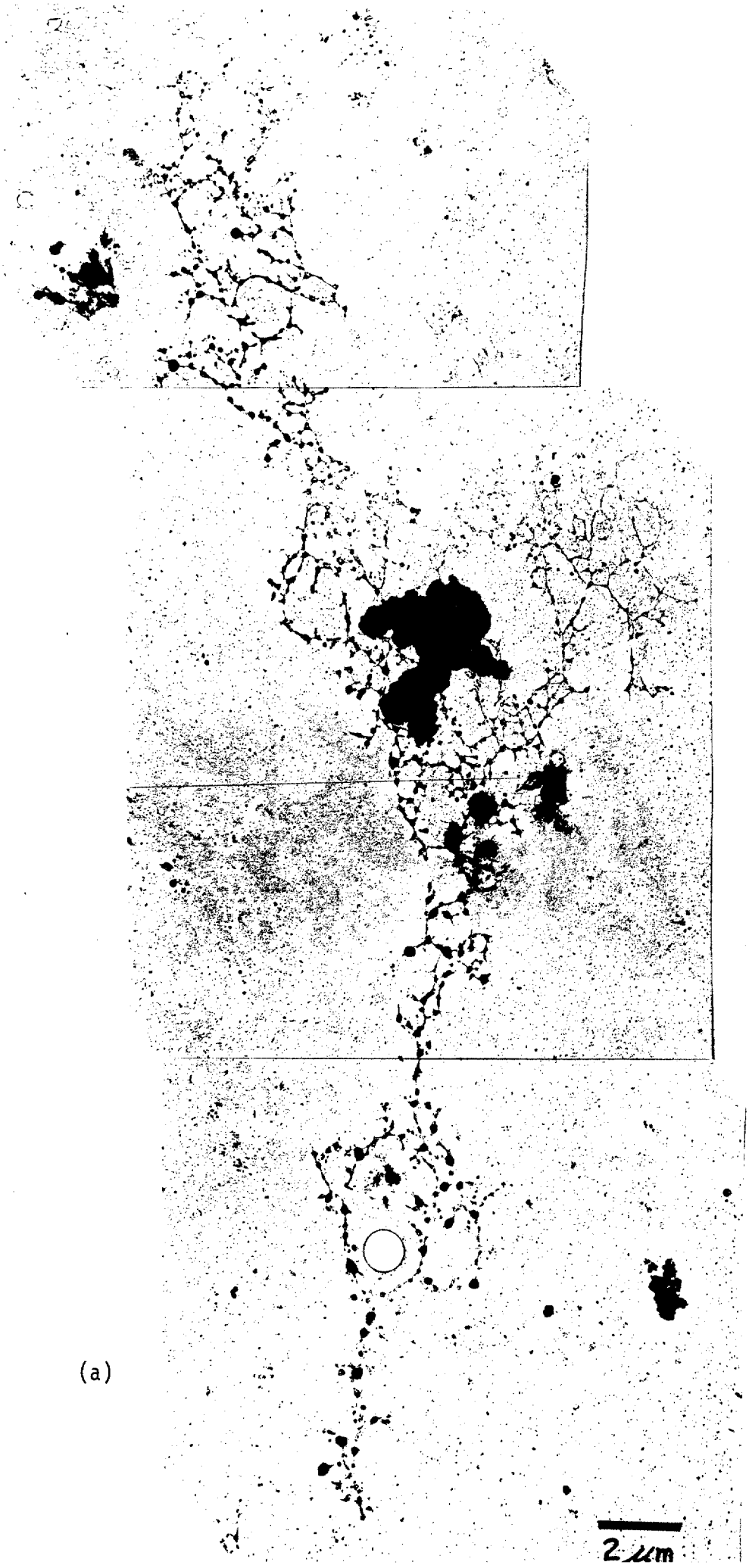
photomicrographs of the preparation are shown in Figure 4. Although a little increase of absorbance (about 0.2) was measured in that fraction, the complexes seen in Figure 4 were not found in other gradient fractions. The dense complexes attached to cell wall fragments are either carbohydrate or protein. There is no possibility that they are membrane-bound DNA fragments because the optical density of 0.2 is too low to explain the presence of as much material as that seen by microscopy. In addition to this, the structure of the complexes is very different from the DNA structure of *E. coli* isolated by the same procedure. It is very unlikely that the particles observed are DNA-membrane complexes. Instead of DNA-membrane complexes, they may be a unit of lipopolysaccharides attached to the outer layer of the cell wall. This would be expected in gram negative bacteria. Nikkaido (43) and Sutherland (64) reported details in the structure of lipopolysaccharides on the outer cell wall layer of gram negative bacteria. The black spots in the electron micrographs Figure 4, (a) and (b) are consistent with fragment of outer cell wall layer of gram negative bacteria.

The chromosomal DNA of *A. vinelandii* is attached to cell membrane as is that of other bacteria (Figure 5). On the other hand,

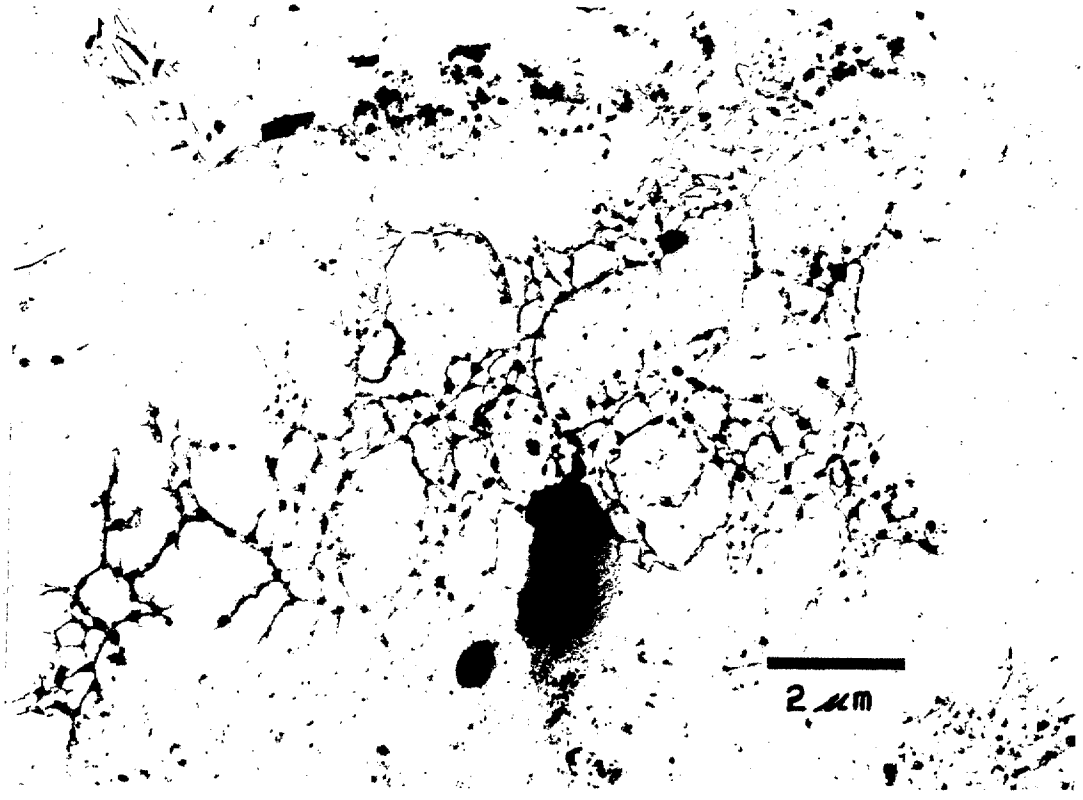
Figure 4. Electron micrographs prepared from the first peak fraction of Type I (Figure 3). (a) It is considered as a unit of lipopolysaccharide attached to the outer layer of cell wall, which was disrupted by lysozyme; (b) It shows more detail in linkage section between the polysaccharide chain and cell wall. This was prepared on the collodion coated copper grids by the same method of formamide techniques for DNA samples.



(a)



(a)

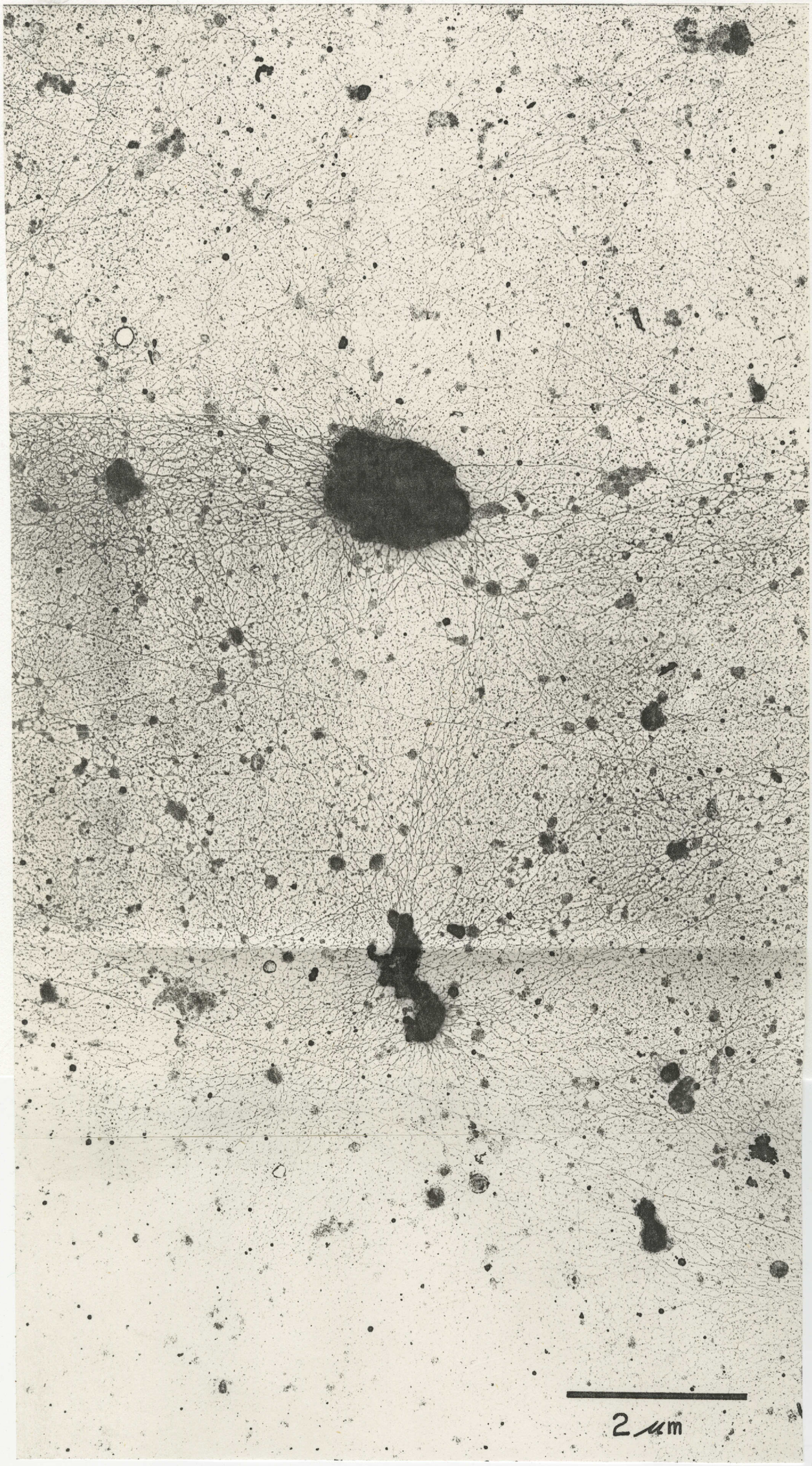


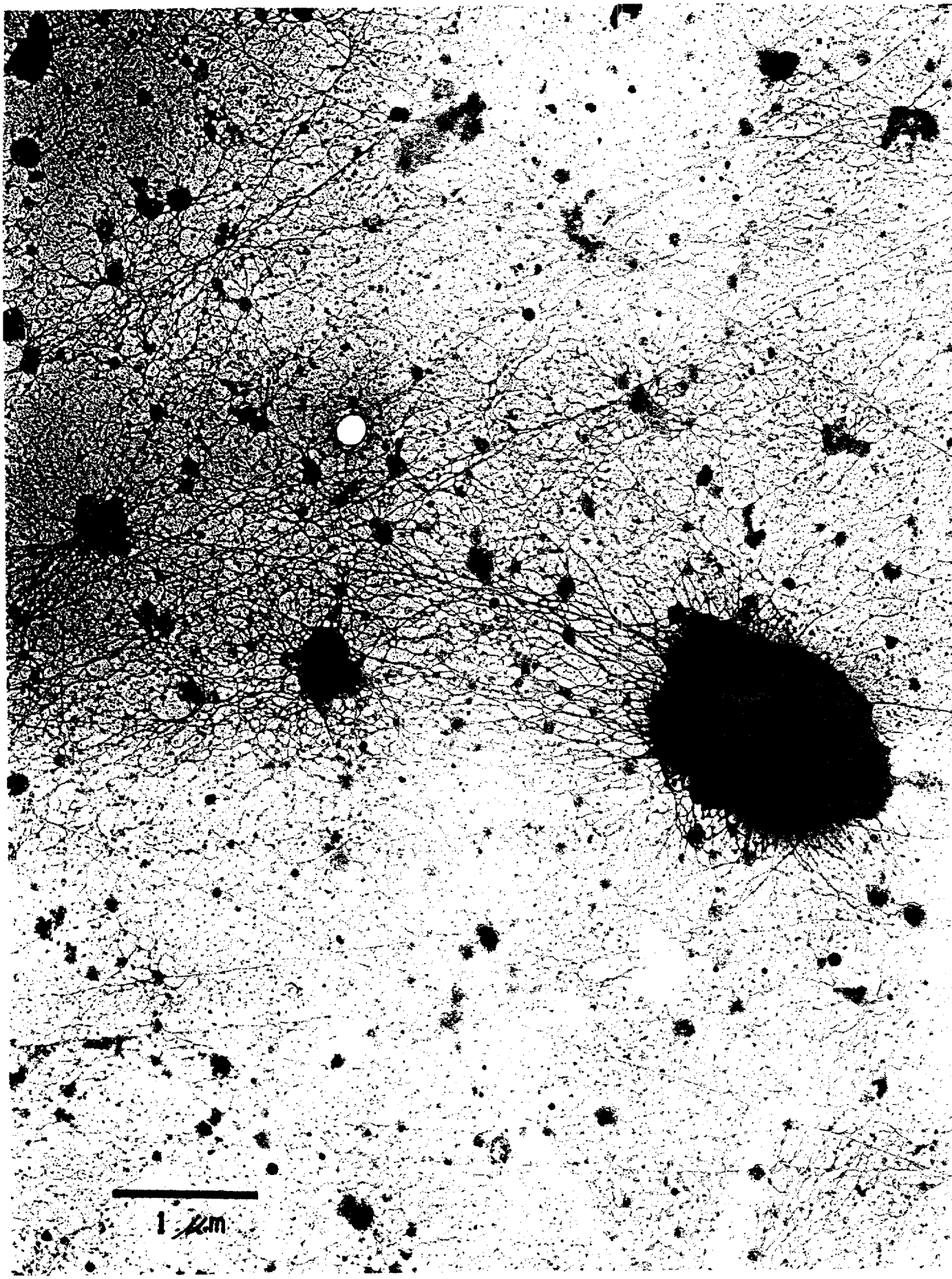
(b)

membrane-bound DNA in the folded state did not appear to be one distinct entity as it is in *E. coli* (16, 71, 72). Since neither the centrifugation experiments, nor microscopy showed clear separation of membrane-bound DNA, it may be that the DNA is of a different and unique type, one intermediate in which the result of centrifugation would give results such as those seen in *E. coli* but of different structure is that of *E. coli* . Figure 5 is an electron micrograph that shows cell membrane particles, black spots, attached to a large amount of DNA spread out from the particles. Particles are seen to be connected to one another in some a still unknown, complicated manner. It is possible that the superimposing DNA structures seen in Figure 5 are in reality an artifact due to the high concentration of DNA employed. It must be recognized, however, that even if the concentration of DNA (about 1.9 μg/ml of the spreading solution) used was large, still there was sufficient space in the prepared grids for each membrane-bound DNA particle to be spread out evenly, forming well separated structures.

In addition, a special characteristic of membrane-bound azotobacter DNA was seen during lysis. That is, after 10 minutes of lysis at 0°c, the lysate showed a clean, chestnut color. Even after 5

Figure 5. Membrane-attached chromosomal DNA of *A. vinelandii* . DNA concentration of spreading solution was about 19 μg/ml. (a) Each cell membrane fragment is connected to another by DNA strands. Homologous gene pairing between multiple copies of genes and branch conformation of DNA strands may be concerned to keep each membrane-attached DNA unit holding together. (b) Magnified micrograph of (a). Several long straight DNA fibers can be detected. They are stretched independently of other DNA strands from cell membrane fragments. It implies that they may carry single-copy gene groups such as *nif* genes.


2 μm



(b)

minutes of centrifugation at 4,000 X G, no precipitation was evident in the lysate. In *E. coli,* on the other hand, the lysate showed turbidity even after 20 minutes of lysis time. This preparation from *E. coli* cells gave a large precipitate of cell debris after centrifugation at 4,000 X G. It can be said that almost all of the membrane fragments disrupted by lysis were bound to DNA molecules in *A. vinelandii.* That is, all DNA molecules of *A. vinelandii* are attached to the cell membrane at many different sites. In this sight then, the pictures in Figure 5 can be interpretated as follows: DNA strands attached to membrane particles at several sites stick to one another forming large aggregates of various sizes which give a spectrum of different sizes and weights. This being so makes it impossible to separate *A. vinelandii* into folded DNA and membrane-attached folded DNA as in *E. coli.*

This interaction between DNA strands may come from homologous gene pairing between the DNA strands having each a copy of a large segment of DNA with multiple genes. The complex structures between membrane fragments and DNA strands shown in Figure 5 cannot be held together only by the weak forces of homologous gene pairing in a centrifugal field of 17,000 rpm. Each membrane-

bound DNA particle is dense enough to be separated from the others in the centrifugal field. Therefore, a strong interaction between DNA strands and membrane fragments would hold the particles of the complex together. It is seen in the electron microscope as complex branching units of DNA spread out from different membrane fragments.

From these results, it is reasonable to assume that the protein-DNA complex will not be of constant size and weight and will consequently not separate in a sucrose gradient. To the contrary, since the DNA strands break at different points due to tensile stress in the centrifugal field, a spectrum of different size DNA strands attached to different membrane fragments is postulated. This postulate is in agreement with all observations of the DNA of *A. vinelandii.*

A specific feature seen in the micrographs shown in Figure 5 is the presence of several long, straight DNA strands in the DNA "net". These DNA strands may represent a relatively independent state of DNA molecules of different configuration, probably without homologous gene pairing. On the basis of evidence presented here, this is reasonable even though the strands are linked with other DNA

strands.

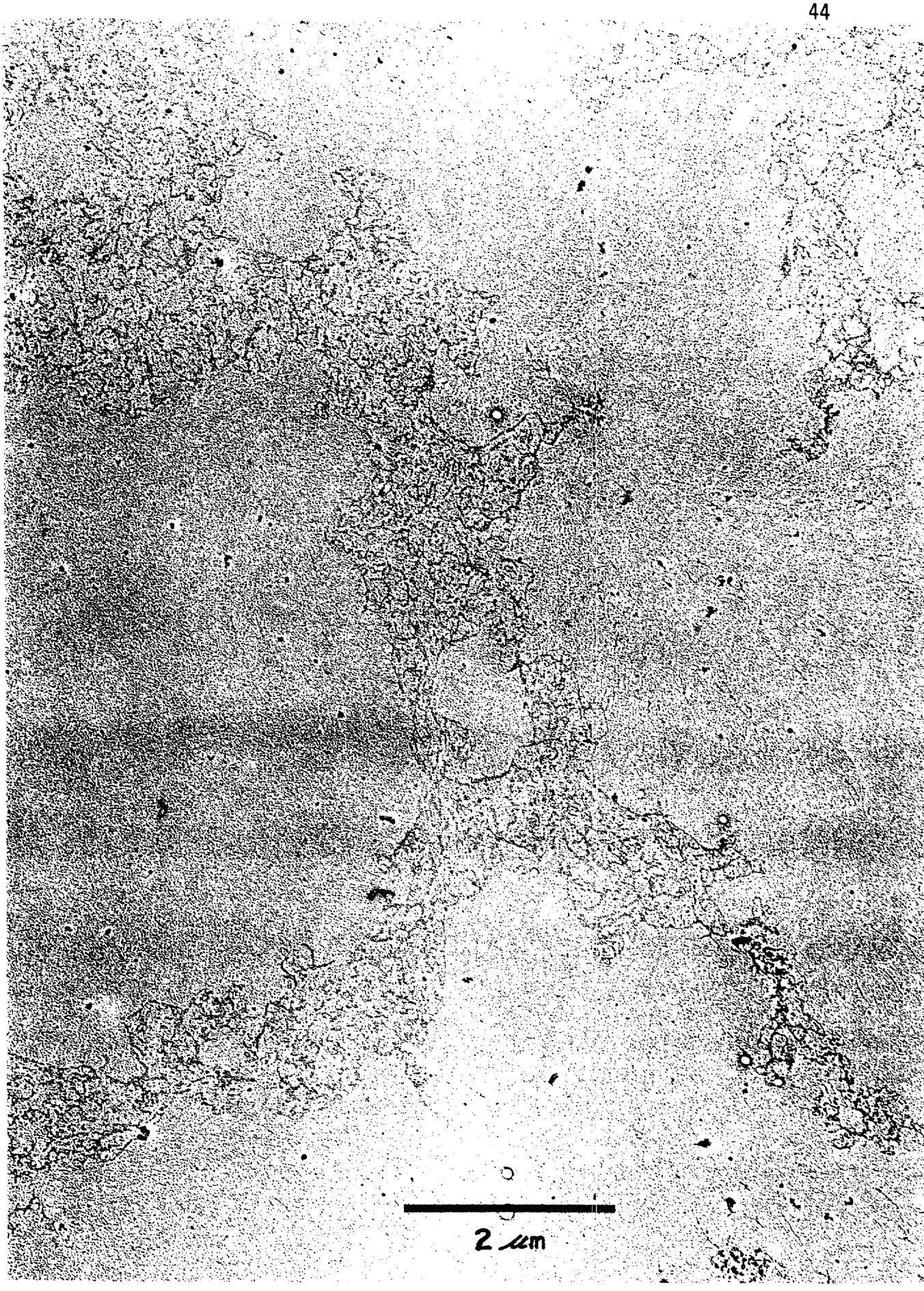
The configuration of the released, folded chromosome of *A. vinelandii* is quite different from that of *E. coli* (16, 71, 72). Although distinctive DNA strands are not completely visible, and even if lots of nicking points are observed in the material shown in Figure 6, fairly long strands of DNA go along the line of the circumference of the spread molecules, and circumscribe a complicated DNA net. The complexity of that configuration can be matched with that of Figure 5 in some ways. The presence of remarkably long strands, despite many other smaller strands showing the complicating interactions with other strands, and the absence of evenly-spread and well described double-strand DNA fibers very similar to those seen in *E. coli*. These unusual properties of chromosomal DNA in *A. vinelandii* imply that the conformation and the organizing manner of chromosomal DNA in *A. vinelandii* can not be directly compared with the well established bacterial chromosome characteristics seen in *E. coli* (7, 8, 9, 11, 20).

In conclusion, the data presented here show in several ways that the DNA of *A. vinelandii* is different from that of *E. coli*. First: the mutation kinetics of *E. coli* follow a one critical target kinetics

Figure 6. Released configuration of the chromosomal DNA. Specific conditions for preparing this micrograph are as follow: Twelve minutes of centrifugation at 17,000 rpm at 4 °C. Spreading solution picked up onto collodion coated grids by aqueous method. (a) Although many nicking points show, the DNA molecules are fairly well spread and represent a truly large chromosome. The arrow indicates the possible nicking point of a long DNA fiber surrounding other complicated DNA molecules. At the upper side of the nick point, another group of released DNA having similar features and size was seen but it showed more nicking points and more DNA in the released condition. (b) Partial magnified view.

(a)

2 μm



(b)

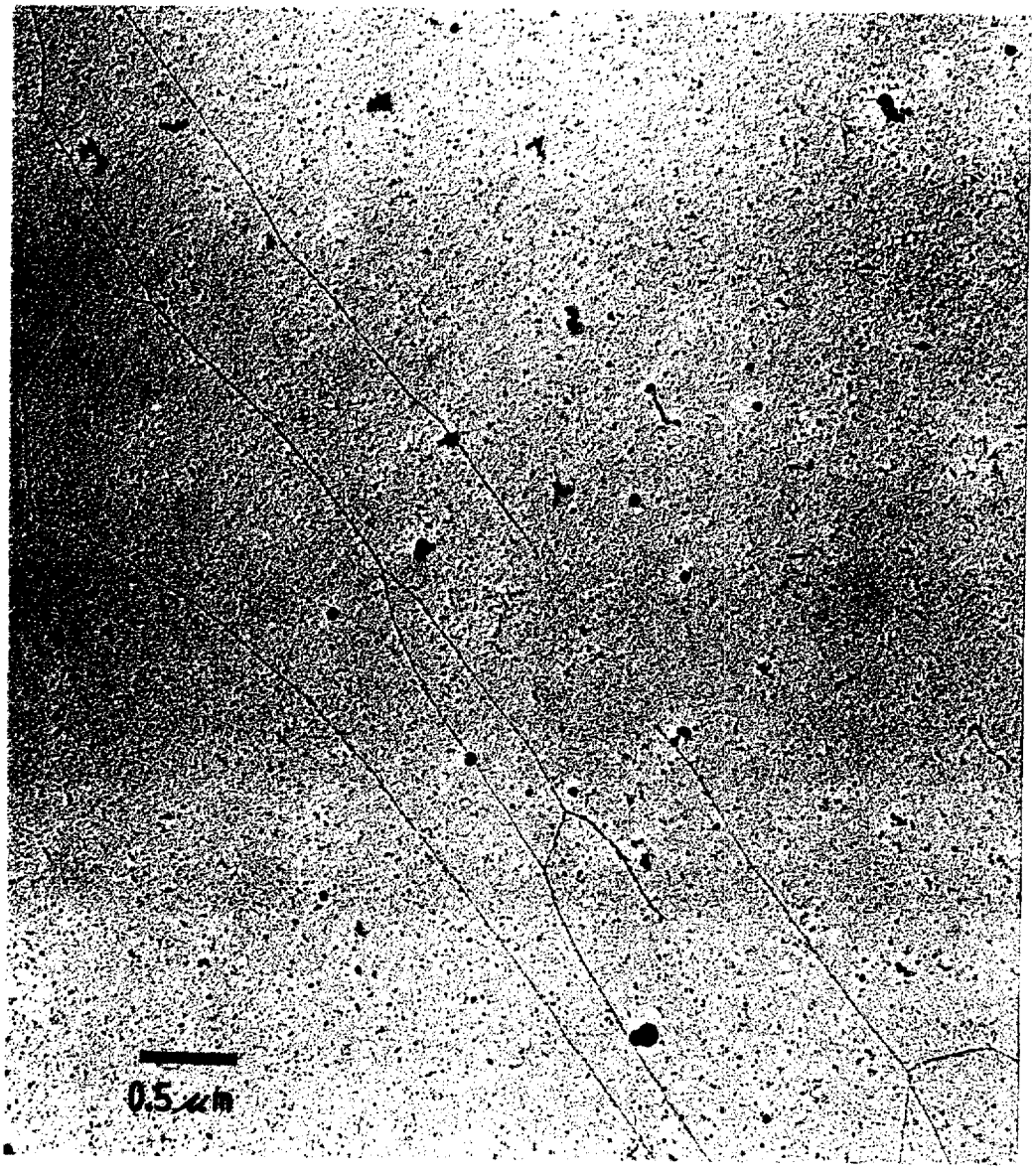
or 1 n function while that of *A. vinelandii* shows the same with regard to *nif,* and hypoxanthine, adenine, methionine, and uracil genes. On the other hand, mutations regarding antibiotics, amino acid requirements, and metabolic pathways show kinetics representative only of allelic multiplicities. Second: the behavior of DNA from *A. vinelandii* is quite different from that of *E. coli* in a sucrose gradient in some ways but very similar in others; that is, no clear separation between slow sedimented DNA particles and fast sedimented DNA-membrane particles in the gradient, no appearance of each membrane-bound DNA particle as one distinct entity, and formation of "net" complex among the membrane-bound DNA particles in the folded state. Third: the structure of *A. vinelandii* chromosomal DNA as seen with the electron microscope is quite different from that of *E. coli* in some respects but similar in others; that is, the presence of chromosomal DNA sub-units each of which is attached to cell membrane at different sites from one other, the presence of long, large DNA strand which stretches out independently from a cell membrane fragment in the DNA "net" complex, the presence of branching conformation of the long, large DNA strand with small sub-units of chromosomal DNA, and the presence of

sticking among the small sub-unit DNA strands probably due to the existence of homologous gene segments on the sub-unit DNA strands.

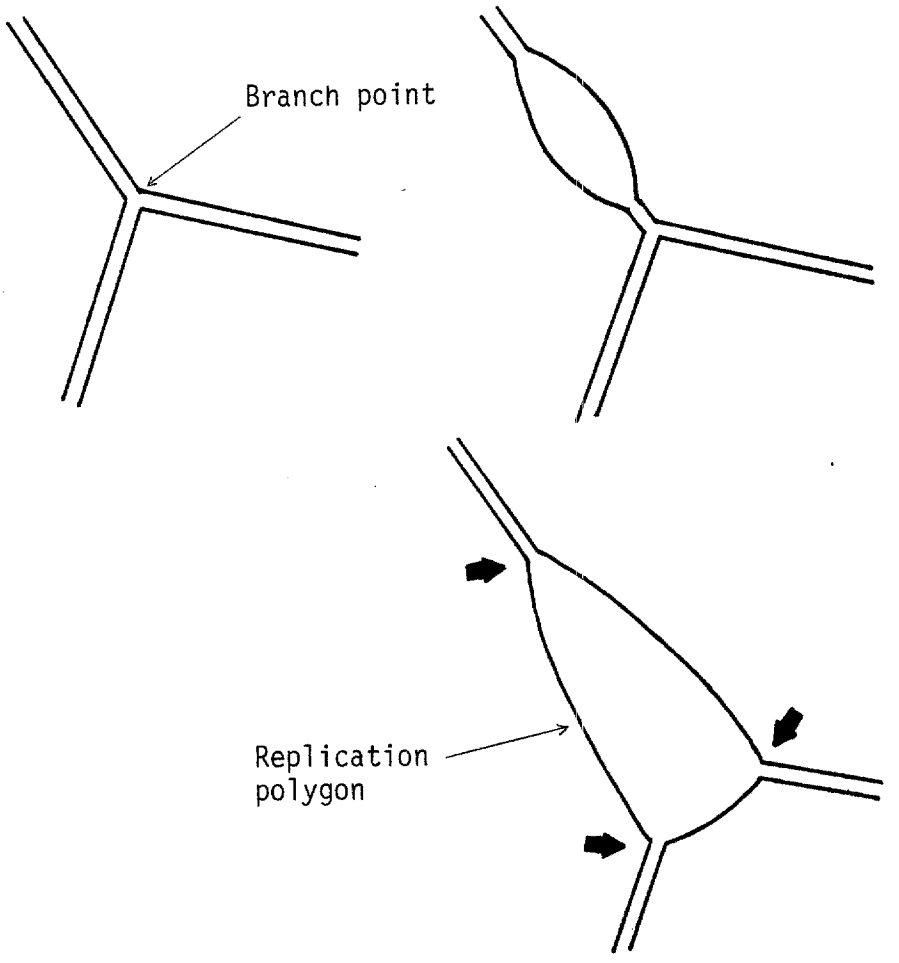
The most significant feature of DNA conformation is seen in the micrograph of Figure 7. In those micrographs, the postulation of branch conformation in *A. vinelandii* chromosomal DNA has a reliable possibility. The key feature is seen in Figure 7, (a), which shows a possible, novel configuration, called "replication polygon" in this study (Figure 7, (b)), observed during the replication process. This is a three-strand branch point of DNA. How can we interpret the bridge between the two strands if it is just considered to be a normal DNA replication fork? It appears almost certain that the strands are not superimposed on one another. The only way of an interpretation for the feature was shown in Figure 7, (b) with a schematic diagram.

There are two interpretations for the electron micrographs shown in Figure 7, (d) and (e) (see also Figure 7 (c)). First: the configuration of DNA observed can be caused by one DNA strand. It is assumed that this can be accomplished by self-circling. When the high magnification of the DNA strands (Figure 7 (e)) is examined, however, it does not look like a place where two linear strands

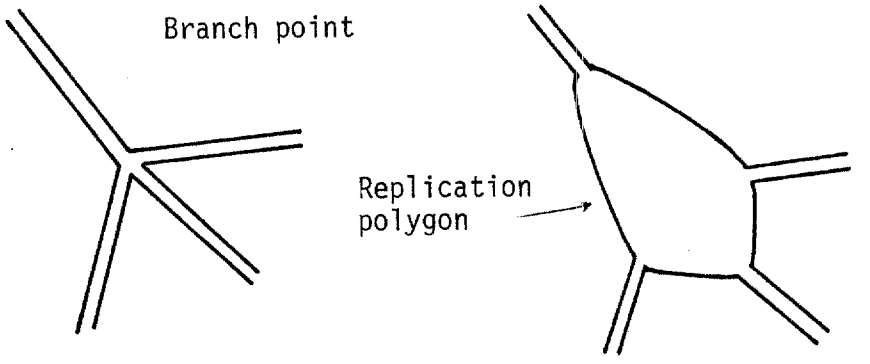
Figure 7. Electron micrographs of branch conformation of chromosomal DNA in *A. vinelandii* . This came from the same preparation shown in Figure 6. (a) Possible feature occurring during replication process of three-strand branch structure of the DNA molecule. Arrows indicate unwinding points for replication at each branch strand. The strand forming triangle is called a "replication polygon" in this study. The replication polygon in this figure seems to be a single strand molecule because it looks thinner than other branch strands having a double helix form. (b) The schematic diagram of the replication polygon is shown with detail of the branch strands. Arrows indicate same position with those in (a). First unwinding of double-helix. occurs in a strand of three branch strands, resulting in a "replication eye." This is followed by unwinding to the other branch strands of the replication polygon. The bottom part of the diagram shows that the same situation is possible for four-strand branch conformation. This structure was not found in these studies. (c) View of the location of the polygon. (d) and (e) Magnification of a branch point of *A. vinelandii* DNA.



(a)

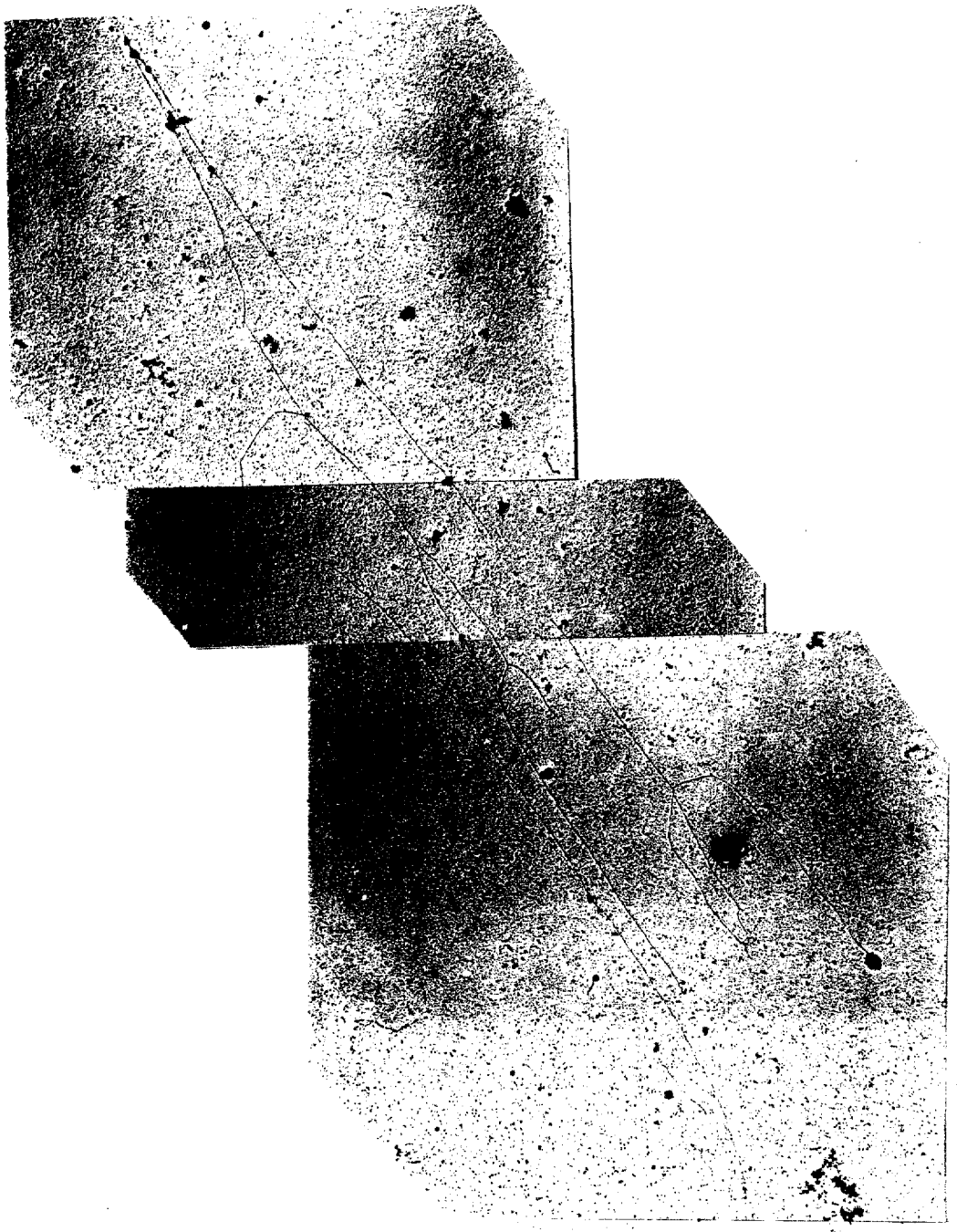


In three-strand branch conformation



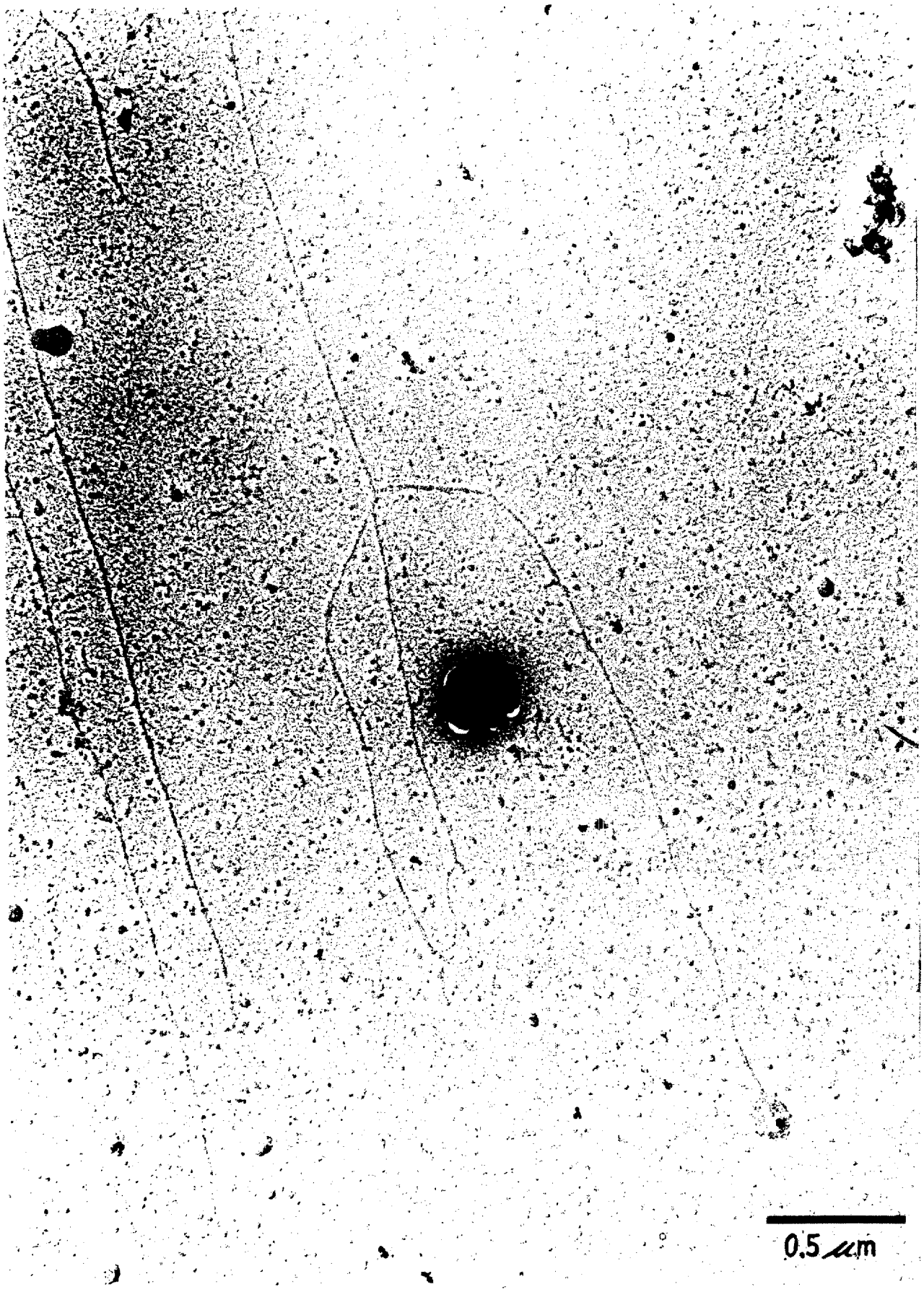
In four-strand branch conformation

(b)

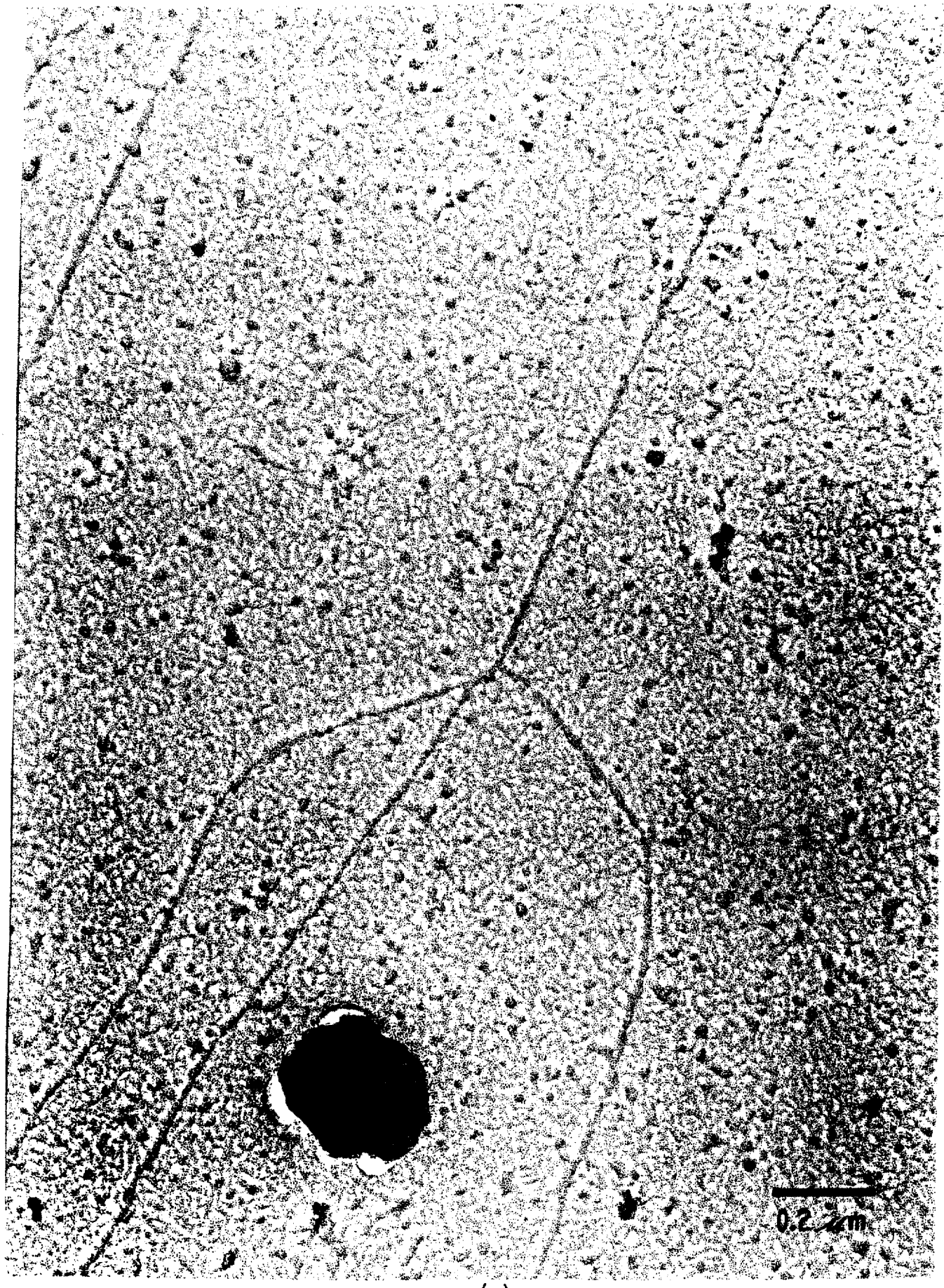


(c)

1 μm



(d)



(e)

cross superimposed one on the other. After careful study, it is obvious that the picture shows a three-strand branch point. One of the branch strands is forming a replication eye whose bottom side strand was broken off from the replication eye, and the others still remain as unreplicated branch strands.

Those pictures in Figure 7 suggest strongly that *A. vinelandii* has a branching chromosomal DNA molecule. This branch conformation of chromosomal DNA may give a strong force to hold each membrane-bound DNA particle together in the "net" complex.

CHAPTER IV

DISCUSSION

Many studies have been reported on the genetics of bacteria but much work still remains to be done. One of the most intriguing problems which remains unsolved concerns the genetics of *A. vinelandii* . It is an organism that may have as many as 80 copies of some of its genes (47) but only one copy of others. *Azotobacter vinelandii* also seems to have some 40 times as much DNA per unit of protein as does *E. coli* (47).

Although *E. coli* has seven sets of rRNA coding genes (38), the remainder of its genome is haploid with a gene number of 1 n. The work of other investigators has shown that there is probably a multiplicity of chromosomes in *A. vinelandii* (49). The difficulty in isolating mutants can be understood only if this is the case. Some mutants, however, including especially those at the *nif* genes, have been relatively easy to find by many workers.

In addition, there is no tenable hypothesis to explain the form in which DNA exists inside the *A. vinelandii* cell nor the reason why

such a large amount of DNA is present. The possibility that there are tandem, reiterated genes in a single, circular large chromosome was excluded by the work of Punita *et al.* (47). They suggested the existence of almost 80 identical chromosomes as big as half of the chromosome of *E. coli* . But this cannot be so *a priori* since *nif,* hypoxanthine, adenine, methionine, and uracil genes behave as haploid genes of 1 n number and not allelic genes of 80 n number.

In this work, a model of the chromosome of *A. vinelandii* is presented. This is the only model which can be reconciled with all the data available on the genetics of *A. vinelandii* . In this model, all genes are assumed to be reiterated but some exist only in a single copy. This explains why mutants of *nif,* hypoxanthine, adenine, methionine, and uracil are found frequently but those of antibiotic resistance and amino acid auxotrophs are never isolated (33).

Punita *et al.* reported *nif* HDK genes existed as almost 80 allelic copies in the *A. vinelandii* cell. However, they never mentioned the fact that mutants of the *nif* genes had been easily isolated by many workers. How can one solve this contradiction? It has been known that this nitrogen-fixing microorganism does not fix nitrogen in soil culture (24) as long as such other nitrogen sources

as NH_4^+, urea, and NO_3^-, even in trace amounts, are available (74). This means that *A. vinelandii* does not fix nitrogen gas in nature due to the abundance of other nitrogen sources in the soil. In laboratory cultures, in a nitrogen-free medium, nitrogen-fixation occurs readily as a result of the inducible enzyme system present in the cell. This requires synthesizing many enzymes like nitrogenase (26, 33, 53, 67) and nitrogenase reductase. These enzymes are oxygen labile and must be protected from oxygen (19, 69, 70).

Inactive nitrogen-fixing cells of *A. vinelandii* in nature probably have a single set of *nif* genes on the chromosomes since these are rarely used. For unknown reasons, the genes perpetually in use exist in, say 80 n number in order for the cell to survive. Gene amplification in both eukaryotes and prokaryotes has been reported (54, 61, 77). The multiplicity of *nif* genes reported by Punita *et al.* (47) can be understood if it is assume that nitrogen-fixation takes place in azotobacter only after the *nif* genes are amplified to the 80 n number. They do not explain why 80 copies of a gene or a metabolic pathway are required for that gene or pathway to function. They envision the *nif* genes in the haploid condition when they are examined by some *nif* gene probe but in the 80 n polyploid condition

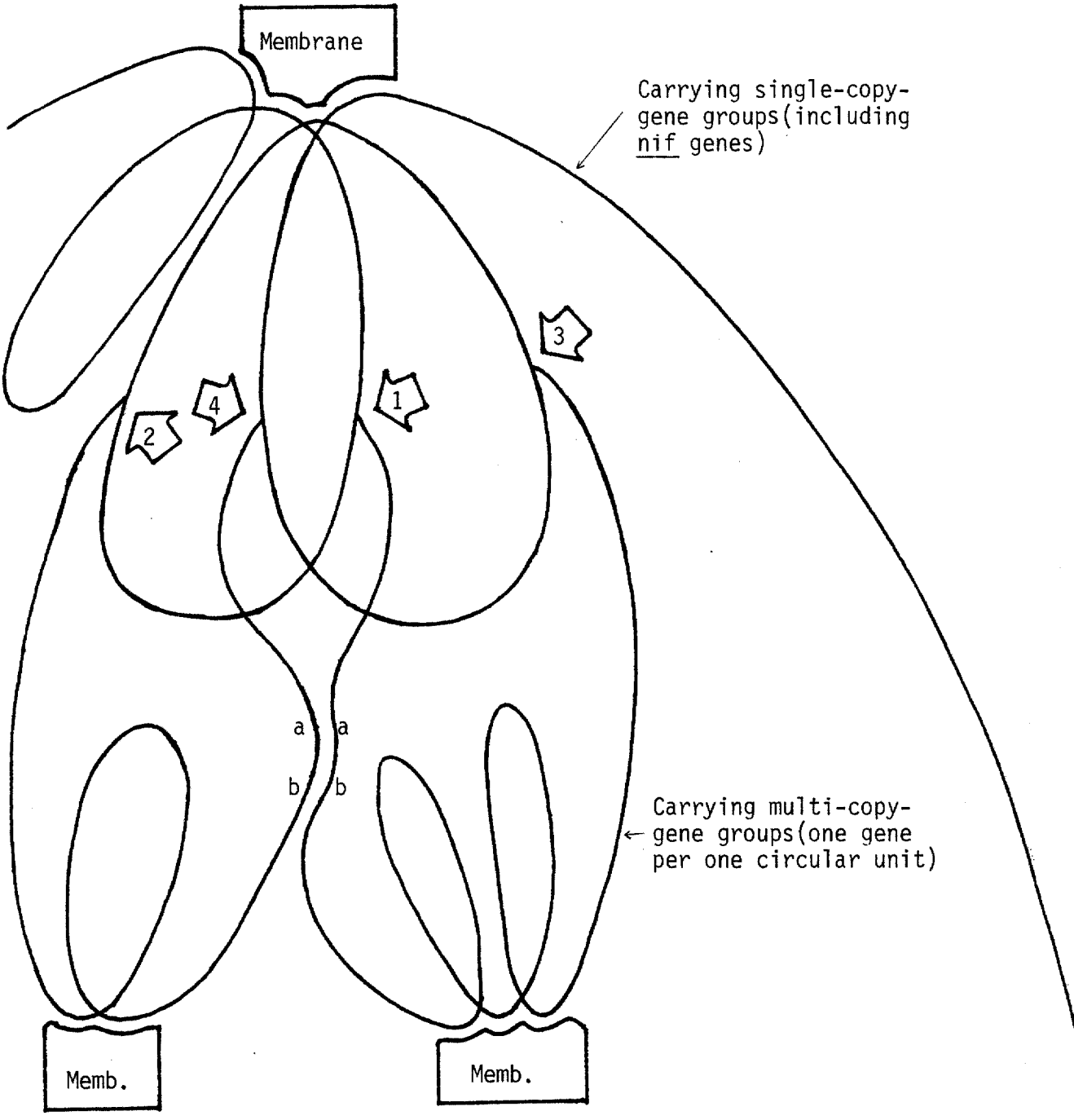
when the cells are growing. This is an erroneous premise since all assays for gene number reported by Punita *et al.* were performed on cultures in log phase or late log phase. Punita *et al.* should have been compelled to compare gene number in cultures fixing nitrogen which should give a multiplicity of 80 n according to their theory and those grown on media with nitrogen where the gene number should be 1 n. Since both cultures gave 1 n (Vela, unpublished data), this theory must be said false.

How can these single-copy gene groups including *nif* genes and multiple-copy gene groups exist on the folded chromosomes of *A. vinelandii* , in inherently stable form? Are they located on identical chromosomes one gene copy per one chromosome as suggested by Punita *et al.* ? If so, are the *nif* genes present on only one specific chromosome among many identical chromosomes all of which lack *nif* genes? In the postulated model of this study, the *nif* genes are placed on a large circular DNA, and other multi-copy genes are placed on each member of multiple circular DNA segments, one gene per one circular unit of large circular DNA carrying many other "branch" loops (Figure 8). This configuration of chromosomal DNA is reconciled with the results obtained in this study.

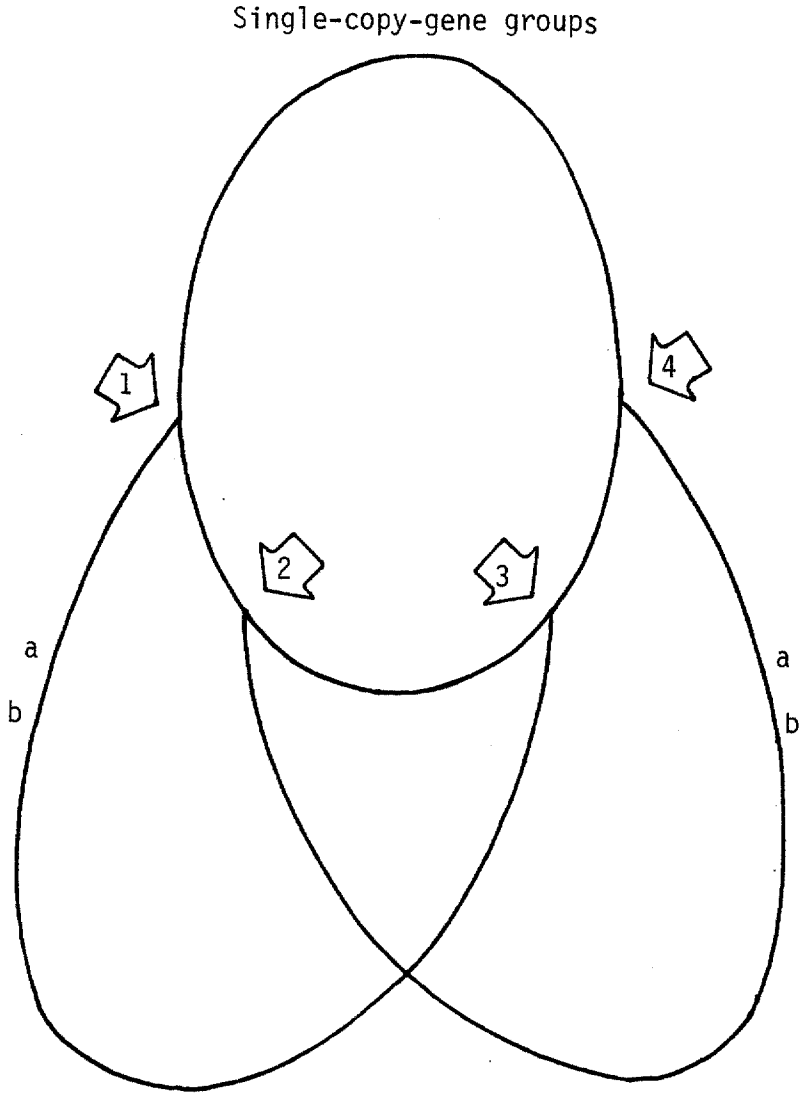
From all the results of this work, a model is postulated of folded chromosomal DNA in *A. vinelandii* that can be simply drawn as in Figure 8. Each folded circular DNA unit is attached to a different site of the cell membrane, and a long folded circular chromosome carrying single-copy genes such as *nif* are also attached to other sites of the cell membrane. The long circular DNA carrying single-copy gene groups is linked with all other smaller circular DNA units carrying multiple-copy gene groups at specific points by branch formation as shown by the arrows in Figure 8.

Details on the branch points shown in heteroduplex DNA and genetic recombination processes have been reported in viruses (6, 12, 34). These branch points came from the renaturation process of heteroduplex DNA or an intermediate stage of the recombination process. However, they are assumed to not be original branch structures of native chromosomal DNA. The branch structure of the chromosome in *A. vinelandii* is within the realms of possibility when we consider these examples of branch points. All strands shown in Figure 8, (a) are highly super coiled double-stranded DNA similar to that of *E. coli*. The unfolded state of the model structure of chromosome (Figure 8, (a)) is shown in Figure 8, (b). It shows the

Figure 8. Model of folded chromosomal DNA carrying multiple copies of genes of *A. vinelandii.* (a) Membrane attached folded state. Each circular DNA unit is attached to the cell membrane at different sites. Long circular DNA carrying single-copy-gene groups may be stretched out from the site of cell membrane attachment. Arrows indicate branch points from the left side of the chromosome carrying single-copy genes to the right side. Small characters 'a' and 'b' indicate allelic genes on the chromosomal loops. This model shows an example of homologous gene pairing between segments with allelic genes. (b) Unfolded and released state of (a). Arrows indicate the same branch points with (a).


Membrane
Carrying single-copy-gene groups(including nif genes)
3
4
1
2
a
a
b
b
Carrying multi-copy-gene groups(one gene per one circular unit)
Memb.
Memb.

(a)

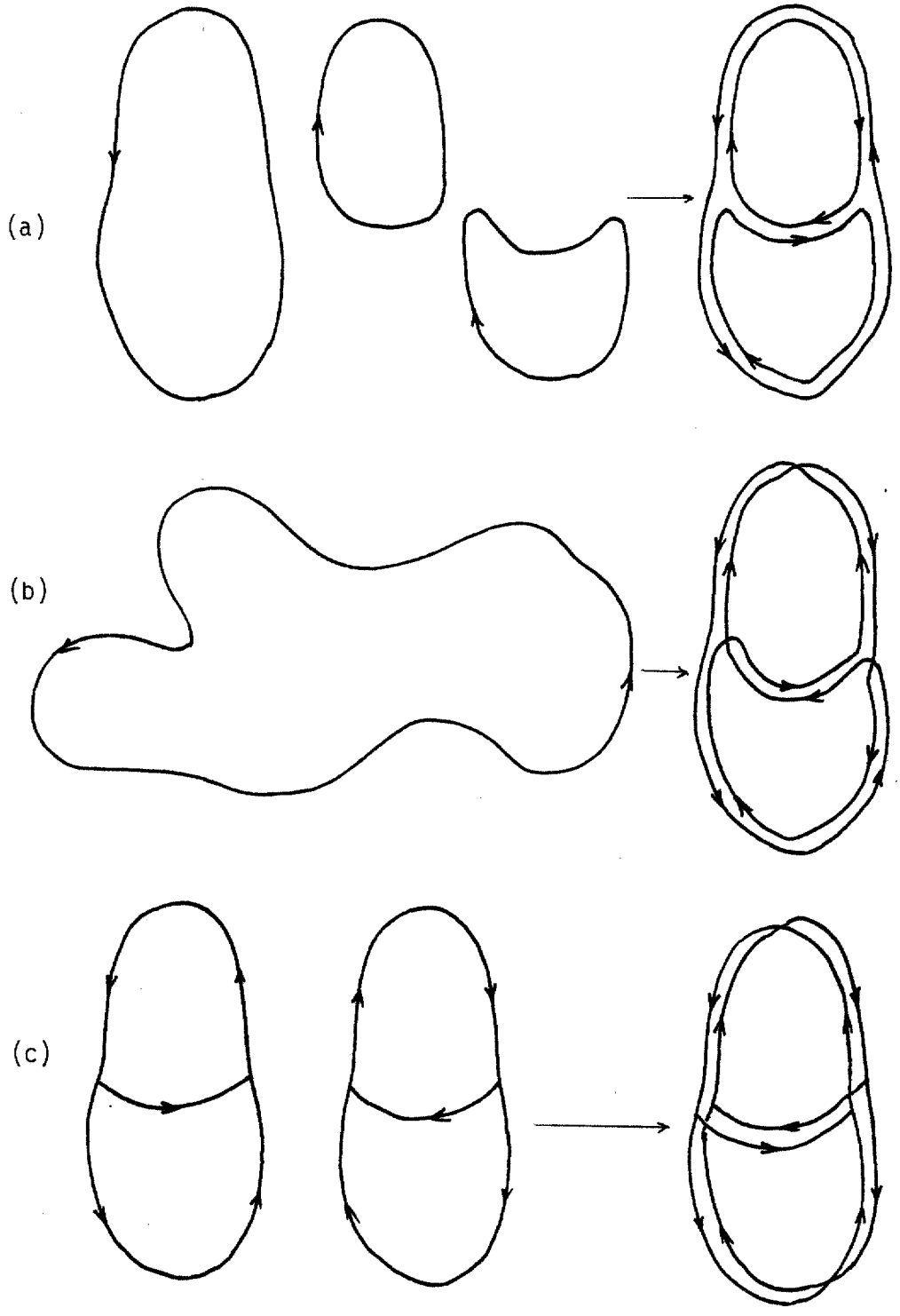


(b)

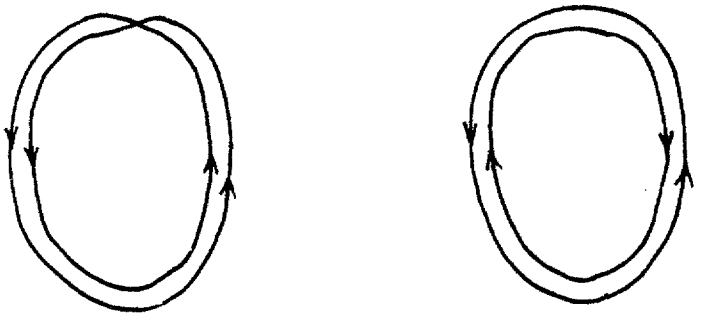
haploid region of the DNA from which circular DNA sub-units carrying multi-copy gene groups are branched out.

In the model proposed by this work, however, one inevitable problem occurs. It is difficult to envision a replication method, that is, this hypothetical chromosome model can not be replicated by the typical replication method of bacterial, circular, DNA as established by Carins (10) and by Salzman *et al.* (50), or even by the rolling circle model proposed by Bastia *et al.* (3), Dressler *et al.* (18), and Gilbert *et al.* (23). To resolve this problem, three possible constructions of branch formation of circular DNA molecules must be considered. The simplest possibility of branch conformation among circular chromosome units is shown in Figure 9. The first case suggests template base pairing of three single-stranded circular DNAs that have different sizes (Figure 9, (a)). The second case suggests that branch formation can be completed by bidirectional self-base pairing between template portions of only one large single-stranded circular DNA (Figure 9, (b)). In this case, the same construction system was applied to models of typical unbranched circular DNA. Bidirectional base pairing growth could not be accomplished in unbranched circular DNA represented by the sketches

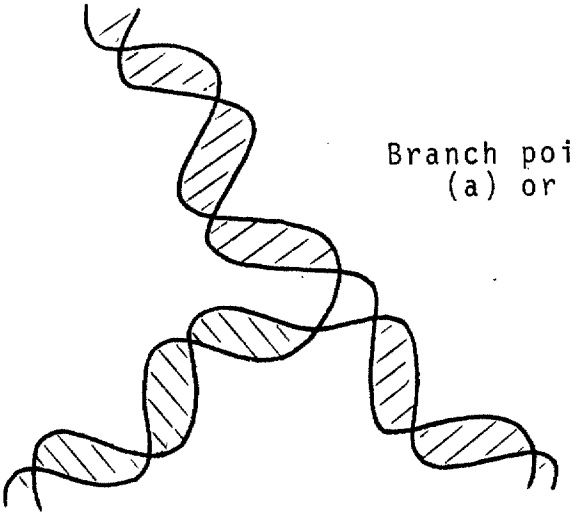
Figure 9. Three possible construction schemes of branch formation in circular DNA. Arrows indicate 5' to 3' directions in DNA molecules. (a) Construction by base pairing of three different sized, single-stranded DNA molecules. (b) Construction by bidirectional self-base pairing between the template portions in one large single-stranded circular DNA. Arrows show bidirectional base pairing. (c) Construction by base pairing between two templates of single-stranded DNA, which themselves have branch in phosphate backbone (see also Figure 13). (d) Applying the construction manner of self-base pairing of a single-stranded circular DNA to typical unbranched circular DNA. The left diagram shows the impossibility of bidirectional pairing. Right side diagram is a typical double-stranded circular DNA with bidirectional pairing between two circular single-stranded templates of DNA. (e) The double-helix feature of branch point in figure (a) and (b). (f) The double-helix feature of branch point in figure (c).


(a)
(b)
(c)

(d)



(e)



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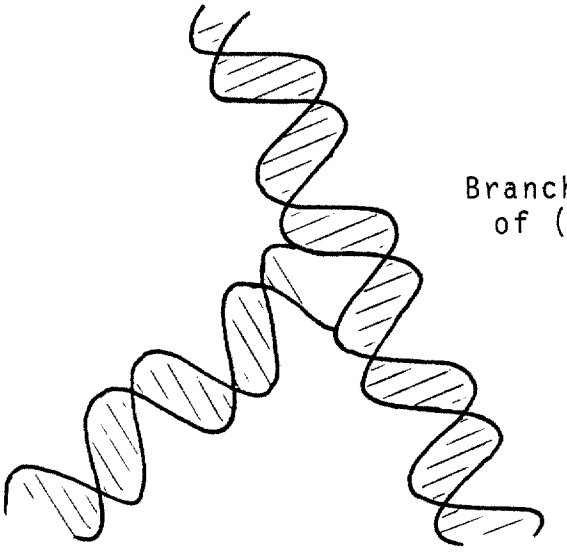
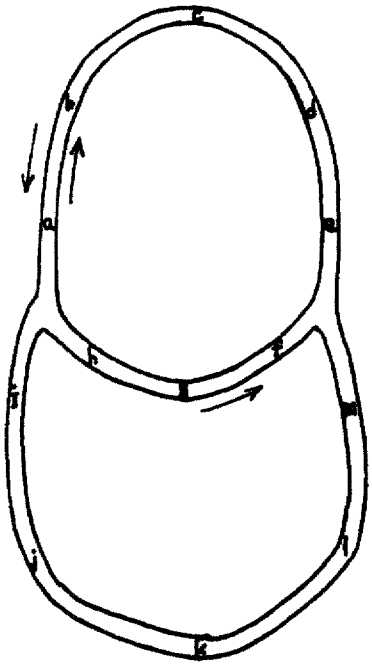


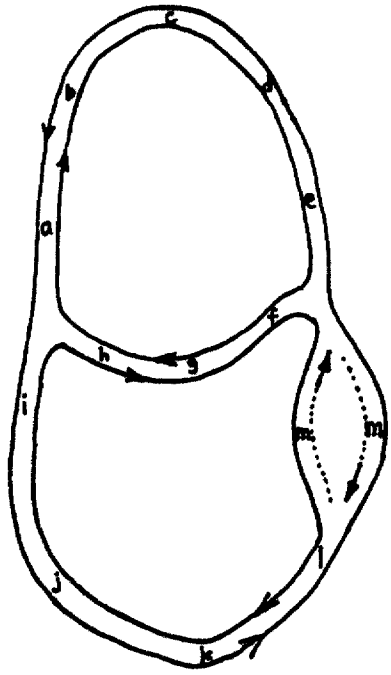
shown in Figure 9, (d). That is, the possible self-base pairing of one single-stranded circular DNA can be a new concept of DNA structures existing in only branch DNA conformations. The third case may be formed from the modification of the DNA structure at the molecular level in which the phosphate backbone of the DNA double-helix has a branching structure (Figure 9, (c)). Two templates of branched single-stranded DNA bind each other by base pairing. This would result in the branched, double-helix structure of chromosomal DNA.

In the first case, the possible replication mode shown in Figure 10 is tenable. In this mode, when a branched circular chromosome completes synthesis of new progeny strands, the chromosome is separated to three unbranched, circular, double-stranded DNA having different sizes. The basic concept of replication is compatible with the method of typical replication mode of bacterial circular DNA (10, 50). The main problem with this replication is how the three separated strands are able to resume their original branched conformation for conserving genetic stability. A possible way is that each circular progeny strand goes through a homologous gene pairing step, and then at each side of the branch

Figure 10. A postulated replication model of circular DNA having branch construction as shown in the scheme of case (a) in Figure 9. (a) Before replication. (b) Starting of replication near a branch point by formation of a replication eye. (c) Further progress of replication resulting in replication polygon. (d) Peeling off replicated strand. (e) Intermediate stage of completely separated three circular DNA units. (f) Homologous gene pairing between replicated circular progeny strand units having the same gene sequences followed by recombination with serial nicking and ligating at branch points by some specific enzymes. (g) Finally, producing complete two progeny chromosomes having the original branch conformation. Arrows indicate 5' to 3' direction. Small letters represent some genes on the chromosome. Doted line indicates newly synthesized DNA molecules. All parts of the double-helix replicated in progeny are composed of one older template strand and one newly synthesized strand.



(a)



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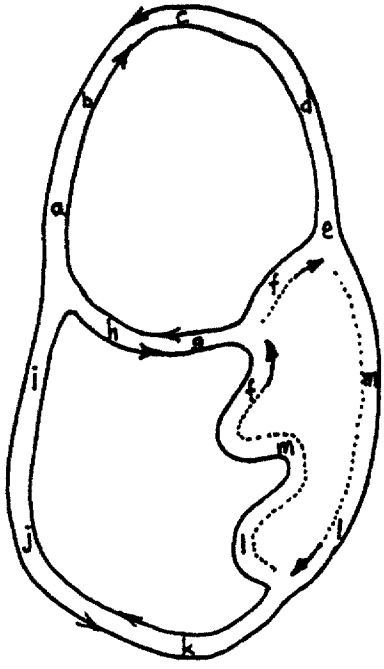
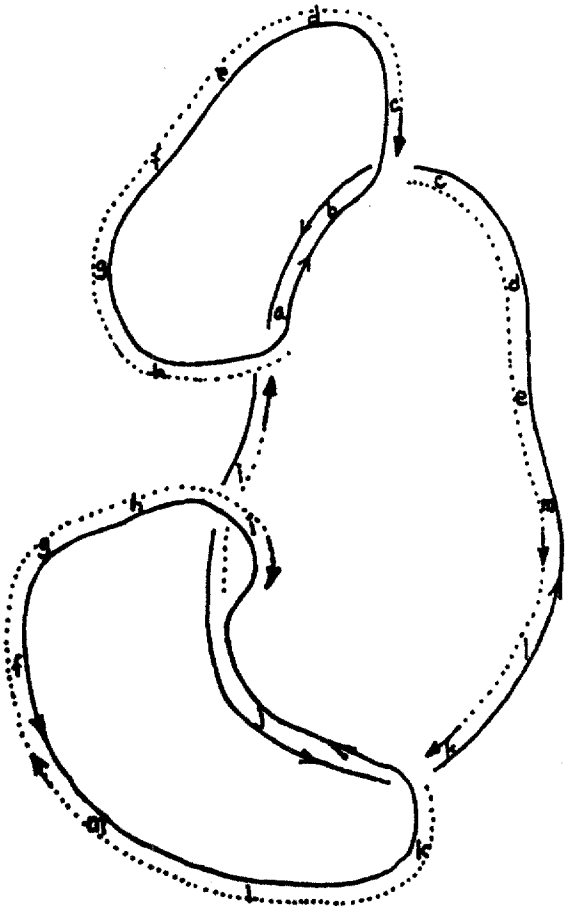
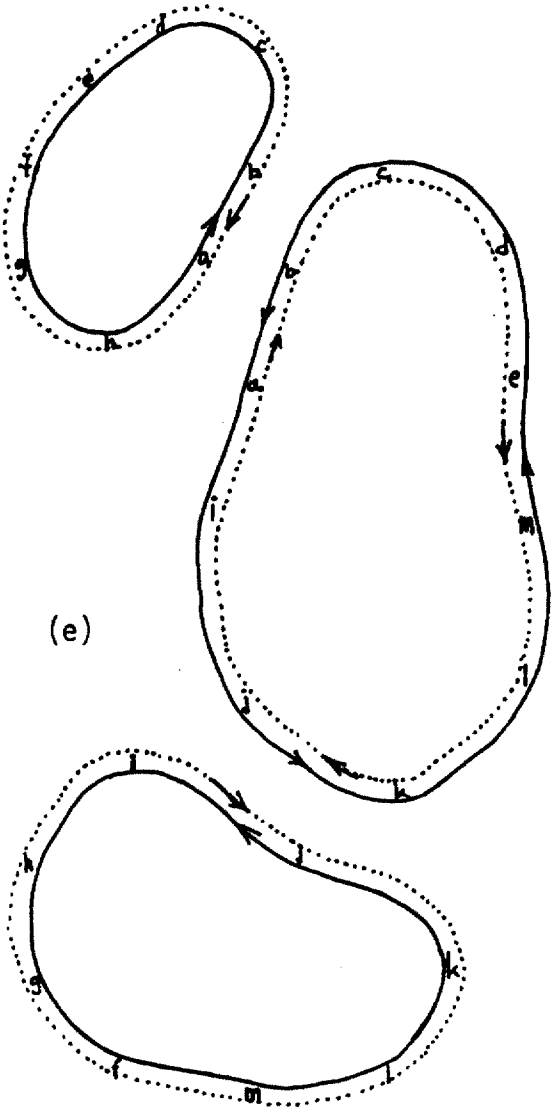


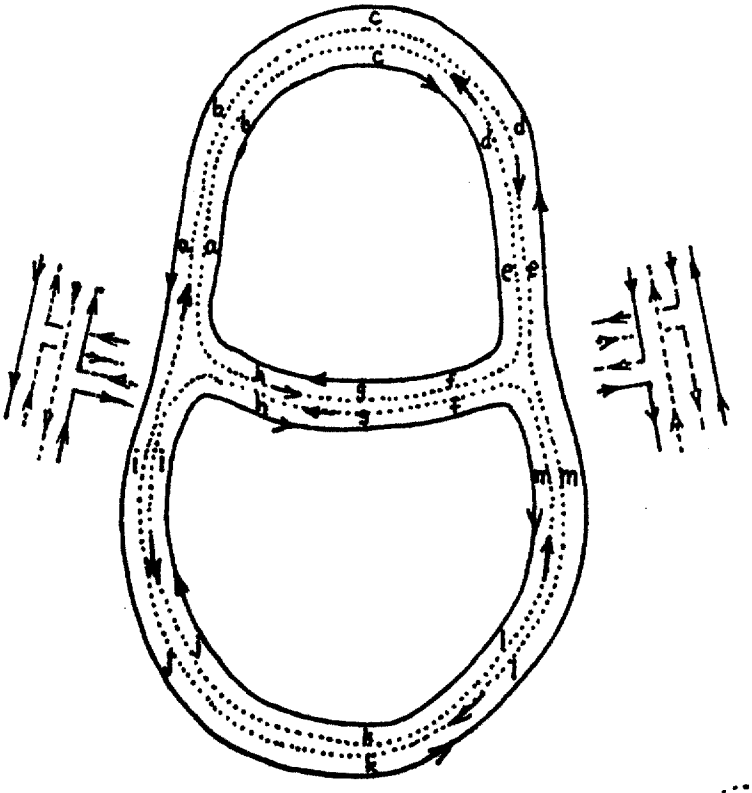
(c)



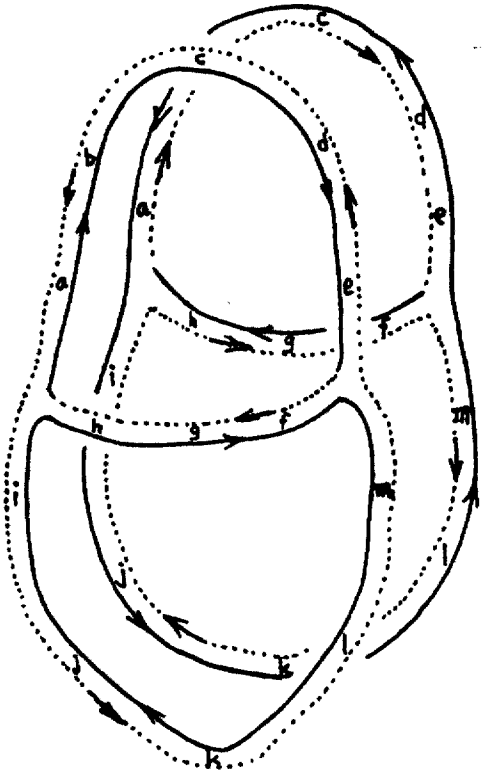
(d)



(e)

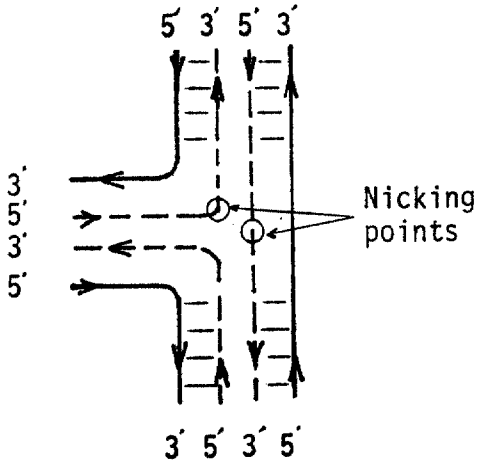


(f)

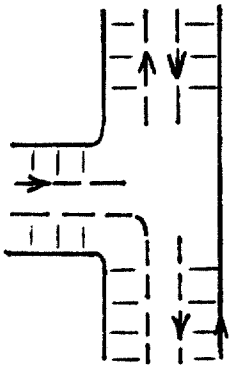


(g)

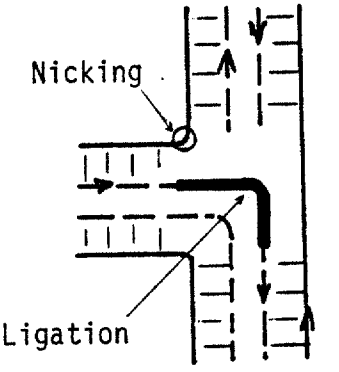
Figure 11. Serial recombinations to get original branch conformation in the replication process. Arrows indicate 5' to 3' direction. (a) The homologous gene pairing at a branch point. (b) First two nicks producing four free ends of DNA molecules. (c) Ligation between only two possible strands. Other cases of ligation cannot occur due to the polarity of DNA molecules. (d), (e), (f), and (g) Sequential nicks and ligation as the same manner. (h) Separated two branch strands in one side of chromosome. (i) Separated two strands in the other side of chromosome.


5′ 3′ 5′ 3′
3′
5′
3′
5′
Nicking
points
3′ 5′ 3′ 5′

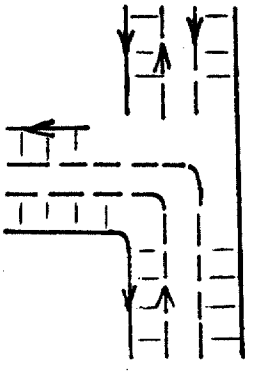
(a)



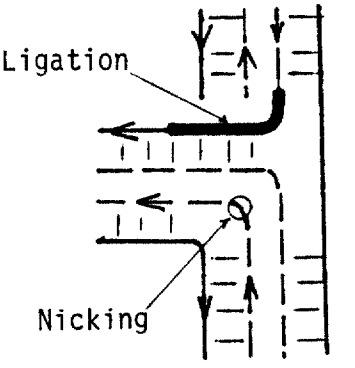
(b)


Nicking
Ligation

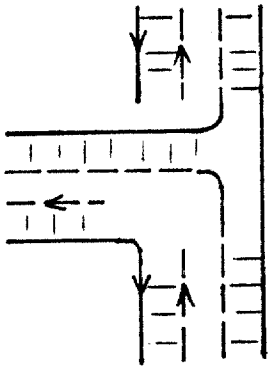
(c)



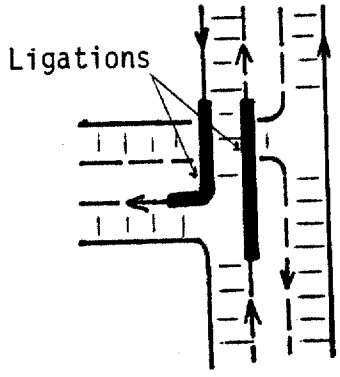
(d)


Ligation
Nicking

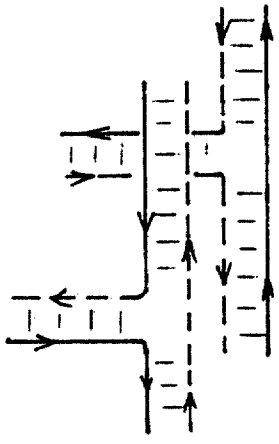
(e)



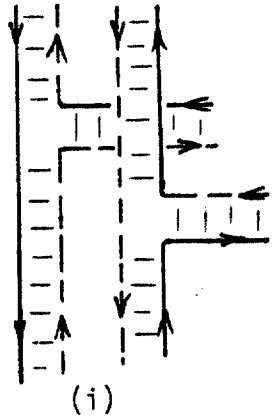
(f)



(g)



(h)



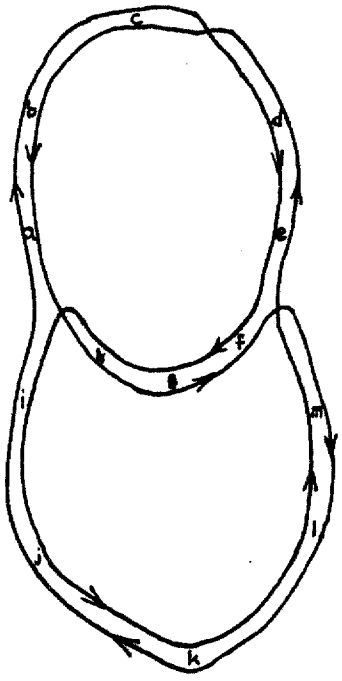
(i)

point, highly regulated, serial nicking and ligating occur (Figure 11). Consequently, two progeny DNA strands are produced having the original branch conformation. The replicated progeny strands contain one older strand and one newly synthesized strand in its double-helix.

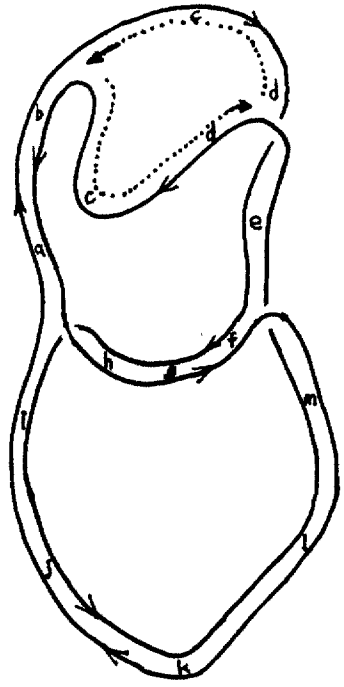
The second case would require a more complicated replication mode (Figure 12). Basic concept is, however, the same with the first one. Starting point of the replication is different from the first case. In this case, "replication polygon" is not formed because the replication started at a position far from branch points. If the starting position of replication were near one of the branch points, the replication polygon would be formed by this means too.

As opposed to the explanations given in the first case (Figure 10), When the synthesis of the new DNA strands is finished, one large circular double-stranded DNA is formed as an intermediate of this replication scheme. This circular DNA contains several pairs of homologous gene segments, and homologous gene pairing occurs among them. Two progeny DNA strands having original branch conformation are produced by recombination steps including serial nicking and ligating, which are exactly the same as in the first case

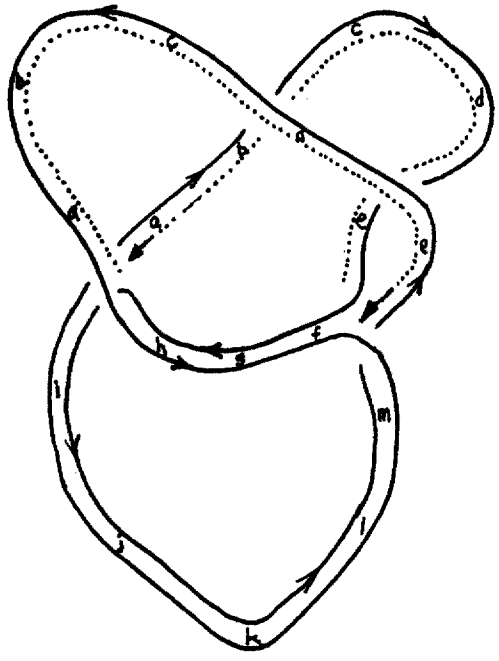
Figure 12. A postulated replication model of circular DNA having branch formations by the self-base pairing of a single large circular chromosome. Replication progresses in the sequence (a)-(b)-(c)-(d)-(e)-(f)-(g)-(h)-(i)-(j)-(k). (b) Starting replication at a point some distance from branch points. (f) One large intermediate single circular DNA during the replication process. (g), (h), and (i) Intra homologous gene pairing. (j) A modified configuration of (i). Recombinations at branch points are completed by exactly the same manner in Figure 11. (k) Separated two progeny strands. Each strand has one part of the older template strand and one part of newly synthesized strand. Arrows indicate 5' to 3' direction, and doted line signifies a newly synthesized DNA strand.



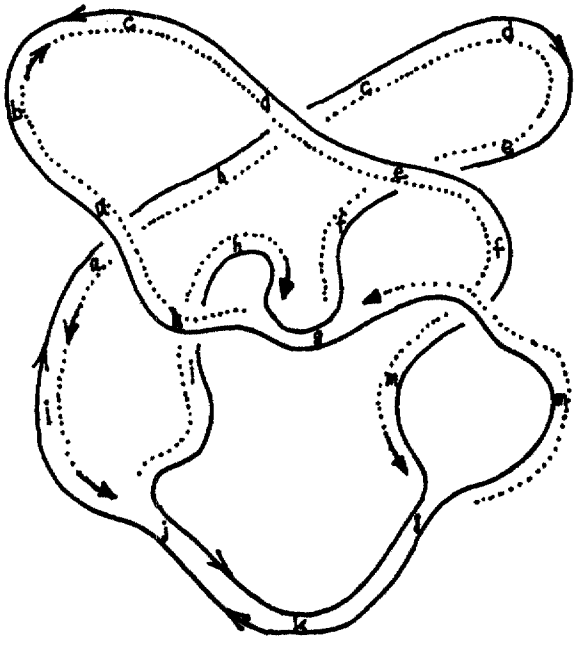
(a)



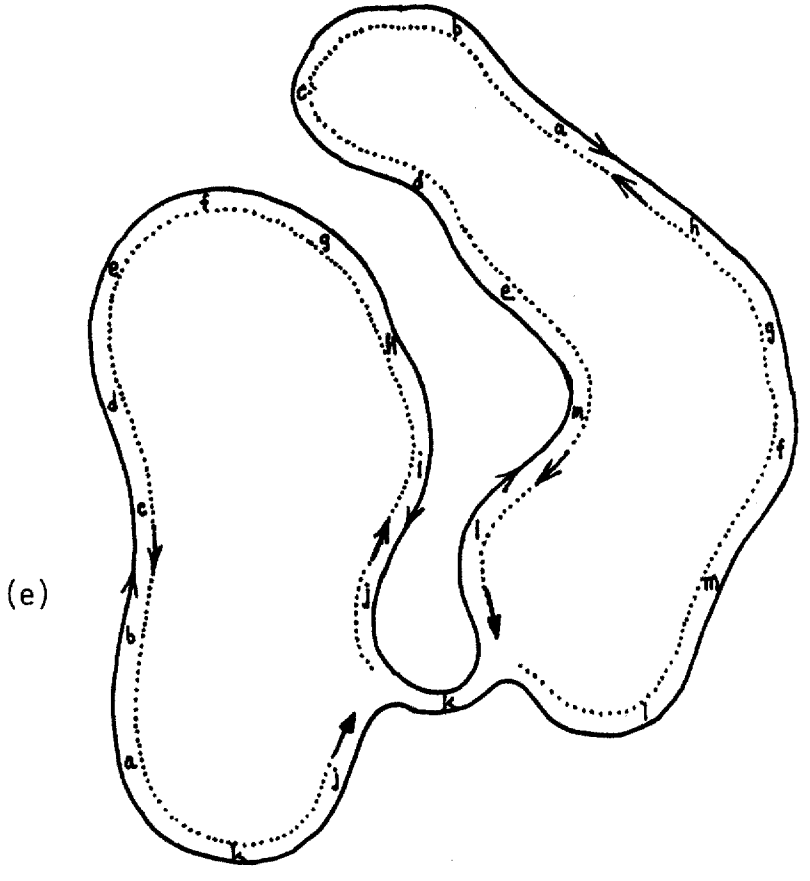
(b)



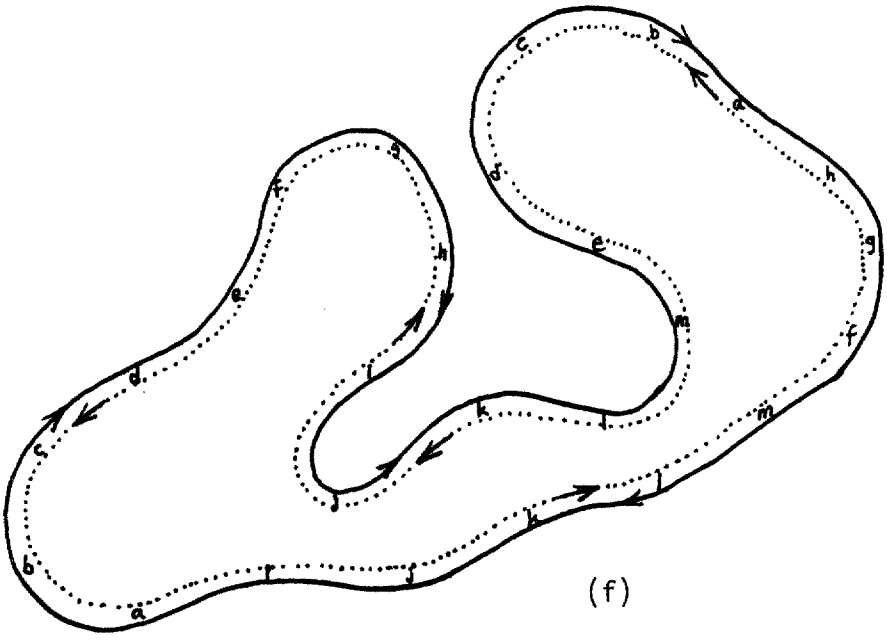
(c)



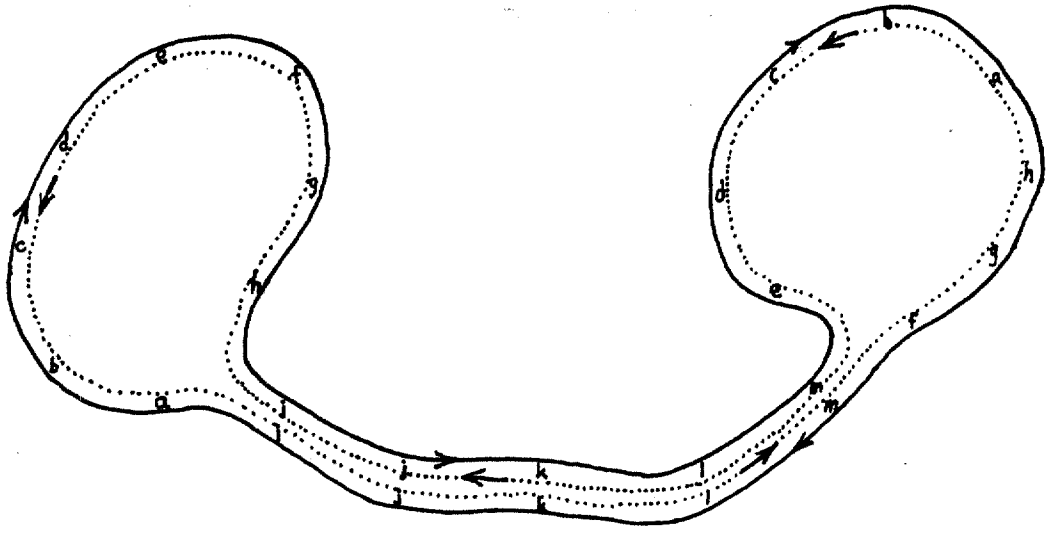
(d)



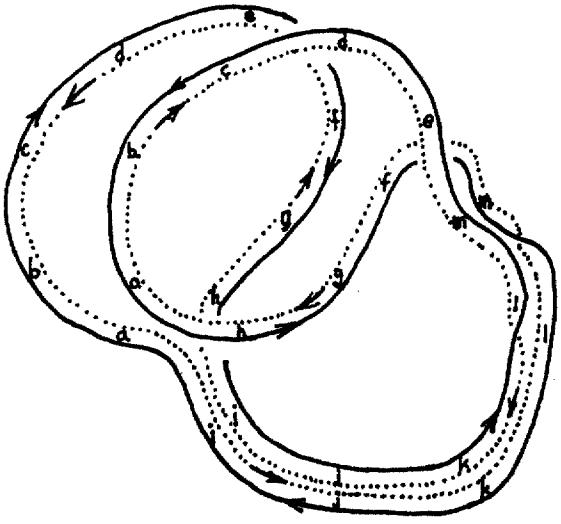
(e)



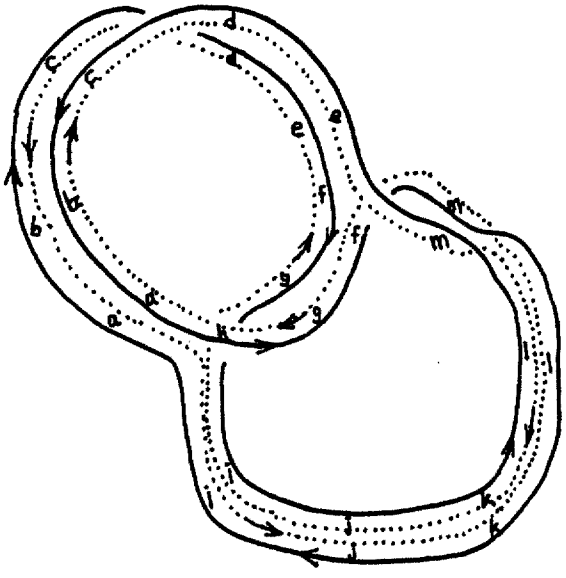
(f)



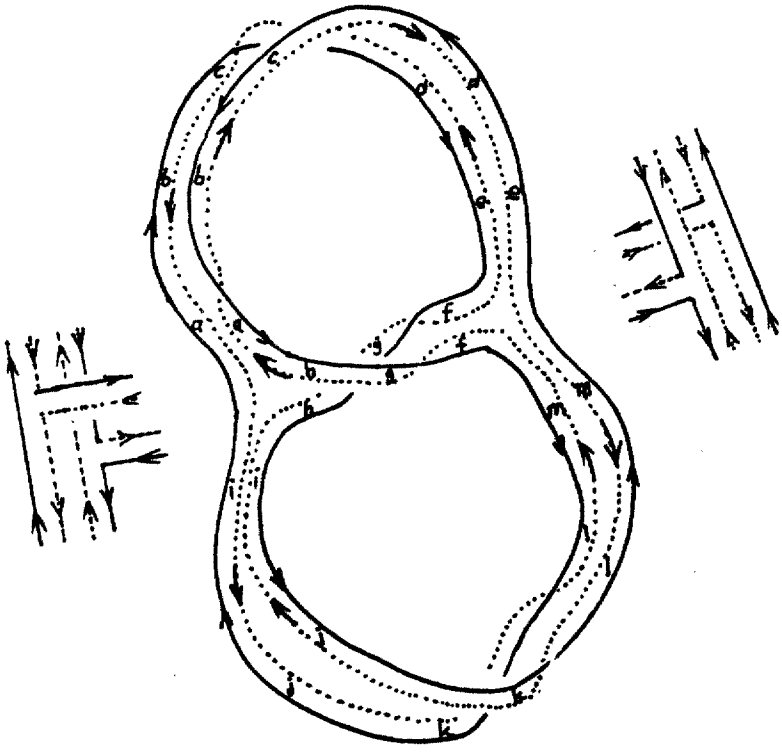
(g)



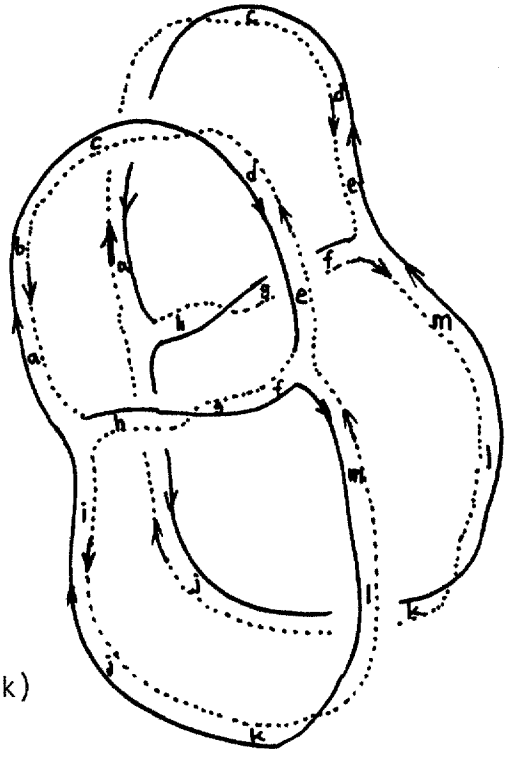
(h)



(i)



(j)

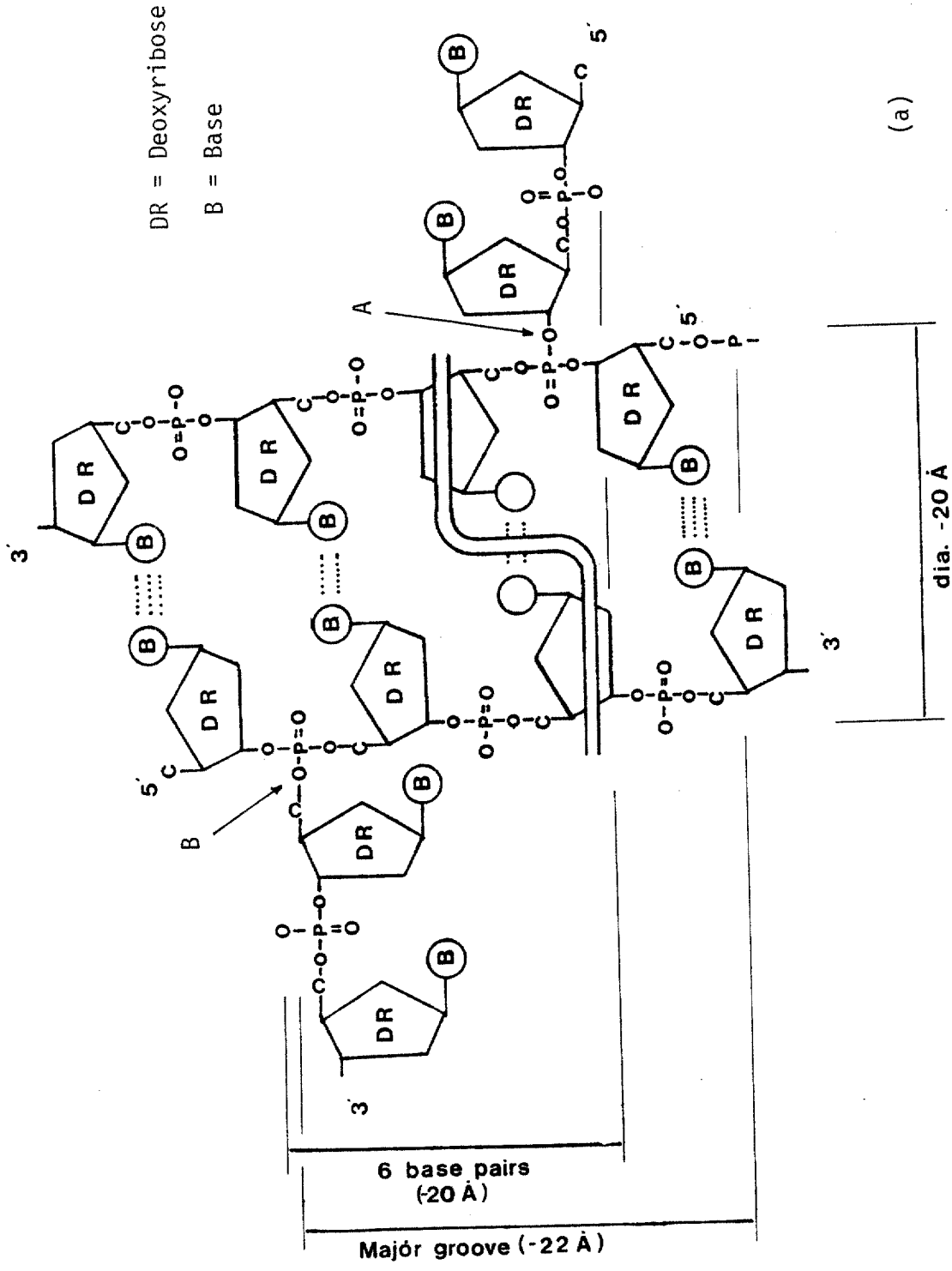


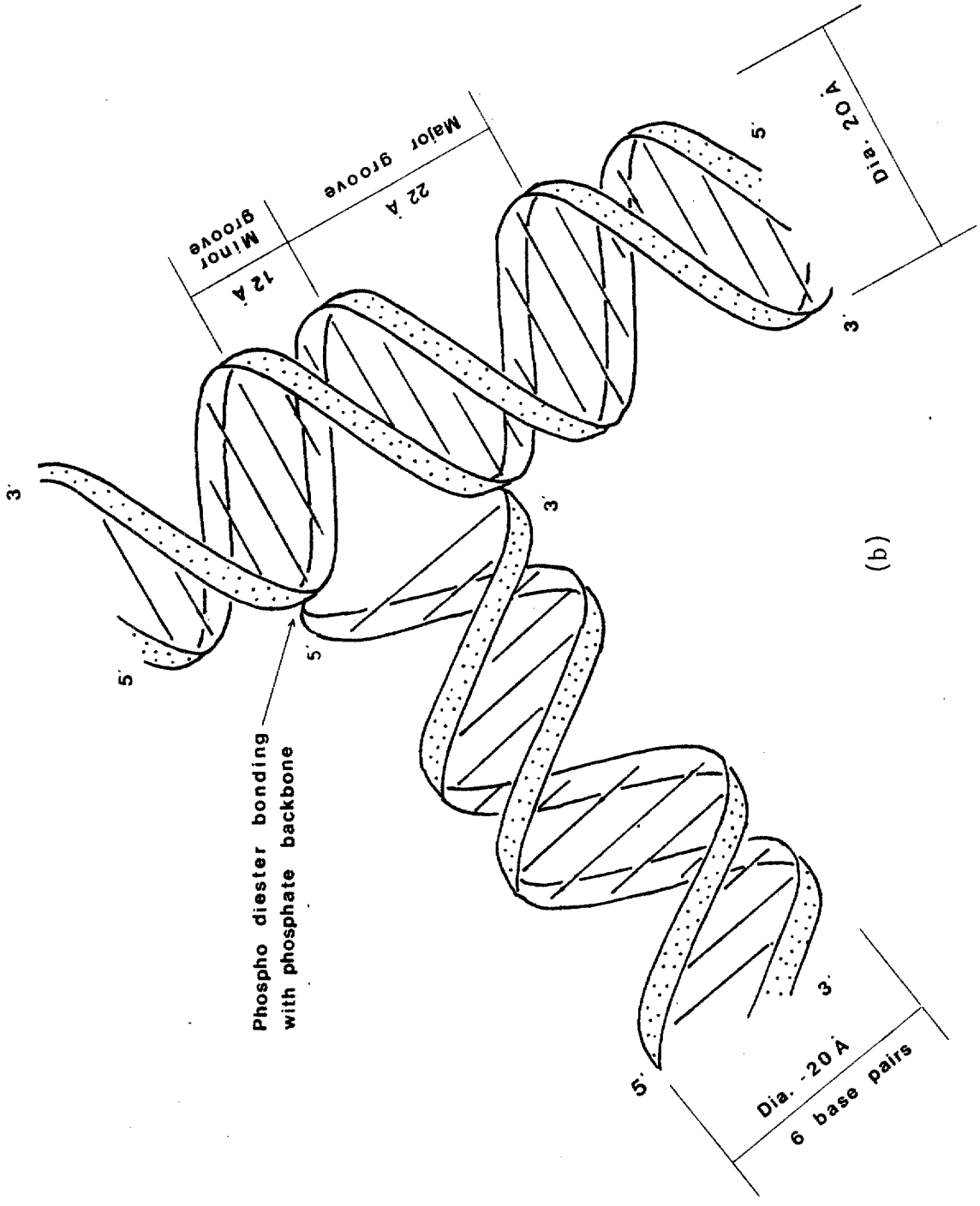
(k)

(Figure 11). In this scheme, two replicated progeny strands are also composed of one older template strand and one newly synthesized strand. Recombination steps including serial nicking and ligating to conserve the original branch conformation of DNA are not random processes. Branch conformation must come from highly regulated serial steps. The most likely way for serial steps to occur in recombination is suggested in Figure 11. As shown in Figure 11, each recombination step has only one case of ligation among the nicked ends. In this case then, sequential nicking and ligating would be the mechanism determining DNA structure.

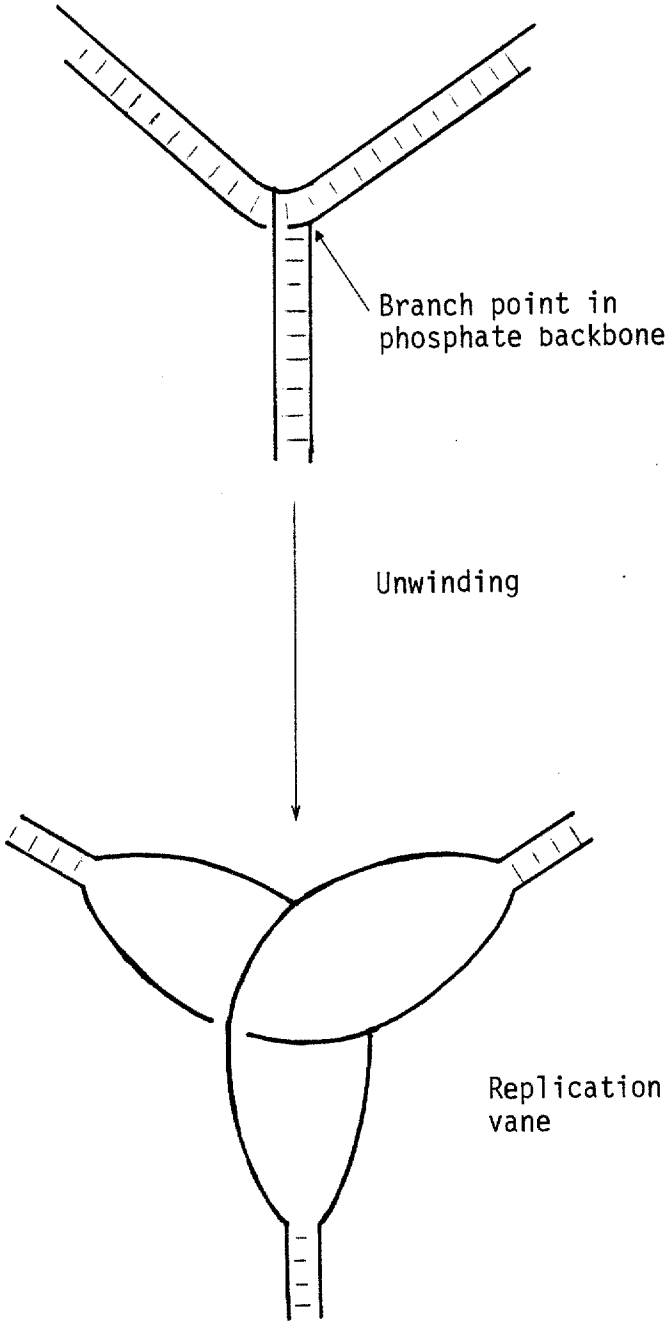
In the third case, each side of the phosphate backbone of the double helix has a branched structure at the molecular level (Figure 13). Phosphate molecules have four ligands, which are covalently bound to four oxygen molecules. Each phosphate molecule of the phosphate backbone, however, provides just two ligands to 3' or 5' carbon atoms of deoxyribose in the DNA molecule by forming phospho-diester bonds with oxygen molecules. One of the other two ligands is present as a negatively charged binding-free oxygen molecule. If this free oxygen molecule is available to a 3' or 5' carbon end of another single DNA strand, it will be possible that the phosphate

Figure 13. Possible branch structure at the molecular level of the DNA double-helix. (a) Chemical basis of the branch structure. The branch strand is bound to the phosphate backbone of the main double-helix strand by forming phospho-diester bonding between an outside binding-free oxygen molecule in the phosphate backbone of the main strand and 5' or 3' end carbon atom of the branch strand. These points are matched by A and B in figure. Two template branched single-strands are stretched out from each side of two phosphate backbones of the main strand. The branch points are at a distance of six-base-pairs from each other in main strand. The two template branch strands form another double-helix by template base pairing sequence. (b) Double-helix featuring the branch structure. It shows the size compatibility of the double-helix structure for branch conformation. Two phosphate molecules between major groove in the main strand give good sites for branch strands. The length of 22 Å (6.5-base-pair length) of the major groove is similar with the diameter of the DNA double-helix, about 20 Å (six base pairs in length). The molecular structure of the double-helix in the main strand is not affected by the binding of the double-helix branch strand. (c) Possible specific configuration called "replication vane" in this study shown during the replication process of this model. It can occur when the replication is started close to a branch point.


DR = Deoxyribose
B = Base
(a)
A
B
5'
3'
dia. -20 Å
6 base pairs
(-20 Å)
Majór groove (-22 Å)


Minor groove
12 Å
Major groove
22 Å
Dia. 20 Å
3'
5'
Phospho diester bonding
with phosphate backbone
Dia. ~20 Å
6 base pairs

(b)


Branch point in
phosphate backbone
Unwinding
Replication
vane

(c)

backbones provide a branch point. That is, one of the phosphate molecules in the main backbone gives a binding site to a 5' or 3' end carbon atom of the third single-stranded DNA molecule, and this results in a third phospho-diester linkage of the phosphate molecule. As a result, a branch structure on the side of the phosphate backbone of the main double-helix DNA strand is formed (Figure 13). On the other side of the phosphate backbone of the main DNA strand, branch structures could be formed by the same linkage mechanism. These two branch strands could be templates to each other and form template base-pairing. Finally, the complete branch structure of DNA double-helix may be formed as shown in Figure 13.

The structure of the phosphate backbone is another possibility for branching in the *A. vinelandii* chromosome. Details of the possible molecular structure and size compatibility in the DNA double-helix of this suggested branch structure are shown in Figure 13. In this case, the replication can occur with typical circular DNA replication mechanisms of bacterial chromosomes (10, 50). Although a specific configuration called "replication vane" occurred during its replication process is proposed in this study (Figure 13, (c)), it was not found in the studies reported here. Therefore, the

first or second case proposed is more likely to be a proper model of branch structure in the *A. vinelandii* chromosome.

The unique and heretofore unexplainable genetic characteristics of *A. vinelandii* can not be solved with theories. Neither can they be explained by the mechanisms of bacterial genetics accepted at this time.

This study was initiated to find a solution which would reconcile genetic observations with DNA structure. Two possible mechanisms which would do this are proposed. These are presented as the conclusion of this work and represent the only tenable explanations of azotobacter genetic systems. The data presented here do not permit selections of one over the other.

This work is in agreement with all reports in the literature regarding azotobacter mutations and also with reports on DNA branch points in genetic recombination (6, 10, 12, 30, 34, 50, 56). Experimentally, electron micrographs from this study gave a more reliable aspect of branch structure than that available in the literature.

Branch conformation of DNA molecules is a new concept in both eukaryotes and prokaryotes. The major parts in this study were

made on assumptions based of experimental studies on the electron microscope. This study, then, provides a good basis for other studies of branch conformation in chromosomal DNA structures. Experimental results which can show the specific mechanisms involved must be carried out vigorously to establish the solution to the problems of *Azotobacter* genetics.

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