Senior Research Thesis

Electron Microscopy of DNA

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Studies of the pre-formed film technique, used to prepare DNA for electron-microscopic investigation, gave results of generally poor reliability, but some data and analyses contradict a conclusion that the average DNA molecule projects its spatial conformation onto a plane during adsorption at the protein-water interface in the pre-formed film technique, which was presented in a paper by Lang, Bujard, et.al.

Electron-microscopic investigation of single-stranded regions of λ DNA produced by reaction with methylmercuric hydroxide (CH_3HgOH) gave promising results which indicate this method may be an effective one for locating and examining A-T rich regions in DNA.

I. INTRODUCTION

The research described in this thesis, performed from October 1966 to May 1967 under the direction of Dr. Norman Davidson and Mr. Douglas Mohr, may be divided into two distinct areas, each of which will be treated separately in the various sections below. It involved 1) the investigation of diffusion-controlled adsorption of DNA from bulk solution onto a monolayer of surface-denatured cytochrome c as a means of preparing DNA for investigation with the electron microscope, and 2) electron-microscopic studies of the partial denaturation of DNA produced by reaction with methylmercuric hydroxide (CH_3HgOH), hereafter referred to as mmh. The diffusion-controlled adsorption technique of preparing DNA for electron-microscopic investigation is also known as the pre-formed film technique, and will be referred to as the pfft in the remainder of this thesis.

A. Pre-Formed Film Technique

Kleinschmidt and Zahn[1] developed the well-known Kleinschmidt method of spreading monomolecular surface films from DNA-containing protein solutions onto water as a means of

preparing DNA for e.m. investigation.

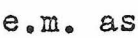
Lang, Kleinschmidt, and Zahn[2] modified this method to obtain adsorption of DNA onto a protein surface film (pfft). In this technique a crystal of cytochrome c is touched to the freshly-swept surface of a DNA-containing solution, forming a protein monolayer on the air-water interface. The DNA diffuses to this monolayer after its formation and is bound to it. Carbon films or parlodion-on-copper grids are then touched to the protein film, a layer of which remains on them as they are further stained or shadowed, and thus ready for viewing under the e.m. As opposed to the Kleinschmidt technique where the DNA becomes entrapped in the film as it is formed, the monolayer film in this technique is formed before the DNA becomes bound to it: thus the name pre-formed film technique.

In the pfft external forces such as shearing forces during spreading and solution convection are excluded, so it would seem an ideal technique for the study of partially-denatured DNA, where the rough Kleinschmidt treatment tends to tangle the single-stranded regions and allow them to hydrogen bond with themselves, thus preventing them from being distinctly visible under the e.m. The pfft should also give a much more accurate description of the true physical nature of DNA in solution, including such characterizing parameters as average end-to-end distance, statistical segment length, etc.

Lang, Bujard, et.al.[3] reported that the average DNA molecule projects its spatial conformation onto a plane during adsorption at the protein-water interface in the pfft. This

seemed a somewhat questionable conclusion, and certain of the parameters used in arriving at this result differed from those generally accepted. Part of the work described below attempted to investigate these discrepancies of Lang, Bujard, et.al., and also to determine the applicability of the pfft for studying various types of DNA, especially DNA which had been partially denatured.

B. Partial Denaturation with CH_3HgOH

Inman[4] reported that heating of DNA in formaldehyde solutions caused partial denaturation of the DNA, reaction of the formaldehyde with denatured regions preventing renaturation. The partially denatured areas of the DNA were visible under the e.m. as loops () in the otherwise thread-like appearance of the DNA. Because the thermally-induced helix-random coil transition occurs at lower temperature in DNA's with higher A-T base pair content, Inman concluded that the partially denatured areas visible with the e.m. represented A-T rich regions of the DNA molecules. Using his electron micrographs, he constructed a crude denaturation map of λ DNA, which he also supposed to be a map of the A-T rich regions in the molecule.

Gruenwedel and Davidson[5] found that the reagent methylmercuric hydroxide (CH_3HgOH) reacts with native DNA by causing prior denaturation which is, under the usual experimental conditions, irreversible. The mmh was found to denature A-T rich DNA's preferentially, the first site of its reaction being the hydrogen bond involving the $N_{(3)}$ atom of thymidine.

Combining the findings of Inman with those of Gruenwedel

and Davidson, attempts to produce and examine mmh-induced partially-denatured regions of DNA are described below. It was hoped that a method in addition to that of Inman for locating and studying A-T rich regions of DNA, agreeing with his results, could be developed.

II. MATERIALS AND METHODS

A. Materials

Calf thymus DNA used for pfft studies was purchased from the Worthington Biochemical Company. λ DNA was kindly donated by Dr. Stanley Cohen of the Biochemistry Department, Albert Einstein Medical Institute, New York, New York. It was obtained by the standard method of phenol extraction from λ wild type bacteriophage.

Methylmercuric hydroxide ("19% Hg Equivalent") was supplied by The Morton Chemical Company, Woodstock, Ill. A stock solution 1×10^{-2} F. in Hg was prepared by diluting the concentrated solution 1/100. Its exact concentration was not determined, but this can be done using the method of Waugh, Walton, and Laswick[6].

Before e.m. investigation grids were stained in a solution made by adding 0.1 ml of a solution containing 5×10^{-3} F. UO_2Ac_2 and 5×10^{-3} F. HCl in absolute ethanol to 10 ml of 95% ethanol.

The DNA was examined on 200 mesh copper grids supplied by Ladd Industries, coated with a parlodion film. The grids are coated by raising them to a water surface upon which a drop of 3% parlodion in amyl acetate has been placed and the latter allowed to evaporate. Grids used in the pfft investigation were made from 1.3% parlodion solution and carbon coated with a carbon arc in a vacuum evaporator.

Grids were shadowed using platinum - palladium foil on tungsten wire, supplied by Ladd Industries, while on a rotating platform in a Kinney vacuum evaporator.

A Philips Model 200 electron microscope was used for all investigations, pictures being taken by a camera in the e.m. column with Kodak general purpose 35 mm film. Enlargements were made with an Omega enlarger onto Kodak Kodabromide F-5 paper.

To determine exact magnifications several pictures of a carbon replica of a 21,600 lines/in. diffraction grating were taken, developed, and enlarged at the same time and settings as the DNA pictures. Measurements of prints of the grating were then used to compute correct magnifications.

B. Procedure

1. Pre-formed Film Technique

Initial attempts to carry out this technique in a Langmuir trough such as that described by Lang, Bujard, et.al.[3], were unsuccessful and terminated because of the bulkiness and general intractability of the trough, large amounts of DNA needed, etc. The following technique was used.

An approximately 125 ml. Petri dish is waxed around the edges and filled with a solution of 0.02 µg/ml DNA in 0.2 F. NH_4Ac until a meniscus is formed at the top. The surface is then swept with a lucite bar, dusted with talc, and touched with a cytochrome c crystal on the end of a stainless steel dissecting needle. The film covers about 70% of the surface. About 30-40 minutes is allowed for the DNA to diffuse to the protein surface. Grids are then touched to this surface.

In practice it is quite difficult to keep the protein film stationary, and very poor results were in general obtained

with this technique. Placing the Petri dish inside a large plastic box which can be closed to eliminate air currents in the 30-40 minutes while the DNA is diffusing to the surface may help overcome this difficulty.

2. Kleinschmidt Technique for CH_3HgOH

The "standard" Kleinschmidt technique, using NH_4Ac as a salt, is not applicable for mmh work because NH_3 reacts with the mmh to such an extent that the latter is prevented from causing partial denaturation of the DNA. $NaNO_3$ was thus chosen as the salt used in Kleinschmidting, and after experiments with different buffer systems, such as borate and phosphate, the following standard procedure was adopted.

A solution of 0.5-1.0 µg/ml DNA, 1.0-1.5 F. $NaNO_3$, and 5-10 x 10^{-4} F. in Hg CH_3HgOH, all in 10^{-3} F. phosphate buffer, pH 7.5 is made up to 1 ml total volume. Immediately before layering, o.1 ml of 1.0 mg/ml cytochrome c solution is added and the resultant solution mixed. This solution is then run down a microscope slide and layered onto the talc-dusted surface of a solution 0.1 - 0.5 F. $NaNO_3$ in 10^{-3}F. phosphate buffer, pH 7.5. As soon as possible after the film spreads grids are touched to it, since there is a slight possibility of the film degenerating in the presence of excess CH_3HgOH.

It will be noted that variable concentrations are given above for the DNA, $NaNO_3$, and mmh. Ordinary DNA concentration for Kleinschmidting is 1.0µg/ml, but this produces quite a preponderance of DNA on the grids and may be lowered to 0.5 µg/ml to reduce interactions between molecules without hindering the ability to locate many molecules with the e.m. Concentrations

of 1.5 $NaNO_3$ in solution, 0.5 F. $NaNO_3$ in the dish are reported to be optimum for preparing DNA with a minimum of tangles and loops as viewed with the e.m.[7]. However, DNA was not seen as reliably with these concentrations as with the lowered ones of 1.0 F. $NaNO_3$ in the solution and 0.1 F $NaNO_3$ in the dish, and the latter appear more suited for mmh investigations. Methylmercuric hydroxide concentrations may be varied from 5×10^{-4} to 10^{-3} F. in Hg. The former appears to produce only a few denatured areas per molecule, while the latter causes quite extensive denaturation. Concentrations of 10^{-4} F. in Hg and lower appear to produce no denaturation.

III. THEORY AND RESULTS

A. Pre-formed film technique

1. Theory

Several representative dimensions may be used to characterize coiled polymers, such as DNA, in solution. These include average end-to-end distance, contour length of the molecule, and the statistical segment length. Using a statistical treatment one can derive[8] the following distribution function for the end-to-end distance of a flexible polymer in two dimensions:

$$W(h)dh = \frac{2}{Lb} e^{-h^2/Lb} h\, dh \qquad \text{.....(1)}$$

where h is the end-to-end distance, L the contour length, and b the statistical segment length of the polymer in question. The polymer is assumed to be composed of rigid segments of length b, with free rotation about the point of connection of any 2 segments.

The statistical segment length, b, is frequently used to

characterize the DNA molecule, and it may be derived from experimental data in two ways: a) the relation

$$h_{max} = \sqrt{\frac{Lb}{2}} \qquad \text{.....(2)}$$

where h_{max} is the value of h for which W(h) attains its maximum, may be obtained by setting $\frac{d\,W(h)}{dh} = 0$, and thus, from a plot of W(h) vs. h made from experimental data, knowing L, b may be obtained through equation (2); or b) from equation (1) it may be seen that a plot of $\frac{\ln W(h)}{h}$ vs. h^2 should give a straight line of slope $-\frac{1}{Lb}$, and again, knowing L, b may be obtained.

2. Results

A number of pictures of calf thymus DNA, prepared using the pfft, were taken, and the length and end-to-end distance of the DNA molecules were measured. The resultant plot of W(h) vs. h was quite broad, and the exact position of h_{max} could only be guessed; a value of $h_{max} = 1.2\ \mu$ was found. Using method a) described above, a value of $b = 3.0 \times 10^{-5}$ cm. was found.

Both methods a) and b) were applied to data given in the paper by Lang, Bujard, et.al.[3] for T-3 DNA. Using method a) with $h_{max} = 1.5\ \mu$ from an experimentally-determined curve given in their paper, and their reported value of $L = 11.6\ \mu$, a value of $b = 3.9 \times 10^{-5}$ cm. was found. And using method b) and data from a curve in their paper, $b = 4.3 \times 10^{-5}$ cm. was found.

B. Partial Denaturation with CH_3HgOH

Plate 1 shows a fragment of λ DNA which has been partially denatured by treatment in 10^{-3} F. (in Hg) CH_3HgOH. Denatured regions are marked with an arrow (⟶) and can be distinguished from mere crossing of DNA over itself (*⟶).

Plate 2 shows what seems to be a complete λ DNA molecule, with several small denatured areas, produced by 5×10^{-4} F. (in Hg) CH_3HgOH. These areas are located ca. 51%, 81%, and 99% of the total distance from one end. On a second seemingly intact molecule denatured areas were found at 50-57%, 75%, and 97% of the total distance from one end. These values compare favorably with those of Inman's denaturation map[4], 52%, 73%, and 98%, but many more molecules will have to be photographed and measured to determine conclusively if mmh denaturation can reproduce Inman's work.

Plate 3 shows several λ DNA molecules Kleinschmidted under the same conditions as the previous two plates, but with no CH_3HgOH present. The complete absence of what have been interpreted as single-stranded regions can be seen. No single-stranded regions were seen in any of the molecules prepared under these conditions and examined with the electron microscope.

Work is currently being carried out to determine the optimum concentrations of CH_3HgOH, $NaNO_3$, and DNA for reproducibly producing and viewing single-stranded regions in intact DNA molecules.

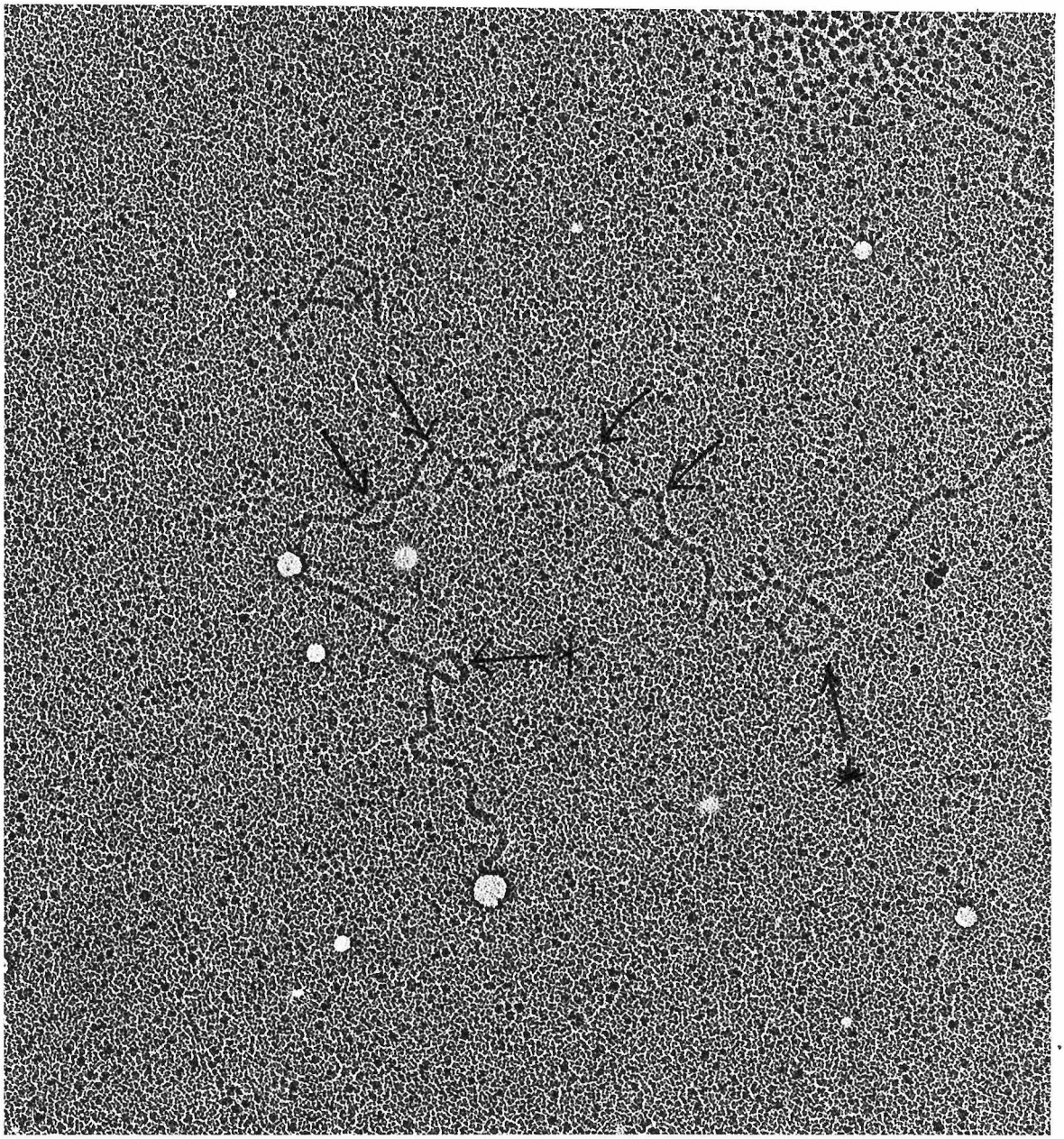


PLATE 1. DNA treated with 10^{-3} CH_3HgOH before Kleinschmidting
Magnification ca. 108,800
(⟶) - Partially denatured areas
(*⟶) - Loops caused by DNA crossing over itself

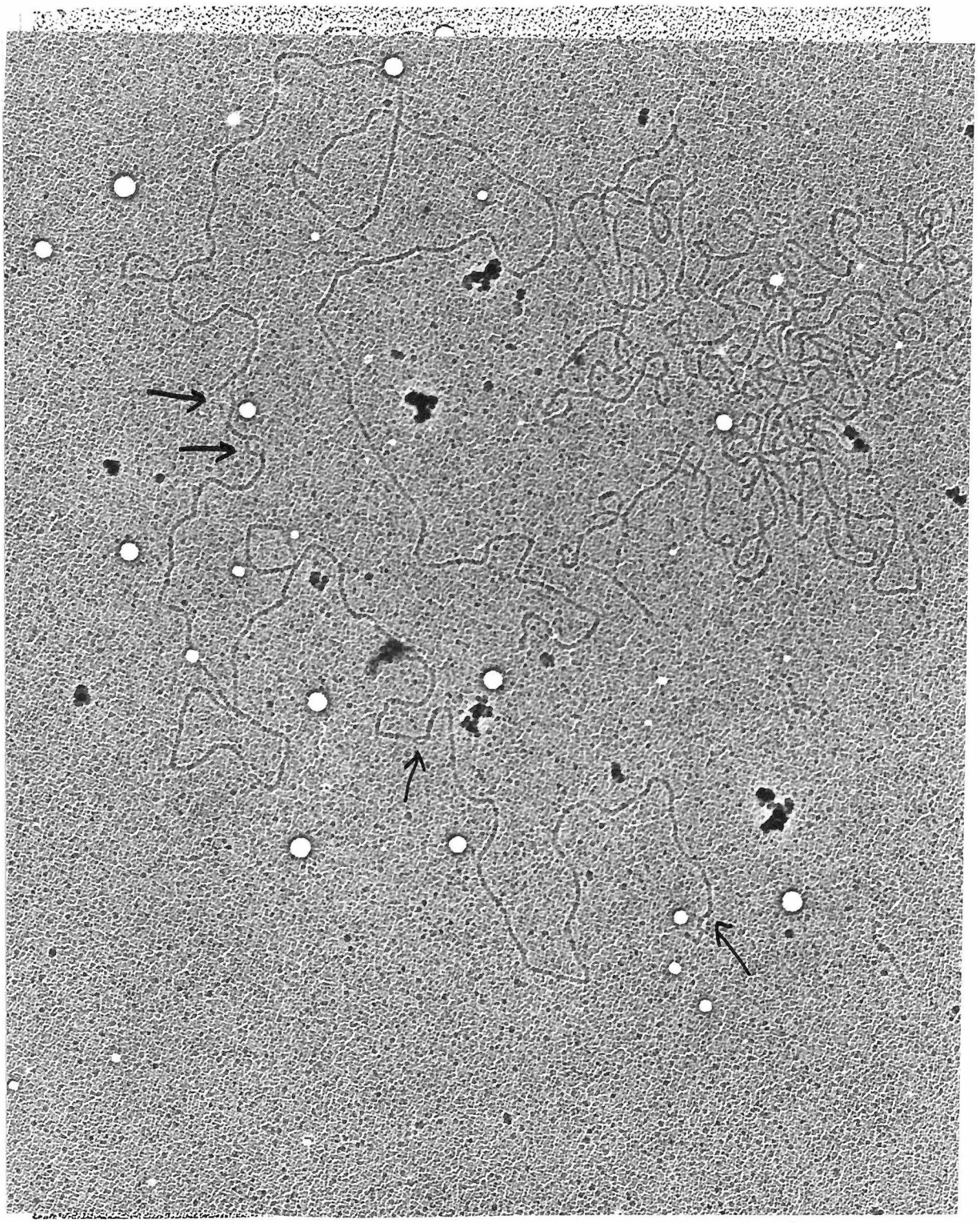


PLATE 2. DNA treated with 5×10^{-4} CH_3HgOH before Kleinschmidting
Magnification ca. 57,800
(⟶) - Partially denatured areas

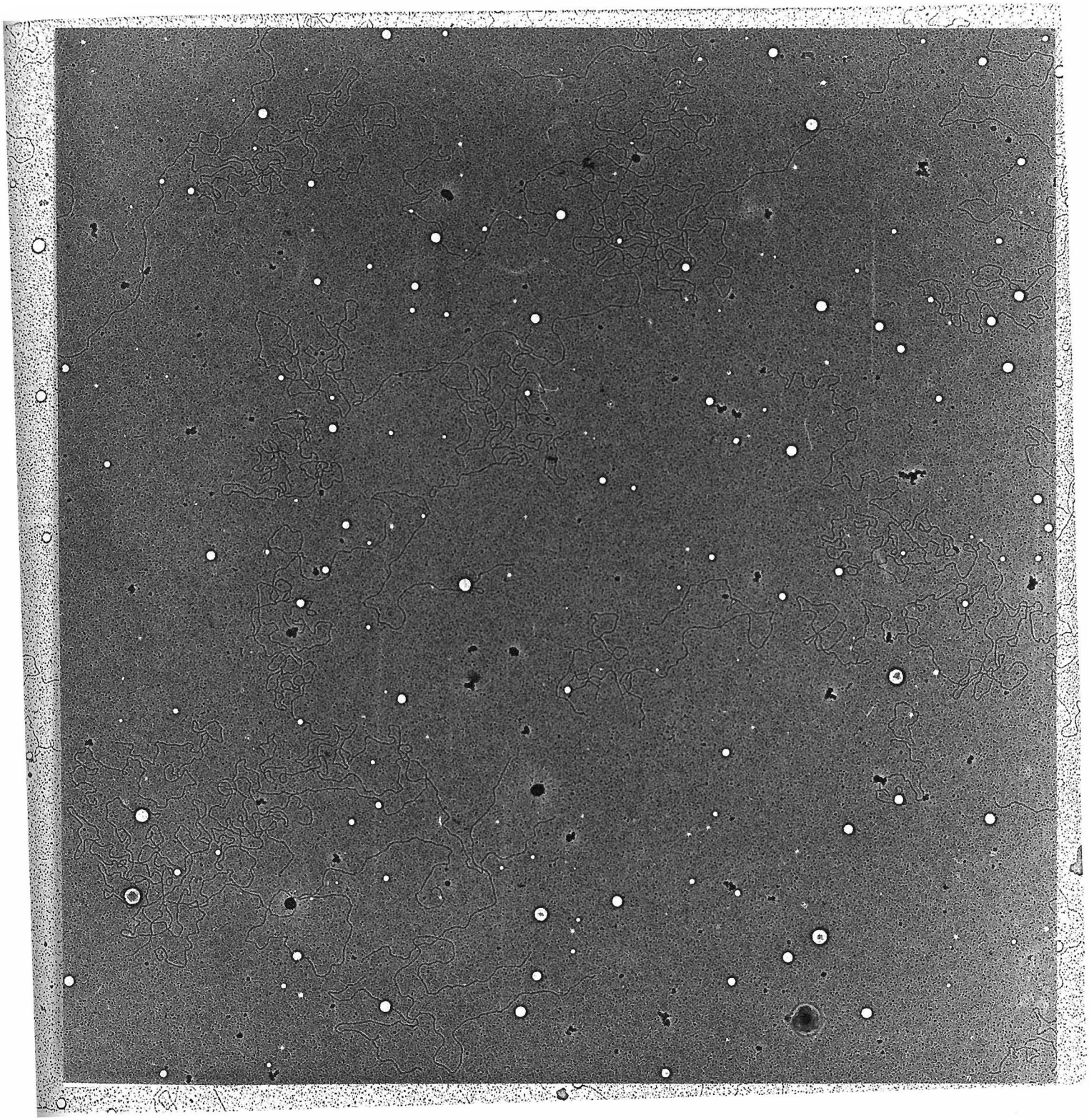


PLATE 3. DNA treated with 0 CH_3HgOH before Kleinschmidting
Magnification ca. 25,840

IV. DISCUSSION

A. Pre-formed Film Technique

The value for the statistical segment length, b, found in this work compares favorably with that obtained from the data of Lang, Bujard, et.al.[3]: 3.0×10^{-5} cm. vs. 3.9×10^{-5} cm. and 4.3×10^{-5}cm. However, the accepted b value for DNA in solution, obtained by other physical methods such as ultracentrifugation, is 7×10^{-6} cm. This does not support the contention of Lang, Bujard, et.al. that the average DNA molecule projects its spatial conformation onto a plane during adsorption at the protein-water interface. If that were the case, the b value found from measurements of DNA prepared by pfft should agree more closely with that obtained for DNA in solution using other physical methods.

Because of the above considerations it must be concluded that although the pfft may eliminate certain external forces in preparation of the DNA for e.m. investigation, it does not give a true picture of the physical characteristics of DNA in solution. Also, because of the difficulty encountered in this work in getting reproducible results with pfft, it does not seem to compare in reliability with the standard Kleinschmidt technique.

B. Partial Denaturation with CH_3HgOH

Plates 1,2, and 3 give reasonably convincing evidence that reaction of mmh with DNA produces partial denaturation visible with the electron microscope. The pictures of supposedly single-stranded regions are of as good quality as those presented by Inman, if not better. Further studies should be made to attempt

duplication of Inman's work.

The measured contour length of λ DNA molecules in this study is shorter than that reported by other investigators, the longest molecules being about 13.5 μ as compared with values of 16-17 μ reported by Inman[4]. This discrepancy may arise because DNA in this work was prepared on parlodion grids, which are reported[9] to give length measurements ca. 25% shorter than those for DNA mounted on carbon films, such as were used by Inman. Some pictures of λ DNA partially denatured by CH_3HgOH on carbon films should be made in the future to investigate this discrepancy.

Partial denaturation of DNA with mmh appears to be a most promising method for detecting A-T rich regions in the DNA and for examining the single-stranded areas produced by denaturation. The results given in this thesis are by no means conclusive, but indicate that this area is definitely worthy of further investigation.

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