ELECTRON MICROSCOPY OF DNA MOLECULES "STAINED" WITH HEAVY METAL SALTS

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ABSTRACT

Single DNA molecules can be rendered visible in the electron microscope by "staining" with water-soluble salts of heavy metals. The best results were obtained with lanthanum nitrate, uranyl acetate, and lead perchlorate. The molecules appear as filaments approximately 20 A wide. Their length was not determined, but it could be shown that it varied with the molecular weight of the DNA used. The same heavy metal salts will preferentially "stain" the nucleic acid in a protein-DNA complex. Evidence is provided for the possibility of a partial separation of a double-stranded molecule into single strands on adsorption to the supporting film.

Mounting interest in the role of nucleic acids in biology and increasing knowledge of their molecular structure have prompted numerous attempts to supplement the information gained through indirect methods by direct observation of single molecules in the electron microscope. Several techniques have been used to obtain a suitable distribution of single molecules and to increase contrast in the preparation. After drying from a drop of dilute solution on a supporting film of collodion, Formvar, or carbon, DNA is usually found in large aggregates, in which little detail is visible. Spraying the solution from an atomizer onto a hydrophilic surface will result in a more homogeneous distribution of the DNA over a larger area and will allow the visualization of single molecules. Hall (10) and Hall and Litt (11) used the surface of freshly cleaved mica, which has the additional advantage of providing a very smooth background for subsequent shadowing, but transfer of the preparation to a grid presents some difficulties. Birbeck and Stacey (6) obtained satisfactory results by using a polymer with positively charged groups as the supporting film, so that DNA could be sprayed directly on the grid. Neither method, however, seems to be well suited for work with material of the very high molecular weight found in native DNA from many sources.

A new technique recently published by Beer (4, 5) offers definite advantages in this respect. By streaking a film-covered grid over the surface of a DNA solution and by carefully draining off, in the same direction, the surplus adhering to the grid, he succeeded in attaching to the film single molecules in the form of nearly straight and parallel strands.

Still another interesting approach is that of Kleinschmidt and Zahn (16, 17), who spread DNA in a monomolecular film of protein on a water surface. The protein is necessary since DNA alone will not form a film, and the protein, furthermore, keeps the molecules of DNA separated so that they can be individually resolved.

In all these investigations shadowing with heavy metals has been used to render the nucleic acid visible in the microscope. This procedure, however, limits the resolution to such a degree that it is difficult if not impossible to distinguish clearly a single molecule from two molecules in close contact. In addition, molecules are not visible when their long dimension is parallel to the

direction of shadowing. More recently it has been shown that contrast of nucleic acid–containing structures can be enhanced *in situ* by staining with heavy metal salts (12, 14, 26, 27), and work with tissue sections, bacteria, viruses, and subcellular particles has shown that the nucleic acid itself is mainly stained in these structures (12–15, 24). Our own work with heavy metal staining of small viruses has convinced us that it should be possible to render single molecules of nucleic acids clearly visible in the microscope, and the results obtained so far are reported in this paper. Since the secondary structure of DNA and its physicochemical properties are better known than those of RNA, the former was chosen for this investigation.

MATERIAL AND TECHNIQUES

Materials Used

A commercially available calf thymus DNA (Worthington Biochemical Company, Freehold, New Jersey) was used for most of the work to be described. Its sedimentation constant was found to be S_{20w} = 21.0 *SU*.[1] Treatment with formaldehyde, as described by Fraenkel-Conrat (8), did not increase the absorption at 260 mμ, thus indicating that no large amount of denatured material was present. In some of the experiments a lithium DNA, also prepared from calf thymus, was used. It had an average molecular weight of approximately 16 × 10^6.[2] A material of lower molecular weight was prepared from the Worthington DNA by exposure to sonic vibration, as described by Doty *et al.* (7). Its sedimentation constant was found to be S_{20w} = 4.3 *SU*.[1]

One milligram per milliliter of DNA (Worthington) was dissolved in 1 M NH_4Cl or NaCl, by letting it stand at 4°C overnight and then slowly stirring it, at room temperature, for several hours. A small amount of insoluble material which caused a slight turbidity of the solution was removed by filtration through a medium grade sintered glass filter. This stock solution was stored in small aliquots at −20°C until used. The lithium DNA was obtained as a clear solution containing 2 mg/ml of DNA in 0.01 M LiCl and stored under the same conditions.

The proteins used, with the exception of histone chloride,[2] were commercially available preparations. Cytochrome *c* was obtained from the Sigma Chemical Company, St. Louis (type III from horse heart, lot 70B-692) and from Mack (Illertissen, Germany). The methoxylated serum albumin was prepared according to the technique given by Mandell and Hershey (19). Stock solutions containing 1 mg/ml of protein were made up in 0.1 M or 1.0 M NaCl or ammonium acetate and stored at 4°C.

Supporting Membranes

Supporting films of Formvar and carbon were prepared in the usual way and mounted on Siemens type platinum iridium grids. The copolymer of styrene and vinyl pyridine was synthesized as described in the literature (6, 9). Films cast on glass slides from a 1 per cent solution in chloroform, as suggested by Birbeck and Stacey (6), were difficult to float off and not of uniform thickness. Films cast on a water surface from a 0.5 per cent solution gave more satisfactory results, but in both cases films thick enough to withstand drying and the electron beam were found to have a rather high contrast in the microscope. Repeated extraction of the polymer with toluene to remove material of lower molecular weight left a fraction from which films, both thin and stable enough, could be cast on water.[3]

Preparations

Either the DNA was sprayed onto a grid with an all glass nebulizer (DeVilbiss Company, Somerset, Pennsylvania), as described by Hall (10), or the technique of Beer (4, 5) was used. Latex spheres or ferritin were added as reference particles to the solutions to be sprayed. No ammonium carbonate was used if the preparations were to be stained later.

For spreading the nucleic acid in a protein monolayer, Kleinschmidt's technique (16) was essentially followed. It consists, briefly, in spreading a solution containing DNA and protein in a ratio of 1:10 to 1:100 on the water surface of a Langmuir trough. The protein forms a monomolecular film and keeps the DNA in the surface. This film is then picked up on electron microscope grids. A glass rod was used to deliver the protein solution to the water surface, as described by Trurnit (23). The film was compressed by hand and picked up on grids at a surface pressure of 0.6 to 1.0 dyne/cm. The specific areas at this pressure were usually 0.6 to 0.8 m^2/mg. No force-area curves were recorded. Sometimes, and especially with cytochrome *c*, smaller specific areas were obtained. This is probably due to the difficulties of compressing a film of a rather soluble protein by moving the barrier by hand, and also perhaps to

[1] Our thanks are due to Dr. Yphantis, of The Rockefeller Institute, for determining the sedimentation constants.

[2] This material was a generous gift from Dr. L. D. Hamilton, of the Sloan Kettering Institute for Cancer Research. Its preparation from calf thymus and its properties have been described elsewhere (18).

[3] The synthesis of the polymer and the fractionation were carried out by Mrs. S. Mahr in this laboratory.

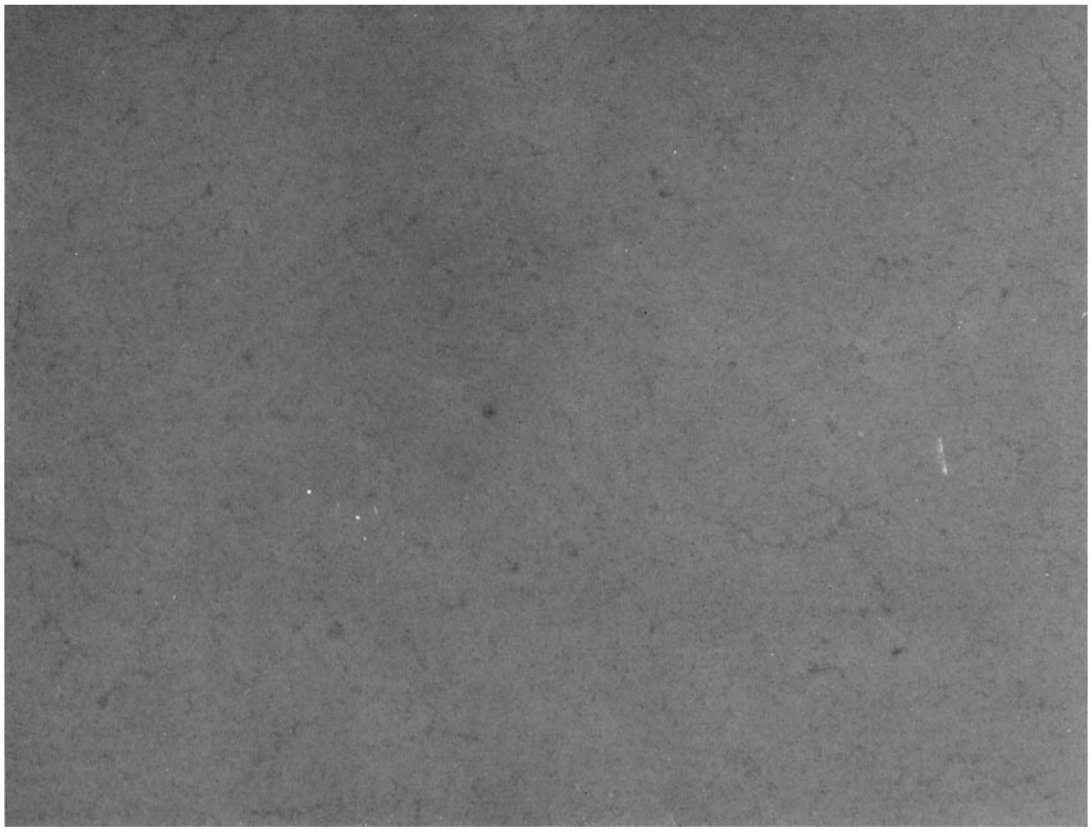


FIGURE 1

(428/61). DNA (Worthington) broken down by sonication. Spread on 0.25 M ammonium acetate from a 0.01 M NaCl solution containing 6×10^{-2} mg/ml of histone chloride, 2.5×10^{-3} mg/ml of DNA. The preparation was stained in 2 per cent uranyl acetate at pH 5.5. Only short pieces of DNA molecules are present. The lower limit of length cannot be established because it cannot be distinguished from staining of the protein film and possible precipitates in the staining solution. × 140,000.

occasional disturbances of the laminar flow of the solution down the glass rod. But since the technique was not used for quantitative work, this seems to be of little importance.

Staining

After the nucleic acid was applied to the grids, the latter were immediately immersed in the "staining" solutions for times varying from 10 minutes to 4 hours, at room temperature. No clear-cut influence of the "staining" time was observed within these limits. The grids were then rinsed in three changes of redistilled water (pH 6.0) and stored in an evacuated desiccator over P_2O_5 until they could be examined in the microscope. If the pH of the "staining" solution was higher than 6.0, the pH of the water used for rinsing was adjusted to that pH with NaOH or NH_4OH. In every experiment some of the grids were shadowed with uranium at an angle of 10°, to check the amount of DNA present and its distribution over the grid.

Microscopy

A Siemens Elmiskop I was used, working with 80 kv, with a beam current of 8 μA and initial magnification of 8000, 40,000, or 160,000 times. The illuminated area was restricted to the field of view as closely as possible. Micrographs were taken of every grid, even if no structures resembling DNA were visible on the screen. Ilford N40 plates developed in Kodak D19 were used throughout this work. Enlarged prints of them were obtained on Kodabromide F.4 or F.5 paper.

RESULTS

Comparison of Different Staining Techniques

Salts of uranium, lanthanum, lead, thorium, thallium, and silver in aqueous solutions were all found, under certain conditions, to impart enough contrast to the DNA to render single molecules visible in electron micrographs, if not on the screen. But the results were somewhat erratic. Often, for no obvious reason, no structures resembling DNA could be found in the "stained" preparations even though the shadowed preparations indicated that they should be present, or heavy precipitates obscured all details in the pictures. Moreover, visibility on micrographs but not on the screen was thought insufficient, if the technique were to be applicable to biological investigations. Therefore work was concentrated on three salts that seemed to hold the best promise, lanthanum nitrate, uranyl acetate, and lead perchlorate.

Lanthanum nitrate was used as a 3 per cent solution in redistilled water. The pH of this solution measured with a glass electrode was 4.2. Increasing the pH to 5.8 with NH_4OH did not seem to increase the contrast in the preparations. Lanthanum nitrate gave the best results with DNA spread in a protein film. It showed less staining of the protein in the background than uranyl acetate, so that the DNA was more clearly visible. It was less useful in preparations without protein, owing to a lack of contrast and a tendency to form precipitates around the DNA strands.

Uranyl acetate was used as a 2 per cent solution in redistilled water which had a pH of 4.2, or as a 0.5 to 2.0 per cent solution, the pH of which was adjusted to 5.2 to 5.5 with 0.1 N NH_4OH. Unless this is done slowly under constant stirring, a delayed formation of heavy precipitates will occur. At a pH above 5.6 part of the uranyl always precipitates.

The solution with a pH between 5.2 and 5.5 always gave a heavier staining of the DNA than the solution with a pH of 4.2. Raising the pH to 6.8 and filtering off the precipitate before use did not result in a further increase in contrast. Therefore the 0.5 per cent solution with a pH between 5.2 and 5.5 was mainly used. It gave good results with both pure DNA and DNA in a protein film, but was rather sensitive to the conditions of rinsing. Prolonged rinsing in distilled water lowers the contrast drastically, whereas too short a rinse leaves too much contrast in the background. Gently agitating the grids for 5 seconds in 3 changes of freshly redistilled water usually gave good results, but for unknown reasons longer rinses would sometimes give a clearer background without much loss in density of the nucleic acid.

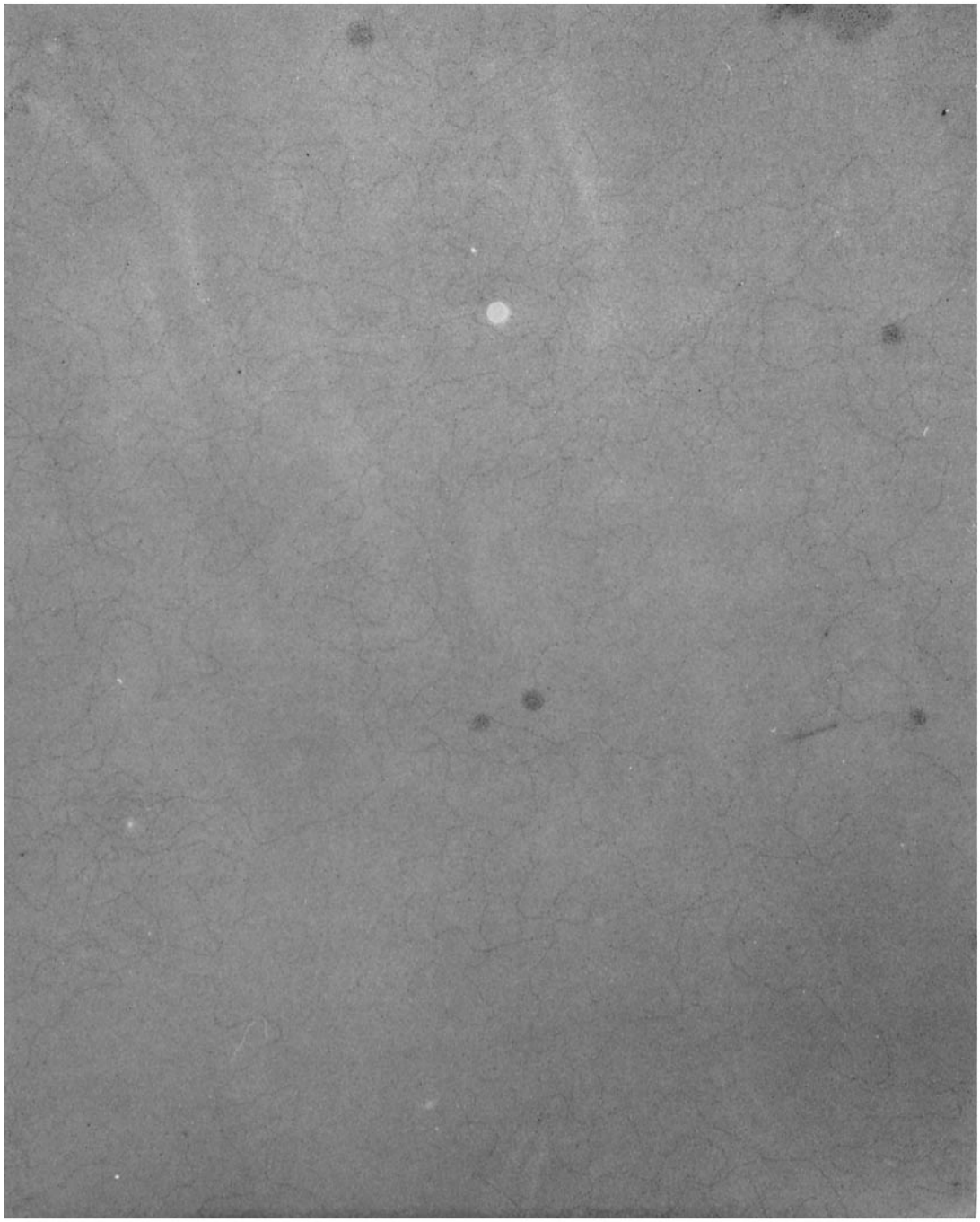
Lead perchlorate gives some staining of pure DNA if a 2 per cent solution in redistilled water is used (pH 5.5), and so does lead acetate (pH 5.7). Single molecules can just be made out on the screen. Better results were obtained with a 1 per cent solution of the perchlorate after raising the pH to 7.3 and filtering off the precipitate. So far no satisfactory results have been obtained with lead staining on DNA in protein films.

General Appearance of Single DNA Molecules

The pictures from shadowed preparations were essentially identical in appearance with those published by Hall (10), Hall and Litt (11), Birbeck and Stacey (6), Beer (4, 5), Kleinschmidt and Zahn (16, 17), and others. With all staining techniques used the molecules appeared as long, thin filaments approximately 20 A wide and of varying lengths up to several microns. No effort has been made so far to measure the length of a greater number of individual molecules, but it was obvious that the lithium DNA and Worthington DNA contained, for the most part, very long molecules, whereas the sonicated DNA showed only very short pieces, mostly between 100 and 500 A in length (Figs. 1 and 2), still shorter pieces being difficult to distinguish from nonspecific "background staining." The outline of the molecules is not smooth and sharp enough for precise width measurements. Any value between 15 and 30 A may be obtained at different

FIGURE 2

(1603/60) Lithium DNA spread on 0.25 M ammonium acetate from 1.0 M ammonium acetate solution containing 5×10^{-1} mg/ml of cytochrome *c*, 2.5×10^{-3} mg/ml of DNA stained with 2 per cent uranyl acetate at pH 5.5. Only very long molecules are present. × 140,000.



places along the molecule. In shadowed preparations of the DNA protein films the filamentous structures show a uniform width of ~50 A. This is thought to be due to a coat of protein around the single DNA molecules, as already suggested by Kleinschmidt and Zahn (16).

Mixed Surface Films of DNA and Protein

Mixed films made from concentrations of DNA between 10^{-2} and 10^{-3} mg/ml and a ratio of DNA to protein between 1:10 and 1:100 gave a suitable distribution of DNA over the grid (Figs. 2 and 3). When DNA of high molecular weight was used in combination with cytochrome *c*, and spread from a solution of 1 M NaCl or NH_4OH on 0.25 M ammonium acetate, there was practically no aggregation of the DNA molecules, and all molecules showed a uniform width. In spite of this it was seldom possible to trace one molecule from end to end, owing to frequent contact points or crossings of molecules. At low magnifications one usually could not trace an individual molecule at these points, and at high magnification the field of view was too small to contain a whole molecule. However, for the testing of staining solutions and similar purposes these preparations were very useful, since any field of view would contain at least part of one or several molecules.

If the same concentration of DNA and any of the proteins were spread from a 0.01 to 0.1 M solution of salt, a different picture was obtained (Figs. 4 to 6). Besides a few single molecules, "spider-like" structures were most frequently found. Evidently, several DNA molecules usually participated in their formation. From a dense aggregate in the center, in which little detail was visible, loops and occasional free ends of single molecules extended in a roughly radial direction covering an approximately circular area of up to several square microns (Figs. 4 and 5). Similar structures containing several such dense centers also occurred. If esterified serum albumin was used instead of cytochrome, structures of this kind were found in preparations spread not only from low but also from high salt concentrations. In DNA-histone films spread from high salt concentration, they would also occur but less frequently.

DNA of lower molecular weight obtained by sonication for 2 hours did not show such aggregates.

Sprayed Solutions of DNA

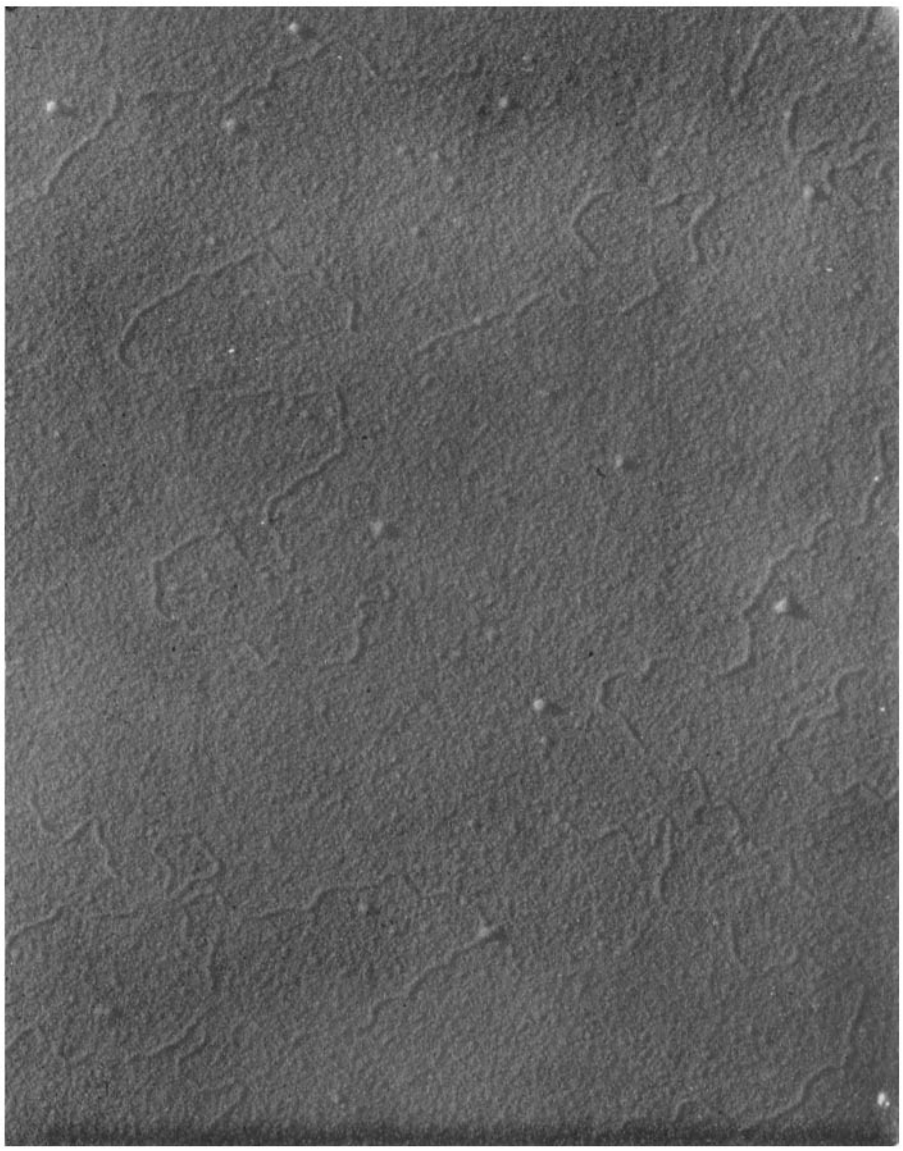
The spraying of high molecular weight DNA solutions on copolymer films usually resulted in an entangled mass of DNA molecules in areas where it was obviously concentrated during drying of a droplet of the sprayed solution. The packing here was not so dense as in the center of the "spiders," and single molecules often could be traced over short distances. Around this area straight bundles and occasionally single straight molecules were found oriented radially. These had probably become attached to the supporting film during drying while the droplet was retracting, and had thus been oriented. The sonicated DNA showed a more even distribution and less aggregation, giving somewhat better pictures.

Oriented DNA Molecules

Beer's technique proved to be very useful for material of high molecular weight. In this preparation all the molecules were deposited nearly straight and parallel to one another (Figs. 7 and 8). Since the purpose of this work was not to measure the length of single molecules, we used a rather high concentration of DNA, 0.1 mg/ml. Therefore, in many places two or more molecules could be found in close contact over long distances until a sudden decrease in width or a branching of the structure indicated the end of one molecule or a separation of the constituent molecules of the bundle. The "branches" usually continued in the same direction and eventually divided again until the smallest diameter of approximately 20 A was reached, indicating that only a single molecule was present. Sometimes these would show, for a

FIGURE 3

(110/61) Same preparation as in Fig. 2 shadowed with uranium under an angle of 10°. Only the protein shell of the molecules is visible. The molecules appear much shorter because they cannot be traced where they run approximately parallel to the direction of shadowing. × 83,000.



very short distance, a branching into two still narrower strands which would then reunite (Fig. 8)—a feature that was not observed in the larger strands. It probably corresponds to pictures of shadowed preparations where the slender thread-like molecule seems to disappear over short distances, its structure merging with the background, which in these preparations is not ideally smooth. We assume that this indicates a partial separation of the double-stranded molecule into the constituent single strands. Miller has recently obtained evidence that this can occur on charged surfaces (20).

In general, the shadowed grids of these preparations seemed to have more material attached than the "stained" ones. This probably means that some molecules float off in the "staining" solution. This would also account for occasional disoriented molecules in the "stained" preparations.

DISCUSSION

The conclusion that the structures described actually represent DNA is based mainly on the fact that their number varies according to the concentration and their length according to the molecular weight of the DNA used (compare Figs. 1 and 2). The pictures of shadowed preparations are essentially identical with electron

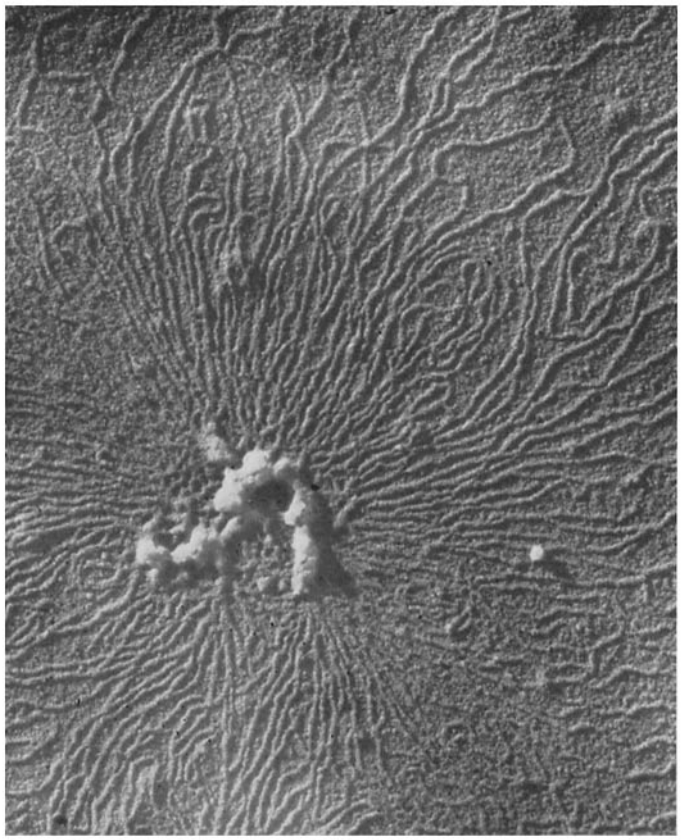


FIGURE 4

(602/61). Typical "spider" formation as obtained when a high molecular weight DNA cytochrome *c* complex is spread from a low salt concentration. In this case 2.5×10^{-3} mg/ml DNA (Worthington), 5×10^{-2} mg/ml cytochrome *c* in 0.04 M ammonium acetate was used, and the preparation shadowed with uranium under an angle of 10°. The protein shell of the molecules is best seen where the long axis is approximately perpendicular to the direction of shadowing; the structure practically disappears where it is parallel to it. × 80,000.

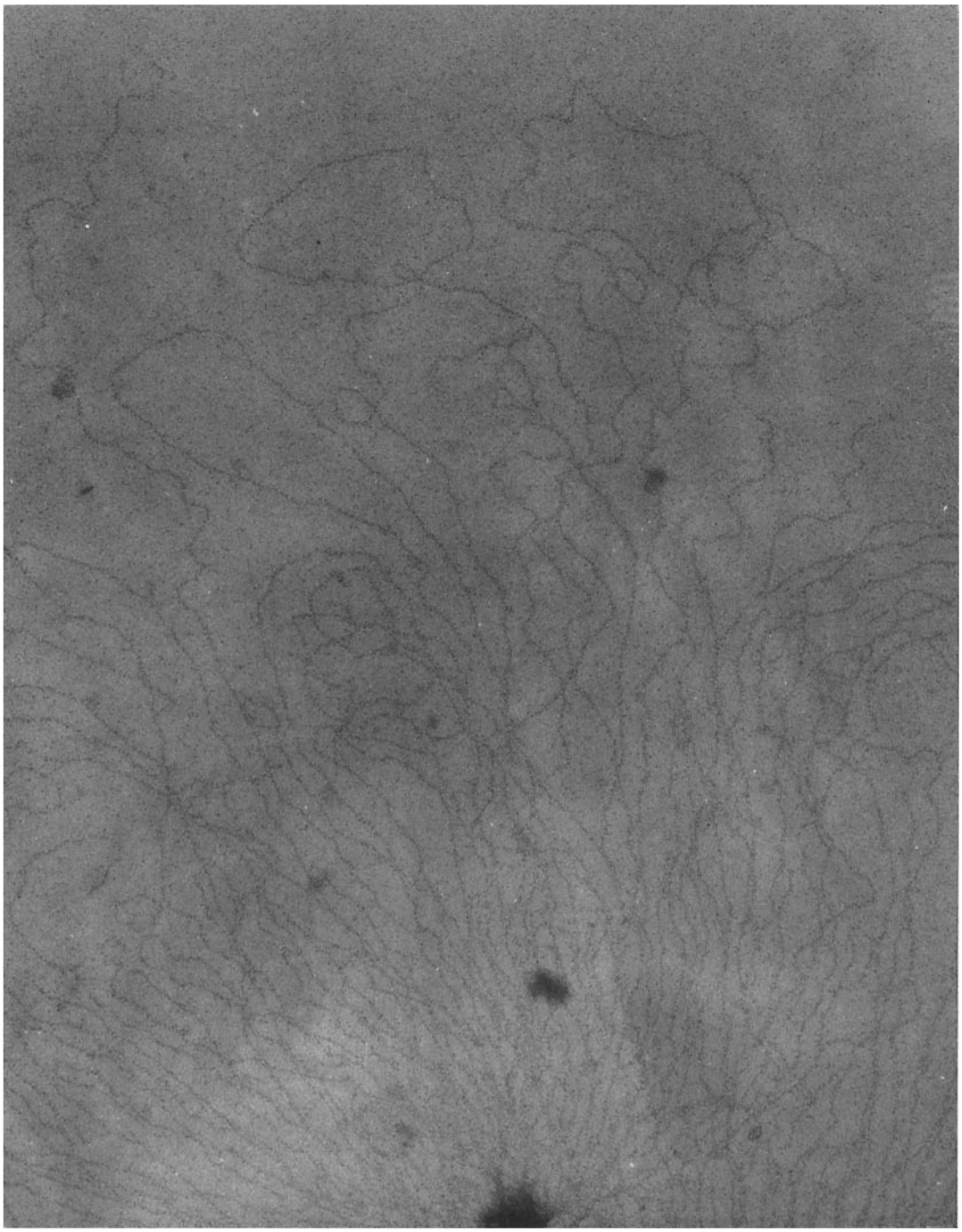


FIGURE 5

(607/61). Same preparation as in Fig. 4 but "stained" with $La(NO_3)_3$. The DNA part of the complex becomes visible. × 140,000.

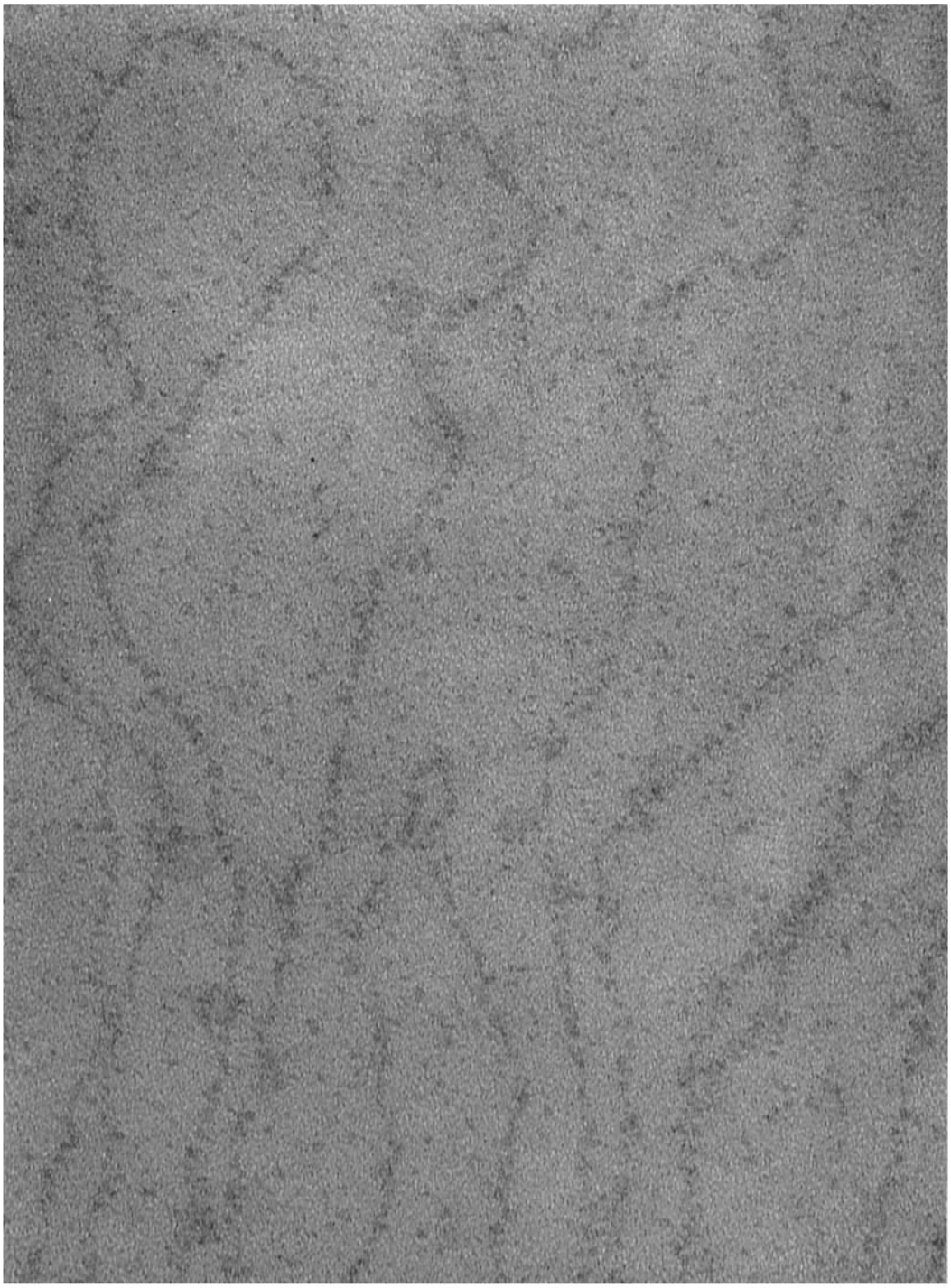


FIGURE 6

(624/61). Same as Fig. 5 at higher magnification. × 560,000.

micrographs of DNA published by others (4–6, 10, 11, 28), and the "stained" preparations show the same general shape and distribution of material as the shadowed ones. Most of the heavy metal salts used are known to stain nucleic acid or at least nucleic acid–rich structures in cells and viruses (12–15, 24, 26, 27), and Terada (22) has shown that $La(NO_3)_3$ and $AgNO_3$ render thin strands of pure DNA visible in the electron microscope, although he has not resolved single molecules in his "stained" preparations. We assume that the thin filaments in our preparations represent single molecules because they are the smallest units found and their width of ~20 A is in good agreement with the width for a double helix of DNA as determined by x-ray diffraction (18, 25).

Little is known about the binding of heavy metal ions to nucleic acids. For uranyl salts the subject is discussed in several recent publications (12, 14, 28, 29). It appears that in solution at pH 3.5 a very stable complex between uranyl ion and DNA is formed with a mole ratio UO_2:P = 1:2. Increasing the pH leads to the binding of additional uranyl or complex uranyl ions which are less firmly attached to the DNA. Using 2 per cent uranyl acetate at pH 4.2, Huxley and Zubay (14) obtained a ratio UO_2:P close to 1:1. They could also show that under the same conditions a nucleohistone will take up a considerable amount of uranyl ions and most of it seems to be bound by the DNA. Even though we were working under somewhat different conditions, our results are in general agreement with these findings. Uranyl acetate gave an increase in contrast with increasing pH of the "staining" solution. The rinsing with water diminished the contrast, indicating that part of the heavy metal bound could be easily removed. In mixed films of DNA

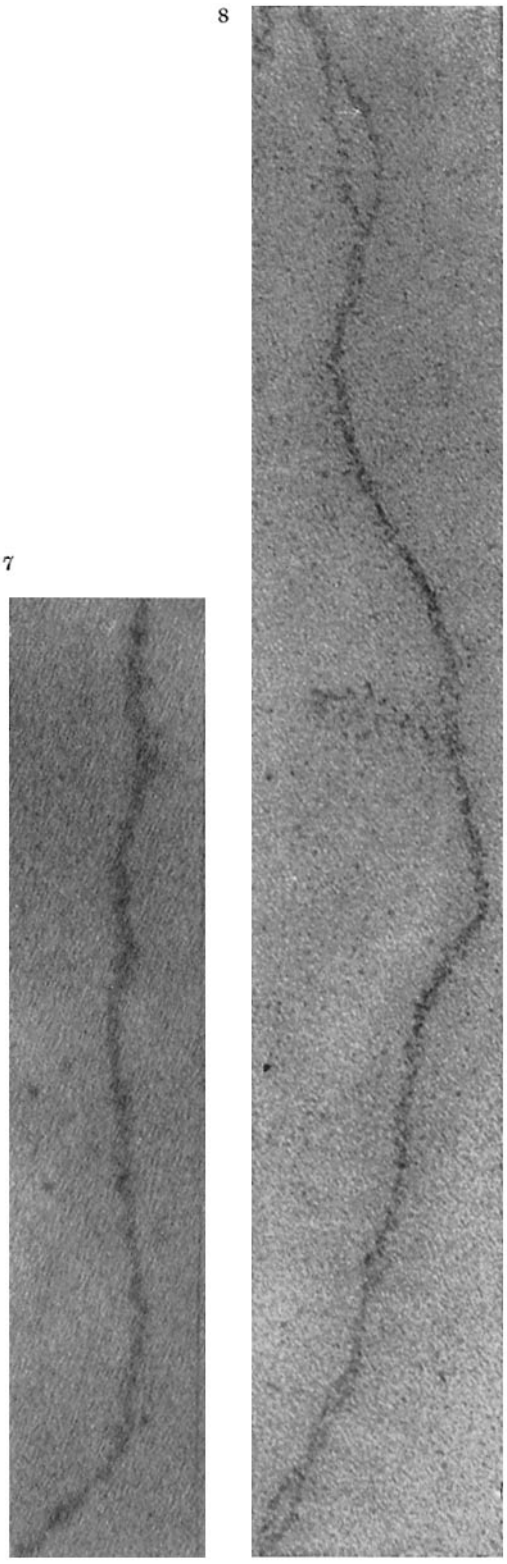


FIGURE 7

(927/61). DNA (Worthington) deposited on the supporting film using Beer's technique (see text) and stained with $Pb(ClO_4)_2$ at pH 7.5. × 560,000.

FIGURE 8

(791/61). Lithium DNA prepared as described in Fig. 2 but stained with 0.5 per cent uranyl acetate at pH 5.2. Note the partial separation into two strands close to the upper and lower edges of the picture. × 560,000.

and protein the "staining" lent contrast to structures which, by comparison with similarly treated preparations of pure DNA, appear to be single DNA molecules, thus indicating that a preferential "staining" of DNA in a nucleoprotein is possible.

But in the latter case a possible contribution of the protein to the "staining" deserves consideration. It has been shown that histone can take up at least one-fifth and serum albumin at least one-seventh of the amount of uranyl which DNA will take up under the same conditions (14, 28). We assume that most of the "background staining" in our preparations can be attributed to this binding of heavy metals mainly by the carboxyl groups in the protein film. This is borne out by the observation of a lower "background staining" in the esterified serum albumin films as compared with films of unesterified proteins. As Kleinschmidt and Zahn (16) have already pointed out, the structures revealed by shadowing of the DNA-protein films, although uniform and similar in their general shape to DNA molecules, have a diameter of approximately 50 A. They probably represent nucleoprotein molecules where the DNA forms the core of the filament and is surrounded by an outer "shell" of protein. One could assume an orientation of the protein in the "shell" with most of its reactive groups located on the inner surface, where binding of a heavy metal would then produce an image in the electron microscope indistinguishable, at the present level of resolution, from a true "staining" of DNA. But DNA in esterified serum albumin films, in which the "background staining" is reduced, gives essentially the same pictures as the other proteins. Moreover, treatment of films with ethyl alcohol at room temperature for 30 seconds apparently removes the DNA from its protein shell. Staining with heavy metal salts after this treatment no longer shows the long, thin filaments, but patches of dense material distributed more or less randomly over the film. If the same preparation is shadowed with a heavy metal, the characteristic pattern of the protein shells can still be seen, apparently unaltered. These observations also suggest that the thin filaments seen in "stained" DNA-protein preparations actually represent DNA molecules, but some contribution of the protein to the stained structures cannot be ruled out.

In this connection another observation should be mentioned. If we "stained" with uranyl before the alcohol treatment, the latter did not affect the appearance of the DNA in the microscope. Evidently the heavy metal stabilizes the nucleic acid, an effect which Kellenberger (15) has used in his method for fixation and staining of bacteria. This observation lends support to the contention that his technique gives a better preservation of the bacterial nucleoid than the conventional procedures.

The general shape of our nucleoprotein molecules is the same as that observed by Zubay and Doty (30) in shadowed preparations of sprayed nucleohistone solutions. But they found a diameter of only 30 A—which is in good agreement with their x-ray data—as compared with approximately 50 A in our DNA-histone films. No conclusive explanation for this discrepancy can be given at the moment. It could easily be due to one of the many known sources of error that occur in the determination of sizes from electron micrographs, especially from shadowed preparations.

The differences observed in DNA-protein films spread from solutions of high and low salt concentrations may tentatively be explained in the following way. Most observers agree that in high salt concentrations the nucleoprotein molecule is dissociated into nucleic acid and protein and that actual nucleoprotein molecules exist in solution only in low salt concentrations. At intermediate salt concentrations the nucleoprotein usually is insoluble (1, 21, 30). If DNA and a basic protein are mixed at very low salt concentrations an insoluble precipitate is formed, unless the concentration of both nucleic acid and protein is very low. This is thought to be due to cross-linking of the DNA by the protein (1, 21). Even though in our experiments the DNA and protein were mixed at concentrations of only 10^{-2} mg/ml and no precipitate was visible in a 0.01 M salt concentration, the "spiders" seem to indicate that some cross-linking occurred. The central dense knots in these formations are thought to represent segments of several DNA molecules linked by the protein, from which free ends or long loops of single molecules extend in all directions. If, on the other hand, the DNA-protein mixture is spread from a high salt concentration onto a substrate where the nucleoprotein is insoluble (0.25 M ammonium acetate), the nucleoprotein complex is actually formed on the surface in a rapidly expanding film, where cross-linking is much less likely to occur. That extensive "spider" formation does occur even under these conditions with the

esterified serum albumin may be due to the much higher molecular weight of this protein as compared with cytochrome *c* or histone. No such indication of cross-linking was found even in films spread from low salt concentrations if the sonicated DNA was used.

The results presented here thus indicate that "staining" with heavy metal salts can be used to render single molecules of DNA visible in the electron microscope. Similar results have been obtained independently by Beer (2, 3) using uranyl nitrate at pH 3.5. The contrast in his pictures apparently is lower, and from his chemical studies (Zobel and Beer, 28, 29) it can be assumed that the UO_2:P ratio in his preparation is $\sim$1:2, whereas according to the results of Huxley and Zubay (14), in our preparation, the ratio should be 1:1 or higher, if not too much of it is lost during the washing of the grids. Even though the additional uranyl does not seem to be bound as firmly, it obviously is useful in increasing the contrast of the preparations. In nucleoproteins the described "staining" procedures evidently render the nucleic acid part visible, which is in good agreement with the results of Huxley and Zubay (14) obtained on uranyl acetate–stained sections of a nucleohistone isolated from thymus tissue.

This work was greatly aided by frequent discussions with Dr. G. Zubay. My thanks are due to him and Dr. H. E. Huxley and also to Dr. M. Beer for making the manuscripts of papers available to me before publication. The use of lead perchlorate as an electron stain was suggested to us when we learned from Dr. A. Bendich about his work on the reaction of DNA with lead salts.

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