STUDIES ON THE FIXATION OF ARTIFICIAL AND BACTERIAL DNA PLASMS FOR THE ELECTRON MICROSCOPY OF THIN SECTIONS

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ABSTRACT

The process of fixation of DNA-containing plasms is investigated by macroscopical and electron microscopical observations on solutions of DNA, nucleohistones, as well as on bacterial nuclei. The following treatments were found to produce a gelation of a solution of DNA or nucleohistones: (*a*) OsO_4 fixation at pH 6 in the presence of amino acids (tryptone) and Ca^{++}. (*b*) Exposure to aqueous solutions of uranyl acetate. (*c*) Exposure to aqueous solutions of indium chloride. Observed in the electron microscope, these gels show a fine fibrillar material. From experiments in which solutions of DNA or nucleohistones are mixed with bacteria and treated together, it is concluded that the behavior of the bacterial nucleoplasm is similar to that of the DNA solutions. The appearance of birefringence indicates that uranyl acetate and indium chloride produce an orientation of the molecules of a DNA solution during gelation. Bacterial chromosomes fixed by these agents also show a certain order, while those fixed by the OsO_4–amino acid–Ca^{++} formula do not. Whether or not the order can be considered to be artificial is discussed, and a tentative conclusion is presented: (*a*) Uranyl acetate may induce artificial order. (*b*) Fixatives which do not gel DNA probably result in the grossest artifacts. (*c*) OsO_4 fixation at pH 6 in the presence of amino acids (tryptone) and Ca^{++} may give the most accurate preservation of the *in vivo* disposition of DNA (RK^+ fixation).

INTRODUCTION

This study deals with two different methods for the fixation of DNA solutions. Both treatments have, in the past, been considered to yield comparable results, and little, if any, clear differentiation in the use of the methods singly or in combination has been emphasized in previous investigations.

The interaction of osmium tetroxide fixatives with DNA solutions can be shown, under certain conditions, to lead to gelation of these solutions. If DNA solutions are brought in contact with uranium salt solutions, gelation of DNA solutions is also obtained; however these gels seem to be of a different nature.

The regions of cells or viruses in which DNA is the most important component have been considered as "DNA plasms." In nature, a number of such DNA plasms exist. These seem to be distinctly different from one another and may be arranged

in an order of increasing complexity. These are: the pool of vegetative bacteriophage DNA, the bacterial chromosome, the mature phage, and finally the chromosomes of higher cells (see Kellenberger, 1962 *a* and *b*). The electron microscopical observation of thin sections of these DNA plasms seems to be a most promising way to obtain information on their organization; however, certain difficulties are encountered. Preparation for the ultramicrotomy involves treatment which results in rather profound modifications of the physicochemical state of the components. By "fixation" we chemically alter most substances so that, we hope, the structure is not grossly distorted by the necessary replacement of water by organic liquids. The dehydrated specimen is then placed in a monomeric embedding material which is polymerized in order to give a block solid enough to be cut.

The present paper presents some observations on the effect of a number of preparation procedures on several natural and artificial DNA plasms. We will consider any solution of DNA, nucleohistone, or other chemically defined DNA compounds as an artificial DNA plasm. We have made the initial assumption that in such a solution the DNA molecules are randomly oriented and individually separated. The microscopical image of such randomly arranged, artificial plasms is to a certain extent predictable. On the other hand, very little is known about the degree of order in highly dehydrated, natural DNA plasms, and predictions would be no more than pure speculation (see Kellenberger, 1962 *a*; Ris, 1962).

The study of artificial DNA solutions shows that one of the main obstacles to overcome in preparation for electron microscopy is the aggregation of DNA macromolecules which occurs in organic solvents during embedding. One of the roles of fixation is to modify the highly hydrated DNA plasms in such a way that they can withstand the replacement of water by organic fluids without forming coarse aggregations. The results described in the present paper indicate that the conditions of fixation which reveal the finest fibrillar structure in electron micrographs are those in which a complete gelation of the DNA solutions can be macroscopically observed. Such a gelation, however, may introduce new structure or destroy structural arrangements in natural DNA plasms. In order to maintain faithfully the "prefixed" state of DNA plasms during structure-conserving fixation, aggregation as well as rearrangements or the destruction of existing arrangements must be rigorously avoided. That the ideal solution has not yet been found is shown by the fact that it cannot be unequivocally demonstrated for the present whether the ordered appearance of the DNA plasm of the bacterial nucleus is real or an artifact.

A variety of studies with several DNA plasms demonstrating variations in response to fixation procedures were carried out. These studies include the following: (*a*) Macroscopic observations on DNA plasm reacting to various fixation procedures. (*b*) Studies of interaction of fixative and DNA plasm on the molecular level by means of polarized light. (*c*) Morphological effects of fixatives as determined in the electron microscope. (*d*) Comparative studies on the bacterial nucleus and artificial DNA plasms, mixed together to form a single preparation, in their responses to the different fixations. (*e*) Observations on the ordered arrangement of the fibrils in the bacterial nucleus.

TERMINOLOGY

Some of the terms which will be used in this paper and in further publications are defined as follows: by *sol* we mean a solution of organic macromolecules, for example a solution of DNA, in which the molecules move freely in relation to each other. If sufficient time is allowed, a given molecule can be found at any point of the solution. In a *gel*, the molecules are cross-linked so as to form an uninterrupted network. Individual molecules are immobilized. Thermal agitation is reduced to random vibrations around a median position, the framework of the gel is physically a solid, and the solvent which it contains is still a liquid. Gels made of fibrillar components can show different properties depending on the mean distance between cross-links measured along linear molecules and also depending on the size of the linked zones. One can distinguish between *reversible gels*, in which the maintenance of cross-linkages depends on outside conditions, such as temperature (*e.g.* agar-agar) or ionic composition of the solvent, and *irreversible gels*, in which the cross-linkages cannot be destroyed without damage to the macromolecule itself.

In a sol, macromolecules are electrically charged so that they tend to repel each other. Under some conditions, for example at the isoelectric point, these charges are in effect neutralized, and the molecules tend to aggregate or coalesce. The *aggregates* have a higher local concentration of the

molecular substance, which leads also to a higher refractive index. Hence, optically, these aggregates manifest themselves as a turbidity of the suspension or even by visible flocs. This phenomenon is known as "flocculation." Typical flocculation occurs when solutions of about 1 per cent DNA or 1 per cent gelatine are mixed with about two volumes of alcohol or other water-miscible solvents.

MATERIALS

Escherichia coli Strains

B, BP. Cultures were grown either in aerated tryptone medium (1 per cent Bacto-Tryptone, 0.5 per cent NaCl, pH 7–7.2, Difco Laboratories, Inc., Detroit, or in synthetic medium M9 (0.7 per cent Na_2HPO_4; 0.3 per cent KH_2PO_4; 0.1 per cent NH_4Cl; 10^{-4} M $CaCl_2$; 10^{-3} M $MgSO_4$; 2×10^{-6} M Fe^{+++}-citrate; 0.4 per cent glucose; pH 7.0).

Artificial DNA Plasms

1. Pure trout sperm DNA, kindly provided by Dr. Zahn, Physiologisch-Chemisches Institut der Goethe–Universität Frankfurt, was dissolved in acetate-Veronal buffer of pH 6.2 in a concentration of 2 to 3 mg/ml. Colorless, viscous solutions are obtained. (For isolation methods see Kiefer *et al.*, 1961; Zahn *et al.*, 1962). Physicochemical data on this preparation provided by Zahn are as follows: mol. wt. $6.1 \cdot 10^6$. Intrinsic viscosity $(\eta)(c^+ = 0) = 36$ ml/gm for c = gm/dl. Sedimentation constant pH 5 at 25°C: s = $19.3 \cdot 10^{-13}$ sec. Partial specific vol.: $V^+ = 0.55$.

2. DNA, "highly polymerized," from Nutritional Biochemicals, Inc., Cleveland. This DNA required gentle shaking at 4°C for 36 to 100 hours to go into solution in acetate-Veronal buffer.

3. DNA from calf thymus, kindly provided by Dr. O. Klamerth, Institut für Virusforschung, Heidelberg, Germany.

4. DNA, obtained from commercial source Fluka Inc., Buchs, Switzerland, described as "DNS, niedermolekular (low molecular weight DNA) aus Hering, purum," described in Fluka catalog No. 5, 1961, No. 52656, could be dissolved in acetate-Veronal buffer up to 200 to 400 mg/ml and gave only at that concentration a DNA sol of the viscosity of 1 per cent boiling agar.

5. Nucleohistone from Worthington Biochemical Corporation, Freehold, New Jersey, in amounts of 6 to 9 mg/ml to distilled water. After gentle shaking at 4°C for 36 to 100 hours the undissolved material was removed by means of filtration through sintered Schott glass filters No. 3G3 and the filtrate adjusted by adding distilled water to give a viscosity comparable to that of the trout sperm DNA.

The above mentioned DNA preparations (items 1 to 3) failed to show any distinct differences. Trout sperm DNA was employed throughout the study since this preparation was more thoroughly examined by physicochemical methods.

Solutions for Fixation and Different Treatments

ACETATE-VERONAL BUFFER BASIC MIXTURE: 19.428 gm $CH_3COONa \cdot 3H_2O$, 29.428 gm sodium diethylbarbiturate, 34 gm NaCl in distilled water to 1000 ml. 5.0 ml of this mixture is mixed with 13.0 ml distilled water, 7.0 ml HCl N/10, and 0.25 ml $CaCl_2$ M. The pH of this buffer is 6.1. In cases where no Ca^{++} is desired, the $CaCl_2$ is omitted. For a pH of 7.2 the mixture is: 5.0 ml of basic mixture, 14.5 ml distilled water, 5.5 ml HCl N/10, with or without 0.25 ml $CaCl_2$ M. For a pH of 8.1 the mixture is: 5.0 ml of basic mixture, 18.0 ml of distilled water, 2.0 ml HCl N/10, with or without 0.25 ml $CaCl_2$ M. In all cases adjustment of pH is made with HCl N/10.

URANYL ACETATE: 0.5 per cent dissolved in the acetate-Veronal buffer of pH 6.1, the pH drops to *ca.* 3.5.

OSMIUM TETROXIDE FIXATIVES: OsO_4 was dissolved in acetate-Veronal buffer of desired pH, to give a 1 per cent solution. In cases where osmium tetroxide fixative was mixed with an equal volume of DNA sols, the osmium tetroxide fixatives contained 2 per cent OsO_4 in the buffer.

(*a*) OSMIUM TETROXIDE FIXATION RK$^+$ (Ryter Kellenberger fixation): This means an osmium tetroxide fixation at a final concentration of 1 per cent OsO_4 with 0.1 per cent $CaCl_2$ and 0.1 per cent tryptone in the solution at a pH of 6.2. Since tryptone reacts immediately with the OsO_4 solution, the tryptone is usually added first to the DNA or bacterial samples, followed 30 to 60 seconds later by the addition of OsO_4. The tryptone solution is made up with 1 gm Bacto-Tryptone (Difco Laboratories, Inc.) or 1 gm Bacto-Casamino Acids (Difco Laboratories, Inc.), 0.5 gm NaCl to 100 ml H_2O. Casamino acids reacted more readily with OsO_4.

(*b*) OSMIUM TETROXIDE FIXATION RK$^-$ (RK minus): This means an osmium tetroxide fixation at a final concentration of 1 per cent OsO_4 with omission of either $CaCl_2$ or tryptone in the fixing fluid or final fixing situation, or with a shift of the pH from pH 6.2 to 7, 8, or 9. In the text and in the figure legends the change with respect to the RK fixation will be indicated in parentheses.

All experiments with fixatives were carried out at room temperature; experiments described in Table I were repeated also in the cold room at 4°C.

INDIUM CHLORIDE: $InCl_3$ (Merck and Company, Rahway, New Jersey) dissolved at 0.5 per cent

in acetate-Veronal buffer at pH 6.2. The pH drops usually to 3.5–3.7.

STREPTOMYCIN: Combistrep 40 mg (Chas. Pfizer and Co., Brooklyn, New York) in 1 ml. acetate-Veronal buffer of pH 6.2.

POTASSIUM PERMANGANATE FIXATIVE: $KMnO_4$ 1 per cent in acetate-Veronal buffer pH 6.2.

ACETONE FOR DEHYDRATION: "Acetone for chromatographic purposes," Merck and Company, dried over freshly prepared anhydrous $CuSO_4$.

METHODS

EMBEDDING: Bacteria were grown under continuous aeration at 37°C in 1000-ml flasks to a density of 1 to 2 × 10^8 cells/ml. Buffered OsO_4 was added to these cells in the logarithmic growth phase so that the final concentration of OsO_4 was 0.1 per cent and the suspensions were immediately spun at 3000 RPM.

The pellets of "prefixed" cells were mixed with 2.5 per cent agar, dissolved in corresponding buffers, and the agar was cut after solidification into small blocks. The fixation procedures were then applied to these cubes. Other specimens, mainly artificial DNA plasms, were cut after fixation into 0.5 mm cubes and dehydrated in successive solutions containing 30, 50, 70, and 90 per cent acetone in water. The blocks were placed for about 2 hours in dried acetone; then, a few drops of Vestopal W, containing initiator and activator, were added. The acetone was evaporated slowly (overnight) with the help of a fan. This procedure insured regular impregnation with the Vestopal. The blocks were then transferred to fresh Vestopal (containing initiator and activator) where they were kept for several hours before transferring them to gelatine capsules. The filled capsules were kept at room temperature overnight and then polymerized at 60–75°C for at least 36 hours.

ULTRAMICROTOMY: Thin sectioning was done with the LKB Ultratome using glass knives.

ELECTRON MICROSCOPE: Siemens Elmiskop I, 40 kv, objective aperture diaphragm 50 μ condensor 1 adjusted to give an illuminated area on the specimen of 7 μ in diameter.

STAINING ON THE GRIDS: Some of the preparations were stained by the lead-cacodylate method of Karnovsky (1961).

BIREFRINGENCE: Birefringence was observed on an optical bench, on which the samples were arranged in rectangular 1 x 1 cm optical cuvettes. Light from a low voltage lamp with a condensor was polarized with a polaroid filter. A suitable lens system produced an approximately collimated beam through the cuvette. After passage through an analyser-polaroid filter, an image of the cuvette was projected into a mirror-reflex camera without objective lens.

TERMINOLOGY OF FIXATION PROCEDURES

As described under Materials and Methods, the RK^+ (RK stands for Ryter and Kellenberger, 1958) fixation must fulfill the following conditions: 1 per cent OsO_4, presence of 0.1 per cent $CaCl_2$, presence of 0.1 per cent tryptone (calculation based on dry weight) in the final fixing fluid. The pH required is 6.2. The RK^+ term does *not* include any uranyl acetate posttreatment. The term RK^- fixation indicates that at least one of the three conditions (0.1 per cent $CaCl_2$, 0.1 per cent tryptone, pH 6.2) is *not* fulfilled (therefore RK minus). We strictly differentiate these two fixation systems from each other as well as from a uranyl acetate fixation. Uranyl acetate however can be used either as second step of fixation to follow an RK^+ or RK^- fixation or it can be the fixative of a single step uranyl acetate fixation alone. In each case the circumstances will be clearly indicated with respect to fixation with uranyl acetate or its equivalent, indium chloride.

RESULTS

Macroscopic Observations on DNA Solutions Reacting with Different Fixatives

Artificial DNA plasms[1] were mixed 1:1 with fixative in a small test tube. The final concentration of OsO_4, when used, was 1 per cent and thus the same as that used in experiments with bacteria. The solutions of DNA were made in the same buffer used for fixation. Nucleohistones were dissolved in some experiments in corresponding buffer and in other experiments in distilled water, where they go into solution more readily. The use

[1] The experiments showed that different concentrations of pure DNA gave the same results. Since some of the samples required gentle shaking for several hours or days for dissolution while others needed filtration through sintered glass filters, the viscosity of a boiling 1 per cent agar solution is given as reference. While even gently shaking as well as filtration may affect the molecule length and filtration the concentration of DNA in the solutions, and since the same results were obtained with a wide range of DNA concentrations as well as with nucleohistone concentrations, only this rough qualitative reference is given. The range of concentrations initially investigated can only be given as: from barely visible viscosity (upon shaking of flasks) to a honey-like viscosity.

TABLE I

Fixing Fluids

	2 per cent OsO_4, pH 6 minus Ca^{++} minus tryptone (fixation RK^-)	2 per cent OsO_4, pH 6 plus Ca^{++} plus tryptone (fixation RK^+)	0.5 per cent uranyl acetate in Veronal-acetate buffer, pH ~ 3.5 plus Ca^{++}	1 per cent $KMnO_4$ in Veronal-acetate, pH 6.3, plus Ca^{++} plus tryptone	1 per cent $KMnO_4$ in Veronal-acetate, pH 6.3, plus Ca^{++} minus tryptone	0.5 per cent $InCl_3$ in Veronal-acetate, pH ~3.6, plus Ca^{++}	Streptomycin 40 mg/ml in distilled water
Pure DNA, as viscous solution in Veronal-acetate, minus Ca^{++}	After several hrs. slight yellow coloration, *no gelation* even after 48 hrs.	After several hrs. (4 to 7) a black-brown *solid gel* is formed. Can be destroyed by stirring into flocs and liquid.	*Immediate gelation* to colorless gel. When DNA added to uranyl acetate, drop-shaped gels are formed. Destruction by stirring not possible.	Within 5 min. half-solid gel, after some hrs. separation in flocs and liquid. Sometimes aggregation of flocs to rather compact sediment.	Within 5 min. half-solid gel, after some hrs. separation in aggregation of flocs and liquid.	Immediate gelation to colorless gel with whitish surfaces. DNA drops form drop-shaped gels. No destruction by stirring.	Immediate flocculation. DNA drops can also form drop-shaped gels; destruction by stirring.
Nucleohistone as viscous solution, either in Veronal-acetate minus Ca^{++} or in distilled H_2O.	After several hrs. slight yellow coloration, *no gelation* even after 48 hrs.	After several hrs. (4 to 7) a black-brown very *solid gel* is formed. Can be destroyed by hard stirring into flocs and liquid.	*Immediate gelation* to colorless gel. When added as nucleohistone drops to the uranyl acetate, drop-shaped gels are formed. No destruction by stirring possible.	Within 5 to 10 min. half-solid gel; after some hrs., separation in flocs and liquid. Sometimes aggregation to sediment.	Within 10 min. a half-solid gel, less solid than in presence of tryptone. After hrs, separation in flocs and liquid. Aggregation to solid sediment.	Immediate gelation to colorless gel with white surface phenomenon. Nucleohistone drops form drop-shaped gels, resistant to stirring.	Immediate flocculation, drop-shaped gels also formed; destruction by stirring.

of these different solvents did not result in differences in the subsequent reactions of nucleohistones.

FIXATIVE RK^-: As one can see from Table I, pure DNA, dissolved in buffers of pH 5–9, was virtually unreactive with OsO_4. The solutions remained as sols and did not change significantly in color. The same was true for nucleohistones, where only a very slight yellow color appeared after several hours at room temperature.

FIXATIVE RK^+: If a DNA solution was mixed with fixative RK^+ 1:1 (2 per cent OsO_4, at pH 6.2, containing 0.1 per cent $CaCl_2$, and 0.1 per cent tryptone), the following events are observed (Table I):

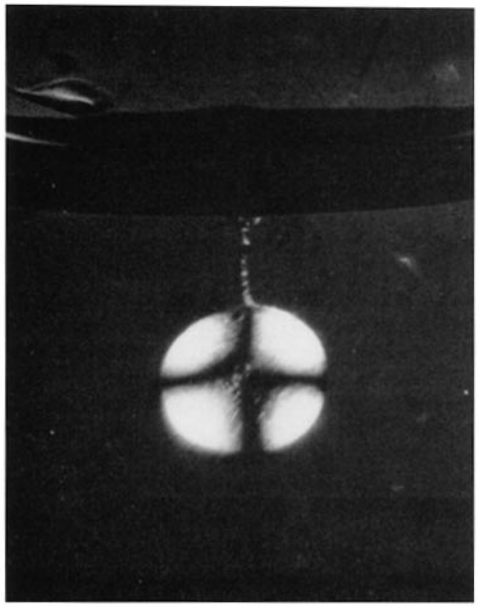


FIGURE 1 Spherite cross appearing in a drop of DNA gelated in uranyl acetate and observed between crossed polaroid filters. The photograph was taken 35 minutes after the start of the reaction. × 7.

One to 2 hours after mixing, the solution became yellow, then brown and gradually gelled. After 6 to 8 hours it had become nearly black and formed a relatively stable gel. The reaction time was highly variable. Some reactions seemed to start only after a lag period, which may depend on parameters which are difficult to control. The chain length of the DNA molecules seemed to have influence. Low molecular weight DNA could not be completely gelled, even in high concentration (see Note Added in Proof). The partial gels obtained had a behavior which could macroscopically be compared with "thixotropic" gels of aged metal hydroxids (Alterungsgele, Stauff, 1960).

Gelation by the RK^+ method requires three essential conditions: a minimum concentration of

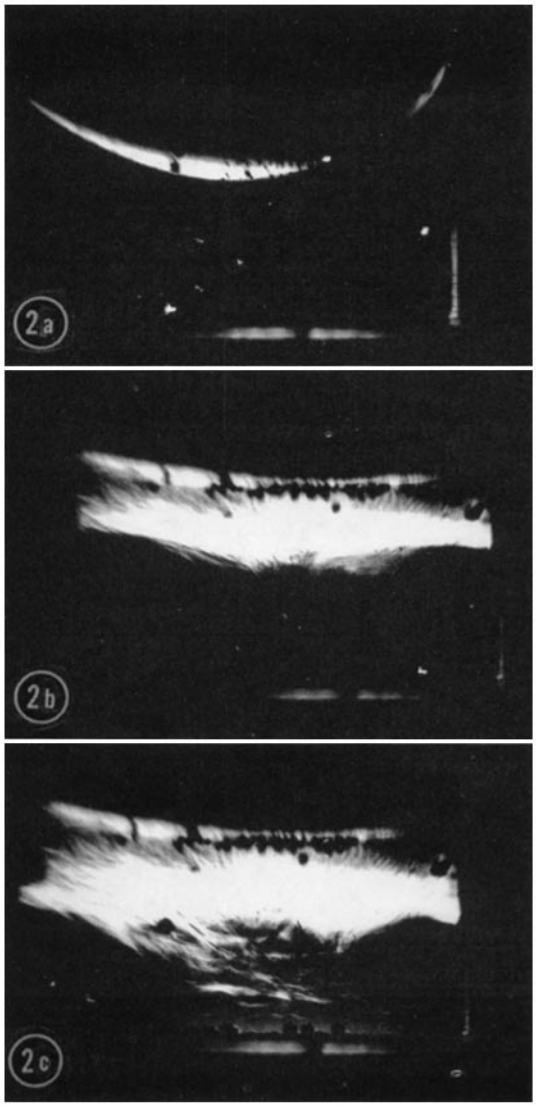


FIGURE 2 The phenomenon of birefringence as observed in a solution of pure DNA overlayered with uranyl acetate as a function of time. The square glass cell is placed between crossed polaroid filters and the photographs taken (*a*) 1 minute, (*b*) 120 minutes, and (*c*) 180 minutes after the beginning of the reaction. The birefringence is not homogeneous but has a fibrillar or layered appearance macroscopically. × 3.

0.05 per cent $CaCl_2$, a minimum concentration of 0.05 per cent tryptone, and a pH of 6.0–6.5. Complete absence or low concentration of either Ca^{++} or tryptone completely prevents gelation. If Ca^{++} and tryptone are used in excess (concentrations above 0.2 per cent) the influence of the third condition of the RK^+ method, the pH of 6.0–6.5, becomes less critical and gelation can occur between pH 6 and 8.

URANYL ACETATE, INDIUM CHLORIDE: It had been shown previously (Kellenberger *et al.*, 1958; Ryter and Kellenberger, 1958) that post-treatment with uranyl acetate after any type of OsO_4 fixation has "strong stabilizing" effects on bacterial nucleoplasms. After OsO_4 fixation under different conditions, the uranyl acetate "post-treatment" always resulted in a fine fibrillar texture in the nucleus. It has been shown (Beer and Zobel, 1961; Zobel and Beer, 1961; Huxley and Zubay, 1961) that the staining effect of uranyl acetate is due to its reaction with nucleic acids. Similar findings are reported by Stoeckenius (1961). Indium chloride has also been used for staining (Watson and Aldridge, 1961), thus indicating that it probably reacts similarly. These two substances have therefore been investigated and found to gelate DNA solutions very rapidly (Table I). Independently, Watson (1962) has shown that indium chloride gelates DNA solutions.

POTASSIUM PERMANGANATE: It had been also shown that $KMnO_4$ fixation according to Luft produced fine stranded DNA plasms in bacteria (Luft, 1956; Mercer, 1958). In the present studies (Table I) $KMnO_4$ fixation was also found to produce a gel with DNA solutions; however, it was much less solid, it flocculated after a few hours, and the sediment dissolved instantly in Versene solution. This is contrary to the RK^+ gels as well as the uranyl acetate and indium chloride gels, which did not dissolve within 2 to 4 hours in Versene solution.

STREPTOMYCIN: Streptomycin is a basic substance which is frequently used to precipitate DNA out of biological homogenates (Cohen and Lichtenstein, 1960). The ability of streptomycin to gel DNA solutions was therefore examined. Streptomycin treatment of DNA solutions results in immediate flocculation; the flocs were readily soluble in versene solution.

In all these experiments, nucleohistones reacted generally like pure DNA; small differences are reported in Table I.

Studies on the Interaction of DNA Plasms with Fixatives at the Molecular Level Using Polarized Light

To obtain more knowledge on the structure of the DNA solutions fixed by the different methods, DNA solutions undergoing gelation were examined with polarized light. Results obtained with pure DNA are similar to those shown by nucleohistone solutions.

TABLE II

Birefringence after Different Treatments of DNA Sols

Fixative	None	RK^-	RK^+	Uranyl acetate	RK^- followed by uranyl acetate	RK^+ followed by uranyl acetate
Birefringence	−	−	−	+	+	−

A DNA sol can be defined as a colloidal solution of dispersed *anisotropic* particles. Such a colloidal system as a whole is not necessarily anisotropic, however, because of statistical disorder. (Therefore, the DNA solution has no optical anisotropic properties and appears to be *isotropic* as long as no external influence exists.) Then, in the absence of external influences, solutions of DNA do not show birefringence when observed between crossed nicols or crossed polaroids. Even rather highly concentrated DNA solutions (0.4 to 0.6 per cent of Dr. Zahn's DNA) of honey-like consistence did not show birefringence. As expected, flow-birefringence does appear upon external agitation with all our DNA sols.[2]

[2] DNA sols were not observed on the optical bench for prolonged periods of time (*i.e.* several days). Phenomena described by Robinson (1961, p. 233) have not been observed. It seems possible that, on standing for long periods of time, DNA solutions undergo changes due to enzymatic activities and that the DNA macromolecules might be, at the time of his observations, already very short. Also, concentrations of 6 per cent in his solutions indicate that the DNA used might have been of rather low molecular weight if not degraded.

RK$^+$-FIXATIVE GELS: Gels obtained with fixative RK$^+$ do not show a birefringence either after or during gelation. Gels obtained by mixing fixative RK$^+$ with the DNA sols or by putting a layer of the RK$^+$ fixative over the solution were studied. In these experiments the tryptone (of the defined RK$^+$ conditions) in a final concentration of 0.1 per cent was mixed into the viscous DNA sol before addition of the fixing fluid or before overlaying the DNA with the OsO_4. When dehydrated with acetone these gels *did not* become birefringent; the cuvette remained black between crossed polars.

URANYL ACETATE GELS: Uranyl acetate gelation is a very rapid process, thus permitting the sol-gel transformation to be followed during all phases on an optical bench. The fixative (pH 3.5) was first poured into a square cuvette and placed on the optical bench between crossed polaroids. A drop of the DNA solution was then carefully added to the uranyl acetate and the reactions observed. The drop, when carefully placed with a pipette on the surface of the fixative, maintained its form and floated just beneath the surface of the liquid. After a few minutes, distinct birefringence appeared at the periphery of the drop as a bright circumference around the invisible drop which then spread continuously toward the center. The entire process was completed within 15 to 45 minutes, a time corresponding to the complete gelation of the drop. (This gelation can be tested by the mechanical resistance of the drop as a whole.) Figures of "spherite crosses" appeared (Frey-Wyssling, 1953) which rotated with rotation of the system of polarization (Fig. 1).

In another series of experiments the solution of DNA was first poured into the cuvette and then overlayered with uranyl acetate fixative. A region of birefringence appeared rapidly in the zone between the two solutions and spread throughout the whole DNA solution after some hours (Fig. 2 *a*, *b*, and *c*). The DNA became a firm gel. When the gel was dehydrated in acetone, no change of the type of birefringence was observed.

COMBINATION OF RK$^+$ WITH URANYL ACETATE FIXATION IN POLARIZED LIGHT: If a gel, obtained by fixation RK$^+$ was overlayered with uranyl acetate, a birefringence could *not* be observed; the molecular structure of the RK$^+$ gel was unchangeable.

If a birefringent uranyl acetate gel was "posttreated" with RK$^+$ fixative, the birefringence persisted and could not be removed; the molecular orientation was part of the gelation.

A treatment with RK$^-$ type fixative does not gel a DNA sol. As already shown, if such a DNA-RK$^-$ fixative mixture is overlayered with uranyl acetate, birefringence appears in the manner described above.

From the results summarized in Table II one can conclude that the molecules of the DNA solutions are randomly oriented and that the birefringence observed after uranyl acetate fixation is due to an order introduced by this fixative.

From the experiments depicted in Fig. 1 (uranyl acetate fixation) we must conclude that the arrangement of the fixed DNA molecules has a spherical symmetry. The observation that birefringence appears first in a surface layer around the DNA drop or at the surface of the overlayered DNA sol, and then spreads into the DNA phase, led to the tentative assumption that the DNA molecules are oriented parallel to the surface, or, what is the same, perpendicular to the gradient of diffusion of uranyl acetate into the DNA.

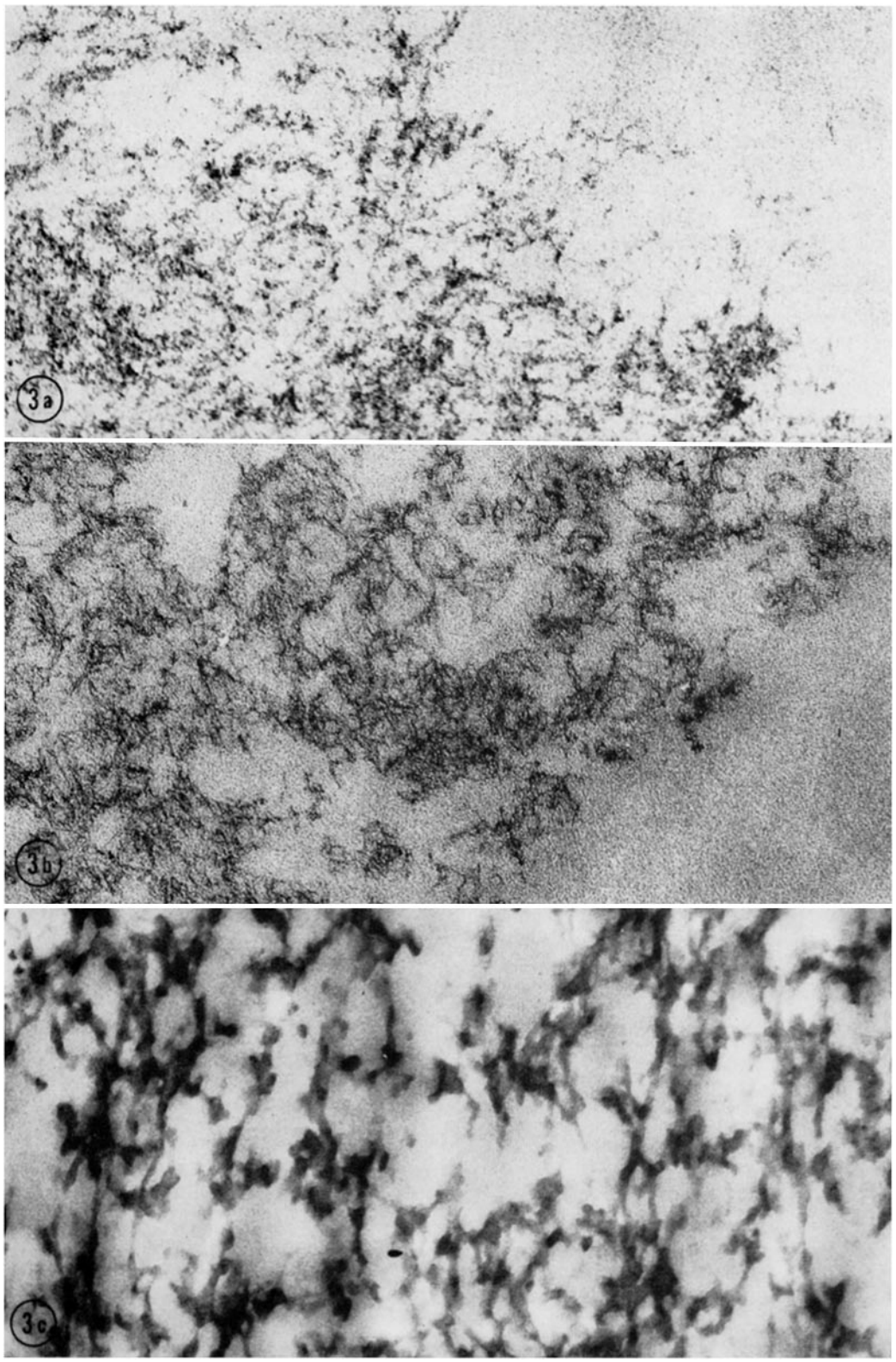
Morphology of Effects of Fixatives as Seen in the Electron Microscope

Pieces of the gels obtained in the macroscopic experiments described above can be embedded

FIGURE 3 *a* An electron micrograph of pure DNA after fixation RK$^+$, followed by uranyl acetate posttreatment. Processed through dialyzing membrane. Fine fibrillar aspect, no visible orientation. × 89,000.

FIGURE 3 *b* Electron micrograph of nucleohistone after fixation RK$^+$ and followed by uranyl acetate posttreatment. Processed through dialyzing membrane. Very fine fibrillar aspect without any visible orientation. × 100,000.

FIGURE 3 *c* Electron micrograph of pure DNA after fixation RK$^-$ (no Ca^{++} pH 7.2) and without uranyl acetate posttreatment. Processed through dialyzing membrane. Very coarse aggregations are visible. × 85,000.


3a
3b
3c

directly. However, another method was also used, in which the different fixative fluids acted through a dialysis membrane placed at the end of a small open tube containing the DNA solution (Schreil, 1961). Even dehydration can be carried out by this method, which has the advantage that not only gels but also highly concentrated sols can be treated. After dehydration the sample was removed from the cellophane membrane for embedding in Vestopal.

FIXATION RK^+: Solutions of pure DNA (Fig. 3 *a*) and of nucleohistone (Fig. 3 *b*) fixed by RK^+ showed a fine fibrillar network arrangement. The arrangement was the same, whether or not a posttreatment with uranyl acetate (aqueous) was made. The micrographs shown here came from uranyl acetate–treated samples, since contrast was otherwise rather low.

FIXATION RK^-: Fig. 3 *c* shows the appearance of pure DNA embedded after RK^- fixation (Ca^{++} omitted, pH 7) and without posttreatment with uranyl acetate. The DNA molecules are coarsely aggregated and no individual fibrils are visible. Nucleohistone gave similar pictures.

Depending on the extent of the deviation from the critical conditions of fixation RK^+, a more or less coarse aggregation of the fibers is obtained. Omission of either Ca^{++} or tryptone, or a pH above 6.2, was sufficient to initiate the aggregation. As in the gelation experiments, the pH was less critical if an excess of Ca^{++} and tryptone was used (0.2 to 0.4 per cent). No significant differences in behavior between DNA and nucleohistone were detectable.

URANYL ACETATE: Uranyl acetate used alone as a fixative or employed as posttreatment after RK^- fixation procedure also preserved the DNA fibrils against coarse aggregation, as can be seen in Figs. 4 *b* and 4 *c*. Instead of the random arrangement observed after fixation of RK^+ (Fig. 4 *a*), however, uranyl acetate treatment results in a sort of orientation of the fibers. This orientation is more pronounced in Fig. 4 *b* (which represents a thin section of a drop of a DNA solution gelated in the fixative) than in Figs. 4 *c* and 4 *d*, which were obtained by the method of the cellophane membraine dialysis. For this latter figure, the direction of cutting was known to be parallel to the dialyzing membrane through which the fixative entered.

No samples treated with other fixatives were embedded. From the above results one can see that DNA solutions fixed with a gelating agent show a fine fibrillar structure, while in the case of fixative RK^-, in which no gelation occurs, the DNA fibers are aggregated.

Comparative Electron Microscope Studies on Simultaneously Fixed Bacterial Nuclei and Artificial DNA Plasms

In the previous section on morphology of effects of fixatives it was shown that the appearance of fixed artificial DNA plasms varied according to the conditions of fixation. The plasms were fine stranded under the same conditions found previously to produce fine strands in bacterial nuclei (Ryter and Kellenberger, 1958). The handling of these DNA or nucleohistone solutions for embedding, especially the fixation through a dialyzing membrane, is rather delicate and the results are not always completely reproducible. Experiments were therefore performed in which bacteria and DNA or nucleohistone were mixed before fixation. In this way the conditions for the fixation of both constituents were strictly identical. The technique used is based on the fact that when DNA is mixed

FIGURE 4 *a* Electron micrograph of a DNA gel obtained after fixation RK^+ followed by uranyl acetate treatment. Moderately finely stranded but with a granular character. This granularity occurs frequently when RK^+ fixation is used in solutions. We believe that it is due to deposited reaction products of the fixation. × 98,500.

FIGURE 4 *b* Electron micrograph of a DNA gel obtained by uranyl acetate fixation only. The fibrils are slightly aggregated; the whole shows some orientation. Such gels show birefringence. × 75,000.

FIGURES 4 *c* and 4 *d* Electron micrograph of a nucleohistone after fixation RK^- (tryptone omitted) and posttreated in uranyl acetate for 4 hours. Processed through a dialyzing membrane. To increase the contrast, the section was stained 12 minutes with Karnovsky's fluid. Some orientation of the fibrils can be detected. 4 *a*, × 55,000; 4 *b*, × 75,000.

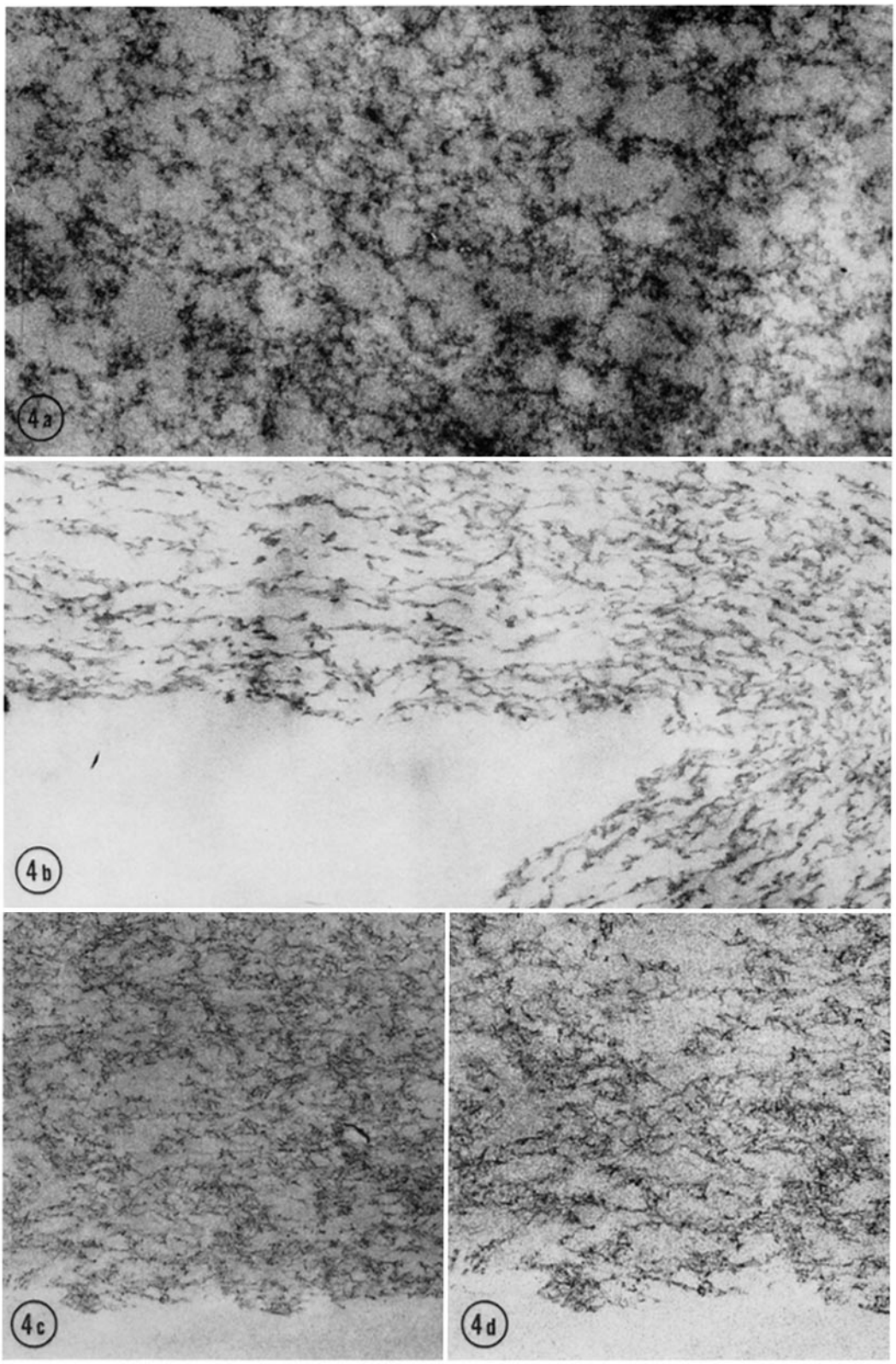

4a
4b
4c
4d

FIGURE 5 *E. coli* grown in M9 mixed with nucleohistone in agar. Fixation RK^+ followed by uranyl acetate. Fine stranded appearance of both natural and artificial DNA plasms. × 70,000.

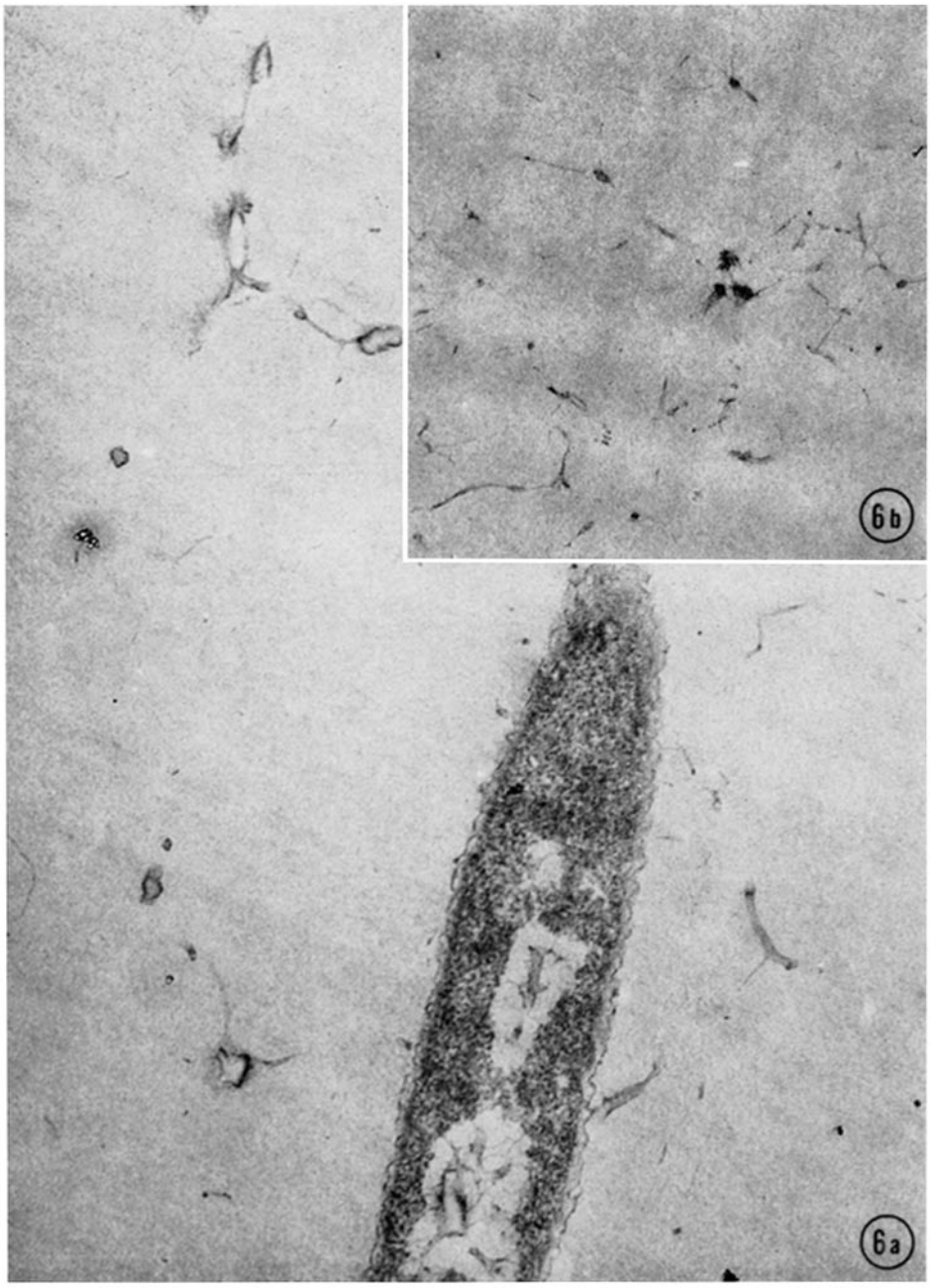


FIGURE 6 *a* *E. coli* grown in M9 mixed with pure DNA in agar. Fixation RK⁻ (tryptone omitted). No postfixation. Coarse aggregations of both natural and artificial DNA plasms. × 52,000.

FIGURE 6 *b* Aspect of the aggregations outside the bacteria in the corresponding experiment with nucleohistone. × 52,000.

with liquid agar at 45°C it remains absorbed in the agar gel, and only negligible amounts are lost by diffusion during the successive steps of fixation and embedding (Schreil, 1961; Bolton and McCarthy, 1962). A further advantage of the method is that we start from uniform material (agar cubes containing the same ratio of DNA:bacterial cells), and with a single pellet several parallel experiments can be made in which only one condition is varied at a time.

The preliminary results obtained with DNA (Schreil, 1961) were confirmed and the observations extended to nucleohistones. No difference in behavior between DNA and nucleohistone has been found, so that the following summarized results are valid for both. In all cases observed, it was found that the appearance of the bacterial nucleoplasm was consistently comparable to that of the surrounding solution of DNA or nucleohistone. After RK^+ fixation the fine fibrillar texture was observed, which is not changed by a subsequent uranyl acetate fixation (Fig. 5). After RK^- fixation (Figs. 6 *a* and 6 *b*) coarse aggregates appeared. RK^- fixation followd by uranyl acetate postfixation also produced fine fibrillar bacterial nucleoplasms and fine fibrillar "outside" DNA or nucleohistone (Fig. 7).

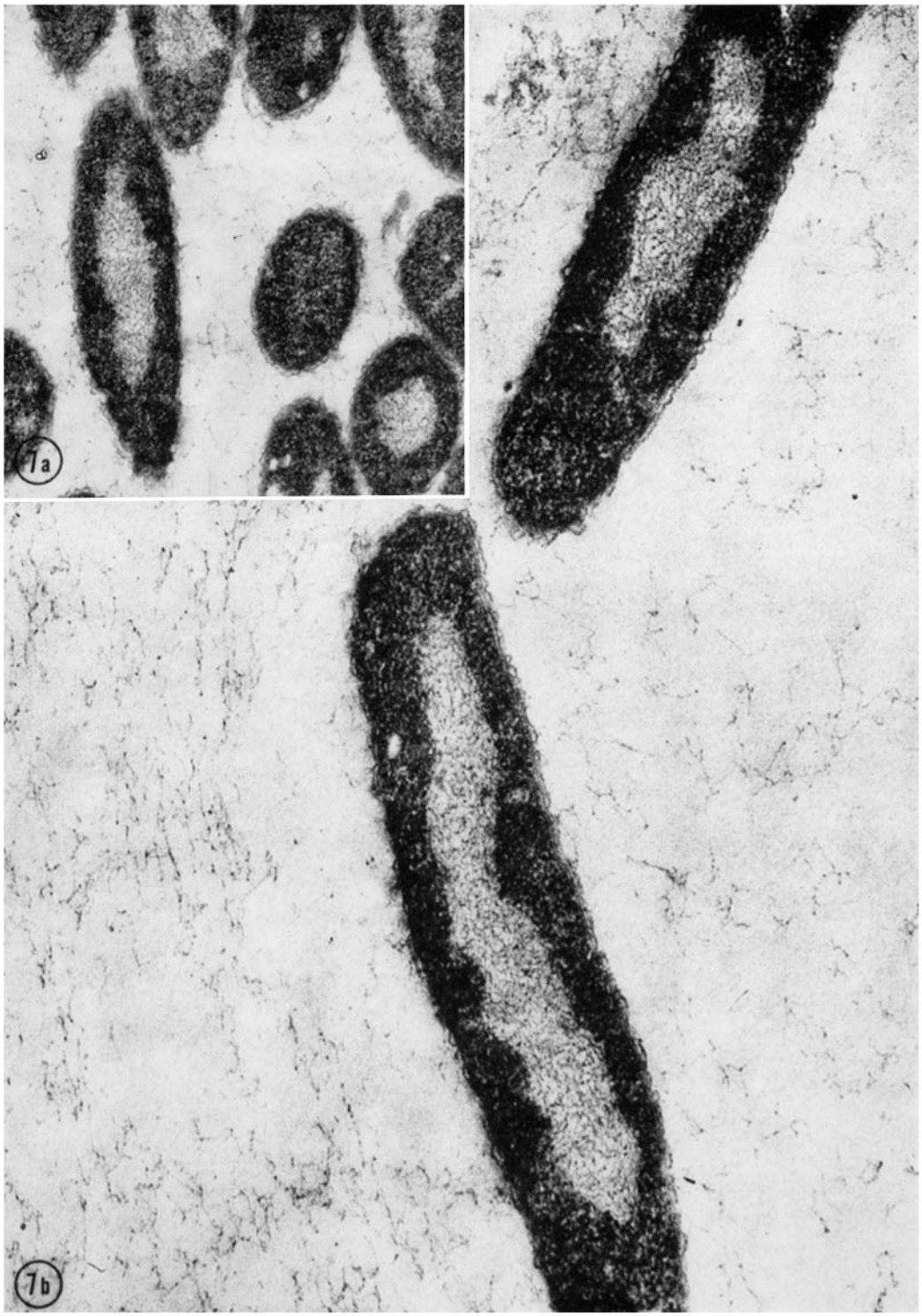
From these experiments it may be concluded that the fine fibrillar appearance of both the DNA plasms of the bacterial nucleus and of a solution of DNA or nucleohistone depends on the conditions of fixation. This would favor the hypothesis that the DNA plasm of bacteria is present as a hydrated sol of DNA in its simple salt form or associated to substances comparable to histone.

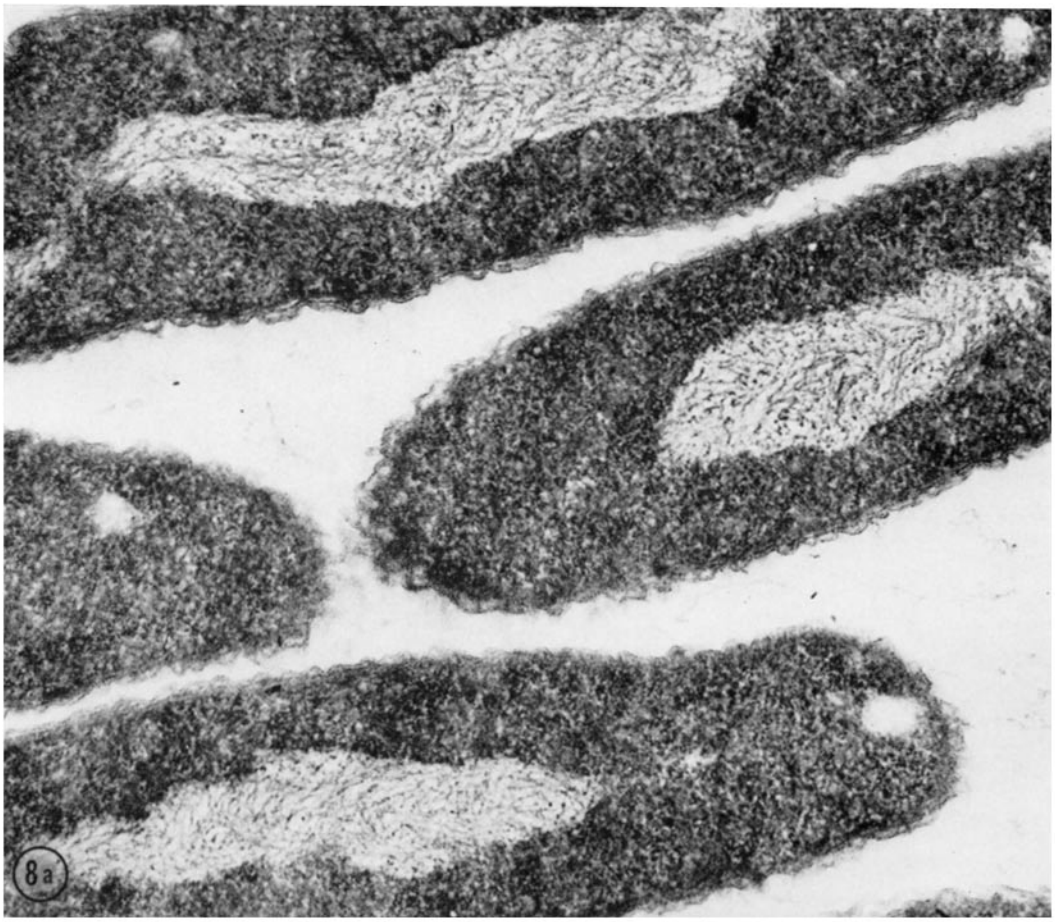
Observations on the Ordered Arrangement of Fibrils in the Bacterial Nucleus

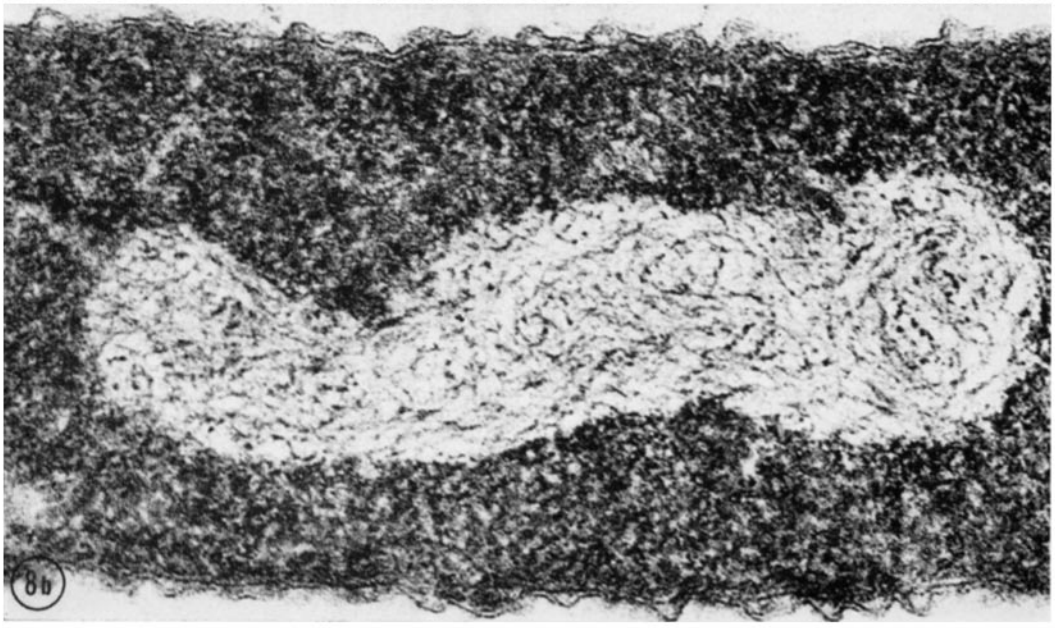
In the previous section only the degree of fibrillar fineness of the fixed DNA plasms of bacteria has been considered. It has been frequently observed that the fibrils of the bacterial nucleus show an ordered arrangement. Since uranyl acetate introduces birefringence in a "macroscopic" gel, experiments were carried out in order to clarify whether the order observed in the bacterial nucleus is an artifact or not.

The following preliminary observations are worth noting: A great proportion of bacteria fixed by RK^- fixation followed by uranyl acetate postfixation showed a distinct degree of organization of the nuclear fibrils (Fig. 8). In the few experiments in which the bacteria had been grown in tryptone and fixed under RK^+ fixation, such arrangements did not occur, whether or not uranyl acetate postfixation had been used (Fig. 9). In those experiments in which the bacteria had been grown in synthetic phosphate-containing medium M9, some order was observed even with RK^+ fixation, when uranyl acetate postfixation followed the RK^+ treatment. It could be that in this case the RK^+ conditions had not been completely fulfilled, since it has been shown (Ryter and Kellenberger, 1958) that even small amounts of phosphate carried over into the fixative with the bacterial sediment after centrifugation hinder the RK^+ fixation and produce aggregation of DNA molecules. Indeed, the low solubility of calcium phosphate must reduce the concentration of free Ca^{++} ions. Since the concentration of 0.1 per cent $CaCl_2$ in the RK^+ fixative is near the critical minimum concentration, it might well be that in some cases the RK^+ conditions are not fulfilled. If the RK^+ conditions are not fulfilled, then no gel is formed from the DNA plasm before uranyl acetate reaches the plasm. In this case, uranyl acetate could introduce artificial order. Indeed, the author strongly suspects that this is frequently the case with some published results. Other experiments, however, in which uranyl acetate follows RK^- fixation (tryptone omission), do not show order in the bacterial nuclei (Figs. 7 *a* and 7 *b*), and therefore definite conclusions cannot be drawn as yet. But it should be noted that the omission of tryptone in the fixative can, under certain circumstances, be partially compensated for by the carrying over of some tryptone broth,—when bacteria were grown in tryptone broth,—with the pellet, since washing procedures of the "prefixed" pellet are to be avoided. When all these conditions are under control (bacteria grown in synthetic medium M9), RK^- fixation (tryptone omitted) and RK^- fixation (Ca^{++} omitted) have the identical effects with the bacterial nucleus.

FIGURES 7 *a* and 7 *b* *E. coli* grown in M 9 mixed with pure DNA in agar. Fixation RK^- (tryptone omitted) followed by uranyl acetate. Fine fibrillar aspect of DNA and of the DNA plasm of the bacteria. 7 *a*, × 40,000; 7 *b*, × 70,000.


7a
7b


8a


8b

DISCUSSION

Two procedures have been described whereby DNA-containing material (DNA plasms) in solution can be transformed into a gel which will resist acetone dehydration, with little evidence of shrinkage and without the formation of coarse aggregations.

This material, when embedded and sectioned, exhibits a fibrillar structure in which individual fibrils have diameters in the neighborhood of 30 to 40 A. In contrast, if one of these procedures is not used, but only conventional fixation applied, gelation of the DNA plasm does *not* take place. As a consequence, very coarse aggregation occurs during dehydration, and in the electron microscope there is found a structure of coarse elements which are in the range of 300 to 400 A, as earlier demonstrated by Ryter and Kellenberger, 1958. It is suggested that the clear gel obtained on RK^+ fixation (OsO_4–Ca^{++}-tryptone, pH 6) or on RK^- fixation followed by a uranyl acetate fixation or on uranyl acetate fixation alone more nearly approximates the arrangement in solution of the DNA-containing substances than does the closely (coagulated) aggregated product resulting from dehydration of conventionally fixed material. It is further suggested that, on this basis, the two procedures may be considered as preferred fixatives for DNA plasms.

The appearance of embedded DNA-containing plasms can be roughly classified either as finely fibrillar or as more or less coarsely aggregated. Employing this as a criterion, it has been found that solutions of DNA or of nucleohistones behave in the same way as the bacterial nucleoplasm. Moreover, those procedures which produce gelation prior to dehydration result in preparations which are finely fibrillar in the electron microscope. OsO_4 in presence of tryptone and Ca^{++} at pH 6 (fixation RK^+) as well as uranyl acetate or indium chloride are such gelating fixatives. It may be concluded from these observations that DNA fibers tend to be aggregated during the dehydration and that this can be minimized, if, prior to dehydration, a gel is formed which acts mechanically against aggregation.

Although such procedures avoid the coarse aggregation of DNA on dehydration, it cannot be assumed that the finely fibrillar structure accurately reflects the arrangement of DNA before fixation. In the gross, a change has been produced by the formation of a gel where none existed before.

From the work of Zobel and Beer (1961) it is known that UO_2^{++} ions are taken up by nucleic acids in a constant ratio (UO_2:P = 1:2 at pH 3.5). This suggests that the bivalent ion binds two phosphate groups. Such binding reduces the net charge of DNA molecules, and therefore they may tend to stick to each other. It is also possible that the uranyl ion forms bridges between the phosphates of two molecules. But it must be remembered in addition that uranyl acetate also forms complexes with the veronal buffer; therefore a variety of uranyl ions of different complexity and of different charge can be expected in this fixing fluid. For indium chloride the possibility of similar reactions with the DNA molecules can be assumed.

For the fixative RK^+, however, nothing is known about the chemical interactions. If a solution of short DNA molecules is treated with this fixative, no gel is obtained, but only a dark brown to black color appears, most probably due only to the reaction of OsO_4 with tryptone. Addition of alcohol or acetone produces a dark precipitate, which can no longer be dissolved. It seems therefore that colored material is now linked to the DNA. Whether this colored material is solely metallic osmium or a combination of reduced OsO_4 of different reduction levels and whether this wholly or partially reduced OsO_4 is in combination with amino acids has not yet been determined.

Although the precise mode of action of these fixing agents cannot be predicted theoretically, some further information from the observation of

Figs. 8 *a* and 8 *b* are thin sections of *E. coli* grown in M 9 after fixation RK^- (tryptone omitted, pH 9) followed by uranyl acetate. Distinct ordered arrangements of the DNA threads. The points visible in the nucleus are probably cross-sections of the fibrils.

FIGURE 8 *a* In this micrograph, which is stained by Karnovsky's technique, darker areas are visible, the meaning of which is not yet clear. × 82,500.

FIGURE 8 *b* A field of the same preparation at higher magnification. × 156,000.

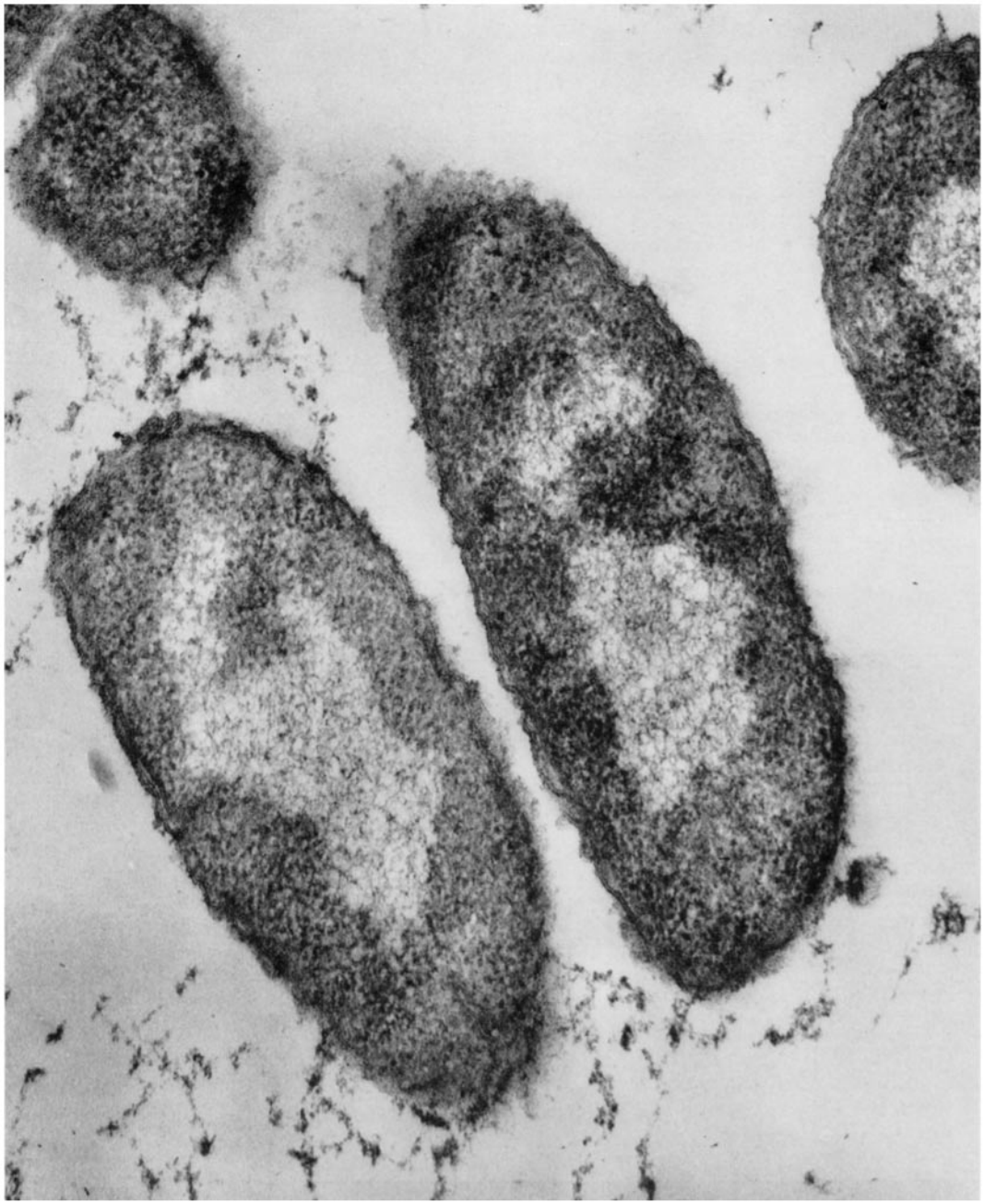


FIGURE 9 *E. coli* grown in tryptone broth, fixed with RK^+ and posttreated with uranyl acetate. The sections had been stained according to Karnovsky. Here the DNA plasm of the nucleus forms a network, and no preferential orientations are visible. Note the "double" structure of cell wall and cytoplasmic membrane. × 90,000.

the organizational pattern of the fixed DNA plasm can be obtained. The following results show that the gel obtained by RK^+ fixation is different from the one obtained by uranyl acetate.

1. The "uranyl gel" is birefringent, but the RK^+ gel never is.

2. The electron micrographs of the gels obtained by uranyl acetate sometimes show an organization in layers.

3. Bacterial nucleoplasms also show a difference in pattern between those fixed by RK^+ fixative and those where the RK^- fixation or any other conventional fixation is followed by a uranyl acetate fixation process. These observations suggest that the uranyl acetate fixation does introduce an artificial order, at least for artificial plasms.

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Note Added in Proof: Further experiments with sonication indicate that DNA's with a sedimentation constant (S^0_{20}) of less than 13 will not form gellable complexes with osmium or uranyl nor will the uranyl complexes exhibit birefringence. More detailed studies will be described elsewhere.

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