HIGH RESOLUTION ELECTRON MICROSCOPE STUDIES OF CHROMOSOMAL FIBERS

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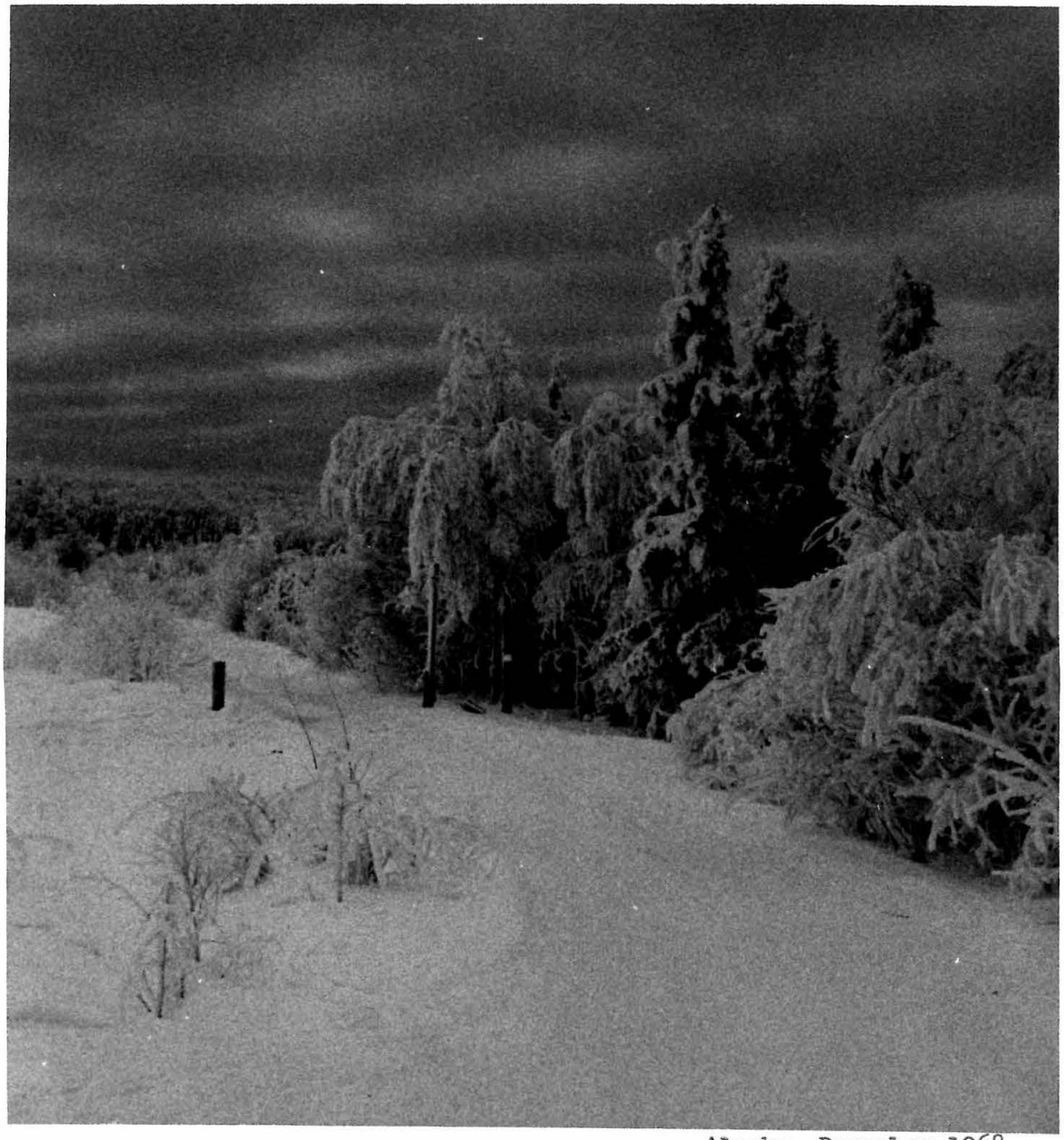

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ABSTRACT

Techniques are described for mounting and visualizing biological macromolecules for high resolution electron microscopy. Standard techniques are included in a discussion of new methods designed to provide the highest structural resolution. Methods are also discussed for handling samples on the grid, for making accurate size measurements at the 20 Å level, and for photographically enhancing image contrast.

The application of these techniques to the study of the binding of DNA polymerase to DNA is described. It is shown that the electron micrographs of this material are in agreement with the model proposed by Dr. Arthur Kornberg. A model is described which locates several active sites on the enzyme.

The chromosomal material of the protozoan tetrahymena has been isolated and characterized by biochemical techniques and by electron microscopy. This material is shown to be typical of chromatin of higher creatures.

Comparison with other chromatins discloses that the genome of tetrahymena is highly template active and has a relatively simple genetic construction.

High resolution electron microscope procedures developed in this work have been combined with standard biochemical techniques to give a comprehensive picture of the structure of interphase chromosome fibers. The distribution of the chromosomal proteins along its DNA is discussed.

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METHODS OF HIGH RESOLUTION ELECTRON MICROSCOPY

I. Introduction

Biology is now in the infancy of a very exciting period of macromolecule structure solving. X-ray diffraction techniques have solved the three dimensional structure of a few simple macromolecules such as myoglobin, lysozyme and hemoglobin. Whether larger molecules can be fruitfully studied by this method remains to be seen. However, molecules which cannot be crystallized cannot be studied by X-ray diffraction. For large molecules and molecules which are not in crystalline environments, electron microscopy is the tool which will give us structural information. High resolution electron microscopy of macromolecules is not new. Visualizing any structural detail but the gross molecule itself, however, is new. With this method, the conformation of the important macromolecules, their significant complexes with each other and the nature of their biologically active regions will be visualized. New techniques for delicately mounting a representative sample of molecules from solution onto the supporting grids will be developed. These molecules will be visualized directly or with highly specific stains or sophisticated shadowing. Focus will be controlled by the machine, and the images will be handled by television systems for direct computer image enhancement. This may be done by taking multiple stereo images of many similar molecules and asking the computer to construct the best model. The model will be projected on a T.V. screen and can be rotated in space for any desired view.

Most of these sophisticated techniques have not been worked out, but the fundamental methods on which they will be based are available.

Computer image enhancement will be a very powerful tool, but on a simple level it can be duplicated by photographic techniques at hand. The door to macromolecule structure solving is open now. Ten angstrom resolution on most macromolecules can be achieved. If the molecules have a very regular structure or crystallize as does catalase, finer detail can be observed. Under ideal conditions, the two strands of DNA can be separately resolved. DNA polymerase from Dr. Kornberg's laboratory at Stanford can be visualized bound to DNA and several problems related to its binding have been answered.

The object of this paper is to describe in a very detailed way the techniques which are unique to this new field of microscopy. Some of these techniques are standard, others are refinements of old methods that have been worked out in this laboratory during a detailed study of chromosomal DNA-protein fibers.

The following presents an overview of the field followed by a detailed description of the techniques. Emphasis has been placed on discussing the appropriate method for different types of samples and the pitfalls of these methods.

II. General

High resolution microscopy holds many problems. The sample must be taken from a free aqueous environment to a flattened, dehydrated state. The macromolecules must be spread out but not sheared on one hand or aggregated on the other. The fine detail of the molecules must be preserved and contrast enhanced with the use of heavy metal shadowing or staining.

Because of their size and delicate structure, most macromolecules are difficult to handle. Typical DNA molecules studied have a length to width ratio of 10,000 to 1. Spreading them without breaking them requires a great deal of finesse. Larger structure such as nucleohistone macrofibers or chromosomes may be so delicate that even the most gentle mounting procedures will cause serious damage.

During mounting, the aqueous phase is removed and the sample deposited on the grid. In this process, local high ionic conditions may occur, and surface tension or other two-phase boundary effects are always present. These short term conditions are difficult to predict or control and may cause damage. Conditions of high salt concentration will remove histone proteins from nucleohistone fibers or DNA polymerase from DNA. Air drying and slow freezing of DNA solutions produce side-by-side aggregates.

The touchstone of mounting is getting the sample to bind to the grid. Poor binding generally requires high concentrations of the sample and leads to aggregation and nonrepresentative selection of molecules in the sample. Streaking chromosomal fibers on untreated carbon grids

selects for long molecules and produces "Christmas tree" aggregates. Good binding assures representative sampling and provides good spreading with the least amount of damage.

Heavy metals must be used to enhance the contrast of the image in the microscope. Achieving the highest resolution requires balancing high contrast produced by large amounts of heavy metal, against good detailing obtained at low amounts of metal while keeping the characteristics of the photographic films in mind.

The purity of the sample and the amount of other biochemical and biophysical data about the sample ultimately determine how much information can be derived from high resolution electron microscopy. Small amounts of nonvolatile salts can mask fine structure and the bits and pieces of debris always present in cell fractionation preparations can completely confuse the interpretation of micrographs. There is no substitute for purity in obtaining unambiguous results.

III. Preparation of Supporting Grids

Preparation of the supporting films is extremely important. At the present time, the inherent roughness of the supporting films limits the resolution obtainable by shadowing or by unstained dark field microscopy. They must be both smooth and "sticky" to provide good spreading, representative selection, and high resolution.

The Philips microscope uses supporting screens 3 mm in diameter to support the films. These are available from Ladd or Pelco (1). For general use, the Pelco copper CG-300 300 mesh grid has been used. Meshes larger than 300 will not support the very thin carbon films and finer meshes are too small for studying long fibers.

Two general types of films are in current use. The first class comprises the plastics, formvar and parlodion. They are formed as a thin film over water, mica, or glass surfaces then stripped off and placed over copper screens. The second type is the pure carbon film formed by vacuum evaporation onto mica.

The sample generally determines which class of film is best. Formvar and parlodion are easy to form and work with and have the advantage of being continuous. Most material adheres well to these films and negative stains spread well on them. Their disadvantages appear in high resolution work. Intense beam currents cause them to melt and move in the field. For a given tensile strength, they are more electron opaque than carbon films. For high resolution shadowing, standard parlodion and formvar films are too rough. This can be circumvented by using the smooth parlodion film discussed below.

Carbon films are the strongest commonly used films. They also afford the best possible contrast. The coefficient of thermal expansion of graphite is close to zero so that there is little film movement in the beam. Carbon films have several disadvantages. They are brittle, hard to work with, and difficulty is often encountered in getting a negative stain to wet the surface and material to stick. When the sample does stick, aggregation is usually a problem. Methods to overcome this handicap are discussed below.

Figure 1 shows how carbon films are prepared. Mica sheets are cleaved with a razor blade or scotch tape. Mica cleaved along molecular planes is believed to provide the smoothest obtainable surface for replication. The mica is immediately placed in a Denton vacuum evaporator or similar device and a vacuum of at least 1×10^{-4} mm Hg obtained. Spectrographically pure 1/8 inch thick, 1 inch long carbon rods with 1/16 inch thick x 3/16 inch long points from Ladd are used. One pointed rod is spring butted against the flattened end of another and part or all of the point evaporated onto the fresh mica surface by passing at least 25 amperes through the rods. In general, evaporation of 1/2 of a point at a distance of eight inches from the mica sheet will produce a carbon film of moderate thickness.

The carbon coated mica sheets are stored in a humid atmosphere. They are placed on wet filter paper pads in covered petri dishes and left in a refrigerator until use. The carbon films may be kept this way for several months. The remaining steps, however, should be done each time grids are needed.

Making Carbon Grids

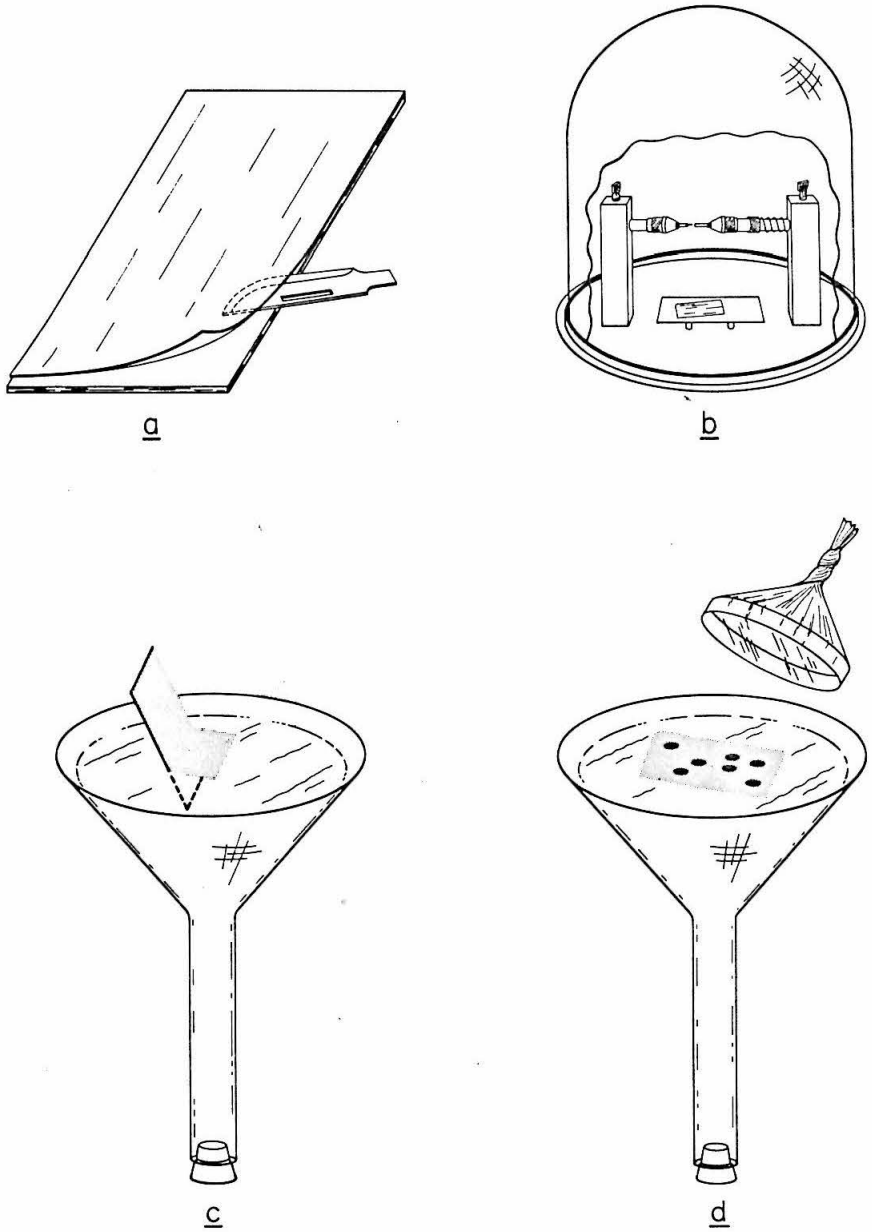


figure 1 (a) Using a razor blade, a sheet of mica is cleaved to obtain a fresh surface. (b) A thin layer of carbon is deposited by vacuum evaporation. (c) After one hour in a humid atmosphere the carbon film is stripped off onto a water trough. (d) Copper screens dipped in adhesive are dropped onto the floating carbon film and rescued with a cellophane-petri dish drum.

The carbon film can be stripped from the mica sheet after it has been in a humid atmosphere for at least one hour. A 1 cm x 3 cm piece is cut from the sheet and lowered at a 45 degree angle, carbon side up, onto the surface of a water trough. The carbon film should remain intact. If it does not, the mica was not properly cleaved or the carbon film is too thin. Very thin films are often difficult to see on the trough. Cold water should be used; condensation on the surface of the carbon makes the very thin film visible. Carbon films will not adhere to copper screens and are often washed off during the mounting steps. To avoid this, grid adhesives are necessary. The adhesive in use now is a 1% solution of polybutene in xylene (2). The copper screens are dipped in this solution, drained off on filter paper then dropped directly onto the carbon film floating on the trough. It is essential to allow at least 30 minutes for adhesion to take place. The grids are removed from the trough with a drum made from Saran wrap and a petri dish. The drum is placed rapidly down on the floating film then lifted up from the water surface edgewise carrying the film and grids with it.

The carbon grids are air dried on the drum then placed back into the evaporator and a 50 micron vacuum obtained. A continuous discharge condition is created by applying a tesla coil across two terminals in the chamber. The grids are left in the discharge for at least fifteen minutes. This final step is very important. It is believed that the bombardment by charged molecules produces chemically active groups on the surface of the film to which molecules will bind. These "ionized grids" must be kept dust-free and the sample mounted within one-half

hour of ionization.

Parlodion films are formed on a water or mica surface. A stock solution of 3% parlodion in amyl acetate is prepared and is stable for several months. In the standard procedure, one drop of the stock solution is placed onto the surface of a water trough. After spreading to form a thin film over the surface, the parlodion polymerizes as the amyl acetate evaporates. This takes one to two minutes. As soon as the film shows a loss of the polymerization rainbow patterns, grids may be dropped onto the film in the manner used for making carbon grids. No adhesive is necessary. The grids are rescued with a Saran wrap drum and ionized before use.

Smoother parlodion films are produced by a combination of the carbon and parlodion techniques. A freshly cleaved mica surface is wetted with a parlodion solution and then set on edge to drain and allowed to dry. Carbon may be evaporated on this for strength. The parlodion or carbon-parlodion film is then handled in the same manner as the carbon films. Selection of the concentration of parlodion is determined by the thinness of the film required. A thin self-supporting film is produced by using 0.3% parlodion and backing it with a small layer of carbon for strength. A 1.0% parlodion film is self-supporting (Fig. 5a).

Formvar films are normally made by wetting an acid-cleaned glass microscope slide with a 0.5% solution of formvar in ethylene dichloride. The slide is tipped on edge to drain and air dried 30 minutes. The film is stripped off onto a water trough, and handled in the same manner as

the other films. If trouble is encountered in stripping, the coated slides may be steamed briefly. It is important to scrape the formvar from the edges of the glass slide prior to stripping.

Formvar or parlodion grids containing many small holes are commonly used to correct microscope stigmatism. These may also be used as extreme fine-mesh grids to support extraordinarily thin carbon films, or to support material to be observed with no supporting film such as collagen rods laid across a hole. Films containing many small holes may be made using a 1% emulsion of glycerol in 0.5% formvar or 3.0% parlodion solutions.

A DuPont polymer, paralene (3) has been tested. This is formed by thermal cracking and repolymerization on a surface in a vacuum. The plastic does not appear useful as a simple substitute for parlodion or carbon. However, its ability to be formed over complex surfaces could be extremely useful in replication of large surfaces.

IV. Mounting Techniques

Mounting may be very easy or extremely difficult depending on the sample. For example T^4 phage are mounted successfully by almost any haphazard method whereas mounting chromosomes requires a very delicate technology. Some of the factors determining the procedure used are the size and shape, the tendency to aggregate, and the vulnerability to physical and chemical damage. Often the experiment itself will dictate the procedure to be used. Quite different methods are used to make width measurements or length measurements on DNA molecules. It is important to have a variety of mounting methods at hand. This provides the best micrographic data and ensures that a result is not a unique artifact of one particular mounting method.

The simplest and most commonly used mounting procedure is streaking. A drop of solution containing the sample is placed on a prepared grid then drawn off by touching it to the edge of a piece of filter paper. The remaining film of liquid is air dried or dehydrated in ethanol. The exact details of this method must be worked out for each sample. If the molecules do not stick readily to the grid, several streakings may be used. If the solution contains nonvolatile components such as sucrose, glycerol or NaCl, one or two water washes may be necessary. Washing is done in the same manner as streaking.

For proper streaking, the grid must be hydrophilic so that a thin film is formed on the grid when the excess solution is removed. If the surface does not wet properly, the drop will appear to roll off as a

bead. In this case the grids should be ionized. Sometimes breathing on the grids will make them wet properly.

Streaking works for most any sized molecule or structure provided that it is rugged enough to stand the rather violent surface tension and shearing forces. Molecules which aggregate readily will do so unless they stick very well to the grid. Chromatin fibers or DNA should not be streaked (Fig. 2a) as they will form side-by-side or Christmas tree aggregates and are broken by the shear forces. Only in the few cases where it is necessary to line up the DNA molecules should this be used. Phage particles, hemoglobin, ferritin and ribosomes are all successfully mounted by this procedure. Solutions of 10 µg/ml to 100 µg/ml of protein are streaked once across parlodion grids, washed once and air dried.

A less commonly used mounting procedure is spraying. In this technique, the solution is placed in an atomizer which is used to spray fine droplets onto the grid. The object is to cover the grid with single unmerged drops whose original extent can be observed by the material left behind after drying. The grid is air dried. This has no advantage over streaking, except when used to determine the concentration of particles in the solution. In a typical experiment, a solution of phage is assayed for infective titer by plaque formation and then a known concentration of 880 Å diameter polystyrene spheres is added and the mixture is sprayed on a grid. The grids are shadowed, and a count of phage particle per polystyrene sphere per drop is made. This gives a

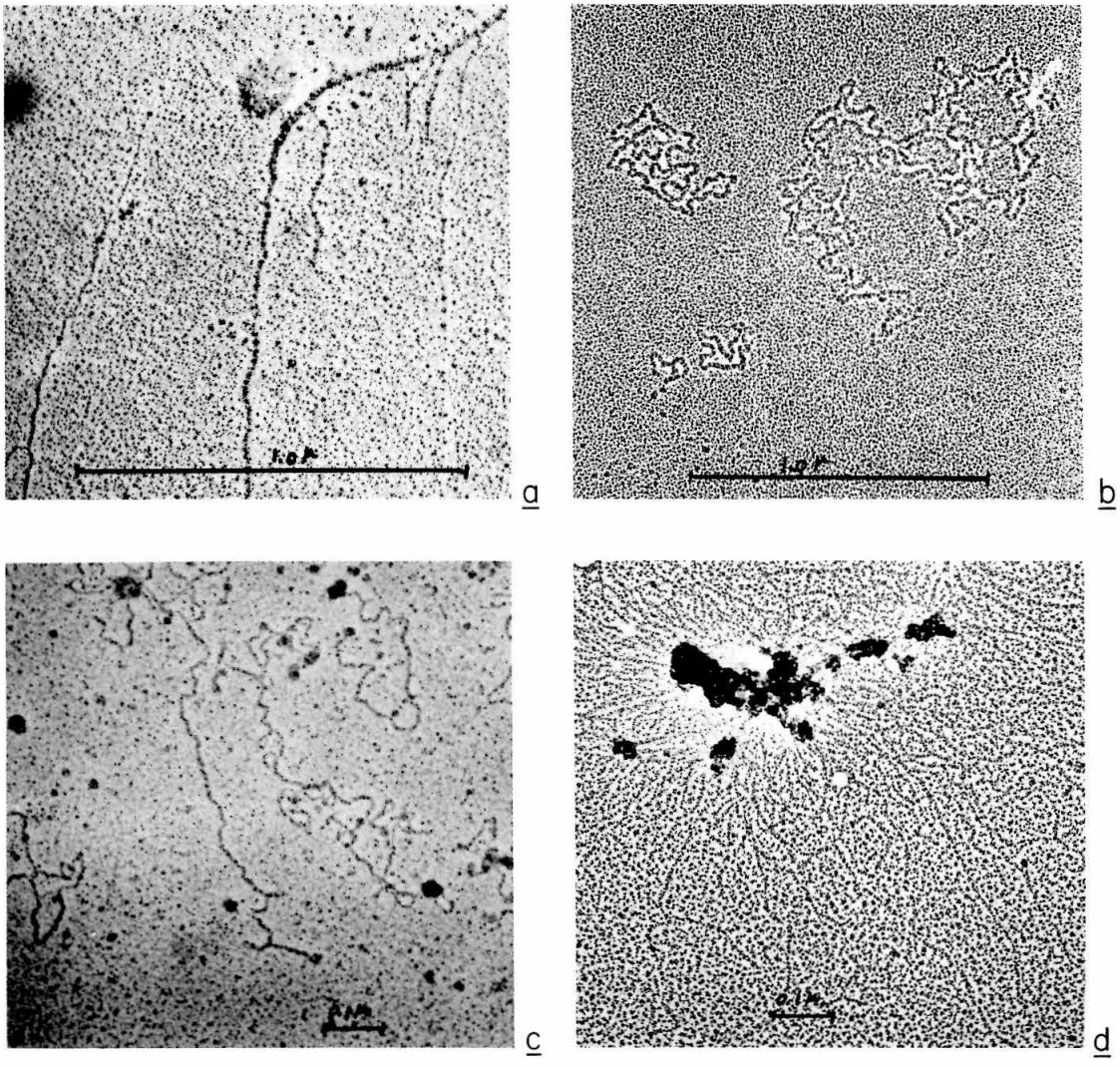


figure 2. Chromosomal fibers mounted by (a) streaking, (b) freeze drying, (c) rapid ethanol immersion, (d) on-grid lysis of nuclei.

measure of infective to total phage particles. Spraying may be combined with freeze drying if the material is disrupted by liquid drying effects.

Two methods are available for depositing the sample on the grid as a liquid and dehydrating with the least damage due to shearing and surface tension forces. In the first method, devised here for chromosomal fibers, dehydration is done by direct immersion into 95% ethanol. The second procedure is a modification of the Anderson critical point drying method in which dehydration is carried out step-wise from 25% ethanol to 100% ethanol.

In the first technique, rapid ethanol immersion, the most critical step is the actual grid preparation. For small structures or fibers, freshly prepared, ionized, thin carbon grids must be used. A drop of solution containing the sample in 0.005 M ammonium acetate is placed on a sheet of Saran wrap. The grid is floated face down on the drop for 10 minutes. During this time, the molecules bind to the active grid surface. The grid is then lifted from the drop carrying a droplet with it and plunged face down into a trough of 95% ethanol. In this step one should avoid streaking the water off, but rather to try to form an ethanol-water interface which will collapse evenly onto the grid. Following one minute in the ethanol, the excess liquid is removed with the edge of a piece of filter paper and the sample air dried.

This procedure has been used for most all the work done by the author on nucleic acid-protein complexes. It is simple and reproducible as long as strict attention is paid to using fresh grids and working

rapidly after ionization. Grids should be used within 15 to 30 minutes of ionization. This method is recommended for all high resolution shadowing studies and any DNA preparations where cytochrome-C spreading cannot be used. It is highly useful for any sample where shearing and ionic forces must be avoided. Results obtained by this method appear similar to those obtained by the much more tedious method of freeze drying (see Fig. 2b,c). When done properly, the material left on the grid is probably a good representation of the material in the solution.

The Anderson critical point drying method has been extensively used by E. J. DuPraw (4, 5). This procedure has been simplified by Dr. John Hearst and Dr. Robert Cummings for chromosome studies. In this method, the sample, normally a large structure such as a chromosome is placed on a carbon or formvar grid in a drop of buffer. The grid is then taken through the following concentrations of ethanol: 25%, 35%, 50%, 70%, 95%, 100%. At each step the grid is simply immersed in a small beaker of the solution for five minutes. Following the 100% ethanol step, the sample is placed in amyl acetate for five minutes. In the critical point drying method, the amyl acetate is replaced with liquid CO_2 under pressure and then passed through the critical point of CO_2 for drying. Hearst and Cummings have eliminated the last step; the sample is simply air dried following five minutes in amyl acetate.

This procedure is almost as simple as rapid ethanol immersion and for any sample should be compared with that procedure to determine which gives the best results. In general the latter procedure is best suited for preserving detail in structures such as chromosomes.

A review of the mounting procedures used for protein-nucleic acid complexes indicated that an extremely gentle method was needed. One attractive method that had been used with some success was freeze drying.

In concept, freeze drying is quite simple. An aqueous solution of the sample and a volatile buffer such as ammonium acetate is spread across the surface of a mica sheet and rapidly frozen. In a vacuum, the water and salts are removed by sublimation. The sample is visualized by a replica technique. The mica sheet with the molecules is first shadowed with a heavy metal, then covered with a layer of carbon also by evaporation. The mica-metal-carbon sandwich is placed in a warm, humid atmosphere for 1 hour and the carbon plus metal film stripped from the mica in a water trough. Grids are made as if it were a simple carbon film.

In most of the earlier procedures, a freshly cleaved mica sheet was simply dipped into the solution, plunged into liquid nitrogen, and then transferred in the nitrogen to a dessicator. After 3 to 12 hours, the mica sheet was transferred to an evaporator for the shadowing steps.

From an examination of the results obtained by this method, it was clear that there were two serious limitations. Whereas ideally there should be little loss of sample molecules from the mica during the dessication and shadowing steps, it was found that 99%-99.9% of the sample placed on the mica was lost. Secondly it was clear that often the freezing was far too slow, producing aggregation (Fig. 4c).

The apparatus and procedure described below successfully overcomes these problems and has been found to produce routinely good results.

It was found that most of the sample loss occurred during the steps between dessication and shadowing. To eliminate this loss, these steps have been combined into one operation. Furthermore, more careful attention to the details of freezing has reduced the amount of freezing artifacts.

The apparatus is shown in Fig. 3. A cup with screen and retaining ring for holding the mica sheets, is held in the evaporator by a holder and transferred from a styrofoam insulated can with a cup holder. The cup is made so that the mica sheet with frozen sample may be kept immersed in liquid nitrogen until it is in the evaporator and evacuation has begun. For this, the cup has a set of wire screens which hold the mica at variable distances below the lip of the cup.

The apparatus is used in the following manner. A drop of solution is spread over a thin sheet of freshly cleaved mica. The solution is then frozen in one of the manners to be described below. Under liquid nitrogen, the mica with frozen sample is placed in the cup and secured by the retaining ring. The cup has been previously placed in the insulated can filled with liquid nitrogen. The cup is rapidly transferred from the can to the evaporator with the holder and evacuation begun.

The mass of the cup provides the desired slow rate of warming over a period of 3 to 4 hours. The mica sheet and sample are rotary

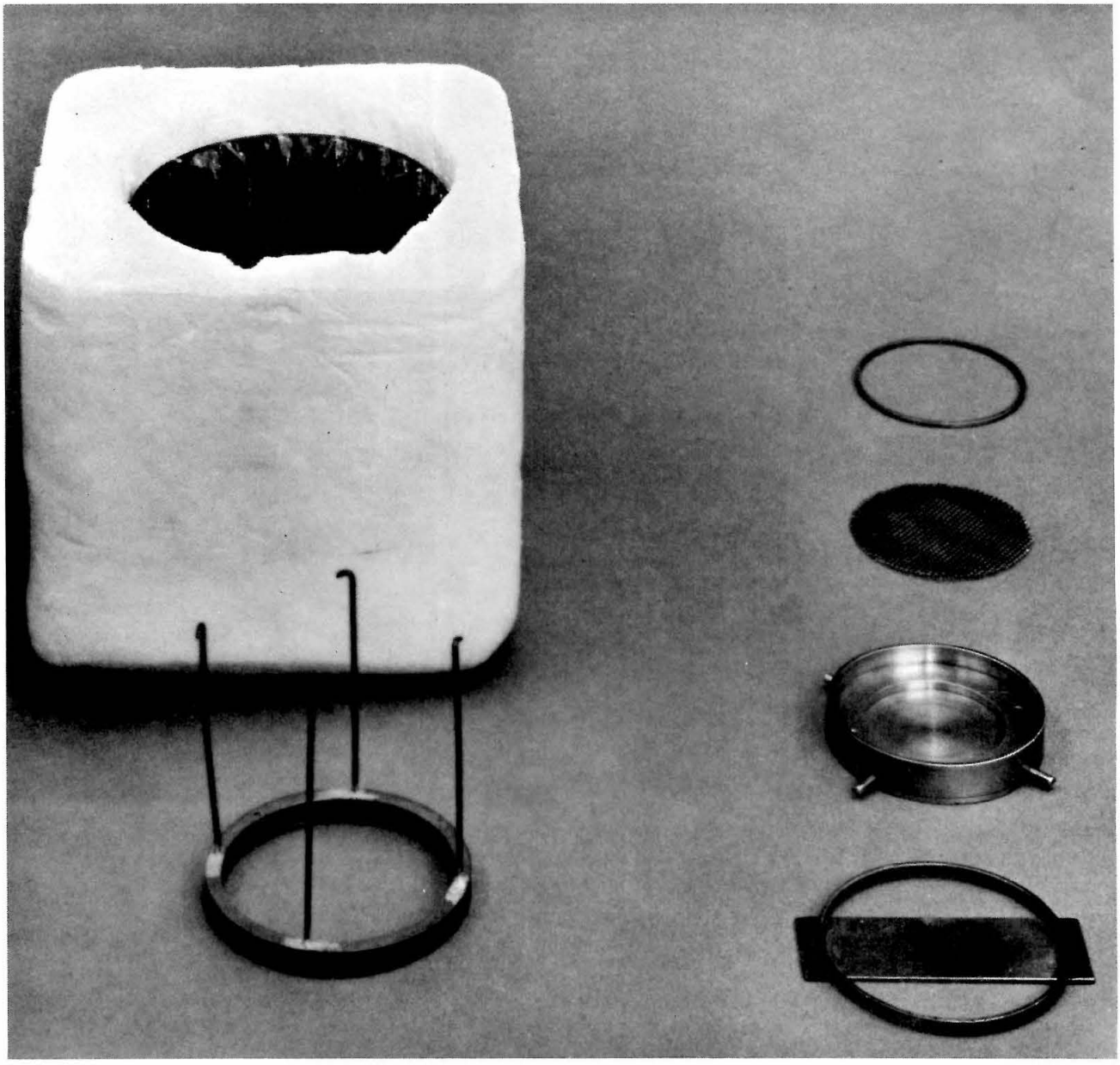


figure 3 Freeze-dry apparatus

shadowed and carbon coated using 2 sets of electrodes. The mica-metal-carbon sheets are handled in the manner described in the first procedure. It is important to work as rapidly as possible so that the sample remains immersed in liquid nitrogen until evacuation has begun.

Freezing may be done by several different methods. In the simplest, the solution is spread on the mica and the mica plunged into the liquid nitrogen in the cup. Due to the poor thermal conductivity of liquid nitrogen, direct immersion provides a relatively slow freezing rate. A faster rate can be achieved by rapidly placing the mica sheet on a polished silver or copper block which is chilled in liquid nitrogen and then immersing it in the nitrogen filled cup. The block must be kept frost-free. Even faster freezing should be achieved using isopentane chilled in liquid nitrogen. All isopentanes tried have proven to contain too many nonvolatile components to be satisfactory. Lower temperatures and faster freezing rates than those produced by liquid nitrogen are available. It is the author's belief, however, that with thin films of solutions on mica, that the freezing rates produced by liquid nitrogen-chilled silver blocks are sufficient to produce a glass and that the most important consideration is producing even cooling over the mica sheet. Uneven cooling forms moving freezing interfaces which will cause aggregation.

Freeze drying done in this manner is a true replica technique. The macromolecules, unless very large, remain on the mica after stripping. Only the metal-carbon film is removed. This was demonstrated by stripping the films onto uranyl acetate or phosphotungstic acid solutions

followed by water washes. In regions where the shadow clearly showed the former presence of DNA or protein molecules, no DNA or protein specific stain was detected. It would have been visible had the molecules been present in the stripped film. Clearly, this method is not suitable for shadow-plus-stain techniques, for some variations of electron microscope autoradiography or suitable for the highest resolution shadowing in which a very small amount of tungsten is used.

If these limitations make this method unusable, the freeze drying may be done directly on the grids. Medium thickness carbon films should be used. The grids are handled as mica sheets, but carried only to the metal shadowing step. This method has the added advantage of allowing more samples to be done at once. Chromosomal fibers freeze dried in the replica method is shown in Figure 2b.

The use of a protein surface film for spreading DNA was first investigated by Kleinschmidt and Zahn (6). Since then many variations of this procedure have been developed (7).

The normal procedure involves forming a protein-DNA complex on the surface of a water trough. A drop of a solution of DNA and protein, usually cytochrome-C, in a high enough salt concentration to keep the two components disassociated is layered over the surface of a water trough buffered at a lower ionic strength. It is believed that the protein surface denatures and binds to the DNA. The binding performs two roles: it spreads the DNA out over the surface and it increases

the apparent fiber width from 15 angstroms to about 150 angstroms. This makes its visualization by shadowing very easy.

Although this technique is normally used for visualizing long double-stranded DNA's, variations may be useful for DNA-protein complexes. A study of the removal of histone proteins from DNA was carried out by a modification of this technique in which the salt concentration in the cytochrome-C-DNA mixture was varied over the range in which histones are removed from DNA. Thick segments along barely visible fibers suggested regions of DNA bare of histone proteins. Surface spreading with formamide has been recently developed for spreading single-stranded regions of DNA or RNA. These techniques have been reviewed by Ron Davis (8).

Investigations of the structure of the nucleus and the large chromosomal fibers required very gentle mounting techniques. To this end, a new methodology was devised for handling organelles such as nuclei directly on the grid. By these methods, it is possible to lyse nuclei on the grid and to spread out the entire nuclear contents (Fig. 2d). It is also possible to treat the nuclei spread on the grid with various chemical agents. These methods have been extremely useful in the study of the chromosomal material as it occurs in the nucleus. The same procedures can be applied to other large structures. Mitochondria can be broken open and studied. This method would be useful in studies

of: photosynthetic bacterial chromatophores, chromosome superstructure, or viral infection.

For large organelles which are visible in the light microscope, a phase contrast microscope can be used to great advantage in monitoring the mounting steps. In a typical nuclear lysis experiment, nuclei are prepared in the normal manner and suspended in a buffered 25% glycerol medium. Carbon or carbon-parlodion grids (200 mesh) are prepared in the normal way. Glass needles are made from 2 mm glass tubing. Several grids are placed on a sheet of dental wax and a glass needle is used to hold the grid while 5λ to 20λ of distilled water is placed on the grid with a micropipette. A glass needle is dipped into the suspension of nuclei and drawn through the drop on the grid. The number of nuclei placed on the grid in this way and time required for lysis is determined with the phase contrast light microscope. Following lysis, a drop of 95% ethanol is dropped onto the grid or the grid is immersed in a dish of 95% ethanol. The grids are shadowed or stained.

This method is extremely useful when combined with chemical treatments. For selective disruption of the nuclear contents, the distilled water may be replaced by 0.2 N H_2SO_4, 0.1% SDS to 1% SDS in distilled water or solutions of Na DOC.

V. Visualization Techniques

Present day high resolution microscopy requires that the electron scattering power of the sample against the background of the supporting film be multiplied through the use of heavy metals. This is done either by heavy metal staining from solution or by heavy metal shadowing.

To stain a sample, as in light microscopy, an opaque "dye" is bound to the sample. There are few stains available to electron microscopists and they are much less specific than those used in light microscopy. In addition, they lack the distinguishing dimension of color.

Heavy metal shadowing provides a method for visualizing the surface of the film bearing the sample. A metal such as uranium is evaporated in a vacuum and at a low angle directed as a beam onto the sample. Because of the low angle of incidence and the elevation of the sample above the flat, smooth, supporting film, the sample collects more metal than an equivalent area of the film. Figure 1 shows a typical apparatus. The sample is usually separated from the source by at least 8 cm, and angles of between 1:5 and 1:10: (vertical:horizontal) are used. The sample may be held fixed or rotated in the plane of the grid during evaporation. Generally, rotary or multiple angle shadowing is used to provide the most easily interpreted micrographs.

Metal shadowing is not a simple technique, and the resolution achieved by this method depends on many variables. It is highly dependent on the angle of shadowing, on the amount of metal deposited, on the smoothness of the supporting film, and on the particular metal used.

In addition, high sample contrast is achieved at the expense of high resolution. The interplay of these factors will be discussed later. Care must be made in selecting the particular metal, supporting film, angle and level of contrast to be used for a given sample.

Metals Platinum - Palladium

A very commonly used metal for shadowing is an alloy of 80% platinum, 20% palladium. The palladium is present to reduce alloying of the platinum to the tungsten heating rod.

In the standard procedure, a 30 mil tungsten wire is formed into a 3 cm rod with a small v in the center. About 3 cm of 1 mil platinum-palladium wire is wrapped about the v. After a vacuum of at least 1×10^{-5} mm of Hg is obtained, a current of 32 amps is passed through the 3 cm 30 mil tungsten wire. This will just melt the Pt-Pd wire which then forms a bead at the bottom of the v. For best results the bead is evaporated as slowly as possible over at least 1 to 2 minutes. Too rapid an evaporation produces large metal grain and breaks the tungsten wire.

Platinum

Platinum is most useful in the form of a 1 mil wire. Because pure platinum will readily form an alloy with tungsten, when used in the v method the high concentration of platinum in the v will form enough platinum-tungsten alloy to cause the wire to break from heating. To avoid this, a 3 cm piece of 1 mil platinum wire is wrapped tightly

around a 1 cm segment of a straight 3 cm, 30 mil tungsten wire. The wire is heated to the minimum temperature for melting the platinum (about 32 amp) and the current is then slowly increased over a 5 minute period to 36 amp. This procedure produces the finest detailing possible with platinum and has the advantage of being highly reproducible. Pure platinum provides the greatest contrast of any of the easily used metals. For this reason, when high contrast, reproducibility, and reasonably fine grain are desired this procedure should be used. It is the author's belief that this is definitely superior to the platinum-palladium v method.

Uranium

In this laboratory we have had little success with uranium. It should, in theory, provide very high contrast. However, because it is obtained in the form of uranium oxide, which is a soft flaky material, it must be evaporated in tungsten wire baskets. Evaporation in this manner is very difficult to control.

Tungsten Shadowing

Tungsten shadowing has been used by a number of workers. It unquestionably gives the finest detail of any heavy metal, but provides poorer contrast and is more difficult to control during evaporation than platinum.

In the procedure used by the author, a 3 cm (electrode separation) length of 20 mil pure tungsten wire from Ladd, is heated to a

predetermined temperature which produces the following three phases: (a) a 2-3 minute heating phase during which little or no evaporation occurs, followed by (b) a 5-10 minute period of slow evaporation and finally (c) rapid evaporation and breakage of the wire. In our apparatus this condition is obtained by passing exactly 32 amperes through the wire. Once the amperage is set it should not be changed. As soon as phase "b" begins, the amperage will begin a slow drop. This may be used to calibrate the relative amount of metal evaporated. In our evaporator, a drop from 32 amp to 31.5 amp corresponds to a light shadow, from 32 to 30-31 amp a medium to heavy shadow and at 29 amps the wire breaks.

The fine detail provided by tungsten shadowing is probably due to a number of effects. Because of its very high melting temperature, it is less likely that beads of metal leave the wire as is supposed to occur with platinum. The metal is highly reactive, so that tungsten to sample bonding may occur. Finally because of the high melting temperature, there is little chance of the electron beam itself melting and puddling the metal on the film.

Tungsten evaporation with a 20 mil wire may undesirably overheat the sample. In this case, smaller wires and shadowing aperatures should be used.

Due to the roughness of standard parlodion, resolutions better than 50 Å cannot be obtained on this film. Smooth parlodion or carbon should always be used for high resolution shadowing. A shadowing angle

of 1:8 is a good compromise. At smaller angles the contrast may be better but the metal will also pile up on itself on the background.

Stains

At the present time staining is limited by the few stains available. Only one negative stain, phosphotungstic acid, is used. Except for the base specific stains of Dr. Michael Beer (9) none of the positive stains are very specific. Uranyl acetate, tantallum chloride, phosphotungstic acid and osmium tetraoxide are all currently used, but most work is done with uranyl acetate. This is an area where technological advance is needed.

Care must be taken staining structures which are disrupted by high ionic conditions. Many DNA-protein complexes are disrupted by normal staining procedures.

A 1% to 2% aqueous solution of phosphotungstic acid is used for negative staining. Over a period of a minute the amount of stain deposited on the grid by a drop placed on it is roughly proportional to the time the drop is left on the grid. The stain is then removed with the edge of a piece of filter paper. Excess stain is removed with a quick streaking wash.

Uranyl acetate as a positive stain is normally used as a 1% aqueous solution. The nonaqueous method of Wetmur (10), with a saturated solution in acidic 95% ethanol, is very useful for nucleic acid complexes.

Any of the mounting procedures except freeze drying may be used prior to staining. The staining solution should always be removed from the grid in the same manner as the sample drop.

VI. Achieving the Highest Resolution

Achieving the highest resolution means producing the greatest information transfer from the sample to an electron image, usually a photographic print. This is not a simple matter. The contrast of the sample over the background must be enhanced to a level at which the finest resolvable detail will be recorded on the film. Yet contrast is always gained at the expense of resolution.

There are several levels of imaging at which the contrast, resolution and information transfer can be discussed.

Imaging Levels

1) level of the sample
2) level of the magnified electron beam
3) level of the developed film
4) level of the developed photographic print
5) level of the fluorescent screen or T.V. camera-screen

The potential resolution differs from level to level. Obtaining the best resolution at one level does not mean simply maximizing the potential resolution at each preceding level. The best resolution on a photographic print is achieved by maximizing the information transferred to the developed negative. This, however, requires balancing maximum contrast at the sample level produced by large amounts of heavy metal and low accelerating voltages plus small apertures at the beam level against high resolution and detailing at the sample level (small amounts of metal) and high voltages and large apertures at the beam level.

The T.V. or fluorescent screens both require a much greater level of beam contrast to produce an image than does film. Producing the best resolution image on the screen requires sacrificing much more resolution than is necessary for photographic images. The contrast-resolution balance and information transfer for each level will be discussed to make the general procedures meaningful.

The best resolution at the level of the sample is achieved by using no heavy metal enhancement. This, however, gives little information transfer to the next level. At the present time, the best total resolution is achieved in the transmission mode of microscopy with the use of heavy metal contrast enhancement or negative staining. For most structural studies this is done by rotary shadowing with tungsten. Any metal deposition on the sample will cover up some detail, and the greater the amount of metal (contrast) the less potentially resolvable detail will be present. It is important to use the absolute minimum amount of metal necessary to transfer the finest possible detail to the level of the developed film. Contrast may be gained in the succeeding steps. Contrast may be gained by using the most advantageous combination of accelerating voltages and apertures and highly contrast-sensitive film. The aim of contrast enhancement should be achieving the greatest information transfer.

The contrast of the image ultimately depends on the number of scattered electrons relative to the total magnified beam. Because the atomic scattering cross sections increase dramatically with decreasing electron energy, the inherent contrast in a sample will be much greater

at 20 K.V. than at 100 K.V. accelerating voltage. However, the theoretical resolving power is a function of accelerating potential also and decreases markedly with decreasing potential. For the Philips 300 for example, the best resolving power of 3 Å is obtained at 100 K.V. and decreases to perhaps 15 Å at 20 K.V.

The resolving power and contrast at any particular voltage depends on the objective apertures used. Large apertures transmit more information hence provide better resolving power than small apertures. Small apertures provide better contrast but poorer resolution and illumination.

To obtain the best information transfer from the sample to the film, these factors must be taken into account. If 10 angstrom resolution is all that is required, then the combination of accelerating voltage and apertures providing the best contrast at a resolving power of 10 Å should be used. In general, tungsten shadowing is capable of at best 8 Å resolution, and potentials in the range of 60 K.V. to 80 K.V. are most useful.

High resolution-high contrast sensitive films directly exposed by the electron beam are at present the best method of transferring information from the beam to a photographic image. In the future beam intensifiers and high resolution T.V. scanners coupled to computers may provide a better system.

The minimum contrast differences to which the photographic emulsion is sensitive sets the minimum contrast level which must be achieved by sample contrast enhancement and beam contrast control. Below this

level, potentially resolvable information will not be transferred to the film, and above this level, useful information will have been sacrificed to gain extra contrast.

Most of the films used for microscopy are very similar. Since developing is a contrast enhancing step, this should be treated with great care. In general the manufacturers recommendations should be followed. Continuous rather than intermittent agitation may significantly increase the contrast, and poor agitation can produce extremely flat negatives.

To obtain the best resolution, one strives for the maximum information transfer to the level of the developed film. If such a high information content negative is printed directly onto photographic paper, most of the information will be lost. The image on the film is a very low contrast image. Photographers refer to this as a flat negative. The information is concentrated in a small region of the density and contrast ranges to which the film is sensitive. Photographic films are much more sensitive to contrast and density differences than are photographic papers. If, for example, over a particular density range, a developed film is capable of resolving 100 levels of difference in intensity, a photographic paper may, over that range on the film, be capable of resolving only 10 levels of film density. If only 10 levels of intensity or density differences were required to transfer all of the information onto the film, no loss of information will occur on printing the image onto the photographic paper. If, however, all 100 levels were required, a 90% loss of information may occur on direct printing.

The solution to this problem requires spreading the information on the film over a greater density range so that enough paper density ranges are covered to provide the maximum information transfer to the paper.

Generally what is done is to increase the contrast in the electron image. At extreme contrast levels of shadowing, an image is produced which appears equally sharp on the photographic negative, print and on the fluorescent screen. Except for a gain in contrast this procedure is just as bad, in terms of information transfer, as directly printing the high information-low contrast negative. The proper solution is to begin with a high information content negative and to expand the range of film density over which the information is stored. This is most easily done by printing the negative onto a high contrast film. The center of the gamma curve of the second generation film is placed in the center of the density range over which the information lies in the original negative. Printing in this manner from film to film preserves the information but expands it over a greater range of film density. After one or more expansions, the second or third generation film is printed on a normal to low contrast grade paper.

The procedure used here is to print the original negatives onto 4 x 5 sheet film. We use DuPont Ortho-A-Litho, and develop in Kodak D-11 developer. One yellow safelight, 10 feet distant is used to monitor development and the film is kept shielded from it most of the time. After one or two expansions, the final film is printed onto a Kodak or Agfa 2 or 3 grade paper. An example is shown in Figure 5b.

Achieving the highest resolution requires a careful balance of the factors discussed above. In addition there are a plethora of practical problems related to machine operation. The high magnification and low illumination and contrast make precise focus and astigmatism adjustments difficult. The inevitable sample damage and contamination due to heating in the beam require rapid operation for the best results.

VII. Practical Problems

The motive for writing a detailed account of methodology in a technical field such as electron microscopy is to transmit knowledge gained through practical experience to people less experienced in the field. One of the marks which distinguishes an experienced microscopist from the learner is his ability to recognize artifacts. An artifact is something which looks like what you want the sample to look like, but which isn't the sample.

For this reason, a description of new techniques is not complete without a warning as to their pitfalls. In the hope that this may save time for others, a brief description of some of the major artifacts of these techniques follows.

The supporting films have real surface features which will be visualized by high resolution shadowing or negative staining. Both formvar and carbon films are replica surfaces. Glass always has many grooves and scratches which are replicated on the formvar surface and may, under staining or shadowing, appear as fibers. Fortunately, formvar is seldom used for high resolution microscopy. Carbon however is used, and mica surfaces,though smooth, will always show a pebbly granularity of about 5 Å size. Besides this, cleavage lines where the surface jumps from one plane to another are always present and may appear like DNA fibers. These may be straight or curved and appear as one line or several intertwined (Fig. 4d).

Parlodion formed on a water surface will always show a rippled or grooved appearance. Because these micro-grooves are often

indistinguishable from DNA fibers, and are often long, parlodion formed on water should not be used as a supporting film for high resolution microscopy of nucleic acids.

All mounting procedures produce some undesirable effects on the samples. The Christmas tree aggregates produced by streaking DNA fibers are common and are easily recognized. Freeze drying, however, produces aggregates which can be much more deceptive. If the freezing rate is too slow, the growing ice crystals exclude the salt and macromolecules from the ice. When several interfaces merge, the molecules are forced together. An example of this effect on chromosomal fibers is shown in Figure 4c.

Shadowed freeze dried preparations always show a number of small circles distinguished by their perfect circularity. These are presumably nonvolatile salt rings.

Fixed angle shadowing images are always carefully interpreted. Rotary shadowed images however are often unskeptically taken as perfect three dimensional surface representations without regard to shadowing effects.

The most troublesome shadowing effect may be illustrated by rotary shadowing tobacco mosaic virus, rods of 150 Angstroms diameter. By the nature of the process, the center of the rod accumulates less metal than the sides. The image appears as two smaller rods lying side by side. Normally this is easily recognized for what it is. In cases where there is possible confusion, stereo pairs will immediately

distinguish the two possibilities. When investigating new samples, stereo micrographs are invaluable.

The comment has been made by a well known microscopist that there probably have been more proteins "discovered" by phosphotungstic acid and uranyl acetate staining than could ever be coded for by all the possible structural genes. Small globular proteins appear as clear circles when negatively stained with PTA. PTA alone on any of the supporting films will form myriads of small circular clear areas. This is probably due to incomplete wetting of the surface or bubbling.

Uranyl acetate used as a positive stain presents less trouble in interpretation than PTA. It has been shown by the author, however, that under certain conditions uranyl acetate will form polymerized aggregates of varying size, shape and electron density (11). In earlier work on chromosomal fibers, several authors mistook these artifacts for chromosomal material. They have also appeared in many other published works (12, 13).

The large tubular structures shown in Figure 4a,b form in aqueous solutions of uranyl acetate at neutral pH. They are an aging phenomena and are seldom present in 1-week-old solutions. Their formation is stimulated by salts such as NaCl or CsCl and is inhibited by acid pH. To avoid these artifacts, one should use fresh solutions at acid pH or the acid, nonaqueous method described by Wetmur (10).

It has been assumed above that the microscope itself will be used under optimal conditions. This means no imperfect focus, astigmatism or blurr. Low magnification microscopy with high contrast objects

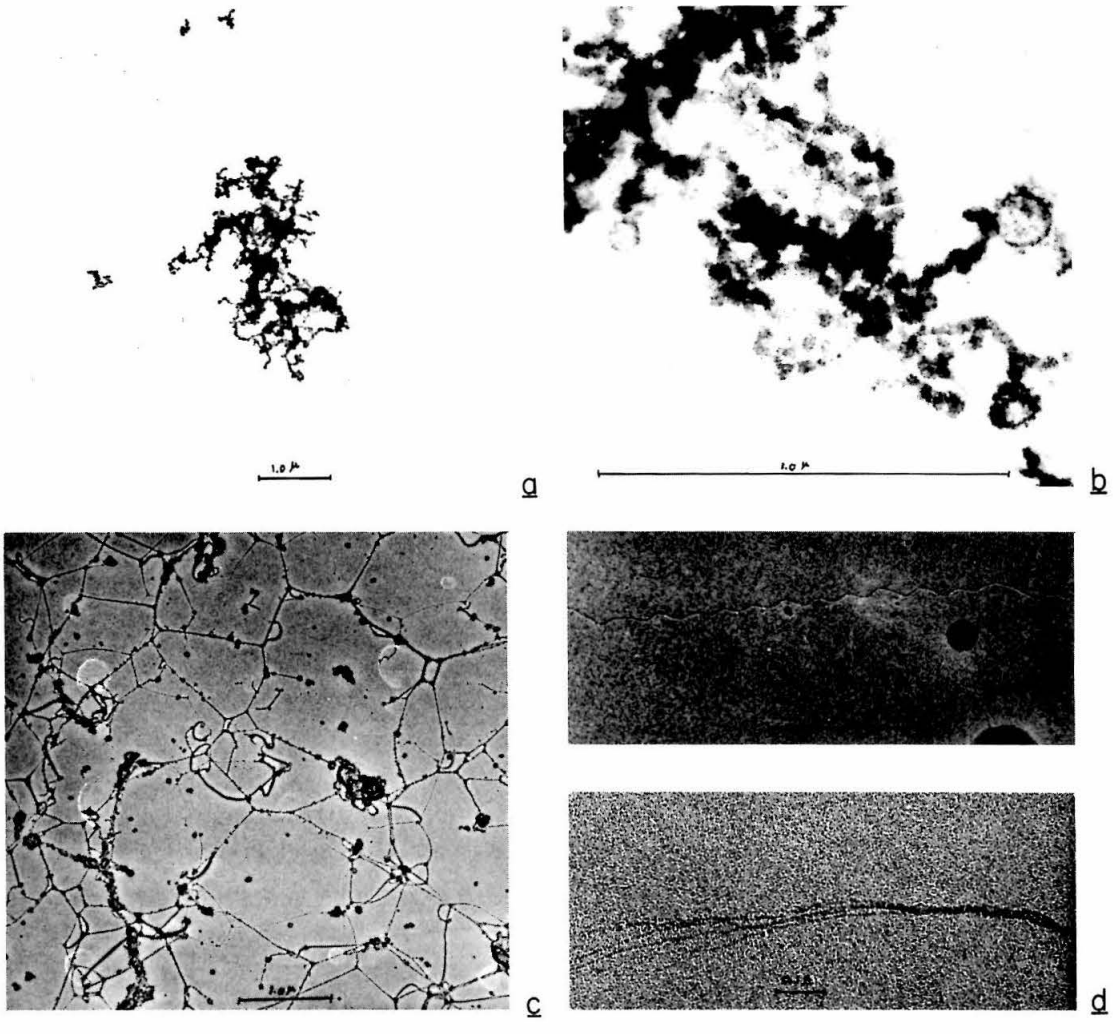


figure 4. Common Artifacts. (a, b) Uranyl acetate complexes from aged aqueous solutions at pH 7. Direct visualization. (c) The effect of slow freezing a solution of nucleohistone fibers. Rotary shadowed. (d) Carbon replicas of mica cleavage lines visualized by shadowing.

such as cytochrome spread DNA is seldom bothered by these problems. With such objects, good focus and stigmatism are easily recognized on the screen. Because such micrographs are seldom printed at a magnification higher than that observed in the viewing screen, if an object looks good in the screen it will look sharp on the print.

High resolution microscopy, however, requires high machine magnification, up to thirtyfold photographic enlargements and low contrast.

The dim illumination of high magnification and low sample contrast result in an image that is at best barely visible in the magnified fluorescent screen. The fine detail cannot be seen but is brought out by photographic enlargement and contrast enhancement. Enlargement and enhancement also magnify out-of-focus, astigmatic, and blurr effects which were not obvious in the image on the fluorescent screen. These effects are very apparent in the 35 mm negatives when studied in a low power viewing microscope.

Because of these difficulties, the greatest care must be taken in focusing and correcting stigmatisms. Several through-focus micrographs should be taken.

Out-of-focus images show an apparently greater contrast than in-focus ones, but the resolution is greatly reduced. Out-of-focus images are easily recognized. Poorly corrected stigmatic images can be more deceptive. In such an uneven magnetic field, a circular string-of-pearls molecule may appear to be composed of helical elements whose pitch angle varies along the strand but is always the same in relation to the whole field.

VIII. Determination of Physical Parameters

Dimensions on a micrograph are determined by comparing it with a similar micrograph of a ruled grating. This is sufficient for most subjects. The development of the technique for mounting single chromosomal fibers and high resolution shadowing has emphasized the need for more accurate methods of measuring very small dimensions such as these fiber widths. The method described below was devised for accurately measuring the widths of DNA and nucleohistone fibers which range from 15 Å to 30 Å.

This method uses fixed-angle-shadowing to magnify the molecules width, and multiple measurements with internal standards to provide accuracy.

Fixed angle shadowing is a common method for size measurement. One simply measures the shadow length and divides this by the shadowing ratio. This works well on large objects such as phage but is subject to two limitations. First, the measurement of the shadow must be made along the direction of shadowing. This is simple for large objects but the direction of shadowing may not be obvious for small, randomly arranged fibers.

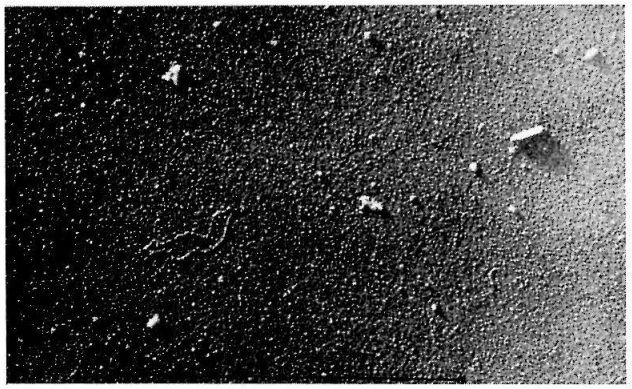
Secondly, the real shadowing angle is dependent on local conditions. The supporting films always sag and bulge so that the surface on which the molecule lies may be far from parallel to the plane of the grid. The shadowing angle and direction must be determined at each point on the grid. To do this, a rod shaped standard, tobacco mosaic virus (TMV) was selected. The width of TMV is roughly 150 Å. Because

the ends are abrupt, the molecule casts a sharp-edged shadow, whose edge gives the direction of shadowing and length, the real local angle of shadowing.

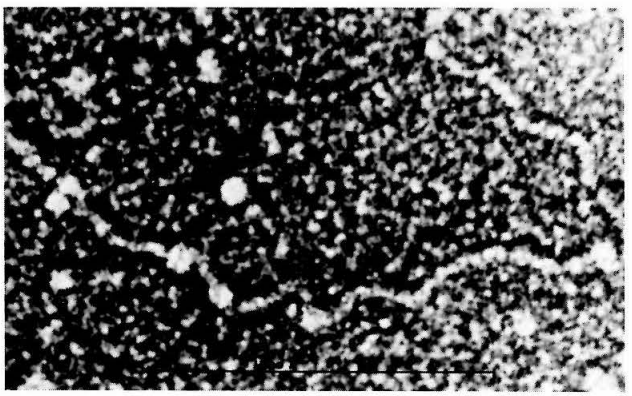
Chromosomal fibers and TMV are mounted separately or together on a carbon grid and shadowed with platinum for best contrast at an angle of 1:8. Multiple micrographs are taken of fields of fibers in which one or preferably more TMV lie perpendicular to the direction of shadowing (Fig. 5c). At the same time and magnification, micrographs are taken of the replica grating. All negatives are printed onto 4 x 5 Ortho-A-Litho sheet film. These sheets are transparent and large enough so that very accurate measurements on them may be made with a Nikon measuring microscope. For this method to be accurate, good values for the grating spacing and width of the TMV sample must be obtained. The first is done simply by making multiple measurements on diffraction gratings. The average TMV width is best obtained by negative staining with PTA.

Knowledge of the average TMV width and measurements of the shadow from the center of the TMV determines the local shadowing angle. This value is then used for that field of molecules. The long TMV shadow allows good alignment of the shadow edge to the rectangular grid on the Nikon viewing screen. This establishes a direction of shadowing for measuring fiber shadows.

Replica gratings are accurate to within 5%. Measurements made on TMV indicate that the width is very uniform, to perhaps 5%. Simplistically then, one might be able to determine any particular



a



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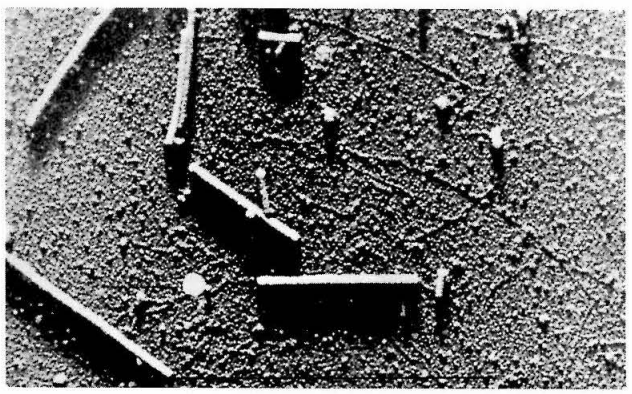

c

figure 5. ((a) SV 40 DNA and TMV fragment mounted by rapid ethanol immersion on smooth parlodion. Platinum shadowing. (b) DNA complexed with several DNA polymerase molecules. Rapid ethanol immersion mounting on carbon grid. Light tungsten shadowing with multiple printing enhancement. (c) TMV particles and nucleohistone fibers mounted and shadowed for width measurement.

measurement to within 25% and by multiple measurements on identical objects, obtain a much better value. This method was used to determine the average fiber width of nucleohistone fibers as increasing amounts of histone protein was removed by NaCl. The width distribution profiles show a smooth shift from the 30 Å average width of the nucleohistone fibers to the 15 Å width of DNA. The results also indicate that measurements of 20 $\pm$ 5 Å can be obtained by this procedure (14).

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INTRODUCTION

This work began as an effort to fill a particular need in the studies of isolated interphase chromosomes (chromatin). The fundamental biochemical and biophysical nature of chromatin was known from studies by Bonner and co-workers (1), Zubay and Doty (2), and others. Little was known however about the physical structure of this material. Clearly an answer was needed to the question "what is the physical structure of chromatin both isolated and as it occurs in the nucleus?" To what extent the physical integrity of the material was retained during isolation was crucial in interpreting the biological studies. With more sophisticated studies of gene activation, knowledge of the molecular structure of chromatin and the nature of the arrangement of the chromosomal proteins along the DNA would be increasingly important. The work of Ris (3), Gall (4), and Bonner (5) had shown by electron microscopy that chromatin contained fibers 30 Ångstroms wide. Other fiber sizes were observed, notably widths of 100 Å and 250 Å (3, 4).

Further electron microscope studies had not been fruitful because the mounting and visualizing procedures used proved far too harsh and crude to retain meaningful structure and provide the required level of resolution.

Development of techniques for gently mounting the isolated chromatin fibers from solution and for achieving higher structural resolution were needed. Such general methods would be valuable in many other areas.

The object of this thesis work was to develop these methods and to use them to describe the nature of isolated chromatin. During the course of this work it became possible to begin to answer a larger question -- "what is the nature of the structural changes involved in gene activation?" In addition, collaboration with Dr. Arthur Kornberg's group at Stanford provided an ideal test system for the new methods and an opportunity to learn more about the nature of DNA replication.

The first chapter of this thesis describes the methods, both new and old, used in this work. This is followed by the DNA polymerase work done with the Stanford group. The structure of interphase chromatin fibers is discussed in depth in the following two papers. In addition these papers discuss our findings about how chromosomal fibers change in appearance as they go from a dormant, heterochromatic state to a state of active transcription.

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Chapter 2

ELECTRON MICROSCOPY OF DNA POLYMERASE

The methods described in the last chapter were designed to provide high resolution electron microscopy of protein-nucleic acid complexes. A valuable test of these methods has been provided through the collaboration of Dr. Regis Kelly, Dr. Joel Huberman and Dr. Arthur Kornberg at Stanford. Their material is simpler and better defined than chromatin fibers. It was hoped that a study of the appearance of DNA polymerase-DNA complexes by these methods would lead to a better knowledge and improvement of the techniques and answer certain questions about the polymerase molecule itself.

A good understanding of the enzymatic properties of DNA polymerase and its mode of binding to DNA has been provided by the work of Dr. Arthur Kornberg and his co-workers at Stanford. This information has been reviewed recently in Science (1), and will be discussed in more depth in a series of forthcoming articles (2).

DNA polymerase has been shown by centrifugation to be an approximately spherical protein of 109,000 molecular weight. It consists of a single polypeptide chain. There are no subunits, and only one sulfhydryl group, believed to be exposed to the surface. The sulfhydryl may be labelled with mercury without affecting the enzymatic properties, and two molecules can be linked through these groups to form dimers.

The polymerase molecule binds to DNA in a highly specific

manner. It will bind to DNA duplexes at free 3' ends of single strands. Polymerase binds to both ends of linear lambda DNA or to Hershey Circles, but not to Hershey Circles treated with ligase to close the 2 single strand breaks. The DNA binding site is supposed to lie on the surface of the molecule and it has been suggested that the molecule has a groove in which the DNA duplex lies. This groove would contain the 3' binding site.

All components of this system have been highly purified. 95% of the protein in the polymerase preparation sediments as a single component; the remainder is of much lower molecular weight. Highly purified λ and ØX-174 RF DNAs have been isolated. For binding studies however, synthetic polynucleotides have been used. Polydeoxyadenylic acids and polydeoxythymidylic acids of varying size have been synthesized and purified. In this work, Poly dA_{4000} (average of 4000 bases per chain) and Poly dT_{200} have been used. They are annealed to form a DNA of a defined composition and length, and possessing a defined number of single strand breaks per molecule.

The initial object of this investigation was to see if the mounting and visualizing procedures developed above would be able to present a picture of polymerase-DNA complexes in agreement with what was known. Because of the delicate and aggregating nature of such complexes this presented a very critical test of the mounting techniques. Once it had been established that the electron micrographs could be interpreted in a useful way, several questions could be answered. The exact size and shape of the polymerase needed to be determined. Whether the DNA binds to the surface of the molecule or is enclosed by it could

be demonstrated. Use of mercury labelling or dimer formation could potentially locate the relative positions of the sulfhydryl group, 3' binding site, and groove.

Preparation of the Complexes

The polymerase, polymerase dimers, and polynucleotides were prepared by Kornberg and co-workers by methods described elsewhere (3).

The synthetic DNA was formed by the following procedure. Solutions of poly dA_{4000} and poly dT_{200} at concentrations of 0.1 mM in A or T residues are prepared, buffered in 0.1 M KPO_4 (pH 7.4), 0.001 M EDTA. The two polynucleotides were mixed with stirring at 60°C. The exact amounts were selected to give the desired T to A ratio. The mixture was then slowly cooled from 60° to room temperature over 3 hours. The DNA solutions were stored at ice temperature. If DNA polymerase was to be complexed with the DNA, the DNA was warmed to room temperature, the polymerase added slowly with stirring and the mixture allowed to stand at room temperature 20 minutes before cooling. Prior to mounting, the solutions were dialyzed against 0.005 M ammonium acetate (AmAc) pH 7.4, and 0.001 M EDTA for two changes followed by one dialysis against 0.005 M AmAc. pH 7.4.

Mounting and Visualizing Techniques

The mounting and visualizing techniques employed are described in the preceding chapter. Thin surface-active carbon grids were used. Samples were placed as 50 λ drops on pre-cleaned Saran Wrap and the grids floated face down on the drops for 10 minutes. Triple distilled

100% ethanol was used for dehydration. Shadowing was done with tungsten (rotary) except for fixed angle shadowing where platinum was employed. Photographic enhancement was necessary and followed the procedures described above.

A new procedure for mounting was developed late in this work. In this variation, the sample in 0.005 M AmAc, pH 7.4 was mixed with formamide and AmAc to make 30% formamide and 0.03 M AmAc. The sample is mounted from this solution in the standard manner. The DNA, formamide and ammonium acetate must be mixed at the time of mounting and the mixture used only once. A slow pH drop is known to occur when formamide is diluted in this manner. Immediate use avoids any possibility of low-pH damage to the sample.

Visualization of the Individual Components

The appearance of the individual polynucleotides, poly dA_{4000} and poly dT_{200} was studied by the standard mounting procedures. Poly dT_{200} molecules mounted in this manner appear as a field of individual spherical particles 50 Å-80 Å in diameter. The longer poly dA_{4000} molecules form tightly knotted twisted rods whose size is close to that of TMV rods.

Complexes of poly dA_{4000} and poly dT_{200} have been formed with T to A ratios of 0.075:1, 0.50:1 and 0.75:1. Because of the phenomenon of "creep," the complexes will be completely double stranded followed by a single-stranded region. The only difference in appearance of the 0.075:1 and 0.75:1 complexes are the relative lengths of duplex

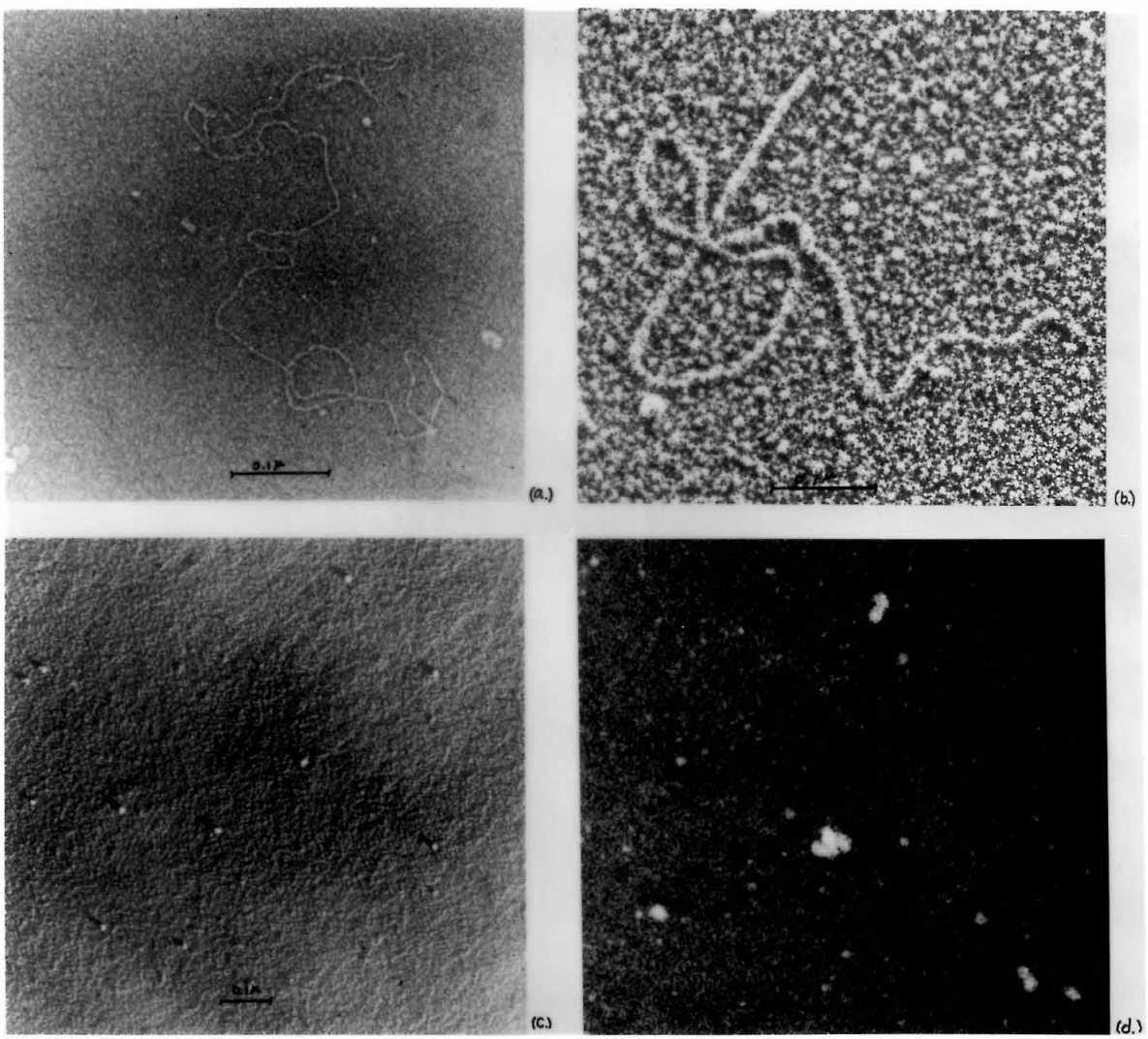


Figure 1 (a,b,c,d). (a) Polyda-polydt DNA mounted by rapid ethanol immersion, and visualized directly with tungsten and photographic enhancement. (b) Polyda-polydt DNA mounted by standard Kleinschmitt procedure, same magnification as (a). (c) DNA polymerase shadowed with platinum. (d) DNA polymerase dimers stained with uranyl acetate, reverse contrast.

over knotted single stranded regions. For this reason the 0.75:1 ratio has been used for most of this work. Ratios of greater than 1A to 1T form triple stranded poly A structures. Fig. 1a shows a typical 0.75:1 complex prepared by the standard mounting procedure. These complexes have also been mounted by the Kleinschmitt procedure (Fig. 1b) and the length distribution determined. A broad distribution from 3000 to 6000 bases in length with a significant number of much larger size was found. This agrees with the observed lengths by direct mounting. This broad dispersity in length may be due to end-to-end duplex aggregation rather than an actual polydispersity of this degree in the poly dA_{4000} samples. The polydispersity of the poly dT_{200} cannot be obtained except by inference from the binding experiments. In general, the complexes spread very well, show a minimum of aggregation and seem identical in appearance to other DNAs. Some knotted rods and 80 Å balls are always seen in complex preparations indicating that there was incomplete annealing.

The individual DNA polymerase monomers and dimers (formed with a mercury bridge) have been visualized by a variety of techniques. Fixed angle shadowing provides a direct, and usually low estimate of the size. Such a field of molecules is seen in Fig. 1c. Measurement of the shadow lengths of 50 molecules gives a best value of 53 ± 5 Å as a mean diameter if it is considered spherical, or 50 Å x 55 Å if ellipsoidal. Molecules may be directly stained with uranyl acetate. A dimer stained in this manner is shown in Fig. 1d. The dimensions obtained from 5 molecules by this method are 130 Å x 65 Å.

Very lightly shadowed (tungsten) monomers appear to be nearly spherical balls 60 Å x 70 Å. Dimers may be visualized directly on a thin carbon film (at 60 kv, no shadowing or staining with 2 cycles of photo-enhancement). Three such molecules were measured giving a dimer dimension of 130 Å x 60 Å. PTA negative staining has been tried without success.

The individual components appear as it was hoped they would on the basis of sedimentation and other biophysical studies. The polymerase, a sphere of 60 Å, is large enough to be easily visualized bound to DNA, and the dimers are clearly resolved as such. The annealed DNA looks like normal DNA while the individual polynucleotides do not.

Visualization of DNA-Polymerase Complexes

Initial attempts indicated that the 60° annealing procedure described above rather than 37° annealing was necessary. It was also observed that extreme aggregation of the DNA-polymerase complexes occurred if EDTA was omitted in the complexing step.

Samples were prepared from DNA annealed at 60°C and complexing done in the presence of EDTA although the EDTA was immediately removed by dialysis. Complexes formed in this manner are shown in Fig. 2(a,b). It is seen that the enzyme is clearly visible bound to the DNA and that the DNA is, in general, well spread. These two micrographs are of the same sample, a 0.75:1 T to A DNA with an excess of enzyme over 3' ends in the complexing mixture.

These two represent extremes in the numbers of polymerase bound per length of DNA in this preparation. It was concluded that

the polymerase-to-polymerase distance appeared reasonably constant within any complex, but varied over a factor of five among complexes on the same grid. The complex in Fig. 2b corresponds to 3' end saturation if the size of poly dT_{200} is of an average of 100 to 150 bases. This difference could be attributed to removal of polymerase from DNA during dialysis or mounting, or to uneven and incomplete complex formation.

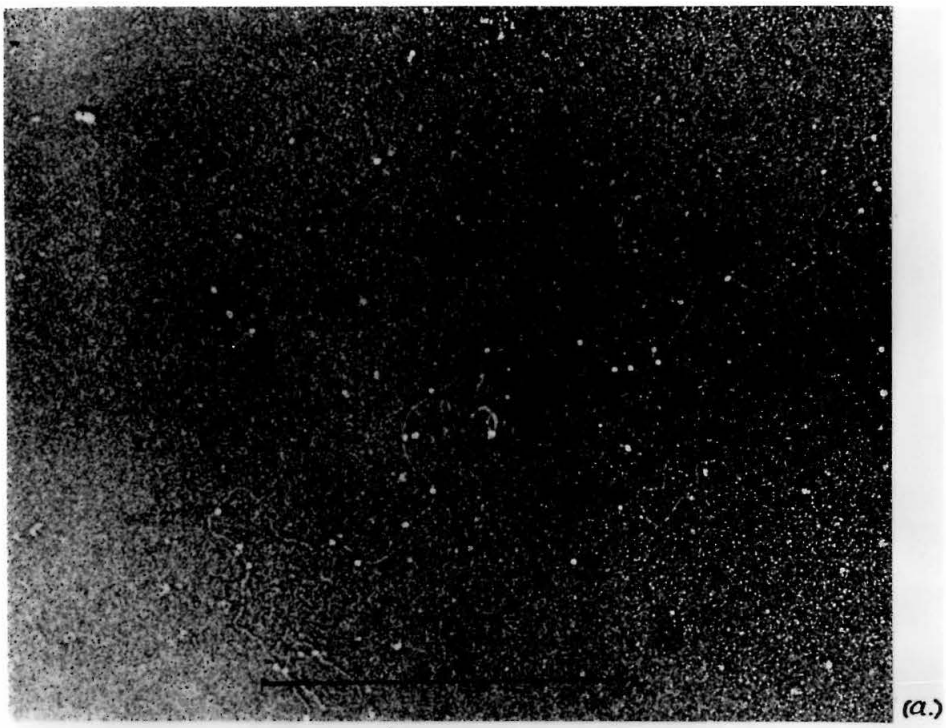
Several conclusions however, were drawn. The general appearance of the complexes agreed with the biophysical evidence and the DNA was seen to bind to the surface of the polymerase.

Polymerase Dimers Bound to DNA

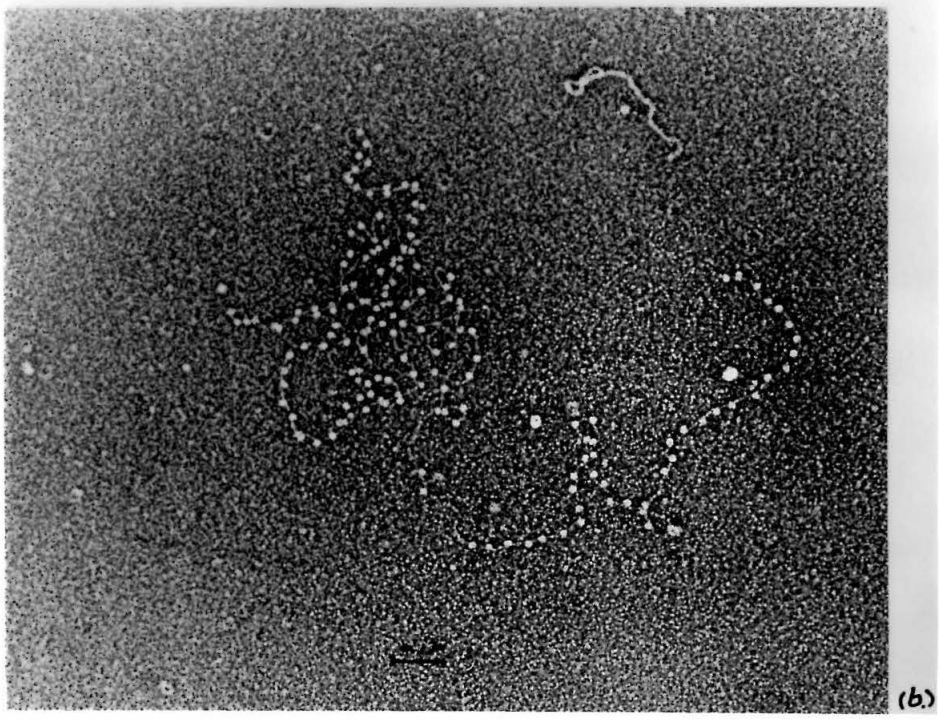
Following the work described above, a meeting was held at Stanford. It was decided that EDTA should be retained in the mixture as long as possible; metal ions are believed to remove polymerase from DNA. DNA polymerase dimers were prepared by Dr. Huberman. The dimer samples were isolated on a sucrose gradient, hence contained both monomers and dimers.

DNA was prepared by the usual procedures with a 0.75 T to 1.00 A ratio. Dimers were bound to the DNA with a 2-fold excess of dimers over 3' ends. Extreme care was taken to avoid metal ions. One set of samples had EDTA present at 10^{-4} M through the mounting steps. The formamide procedure was tested against the standard mounting procedure.

The results of these experiments showed that the formamide



(a.)



(b.)

Figure 2 (a,b). DNA-DNA polymerase complexes.

procedure is a definite help in preventing intra-complex aggregation and facilitating spreading. Both sets of samples showed almost complete saturation of 3' sites by polymerase (if the 3' sites are spaced about 150 base pairs apart). The set in which the EDTA was removed in the last step appeared to show better overall resolution.

The general results are reproducible. Figure 3a shows part of a typical complex mounted from 0.03 M AmAc, 30% formamide.

The average distance between polymerase molecules (monomers or dimers) on the DNA was measured on nine successive complexes viewed in the microscope. Unlike the previous results, this spacing was highly regular among the nine complexes. The following values give the average spacing for each complex in angstrom units and represents a total of 394 bound sites: 575, 520, 640, 585, 655, 660, 450, 435, and 500. Two-thirds of the individual values fall within 550 $\pm$ 75 Å.

If it is assumed that all 3' sites are bound by polymerase in these preparations, then the average poly dT is 160 $\pm$ 20 bases long. This size is very reasonable considering the synthesis conditions.

It may be concluded that the previous differences in polymerase per complex were due to the presence of metal ions and not to some general artifact of the mounting procedure.

Monomers of polymerase bind DNA at only one site, and bind the DNA through free 3' single strand ends. Such free ends are far apart in the DNA used, and one would expect that dimers would be bound to DNA by only one of the two monomers. Assuming this to be the case, and knowing that the DNA binding sites and sulfhydryl sites can be located by seeing where the DNA and one monomer, and both monomers touch, respectively,

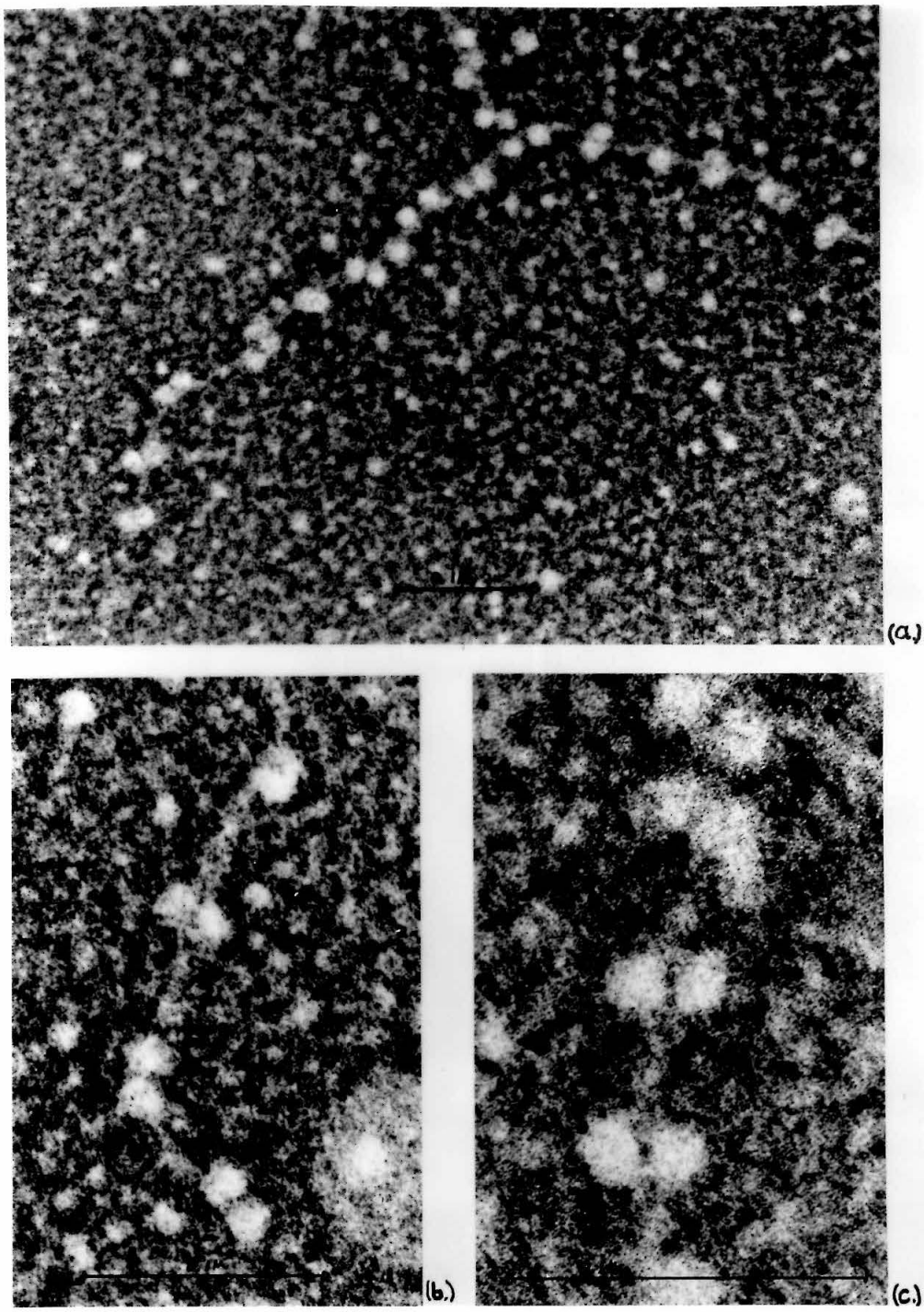


Figure 3 (a,b,c). (a) Monomers and dimers of DNA polymerase bound to DNA. (b,c) Dimers bound in side-by-side and straddle modes. Prepared by direct mounting with foramide, with tungsten and photographic contrast enhancement.

the appearance of the dimers bound to DNA could potentially locate these two sites relative to each other. Consider for example, the monomers visualized as globes with the two monomers bound through the sulfhydryl groups at 0° latitude, 0° longitude, with the DNA binding sites opposite at 0°, 180°. In this case modes such as those shown in Figure 4a, b, would be observed. If on the contrary, both sites are located at 0°,0°, then modes such as Fig. 4b,c, would be seen in the micrographs. Thirdly, if the sulfhydryl is at 0°,0° and the binding site at 0°,90° (at one pole of the globe), then modes such as 4d and 4e would be observed. In the micrographs of dimers bound to DNA, all of these modes have been observed. The modes referred to as straddle and side-by-side, however, are predominant over modes such as 4a. It may be reasoned that the two groups are located closely rather than far apart.

Conclusion

This study has increased the confidence levels of two different projects. In our study of the nature of chromosomal DNA and its associated proteins, this work has defined the limits to which the mounting and visualizing techniques may be trusted to give quantitative and artifact-free results. Secondly, it has shown the Kornberg group that the electron microscope picture of their system is in good agreement with the model of polymerase and polymerase binding derived from other biophysical data.

Several refinements and improvements in mounting techniques such as the formamide procedure have been developed during this study.

It has been demonstrated that DNA binds to the surface of DNA polymerase, and only one enzyme binds at each 3' break. In addition, dimers of polymerase bound to DNA have been visualized.

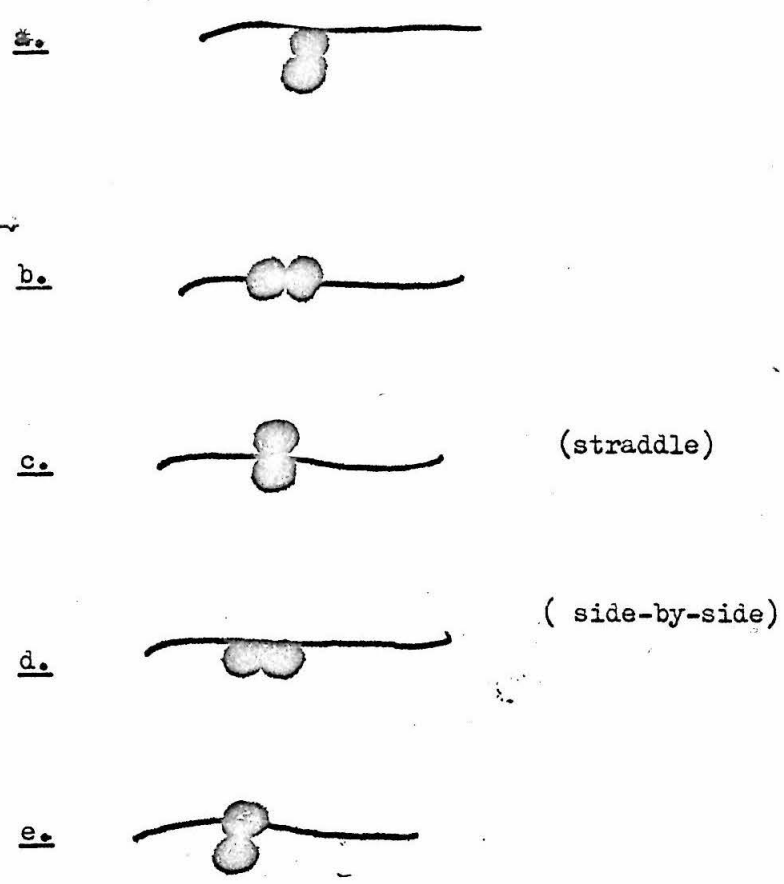


figure 4. (a,b,c,d,e). Diagramatic modes of Polymerase dimers bound to DNA.

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THE STRUCTURE OF ISOLATED INTERPHASE CHROMOSOMES

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INTRODUCTION

It is known that interphase and metaphase chromosomes are chemically similar and visually dissimilar. Recently, electron micrographs have indicated that a fiber 30Å wide may be basic to the structure of interphase chromosomes chromatin (Bonner and Huang, 1962). DuPraw and Bahr (1969) have demonstrated that human metaphase chromosomes are constructed of 230 Å wide fibers. The 30 Å fiber appears to be a single DNA duplex complexed with a nearly equal amount of histone protein (Bonner et al., 1968a). X-ray diffraction data demonstrate that the presence of these proteins confers a new level of supercoiling on DNA, a structure with a pitch of 120 Å (Wilkins, Zubay and Wilson, 1959; Pardon, Wilkins and Richards, 1967). The 230 Å macrofiber of metaphase chromosomes contains an amount of DNA equivalent to 50-100 duplexes per unit length of macrofiber (DuPraw and Bahr, 1969). The structural relation of the 230 Å macrofiber, believed to condense from the smaller fiber, has not heretofore been known.

Interphase chromatin has been isolated from a variety of organisms and its biochemical properties well catalogued (Bonner et al., 1968a). In the past, a thorough structural study of this material by electron microscopy has been hindered by the lack of good specimen mounting techniques. In addition, it has been difficult to correlate mode of preparation, and biochemical properties with the appearance of the material.

In this work, a variety of new techniques have been developed

for mounting chromosomal fibers from solution, and for visualizing the structure at a high level of resolution. With these techniques, chromatin isolated and characterized by the methods of Bonner (Bonner et al., 1968b) has been studied by high resolution electron microscopy.

Bonner (Bonner et al., 1968a) has shown that the inactive portion of the genome consists of DNA complexed with a somewhat equal mass of histone. The template active portion is more rich in non-histone protein and contains less histone. The histones are well-characterized. They comprise eight species of molecules, all rich in basic amino acids but each different from the others (Fambrough and Bonner, 1966). For one, histone IV of pea bud and calf thymus tissues, the amino acid sequence is known (Delang, Fambrough, Smith and Bonner, 1968). Methods have been developed for the selective removal or attachment of various of the histone species or the chromosomal DNA (Smart and Bonner, 1969).

We have used several approaches to investigate the structure and composition of interphase chromosomes. First, chromatins from two tissues have been studied in detail throughout their purification. Second, chromatins of a wide variety of organisms of different evolutionary status and overall genetic activity have been compared. Third, purified nucleohistone has been subjected to a variety of biochemical treatments to provide information about the distribution of the various proteins along the chromosomal DNA.

MATERIALS AND METHODS

Electron Microscopy

The methods developed for mounting and visualizing chromatin samples for high resolution electron microscopy are described in detail in a forthcoming publication (Griffith, 1969a). An outline of the major methods used here is presented below.

Preparation of Carbon or Smooth Parlodion Supporting Films

Mica sheets from Ladd are cleaved with a razor blade or with double-stick tape and a thin layer of carbon is evaporated onto the fresh surface in as good a vacuum as can be obtained. To make smooth parlodion, a fresh mica surface is dipped into 0.3 to 1.0% parlodion in amyl acetate and allowed to dry. This is then backed with carbon in the above manner for strength.

Both films are removed from the mica by stripping into a water trough. Grids are first dipped into 1% polybutene in xylene, drained on filter paper, then placed on the floating film (Jamesson, 1968). It is important to wait one-half hour to allow the adhesive to work.

We have a simple method for rescuing the grids. A drum of Saran-wrap with a petri dish is used (Fifkova, 1968). This is placed firmly down over the floating film and the grids. Picked up on the slant, the drum will lift the film and grids from the water surface.

Forming Surface-active Grids

After they are dried, the grids are placed in a 50 μ vacuum

and a Tesla coil is applied to a terminal of the chamber. The grids are "ionized" in this way for 30 minutes. The procedure appears to produce temporary active groups on the film surface. To take advantage of this state, the grids must be used immediately.

Mounting the Sample

Samples have been mounted by two different methods, freeze-drying and rapid ethanol immersion. The two methods yield similar results and the latter, and simpler method has been used for most of this work.

For rapid ethanol immersion, the sample must be exhaustively dialyzed against a volatile buffer. Drops of a solution of the sample are placed on a sheet of pre-cleaned Saran-wrap. A grid prepared in the above manner is placed on the drop face-down for 10 minutes. It is then lifted off, carrying a droplet with it, and plunged face-down into a tray of redistilled 100% ethanol for 30 seconds and air dried.

Nuclei may be lysed and gently spread on the grid by the following method. Purified nuclei are suspended in 25% glycerol, 0.01 M tris, pH 8.0. A few nuclei are transferred with a glass needle to a grid on which 20 λ of distilled water has been placed. Lysis is monitored with a phase contrast light microscope. Following lysis the grid is dehydrated in ethanol in the above manner.

Contrast Enhancement

For the best resolution, samples are lightly shadowed (rotation) with tungsten at an angle of 1:8. For staining, the nonaqueous uranyl

acetate method of Wetmur (Wetmur, Davidson and Scaletti, 1966) is incorporated into the dehydration step. Photographic contrast enhancement by multiple printing on high contrast film is used.

Measurement of Fiber Widths

A method has been developed for the accurate measurement of nucleohistone fiber widths. This method uses fixed-angle shadowing to magnify the molecule width and multiple measurements with internal standards to provide accuracy. The real shadowing angle is dependent on local conditions. The supporting films always sag and bulge so that the surface on which the molecule lies may be far from parallel to the plane of the grid. The shadowing angle and direction must be determined at each point on the grid. To this end we use a rod-shaped standard, tobacco mosaic virus (TMV). The width of TMV is uniform. Because the ends are abrupt, this molecule casts a sharp-edged shadow, whose edge gives the direction of shadowing and length, the real local angle of shadowing.

Chromosomal fibers and TMV are mounted separately or together on a carbon grid and shadowed with platinum for best contrast at an angle of 1:8. Multiple micrographs are taken of fields of fibers in which one or preferably more TMV lie perpendicular to the direction of shadowing. At the same time and magnification, micrographs are taken of a replica grating. All negatives are printed onto 4" x 5" Ortho-A-Litho sheet film. These sheets are transparent and large enough so that very accurate measurements on them may be made with a Nikon

measuring microscope. For this method to be accurate, good values for the grating spacing and width of the TMV sample must be obtained. The first is done simply by making multiple measurements on diffraction gratings. The average TMV width is best obtained by negative staining with PTA. Knowledge of the average TMV width and measurements of the shadow from the center of the TMV determines the local shadowing angle. This value is then used for that field of molecules. The long TMV shadow allows good alignment of the shadow edge to the rectangular grid on the Nikon viewing screen. This establishes a direction of shadowing for measuring fiber shadows.

Isolation of Nuclei and Chromatin

The isolation, purification and chemical characterization of chromatin are described in detail elsewhere (Bonner et al., 1968b).

Partially dehistonized pea bud chromatins were obtained from Mr. John Smart and prepared by methods to be described by Smart (Smart and Bonner, 1969). Tetrahymena nuclei and chromatin were prepared from the a-micronucleate strain, Tetrahymena pyroformis W. The methods used are described elsewhere (Griffith, 1969b).

Chicken erythrocyte and sea urchin nuclei were prepared by Mr. Richard Firtel using the general procedures of Bonner (Bonner et al., 1968b).

RESULTS

I. Composition and Appearance of Chromatin at Each Level of Purification

For studies of isolated chromatin, it is important to know how its structure and chemical composition changes during isolation. Two typical tissues, pea bud and rat liver, have been selected for such study. Table I shows the change in chemical composition from crude nuclear lysate to purified chromatin (nucleohistone) relative to DNA. It may be seen that the primary effect of purification is to remove RNA and protein. From the behavior of the lysates on sucrose gradients and protein isolated from the gradients on polyacrylamide gels, the RNA and protein removed from the chromatin fibers are identified as ribosomal and membrane contaminants. Electron microscopic examination of crude nuclear lysates also shows the presence of large amounts of membrane and ribosomal material. The ribosomal contaminants appear to be extranuclear. Nuclei washed with Triton X100 to remove membranes and ribosomes followed by on-grid lysis show no ribosomes within the nucleus. This finding demonstrates the importance of this particular step in chromatin isolation.

The micrographs in Figure 1 show typical fields of nuclear fragments. The general appearance of the crude nuclear lysate mounted from solution (Fig. 1a) agrees with what is seen in light microscopy by orcein stain. The material appears highly aggregated, due in part to membrane contaminants. Thin fibers 20-30 Å are always visible.

Table I

Chemical Composition of Rat Liver Chromatin

at Various Levels of Purification

Fraction	% DNA	% RNA	% Protein
Whole nuclei[1]	20	5	75
Crude chromatin[1]	31	5	64
Purified chromatin[2]	37	1	62

[1]From Steele, W. J., and H. Bush, Cancer Res. 23, 1153 (1963).

[2]From Marushige, K., and J. Bonner, J. Mol. Biol. 15, 160 (1966).

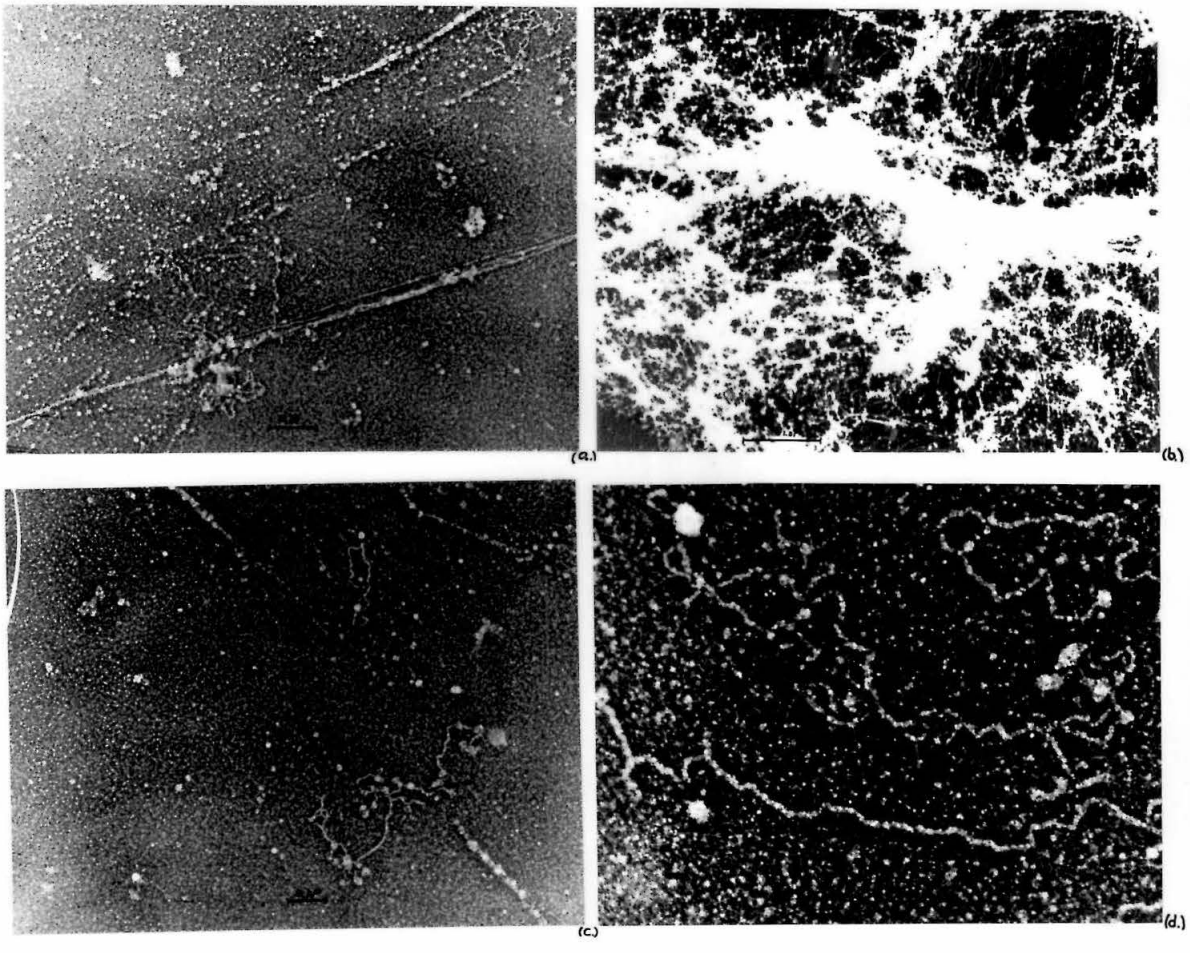

Figure 1 (a,b,c,d). (a) Crude pea bud nuclear lysate. (b) Rat liver nucleus spread by on-grid lysis. (c) Purified pea bud chromatin showing macrofibers and knobbed fibers. (d) Purified pea bud chromatin showing supercoiled 30 Å fibers.

On-grid lysis of nuclei provides very gentle mounting conditions. A nucleus spread in this manner is shown in Figure 1b. It is clear that the nuclear material is highly condensed but that areas showing thin DNA-like fibers are visible. Both pea bud and rat liver nuclei show the same appearance at this stage.

Nuclei lysed on the grid show a very clean separation of the nuclear contents from the nuclear membrane. It appears that there are few areas of the membrane to which chromosomal fibers are bound.

Purified chromatin is prepared from nuclear lysates by sucrose gradient sedimentation. The chromatin pellets free of membrane contaminants. Purified chromatins from various sources may still differ in their general appearance at this stage due to amounts of residual cell debris. Calf thymus, chicken erythrocyte and rat ascites chromatins are always purer at this step than rat liver or Drosophila chromatins.

Purified chromatins mounted by the rapid ethanol dehydration procedure and shadowed with tungsten are shown in Figures 1c and 1d. They are typical of both rat liver and pea bud purified chromatin. Three general classes of fibers have been found: 1) large macrofibers 230 Å in diameter. These fibers may be quite long and show a periodic condensed substructure; 2) long, uniform fibers 30 Å in width. These fibers are often found in a supercoiled configuration. The supercoil has a pitch of 120 Å; 3) Fibers 20-30 Å in diameter characterized by the presence of attached knobs 100-250 Å in diameter. Both rat liver and pea bud tissues show these three classes of fibers.

Nucleohistone is prepared from purified chromatin by shearing in a Virtis homogenizer "60" at 45 volts for 90 seconds followed by precipitation of the unsheared or aggregated debris by centrifugation at 10,000 g for 10 minutes. From Table I it is clear that this step does not have a marked effect on the chemical composition of the material. Nucleohistone is, however, more soluble than is purified chromatin and is therefore easier to work with. Its template priming capacity is only slightly higher than that of purified chromatin.

Electron microscopy of nucleohistone from rat liver and pea bud show that shearing eliminates the 230 Å macrofibers. These are broken down into fibers of the other two classes, primarily into the 30 Å fibers. Pea bud nucleohistone has been studied extensively by various mounting and visualization procedures. Its fibers show the same appearance when mounted by the freeze-dry technique described as when mounted by rapid ethanol dehydration.

II. Composition and Appearance of Chromatins from Other Organisms

A variety of chromatins from a variety of different higher creatures have been studied to try to obtain more insight into the function of the three classes of chromosomal fibers. The general results of these studies are shown in Table II. The chemical composition, template priming capacity, and appearance of such chromatins are tabulated. Isolation and chemical characterization of each was done by the authors or by others in this laboratory and with standard procedures.

Calf thymus chromatin resembles pea bud and rat liver chromatins in all important aspects. All three of these chromatins are from differentiated tissues and have a relatively low level of genetic activity. To provide a wider range of samples, the chromatins of the protozoan Tetrahymena, of sea urchin embryos, and of chicken erythrocytes have been included.

The results of the work with Tetrahymena are described in detail elsewhere (Griffith, 1969b). Tetrahymena pyriformis W is a non-differentiated organism and forms no chromosomal structures. Template assays and separation of the active from the inactive portion of the genome indicate that one-half of the genome is in the active state of transcription at any time during the normal growth period. Tetrahymena chromatin is similar to that of all other higher creatures in general chemical composition. Its lower histone content and larger content of non-histone protein is as expected according to current ideas of the function of these two classes of protein. Extensive analysis of Tetrahymena macronuclear chromatin by on-grid lysis indicates that it contains no 230 Å macrofibers. Only the thin single fibers characteristic of nucleohistones of pea bud and rat liver have been observed. Figure 2a shows a typical lysed nucleus and its 30 Å chromosomal fibers. We have not found any anomalous chromosomal structures in Tetrahymena. We believe that those reported (Wolfe, 1967) may be attributed to difficulties in staining (Griffith, 1969c) or aggregation.

On-grid lysis investigation of nuclei from the pluteus stage of sea urchins shows that all three classes of fibers are present, and that

they are at a molecular level indistinguishable from similar classes of fibers of other creatures.

The chromatin of avian erythrocytes is known to be entirely heterochromatic and to stain densely with orcein. The data here presented show that the structure of this chromatin is similar to that of other heterochromatic chromatins and, in keeping with its heterochromatic nature, is genetically inert.

The on-grid lysis method is particularly suited to the study of the chromatin of avian erythrocytes. 230 Å macrofibers are very labile to shear. Two such fibers are shown in Figure 2b,c, the second appearing to have been stretched more than the first. The chromatin of nuclei which show little shear damage appear to be entirely composed of such fibers. These fibers are of the same size and periodic condensation as are fibers of the same class from other organisms.

III. Structure of the Three Classes of Chromosomal Fibers

The general appearance and composition of the 230 Å macrofibers have been discussed above. The have been found to be continuous over lengths of 10 μ. Shearing degrades them into 30 Å fibers identical to those of the second class. No branches or forks have been found in them. When they are subjected to gentle stretching, a basic doubleness becomes apparent (Fig. 2b,c). Treatment on the grid with agents known to remove all or a fraction of the chromosomal proteins, yields partially-degraded fibers showing two (or a multiple of 2) remaining DNA fibers (Fig.6).

30 Å fibers of the second class (Fig. 7) are characterized by a very

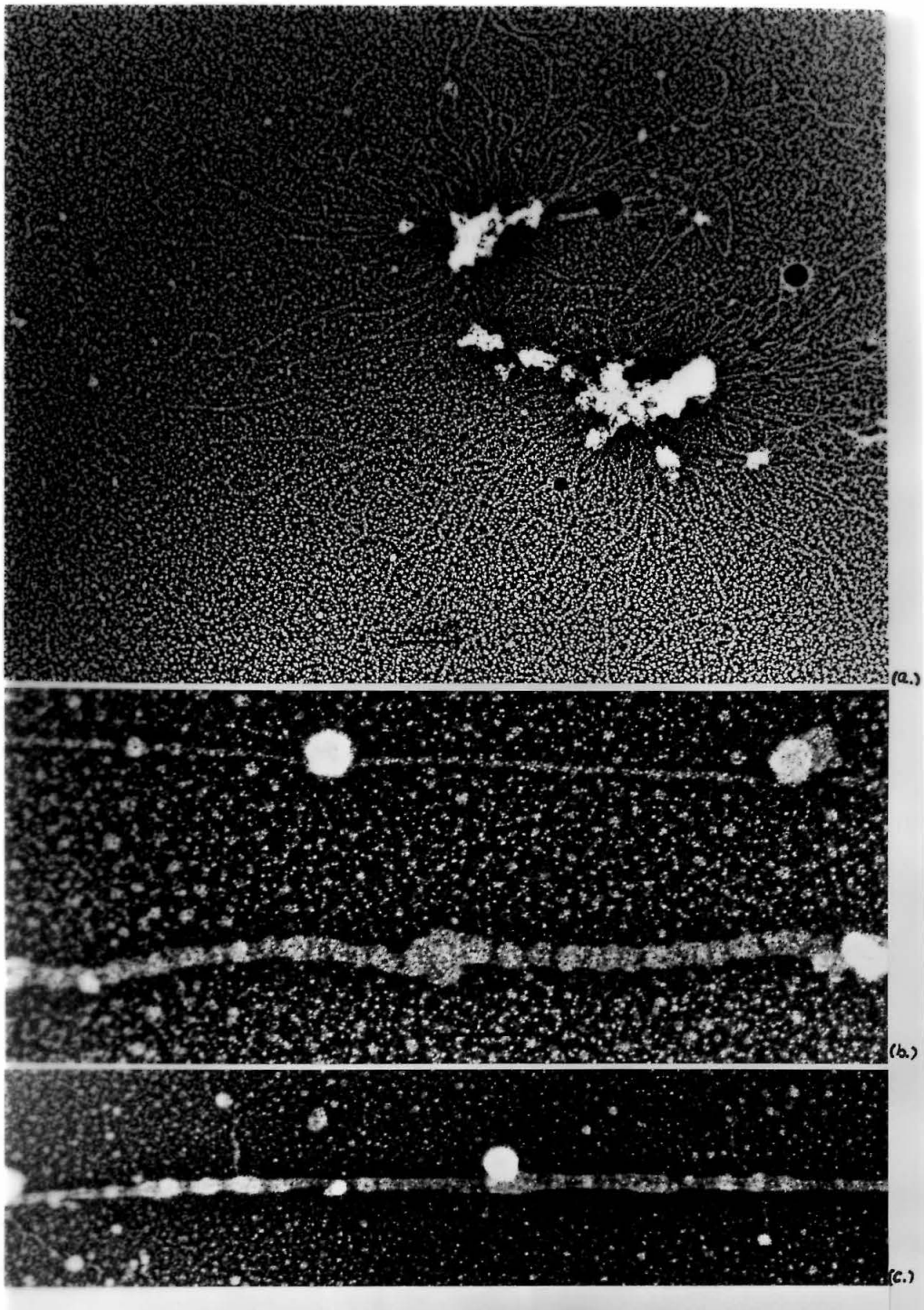

Figure 2 (a,b,c). (a) Tetrahymena nuclear fibers. Prepared by on-grid lysis. (b) Macrofiber and single chromatin fiber from chicken erythrocyte nucleus spread by on-grid lysis. (c) Stretched macrofiber showing double nature.

TABLE II

COMPOSITION AND APPEARANCE OF VARIOUS CHROMATINS

Chromatin	Composition				Template	Appearance		
						Presence of Fiber Type		
	DNA	RNA	Histone	Non-histone	Priming Capacity	230 Å macro	30 Å super-coiled	20-30 Å knobbed
Pea bud[1]	1.00	.10	1.05	.500	20%	+	+	+
Rat liver[1]	1.00	.04	1.00	.67	20%	+	+	+
Calf thymus[1]	1.00[2]	.01	.95	.33	15%	+	+	+
Tetrahymena	1.00	.09	.65	.76	38%	-	+	+
Sea urchin[1] (Pleuteus)	1.00	.08	.86	1.04	20%	+	+	+
Chicken erythrocyte	1.00	-	-	< 0.02	< 1%	+	(Very little)	

[1]Bonner et al., 1968b.

[2]Chalkley and Jensen, 1967.

uniform 30 Å width, and supercoiling of 120 Å pitch. This supercoiling appears to be lost on stretching the fibers or aging of the fiber preparations. 20 Å to 30 Å fibers of the first class are easily distinguished by the lack of supercoiling, and by their possession of clustered knobs. The knobs may be found bound to single fibers in nuclear lysates, as well as in crude and purified chromatins, and nucleohistone (Fig. 3a). They range in size from 150 to 250 Å in diameter, and vary in shape from spherical to oblong or prismatic. Several classes have been found, distinguishable both by their size and shape. The fibers appear bound to the surface of most of these knobs rather than enclosed by them. Occasionally nucleic acid-like tails are seen protruding from the bound knobs (Fig. 3b). Many knobs appear similar to isolated E. coli RNA polymerase bound to DNA. The knobs are usually clustered within gene-length distances of each other but are almost always separated by at least 200 Å. These knobs have been identified as non-histone proteins by the following procedure. Treatment of chromosomal fibers with 0.2 N H_2SO_4 has been shown to remove 80% of the proteins of the histone class, leaving the non-histone proteins bound to the chromosomal DNA (Bonner et al., 1968a).

Nucleohistone is treated with acid in this manner, followed by centrifugation into 1.2 M sucrose to separate the DNA non-histone protein complex from the histones. This treatment does not remove the knobs (Fig. 3b,c). Other methods of removing proteins from chromosomal fibers have also been studied. When chromatin is pelleted from solutions of NaCl over the concentration range of 0.6 M to 3.0 M, all histone and

most non-histone are removed (Bonner et al., 1968a). Extraction of nucleohistone with increasing concentrations of NaCl causes a disappearance of the knobs in agreement with the known removal of non-histone by this method. Similarly centrifugation of chromatin in 6 M CsCl, a method known to remove all protein from DNA, removes the knobs. The knobs are removed by treatment of the chromatin with preincubated pronase at 2 mg/ml. for 2 hours at 32°C.

The non-histone proteins are primarily confined to fibers of the first class and their large size has simplified studies of their distribution in these fibers. The histone proteins are believed to be complexed stoichiometrically to fibers of the second class from which evidence indicates 230 Å macrofibers form. The following experiment demonstrates that fibers of all three classes are complexed with histone protein.

A widely-used method for spreading bare DNA is the protein-surface-film method developed by Kleinschmidt and others (Kleinschmidt and Zahn, 1959; Van Bruggen, Borst, Ruttenberg, Gruber and Kroon, 1966). Bare DNA and a protein such as cytochrome c are mixed in a solution of salt sufficiently concentrated to prevent protein-DNA association. When this is spread on a hypotonic water trough, the charged DNA binds a coat of denatured protein. This DNA-protein complex is 150 Å wide. Because the binding is ionic, DNA which had previously been neutralized by histone, for example, will not form such a 150 Å complex. Thus a 30 Å wide nucleohistone fiber in the cytochrome film would hardly be visible when spread by this method. Any region along the fiber 200 Å or longer and bare of histone should, however, form a complex with the cytochrome c and be clearly visible (i.e., 150 Å in width). In addition,

if the salt concentration in the nucleohistone-cytochrome mixture is in the range in which histones are dissociated from DNA (0.3 M to 3.0 M NaCl), the amount of cytochrome bound to the fibers at various salt concentrations will tell us about the distribution of the histone.

Nucleohistone and purified DNA of pea bud, rat liver and Tetrahymena have been spread by this method. The concentration of NaCl in the layering solution was varied from 0.10 to 3.0 M NaCl while the ionic strength of the trough remained 0.1 M ammonium acetate.

The results of these experiments show that there are few if any regions of bare DNA 200 Å or longer in native chromatin. The fiber widths appear to increase uniformly along the fiber length as cytochrome c is bound. With treatment with 3.0 M NaCl there is no difference between the width of DNA from nucleohistone and that of previously purified DNA (Figure 8). In the region in which histone I is selectively removed (0.4 to 0.6 M NaCl), no non-uniformity in the fiber widths has been observed.

The distribution of the histone proteins along the nucleohistone fibers (classes I and II) has been further studied by removal of histone I in the manner described above, and selective removal of most of histone II (50% of the total protein) by treatment with 0.375 M sodium deoxycholate. These fibers were then examined by high resolution (tungsten shadowing) electron microscopy.

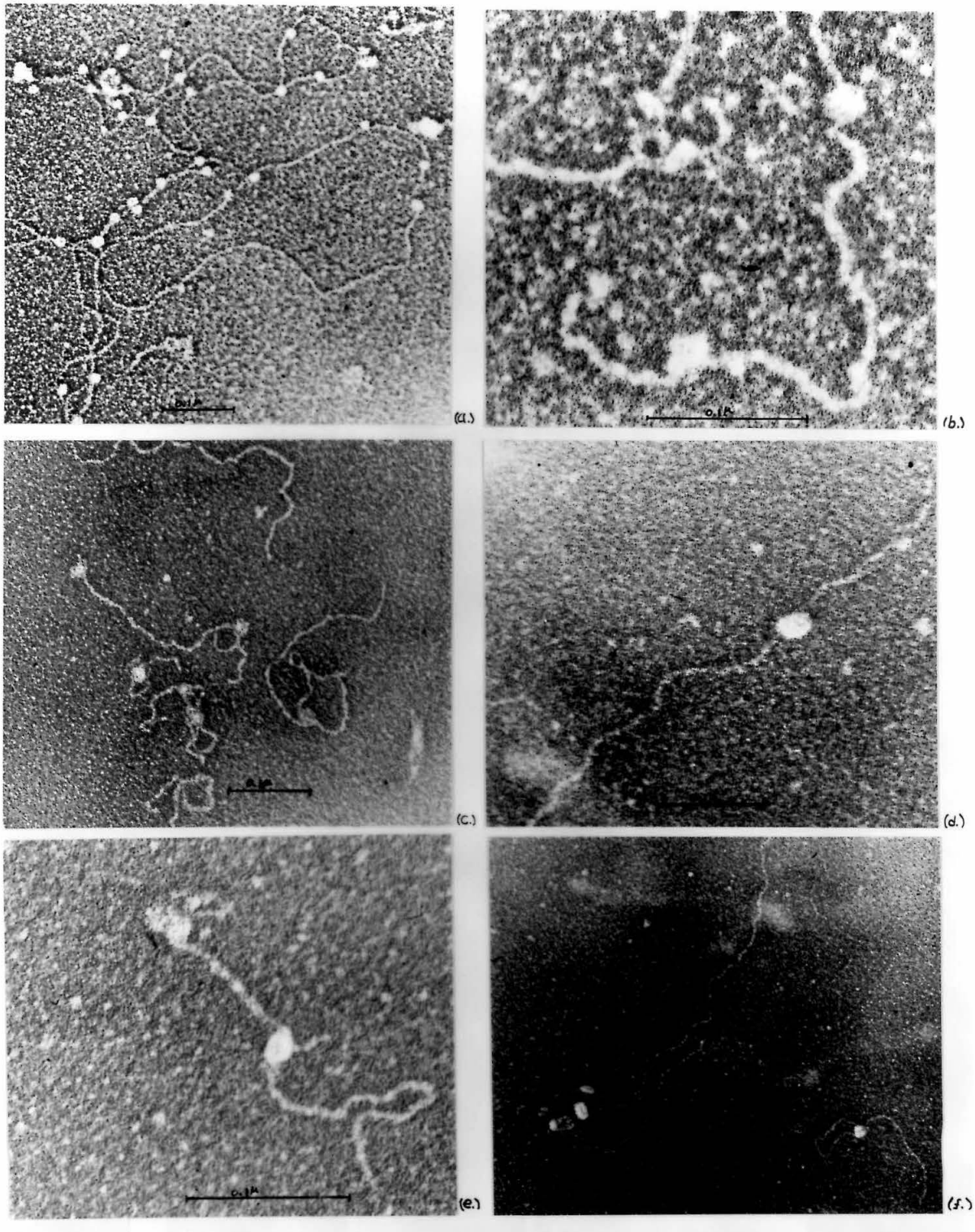

Figure 1 (a,b,c,d,e,f). (a,b,c) Protein knobs on native chromatin fibers. (d,e,f) Knobs remaining after extraction of histone protein with 0.2 N H_2SO_4. Rapid ethanol mounting with tungsten and photographic enhancement.

No gaps of 50 Å or larger in the 30 Å (native) fiber corresponding to a bare 15 Å DNA were observed in the case of selective histone I removal. Similarly the selective removal of most of histone II did not yield any obvious gaps in the histone coat.

To further characterize the nature of histone removal and the manner in which this protein is bound to and distributed along the DNA, the width of nucleohistone fibers was accurately measured as histone protein was progressively removed with DOC. The method by which this was done is described above. A preparation of pea bud nucleohistone was extracted with twelve different concentrations of DOC from 0 to 0.15 M covering the entire range of protein removal. Each fraction was characterized for the following properties: total chemical composition, type and amount of histone remaining (acrylamide gel electrophoresis), melting profile, and template priming capacity. The preparation and characterization was performed by Mr. John Smart in our laboratory.

Histone is removed with increasing concentration of DOC similar to histone removal by NaCl. Histone II is removed first, followed by III and IV, and lastly, histone I.

Figure 5 shows a typical field of TMV and nucleohistone fibers. For each DOC concentration, micrographs of at least fifty different fields were made. From these, ten were selected for measurement. One hundred measurements were made at random along the fibers to provide width distributions at each concentration of DOC.

Figure 4 shows these plots for 0 M, 0.015 M, 0.0375 M and 0.0500 M. The most important feature is the demonstration that partial

removal of histone yields fibers intermediate in width between pure DNA and native nucleohistone. The fiber shadows are smooth to 20 Å, less than the 60 Å length which one histone molecule would cover if it neutralized each phosphate along the DNA. All traces of supercoiling appear to be lost above 30% to 40% protein removal. Pardon, Wilkins and Richards (1967) have shown that the X-ray reflections characteristic of nucleohistone are lost if the fibers are stretched or the histone proteins are removed. The location of these reflections suggests a helical supercoiling of the nucleohistone fiber with a pitch of 120 Å and width of 100 Å. As they point out, this could account for chromatin fibers of 100 Å width commonly seen by electron microscopy.

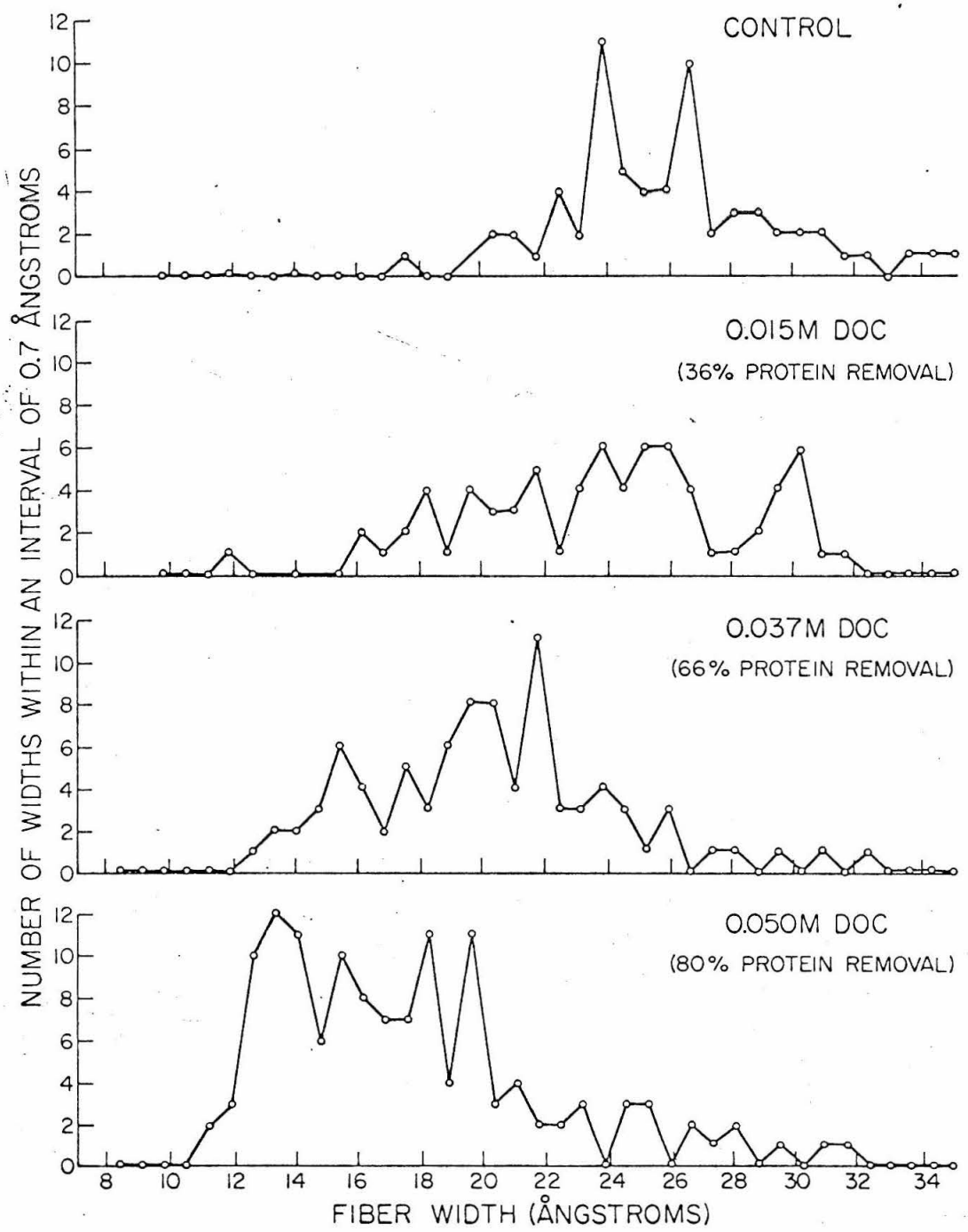

Figure 4. Distribution of nucleohistone fiber widths following removal of increasing amounts of protein with sodium deoxycholate. Measurements have been grouped about values 0.7 Å apart.

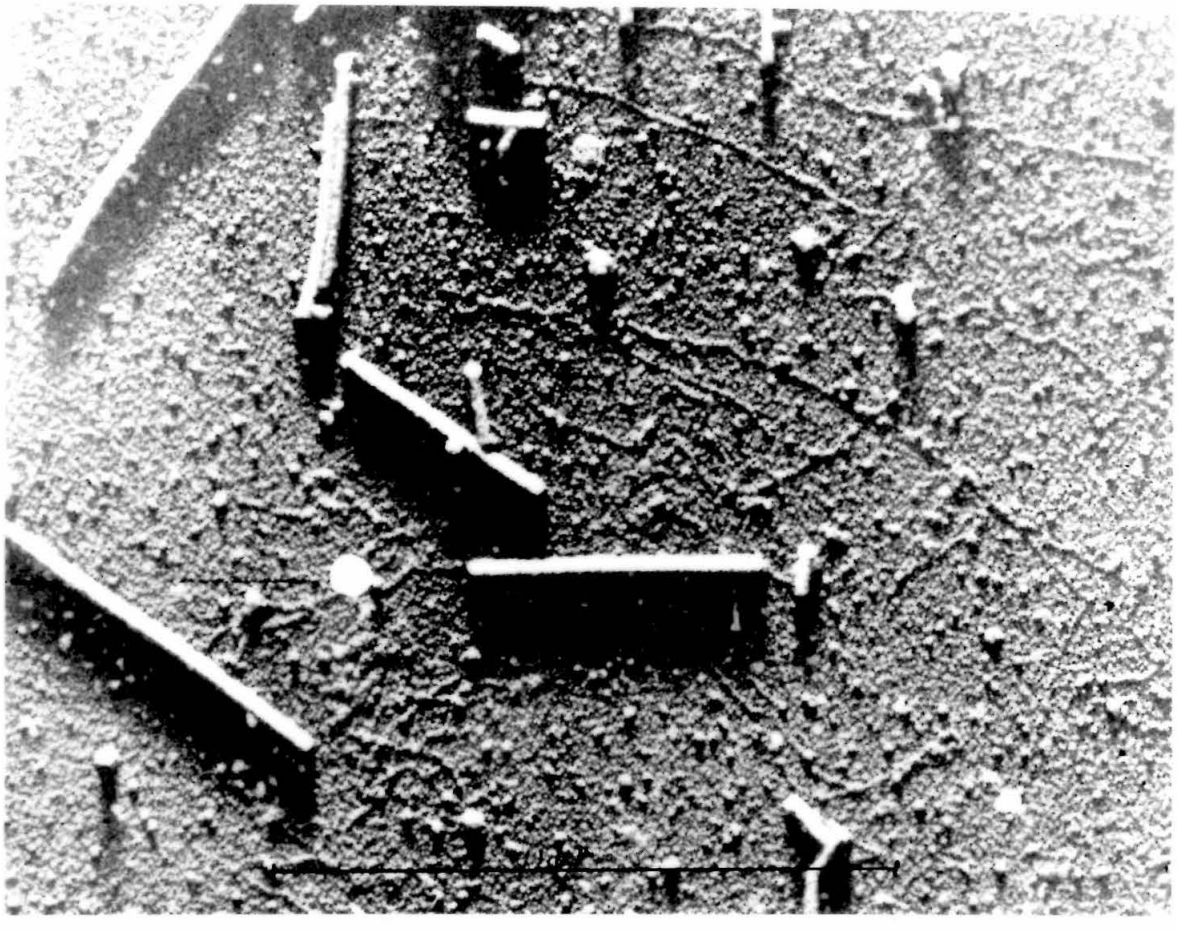

Figure 5. TMV rods and nucleohistone fibers mounted together for width measurement. Fixed angle shadowing with platinum.

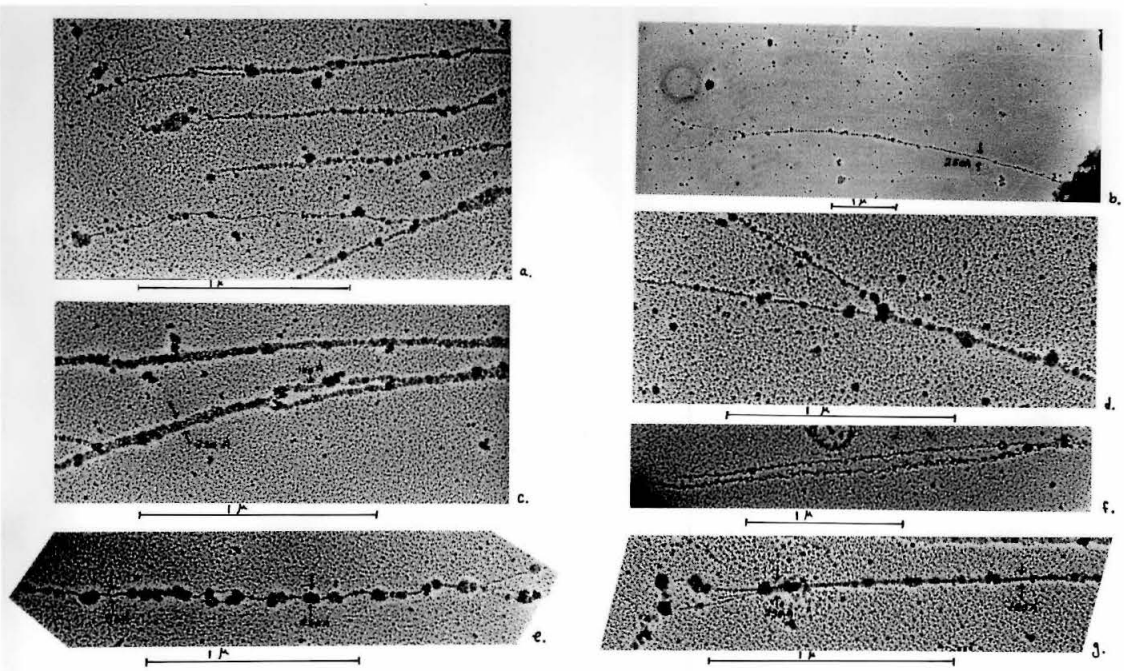

Figure 6. Macrofibers from chick erythrocyte nuclei lysed directly on the electron microscope grid in 0.1 M β-mercaptoethanol in distilled water. Tungsten shadowing. Basic doubleness or quadrupleness of the macrofibers is apparent.

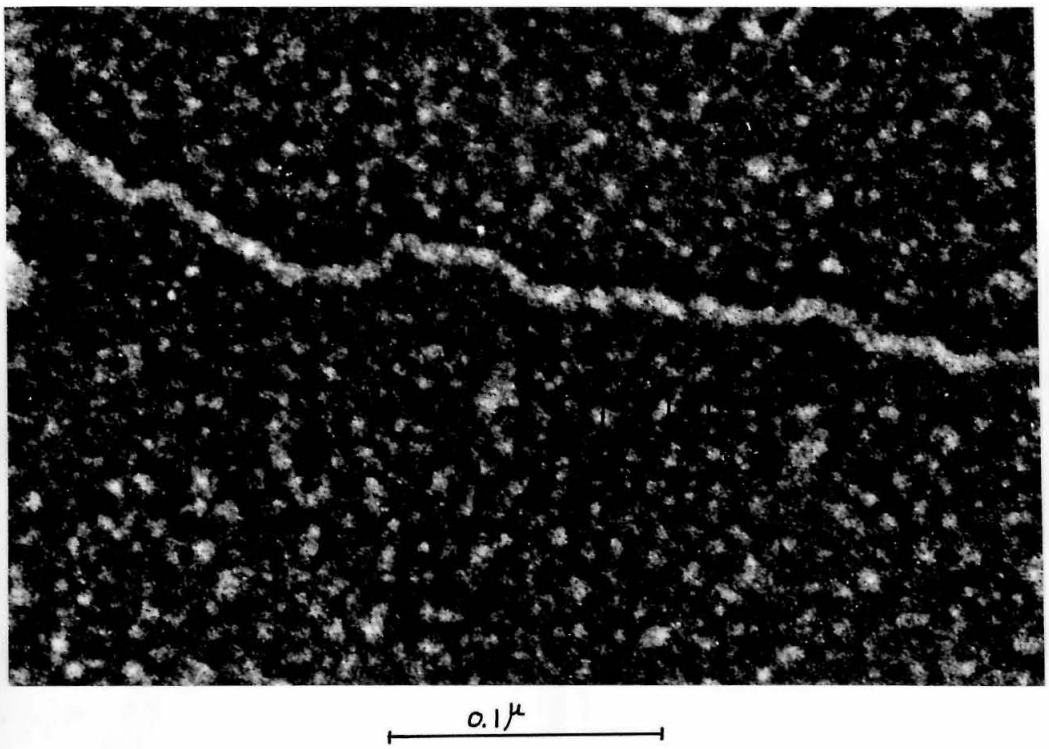

Figure 7. Supercoiling of the 30 Å nucleohistone fiber. Under the best conditions of resolution and mounting, the non-knobbed 30 Å nucleohistone fibers show an apparent coiling whose repeat distance is 100 Å to 150 Å. Fibers which appear to have been stretched during mounting may show larger repeat distances or no coiling at all.

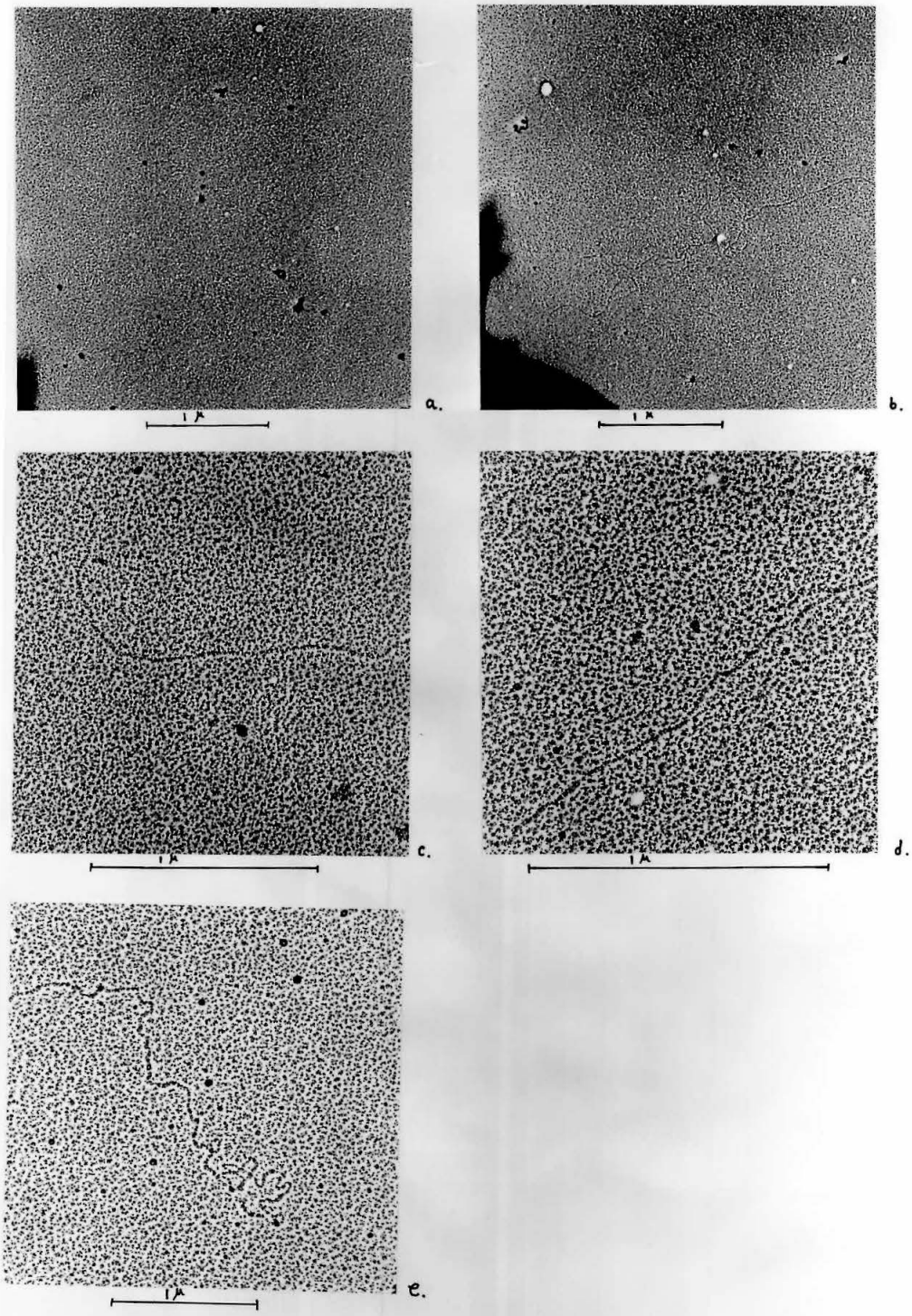

Figure 8. Nucleohistone fibers from pea buds spread with cytochrome in the presence of various concentrations of NaCl. (a) 0.3 M NaCl; (b) and (c) 1.0 M NaCl; (d) 2.0 M NaCl; (e) purified pea bud DNA spread from 1.0 M NaCl.

DISCUSSION

Our knowledge of chromatin structure can be summarized by the following two statements:

1. The interphase chromosomes of all creatures possessing histone proteins (all eukaryotes) have the same fibral structure.

2. There is one basic chromosomal fiber, a single DNA duplex complexed with an approximately equal amount of histone protein. Fibers which are being transcribed contain RNA polymerase-like proteins. In heterochromatic or metaphase states, the basic fiber condenses into macrofibers.

The universal presence of histones in the chromosomes of all higher creatures has been proposed by Bonner (Delang, Smith and Bonner, 1969) on the basis of detailed studies of the chromosomal composition of higher creatures. In addition, it has been demonstrated (Delang et al., 1968) that the amino acid sequence of one of the histones, histone IV of pea bud tissue, is identical (only 2 conservative replacements in 102 amino acids) to the sequence of histone IV of calf thymus. It appears that this extraordinary structural conservation may be true for all of the histones. If the composition and relative amount of the 8 histones remain the same in all higher creatures, it would seem reasonable that the manner in which they complex with the DNA would not vary. This is what has been found.

We have separated the chromatin into three classes for ease of discussion. The distinctions are made on the basis of appearance, and,

we believe, function. It should be apparent, however, that an entire spectrum of fibers is present and that any particular gene may be found in any of the fiber classes depending on the state of the cell.

The comparison of Tetrahymena and chicken erythrocyte chromatin has been extremely valuable. From this comparison we can conclude that the heterochromatin seen by light microscopists is normal chromatin condensed into 230 Å macrofibers. Heterochromatin does not differ greatly in chemical composition from diffuse chromatin. The macrofibers are inactive because they are too highly condensed to support RNA synthesis. This is probably due to the inability of RNA polymerase to reach its binding site. There is too much material in the macrofibral form in tissues such as pea bud to be attributed solely to metaphase chromosome relics. The macrofibers seem rather to be associated with the differentiated nature of these tissues.

Of the possible models of the structure of the 230 Å fibers, several have been ruled out by these studies, but one in particular seems attractive. The work of DuPraw (DuPraw and Bahr, 1969) on these fibers indicates that the DNA is then condensed to a level of 50 to 100 DNA duplexes (as nucleohistone fibers)/unit length of macrofiber. One possible arrangement is that 50 to 100 separate duplexes lie side-by-side to form the macrofiber. Our studies on the selective disruption of these fibers, and the high resolution micrographs of the native fibers show that this cannot be the case. Alternatively, one 30 Å nucleohistone fiber could be tightly supercoiled to form this fiber. The common appearance of two separate fibers lying side-by-side

in acid-extracted macrofibers precludes this model. The more attractive model is that the macrofiber is condensed from one or two DNA duplexes which lie along the length of the macrofiber, loop about at the end, and run back to end at the origin. Similar models have been suggested by others (Hearst, 1969). The DNA, in the form of a nucleohistone fiber, is highly coiled producing a 20 to 50-fold foreshortening of the total duplex length. This allows the DNA of the chromosome to be organized into discrete bundles.

The structure of the basic nucleohistone fiber can be described with assurance as a single DNA duplex covered with a stoichiometric amount of histone protein. The presence of histone produces a super-coiling of 120 Å pitch seen by X-ray diffraction (Wilkins, Zubay and Wilson, 1959; Pardon, Wilkins and Richards, 1967) as well as by electron microscopy. During transcription, these fibers are marked by the presence of large non-histone protein units which we believe to be primarily composed of RNA polymerase.

The results of our histone distribution studies indicate that the histones are thoroughly intermingled, and that at any one point on the DNA, more than one histone of a particular class is complexed to the DNA.

These studies also demonstrate that there are no lengths of DNA in chromatin even 10% of the average gene length (2000 Å) which are entirely bare of histone. This is even true for Tetrahymena chromatin in which one-half of the genes at any one time are in the active state. It may be necessary, therefore, to restructure our thinking of DNA transcription by RNA polymerase in higher organisms to include the

intimate association of histones.

CONCLUSION

The interphase chromosomes of all higher creatures possessing histone proteins have the same fibral structure. There is only one basic fiber, a single DNA duplex complexed with an approximately equal amount of histone protein. During transcription these fibers are distinguished by RNA polymerase-like proteins; during metaphase or periods of dormancy they condense into macrofibers.

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TETRAHYMENA: A BIOPHYSICAL AND BIOCHEMICAL STUDY OF ITS GENOME

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Recent studies of genetic regulation in higher creatures have used the great variation in genome size of such creatures as a tool for developing model of control (Britten and Davidson, 1969). The DNA content per genome of the simplest of higher organisms is only a few times that of E. coli (0.004 μμg) while at the other end of the scale are organisms possessing of the order of 100 μμg DNA per genome. Extreme conservation in the composition of the histone proteins of chromosomes has also been found. These basic proteins are complexed to the chromosomal DNA of all higher creatures. For studies of gene regulation, a simple higher organism would be useful. Such an organism was suggested by the protozoan Tetrahymena. Lee and Scherbaum (1966) have shown that the macronucleus contains histones similar to those of calf thymus and rat liver tissues. Tetrahymena retains many of the advantages of bacterial systems: rapid growth, ease of radioactive labeling and suspension culture. Furthermore Tetrahymena is essentially non-differentiated and forms no chromosomal structures (amicronucleate strain).

In this paper a method for isolation of macronuclear chromatin is described. The physical-chemical and genetic properties of this chromatin are compared to those of other previously studied higher creatures.

MATERIALS AND METHODS

Culture of the Cells

Tetrahymena pyroformis W cells were cultured in a 1% (w/v) proteose peptone (Difco), 0.25% (w/v) yeast extract (Difco) medium with vigorous aeration. Thirty liter batches were concentrated by continuous flow centrifugation yielding one hundred grams of packed cells (3-4 day culture).

Isolation of Nuclei and Nucleohistone Fibers

The macronuclei were isolated in an isolation medium containing: 0.25 M sucrose, 0.002 M ($CaCl_2$), 0.003 M ($MgCl_2$), Tris 0.01 M, pH 8.0. Lysis was accomplished by a modification of the technique of Lee and Scherbaum (1966); 100 grams of cells were suspended in 300 ml of isolation medium with 0.3% (v/v) triton x 100. The cells are shaken until lysis is complete. This is monitored with a standard Leitz phase contrast microscope. The macronuclei are collected by centrifugation at 5,000 g for 10 minutes, then washed twice by suspension in isolation medium and centrifugation for 10 minutes at 3,000 g. The nuclear pellet is then suspended in 45 ml of isolation medium made 1.5 M in sucrose. 15 ml volumes are layered over 5 ml volumes of 2.3 M sucrose (in isolation medium) in 3 Spinco SW25 rotor tubes. Centrifugation at 22,000 rpm in the SW25 rotor for 1 hour pellets the macronuclei, freeing them of all membrane contamination.

For electron microscopy, the nuclei are suspended in 25 (v/v) % glycerol, 0.005 M EDTA, pH 7.5.

For the isolation of nucleohistone fibers, the nuclei are washed in 0.15 M NaCl, 0.005 M EDTA pH 8.0 by suspension and centrifugation at 10,000 g for 10 minutes. The nuclei are lysed by suspension with stirring for 30 minutes in 200 ml of 0.01 M Tris pH 8.0. After lysis is complete, the crude chromatin is washed by repeated suspension and pelleting, by centrifugation, in 0.15 M NaCl, 0.005 M EDTA pH 8.0.

The crude chromatin is sheared and debris pelleted yielding a solution of nucleohistone fibers. The crude chromatin is suspended in 50 ml of 0.01 M Tris pH 8.0 and sheared in a Virtis "45" for 90 seconds at 45 volts. The debris is pelleted by centrifugation for 10 minutes at 10,000 g. The fibers may be further purified by repeated precipitation in 0.15 M NaCl, 0.005 M EDTA pH 8.0, and collection by centrifugation at 10,000 g for 10 minutes. The fibers are dissolved in 0.01 M Tris pH 8.0.

Electron Microscopy

Samples were prepared for electron microscopy by methods described in detail elsewhere (Griffith, 1969a).

On-grid lysis was accomplished by transferring with a glass needle a few nuclei from a nuclear suspension to a carbon covered grid supporting a 20 λ drop of distilled water. Lysis may be monitored by light microscopy. Following lysis, dehydration is performed by rapid immersion in 95% ethanol for one minute. The grids are rotary shadowed with platinum or tungsten at an angle of 1:8. Samples are stained with uranyl acetate using the procedure of Wetmur, Davidson and Scaletti (1966) or with fresh 1% aqueous solutions at acid pH.

Composition of Nucleohistone, Template Assays

Chemical composition of the nucleohistone fibers was determined using the exact procedures of Bonner et al. (1968a). Histones are extracted with 0.2 N H_2SO_4 rather than HCl which has been found to extract nonhistone acid-soluble proteins. Characterization of Tetrahymena histones by polyacrylamide gel electrophoresis is always carried out with pea bud or calf thymus histones for comparison.

Template capacity was measured using the low salt incubation medium of Bonner et al. (1968a). RNA polymerase is isolated as fraction 4 from E. coli by the method of Chamberlin and Berg (1962) and Bonner et al. (1968a).

Highly purified DNA was prepared by the method of Ho and Bonner (1969) using the Sevag procedure of Marmur (1961) followed by RNAse treatment and CsCl sedimentation.

Renaturation of Tetrahymena DNA

The melting and renaturation of Tetrahymena DNA was done following the methods of Wetmur (1967) and Ho and Bonner (1969). DNA at a concentration of 90 µg/ml in buffer was sheared to an average molecular weight of 600,000 and melted by boiling for 10 minutes. The DNA was cooled to 68°C and renaturation followed by the change in optical absorbance at 260 mµ, using a Gilford 2000 recording spectrophotometer.

RESULTS

Electron Microscopy

Tetrahymena nucleohistone fibers were found to aggregate and precipitate from solution at ionic strengths below that of the 0.01 M Tris buffer. Selective removal of the histone proteins by 0.2 N H_2SO_4 does not alter this effect. Since low ionic strength, volatile buffers are normally used for mounting DNA or nucleohistone fibers for high resolution electron microscopy the nuclei were lysed directly on the grid. Nuclei spread in this manner are shown in Fig. 1a,b. The chromosomal material appears to be composed entirely of long fibers 30 Å thick. No 230 Å macrofibers have been observed. On-grid lysis with a 0.1% SDS solution (Fig. 1c) yields apparently bare DNA fibers spread in the same manner as the chromosomal fibers. Typical 30 Å nucleohistone fibers from pea bud nuclei are shown in Fig. 1d.

Chemical Composition

Chemical analysis of purified nucleohistone isolated from 500 grams of cells taken from 1, 2, 3, 4 and 7 day old cultures has been performed. Under our growth conditions, stationary phase is reached between the 3rd and 4th day. The chemical composition has been found to remain essentially constant from the 1st to the 7th day. Only a slight relative increase in RNA content has been found. The 3rd day composition is shown in Table 1. The values for other tissues were obtained by others in this laboratory using procedures identical to those used for this work.

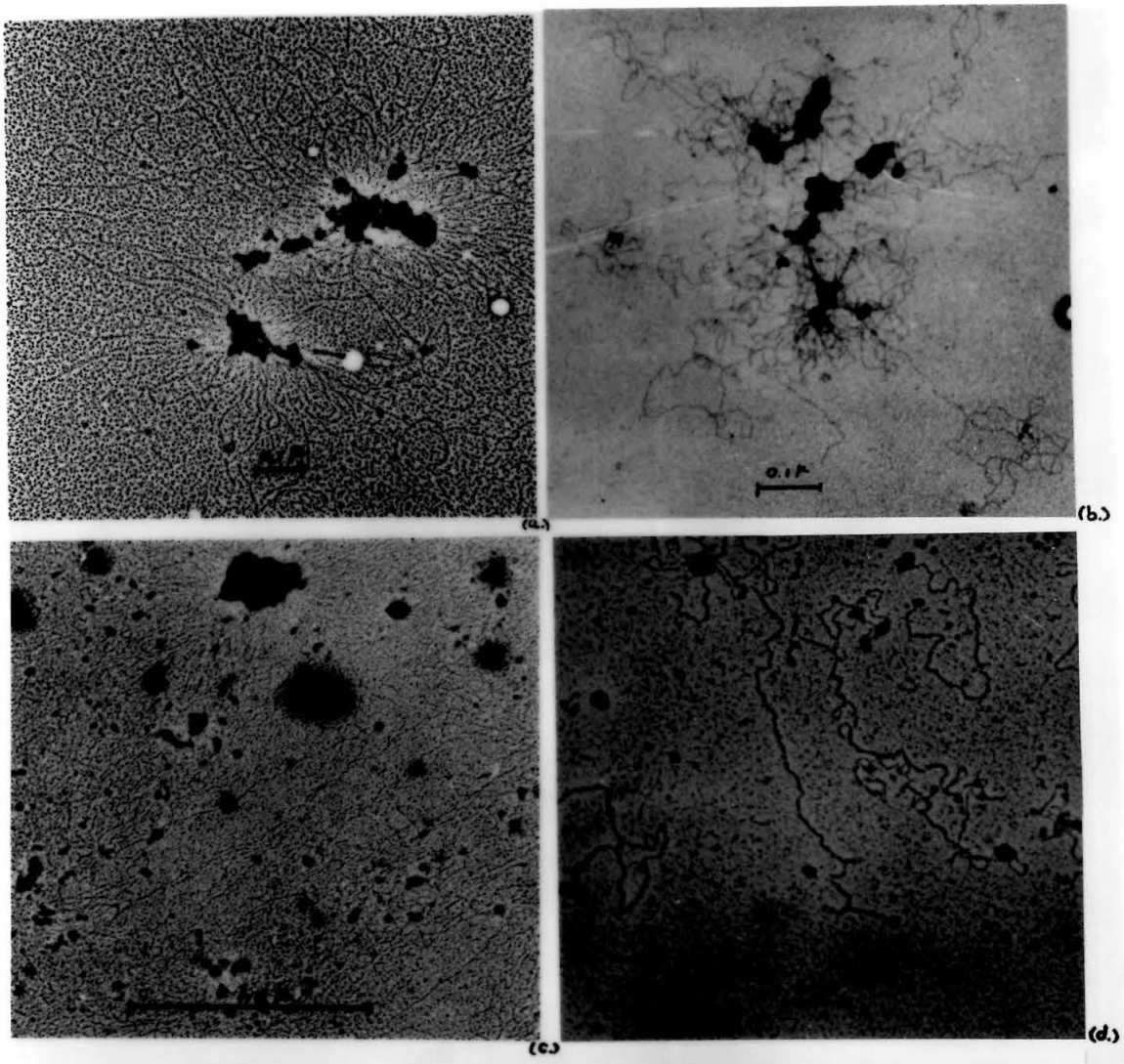

Figure 1 (a,b,c,d). Tetrahymena chromosomal fibers spread by on-grid lysis; (rotary) shadowed with platinum (a) and stained with uranyl acetate (b). Tetrahymena nucleus lysed on grid with 1% SDS solution to remove protein, platinum shadowing. (d) Typical pea bud chromosomal fibers mounted directly from solution; tungsten rotary shadowing.

TABLE 1

CHEMICAL COMPOSITIONS OF VARIED NUCLEOHISTONES

Nucleohistone	Content Relative to DNA of: DNA	Histone	Non-histone Protein	RNA	Template Activity: % of DNA
Tetrahymena	1.00	.65	.76	.09	38%
Sea Urchin Pluteus[1]	1.00	.86	1.04	.08	20%
Pea Vegetative Bud[1]	1.00	1.05	.500	.10	20%
Rat Liver[1]	1.00	1.00	.67	.04	20%
Cow Thymus[2]	1.00	.95	.33	.01	15%
Chicken Erythrocyte[3]	1.00	-	-	<.02	<1%

[1]From Bonner et al. (1968b)

[2]From Chalkley and Jensen (1968) and Smart and Bonner (1969)

Histones

Acid extracted histone proteins from purified nucleohistone were separated by electrophoresis on 7-1/2% polyacrylamide gels. Following staining with Buffalo Black the gels were densitometered with a Canaleo Model 2000 densitometer. A typical trace from a 3-day culture is shown in Fig. 2. No differences in the electrophoretic patterns of histones extracted at various stages of the growth period were observed. The patterns show multiple histone III bands typical of oxidized animal histone III.

Template Activity

The ability of purified nucleohistone to serve as a template for RNA synthesis with added E. coli RNA polymerase was measured relative to purified DNA. The reactions were carried out under standard low salt conditions with increasing amounts of DNA to saturation of the reaction (Bonner et al., 1968).

A typical result of these experiments is shown in Fig. 3. The template capacity of the nucleohistone relative to purified DNA at saturation is 38%. Saturation values for 1-1/2, 3, 4 and 7 day cultures are given in Table 2.

DNA Renaturation

Highly purified Tetrahymena DNA was sheared to a molecular weight of 600,000. The DNA was melted by boiling 10 minutes in a 1 M NaCl Tris buffer and cooled to 68°C. Renaturation was monitored by hyperchromicity change at 260 mμ in a Gilford 2000. Two distinct components are observed. A rapidly renaturating component comprising

TABLE 2

TEMPLATE CAPACITY OF NUCLEOHISTONES

FROM TETRAHYMENA CULTURES

Age of Culture: Days	Template Capacity at Saturation Relative to DNA
1-1/2	39%
3	36%
4	38%
7	42%

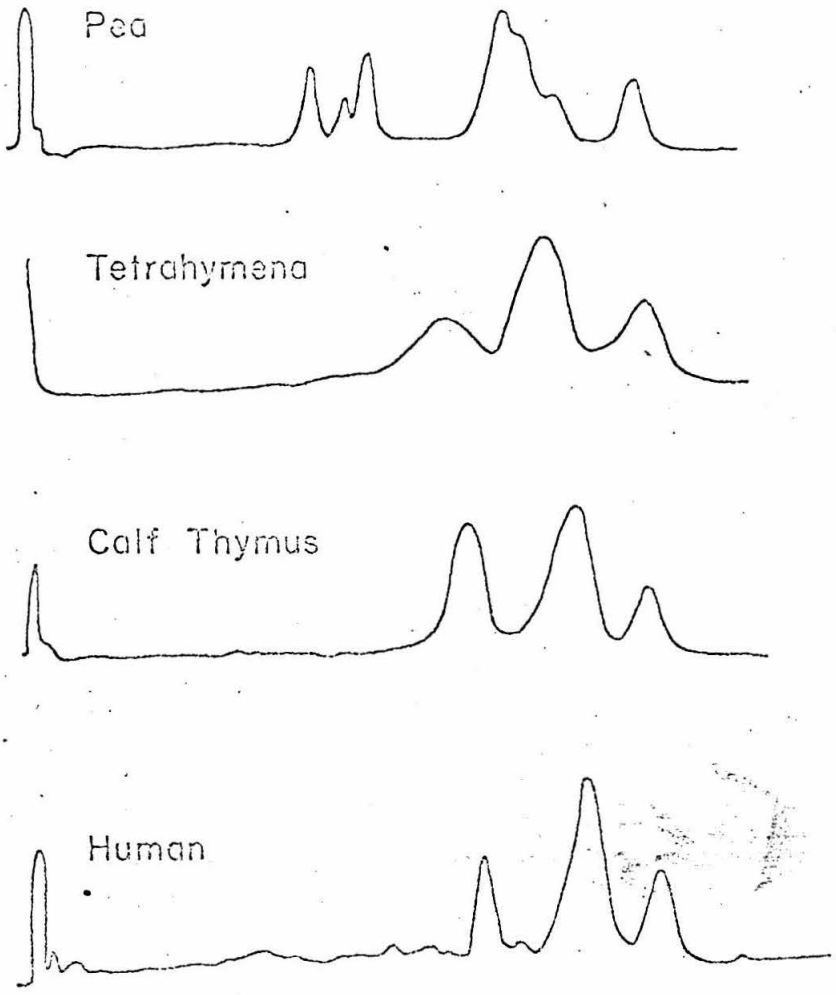

Figure 2. Polyacrylamide gel electrophoresis patterns of histones isolated in this laboratory from a variety of sources.

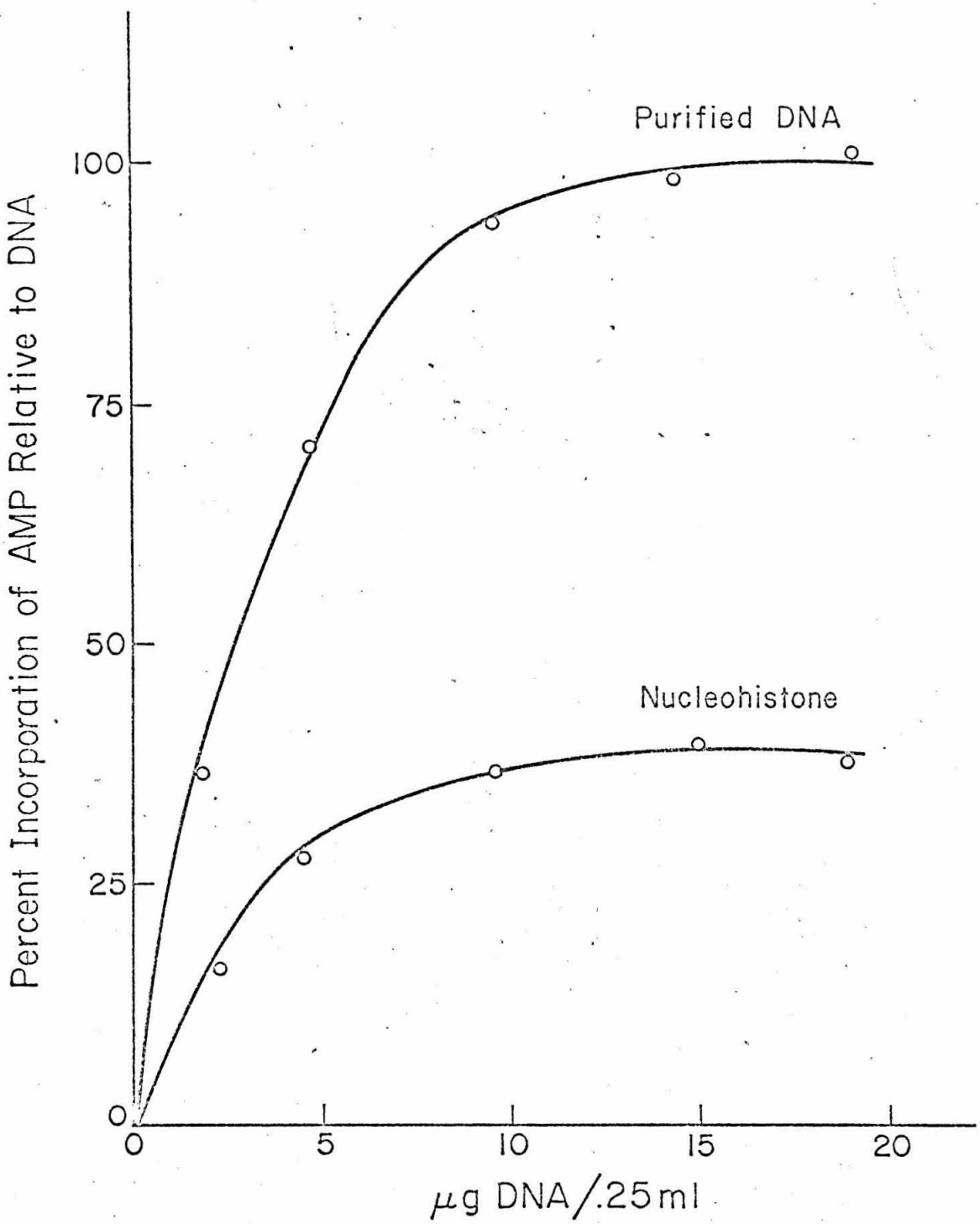

Figure 3. Template activity of Tetrahymena Nucleohistone and DNA.

7% of the total DNA renatures with a half time of about 40 seconds. The remaining 93% of the DNA renatures as one family with a half time of 5.5 hours (Fig. 4). From the slope of the reciprocal renaturation plot, the second order rate constant can be calculated by the relation K_2 = (slope 1.9×10^{-4}). The second order rate constant is dependent on G + C content (Wetmur, 1967). Correction for the low G + C content of 30 mole percent to 50 mole percent G + C gives K_2 fast = 12 liter/mole-sec, K_2 slow = 0.96 liter/mole-sec. From the half times for renaturation, and the initial concentration of DNA (1.8 OD_{260}/ml), the Tetrahymena DNA may be compared with other creatures. Drosophila contains 0.2 μμg DNA/haploid nucleus and the rate of renaturation of the slowest component of Drosophila DNA is such that at a concentration of 1.8 OD_{260}/ml $t_{1/2}$ slow = 30.5 hrs. By comparison we calculate that the DNA content per haploid nucleus of Tetrahymena must be 0.04 μμg/hap. nuc. Since $t_{1/2}$ (slow Tetrahymena) = 5.5 hours, 5.5/30.5 x 0.2 μμg DNA/nucleus = 0.04 μμg/haploid nuc. Comparison of the reannealing rates for the fast and slow components and their relative proportions suggests that the fast component is 42-fold redundant over the slow renaturing fraction. Structural genes may be considered to be of the order of 2000 base pairs in length. We have, in work to be published elsewhere, measured the length of the redundant genes by electron microscopy and found them to be close to 200 base pairs in length. Using these values leads to the estimates in Table 3 for the number of unique and structural genes in the Tetrahymena genome.

Figure 4. Second order rate plot for renaturation of sheared tetrahymena DNA. The slope of the fast component is 1.2×10^{-3} sec^{-1}. K_2, the second order rate constant is obtained by dividing the slope by 1.95×10^{-4} (Wetmur, 1967). K_2 (fast) = 6.0 liter/mole-sec. Similarly, slow (slow) = 9.3×10^{-5} sec^{-1} and K_2 (slow) = 0.48 liter/mole-sec.

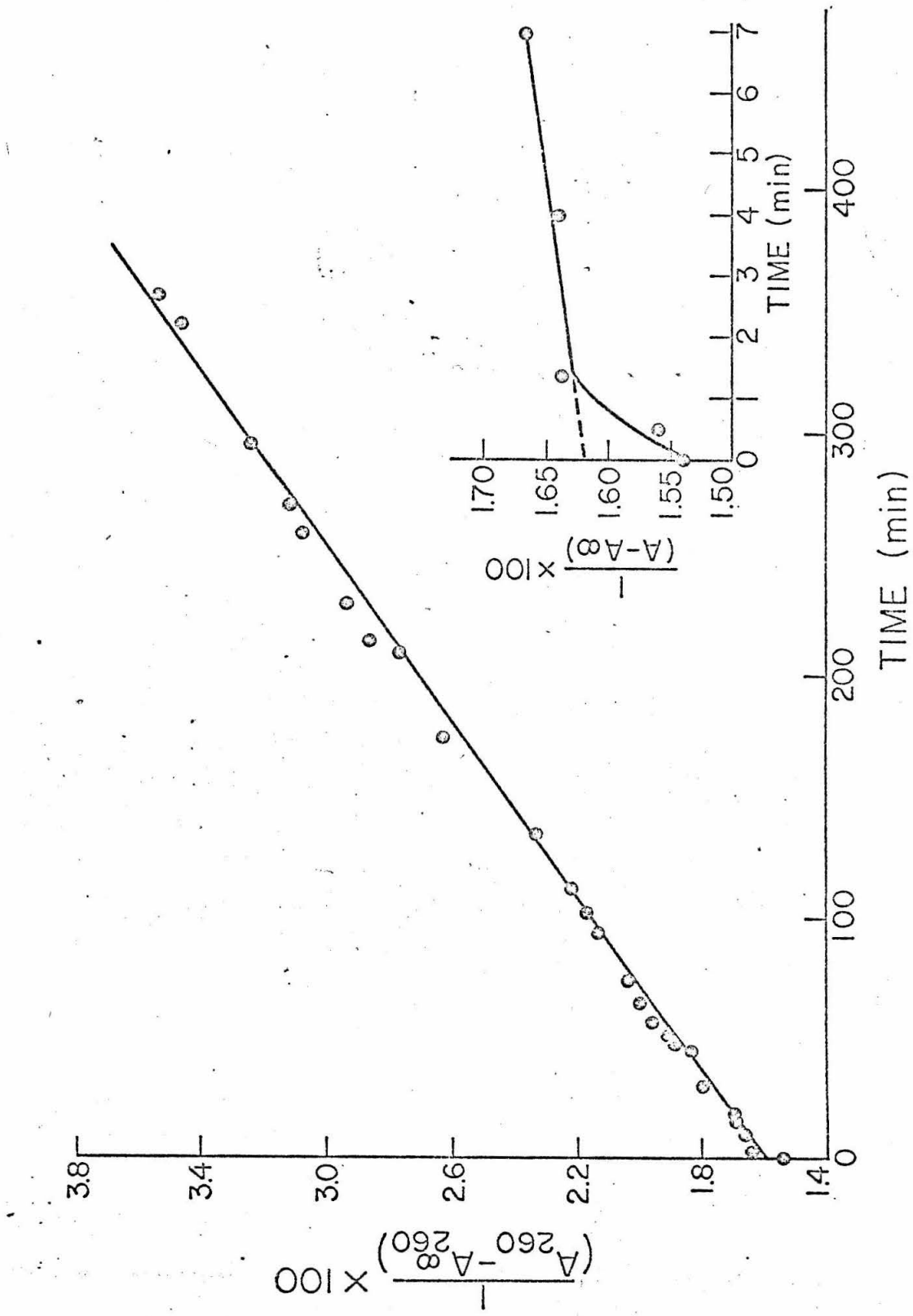
$\frac{1}{(A_{260}-A_{280})} \times 100$
3.8
3.4
3.0
2.6
2.2
1.8
1.4
0
100
200
300
400
TIME (min)
$\frac{1}{(A-A_\infty)} \times 100$
1.70
1.65
1.60
1.55
1.50
0
1
2
3
4
5
6
7
TIME (min)

TABLE 3

COMPLEXITY OF TETRAHYMENA GENOME

	μμg DNA/Haploid Set	Cot. 1/2	Base Pairs/Nucleus	Redundancy
Redundant	0.0027	3.6×10^{-2}	3.4×10^{6}	42
Unique	0.0370	20.4	45×10^{6}	1

	Estimated Size of Genes	Number of Genes	Number of Families
Redundant	200 base pairs	1.7×10^{4}	405
Unique	2000 base pairs	2.2×10^{4}	22,000

Number of redundant genes/structural (unique) genes = 0.7

DNA content/haploid set of other similar creatures

Neurospora 0.17

Yeast 0.245

Dyctoselium 0.04

Tetrahymena 0.04

DISCUSSION

For Tetrahymena to be of use in studies of higher creatures, it is essential to have a comparison of its genome with genomes of well characterized higher creatures. The physical and chemical nature of interphase chromosomes of creatures of the highest orders is well known (Bonner et al., 1968b). The basic chromosomal fiber, termed a nucleohistone fiber, is composed of a single DNA double helix complexed with the relatively homogeneous class of basic proteins, the histones, and with a smaller amount of non-histone protein. It also contains a small amount of RNA known as chromosomal RNA.

These fibers have been shown by electron microscopy to be 30 Å in width, extremely long, and supercoiled with a pitch of 150 Å and width of 100 Å (also shown by X-ray diffraction to have a 110 Å pitch (Wilkins, Zubay and Wilson, 1959; Pardon, Wilkins and Richards, 1967). Isolated interphase chromatin of such tissues as pea bud or rat liver contain two other classes of fibers. These are 1) 230 Å thick macrofibers highly condensed from one or several 30 Å nucleohistone fibers, and 2) single nucleohistone fibers of 30 Å or less width, which lack the 110 Å supercoiling and exhibit numerous knobs shown to be non-histone proteins. The size of the knobs (150 Å - 250 Å) is typical of RNA polymerase (Griffith, 1969b).

The aggregation of Tetrahymena nucleohistone fibers at low ionic strengths has excluded the highest resolution electron microscopy by our methods. These aggregates appear as highly condensed knobular bundles. Further confusion may be added by the presence of the macrocomplexes of uranyl acetate formed in aged aqueous solutions at neutral

pH. These macro-complexes have an electron density typical of stained protein and may appear as a tangle of 300 Å knobular fibers (Griffith, 1969c). These forms may account for reports of anomalous chromosomal structures in Tetrahymena (Wolfe, 1967).

On-grid lysis of purified nuclei provides a simple and least ambiguous picture of the state of the chromosomal material as it exists in the nucleus. Using this method, we have clearly demonstrated that the chromosomal material as it exists in the nucleus is composed entirely of long uniform fibers whose width is 20 Å-30 Å. No large chromosomal structures or 230 Å microfibers have been observed. Removal of protein from the fibers by treatment on the grid with 1% SDS yields similarly appearing bare DNA fibers (Fig. 1a,b,c,). The electron microscopic evidence then suggests that the Tetrahymena genome is constructed of fibers similar to those of higher plants and animals. It lacks the thick macrofibers typical of the more complex differentiated creatures. More stringent tests of the similarity of Tetrahymena nucleohistone to other nucleohistone are the chemical composition and character of the histone proteins. The complexity of the genome may be studied by template assays and DNA renaturation experiments.

The histones of many higher creatures have been studied and show a striking similarity. The amino acid sequence for histone III from pea bud and calf thymus tissues has been determined by Delang, Smith, Fambrough and Bonner, 1968). The sequences differ by only 2 conservative replacements in 102 residues. This strong conservation may be true for all of the histones. The presence of histone protein

serves as an excellent criterion for distinguishing higher and lower creatures.

Tetrahymena has been shown to possess histones whose amino acid composition and gel electrophoresis patterns are similar to the previously characterized histones from plants and animals (Lee and Scherbaum, 1966). In the work reported here, histones extracted from purified nucleohistone have been shown to produce gel electrophoresis patterns identical to those of cow and rat histones and similar to plant histones. The electrophoretic differences between plant and animal histones has been reported by Fambrough and Bonner (1966), and are due to the presence of dimers and multimers of histone III in the animal histones and of dimers alone in plant histones. These are produced by the presence of an additional sulfhydryl group in the histone III of animals. Considering the strong conservation of histones in evolution this suggests that Tetrahymena is more animal-like than plant-like.

From the data on Table 1, it is clear that the nucleohistone of Tetrahymena is similar in all respects to higher plants and animals. The differences, a low histone to DNA ratio, and high content of non-histone protein both reflect the greater level of genetic activity.

Recent studies here have indicated that regions of the genome undergoing transcription are partially devoid of histones, contain chromosomal RNA, and have a proportionately large amount of non-histone proteins of which RNA polymerase is believed to comprise a large fraction. By inference from the template assays, Tetrahymena appears to have perhaps 40% of its genome in the act of transcription at any one time.

Fractionation by the method of Marushige into template active and template inactive fractions indicates that 50% of the nucleohistone is in a high histone, low non-histone (relative to DNA) state. This is separated from the remainder which is poor in histone but contains most of the non-histone protein.

From the work of Wetmur (1967) and others, we have a method for determining the level of genetic complexity of any organism. The amount of DNA per genome present in unique and redundant families may be determined and the number of genes present in one or multiple copies may be estimated by knowing the reannealing rates of the sheared denatured DNAs and the total amount of DNA per genome of that creature. Bonner (1969) has noted a striking correlation between the evolutionary level of an organism and the total amount of redundant DNA. Britten and Davidson (1969) propose that this DNA provides a controlling system for the unique (structural) genes. All higher creatures studied thus far are distinguished from bacteria by the presence of some redundant DNA and histones -- both control factors. The proportion of redundant DNA varies from 10% of the total in Drosophila to 70% in Lily.

The results of these experiments with Tetrahymena DNA indicate that it possesses a small (7%) but highly significant fraction of rapidly reannealing (redundant) DNA. The remaining DNA reanneals very slowly as a single class. Assumptions about the size of regulator and structural genes and that the slowly annealing class is present in one copy of each gene per copy of the genome are necessary. When these assumptions are made, it appears that the Tetrahymena genome is 5 times larger than E. coli and contains a small but significant number of

regulator (redundant) genes.

The overall picture of Tetrahymena is that of a true but extremely simple higher creature. The absence of some of the more complex genomic functions makes it an ideal system for the study of the most basic mode of gene control in higher creatures. These simplifications are: the absence of metaphase chromosome structures (a-micronucleate strains), the absence of cellular differentiation, and the inability to control its overall rate of metabolism to suit the environment. Because of the last aspect, Roth has likened Tetrahymena to cancer cells (Roth, 1965). The lack of control at the environmental level is reflected in the constant template activity of the nucleohistone over the growth cycle.

We attribute the condensed 230 Å macrofibers in other chromatins to metaphase chromosome relics and to the heterochromatin of all differentiated creatures. Their lack in Tetrahymena is understandable.

CONCLUSION

We have found Tetrahymena to be a true but very simple higher creature. The physical structure and chemical composition of its nucleohistone resembles other higher creature nucleohistones in all critical aspects. The character of its histones suggests an animal-like nature of this protozoan. A very high level of genetic activity has been shown. The relative simplicity of Tetrahymena's mechanisms for genetic control has been inferred from DNA renaturation studies. Tetrahymena appears to provide an excellent system for the study of the most basic genic control in higher organisms.

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URANYL ACETATE STRUCTURES

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Uranyl acetate has become a widely used stain for electron microscopy. It is used to positively stain both nucleic acids and proteins, and is finding increasing use for staining macromolecular structures mounted on grids from solution.

We have investigated its use for staining the 30 Å and 230 Å classes of chromosomal fibers. Uranyl acetate has been used for this purpose by others (Ris, 1967; Wolfe, 1967). We have found that under particular conditions the stain itself will form macro-complexes. These complexes vary greatly in size and structure and have an electron density similar to that of stained biological materials. A very common form is shown in Fig. 1 (top, inset). These forms appear as bundles of knobular fibers 300 Å in width. Typical smaller structures are shown in Fig. 1 (bottom). Stereomicroscopy and rotary shadowing shows that the structures are three dimensional and have a filamentous "nylon stocking" structure.

Studies on the formation of these structures has shown that they form in aqueous solutions of uranyl acetate on aging for one week or more. Their formation is enhanced by the presence of salts such as CsCl or NaCl. Formation is inhibited by acid pH. We have not been able to produce these structures in non-aqueous solutions of uranyl acetate.

To avoid these structures, we recommend using fresh aqueous solutions at pH 4 or below or the non-aqueous method of Wetmur (1966).

Because of the resemblance of the knobular fiber form to the macrofibers of chromosomes, and smaller structures to other protein complexes, we feel that extreme care must be taken to avoid possible misinterpretation when using this stain.

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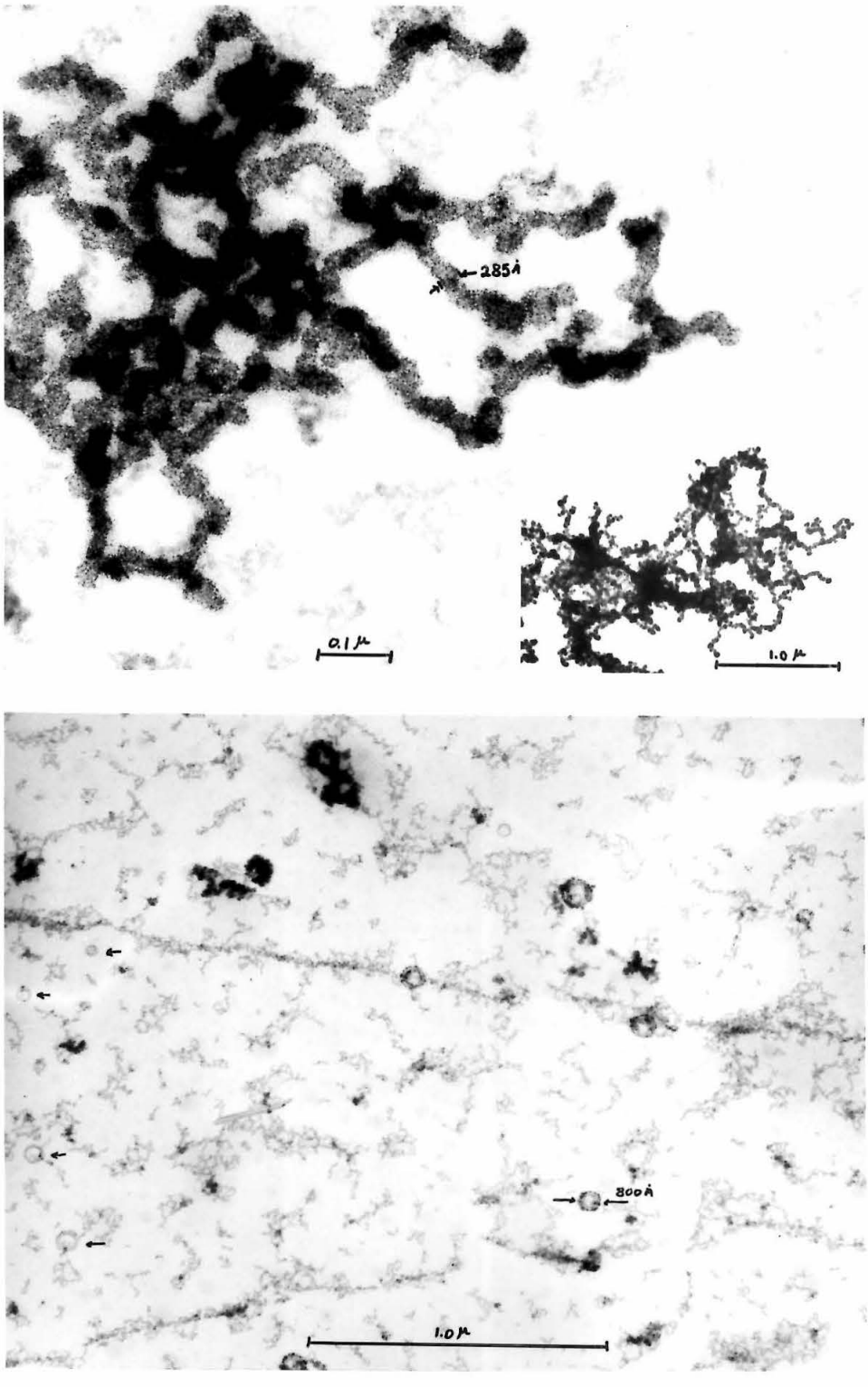

Fig. 1. Uranyl Acetate Complexes.

Large fibrous forms (top and inset) and doughnuts (bottom) present in aqueous solutions of uranyl acetate.