AN ELECTRON MICROSCOPE STUDY OF DNA MOLECULES FROM *CHIRONOMUS TENTANS* AND *CHIRONOMUS THUMMI*

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IN recent years substantial information has been gained concerning the size and topology of DNA (deoxyribonucleic acid) molecules of viruses (for reviews see VINOGRAD and LEBOWITZ 1966; COHEN 1967; THOMAS and MACHATTIE 1967), bacterial plasmic and sex factor (ROTH and HELINSKI 1967; HICKSON, ROTH and HELINSKI 1967), *Mycoplasma hominis* (BODE and MOROWITZ 1967), bacteria (CAIRNS 1963) and mitochondria (see for example, VAN BRUGGEN, BORST, RUTTENBERG, GRUBER and KROON 1966; SINCLAIR and STEVENS 1966; DAWID and WOLSTENHOLME 1967, 1968; HUDSON and VINOGRAD 1967; CLAYTON and VINOGRAD 1967). Few studies, however, have been made on the size and form of DNA molecules of chromosomes of higher organisms, even though such information is an essential prerequisite to an understanding of the way in which chromosomes are constructed. HOTTA and BASSEL (1965) examined rotary shadowed DNA from isolated wheat nuclei and boar sperm in the electron microscope and concluded that the native molecules of both higher plants and animals vary markedly in length, up to more than 100 μ . They also found a number of circles of DNA from boar sperm of contour lengths varying from 0.5–16.8 μ . SOLARI (1965, 1967) reported DNA molecules at least 108.5 μ long in electron microscope preparations from sea urchin sperm and isolated nuclei. By applying the autoradiographic technique of CAIRNS (1963) to Chinese hamster cells grown *in vitro*, HUBERMAN and RIGGS (1966) obtained rows of grains of various length up to about 1.8 mm, which they attributed to single DNA molecules. In similar preparations of HeLa cells (CAIRNS 1966) and human lymphocytes (SASAKI and NORMAN 1966) rows of grains up to 500 μ and 2.2 cm, respectively, were found.

The present report relates the results of experiments on the form and size distribution of DNA molecules from polytene salivary gland chromosomes of larvae of *Chironomus tentans* and *Chironomus thummi*.

MATERIALS AND METHODS

Salivary gland nuclei were isolated from *Chironomus tentans* and *Chironomus thummi* fourth instar larvae according to the method of RISTOW and ARENDS (1968). Salivary glands were isolated from the severed front ends of about 150 larvae by centrifugation for 15 min at 4°C in an

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SW-39 rotor (Spinco Model L) at 35,000 rpm in a discontinuous sucrose gradient (20%, 65%, and 80% w/v) containing 0.01 M Tris (pH 7.0), 0.05 M KCl and 0.0015 M MgCl_2 . The glands were removed from the middle phase, washed in an isotonic buffer containing 0.01 M Tris (pH 7.0), 0.003 M MgCl_2 , and 0.22 M sucrose, and then treated in turn with 0.1% chitinase (Calbiochem) for 5 minutes at 20°C, 1% Tween 80 for 5 minutes at 20°C, and 0.15% sodium deoxycholate at 4°C for 20 minutes. The glands were gently pipetted to release the nuclei and the suspension poured through a 53 μ mesh nylon filter which traps unbroken cells and cell debris. The nuclei were then alternately washed with isotonic buffer and centrifuged at low speed, and finally suspended in 0.5 ml of buffer. SDS (sodium dodecyl sulphate) was added to 0.25% and the mixture very gently stirred. After 10 min a sample was removed with a wide bore (at least 2 mm) pipette and prepared for electron microscopy. A second sample was incubated with 0.5 mg/ml of DNase (deoxyribonuclease) for 4 hrs at 37°C and then prepared for electron microscopy; the pronase had been freed from DNase activity by the method of HORRA and BASSEL (1965). A third sample was shaken with phenol and the aqueous layer treated with 80 $\mu\text{g}/\text{ml}$ RNase (ribonuclease) for 30 min. Half of the latter sample was prepared for electron microscopy directly and the other half after digestion with 1 $\mu\text{g}/\text{ml}$ DNase for 5 min.

The samples were prepared for electron microscopy essentially according to the method of FREIFELDER and KLEINSCHMIDT (1965). Approximately 0.1 ml of a 1 M ammonium acetate solution containing 10 μl of the DNA sample, (about 0.24 μg of DNA) 0.01% cytochrome c, and 0.5% formaldehyde was allowed to flow down an inclined glass slide onto a hypophase of 0.3 M ammonium acetate containing 0.5% formaldehyde. The surface film was picked up on carbon coated formvar films supported on 100 mesh copper grids. The grids were then shadowed on a rotary turntable at an angle of 10° with 7 mg of platinum-palladium-silver-gold alloy wire (Usine Genevoise de Dégrossissage d'or, Genève, Legierung Nr. 1,003) evaporated from a 0.8 mm tungsten wire at a mean distance of 8.5 cm from the grids in a Siemens VGB 500 evaporating unit. Grids were examined in a Siemens Elmiskop I (using combined objective and intermediate lenses, and projector pole piece II) and selection was made in favor of those on which the DNA molecules (see results) were so well separated from each other that the majority did not have any points of crossover with other molecules. For each sample, except that treated with DNase, grid squares were methodically searched from one side to the other and the first 50 DNA molecules seen, which did not run out of view off the grid square, or were not excessively tangled so as to make measuring meaningless, were photographed at original magnifications calibrated with a diffraction grating replica (Fullam, 2,160 lines/mm) to be 9,550 $\times$. Molecules were measured with a map measure on positive prints at magnifications of at least 40,000 $\times$.

Sperm sacs were dissected out of 3 newly emerged imago *C. tentans* males in the same isotonic buffer used for isolating salivary gland nuclei. The sacs were transferred to 20 μl of buffer containing 2% SDS, the sperm quickly squashed out and the sacs removed. It was ascertained by examining the sacs under phase contrast that the nuclei of the sac wall were not broken down by their brief exposure to SDS. After 10 min when most of the sperm appeared to have been lysed, the 20 μl sample was prepared for electron microscopy in the manner described for the samples of salivary gland nuclei. Thirty DNA molecules from the sperm sample were photographed and measured.

To isolate larval DNA, washed larvae were quickly homogenized in 10 to 20 volumes of cold 0.1 M Tris-HCl buffer, pH 7.4, containing 1 mM EDTA; SDS was added to a concentration of 2%, and the mixture shaken with one volume of phenol for 20 min at 0°C. The aqueous phase was collected and the DNA precipitated with two volumes of ethanol. In most cases the DNA cannot be "spooled out" at this stage. The precipitate was dissolved in Tris buffer (one-tenth to one-fifth of the original volume) and treated with 50 $\mu\text{g}/\text{ml}$ of RNase for 30 min at 37°C. The solution was extracted two to three times with phenol and the DNA precipitated with ethanol and spooled out. Chironomus DNA prepared in this way had a molecular weight of about 5×10^6 .

Chironomus larvae (as well as larvae and imagos of some *Drosophila* species) appear to contain very active and stable nucleases. When the original extraction was carried out at room temperature the size and yield of DNA were less. Attempts to use pronase digestion of proteins as the initial step in the isolation procedure failed entirely.

RESULTS

In the samples of salivary gland nuclei treated with SDS alone, with SDS and pronase, and with SDS, phenol and RNase, long non-circular filaments having the well known characteristics of double-stranded DNA (see for example MACHATTIE, RITCHIE, THOMAS and ROBINSON, 1967) were observed (Figure 1). Data concerning the lengths of these molecules are summarized in Figure 2. Molecules from *C. tentans* salivary gland nuclei treated with SDS alone range in length from 8.1 μ to 89.4 μ and have a mean length of 46.7 μ . Molecules from similarly treated nuclei of *C. thummi* have a greater range of lengths, 5.2 μ –154.5 μ and a greater mean length, 60.9 μ . The mean length of molecules from *C. tentans* nuclei incubated in pronase is slightly higher but not significantly different ($P = < 0.2, > 0.1$) from the mean of the sample treated with SDS alone. Treatment with phenol and RNase resulted in an approximate halving of the filament mean length to 23.1 μ . It seems more reasonable that this reduction resulted from shearing of the molecules by the relatively rougher treatment of this sample than from any specific action of either of the reagents. Following DNase treatment (Figure 3) pieces no longer than 1 μ were found confirming that the filaments are in fact DNA.

The range (9.3 μ –241.4 μ) and mean length (76.1 μ) of DNA molecules from *C. tentans* sperm (Figure 4) were greater than any of the corresponding values for DNA molecules from salivary gland nuclei.

Material having a fuzzy appearance, a "puddle" (see for example DAWID and WOLSTENHOLME 1968), was found at the terminal portions of 16 and 23%, respectively, of the salivary gland DNA molecules from the SDS, and SDS and pronase treated samples of *C. tentans* (Figure 5), and 19% of the molecules from the *C. thummi* sample. Similar material appeared at only 7% of the ends of molecules treated with phenol and RNase (Figure 6), and 3% of the ends of sperm molecules. The interpretation of the "puddle" material as single-stranded DNA is supported by its presence after SDS, pronase, phenol and RNase treatment and also by its similarity in appearance to the predominant component of both denatured nuclear DNA (Figure 7), and denatured mitochondrial DNA (DAWID and WOLSTENHOLME 1968) and to material which has been correlated with single-stranded regions produced by *Escherichia coli* exonuclease III digestion in phage T7 DNA molecules (RICHARDSON, INMAN and KORNBERG 1964) and in phage T2 DNA molecules (MACHATTIE, RITCHIE, THOMAS and ROBINSON 1967).

Only one well spread molecule having no visible ends was found (Figure 8). A small region of single-stranded DNA is involved in this molecule (Figure 9).

The buoyant density in CsCl of DNA extracted from whole larvae was found in each of 3 centrifugations to be 1.692 (33% guanine + cytosine) for *C. tentans* and in each of two centrifugations to be 1.693 (34% guanine + cytosine) for *C. thummi* (Figure 10).

DISCUSSION

The length measurement data presented indicate that the minimum mean

FIGURE 1.—Electron micrograph of a rotary shadowed molecule of DNA from isolated salivary gland nuclei of *Chironomus tentans* treated with SDS. The letters indicate identical points of the molecule which is $50.5\ \mu$ in total length. $\times 34,500$.

FIGURE 2.—Frequency distributions of the lengths of rotary shadowed DNA molecules from isolated salivary gland nuclei of *Chironomus thummi* and *Chironomus tentans* following the various treatments indicated. The mean and standard deviation of each sample which comprises 50 molecules are given.

length of continuous DNA molecules in *Chironomus* salivary gland chromosomes is in the order of 50 μ . The molecules seen in electron microscope preparations of lysed *Chironomus* nuclei could be the breakdown products of single DNA molecules which run the whole length of the chromosome. It seems most probable, in fact, that if *Chironomus* DNA were very long, *in situ*, it would be broken down during preparation due to shear forces produced particularly during spreading. Alternatively, the length measurements may be a fair estimate of the actual lengths of DNA molecules in chromosomes. The larger lengths of sperm molecules may indicate some enzymatic breakage of salivary gland molecules or different organization of the DNA in the two tissues.

EDSTRÖM (1964) determined the amount of DNA in the largest salivary gland nuclei of *C. tentans* as about 3340 $\mu\mu\text{g}$ and, their polyteny as 16,384 C. From these data and an estimate of 2,000–5,000 bands for the sum of the four chromosomes (following a consideration of band number in this species by BEERMANN (1952)),

FIGURE 3.—Electron micrograph of rotary shadowed molecules of DNA from a preparation of isolated salivary gland nuclei after treatment with SDS, phenol, RNase and 1 $\mu\text{g}/\text{ml}$ DNase for 5 min. Filaments no longer than 1 μ can be found. $\times 40,000$.

EDSTRÖM (1964) derived a value of 6×10^{-5} μg or 18 μ DNA for the mean haploid amount of DNA per band. By using enzyme controlled, sensitive staining techniques, SWIFT (1962) and WOLSTENHOLME (1965 and 1966) demonstrated that the interband regions of polytene chromosomes contain DNA, but whether or not DNA is actually continuous through interband regions was not deter-

FIGURE 4.—Frequency distribution of the lengths of 30 rotary shadowed DNA molecules from *Chironomus tentans* sperm lysed with SDS. The mean and standard deviation of the sample are given.

FIGURES 5 and 6.—Electron micrographs of the terminal portions of two rotary shadowed DNA molecules at which material having a "puddle" appearance can be seen. 5) From the sample treated with SDS and pronase. 6) From the sample treated with SDS, phenol and RNase. Both micrographs $\times 83,000$.

FIGURE 7.—Electron micrograph of rotary shadowed denatured nuclear DNA from liver of *Xenopus laevis*. The chief component is similar in appearance to the "puddle" material found at the ends of some molecules extracted from *Chironomus* salivary gland nuclei. $\times 83,000$.

mined. If, however, EDSTRÖM's estimate at 18μ for the mean haploid amount of DNA per band is correct, then the present finding that the mean minimum length of DNA molecules from *C. tentans* is about 50μ , indicates that a single molecule of DNA must on the average be involved in at least 3 bands and, therefore, that DNA must be continuous through at least some interband regions. Data from *Drosophila melanogaster* are also in agreement with this interpretation. WOLSTENHOLME (unpublished) has found the mean length of DNA molecules from salivary gland chromosomes of this species to be 72μ , and RUDKIN and SCHULTZ (quoted by EDSTRÖM (1964) and RUDKIN (1965)), have estimated the mean haploid amount of DNA per band to be about $3 \times 10^{-5} \mu\text{g}$, which is equivalent to about 9μ DNA. In this case a single DNA molecule is indicated

FIGURE 8.—Electron micrograph of a rotary shadowed molecule of DNA from isolated salivary gland nuclei of *Chironomus thummi* lysed with SDS. The molecule, which measures $84\ \mu$, is well spread, but no ends are visible; a small piece of single-stranded DNA (arrow) is involved in this molecule. $\times 21,300$.

FIGURE 9.—A higher power micrograph of the region of the molecule including the single-stranded material indicated by the arrow in Figure 7. $\times 74,000$.

FIGURE 10.—Microdensitometric tracings of CsCl buoyant density gradients of *Chironomus tentans* DNA (top) and *Chironomus thummi* DNA (bottom) isolated from whole larvae. The reference band to the right is native DNA of *Pseudomonas aeruginosa*. Centrifugation was for 20 hours at 39,460 rpm and 20°C (MESELSON, STAHL and VINOGRAD 1957).

to be involved in at least 8 bands. The assumption is made in these arguments that during preparation of the DNA there is no selection against the shorter DNA molecules present in chromosomes.

The total haploid lengths of DNA of each of the 4 chromosomes of *C. tentans* calculated from EDSTRÖM's (1964) data, are 20 mm, 19.7 mm, 17.4 mm and 6.9 mm. Therefore, the data presented set the maximum number of non-DNA linkers per chromatid at about 400 for the largest chromosome and 140 for the smallest. This assumes that the DNA molecules appearing in our micrographs do not contain linkers. If such linkers existed, they would have to be stable to detergent and pronase, be relatively short and/or have a diameter close to that of the DNA double helix.

It has been suggested from the results of studies of *Chironomus* salivary gland chromosomes, involving autoradiography, Feulgen microspectrophotometry and staining, (KEYL and PELLING 1963; KEYL 1965; PELLING 1959, 1964) that the bands are individual units of replication and of transcription (PELLING 1966). If this is so, then a consequence of one DNA molecule being involved in a number of bands is that a single molecule must have more than one point along its length at which both replication and transcription can be initiated. The results of autoradiography studies of DNA replication in *D. melanogaster* salivary gland chromosomes made by PLAUT, NASH and FANNING (1966) leave open the possibility, however, that a unit of DNA replication may include a number of bands. In this case the present results need not necessitate the postulation of more than one site of initiation of DNA synthesis per molecule.

The presence of single-stranded regions in DNA not exposed to denaturing treatment has not been previously observed. Our identification of the "puddle"

regions must be considered tentative. If the terminal "puddles" are actually single-stranded DNA they could have arisen in various ways. The simplest explanation would invoke the presence of an exonuclease similar to *Escherichia coli* exonuclease III (RICHARDSON, LEHMAN and KORNBERG 1964). This enzyme would be less abundant in sperm than in salivary glands, leading to the lower number of single-stranded ends in sperm DNA. Alternatively, the rarity of such regions in sperm DNA is consistent with the interpretation that at least some of the single-stranded regions are correlated to points of replication and/or points of transcription *in vivo*. Such points might be mechanically weak and break preferentially upon spreading (see HANAWALT and RAY 1964). This could also explain the observed difference in mean length between sperm DNA and salivary gland nuclear DNA. Shearing of DNA molecules appears to result in straight ends (at least at the present level of observation) rather than "puddle" ends: only 7% of the ends of molecules that presumably were sheared during phenol and RNase treatment to an average of one half their original length are single-stranded.

With the exception of the one molecule having no visible ends, circular DNA such as that described by HOTTA and BASSEL (1965) from boar sperm has not been found. Although circles could of course have been broken during isolation of the DNA, in light of the above consideration it seems unlikely that in general the DNA of bands is, *in situ*, circular, as has been suggested by KEYL (1965).

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SUMMARY

Isolated nuclei of *Chironomus tentans* and *Chironomus thummi* salivary glands were lysed with SDS (sodium dodecyl sulfate), then spread and prepared for electron microscopy by the Kleinschmidt technique. Filaments were observed with a mean length of 46.7 μ (range, 8.1–89.4 μ ; SD $\pm$ 16.5; n = 50) for *C. tentans* and 60.9 μ (range, 5.2–154.5 μ ; SD $\pm$ 39.6; n = 50) for *C. thummi*. That these filaments represent uninterrupted DNA molecules is indicated by the following: (1) Digestion of the *C. tentans* lysate with DNase-free pronase did not result in reduction of the filament mean length, 52.7 μ (range 0.6–130.9 μ ; SD $\pm$ 30.7; n = 50); treatment of the lysate with phenol and RNase reduced the filament mean length to 23.1 μ ; most likely by mechanical breakage; (2) after digestion with DNase filaments no longer than 1.0 μ were found. The mean value for salivary gland chromosome DNA molecules of 50–60 μ is approximately 3 times greater than the mean haploid length of DNA per *Chironomus* salivary gland chromosome band (EDSTRÖM 1964) indicating that DNA is continuous through at least some interband regions. Material having the appearance of single-stranded DNA was found at the terminal portions of 16–23% (n = 100 for each class) of the terminal portions of salivary gland DNA molecules released

by SDS, or SDS and pronase. Similar material was found at 3% ($n = 100$) of the terminal portions of DNA filaments obtained from *C. tentans* sperm. The mean of the sperm filaments was 76.1μ (range, 5.3–241.4 μ ; $SD \pm 51.7$; $n = 30$). The buoyant density in CsCl of DNA extracted from whole larvae was found to be 1.692 (33% GC) for *C. tentans* and 1.693 (34% GC) for *C. thummi*.

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