# *ABNORMAL CIRCULAR DNA MOLECULES INDUCED BY ETHIDIUM BROMIDE IN THE KINETOPLAST OF TRYPANOSOMA CRUZI**

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*Abstract.*—The trypanocidal drug ethidium bromide induced abnormal circular DNA molecules with high frequency in the kinetoplast of *Trypanosoma cruzi*. These molecules are circular dimers, trimers, or higher circular forms with a contour length which is a multiple of the monomer length usually found in the kinetoplastic DNA of normal trypanosomes. These abnormal molecules represent more than 30 per cent of the kinetoplastic DNA, while in normal kinetoplastic DNA we only observed three circular dimers out of several thousand molecules measured.

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It is now well established that the kinetoplast of trypanosomatids is a part of the mitochondrial apparatus where DNA is found in high concentration. In *Trypanosoma cruzi* the kinetoplastic DNA represents more than 20 per cent of the total DNA. When the trypanosomes are cultured in the presence of ethidium bromide, which is a trypanocidal drug, we described "dyskinetoplastic" trypanosomes.[1] In these trypanosomes the kinetoplastic DNA was progressively lost.[2, 3]

In previous works we described a circular kinetoplastic DNA fractionated from *Trypanosoma cruzi* DNA by Hg-$Cs_2SO_4$ method.[4, 5] Covalently closed circular DNA was isolated by buoyant density centrifugation in ethidium bromide CsCl density gradients. Electron microscope studies have shown that kinetoplastic DNA molecules are in the form of open and closed circles of contour length 0.45 $\mu$. DNA molecules are also in the form of catenanes consisting of two or more topologically interlocked circular units of the monomer size 0.45 $\mu$. Associations of numerous circles have also been described and confirmed by band sedimentation. Long linear DNA molecules were also seen; these molecules are free or are attached to the associations of circles.

In this paper, we report electron microscope studies of the kinetoplastic DNA of trypanosomes (*Trypanosoma cruzi*) treated with ethidium bromide. We noted the presence of abnormal circular molecules; they are circular oligomers with a contour length which is a multiple of the monomer length usually found in the kinetoplastic DNA of normal trypanosomes.

*Materials and Methods.—Culture of trypanosomes:* Trypanosomes (*Trypanosoma cruzi*, strain "Institut Pasteur") were grown according to the previously described method.[5] Ethidium bromide (EB) in distilled water was added aseptically to the culture medium to obtain a final concentration of 1.5 $\mu$g EB by ml. At this concentration EB does not effect trypanosomes' growth.

*Dyskinetoplasty:* Evaluation of the percentage of dyskinetoplastic trypanosomes was performed after spreading of trypanosomes on slides and coloration by Giemsa stain. Trypanosomes were counted as dyskinetoplastic when their kinetoplast was not stained at all. Dyskinetoplastic trypanosomes were also examined with a UV microscope according to the method described in a previous work.[1]

*Preparation and fractionation of kinetoplastic DNA:* DNA was extracted according to the method of Marmur.[6] Kinetoplastic DNA was fractionated according to the previously described methods.[4]

*Equilibrium centrifugation in CsCl:* Buoyant density determinations of DNA were made in CsCl according to the method of Vinograd and Hearst.[8]

*Electron microscopy:* DNA was spread for electron microscopy according to the technique described by Freifelder and Kleinschmidt[9] with some minor modification.[5] Trypanosomes treated with EB were observed with the electron microscope according to the technique described by Delain and Riou.[10]

*Results.—Fractionation of total DNA:* In conditions of these experiments the dyskinetoplasty was about 47 per cent. There is a diminution of the kinetoplastic DNA of about 30 per cent in trypanosomes treated with ethidium bromide. Figure 1 shows the microdensitometer tracings of DNA buoyant density from normal trypanosomes and EB-treated trypanosomes. The buoyant density of kinetoplastic DNA is $\rho = 1.699$ gm/ml. There is no change in the buoyant density after ethidium bromide treatment. The buoyant density of nuclear DNA is $\rho = 1.710$ gm/ml. In some experiments we noted the apparition of a second light satellite DNA of buoyant density $\rho = 1.686$ gm/ml, as previously described.[4]

*Fractionation of kinetoplastic DNA:* Kinetoplastic DNA of trypanosomes treated with ethidium bromide was submitted to the fractionation of its molecular forms as described in a previous paper.[5] Figure 2 shows a fluorescence photograph of an ultracentrifuge tube after kinetoplastic DNA fractionation in the $Cs_2SO_4$ gradient. We obtained two DNA-ethidium bromide bands standing out over the background of dye because of the enhanced efficiency of fluorescence

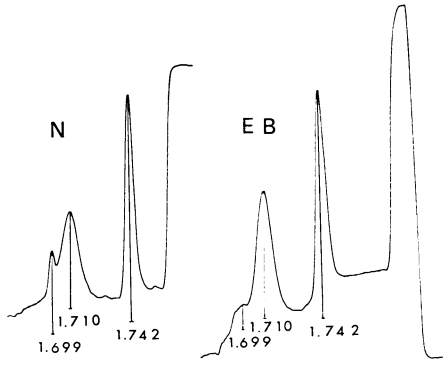


FIG. 1.—Microdensitometer tracings of buoyant density of DNA in CsCl gradient in the analytical ultracentrifuge. Runs are performed in the Spinco model E analytical ultracentrifuge for 24 hr at 25°C at 44,770 rpm. The UV photographs are scanned with a Joyce-Loebl microdensitometer. The buoyant densities of the DNA are expressed relative to the density of *Bacillus subtilis* phage 2C DNA taken as 1.742 gm/ml. *Left:* whole cell DNA of normal trypanosomes. *Right:* whole cell DNA of trypanosomes treated with EB.

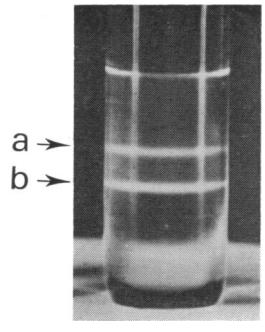


FIG. 2.—Kinetoplastic DNA of trypanosomes treated with EB after centrifugation in a CsCl-EB density gradient. Prior to drop collection the centrifuge tube was examinated in a darkened room with 366-mμ light from a Desaga Uvis lamp and photographed on Kodachrome II film through a Kodak Wratten 2 A filter. The mixture was composed of 20 μg of DNA, 300 μg EB, 2.7 gm CsCl, final vol 3 ml, 24 hr at 44 krpm, 20°C (ultracentrifuge Beckman L2 SW50 rotor).

when ethidium bromide binds to DNA.[11] The upper and lower bands have been labeled *a* and *b*.

*Electron microscopy of kinetoplastic DNA:* Random samples of the DNA were obtained by photographing unselected large fields. We obtained open circles, closed circles, small linear DNA molecules (considered as broken circles), and molecules in the form of catenanes consisting of two or more topologically interlocked circles. Catenated oligomers are DNA molecules usually found in the kinetoplast of normal trypanosomes.[5] In the kinetoplastic DNA of the trypanosomes treated by ethidium bromide we also found circular oligomers (dimers, trimers, tetramers, pentamers) which are abnormal DNA molecules. The length distribution histograms of these DNA molecules observed in bands *a* and *b* are presented in Figures 3 and 4. The electron micrographs of these different types of DNA molecules are shown in Figures 5 and 6. The proportion of DNA molecules and values of their mean length found in different ethidium bromide–DNA bands are included in Tables 1 and 2, respectively. The proportion of abnormal DNA molecules is relatively high; it represents about 31 per cent of the kinetoplastic DNA in each band *a* and *b*. DNA molecules which are found in the band *a* are principally composed of relaxed circles (about 87%). We found 9 per cent of supertwisted circular monomers or oligomers (covalently closed circles) and linear molecules free or attached to the circles (about 4%). In the band *b* we found essentially supertwisted circles (77.2%) and relaxed circles (open circles) corresponding to 22.8 per cent of the kinetoplastic DNA. These results are in agreement with the binding property of the intercalating drug ethidium bromide with closed and open circular DNA molecules. There is a significant difference in the contour length of circular monomer molecules found in band *a* and band *b*.

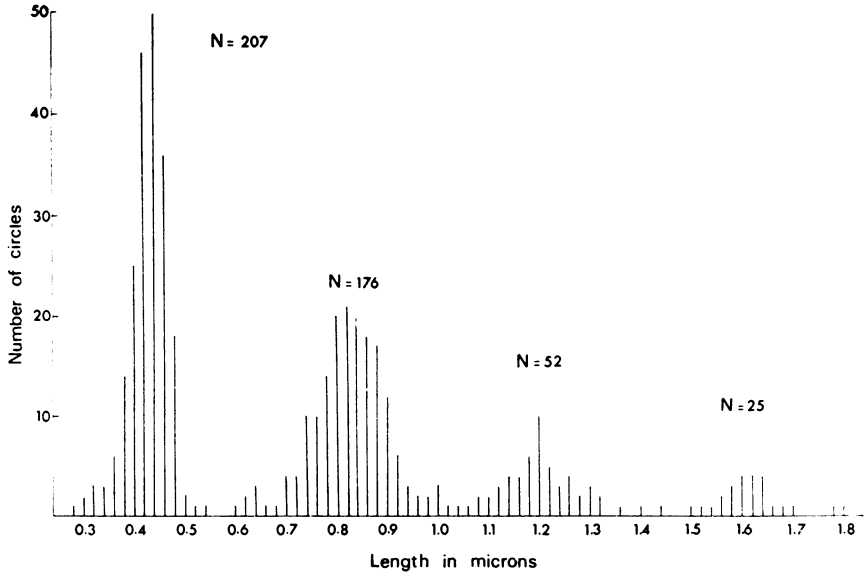


FIG. 3.—A histogram of the distribution of lengths of circular DNA molecules isolated from band *a* as referred to the legend of Fig. 2.

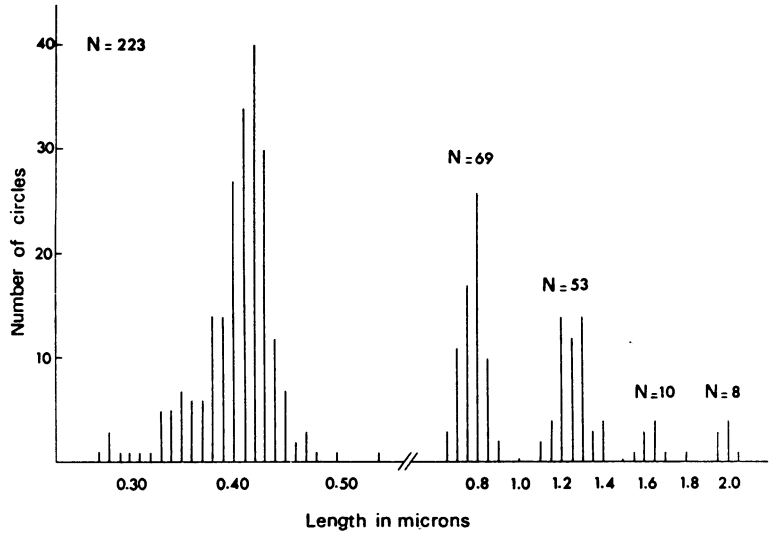


FIG. 4.—A histogram of the distribution of lengths of circular DNA molecules isolated from band *b* (cf. Fig. 2).

We found a mean value of 0.40 $\mu$ ± 0.04 for contour length of molecules from band *b* while in band *a* the mean value is 0.43 $\mu$ ± 0.03. This difference is due to the difficulty of measuring small supertwisted DNA molecules.

*Discussion.*—The method using the complexing by mercuric ions in a $Cs_2SO_4$ gradient is very efficient in removing the nuclear DNA. The kinetoplastic DNA represents about 20 per cent of the total DNA of the trypanosomes. The DNA content of one trypanosome has been evaluated to $1.8 \times 10^{-13}$ gm,[3] so that each kinetoplast contains about $3.5 \times 10^{-14}$ gm of DNA. DNA molecules from the kinetoplast are circular monomers 0.45 $\mu$ long corresponding to a molecular weight of $9 \times 10^5$ daltons.[5] From these data the number of DNA molecules in each normal kinetoplast can be evaluated at about 24,000 (if we neglect the other types of molecules in very low concentration and if we consider that each trypanosome has only one kinetoplast). The role of so much DNA in this organelle is not yet well established.

Electron microscope studies of the ultrastructure of trypanosomes treated with ethidium bromide revealed alterations of the kinetoplastic DNA structure without any modification of the nuclear DNA. Ethidium bromide yields an inhibition of the replication of kinetoplastic DNA[3] with a progressive loss of this DNA and production of dyskinetoplasty. Dyskinetoplastic trypanosomes are not viable; they can only survive for four weeks after transplantation each week in a new medium free of ethidium bromide.[12] Mitochondrial DNA of many organisms is now known to occur in the form of closed circular duplex molecules with a contour length of approximately 5 $\mu$ and a molecular weight of about $10^6$ daltons. Vinograd *et al.*[13, 14] have discovered complex forms of mitochondrial DNA in several mammalian species (rabbit, guinea pig, mouse embryo, HeLa cells, and human leucocytes). These complex forms consisted of catenated oligomers with

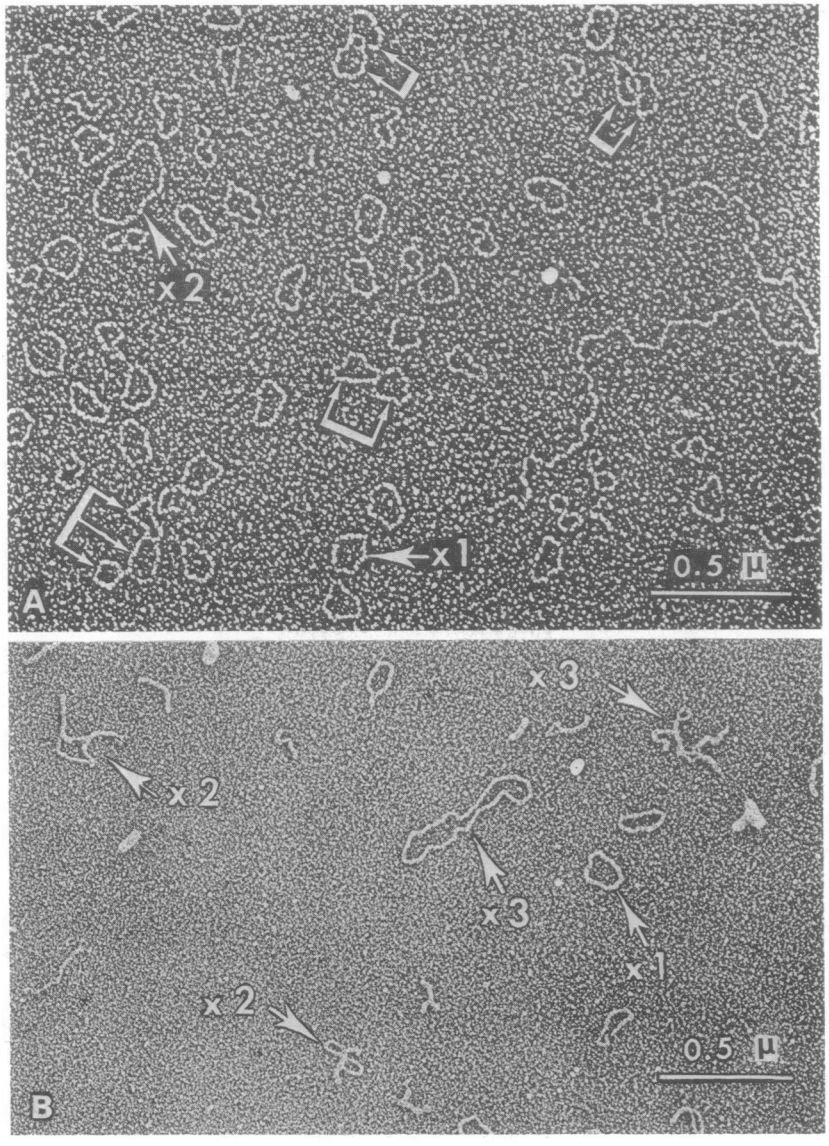


FIG. 5.—Large-field electron micrographs of kinetoplastic DNA after fractionation by EB from band *a* (*A*) and band *b* (*B*). (*A*) Open circular monomers, circular dimer (× 2), catenated dimers (*double arrows*), catenated trimer (*triple arrow*), linear molecule; (*B*) open and closed circular monomers, closed circular monomers, closed circular dimers (× 2), open and closed circular trimers (× 3).

two or more interlocked circular duplexes connected to each other like links of a chain. Vinograd *et al.*[15] described another type of molecule, circular dimers which are circular molecules with twice the molecular weight and contour length of the monomers. This circular dimer form occurs at a high frequency in mitochondrial DNA from human leukemic leucocytes. Circular dimers were not observed in normal human leucocytes. Catenated dimer forms occur in leucocytes

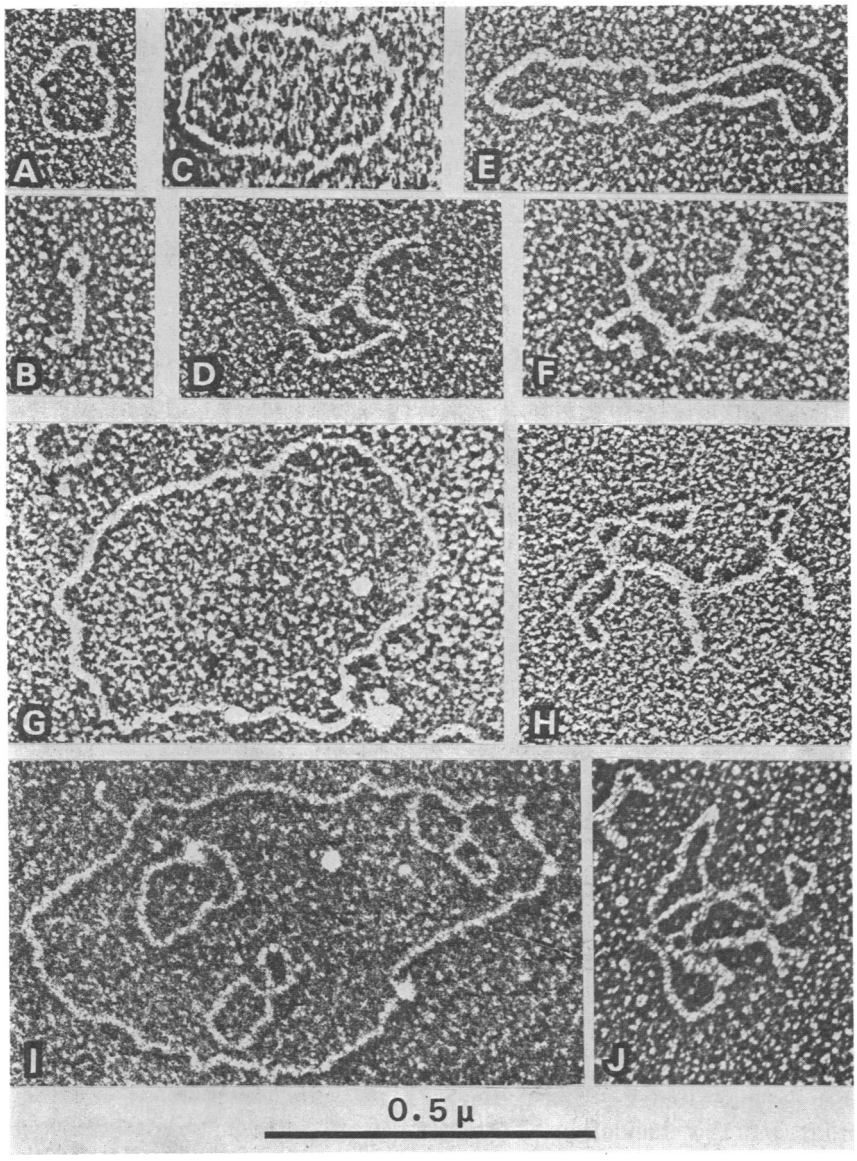


FIG. 6.—Electron micrographs of kinetoplastic DNA after fractionation by EB: (*A* and *B*) Open and closed circular monomers; (*C* and *D*) open and closed circular dimers; (*E* and *F*) open and closed circular trimers; (*G* and *H*) open and closed circular tetramers; (*I* and *J*) open and closed circular pentamers. The scale is the same for all the molecules.

from normal and leukemic human donors. Vinograd established a catenane index (catenated dimers/total dimers), which is different with the evolutionary state of the leukemia. There is a difference in the distribution of types of oligomer in the DNA preparations from the three leukemic patients studied. The preparation from the two patients with clinically more advanced leukemia had a

TABLE 1. *Number and percentage of different molecular forms of kinetoplastic DNA found in bands a and b after fractionation by ethidium bromide.*

| | Band *a* | | | Band *b* | | |
|---|---|---|---|---|---|---|
| | Number of molecules | Percentage of molecules | Percentage* in DNA | Number of molecules | Percentage of molecules | Percentage* in DNA |
| Circular monomers | 1903 | 79.9 | 62.5 | 771 | 81.8 | 63.2 |
| Circular dimers | 259 | 10.9 | 17.0 | 69 | 7.3 | 11.3 |
| Circular trimers | 100 | 4.2 | 9.8 | 53 | 5.6 | 13.1 |
| Circular tetramers | 25 | 1.0 | 3.3 | 10 | 1.1 | 3.3 |
| Circular pentamers | 4 | 0.2 | 0.6 | 8 | 0.8 | 3.3 |
| Catenated dimers | 70 | 2.9 | 4.6 | 25 | 2.7 | 4.1 |
| Catenated trimers† | 19 | 0.8 | 1.9 | 7 | 0.7 | 1.7 |
| Catenated tetramers | 2 | 0.1 | 0.3 | ... | ... | ... |

* Percentage by weight of the kinetoplastic DNA.
† Catenated trimers were always observed interlocked 2 by 2 as shown in Fig. 5*A*.

TABLE 2. *Contour length (mean values and standard error) of the different types of molecules found in bands a and b as referred to the legend of Figure 2.*

| | Band *a* | | Band *b* | |
|---|---|---|---|---|
| | Number of molecules examined | Contour length in microns | Number of molecules examined | Contour length in microns |
| Monomers | 207 | 0.43 ±0.03 | 223 | 0.40 ±0.04 |
| Circular dimers | 176 | 0.82 ±0.07 | 69 | 0.77 ±0.05 |
| Circular trimers | 52 | 1.20 ±0.06 | 53 | 1.25 ±0.07 |
| Circular tetramers | 25 | 1.58 ±0.06 | 10 | 1.64 ±0.06 |
| Circular pantamers | 4 | 2.02 | 8 | 1.99 ±0.03 |

higher catenane index. These results, if they are corroborated, are very important, but the elucidation of the relationship between the occurrence of the oligomers and the physiological state of the cell will require further quantitative investigation with a variety of cell systems.

In our experiments we induced a new physiological state in trypanosomes with a chemical drug which specifically affects kinetoplastic DNA. This high frequency of circular dimers, trimers, tetramers, and pentamers induced by ethidium bromide is quite puzzling, and we must point out that it is balanced with the diminution of the frequency of catenated oligomer as compared with normal kinetoplastic DNA. In the kinetoplastic DNA from normal trypanosomes, we only observed three circular dimers out of several thousand molecules measured.[5] The mechanism of this drug action is unknown, but several hypotheses can be made.

Vinograd *et al.*[13] have formulated a plausible scheme for the formation of oligomer by recombination. If this scheme is plausible, several enzymes could be implicated in this process. Ethidium bromide could disturb one of these enzymes and orient the recombination process to form circular oligomers instead of catenated oligomers. The mode of replication of closed circular molecules is not yet understood sufficiently to permit predictions of the possible structures that might be formed as a result of errors of replication induced by ethidium bromide. Nevertheless, the model presented by Goebel and Helinski[16] for the duplication of Col E1 (colicigenic factor) DNA and the formation of higher multiple circular DNA forms, and the model presented by Kiger and Sinsheimer[17] for the replication of circular DNA molecules could be applied to the duplication of kinetoplastic DNA. In these models, duplication of DNA is considered to involve initially a break in one of the phosphodiester bonds of one of the two strands of the covalently closed double-stranded circle. This endonucleolytic cleavage occurs at a specific site and generates a cohesive end for subsequent cyclization of the duplicated strand. During this process one of the strands remains in the covalently closed cyclic form and serves as a template for the other strand. After the synthesis of the complete sequence of the covalently open strand, a second endonucleolytic cleavage in the initiation region releases the duplicated strand for cyclization. In trypanosomes, ethidium bromide could occasionally block the second endonucleolytic cleavage and produce the formation of circular dimers, trimers, and higher circular molecules.

We frequently observed by electron microscopy the attachment of DNA to the membrane of the kinetoplast,[10] principally in trypanosomes treated by ethidium bromide.[3] These attachments may represent sites where replication is initiated.

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