# *ELECTRON MICROSCOPY OF THE CIRCULAR KINETOPLASTIC DNA FROM TRYPANOSOMA CRUZI: OCCURRENCE OF CATENATED FORMS**

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*Abstract and Summary.*—Circular kinetoplastic DNA was fractionated from *Trypanosoma cruzi* DNA by Hg-$Cs_2SO_4$ gradient. Covalantly 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 $\mu$, the smallest known microbial DNA. 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, their size ranging between 2 and 12 $\mu$. These molecules are free or are attached to associations of circles.

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The amount of kinetoplastic DNA in *Trypanosoma cruzi* represents 15–20 per cent of the total DNA. The kinetoplastic DNA has a higher AT content than the nuclear DNA; this property is used for its fractionation.[1] It renatures at a high rate and consists of small circular duplex molecules of a uniform size of about 0.45 $\mu$.[1] It has been shown that mitochondrial DNA of several vertebrate and avian species occurs in circular form (open circle) and in supercoiled form (closed circle),[2–5] as described previously for circular duplex viral DNA's by Vinograd.[6] More recently, Vinograd *et al.* have shown that a significant fraction of DNA molecules are in the form of interlocked dimers or catenanes in HeLa cells,[7] in leucocytes of leukemic patients,[8] and in unfertilized sea urchin eggs.[9]

We have previously described a circular DNA, 0.45 $\mu$ long, extracted from the kinetoplast of *Trypanosoma cruzi*.[1] In this paper we report the results of additional studies on this DNA: covalently closed circular duplexes and catenated forms of DNA circles sometimes associated with linear DNA. These different molecular forms of kinetoplastic DNA have been fractionated by buoyant density centrifugation with ethidium bromide in cesium chloride.

*Materials and Methods.—Culture of trypanosomes:* Trypanosomes (*Trypanosoma cruzi*, strain "Institut Pasteur") were grown for 6 days at 27°C in 500-ml flasks containing 300 ml of Parker 199 medium supplemented with 10% of the liquid phase of Tobie medium; 250 IU of penicillin/ml and 50 $\mu$g of streptomycin/ml were also added to the medium. The trypanosomes (about $10^7$ cells/ml) were washed five to six times with SSC (0.15 *M* NaCl–0.015 *M* sodium citrate) and collected by centrifugation at 1000 *g*.

*Preparation and fractionation of kinetoplastic DNA:* Trypanosomes were lysed with 1% sodium dodecyl sulfate; DNA was extracted according to the method of Marmur;[10] two treatments with 50 $\mu$g RNase/ml were made under standard conditions. The material was subjected to preparative density gradient centrifugation in $Cs_2SO_4$ in the presence of mercuric ions. Kinetoplastic DNA, which has a base composition different from

that of nuclear DNA, was fractionated according to the previously described methods.[1] Kinetoplastic DNA was extensively dialyzed against SSC to remove $Cs_2SO_4$ and mercury, then mixed with ethidium bromide (EB) and CsCl as described by Radloff and Vinograd.[11] Each DNA band was collected from the bottom of the tube and extensively dialyzed against SSC to remove CsCl and EB.

*Equilibrium centrifugation in CsCl:* Buoyant density determinations of DNA were made in CsCl according to the method of Vinograd and Hearst.[12]

*Electron microscopy:* DNA in 0.1 *M* $NH_4$ acetate, pH 7.6, was spread for electron microscopy according to the technique described by Freifelder and Kleinschmidt,[13] with some minor modification. The DNA solution was diluted in 0.1 *M* $NH_4$ acetate, pH 7.6, to a final concentration of 2 $\mu$g/ml. Cytochrome *c* was added just before the solution was spread on 0.5 *M* $NH_4$ acetate, pH 7.6, to fill a Petri dish (diameter 10 cm). Grids were shadowed while rotating with a platinum-carbon pellet (produced by Ladd-Burlington, Vermont) at an angle of 4°. Several molecules were successively shadowed while the specimen was rotating and again while it was stationary. The specimens were examined in a Philips EM 300 electron microscope, and the lengths of the DNA molecules were measured with a map-measuring device on positive prints at a magnification of 150,000. A carbon grating replica (Fullam 30,000 lines/inch) was used to calibrate the microscope magnification.

*Results.—Fractionation of total DNA:* After the Hg-$Cs_2SO_4$ gradient centrifugation, total DNA was separated into two fractions: fraction I containing kinetoplastic DNA, and fraction II containing nuclear DNA (Fig. 1). The purity of these fractions was assayed by analytical ultracentrifugation in a CsCl gradient (Fig. 2). The buoyant density of kinetoplastic DNA is $\rho = 1.699$ gm/ml.

*Fractionation of kinetoplastic DNA by EB:* Figure 3 shows fluorescence photographs of ultracentrifuge tubes after kinetoplastic DNA fractionation in the $Cs_2SO_4$ gradient (fraction I, Fig. 1). The DNA-EB bands stand out over the background of dye because of the enhanced efficiency of fluorescence when EB binds to DNA.[14] The upper, middle, and lower bands have been labeled *a*, *x*, and *b* (*B*). These three bands are not always found after EB fractionation. Four kinds of fractionation are obtained, depending on the batches of trypanosomes.

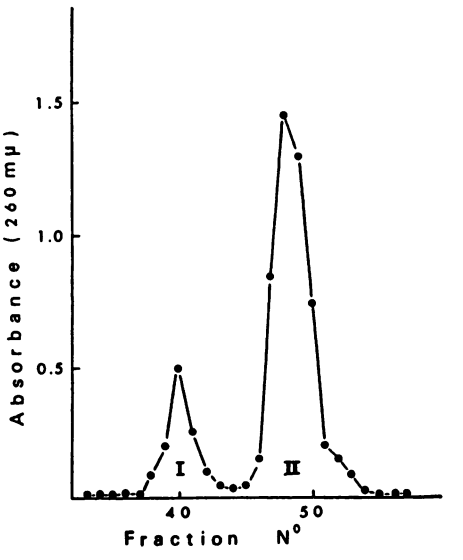


FIG. 1.—Results of a preparative ultracentrifugation experiment in Hg-$Cs_2SO_4$ gradient. DNA is dialyzed against 0.1 *M* $Na_2SO_4$. A solution of suitable concentration of $Cs_2SO_4$ to obtain a final density of 1.520 is mixed with DNA. A borate buffer, prepared from 0.1 *M* $Na_2B_4O_7$ (pH 9.2) is added, with the final solution containing 50 mmoles of borate buffer. $HgCl_2$ in distilled water is mixed by gentle stirring, the final molar ratio $Hg^{2+}$/DNA (phosphate) = 0.12. DNA (100 to 200 $\mu$g/ml) is centrifuged (ultracentrifuge Beckman model L2; Titanium type 50 rotor, 36 krpm for 64 hr at 22° C). Each fraction is two drops diluted into 1 ml of distilled water.

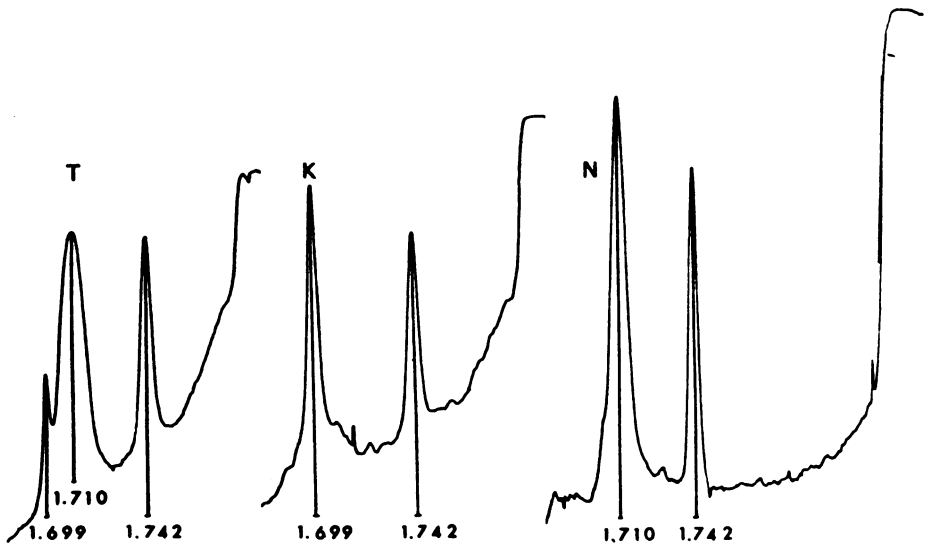


FIG. 2.—Microdensitometer tracings of buoyant density of DNA in CsCl gradient in the analytical ultracentrifuge. Runs are performed in the Spinco model E analytical ultracentrifuge for 24 hr at 25°C at 44,770 rpm. The UV photographs are scanned with a Joyce-Loebl microdensitometer. The buoyant densities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

Figure 3*C* shows the fluorescence emission from the two well-separated DNA bands *a* and *b*. Figure 3*D* shows only the DNA band *a*. Band *x* is very close to band *a* in Figure 3*A* and *B*, with the latter showing band *b* to be very slight.

*Electron microscopy of kinetoplastic DNA:* Random samples of the DNA were obtained by photographing unselected large fields. The different types of DNA molecules are shown in Figures 4 and 5. Small circles such as those shown in Figure 4 (*1*) were obtained. The contour length of 289 DNA circles that were

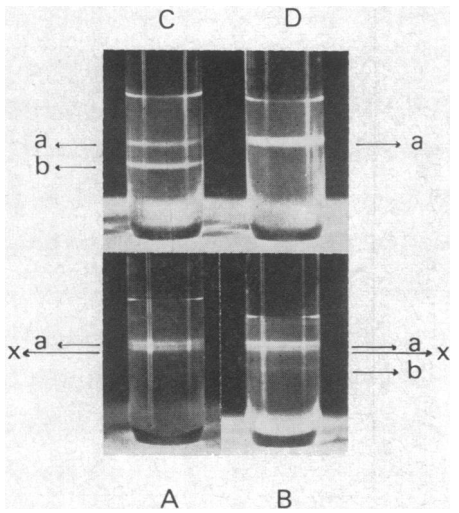


FIG. 3.—Kinetoplastic DNA after centrifugation in a CsCl-EB density gradient. Prior to drop collection the centrifuge tubes were examined in a darkened room with 366 mμ light from a Desaga Uvis lamp and photographed on Kodachrome II film through a Kodak Wratten 2 A filter. The mixture was composed of 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 SW 50 rotor).

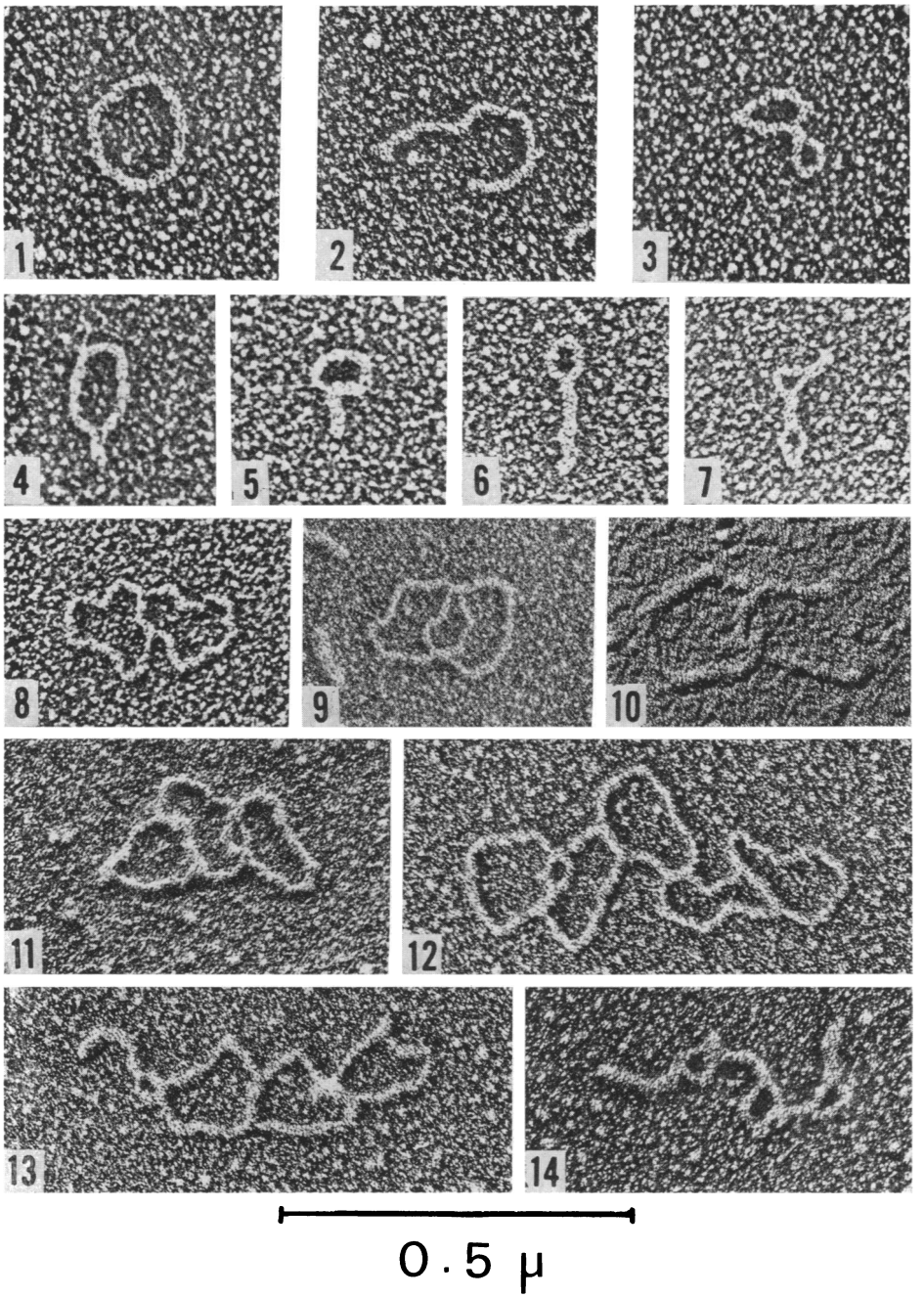


FIG. 4.—Electron micrographs of kinetoplastic DNA after fractionation by EB: (*1*) relaxed circle; (*2*) broken circle; (*3–7*) twisted and supertwisted circles; (*8–12*) catenated molecules of relaxed circles; (*13, 14*) catenated molecules of supertwisted circles.

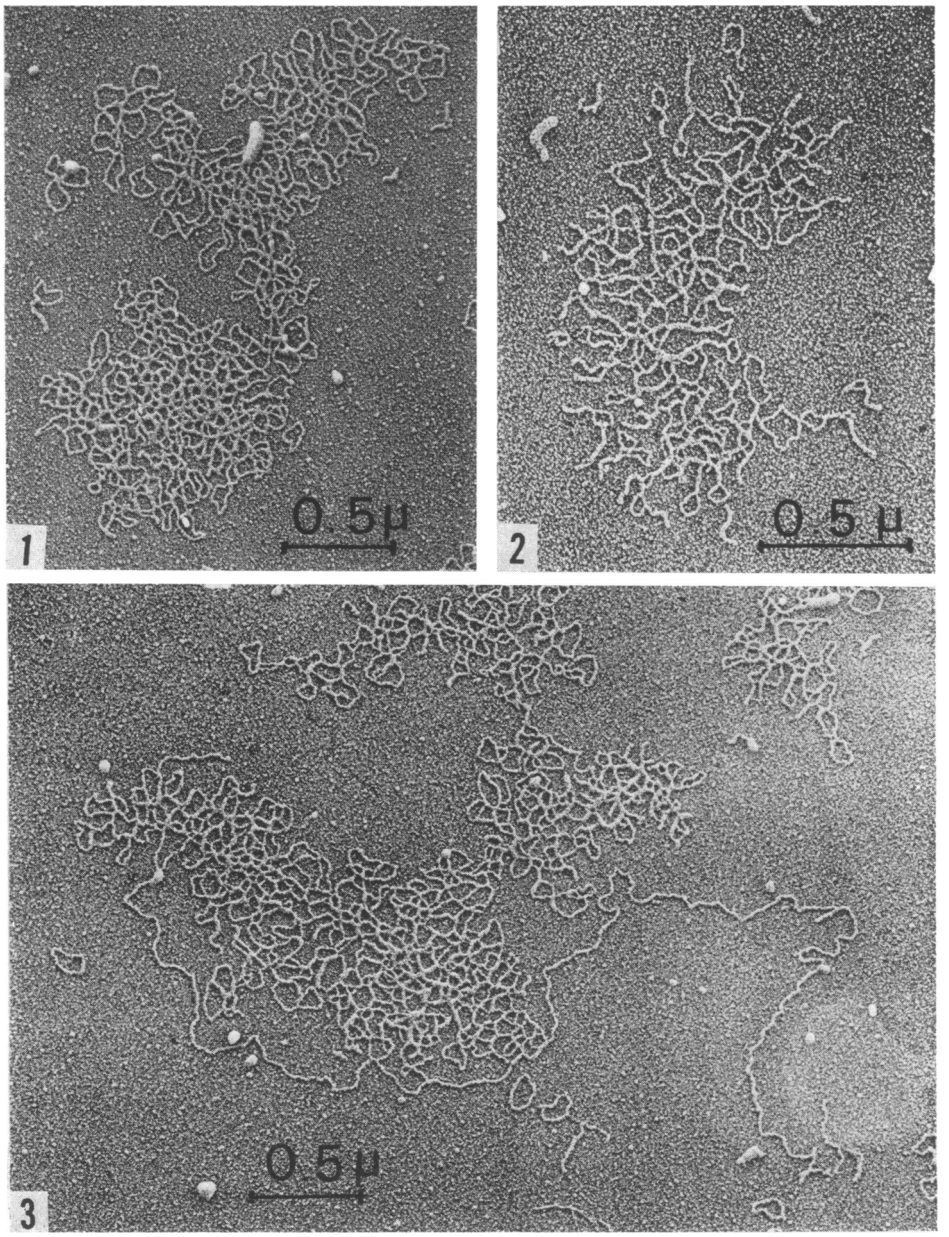


FIG. 5.—Electron micrographs of kinetoplastic DNA after fractionation by EB. (*1*) Associations of relaxed circles; (*2*) associations of relaxed and supertwisted circles; (*3*) associations of relaxed circles with linear molecules.

measured gave a mean value of 0.45 $\mu$ with a standard error of 0.02. Small linear DNA molecules (considered to be broken circles) have the same length as small circles (Fig. 4, *2*). Twisted and supertwisted circles observed in our preparations are shown in Figure 4 (*3–7*). The apparent circumference of these molecules is compatible with the mean value given above for the DNA circles.

Associations of DNA circles were also obtained. These molecules are in the form of interlocked circles or catenanes, as described by Vinograd.[7-9] Dimers, trimers, tetramers, pentamers with relaxed circles (Fig. 4, *8–12*), and supertwisted circles (Fig. 4, *13*, *14*) are observed. Figure 4 (*10*) shows the interlocking nature of two circles. The circles making up the oligomers and the single circle that we consider to be a monomer have the same length (0.45 $\mu$).

The proportion of DNA molecules found in different EB-DNA bands is included in Table 1.

TABLE 1. *Number and percentage of different molecular forms of kinetoplastic DNA found after fractionation by EB.**

| Molecular forms of DNA | Band *a* | | Band *b* | | Band *x* | |
|---|---|---|---|---|---|---|
| | Number | Per cent | Number | Per cent | Number | Per cent |
| Monomers | 380 | 65.0 | 110 | 19.0 | 402 | 57.9 |
| Dimers | 42 | 7.2 | 7 | 1.2 | 32 | 4.6 |
| Trimers | 24 | 4.1 | | | 26 | 3.8 |
| Tetramers | 18 | 3.1 | | | 22 | 3.2 |
| Pentamers | 6 | 1.0 | 3 | 0.5 | 12 | 1.7 |
| Broken circles† | 78 | 13.4 | 62 | 10.8 | 104 | 15.0 |
| Supertwisted circles | 36 | 6.2 | 396 | 68.5 | 96 | 13.8 |
| Total | 584 | | 578 | | 694 | |

* See also Fig. 3*B*.

† These DNA molecules proceed certainly from circles that are broken during DNA extraction or DNA spreading for electron microscopy.

We obtained other types of DNA molecules consisting of associations of many circular DNA molecules (Fig. 5). Three kinds of associations were observed: (1) Associations of relaxed circles, found in EB-DNA bands *a* and *x*; (2) associations of relaxed and supertwisted circles, found in EB-DNA band *x*; and (3) associations of relaxed circles with linear DNA molecules, found in EB-DNA bands *x* and *a*.

Also observed in the three types of EB-DNA band were long linear DNA molecules representing 0.8 to 1 per cent of the DNA. These molecules were 2–12 $\mu$ long.

*Discussion.*—The DNA of the *Trypanosoma cruzi* kinetoplast, which comprises 15 to 20 per cent of the total DNA, is a particularly favorable material for the analysis of DNA structure and properties. Electron microscope studies of *Trypanosoma cruzi*[15, 16] show that the kinetoplast is associated with the mitochondrial apparatus. The Hg-$Cs_2SO_4$ method has proved effective in separating the kinetoplastic DNA from nuclear DNA (it is not yet possible to isolate the kinetoplastic organelles by cell fractionation and then to extract their DNA).

In previous work[1] we reported the presence of a light satellite DNA ($\rho$ = 1.686 gm/ml) which was not detected in these preparations. The source of this light DNA and the variability of its occurrence remain unexplained. The results described in this paper confirm our previous work and indicate that the kinetoplastic DNA ($\rho$ = 1.699 gm/ml) is formed of "minicircles." Several remarks can be made:

(1) Mitochondrial DNA's from many vertebrates (mammals,[2, 3, 7, 8] birds,[4] and amphibians[5]) and from at least one invertebrate (the sea urchin)[9] exhibit a

remarkable structural similarity. All these DNA's were found in the form of closed circular duplex molecules of approximately 5-$\mu$ length, with an estimated molecular weight of $10^7$ daltons. Unlike mitochondrial DNA of other organisms, DNA from yeast mitochondria appears mainly as a linear double helix 4.0–4.5 $\mu$ in length.[17] A small percentage of these molecules consists of superhelical circles with length heterogeneity: 4.0 $\mu$,[17] 0.8–1.5 $\mu$.[18] DNA from *Tetrahymena* mitochondria has recently been shown to be a linear filament of 17.6 $\mu$.[19]

In kinetoplastic DNA from *T. cruzi*, the mean contour length of the 289 DNA circles measured was 0.45 $\mu$ with a standard error of 0.02. Out of the several thousand molecules observed, only three molecules are 1 $\mu$ in length. This DNA is the smallest monodisperse DNA species thus far described,[20] and the smallest microbial DNA of any kind.[21]

(2) The ethidium bromide–CsCl method has proved effective in separating the closed and open circular forms of this DNA. Nevertheless it is worth noting again that kinetoplastic DNA is not always fractionated in several bands (see Fig. 3). The absence of band *b*, in which closed circles are found, can be the result of the action of an enzyme (such as DNase-1) that hydrolyzes one strand of the DNA and makes it react with EB like a nicked DNA circle or a linear DNA. This DNA bands with EB in *a* (see Fig. 3*D*). The liberation of such an enzyme by trypanosomes could correspond to a physiological state presently unexplained.

(3) A third band, *x*, of intermediate density was also obtained (Fig. 3*A* and *B*). The buoyant densities of bands *x* and *a* are not very different. The DNA in *x* is composed by associations of supertwisted and relaxed circles (see Fig. 5, *1*), while, in *a*, associations are formed by relaxed circles (see Fig. 5, *2*).

(4) Associations of DNA circles (see Fig. 5) are probably not an artifact.

Preliminary studies of this DNA by band sedimentation in the analytical ultracentrifuge, according to the conditions described by Bruner and Vinograd,[22] yield the following results: The DNA from band *b* sediments at pH 8 in one component with $S^\circ_{20,w}$ of 16–18*S*. The DNA from *a* sediments at pH 8 into two components, with $S^\circ_{20,w}$ values of 11*S* for the lightest one; the second component, very heterogeneous, sediments in the range of 28–40*S*. The DNA from band *x* also sediments at pH 8 into two components, with $S^\circ_{20,w}$ of 11*S* for the first one; the second component is also heterogeneous and sediments in the range of 35–50*S*.

The fastest components (28–40*S*) and (35–50*S*) represent approximately 50 per cent of the DNA of each band and spread very rapidly in the ultracentrifuge cell. Such spreading is compatible with the heterogeneity of DNA associations. Sedimentation experiments were carried out with 3 *M* CsCl bulk solutions.

Sedimentation experiments in sucrose gradient of $^3$H-thymidine DNA confirm the results obtained by band sedimentation. Preliminary treatment of DNA by pronase does not modify the sedimentation or the electron microscope pictures.

The presence of the long, linear DNA filaments is puzzling. These molecules were present either free or associated with DNA circles (see Fig. 5, *3*). Two explanations for this discrepancy are possible: that the long, linear molecules are a contamination by nuclear DNA; that this DNA is a special feature of kinetoplastic organelles which could have a role in the replication of its DNA.

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