

ISOLATION AND CHARACTERIZATION OF KINETOPLAST DNA FROM BLOODSTREAM FORM OF *TRYPANOSOMA BRUCEI*

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ABSTRACT

We have used restriction endonucleases PstI, EcoRI, HapII, HhaI, and S₁ nuclease to demonstrate the presence of a large complex component, the maxi-circle, in addition to the major mini-circle component in kinetoplast DNA (kDNA) networks of *Trypanosoma brucei* (East African Trypanosomiasis Research Organization [EATRO] 427). Endonuclease PstI and S₁ nuclease cut the maxi-circle at a single site, allowing its isolation in a linear form with a mol wt of 12.2×10^6 , determined by electron microscopy. The other enzymes give multiple maxi-circle fragments, whose added mol wt is $12-13 \times 10^6$, determined by gel electrophoresis. The maxi-circle in another *T. brucei* isolate (EATRO 1125) yields similar fragments but appears to contain a deletion of about 0.7×10^6 daltons.

Electron microscopy of kDNA shows the presence of DNA considerably longer than the mini-circle contour length ($0.3 \mu\text{m}$) either in the network or as loops extending from the edge. This long DNA never exceeds the maxi-circle length ($6.3 \mu\text{m}$) and is completely removed by digestion with endonuclease PstI. 5-10% of the networks are doublets with up to 40 loops of DNA clustered between the two halves of the mini-circle network and probably represent a division stage of the kDNA. Digestion with PstI selectively removes these loops without markedly altering the mini-circle network. We conclude that the long DNA in both single and double networks represents maxi-circles and that long tandemly repeated oligomers of mini-circles are (virtually) absent.

kDNA from *Trypanosoma equiperdum*, a trypanosome species incapable of synthesizing a fully functional mitochondrion, contains single and double networks of dimensions similar to those from *T. brucei* but without any DNA longer than mini-circle contour length.

We conclude that the maxi-circle of trypanosomes is the genetic equivalent of the mitochondrial DNA (mtDNA) of other organisms.

KEY WORDS kinetoplast DNA · *Trypanosoma brucei* · maxi-circles · mini-circles · *Trypanosoma equiperdum* · *Trypanosoma evansi*

Kinetoplast DNA (kDNA)¹ is probably the most spectacular and bizarre form of mitochondrial DNA (mtDNA) in nature. kDNA is found in unicellular flagellates of the order *Kinetoplastida*, which includes the genera *Trypanosoma* and *Leishmania* whose species are responsible for widespread and debilitating tropical diseases such as African sleeping sickness, Chagas' disease, and leishmaniasis. The highly unusual mtDNA of these parasites has received much attention in recent times (reviewed in references 1-3, 5, 6, 22, 30, 31, 37, 41).

The kinetoplast of these organisms can be seen by light microscopy as a small, oval or round body, situated near the base of the single flagellum. Electron microscopy has revealed that the kinetoplast is a large mass of DNA contained within a specialized portion of the single giant mitochondrion. The total mass of DNA in a kinetoplast from representatives of the suborder Trypanosomatina ranges from 4×10^9 to 4×10^{10} daltons (see reference 1), and this mass can be isolated as a single gigantic network of topologically interlocked circular molecules. The major structural element in the network is a small duplex circle of DNA—the mini-circle—varying in contour length from 0.3 μm in *Leishmania* to 0.8 μm in *Crithidia* (see reference 1). In addition, DNA longer than mini-circles has been found associated with purified kDNA networks, and this has variously been interpreted as a more complex form of kDNA, as a replicative form of kDNA, as tandem repeats of the mini-circle sequence, or as contaminating nuclear DNA (see reference 37).

Enzymatic dissection of kDNA networks from the insect trypanosome *Crithidia luciliae* with restriction endonucleases led to the discovery that (virtually) all longer DNA in networks from stationary-phase cells could be accounted for by the presence of a novel structural component, the maxi-circle. These maxi-circles have a unique,

non-mini-circle sequence, an average contour length of 10.2 μm , and they represent 3-5% of the kDNA network (3, 13, 14, 16). Free maxi-circles were first visualized by Steinert and Van Assel (36).

In this paper, we have extended these studies to the kDNA from members of the *Trypanozoon* subgenus, to establish whether kDNA in general possesses maxi-circles. The conveniently small size of the kinetoplast network from these trypanosomes makes them more amenable to study by electron microscopy than the Crithidial kDNA networks. Some of the results of this work have been summarized in recent reviews (2, 3).

MATERIALS AND METHODS

Organisms

Trypanosoma (Trypanozoon) brucei brucei East African Trypanosomiasis Research Organization (EATRO) strain number 427 was obtained from Dr. G. A. M. Cross, Molteno Institute, Cambridge, U. K. This strain produces a fulminating infection in rats 4 days after inoculation; it is still capable of cyclical transmission via *Glossina morsitans* (24).

T. (T.) brucei brucei EATRO 1125 was obtained from Dr. M. Steinert, Free University of Brussels, Brussels, Belgium, and produces a fulminating infection 4-5 days after inoculation.

T. (T.) brucei evansi gives a relapsing parasitemia resulting in death of the infected animal in 2-5 wk.

T. (T.) brucei equiperdum produces a single peak of parasitemia in an animal 4-5 days after infection. The latter two strains were obtained from Professor D. Zwart, Institute for Tropical and Protozoal Diseases, State University Utrecht, The Netherlands.

All four strains of trypanosome had a visible kinetoplast after staining with Giemsa's. They were maintained as stabulates stored at -196°C in 5% dimethyl sulphoxide.

Growth and Isolation of Parasites

Wistar strain rats (~300 g) were infected by intraperitoneal inoculation with 10^6 trypanosomes, and the organisms were harvested by exsanguination at the peak of parasitemia. The trypanosomes were concentrated by centrifugation ($1,000 \text{ g} \times 10 \text{ min}$) and separated from blood cells using a diethylaminoethyl-cellulose column (18). The trypanosomes were again concentrated by centrifugation and washed once in 50 mM sodium phosphate, 45 mM sodium chloride, 55 mM glucose (pH 8.0).

Isolation of kDNA Networks

The pelleted trypanosomes (1-4 g wet weight) were resuspended in 100 mM NaCl, 250 mM sodium EDTA,

¹ Abbreviations used in this paper: kDNA, kinetoplast DNA; mtDNA, mitochondrial DNA; SSC, 150 mM NaCl, 15 mM sodium citrate (pH 7.0); NET-100, 100 mM NaCl, 100 mM sodium EDTA, 10 mM Tris (pH 8.0); EATRO, East African Trypanosomiasis Research Organization.

10 mM Tris buffer (pH 8.0) to give a ratio of 1 g of cells to 20 ml of buffer and incubated with pronase (2 mg/ml, final concentration) and sodium dodecyl sulfate (1% wt/vol, final concentration) at 37°C for 1.5–2 h. The pronase was pretreated for 2 h at 37°C and 15 min at 80°C before use. The incubation mixture was layered over a 20-ml cushion of 20% wt/vol sucrose in 100 mM NaCl, 100 mM sodium EDTA, 10 mM Tris buffer (pH 8.0) (NET-100) in SW-27 cellulose nitrate tubes, previously soaked for 24 h in 0.1% wt/vol Sarkosyl-L (sodium sarkosinate), 1 mM sodium EDTA. After centrifugation at 19,000 rpm for 1 h at 20°C in a Beckman L2-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), the supernate containing the nuclear DNA was carefully aspirated and the pellet containing the kDNA was resuspended in 5–6 ml of NET-100 buffer. The pellet was then incubated with ribonuclease (100 µg/ml, previously heat-treated at 80°C for 15 min to inactivate contaminating deoxyribonuclease) for 1 h at 37°C and subsequently with pronase (0.2 mg/ml) and sodium dodecyl sulfate (0.1% wt/vol) for 1 h at 37°C. The digestion mixture was deproteinized by shaking with an equal volume of chloroform/isoamyl alcohol (24:1, vol/vol), briefly centrifuged to separate the phases, and the aqueous layer was removed. The thin interface of denatured protein was reextracted with a small volume of NET-100, and the combined aqueous layers were dialyzed for 24–48 h against 2 liters of 1 × 150 mM NaCl, 15 mM sodium citrate (pH 7.0) (SSC) or NET-100 (with one to two changes) at room temperature.

The dialysate was further purified by NaI gradient centrifugation. The kDNA in NaI ($N_D^{25} = 1.4335$) containing 25 µg of ethidium bromide per ml was centrifuged at 36,000 rpm in a Spinco model L-50, type 40 rotor (Beckman Instruments, Inc., Spinco Div.) for 48–72 h at 20°C. The sharp upper fluorescent band of kDNA was siphoned off and twice extracted with an equal volume of isoamyl alcohol, then dialyzed against 1 × SSC (2 liters, two changes) for 24–48 h at 4°C. The kDNA was concentrated and washed free of any traces of nuclear DNA by centrifugation at 25,000 rpm for 30 min in sterile Eppendorf microcentrifuge tubes in an SW-50.1 rotor fitted with perspex adaptors. The (invisible) pellet of kDNA was resuspended in 5 mM Tris buffer (pH 8.0) and centrifuged as before. The final pellet was stored frozen at –20°C in the same buffer at a concentration of 100 µg/ml.

Gel Analysis of Endonuclease

Digests of kDNA

The purified kDNA (1–3 µg) was degraded with restriction endonucleases as described by Kleisen et al. (15). In most experiments the large mini-circle associations were removed by centrifugation in microcentrifuge tubes as described above, as they stay on top of the gel and tend to trap the smaller DNA molecules. After

centrifugation, 90 µl of the supernate from the 100-µl digest was layered on top of 0.5 or 1.0% agarose gels and electrophoresed, stained and photographed as described (15). In recent experiments horizontal slab gels were also used (21).

The molecular weights of DNA fragments were determined from their mobility in gels relative to the set of reference DNAs used previously (15, 16). Recently, Ito et al. (12) have carefully redetermined the molecular weights of the five fragments derived from phage $\phi 29$ DNA by digestion with endonuclease EcoRI. We have now used their values (6.11, 3.89, 1.08, 0.54, 0.36 × 10⁶) rather than the values determined by us before.

Electron Microscopy

For the spreading of DNA a modified version (8) of the microdiffusion technique of Lang and Mitani (17) was used. The spreading solution contained 3 µg of cytochrome *c* and 1–2 µg of kDNA per ml, 0.15 M ammonium acetate. Diffusion was for 40 min in an atmosphere saturated with formaldehyde vapor. Phage PM2 DNA, if included as length marker, was added to the spreading solution at a concentration of ~0.05 µg/ml. The DNA-protein film was picked up on carbon-coated 200-mesh grids, rotary-shadowed at low angle with Pt:Pd (80:20) alloy, and photographed with a Philips EM300 microscope at an operating voltage of 60 KV.

The relative length of DNA molecules was determined by projecting negatives of photographed molecules onto paper, tracing the resulting image and measuring the tracings with a Hewlett-Packard Digitizer, model 9107A (Hewlett-Packard Co., Palo Alto, Calif.). The relative lengths of DNA molecules were determined by using the enlargement of the electron microscope (calibrated with a carbon replica of a diffraction grating containing 2,160 lines per mm) and of the projection. The absolute length of the long linear PstI fragment was determined by comparison with co-spread phage PM2 DNA molecules with a contour length of 3.02 µm and a mol wt of 5.91 × 10⁶ (9).

Source of Endonucleases

Endonucleases EcoRI, HapII, and HindII + III were obtained and assayed as described (15, 16). Endonuclease HhaI, isolated and assayed as in reference 29, was a gift from Mr. F. C. P. W. Meijlink of this laboratory. Endonuclease PstI was isolated by the procedure described by Roberts et al. (28) for endonuclease HaeII with omission of the ammonium sulfate precipitation and assayed for 30 min at 37°C in 10 mM Tris (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol. After the incubation the solution was extracted once with an equal volume of buffered phenol; the phenol was removed by ether extraction, the ether with N₂ gas.

S₁ nuclease from *Aspergillus oryzae* was isolated ac-

according to the procedure of Vogt (40), omitting the Sephadex G100 filtration and the sulphoethylsephadex chromatography. The enzyme was assayed for 30 min at 45°C in 0.125 M sodium acetate (pH 4.7), containing 0.1 mM ZnSO₄, 0.4 M NaCl, and 0.04% sodium dodecyl sulfate. After the incubation, protein was removed with phenol as described above.

Other Materials

The source of other materials is given in previous papers (14-16).

For electron microscopy of endonuclease-treated networks, 25 μg of kDNA was digested in a proportionally larger volume of incubation mixture, and the networks and linear components were separated by centrifugation as described above. Both pellet and supernate were centrifuged a second time before dialysis against 5 mM Tris (pH 8.0).

RESULTS

General Properties of kDNA Isolated from *T. brucei*

Several methods for the isolation of kDNA were compared in an attempt to maximize the yield of kDNA from the bloodstream trypanosomes. The published methods of Kleisen et al. (14) and Laurent et al. (19) were found to give no better yields than the method reported here. The total yield of kDNA was ~20-30 μg/g wet weight cells, representing about 30% overall recovery of the total kDNA (calculated from reference 23, assuming 10¹⁰ cells per g wet weight). The method described here has the advantage that it is simple and less time consuming. We find that equilibrium gradient centrifugation in NaI is to be preferred to CsCl/ethidium bromide as considerable losses of kDNA can occur in the latter method due to precipitation and absorption of kDNA onto the walls of the centrifuge tube. The chaotropic properties of NaI may be responsible for this difference. Simpson and Simpson (30) have emphasized the necessity for shearing trypanosomal lysates to increase the final yield of kDNA and decrease contamination with nuclear DNA. Shearing by passing the lysate through a no. 20 gauge needle or through a cream press does marginally increase the yield of kDNA, but networks isolated with this modification showed varying degrees of damage when examined by electron microscopy. For this reason we have not included shearing in the standard procedure.

Networks of kDNA isolated by the standard procedure show a restricted dye uptake in CsCl/

propidium diiodide gradients (Fig. 1) as expected for a complex consisting of closed circles. The kDNA bands rapidly to form a symmetrical sharp peak at a higher density than component I of phage PM2 DNA (Fig. 1 A).

Characterization of Maxi-Circle from *T. brucei* kDNA

After digestion of *T. brucei* kDNA (strain 427) with various endonucleases, the fragments can be separated by electrophoresis in agarose gels as illustrated in Fig. 2. Each endonuclease yields its characteristic maxi-circle "extra bands" in addition to those of the mini-circle component (mol wt 0.6 × 10⁶, [6, 38]). In digests containing less than three extra bands, the molecular weights of these bands were determined by co-electrophoresis with suitable standard DNA mixtures in 0.5% agarose gels (Table I). The sum of the molecular

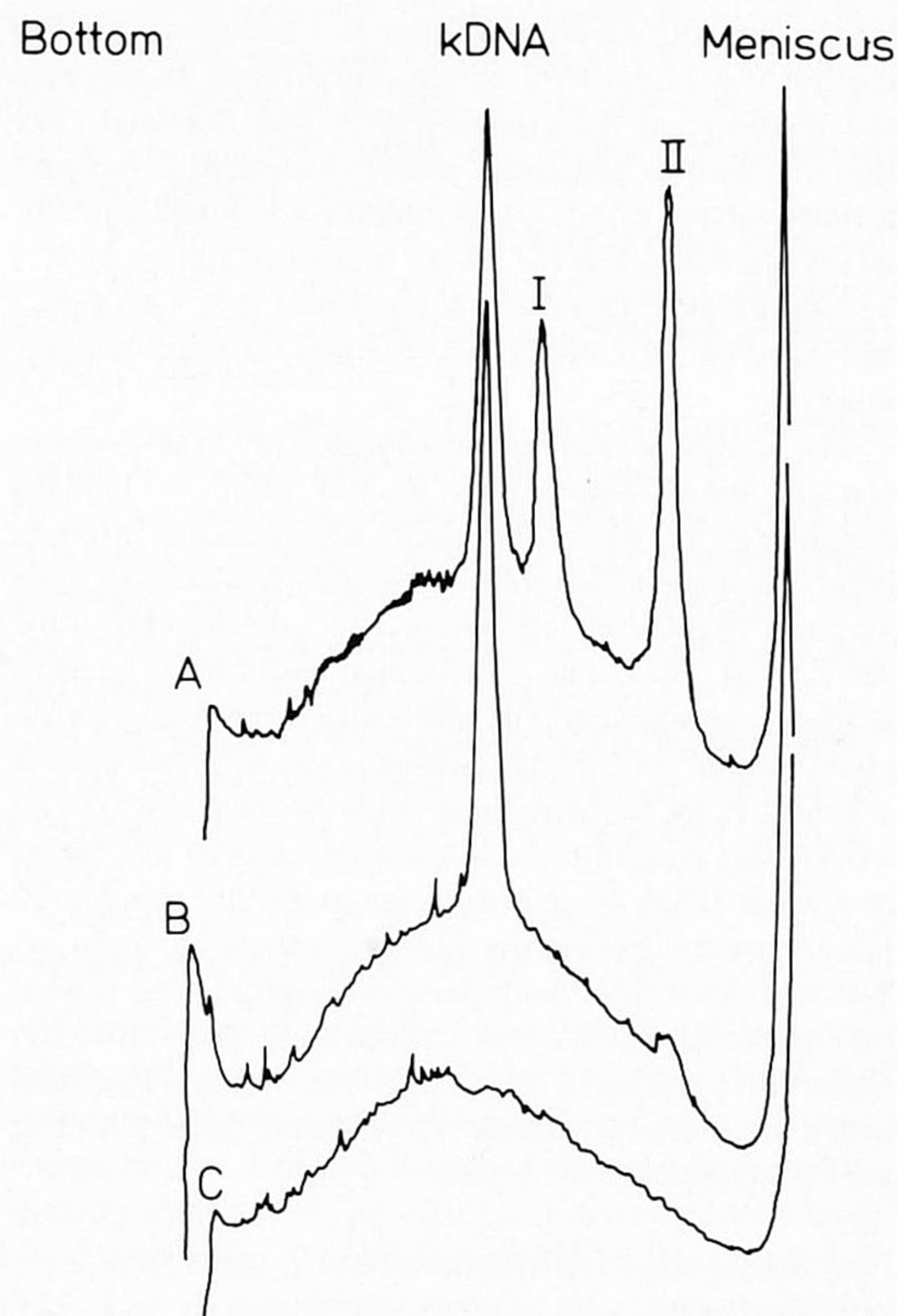


FIGURE 1 Analytical CsCl/propidium diiodide equilibrium gradient of intact *T. brucei* kDNA networks. Gradients were prepared and photographed for scanning as described previously (14). A: *T. brucei* kDNA (5 μg) plus a mixture of covalently closed (I) and open (II) circular phage PM2 DNA (8 μg) as internal control. B: *T. brucei* kDNA (5 μg). C: No DNA present.

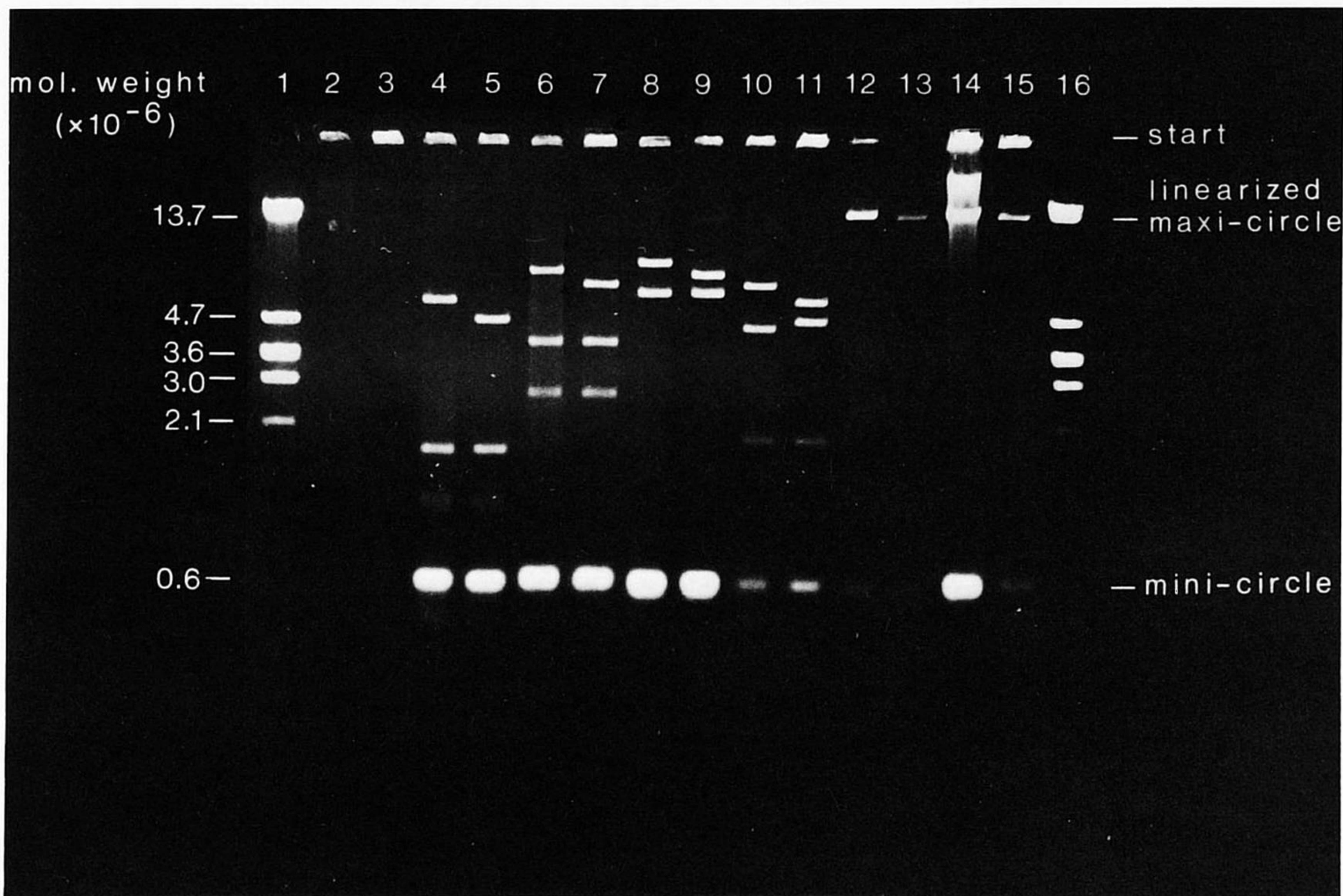


FIGURE 2 Electrophoretic analysis of kDNA from two *T. brucei* strains after complete digestion with various nucleases. Digests of 0.4 μg kDNA were run on a 0.5% agarose flat slab gel for 17 h at 60 V and the DNA was stained and photographed as described in Materials and Methods. The outside lanes 1 and 16 contain an EcoRI digest of phage lambda DNA. Lanes 2, 4, 6, 8, 10, 12, and 14 contain kDNA from strain EATRO 427; lanes 3, 5, 7, 9, 11, 13, and 15, kDNA from strain EATRO 1125. Lanes 2 and 3, undigested kDNA; lanes 4 and 5, kDNA digested with HindII + III; lanes 6 and 7, kDNA digested with EcoRI; lanes 8 and 9, kDNA digested with HapII; lanes 10 and 11, kDNA digested with HhaI; lanes 12 and 13, kDNA digested with PstI; lanes 14 and 15, kDNA digested with S_1 nuclease. The molecular weights of the lambda DNA marker fragments are from reference 39. The slowest band in slots 1 and 16 is a double band; the faster component one is fragment 1 of lambda DNA, the slower a doublet containing the (terminal) fragments 1 and 6 of this linear DNA held together by the sticky ends. The weak slow bands in lanes 8-10 are partial digest products that disappear at higher endonuclease/DNA ratio.

weights of each set of extra bands in each case gives a mol wt of $12\text{--}13 \times 10^6$ for the maxi-circle. The apparent difference in size of the single linear high molecular weight component released by either endonuclease PstI or S_1 nuclease (Table I) is not significant since a mixture of the linear maxi-circles generated by either enzyme could not be resolved into two bands by co-electrophoresis in the same slot (not shown).

The kDNA preparation from strain EATRO 427, used for the experiment in Fig. 2, contained some nuclear DNA contamination. This shows up as a general increase in background in lanes 2, 4, 6, 8, 10, and 12 and as a slow band with an apparent mol wt of 25×10^6 after S_1 nuclease

digestion in lane 14. This slow band was missing in other kDNA preparations from this strain or in the kDNA from strain 1125 (lane 15).

None of the restriction enzymes used in Fig. 2 completely digests the kDNA network, and the remaining mini-circle associations are clearly visible in Fig. 2 as a fluorescent band remaining in the gel slot. We attribute this to the pronounced sequence heterogeneity in the mini-circle population (38), also observed in other trypanosome species (1, 6, 13, 15, 25, 27, 33). We have recently obtained complete digestion, however, with the endonucleases AluI and TaqI (unpublished observations).

For a determination of the molecular weight of

TABLE I

Molecular Weights of Maxi-Circle Fragments Released from *T. brucei* (EATRO 427) kDNA Obtained with Various Endonucleases

Fragment no.	Molecular weights ($\times 10^{-6}$) of fragments released by:				
	EcoRI	HhaI	HapII	PstI	S ₁
1	6.5	5.9	7.2	12.2	12.9
2	4.0	4.4	5.9		
3	2.8	2.1			
Total	13.3	12.4	13.1	12.2	12.9

Molecular weights were determined by co-electrophoresis through 0.5% vertical agarose slab gels with suitable marker DNAs as described in Materials and Methods. Each value is the mean of three separate determinations. The results for endonuclease HhaI are based on two flat gel experiments and are less accurate.

the maxi-circles more precisely, the linearized maxi-circle released from kDNA networks of strain 427 by endonuclease PstI was measured by electron microscopy using circular phage PM2 DNA as internal standard. A single homogeneous class of linear DNA molecules of mean length $6.21 \pm 0.06 \mu\text{m}$ ($n = 28$) compared to phage PM2 DNA standard of mean contour length $2.99 \pm 0.03 \mu\text{m}$ ($n = 21$) was found (Fig. 3). After correction for the published size of phage PM2 DNA (9), the mean length of the maxi-circle is $6.27 \mu\text{m}$ with a mol wt of 12.2×10^6 daltons in agreement with the value obtained in Table I. This is about half the size of the maxi-circle isolated from kDNA of the insect trypanosome *C. luciliae* ($10.2 \mu\text{m}$, 22×10^6 daltons) determined under identical conditions (3).

The Sequence Evolution of Maxi-Circles

Hybridization experiments have previously shown that the kDNAs from two *T. brucei* strains, the strain EATRO 427 used in this work and strain EATRO 1125, show only partial sequence homology (38). For determination of whether this remarkable sequence divergence also affects the maxi-circles, restriction enzyme digests of the kDNAs from both strains were run in parallel (Fig. 2). It is clear that the extra bands for both strains are similar and that, in four of the five restriction enzyme digests, only one band runs faster in the 1125 digest. These results can be explained by the presence in 1125 kDNA of a single contiguous deletion of about 0.7×10^6

daltons. In the HhaI digest, two bands show altered mobility. The simplest interpretation is that the deletion has eliminated one HhaI site and that a new site has arisen in the junction fragment.

We conclude from these results that the large sequence differences found between the kDNAs of strains 427 and 1125 are due to differences in the mini-circles, whereas the maxi-circle sequence is largely conserved. A similar conclusion has been reached in a recent comparison of the kDNAs from *C. luciliae* and *Crithidia fasciculata* (unpublished experiments). In this case, too, total kDNA gives partial cross-hybridization (38), but the maxi-circle sequence is largely conserved as judged from restriction enzyme fragmentation.

Ultrastructure of *T. brucei* kDNA

Single kDNA networks spread by the protein monolayer technique show that the network consists of a large number of interlocking or catenated mini-circles forming an oval-shaped disk with dimensions ~ 4.5 by $3 \mu\text{m}$ in long and short axis, respectively (Fig. 4). In addition to the mini-circle

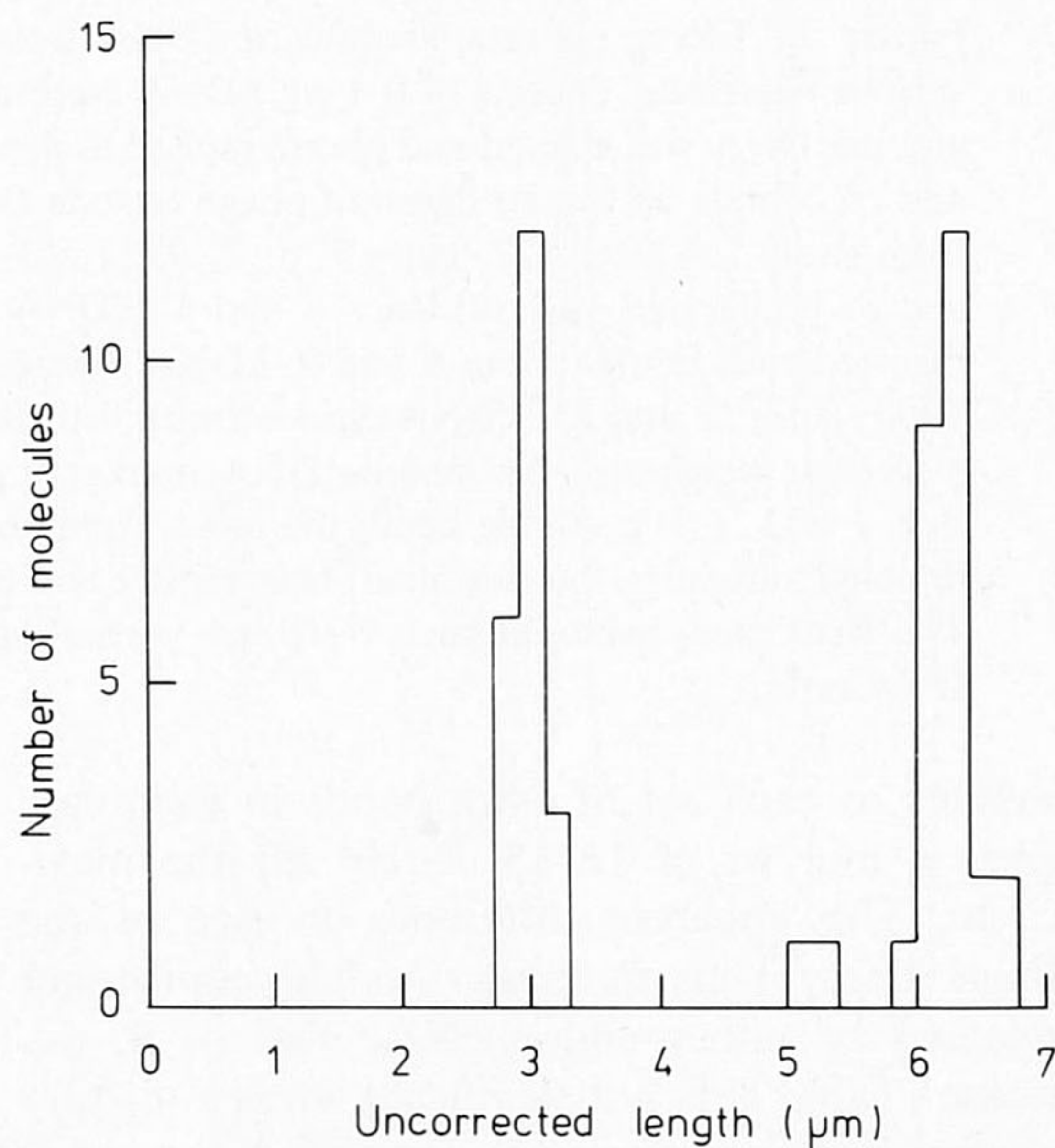


FIGURE 3 Length distribution of long linear molecules released from *T. brucei* kDNA networks by digestion with endonuclease PstI. The networks were digested with PstI endonuclease and the isolated linear molecules were measured with circular phage PM2 DNA as internal control as described in Materials and Methods. The phage PM2 DNA had an uncorrected contour length (\pm SEM) of $2.99 \pm 0.03 \mu\text{m}$ ($n = 21$) and the linear DNA molecules of $6.21 \pm 0.06 \mu\text{m}$ ($n = 28$).

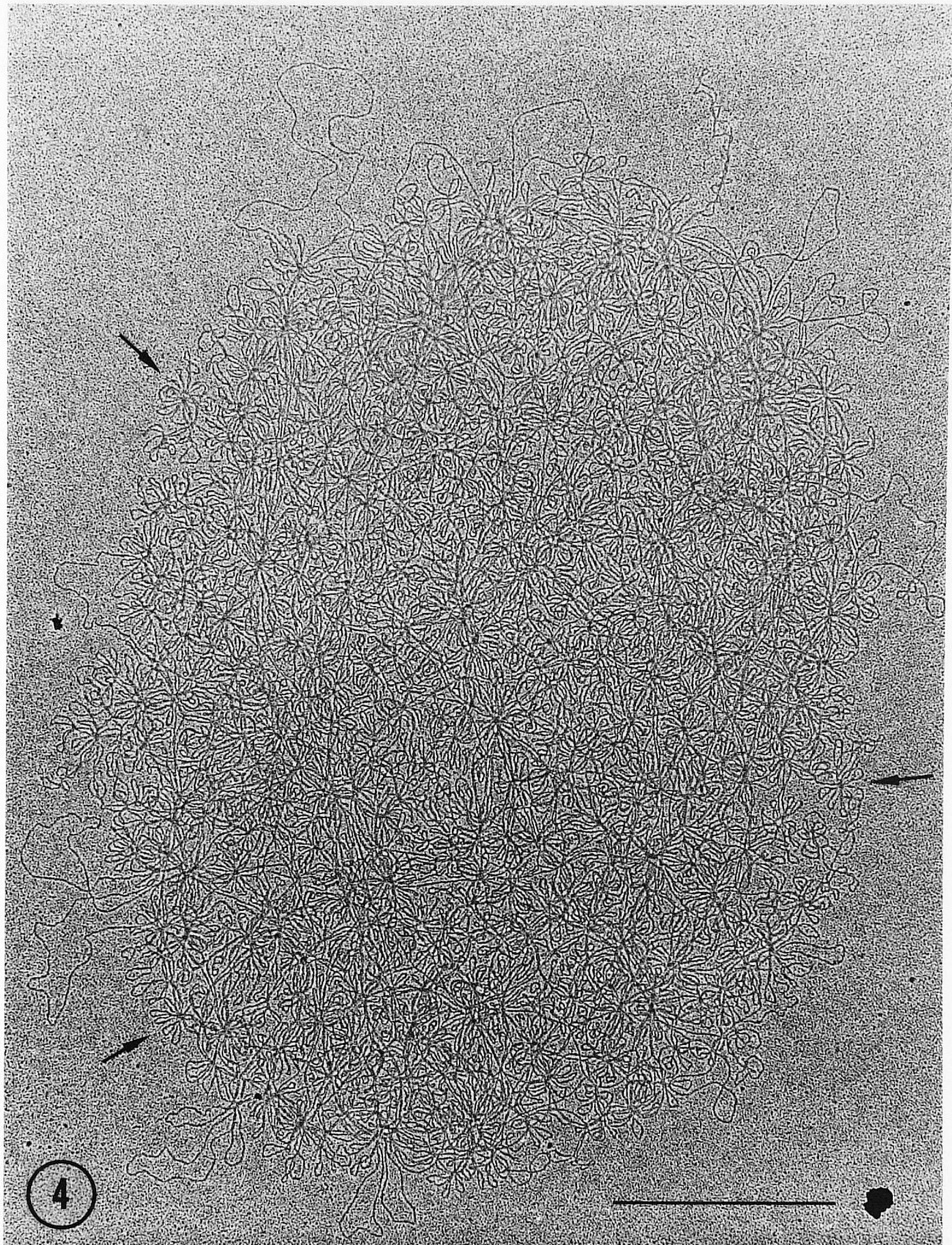


FIGURE 4 Whole kDNA network isolated from *T. brucei* spread by the protein monolayer technique. Relaxed and supercoiled loops can be seen extending from the edge of the network and long linear DNA can be seen running through the interior of the network. At the edge of the network structure a few small groups of interlocking mini-circles ("rosettes") can be seen (arrows). Bar, 1 μm . $\times 33,700$.

components, longer DNA can be seen protruding from the edge of the network and also threaded throughout the interior. A maximum of 23 edge loops has been found associated with a single kDNA network. The length of the edge loops varies according to the extent that the network spreads apart. One preparation revealed a mean length of $1.9 \pm 0.1 \mu\text{m}$ ($n = 30$) when it was co-spread with phage PM2 DNA as standard.

No edge loops greater than the length of the maxi-circle ($6.3 \mu\text{m}$, Fig. 3) have been found by us or by Brack et al. (6). The large edge loop illustrated in Fig. 5 has a free contour length of $5.5 \mu\text{m}$ and probably represents a maxi-circle almost freed from the network. Unfortunately, it is not possible to trace the remainder of the molecule within the network to confirm that it is circular and of maxi-circle size. However, the characteristic twisted appearance of some of these loops suggests that they are part of a closed circular duplex.

Long linear DNA extending from the edge of the network is extremely rare in our standard preparations, but is more common when the trypanosome lysate was sheared during preparation. We have found no free maxi-circles; this is to be expected because our method for the isolation of kDNA strongly selects for the rapidly sedimenting network structures.

Depending on the preparation of kDNA, ~5–10% of the networks were doublets (Fig. 6) and presumably represent intermediate replicating forms of the network. The double networks are connected by a central condensation of DNA from which a large number of loops radiate out on both sides. Each half of the double networks is approximately the same size as a single nondividing network. Again, in the doublets the edge loops which are concentrated between the two halves of the network never exceed the size of the maxi-circle, the longest loop measured in these forms being $4.8 \mu\text{m}$. The number of loops found

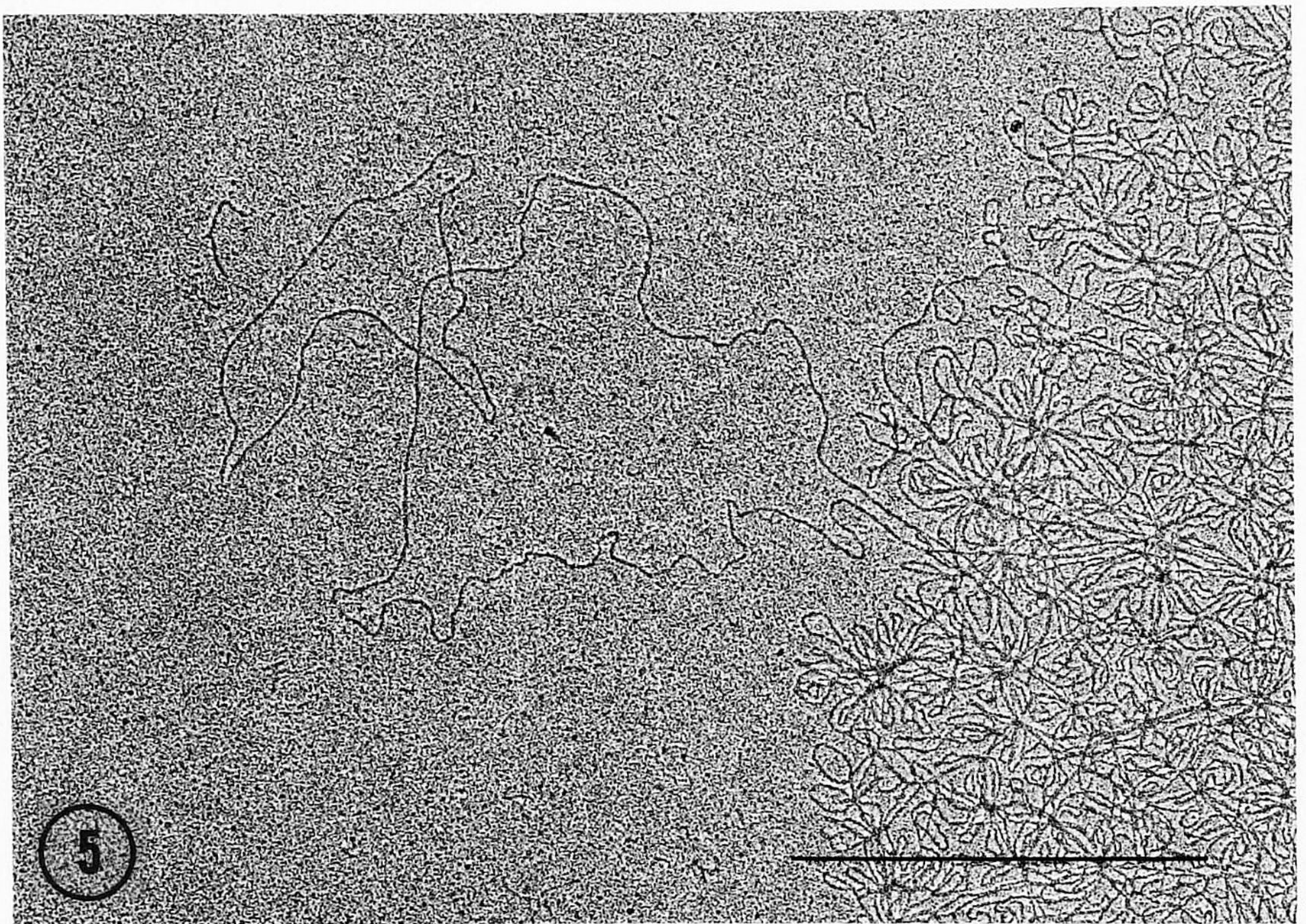


FIGURE 5 Portion of a *T. brucei* kDNA network showing the large circular component (maxi-circle) sticking out from the network. The length of the maxi-circle protruding from the network is $5.49 \mu\text{m}$, using the phage PM2 circular DNA molecule overlying the maxi-circle as internal standard. A single free mini-circle is also visible. Bar, $1 \mu\text{m}$. $\times 45,000$.

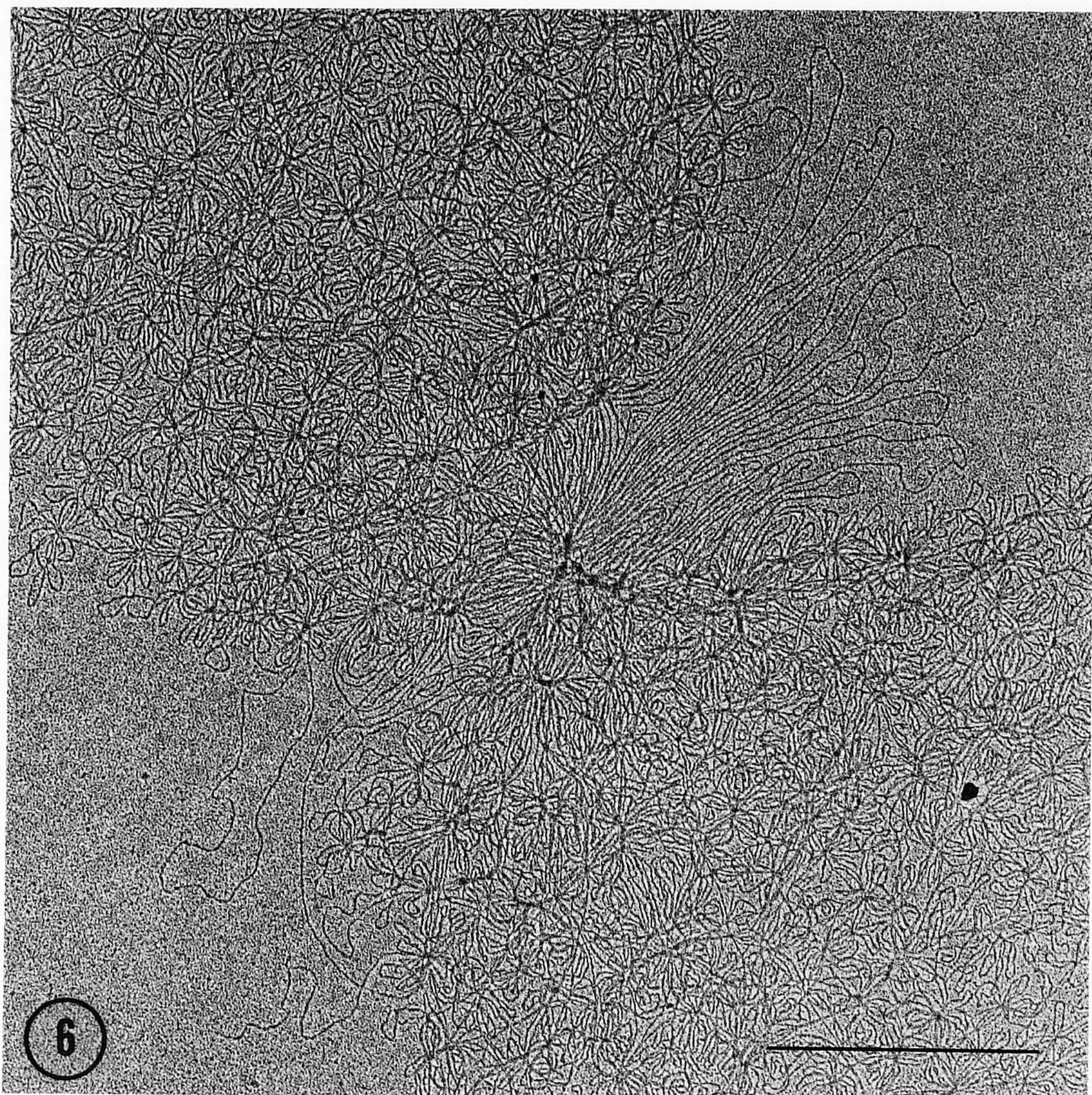


FIGURE 6 Doublet kDNA network from *T. brucei* spread by the protein monolayer technique. Only the central part, where the two networks are in contact, is shown. Bar, 1 μm . $\times 34,900$.

in the doublets is variable, depending on the degree of separation of the two halves of the network, but did not exceed 40 loops/doublet.

Ultrastructure of T. brucei *kDNA after Treatment with* *Endonuclease PstI*

Digestion with endonuclease PstI removes not more than 15% of the total network DNA estimated by ultraviolet absorbance after separation into network and linear fractions by differential centrifugation or equilibrium density centrifugation in CsCl/propidium diiodide. The appearance

of a single and a double network after treatment with endonuclease PstI can be seen in Figs. 7 and 8, respectively. In both cases, all edge loops and long DNA in the network are absent, whereas the overall mini-circle network remains intact. Some general loosening of the network is apparent, but no holes or thinned regions are visible, indicating that the mini-circle class cut by PstI is distributed evenly throughout the network.

Ultrastructure of T. equiperdum kDNA

T. equiperdum is closely related to *T. brucei* and has been shown by Renger and Wolstenholme (26) to contain mini-circles of similar contour

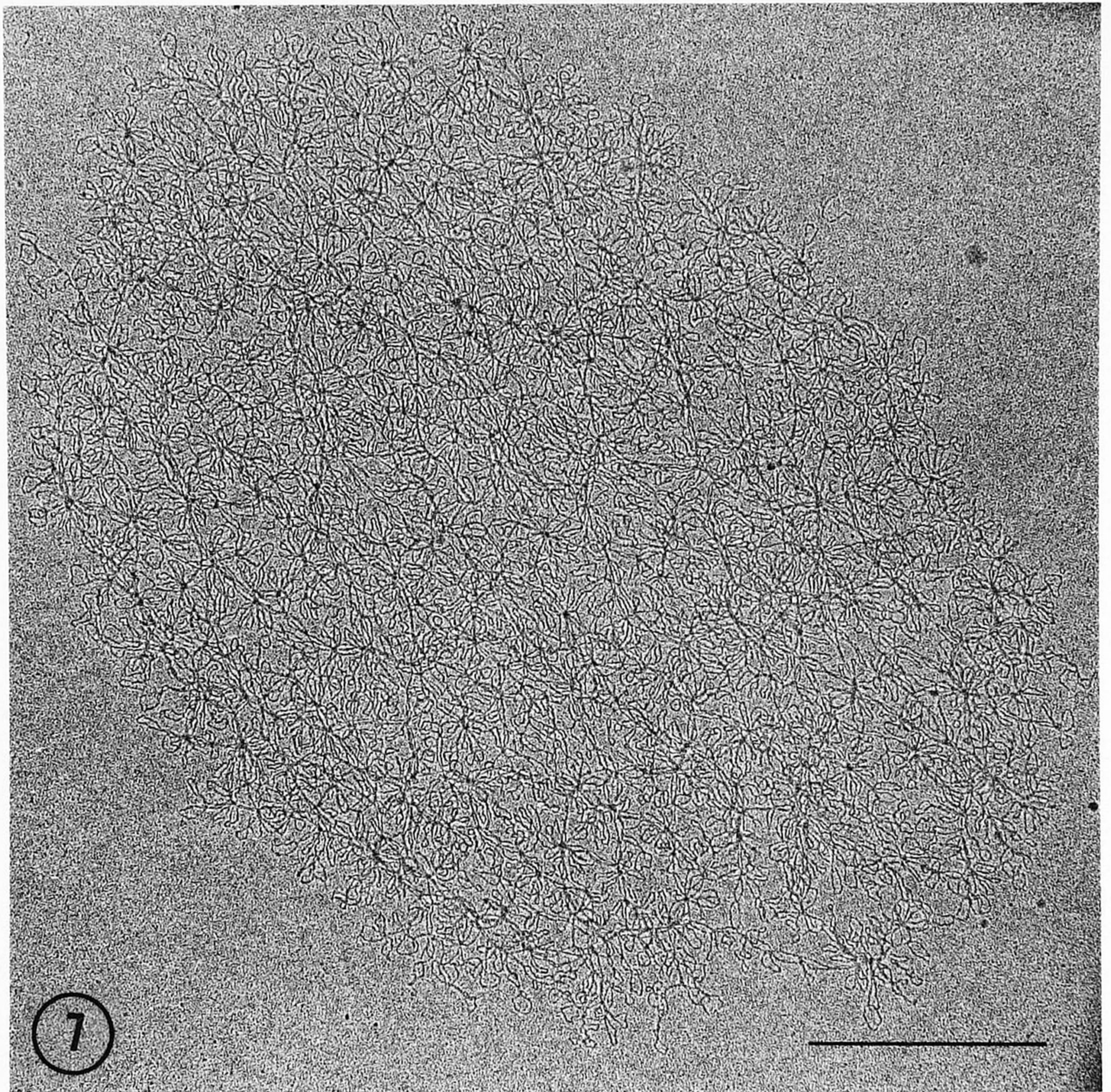


FIGURE 7 Whole kDNA network from *T. brucei* after digestion with endonuclease PstI. kDNA was digested with endonuclease PstI and separated from the mini-circle and linearized maxi-circles by differential centrifugation as described in Materials and Methods. Bar, 1 μm . $\times 30,200$.

length. Preliminary studies on networks isolated from *T. equiperdum* have revealed some interesting differences with respect to those from *T. brucei*. Spread by the protein monolayer technique, the kDNA from *T. equiperdum* forms a flat, slightly oval-shaped disk of dimensions similar to those of *T. brucei* kDNA (Fig. 9). However, the complete absence of long DNA loops at the edge of the network and long linear DNA in the interior of the network is most striking. Moreover, the similarity between these networks and the *T. brucei* kDNA network after treatment with endo-

nuclease PstI (Fig. 7) suggests that the maxi-circle component is absent from kDNA of *T. equiperdum*. In agreement with this suggestion, no linear maxi-circle fragments were detected on agarose gels after endonuclease PstI treatment of *T. equiperdum* networks (not shown).

The doublet networks from *T. equiperdum* are also of dimensions similar to those of the doublets from *T. brucei* (Fig. 10), except for the complete absence of long loops of DNA radiating from the central condensation between the two halves of the mini-circle network. These doublets resemble



FIGURE 8 Doublet kDNA network from *T. brucei* after digestion with endonuclease PstI. The networks were prepared as described in the legend to Fig. 7. Bar, 1 μm . $\times 26,250$.

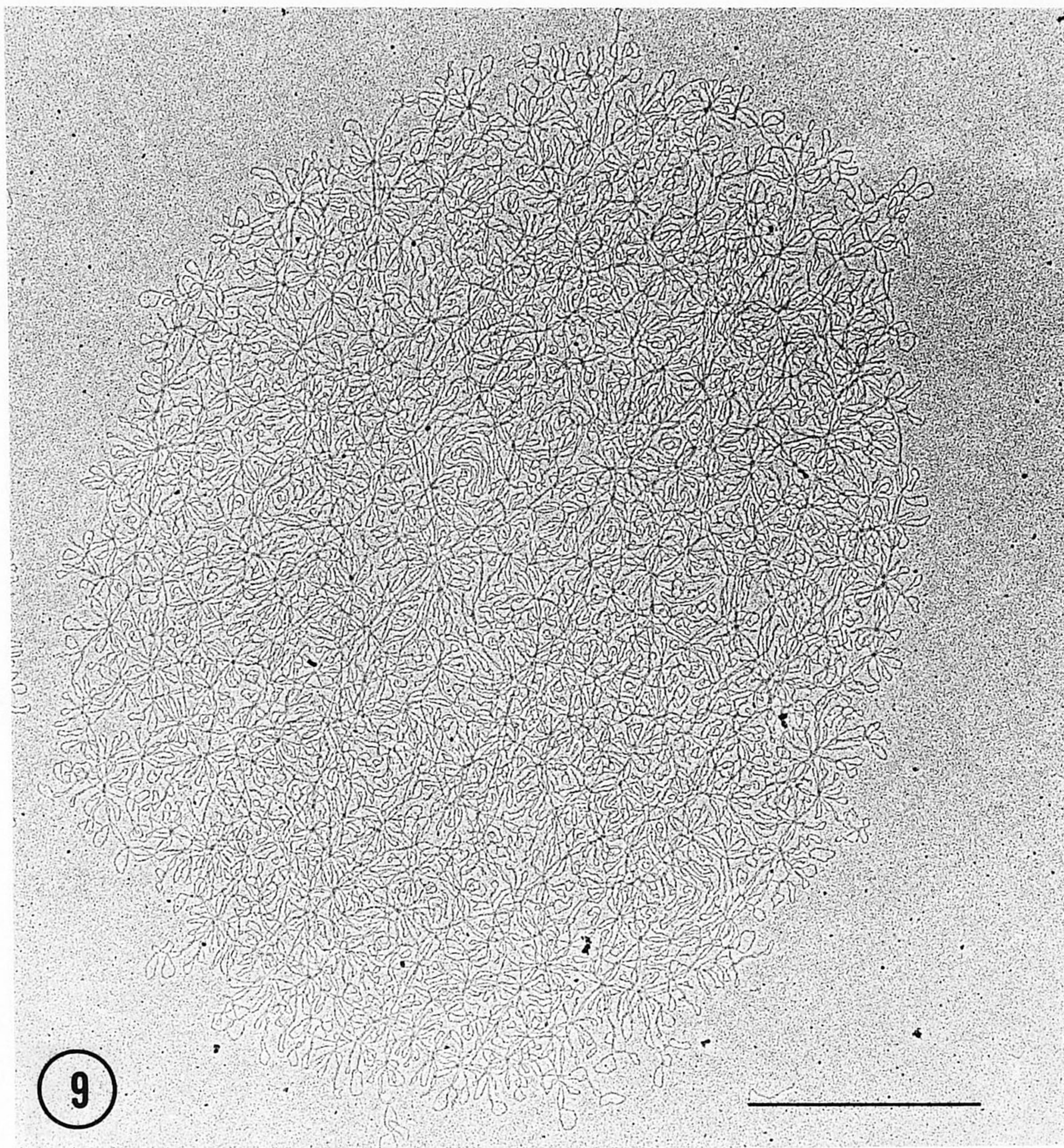


FIGURE 9 Whole kDNA network isolated from *T. equiperdum* spread by the protein monolayer technique. No loops of DNA greater than the contour length of the mini-circle are present. Long linear DNA is not visible within the network structure. Bar, 1 μm . Compare with Figs. 4 and 7. $\times 30,500$.

those of *T. brucei* after removal of the maxi-circle sequences by treatment with endonuclease PstI (Fig. 8).

DISCUSSION

Our experiments show that endonuclease digestion of *T. brucei* kDNA gives rise to DNA fragments longer than mini-circle monomers. Each restriction enzyme yields a characteristic set of

bands on gels, in each case adding up to $12\text{--}13 \times 10^6$ daltons (Table I), and this shows that these extra bands do not represent mini-circle oligomers but are derived from another, more complex DNA with a nonrepeated sequence.

This component is released by S_1 nuclease as a homogeneous population of linear molecules with a mol wt of $12\text{--}13 \times 10^6$. S_1 nuclease is specific for single-stranded DNA but also attacks twisted

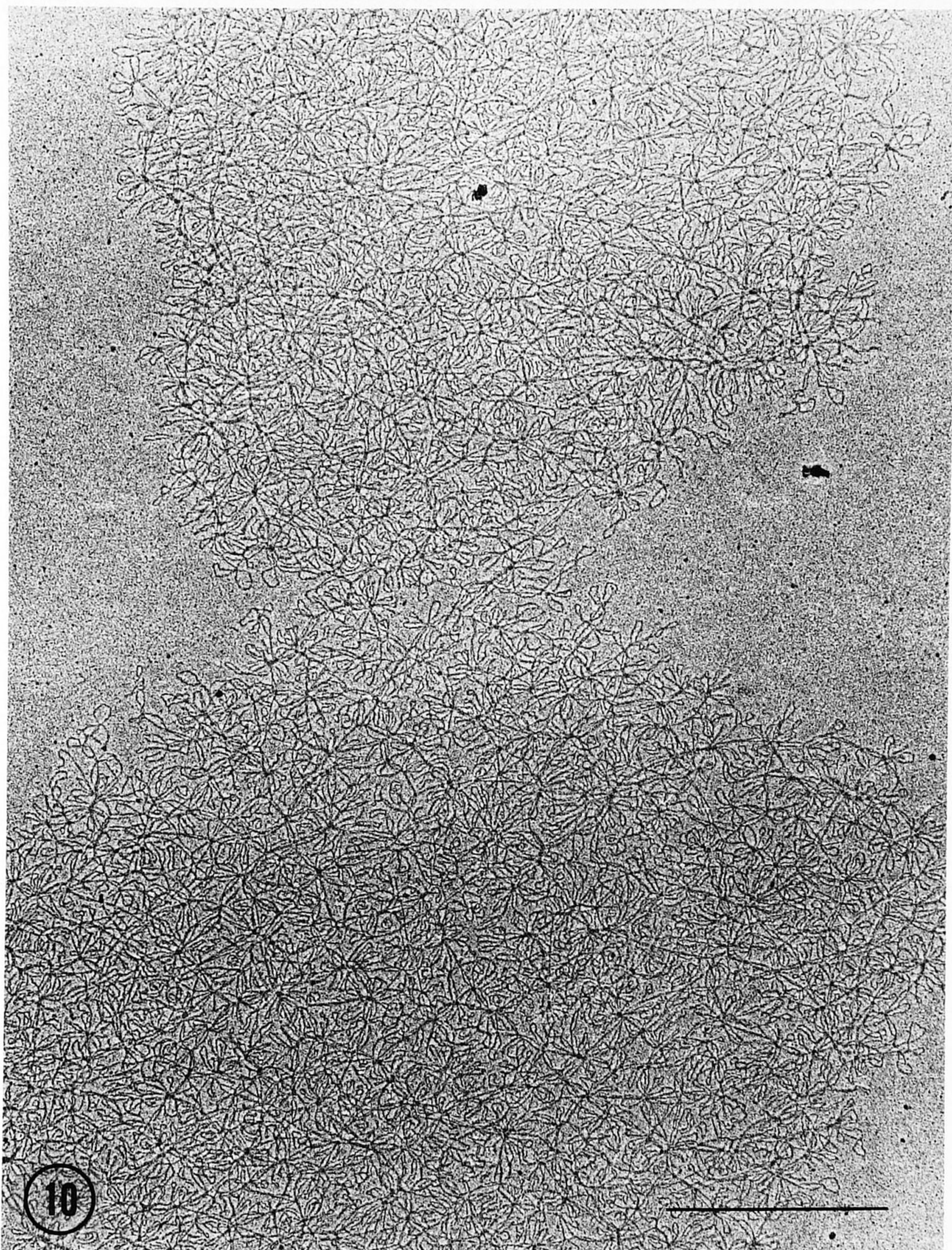


FIGURE 10 Doublet kDNA network from *T. equiperdum* spread by the protein monolayer technique. No long loops of DNA are visible radiating from the area between the two halves of the network. Compare with Figs. 6 and 8. Bar, 1 μm . $\times 32,500$.

circles, first converting them rapidly into open circles by nicking and then more sluggishly into linearized DNA by cutting at the strand opposite the nick (10). We therefore conclude from the results with S_1 nuclease that the complex DNA found in the network is present as large circles catenated into the network, i.e., maxi-circles.

No maxi-circles were found free in any of our *T. brucei* kDNA preparations. However, it is of interest that some circles were found already in 1966 in total cell DNA from *T. brucei* by Sinclair and Stevens (35) and B. J. Stevens (personal communication). Three classes of circular DNA were found: a class of narrow distribution at 7 μm , another with a broader distribution at 13–16 μm , and the third at 24 μm . The significance of the two latter classes of circular DNA is not clear, but the 7- μm class could represent maxi-circles liberated from torn networks.

The intact kDNA networks of *T. brucei* are much smaller than those of *Crithidia* (see also reference 6), and this simplifies their analysis by electron microscopy. A striking feature of the *T. brucei* networks is the large number of long edge loops. None of these loops exceeds the size of maxi-circles, and treatment of the networks with endonuclease PstI, an enzyme that cuts only a small fraction of the mini-circles, results in a complete removal of all edge loops (Figs. 7 and 8) and the concomitant appearance of a homogeneous class of long linear DNA in electron micrographs (Fig. 3) or gels (Fig. 2). We conclude from these results that (virtually) all long DNA in the networks isolated by our procedure is maxi-circle DNA and that the interpretation (see reference 37) that such loops represent tandemly repeated mini-circles, possibly intermediates in the segregation of mini-circles, is untenable. It remains possible, however, that such intermediates do exist but are selectively removed by our isolation procedure.

The trypanosomes used as starting material for kDNA isolation are rapidly dividing and should contain replicating kDNA. The network doublets found by us and by others (6, 37) seem obvious candidates for late intermediates in the replication of networks. Doublets were observed in all kDNA preparations examined in this paper and in more recent work with another *T. brucei* strain (EATRO 31) and *T. evansi* (J. H. J. Hoeijmakers, unpublished observations). Moreover, they were present at 5–10% of the total networks present, irrespective of the DNA concentration

used for spreading or the presence of detectable nuclear DNA. This makes it unlikely that they are artefacts of spreading or isolation. Proof that the doublets are intermediates in replication will require their further characterization by autoradiography and/or biochemical procedures (cf. references 4, 20, 32).

The appearance of the doublets after treatment with endonuclease PstI (Fig. 8) shows that the long loops emerging from the region of contact (Fig. 6) are maxi-circle loops rather than strings of mini-circle oligomers. The appearance of the doublets from *T. equiperdum* provides striking confirmation for this interpretation. If network doublets are indeed late intermediates in network replication, the fact that they are held together by mini-circle monomers would argue against a model for network segregation in which all mini-circles and maxi-circles are converted into one giant circle (see reference 2).

Whereas long edge loops are randomly distributed over the edge of monomer networks, they are entirely concentrated in the region of contact in the doublets. This is compatible with a model in which the maxi-circles are catenated with each other and a small "anchoring" region of the mini-circle network. This anchoring region must broaden sufficiently, however, in the monomer networks to allow the 6- μm circles to extend on all sides from the edge of the $3 \times 4.5 \mu\text{m}$ network.

Representatives of the *Trypanozoon* subgenus are unique amongst the *Kinetoplastida* in being able to survive in the absence of a fully functional mitochondrion (11). In all other subgenera, trypanosome mutants that lack cytologically staining kDNA (so-called dyskinetoplastic mutants) are inviable. Opperdoes et al. (24) have suggested that a defect is present in the maxi-circles in all trypanosomes unable to grow in axenic culture or in insects (I^- strains). Thus, in kinetoplastic I^- strains the maxi-circles would be absent, yet the mini-circle network would still remain intact and, therefore, would stain normally in cytological preparations, whereas in dyskinetoplastic I^- strains the mini-circle network would also be disrupted. The sensitivity of the mitochondrial ATPase to oligomycin was demonstrated to be a suitable criterion for distinguishing kinetoplastic strains with completely repressed mitochondrial biogenesis (I^+ strains) from strains in which a functional mitochondrial genome is absent (I^- strains) (24).

Our results with kinetoplastic strains of *T. brucei* and *T. equiperdum* are of interest in relation to these proposals. *T. equiperdum* is, by definition, I⁻, because it is transmitted between horses during coitus without an intermediate insect vector, and attempts to grow it in axenic culture have been unsuccessful (11). The mitochondrial ATPase of this strain of *T. equiperdum* is insensitive to oligomycin (F. R. Opperdoes, unpublished results), indicating that the mitochondrial genome is defective. In contrast, the *T. brucei* strain EATRO 427 contains an oligomycin-sensitive ATPase (24) and has been demonstrated to undergo cyclical development in the tsetse fly vector (24) and to grow in axenic culture (7), indicating that the mitochondrial genome is intact. Thus, the complete absence of maxi-circles in *T. equiperdum* kDNA, while retaining a mini-circle network indistinguishable from *T. brucei* kDNA, is in complete support of the model proposed by Opperdoes et al. (24). Furthermore, in (unpublished) experiments we have also failed to find any maxi-circles in a kinetoplastic I⁻ strain of *T. evansi*. However, an alternative explanation for our results could be that in kinetoplastic I⁻ strains the maxi-circles are no longer catenated to the mini-circle network and consequently are lost during preparation of the kDNA because our method of isolation involves a stringent sizing step. Further experiments are required to test this possibility and to determine whether free maxi-circles are present in the mitochondrion of I⁺ strains.

The question remains why I⁻ strains contain kDNA networks at all. If kDNA is merely the functional equivalent of mtDNA in other cells, one would eventually expect a complete loss of kDNA, once the maxi-circles are gone. This is also found in yeast in which the rho⁻ mutants that contain altered mtDNA throw off rho⁰ mutants with no mtDNA at all (see reference 24). The faithful preservation of the networks in *T. equiperdum*, therefore, raises the possibility that the mini-circles do not contain genes for mitochondrial components at all. This possibility is underlined by two recent findings. First, mini-circles are heterogeneous in sequence (13), and in *T. brucei* this heterogeneity is extreme (28). It is hard to reconcile this with genetic function. Second, in unpublished hybridization experiments in this laboratory, no RNA complementary to mini-circle DNA has been found in *Crithidia* (E. Sanders and R. A. Flavell, unpublished experiments), raising the possibility that the kDNA

transcription products found by Simpson et al. (34) in *Leishmania* are transcribed from the maxi-circles rather than from the mini-circles. With Steinert et al. (38), we think that mini-circles may not have a conventional genetic function. We have discussed this in detail elsewhere (2).

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