Heterogeneity of the Kinetoplast DNA Molecules of *Trypanosoma cruzi*†

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ABSTRACT: Kinetoplast DNA (kDNA) of the culture form of *Trypanosoma cruzi* is cleaved by restriction endonucleases (*Hpa*II, *Hin*dII, *Eco*RI, and *Hae*III). The analysis of the cleavage patterns proves that the minicircles (free circular genome units) are heterogeneous in base sequences. The same results are obtained with the complex kDNA network which is composed of the association of minicircles and linear molecules. Kinetic studies of the renaturation of kDNA previously cleaved by *Hpa*II into fragments of the genome unit size show at least two populations of molecules. About 75% of these molecules correspond to the fast renaturing population having the molecular complexity of the minicircles. The molecules of the slow renaturing population have a much higher molecular complexity than the minicircles and do not seem to be related to the majority of the long linear molecules.

Trypanosomes are parasitic flagellates characterized by a unique mitochondrion. A specialized region of this organelle, called the kinetoplast, contains a large amount of DNA (kDNA)[1] which represents in the culture form of *Trypanosoma cruzi* 20 to 25% of the total DNA. As revealed by electron microscopy by Riou and Delain (1969a), kDNA is a network of minicircular molecules associated with each other and with long linear molecules. One kinetoplast contains 20 000 to 30 000 interlocked and free minicircles (Riou and Delain, 1969b). The minicircles, the lengths of which are 0.45 μm (about 1440 base pairs), are considered as the kinetoplastic genome units. Similar molecular arrangements of kDNA molecules were described subsequently in several trypanosome species (for a review see Simpson, 1972).

No information is available concerning the genetic content of kDNA. If all the minicircular molecules were to have the same nucleotide sequence, their genetic potential would be very low; they would code for only one or two proteins. The numerous copies of these genome units would reflect a gene amplification. Nothing is known about the coding properties of the linear molecules; moreover, their proportion is difficult to estimate and so far it has not been established if their base sequences correspond to those of the minicircles. Previous results have shown that minicircles do not display the same number of cleavage sites by restriction endonucleases (Riou and Yot, 1975a,b). The purpose of this work is to confirm the heterogeneity of the minicircles, to study the renaturation kinetics of these molecules, and to investigate the relationship between long linear molecules and the minicircles.

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[1] Abbreviations used are: kDNA "form II", kinetoplastic DNA in its relaxed form; kDNA "form I", kinetoplastic DNA in its constrained form; mt- and nDNA, mitochondrial and nuclear DNA, respectively; *Eco*RI, *Hin*dII, *Hpa*II, and *Hae*III, restriction endonucleases respectively isolated from *Escherichia coli* RY13, *Hemophilus influenzae* strain d, *Hemophilus parainfluenzae*, and *Hemophilus aegyptius;* PDI, propidium diiodide; SSC, 0.15 M NaCl-0.015 M sodium citrate (pH 7.2); LM and SM, liquid and solid medium.

Materials and Methods

Trypanosoma cruzi and Culture Conditions. Experiments were performed using the culture form (epimastigote form) of *T. cruzi*, strain Tehuantepec, supplied to us by the Pasteur Institute. The strain was isolated from mouse blood in 1972 (Oct. 10), adapted to the culture medium, and maintained by weekly subcultures. Trypanosomes were cultured in the liquid medium (LM) which overlays a solid medium (SM). LM was composed of 37 g of brain heart infusion, 10 g of glucose, and distilled water to 1000 mL, sterilized in an autoclave for 30 min at 115 °C. SM was composed of 8 g of nutrient broth, 20 g of agar Noble, 8 g of NaCl, and distilled water to 1000 mL, sterilized in an autoclave for 1 h at 125 °C. The medium reactives were from Difco. One-hundred milliliters of SM was melted in a 500-mL Erlenmeyer flask and 10 mL of decomplemented human blood was added under suitable conditions of temperature. When blood medium was solidified, 60 mL of LM supplemented with 200 IU/mL of penicillin and 40 μg/mL of dihydrostreptomycin were added. The trypanosomes were cultured at 27 °C. DNA was extracted from trypanosomes harvested in the exponential phase, namely 6 or 7 days after inoculation. When radioactive DNA was needed, 10 μCi/mL [^{32}P]phosphate (20 Ci/mmol) or 5 μCi/mL [^{3}H]thymidine ([^{3}H]methylthymidine, 25 Ci/mmol) was added to the medium at the beginning of the culture. The specific activity of kDNA was about 8000 cpm/μg for ^{32}P labeling and 32 000 cpm/μg for ^{3}H labeling.

Preparation and Purification of kDNA. Cells were lysed by sarkosyl (final concentration 1%) in SSC for 15 min at 37 °C. The action of Pronase (preincubated 1 h at 37 °C) for 2 h at 37 °C was followed by addition of sodium perchlorate to 1 M. The lysate was deproteinized two times with 2 vol of isoamyl alcohol–chloroform (1:24, v/v); the water phase was dialyzed against SSC and incubated first for 30 min at 37 °C with 10 μg/mL RNase A (pretreated 10 min at 100 °C) and then for 30 min at 37 °C with RNase T1 (10 U/mL). After an additional deproteinization kDNA molecules were fractionated by ultracentrifugation in a PDI–CsCl gradient (PDI, 500 μg/mL; CsCl, 6.36 g; final volume, 8.8 mL; Beckman 50 Ti Rotor; 24 h; 20 °C; 40 000 rpm). The lower band composed of kDNA in its constrained form (kDNA "form I") contains covalently closed minicircles and is often contaminated with

about 5% of nDNA. The upper band contains nDNA and relaxed kDNA (kDNA "form II") composed of open minicircles. kDNAs from "form I" and "form II" were freed of residual nDNA by subsequent ultracentrifugation in a Hg^{2+}–Cs_2SO_4 gradient (Riou and Paoletti, 1967). Sometimes two successive Hg^{2+}–Cs_2SO_4 gradients are necessary. The purity of the kDNA was checked by analytical ultracentrifugation in a CsCl gradient.

Cleavage of kDNA. Several restriction enzymes and S1 nucleases were used to examine the overall composition of kDNA. *Eco*RI endonuclease was purified essentially according to Yoshimori (1971). *Hin*dII and *Hae*III endonucleases, respectively purified by the methods of Smith and Wilcox (1970) and Middleton et al. (1972), slightly modified, were gifts from J. P. Dumas. *Hpa*II endonuclease, prepared as described by Sharp et al. (1973), and S1 nuclease, isolated according to Slor (1975), were kindly provided by P. Nardeux. The activities of the various restriction endonucleases were checked under conditions described in the above corresponding references using λ DNA and SV40 DNA.

The DNA fragments of kDNA were separated by electrophoresis in vertical 2% agarose slab gel as described by Danna et al. (1973). After migration they were located by ethidium bromide staining and photographed. They were eluted from the gel by electrophoresis, recovered by precipitation in the presence of ethanol, and generally further purified by centrifugation in a PDI–CsCl gradient.

Base Composition of DNA. ^{32}P-Labeled DNA was completely digested at 37 °C with pancreatic DNase I (Worthington, RNase free) and snake venom phosphodiesterase (Sigma, type II) under the conditions described by Tapiero et al. (1974). The four nucleoside 5'-monophosphates were separated by paper electrophoresis at high voltage and their radioactivities were determined in a liquid scintillation spectrometer.

Renaturation Kinetic Analysis. Renaturation kinetic analysis was carried out according to the spectrophotometric method of Wetmur and Davidson (1968). The DNA extensively dialyzed against 1 × SSC (pH 7.2) or for some assays against 1.0 M NaCl was introduced into a water-jacketed quartz cuvette which had a circular neck, holding a fitted Teflon stopper. A layer of mineral oil was placed over the surface of the sample before stoppering. Two circulating propylene glycol baths, a denaturing bath and a renaturing one, were connected in parallel through two stopcocks to the cuvette. By turning both stopcocks the DNA solution can be brought from denaturing conditions (98 °C for 2 to 3 min) to the renaturation temperature (T_m −25 °C) within 30 s as measured by a thermocouple. Reassociation kinetic curves were always done twice for each DNA sample and the resulting curves were superimposable. The DNA renatured to the original denatured optical density 260-nm value. The absorbance at 260 nm was recorded as a function of time in an Acta III Beckman spectrophotometer. Sheared phage (PM_2-fd(RF)-T_7) DNAs were used as controls. The molecular weights of these denatured DNAs were determined from the alkaline sedimentation coefficient $s_{20,w}{}^{pH\ 13}$ obtained by band velocity sedimentation (Vinograd et al., 1963). The correlation between alkaline sedimentation velocity and molecular weight was determined as described by Studier (1965). The phage DNAs were generously provided by P. Giacomoni.

Results

Fractionation of kDNA and Preparation of Free Minicircular Genome Units. The purified kDNA from the lower band

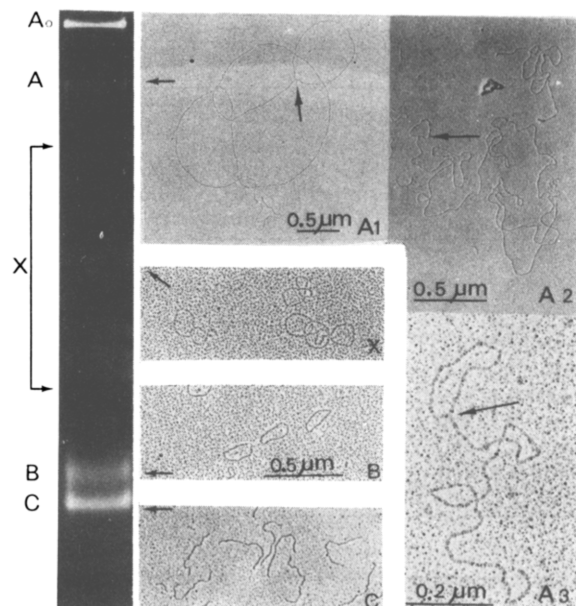

FIGURE 1: Fractionation and analysis of kDNA molecules in the relaxed form (kDNA "form II"). Electrophoretic pattern in a 2% agarose slab gel (0.04 M Tris, 0.02 M sodium acetate, 2 mM EDTA, pH 7.8; 3 V/cm for 12 h). Electron micrographs of the DNA eluted from the corresponding electrophoresis bands. The molecules were examined under a Philips EM 300 electron microscope after being spread according to the method already described (Riou and Gutteridge, 1977). kDNA was cospread with PM_2 phage DNA (9900 base pairs, 6.4 × 10^6 daltons) as internal size standard. A circular pentamer is presented in A1 and monomers in A2 and A3; these molecules are forked with linear DNA. Band A also contains circular molecules (see Figure 2) and associations of minicircles. Bands A_0, X, B, and C are defined in the text.

(kDNA "form I") of the PDI–CsCl gradient represents about 60% of the total kDNA and does not contain any (or very few) free molecules. In contrast, the kDNA in its relaxed form (kDNA "form II") usually contains free minicircular molecules as revealed by gel electrophoresis and electron microscopy. The kDNA "form II" was fractionated by gel electrophoresis as illustrated in Figure 1. Several bands designated A_0 to C in order of increasing mobility are observed. Their respective amount has been determined using ^{32}P- or ^{3}H-labeled kDNA (Table I) assuming that the labeling is uniform. Band A_0 corresponds to kDNA which cannot penetrate into the gel because of the large size of its network (mol wt 2.0 to 2.5 × 10^{10}). Band A mostly contains associations of minicircular molecules and linear molecules of heterogeneous length. When measured by electron microscopy after elution from the gel, the length of linear molecules is found to be between 3.5 and 12 μm. About 8% of these molecules have replication forks and 20–30% of these replicative intermediates are minicircles or circular oligomers forked with linear molecules such as in the rolling circles model of Gilbert and Dressler (1968). Molecules of this type are shown in Figure 1 (photographs A_1, A_2, and A_3). However, considering the position of the band in the gel it cannot be excluded that a part of the linear DNA would be from nuclear origin. Furthermore, a low proportion of circular molecules (<1%) which could be considered as circular oligomers of the minicircles was detected by electron microscopy of the DNA of band A in 3 out of 4 batches of DNA. Their contour length ranges from 1 to 6 μm for molecules photographed (Figure 2). These circular molecules could be consistent with being circular oligomers of the minicircles. The bands designated X are a series of weak bands composed of low oligomers of catenated minicircles or eight-shaped

TABLE I: Radioactivity Content of the Electrophoretically Separated Bands after Hydrolysis of *T. cruzi* kDNAs with Restriction Endonucleases.

			Radioactivity (%)										
			*Eco*RI			*Hin*dII			*Hpa*II			*Hae*III	
Bands	kDNA form II control	kDNA form I control	kDNA form II	kDNA form I	Free mini-circles	kDNA form II	kDNA form I	Free mini-circles	kDNA form II	kDNA form I	Free mini-circles	kDNA form II	kDNA form I
A_0	57.8	76.5	12.1	7.6		9.8	24.0		4.0	3.7		0.7	0.1
A	15.1	0.5	6.4	3.0		6.9	4.1		3.7	6.5		4.7	1.3
X (catenated minicircles)	5.4	1.0	10.4	14.0		7.4	11.4		8.5	7.1		1.2	0.5
B (minicircles 0.45 μm)	19.1	15.6	10.3	15.1	20.5	10.0	39.2	19.0	20.3	21.7	8.0	3.4	3.0
C (linear 0.42 μm)	2.6	6.4	13.2	16.8	19.0	65.9	21.3	81.0	59.4	61.0	92.0	11.9	11.2
D (linear 0.31 μm)			15.5	15.0	16.2							18.1	17.6
E (linear 0.21 μm)			16.5	14.5	12.1				4.1			33.2	40.0
F (linear 0.10 μm)			12.5	11.4	25.4							26.8	26.3
G (linear 0.09 μm)			3.1	2.6	6.8								

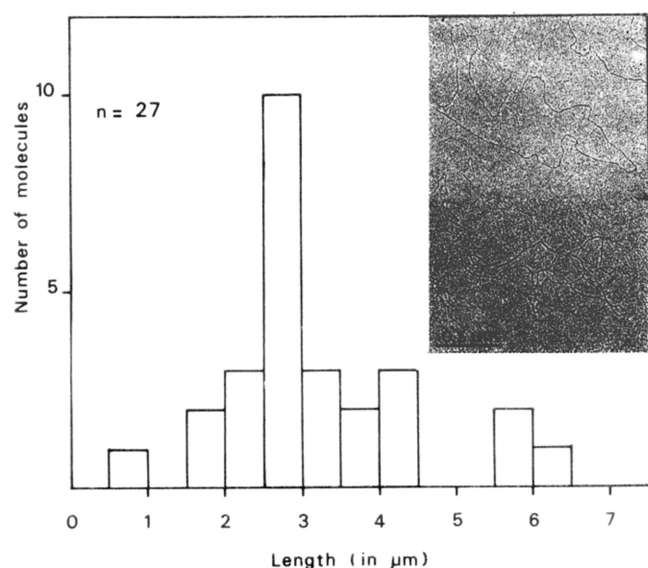

FIGURE 2: Histogram of the contour length of circular molecules found in band A of Figure 1. Electron micrographs of molecules with 5.3 and 4.2 μm contour length.

figures (dimers and trimers). Band B contains free minicircular genome units of 0.45 ± 0.02 μm in perimeter, corresponding to 1436 ± 60 base pairs and to a molecular weight of 0.93 × 10^6 as compared to PM_2 phage DNA. For preparative purposes free minicircles were isolated from kDNA "form II" using such a fractionation and elution of the DNA from band B. The DNA molecules of band C are linear molecules of the length of minicircles. The proportion of both linear molecules and minicircles increases with the age of the DNA preparation. The linear molecules are probably created by mechanical breaking of minicircles. Indeed, old preparations of minicircles afford a much higher proportion of these linear molecules than a fresh preparation. It can be pointed out that after spreading for electron microscope examination the length of these linear molecules (0.42 ± 0.04 μm) is 9% shorter than that of the minicircles from which they are derived. We suggest that the minicircles are tangled as compared to the corresponding linear molecules.

Base Composition of DNA. Because of the molecular complexity and heterogeneous forms of the kDNA, the base compositions of the kDNAs "form II" and "form I" and of the DNA eluted from each gel electrophoresis band were performed (Table II). The results obtained show that the kDNA of each band has the same base composition as the unfractionated kDNA "form I" or kDNA "form II". It is important to notice that the base composition of DNA from band A is in the same order as that of the complex kDNA and of the minicircles. This result excludes the presence of a large amount of nDNA in band A.

TABLE II: Base Composition of *T. cruzi* kDNA and nDNA.

	Base composition (%)				
	C	A	G	T	(A + T)
kDNA "form I"	20.6	31.0	18.5	29.9	60.9
kDNA "form II"	20.4	30.6	18.7	30.3	60.9
nDNA[a]	25.5	23.3	25.1	26.1	49.4
Band A_0[b]	17.6	31.5	20.2	30.7	62.2
Band A[b]	19.2	30.8	19.9	30.1	60.9
Band X[b]	20.1	30.5	17.6	31.8	62.3
Band B[b]	19.0	31.0	19.7	30.3	61.3

[a] nDNA was prepared and fractionated from kDNA after Hg^{2+}–Cs_2SO_4 gradient as described under Materials and Methods. [b] Eluted kDNA from the bands obtained after preparative electrophoresis in a 2% agarose gel.

Endonuclease Cleavage of the Free Minicircular Genome Units and of the Complex kDNA. The cleavage of the free minicircular genome units by *Hin*dII (Figure 3, channel 5) or by *Hpa*II (not shown) occurs at one site as revealed by gel electrophoresis analysis of band C of Figure 3. Indeed the resulting material from these digestions corresponds to linear molecules of 0.42 μm. An appreciable amount of minicircles is not cleaved (Table I). *Eco*RI provides five fragments, C, D, E, F, and G, of respective lengths 0.42 ± 0.03, 0.31 ± 0.02, 0.21 ± 0.02, 0.10 ± 0.01, and 0.09 ± 0.01 μm (Figure 3, channel 1). The length of the different molecules was determined by electron microscopy after elution of each DNA band. Table I gives the proportions of the different fragments and of the uncleaved minicircles. Digestion by *Hae*III provides four fragments, C, D, E, and F (Figure 3, channel 3), of the same length as those obtained with *Eco*RI (Figure 3, channel 1). It is quite remarkable that these two enzymes provide the same cleavage pattern. Double attack of the kDNA, successively by *Eco*RI and by *Hae*III, provides again the same pattern (Figure 4). Thus, the cleavage sites by these enzymes are necessarily located very close to each other.

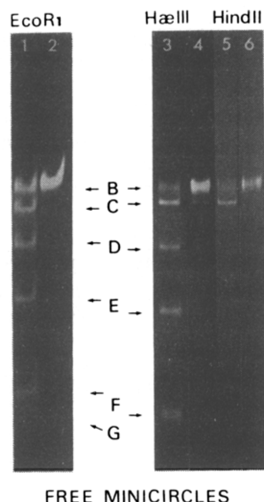

FIGURE 3: Electrophoretic pattern in a 2% agarose slab gel of restriction endonuclease digests of kDNA free minicircles (band B of Figure 1): (1) *Eco*RI; (3) *Hae*III; (5) *Hin*dII; (2, 4, and 6) minicircles without enzyme.

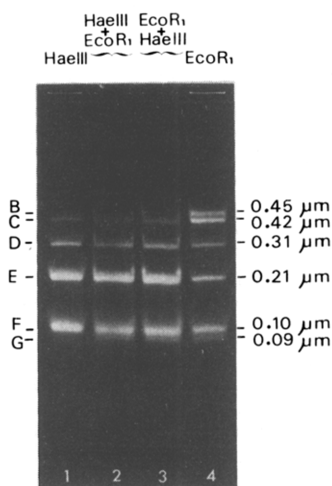

FIGURE 4. Electrophoretic pattern in a 2% agarose slab gel of the products of a double digestion with *Eco*RI and *Hae*III: (1) *Hae*III; (2) *Hae*III + *Eco*RI; (3) *Eco*RI + *Hae*III; (4) *Eco*RI.

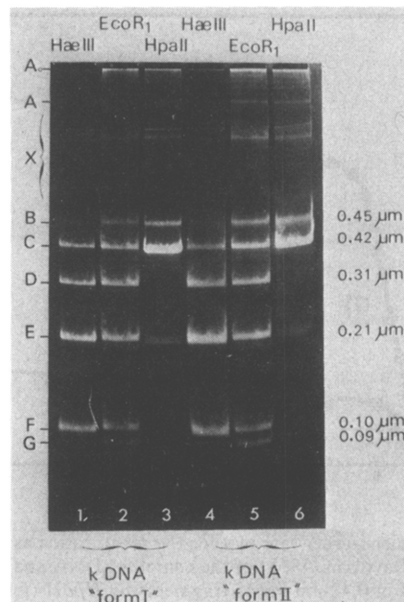

FIGURE 5: Electrophoretic pattern in a 2% agarose slab gel of the cleavage products by restriction enzymes of the complex kDNA "form I" (constrained form), (1) *Hae*III, (2) *Eco*RI, (3) *Hpa*II, and of the complex kDNA "form II" (relaxed form), (4) *Hae*III, (5) *Eco*RI, (6) *Hpa*II. The DNA of the different bands was eluted and its length measured by electron microscopy.

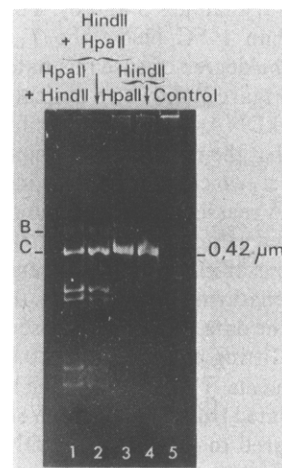

FIGURE 6: Electrophoretic pattern in a 2% agarose slab gel of the successive digestions of kDNA "form I" with *Hpa*II and *Hin*dII: (1) *Hpa*II + *Hin*dII; (2) *Hin*dII + *Hpa*II; (3) *Hpa*II; (4) *Hin*dII; (5) kDNA "form I" control.

The patterns of the digests by these enzymes of kDNA "form II" and "form I" (Figure 5, Table I) are the same as those obtained from the free minicircular genome units with, however, extra bands of lower electrophoretic mobility than that of minicircles. As compared with minicircles the only difference is for *Hpa*II which produces an extra band having the electrophoresis mobility of band E already described with *Eco*RI and *Hae*III. In order to eliminate the possibility of incomplete digestion, kDNA was treated with the enzymes in a quantity 20-fold superior to that required for complete cleavage of λ or SV40 DNAs. In parallel, incubation times were also increased from 2 to 16 h. The kinetic studies of the kDNA digestion by these endonucleases had shown that the plateau is reached in 2 h of incubation under conditions that lead to complete cleavage of λ and SV40 DNAs. Furthermore, the DNA from bands B or C (Figure 5) resulting from a first enzymatic digestion and purified in a PDI–CsCl gradient was submitted to a second incubation with the same enzyme. The results show that kDNA is resistant to the second enzyme treatment (not illustrated).

The comparative results related to the lengths or to the proportions of the *Eco*RI or *Hae*III fragments clearly demonstrate that the number of cleavage sites is not identical in each minicircular molecule. The same conclusion is provided when a double attack of the kDNA was performed, first with *Hin*dII and then with *Hpa*II. Four major new fragments were obtained under these conditions (Figure 6) instead of two fragments which should be expected since these enzymes separately cleave the minicircles into one linear molecule. Consequently, all these results are in favor of heterogeneity of the minicircles which are the major molecular components of the kDNA.

Kinetics of Renaturation. The optical method of Wetmur and Davidson (1968) was used to determine the second-order rate constant k_2. These experiments were carried out with linear fragments of the genome unit size (1440 base pairs) obtained by preparative gel electrophoresis of the *Hpa*II digest

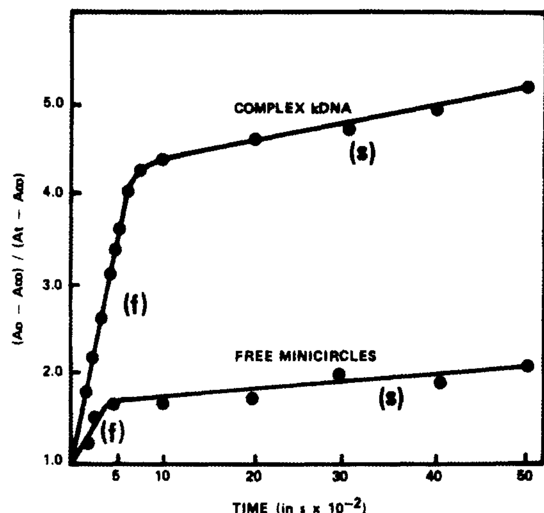

FIGURE 7: Second-order rate plot for the renaturation as described by Wetmur and Davidson (1968) of the complex kDNA and of free minicircles cleaved in 0.42-μm linear fragments by *Hpa*II (fast renaturing component f and slow renaturing component s). The reassociation reaction was studied with two different kDNA concentrations varying by a factor of four, the saline concentration and renaturation temperature remaining constant. The resulting values of k_2 were constant showing that both components follow second-order reaction.

TABLE III: Renaturation Kinetic Analysis of kDNA.

	Fast renaturing component (f)	Slow renaturing component (s)
From complex kDNA		
Mole fraction (%)	61–75	39–25
Rate constant k_2 (L mol^{-1} s^{-1})	3024–4275	62
Kinetic complexity, N_D (daltons)	1.27–0.64 × 10^6	44–42 × 10^6
From free minicircles		
Mole fraction (%)	45–55	45–55
Rate constant k_2 (L mol^{-1} s^{-1})	2800	30
Kinetic complexity, N_D (daltons)	1.4 × 10^6	82 × 10^6

of the free minicircles or of the complex kDNA structures (band C, Figure 5, channels 3 and 6). The T_m of renatured kDNA is less than 1 °C below the T_m of native DNA, suggesting that the degree of base mismatching during renaturation is low. Figure 7 shows an optical plot of the reassociation of such kDNAs. This representation distinguishes unambiguously that the renaturation is biphasic, revealing the presence of at least two classes of nucleotide sequences. About 70% of the kDNA reassociates at a rapid rate. Table III contains the computed values for the rate constants k_2 of the two molecular fractions of kDNA, the fast renaturing component f and the slow renaturing component s. The k_2 values were calculated from the data points by least-squares analysis after tracing the best fitting curve (SD = ±0.001) with two second-order components. The corresponding kinetic complexities (N_D) were calculated from the data of Wetmur and Davidson (1968) as compared to several phage DNAs (Figure 8). It appears from these data that the kinetic complexity of the fast renaturing component corresponds approximately to the molecular complexity of the minicircular kDNA genome unit within the limits of experimental errors. The reassociation rate constant of the slow renaturing component corresponds to DNA molecules of much higher complexities than minicircles. It must be pointed out that the same results are obtained when reassociations are performed from the free minicircular genome units or from the complex kDNA. This slow renaturing DNA population may be the reflection of the heterogeneity revealed by restriction endonucleases cleavage.

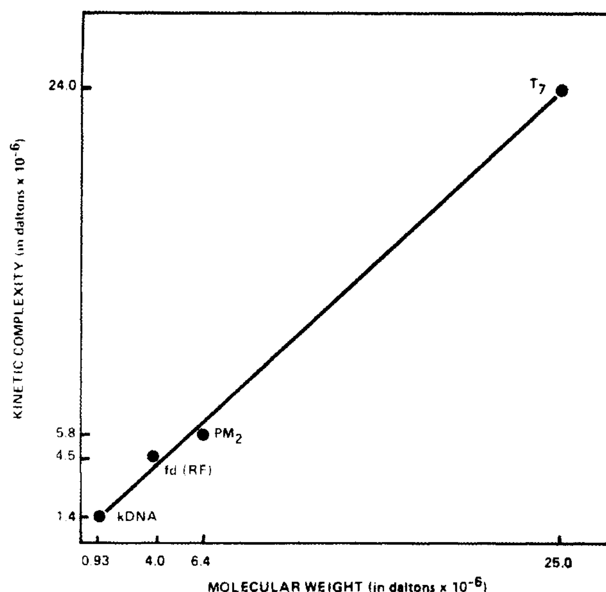

FIGURE 8: Values of the kinetic complexities N_D (in daltons) of phage DNAs calculated from their respective k_2 as described by Wetmur and Davidson (1968) vs. molecular weights.

Discussion

It has already been shown by electron microscopy that long linear molecules are imbricated in the kDNA network (Riou and Delain, 1969a). These molecules could correspond to a part of the DNA of band A observed after fractionation by gel electrophoresis and it is still difficult to exactly evaluate the proportion of linear molecules imbricated in the kDNA network. If they are present in low proportion it is not possible to know if they have the same base composition as minicircles. In contrast, if they exist in high proportion their base composition is the same as that of the minicircles. Our results show that the cleavage patterns obtained with restriction enzymes are the same for free minicircles and for the complex kDNA network which contains these linear molecules. It must be pointed out that a proportion of linear molecules fractionated in band A could have another origin than the kDNA network. For instance, it cannot be excluded that a nonkinetoplastic DNA component might be fractionated by Hg^{2+}-Cs_2SO_4 gradient with the kDNA. Furthermore, the origin of the kDNA "form II" fractionated in its relaxed form must be discussed. This relaxed state can be due to mechanical breakdown and nicking of the network during extraction and fractionation of DNA or to a physiological state such as in the form of a relaxation complex described with colicinogenic factors (Lovett et al., 1974). Price et al. (1976) in *L. tarentolae* have shown that the kDNA of cells in the stationary phase can be recovered in a covalently closed configuration while the kDNAs of cells in the log phase have open structures and could be in a replicative state. We have confirmed these observations (G. F. Riou, unpublished work). The trypanosomes used in this work are collected in the log phase; the relaxed kDNA so fractionated could be the replicative state of the network, corroborated by the presence of forked molecules.

The cleavage by *Eco*RI or *Hae*III of the free minicircular genome units provides, respectively, five or four linear fragments. Their proportions and the sum of their lengths do not agree with the molecular weight of the minicircles, implying that these molecules are heterogeneous. It should be assumed that four sites of cleavage for *Eco*RI and *Hae*III are present in the minicircles. One or more of them are suppressed explaining the heterogeneity of the minicircle population. The amount of fragments obtained as well as their length after cleavage do not permit us to predict the minicircle population which really exists. These supposed sites of cleavage are summarized in the scheme of Figure 9. This base sequence heterogeneity could only be a microheterogeneity; it is not likely due to the heterogeneity of trypanosomes because similar results have been obtained with cloned and uncloned *T. cruzi,* Sonya strain (Riou and Gutteridge, 1977), and recently with Tehuantepec strain (G. F. Riou, unpublished work) and *Crithidia luciliae* (Kleisen and Borst, 1975).

The second-order rate plot for the renaturation of 0.42-μm linear fragments from total kDNA as well as from free circular genome units provides at least two components with different renaturation rates. The proportion of the fast renaturing population found with free circular genome units is, however, lower than that found from the kDNA network. It must be pointed out that experiments of renaturation are performed with free minicircles which represent about 10% of the total kDNA. Two eventualities exist, either the free minicircles are present in the cell or they are liberated from the periphery of network by mechanical breakdown. Nevertheless, the minicircles could not be representative of the molecular population due to the selectivity of their fractionation. In preliminary experiments with sonicate kDNA from *C. luciliae,* Steinert and Van Assel (1972) obtained a similar two-slope curve. The great majority of the fast renaturing component is composed of molecules having the complexity of the minicircle. We cannot exclude that a part of this population contains molecules of highly repetitive base sequences which reassociate at such a rate that the initial hyperchromic drop cannot readily be followed by spectrophotometry due to the relatively high concentration of DNA. When reassociation of the complex kDNA is performed according to the hydroxylapatite method (Britten and Kohne, 1968; Tapiero et al., 1974) the fast renaturing component corresponds to 75–80% of the kDNA. By S1 nuclease treatment 10 to 15% of this DNA fraction is hydrolyzed. Thus, the proportion of the fast renaturing component determined by the optical method is in good agreement with these results. Furthermore, about 15% of the DNA renatured at the zero time of experiments, even for C_0t (mol $\times$ s $\times$ L^{-1}) values as low as 10^{-7}. Similar instant renaturation was reported for yeast mtDNA (Locker et al., 1974), leucoplast DNA (Siu et al., 1975), and recently for the kDNA of *C. acanthocephali* (Fouts et al., 1975). This DNA fraction was considered to contain repeated sequences either in a tandem or in an inverted tandem arrangement.

We have observed the presence of a population of high kinetic complexity (~40 to 80 $\times$ 10^6 daltons) in the complex kDNA as well as in the free minicircles. This fact excludes the only participation of DNA of high molecular weight (long linear molecules) for the low renaturation rate observed. Furthermore, the attack of free minicircles by restriction enzymes gives electrophoresis patterns showing heterogeneity of these minicircles. It could be suggested that the fast renaturing population corresponds to homogeneous minicircles and that the slow renaturing one to heterogeneous minicircles. However, the proportion of these two populations is not in agreement with this hypothesis; such a discrepancy is difficult to explain. It is likely that the fast renaturing population also provides a heterogeneous pattern with restriction enzymes. Experiments are in progress in order to isolate the two renaturing populations and to test them with restriction endonucleases. Wesley and Simpson (1973) reported from renaturation studies that more than 95% of *Leishmania tarentolae* kDNA consists of repeated minicircles base sequences and also possesses a component of higher complexity which could be equivalent to the mtDNA of other organisms. Kleisen et al. (1976) have recently reported the presence of a kDNA compound of high molecular complexity in the kDNA network of *C. luciliae* that they called maxicircles. These authors consider that the presumed maxicircles are mtDNA corresponding to 3–5% of the kDNA and with about tenfold the molecular complexity of the minicircle. The circular molecules we described in band A do not seem to be related to the above maxicircles.

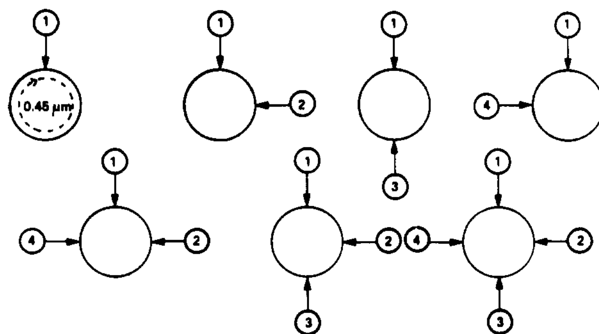

FIGURE 9: Possible sites for *Hae*III or *Eco*RI on the minicircle assuming that the site 1 is fixed. All sites are distant by one-half or one-quarter of a minicircular genome unit.

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Binding of Adenine Nucleotides and Pyrophosphate by the Purified Coupling Factor of Photophosphorylation†

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ABSTRACT: The purified, homogeneous coupling factor for photophosphorylation of spinach chloroplasts binds 2 mol each of ATP, adenylyl imidodiphosphate (AMP-PNP), ADP, or pyrophosphate (PP_i) per mol of enzyme, but very little AMP, and no P_i. The saturation curves for the binding of ADP, ATP, and AMP-PNP to the coupling factor exhibited distinct bimodalities when the enzyme was prepared by a procedure that involved extraction of chloroplast membranes with ethylenediaminetetraacetic acid. These bimodalities were barely evident with coupling factor isolated from chloroplasts that had been previously extracted with acetone. This difference in binding profiles indicated that the two isolation and purification procedures may yield coupling factor in somewhat different conformational states. A study of the competition between ADP, ATP (or its analogue AMP-PNP), and PP_i for binding to the coupling factor revealed that both competitive and allosteric mechanisms may operate in the binding of ligands by this enzyme. The similar nucleotides ATP and AMP-PNP behaved differently in these experiments. ADP was completely inhibited from binding to the enzyme by 2 mol of AMP-PNP per mol of enzyme or by only one mole of intact ATP. This may be related to the ability of the coupling factor to hydrolyze ATP but not AMP-PNP. In addition, the results presented here suggest that adenine nucleotides may interact with the chloroplast coupling factor through both their purine base and their terminal pyrophosphoryl group. Bound nucleotides are enzymatically altered subsequent to their association with the coupling factor, and the details of this reaction, as well as its implications, are presented in the following paper of this issue.

The chloroplast coupling factor 1 (CF_1)[1] is generally accepted as the enzyme that catalyzes the terminal steps in the photosynthesis of ATP (Penefsky, 1974a). Studies of the binding of adenine nucleotides to this enzyme should thus be pertinent to the mechanism of photophosphorylation. The isolated, purified CF_1 has two binding sites for ADP, one with a higher affinity for ADP than the other (Roy and Moudrianakis, 1970, 1971a; Girault et al., 1973; Cantley and Hammes, 1975; Vandermeulen and Govindjee, 1975). CF_1 also has two binding sites for ATP (Livne and Racker, 1969) and for PP_i (Girault et al., 1973). The results obtained from a study of

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[1] Abbreviations used are: CF_1, 13S coupling factor of photophosphorylation; acetone enzyme, CF_1 prepared from an aqueous extract of acetone-treated chloroplasts; EDTA enzyme, CF_1 prepared from an EDTA extract of chloroplast membranes; AMP-PNP, adenylyl imidodiphosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DTT, dithiothreitol; DEAE, diethylaminoethyl; ATPase, adenosine triphosphatase; EDTA, ethylenediaminetetraacetic acid; P_i, orthophosphate; PP_i, pyrophosphate; $\bar{\nu}$, moles of ligand bound per mole of enzyme.