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Electron Microscopy of Kinetoplastic DNA from *Trypanosoma mega*

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Abstract. The electron microscopical aspect of kinetoplast DNA has been studied in preparations obtained by osmotic disruption of isolated organelles. A large amount of this DNA appears to be of large molecular size. This is apparently at variance with the observations, made by others using a different technical approach, of kinetoplast DNA consisting mainly of small circular molecules of constant size. As a homogeneous population of small circles (0.74 μm in contour length) could also be seen in our samples, the possibility that at least two different kinds of DNA exist in the kinetoplast is discussed.

The protozoa of the order Kinetoplastidae are characterized by the presence of a large amount of mitochondrial DNA which is contained in a specialized part of the mitochondrion, the kinetoplast. Kinetoplast DNA has been separated from nuclear DNA by density gradient centrifugation[1–4] and its molecular properties studied. Like mitochondrial DNA in other organisms, it renatures extensively and rapidly,[5,6] but, although mitochondrial DNA is generally found as circular duplexes at least 4.5 μm in length,[7,8] kinetoplast DNA of *Trypanosoma cruzi* has been described as consisting of circles about ten times smaller.[9] Relaxed and supercoiled molecules of such small dimensions have been observed, as have catenanes and a small proportion of linear molecules.[9] Similar observations have been reported for *Leishmania tarentolae*.[10]

Kinetoplast DNA seems to be bound to the kinetoplast membrane[11] and, in total DNA extracts from trypanosomes, the yield of kinetoplast DNA as compared with nuclear DNA tends to be surprisingly low. These facts led us to the supposition that the small kinetoplast DNA molecules described by Riou and Delain[9] and by Simpson[10] are probably not random samples. In order to check on this point, we used a new approach to the problem of the electron microscopical aspect of kinetoplast DNA. Kinetoplasts were isolated and their total DNA content was collected on grids after osmotic lysis. Only a small proportion of the DNA was found to occur as small circles in these preparations: the major part appeared as continuous structures of much larger size.

Material and Methods. *Trypanosoma mega* is grown until late log phase in Boné's medium.[12] The cells are then collected by low-speed centrifugation and chilled. Their disruption is obtained by a slightly modified version of the procedure described by Simpson.[11] The trypanosomes are washed in 10^{-3} *M* EDTA in distilled water and allowed to swell in this medium until they can be readily broken by a few passages through a no. 26

hypodermic needle. The kinetoplast-mitochondrion complex is liberated from the cell envelope as an osmotically swollen vesicle, which shrinks to its approximately normal size upon addition of enough concentrated sucrose solution to make the final concentration 0.25 *M*. The lysate is then centrifuged at 7000 *g* for 10 min and the resulting pellet resuspended in STE buffer (sucrose, 0.25 *M*; Tris 2×10^{-3} *M*; EDTA 10^{-3} *M*; pH 7.2). After two washes of the 7000 *g* sedimentable fraction with STE buffer, this fraction is deposited on top of a 5% Ficoll (Pharmacia, Uppsala, Sweden) solution in STE buffer and spun at 2000 *g* for 15 min to separate the unbroken cells, which sediment to the bottom, from the subcellular fragments. The supernatant, containing most of the kinetoplasts, is then centrifuged at 12,000 *g* and the pellet is resuspended in STE buffer. Further fractionation is obtained by equilibrium centrifugation in a linear 78.5–8.5% sucrose density gradient. This gradient is prepared in such a way as to contain an inverse concentration gradient of the sample in its 38.5–8.5% top portion. It is spun for 3 hr at 24,000 rpm in a SW25 Spinco rotor. The gradient is then collected in fractions of ten drops each, after puncture of the tube. All the steps of this cell fractionation process are performed at temperatures between 0° and 5°C. The distribution of the kinetoplasts and nuclei among the different fractions is estimated by examining small samples of individual fractions with the phase contrast-fluorescence microscope, in the presence of 20 μg/ml ethidium bromide. Kinetoplasts and nuclei are easily identified on the basis of their fluorescence, size, and characteristic morphology. A fairly clean kinetoplast fraction bands at the density level 1.21 gm/ml.

The osmotic disruption of the kinetoplast and the release of its DNA is basically performed by the procedure described by Van Bruggen *et al.*[13] Freshly isolated kinetoplasts are suspended in ice-cold 4 *M* ammonium sulfate. Cytochrome *c* is added to a final concentration of 0.03% and the mixture kept in ice for 30 min. The spreading is made at room temperature with distilled water as the hypophase. The DNA-protein film is picked up on 300-mesh formvar and carbon-coated grids; these are dehydrated in a solution of uranyl acetate 10^{-5} *M* in 95% ethyl alcohol containing 10^{-3} *M* HCl,[14] and shadowed with platinum iridium at an angle of 6 to 8°.

A few samples are incubated with DNase (10 μg/ml) in the presence of 5×10^{-3} *M* $MgCl_2$ before spreading.

The observations and micrographs are made with a Hitachi HS-6 electron microscope that was calibrated with a carbon grating replica.

Results and Discussion. Our kinetoplast fraction still contains a variable but small proportion of nuclei and nuclear fragments, together with a few cytoplasmic debris such as membranes and flagella. We tried to digest contaminating nuclear DNA selectively with DNase, but the kinetoplast membranes appeared to be too permeable to the enzyme to allow any useful and safe use of this method. We found, however, that the presence of fragments of contaminating chromatin is not a complicating factor. Indeed, the electron microscopical aspect of its irregular clumps of very long nucleoprotein fibers, which are usually stretched over several meshes of the grid, is very different from the regular patches of DNA, rather constant in size, which result from the explosion of kinetoplasts. Moreover, in the living trypanosome, the kinetoplast is morphologically connected to the flagellum. This connection is frequently maintained after osmotic disruption and, quite often, the kinetoplast DNA patch remains attached to the remnants of the 9 + 2 axonemal fibers of the flagellum (Fig 1) so that any confusion with contaminating nuclear DNA becomes very unlikely.

After spreading, the kinetoplast DNA forms a round patch (Fig. 1), about 10 μm in diameter, of intricately entangled fibers which are sensitive to DNase digestion. Free ends are very rarely observed. Especially where the filaments

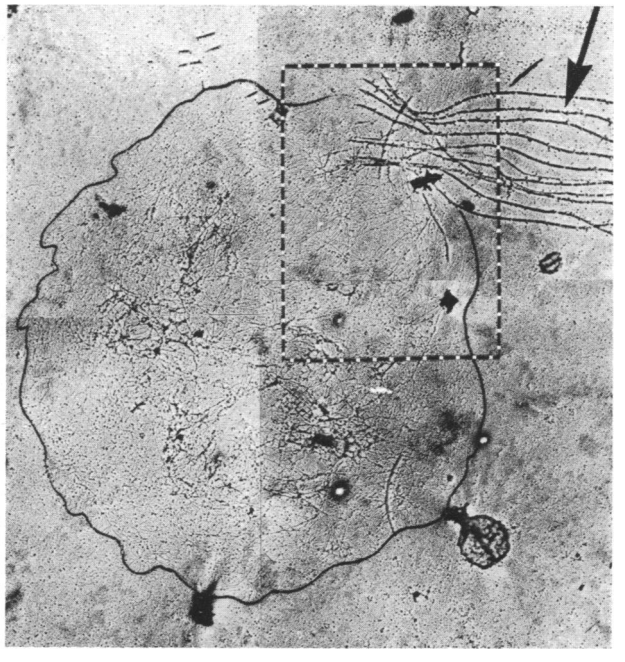

FIG. 1.—Electron micrograph of a patch of kinetoplast DNA still attached to the partially dissociated 9 + 2 fibers of the flagellum (*arrow*). The contour of the patch has been outlined. The broken line frames a part of the picture which is shown enlarged in Fig. 2. ×6900.

are most densely interlaced, a distinct rosette pattern composed of loops of variable size is formed (Figs. 2 and 3). This particular pattern has been observed in phage and bacterial DNA preparations[15, 16] and is probably related to the technique used rather than to the molecular configuration of the nucleic acid itself. The spreading of the DNA is often much better at the edges of the patch, where continuous filaments can be traced along lengths which may exceed 10 μm. Long segments of this kind seem to occur most frequently near the remnants of the basal body. In addition, small circular molecules are found all over the grid (Fig. 3). Thirty-six of these circles, sampled at random, have been measured. Except for one of them, which has a contour length of 0.44 μm, they form a homogeneous population of 0.74 ± 0.03 μm circles, all of the "open" type. Densely supercoiled molecules have not been observed with certainty, hence, there is no evidence for the presence of covalently closed circles. In addition to the DNA filaments, a large number of small bodies, presumably membrane fragments of 250 to 500 Å in diameter, are found either loosely dispersed over the background or more densely intermingled with the DNA fibers of the disrupted organelles.

Our results are in apparent contradiction with those reported by Riou and Delain[9] and by Simpson,[10] in that most of the kinetoplast DNA appears in our preparations to be of considerably larger molecular size than the minicircles isolated by these authors from whole trypanosome DNA extracts. This discrepancy might be due to the presence, in the kinetoplast, of at least two different kinds of DNA: a homogeneous population of easily solubilized small circles and a DNA of much higher molecular weight which would remain strongly bound to insoluble structures, presumably the inner kinetoplastic

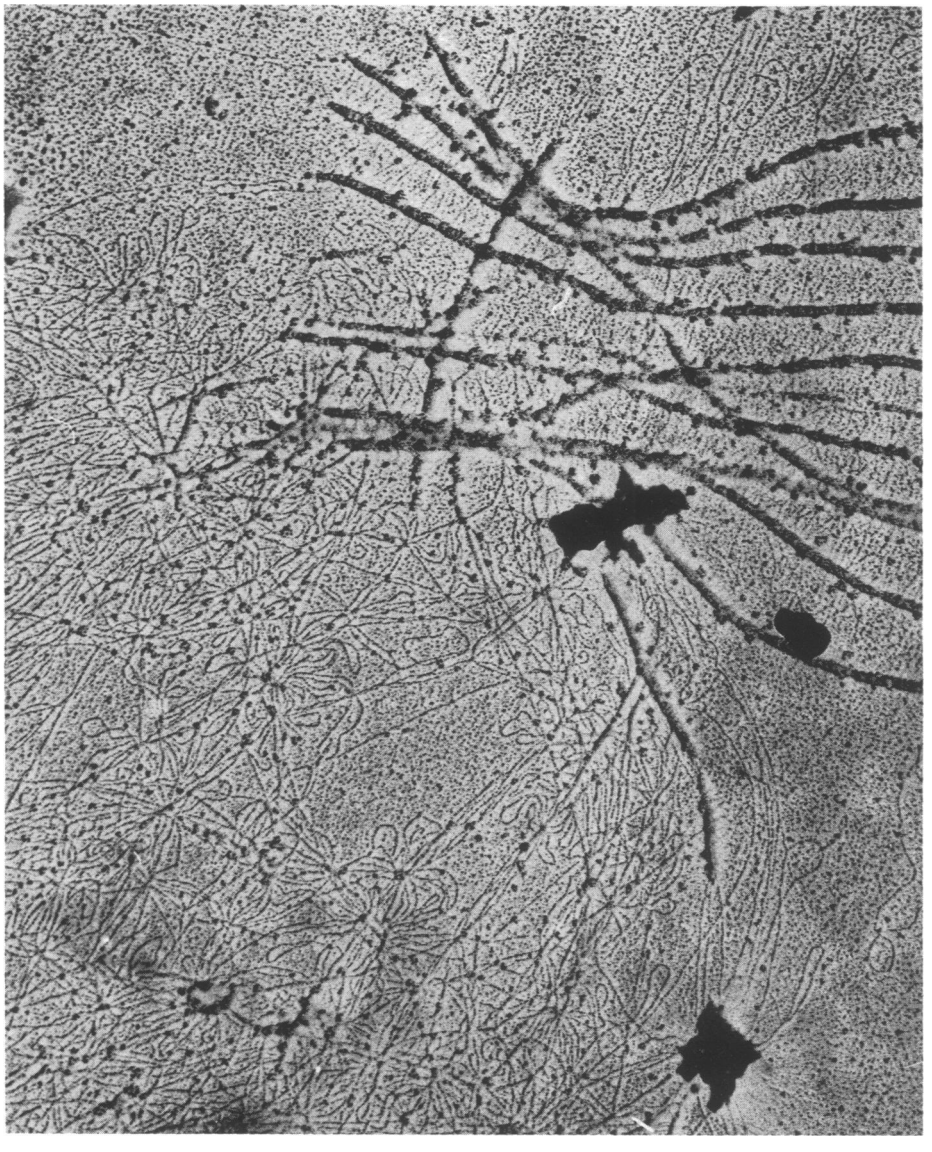

FIG. 2.—Kinetoplast DNA. Note the long continuous fibers and the rosette pattern. ×31,000.

membrane. As we have suggested above, the conventional extraction methods would select the most soluble fraction only, thus yielding (as in Riou and Delain's and in Simpson's works), a high proportion of small circles. Conversely, our own preparation would select the less diffusible fraction of kinetoplast DNA. This raises two important questions: (*a*) Does the high molecular weight fraction contain sequences homologous to the circular molecules and (*b*) does it contain segments of high informative capacity? Answers to these questions could be obtained by the technique of molecular hybridization and by measuring the

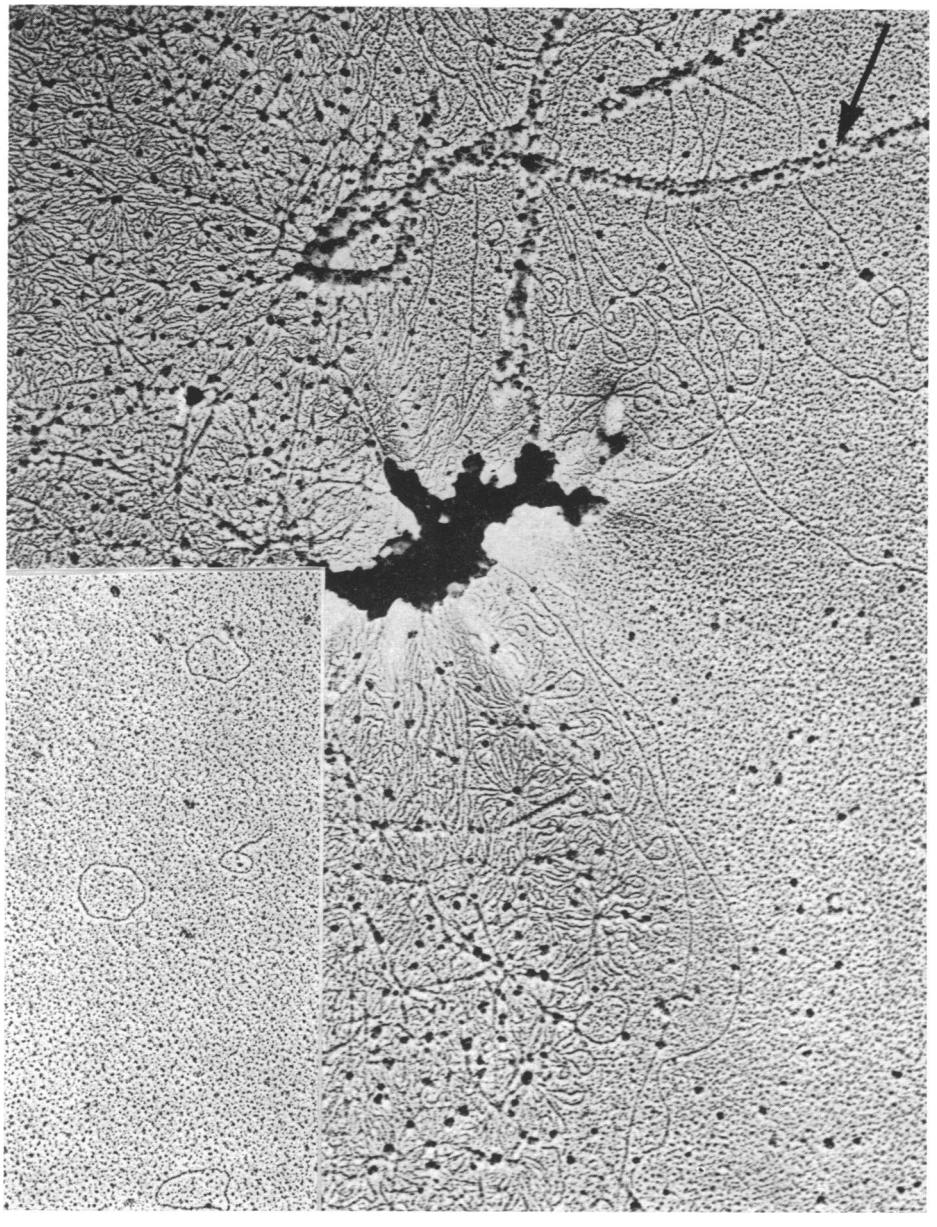

FIG. 3.—Edge of a kinetoplast DNA patch showing long continuous segments of DNA close to the remnants of the flagellar fibers (*arrow*). Intermingled with the DNA filaments are numerous 250 to 500 Å particles. ×31,000. *Inset:* 0.74 μm DNA circles. ×31,000.

kinetic complexity of kinetoplast DNA, provided satisfactory samples of this DNA can be prepared. In relation to question (*a*), one should also consider the possibility that most of or maybe all the kinetoplastic DNA could be composed of short repetitive sequences arranged in units of high molecular size, but that in some circumstances, perhaps in the presence of a specific endonuclease, these

sequences could be disconnected and allowed to form circles, thanks to the presence of sticky ends. That repetitive sequences are present in kinetoplast DNA is consistent with the high rate of renaturation of the "small circle" fraction.[6] The exact role of such an amplified DNA in the kinetoplast remains an open question, but, if the circle corresponds to the basic sequence, the length of this sequence might be species specific. It would be 0.45 μm in *Trypanosoma cruzi*, only 0.22 μm in *Leishmania tarentolae* and, if the small circles observed in our preparations originated from the kinetoplast, which seems most probable, 0.74 μm in *Trypanosoma mega*.

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