

Structure and Function of Kinetoplast DNA*†

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SYNOPSIS. Cells of the order Kinetoplastida possess a single mitochondrion which contains a large amount of a uniquely organized DNA. This kinetoplastic DNA (K-DNA), representing 10-20% of the total cell DNA in different species, has as its major molecular component a small closed circular molecule present in large numbers. The size and thereby the amount of genetic information carried by the minicircles varies from species to species: *Leishmania tarentolae* and the Salivarian trypanosomes have the smallest, the Stercorarian trypanosomes *Trypanosoma lewisi* and *Trypanosoma cruzi* intermediate, and *Crithidia* and also *Trypanosoma mega* the largest minicircles. In *L. tarentolae*, purified minicircles, which are the size of 1 gene, have been shown by renaturation kinetics to consist of only 1 or 2 classes. *L. tarentolae* K-DNA also contains another molecular species—a long molecule which may represent up to 30% of the total K-DNA. The minicircles, nevertheless, represent a gene amplification of the order of 10^4 . In all species that have been examined so far, the K-DNA consists of a single sheet of interlocked closed circular molecules which can be isolated in an intact form because of its resistance to shear forces and its high molecular weight. In addition, at least in *L. tarentolae*, 6-9 % of the K-DNA is either free in the mitochondrion or loosely bound. The main K-DNA structure has been termed a "network" and can be seen in the light microscope after staining in solution with acridine orange or after fixing and staining with Giemsa's, or in the electron microscope. The quaternary structure of such networks in terms of the organization of minicircles and long molecules is not understood. Controlled breakdown of networks from *L. tarentolae* was achieved by sonication, and the release of open and closed monomeric minicircles, catenated dimers, trimers and higher oligomers, and short linear fragments was measured. A maximum of 43% of the total network DNA was released in the form of closed monomers, dimers, and trimers, thus providing a minimal estimate for the percentage of minicircles in K-DNA from this species. K-DNA replicates fairly synchronously with nuclear DNA in all species that have been examined. Replication of DNA molecules in the kinetoplast networks is limited to the periphery, as seen in autoradiographs of networks isolated from cells (*L. tarentolae*, *Crithidia fasciculata*) pulsed with ^3H -thymidine. The molecular implications of this unusual replication pattern remain an open question, as does the genetic function of the K-DNA itself.

Index Key Words: Salivarian trypanosomes; Stercorarian trypanosomes; *Trypanosoma mega*; *Leishmania tarentolae*; *Crithidia*; kinetoplastic DNA; fine structure; function.

THE existence of a prominent dark-stained granule of nucleic acid at the base of the flagellum in all hemoflagellates has intrigued protozoologists for many years. It was known since

1924 (3) that the granule contained DNA, and it was suspected that it also had mitochondrial affinities (16). It was shown also in early studies that experimental elimination of the granule

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was possible by treatment of the cells with certain dyes (21). As a result of these investigations, it was realized that the presence of the granule was essential for the viability of the great majority of the hemoflagellate species (18).

All of these facts came together into a unified theory in the early 1960's, with the application of electron microscopy and biochemical techniques to the study of kinetoplasts. Steinert, Vickerman, Ris and others showed clearly that the classical kinetoplast granule consisted of a large fibrous mass of DNA situated within one region of the single mitochondrion that extended throughout the cell. These workers developed the theory that the K-DNA was essential for the process of mitochondrial biogenesis, found to play a major role in the cyclical transformations that occur in many of these species (see 18).

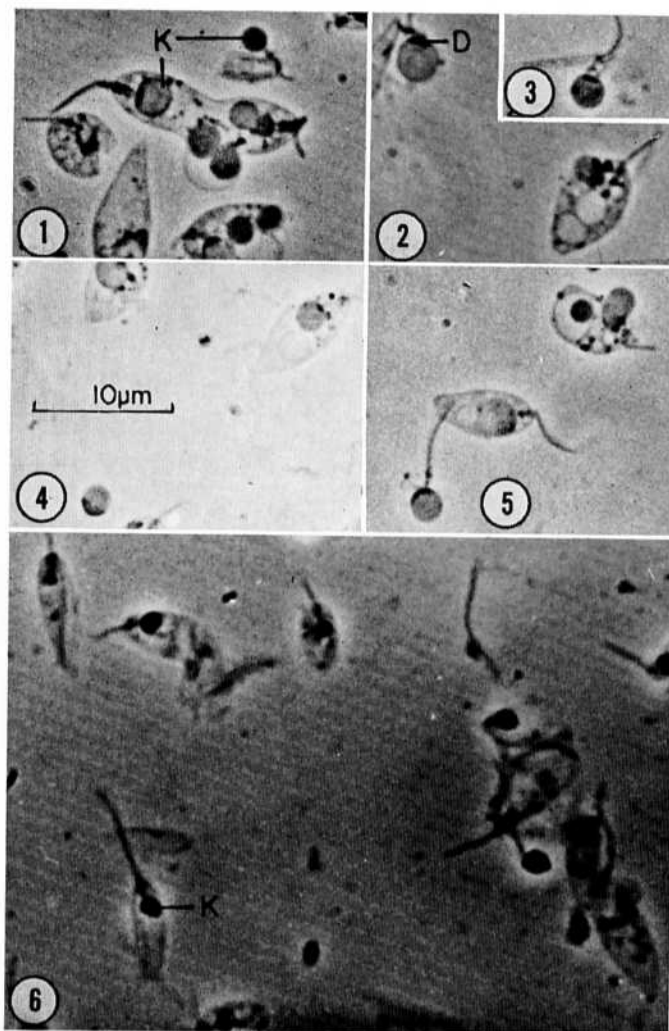
As in many other situations in science, at about the same time it was discovered that a unique DNA occurs in all mitochondria in all eukaryotes, and that this DNA has some genetic role in the process of mitochondrial biogenesis (see 1). The study of mammalian mitochondrial DNA has progressed much faster than the study of the kinetoplast DNA mainly due to the simplicity of the system: mammalian mitochondria contain several identical circular DNA molecules 5 μm in circumference, that have been found so far only to code for mitochondrial ribosomal RNA and mitochondrial transfer RNA (6), and it is clear now that most of the mitochondrial proteins are coded in nuclear DNA and synthesized in the cytoplasm (15). The situation differs somewhat in the case of the fungi (5), higher plants (8), and in the few ciliate protozoa (20) that have been studied where the mitochondrial genome is approximately 4-6 times the size of the mammalian mitochondrial genome.

Structure of the K-DNA In Situ

The kinetoplast DNA proved to be a recalcitrant object for detailed study due to the complexity of the system, but the very complexity itself has made this study fascinating and meaningful. In thin sections examined with the electron microscope, K-DNA appears as a concave sheet of parallel, longitudinally oriented fibers situated near the basal body but within the inner mitochondrial matrix (18). It seems to be membrane bound, and is present in such a high concentration that it can be seen with phase contrast optics in osmotically swollen cells (Figs. 1-6).

Isolation of K-DNA

The 1st indication that the K-DNA could be isolated and was a very large structure came with the finding by DuBuy et al. (7) that the K-DNA of *Leishmania enrietti* rapidly formed a satellite band in CsCl gradient. The separation of K-DNA from several species (*Leishmania tarentolae*, *Trypanosoma cruzi*, *Trypanosoma lewisi*) was achieved initially by the use of preparative buoyant separations in CsCl or Cs_2SO_4 . With *L. tarentolae*, this procedure involved much handling and led to a heterogeneous mixture of several molecular types (Figs. 7-15) (19). The most striking and unusual species was a small minicircle, only 0.29 μm in circumference, containing 838 ± 45 base pairs, approximately the size of 1 gene. This molecule was present in large quantities either free or catenated to one or more minicircles (Figs. 7-14). Also present were presumed fused dimers (termed "figure eights") and "circular oligomers" having several minicircular sized loops but no apparent interlocking (Fig. 10). The major macrospecies was a large association composed of the smaller molecules catenated together and also containing a long nonminicircular molecule (Fig. 15). We assumed



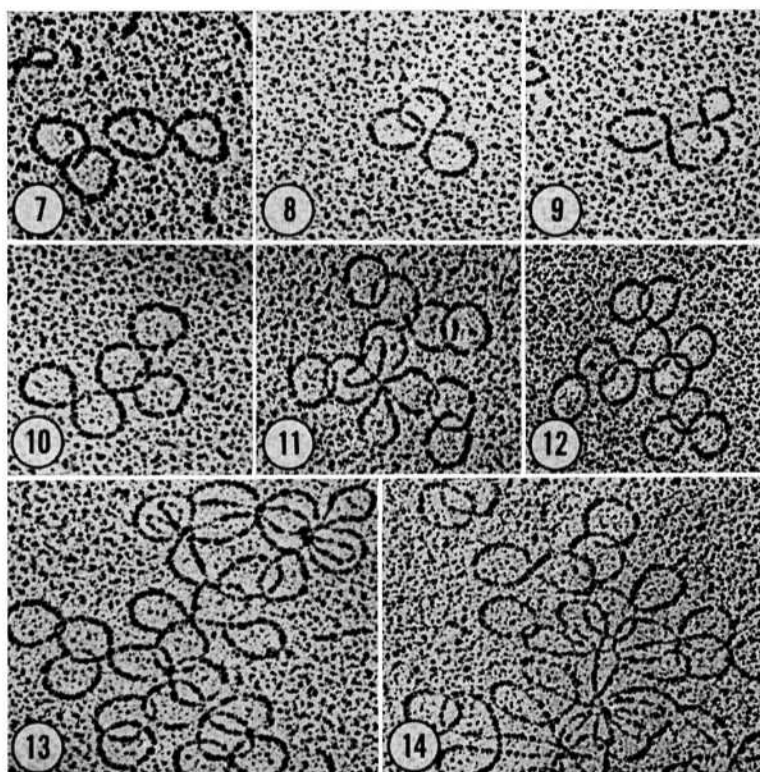
Except for Fig. 16, all figures are of *Leishmania tarentolae* or of K-DNA from this species.

Figs. 1-6. [Promastigote stages lysed in 0.02 M Tris HCl-2 mM EDTA, pH 7.9 (Tris-EDTA). Dark phase-contrast photomicrographs. For magnification see the scale in Fig. 4.] 1-5. Swollen vesicle (K) at the base of the flagellum probably represents the entire kinetoplast-mitochondrion complex, with the K-DNA seen as a dark stripe (D) along the vesicle wall next to the basal body. 6. Lysed promastigote stages after resuspension in 0.25 M sucrose-Tris-EDTA. The shrunken kinetoplast-mitochondrion complexes (K) appear discoid. [Reprinted from ref. 17, by permission of J. Protozool.]

that the smaller species represented breakdown products. Similar descriptions were made of the isolated K-DNA from *T. cruzi* (14) and *T. lewisi* (11), the basic difference being in the size of the minicircle. It is a striking and as yet unexplained generalization that the minicircular size is species specific. The size varies from 0.29 μm in *Leishmania* (19) and the Salivarian trypanosomes, to 0.4-0.5 μm in the Stercorarian trypanosomes, *T. cruzi* (12, 14), and *T. lewisi* (11) to 0.79 μm in *Crithidia* (13, unpublished data).

Isolation of Intact Kinetoplast Genome

Recently several workers have succeeded in isolating the entire K-DNA genome of several species: *Trypanosoma brucei* (K. Stuart, personal communication), *Crithidia luciliae* (9),



Figs. 7-14. [Electronmicrographs of various molecular types of K-DNA purified by successive CsCl equilibrium centrifugations. Each minicircle is $0.29 \mu\text{m}$ in circumference.] 7-9. Monomeric minicircles and fused dimers. 10. Circular oligomer. 11, 12. Small catenanes. 13, 14. Parts of associations. [Reprinted from *J. Mol. Biol.* (ref. 19) by permission of Academic Press, New York.]

and *Crithidia acanthocephali* (13). In line with these reports we have developed a simple method of general utility and applied it to *L. tarentolae*, *Crithidia fasciculata*, and *T. cruzi* (unpublished).

The cells are lysed in sarkosinate or sodium dodecyl sulfate (SDS) at 60 C and digested with pronase. The nuclear DNA is centrifuged at a fairly low speed, the value of which varies from species to species, and the pellet washed once. By this procedure we can recover up to 50% of the K-DNA of *L. tarentolae* and *C. fasciculata* free of nuclear DNA.

Physical Properties of Networks and Associations

The isolated K-DNA consists of a remarkably homogeneous population of DNA particles to which we have given the non-committal term "networks." The K-DNA networks can be seen in solution in the fluorescence microscope. In Giemsa-stained smears examined in brightfield the networks appear as cup-like sheets of DNA (Fig. 16). From observations of networks in the electron microscope, it is clear that the K-DNA remains in the compact 2-dimensional configuration seen in the cell. In agreement with these observations of compact masses of DNA, the networks were found to have an $S_{20,w}$ of $\sim 2 \times 10^3$ for *Leishmania* and $\sim 5 \times 10^3$ for *Crithidia*, their molecular weights being $\sim 10^{10}$ daltons. Networks from stationary phase cells are composed entirely of covalently closed circular molecules, as shown by ethidium bromide CsCl equilibrium centrifugation and alkaline band velocity sedimentation (unpublished data).

The percentage of *L. tarentolae* network DNA in the form of minicircles was estimated previously on the basis of controlled digestion with DNase II as $67 \pm 10\%$ (19). Controlled break-

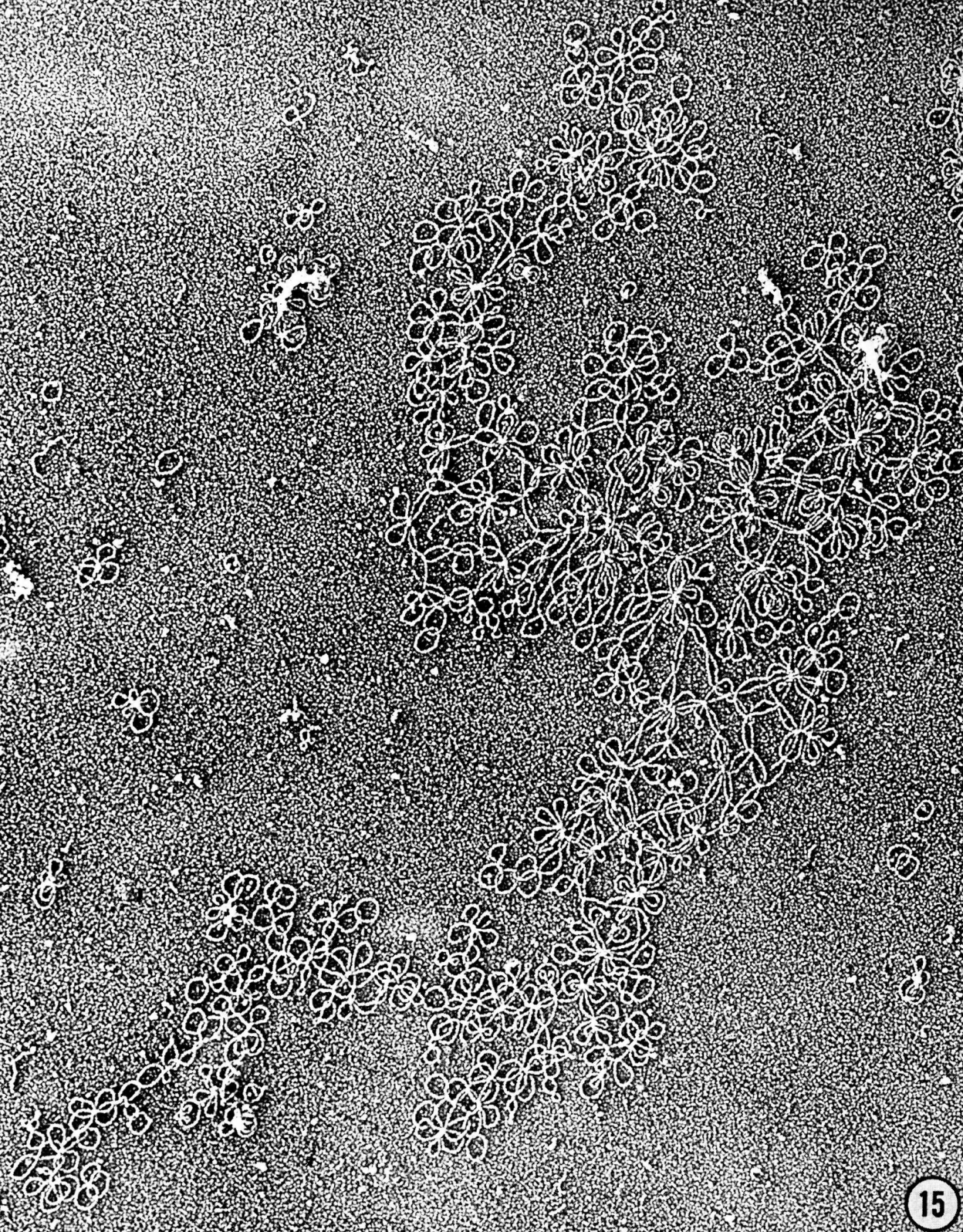
down of associations and networks was achieved also by sonication (Fig. 17). We were able to recover a maximum of 43% of the DNA of the networks in the form of closed monomeric minicircles and catenated dimers and trimers. Sonication of *C. fasciculata* networks is much less efficient, possibly indicating a different type of network substructure. The results with *Leishmania* imply the existence of approximately 10^4 minicircles per network. Similar values have been obtained for *T. cruzi* and *C. acanthocephali*.

The detailed quaternary structure of *Leishmania* K-DNA is unknown, but probably involves a subunit type of organization, including the long molecules as well as catenanes of minicircles. In *C. acanthocephali*, however, Renger & Wolstenholme (13) have reported recently a rosette type of organization, in which catenanes of 30-40 minicircles are linked to other catenanes on several sides in a 2-dimensional plane. No other species has been examined in detail.

Isolation and Physical Properties of Minicircles

The minicircle itself remains the most unusual aspect of the K-DNA system. Ron Wesley in my laboratory has carried out a study of the biophysical properties of minicircles from *L. tarentolae* (unpublished). He found that 6-9% of the K-DNA is either free within the mitochondrion or loosely bound to the network, since it can be isolated from the lysate after complete removal of the networks. This DNA consists mostly of monomeric minicircles, with catenated dimers, trimers, and higher oligomers present in decreasing amounts. The free minicircles are identical in almost all respects with those isolated from networks by sonication.

Open *L. tarentolae* minicircles have the same bi- or triphasic



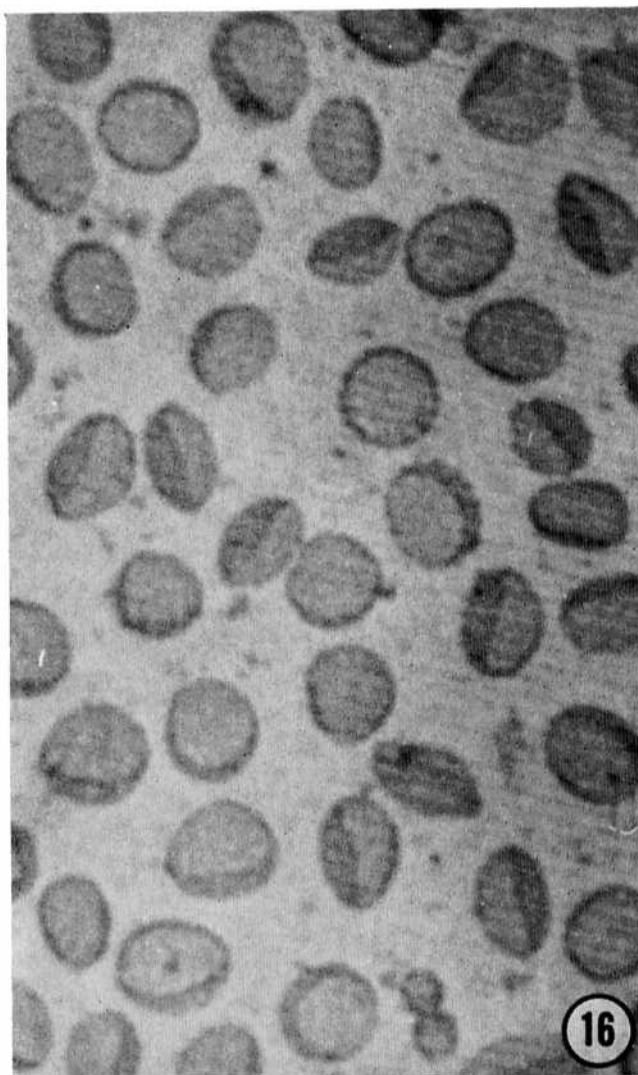


Fig. 16. Photomicrograph of purified D-DNA networks from *Crithidia fasciculata*. Note the lips of the networks which are the result of constraining the cup-shaped structure to lie in a 2-dimensional surface. [The DNA was mixed with 0.25 M sucrose-1% serum albumin, spread onto gelatin-covered slides, air-dried, fixed in absolute methanol, and stained with Giemsa's.]

melting curve as that reported previously for total K-DNA (19). The most likely explanation for this behavior is intramolecular rather than intermolecular heterogeneity, for we never were able to separate 2 classes of minicircles on any physical basis. The only other unusual physical property of *L. tarentolae* minicircles is an extremely low superhelix density (unpublished data).

Replication of K-DNA

The existence of 10^4 minicircles bound into such a complex structure as the network poses interesting problems in terms of DNA replication. Furthermore, K-DNA replication occurs fairly synchronously with N-DNA replication, thus invoking the existence of precise intracellular regulatory mechanisms controlling the 2 genomes.

Previous workers have demonstrated by electron microscopy of thin sections that in most cells the sheet of K-DNA increases

in area, not in thickness, and then divides by constriction (18). In some cells, such as epimastigotes of *T. conorhini* (10) and *T. cruzi* (2), 2 parallel sheets of K-DNA occasionally are seen during replication, but this is not common. To localize the sites of DNA replication in the network, Agda da Silva and I have pulsed *L. tarentolae* and *C. fasciculata* cells with ^3H -thymidine and then let the cells grow in unlabeled thymidine (unpublished). Directly after the pulse, incorporation of label was apparent only at the edges of the purified networks, as shown by light microscope autoradiography. Furthermore the results of the chase were consistent with a peripheral localization of DNA synthesis in the networks. This type of replication pattern would imply that some molecules in the center of the network are not replicated at all and that some molecules on the periphery are the product of more than 1 round of replication in a single cell cycle. To test this prediction, we grew cells in ^3H -thymidine and then let them divide once or twice in the presence of ^{32}P i and 5-bromodeoxyuridine. Closed monomeric minicircles were isolated from networks and centrifuged to equilibrium in CsCl. The minicircles had a normal semiconservative replication pattern—a light/light to light/heavy to heavy/heavy transition in terms of buoyant density. The existence of non-replicating minicircles would call for the retention of the light/light peak and a too early appearance of the heavy/heavy peak, which was not the case. We are left with an unresolved paradox which must be answered by further work.

The replication of the K-DNA is an important problem, but the problem of most interest to protozoologists and cell biologists is its function.

Genetic Function of the K-DNA

What is the purpose of such a large amount of mitochondrial DNA? To answer this we must first answer the question of how much unique genetic information occurs in the kinetoplast DNA and how much repetition. One approach to this problem is that of quantitative renaturation kinetics as pioneered by Roy Britten (4). Ron Wesley in my laboratory has shown by such studies that there is most probably only 1 class of minicircles in *L. tarentolae* K-DNA (unpublished data). These then represent a single gene amplification on the order of 10^4 . He found also, however, that in addition to minicircles, K-DNA networks contain another species which has a kinetic complexity ~ 12.7 times that of a minicircle. We have not isolated this species but the likely candidate is the long molecule.

Hence the K-DNA of *L. tarentolae* is mostly repetitive but there is more information present than represented by the minicircle alone.

Another approach to genetic function is the study of RNA transcription. We have found that purified kinetoplasts from *L. tarentolae* contain 2 unique species of RNA which sediment at 9 and 12 S in sucrose gradients and acrylamide gels (unpublished data). The synthesis of these species is inhibited specifically by ethidium bromide, a known inhibitor of mitochondrial DNA transcription. Furthermore, labeled 9 and 12 S RNA's specifically hybridize to minicircles on filters. This work still is preliminary and we do not yet know whether these RNA species represent mitochondrial ribosomal RNA's or RNA's that are translated, nor do we know how much of the minicircle is represented by these species. Another important question concerns the number of minicircles in the network that are transcribed during the cell cycle. It is, however, comforting to know that minicircles do have some genetic function and are transcribed.

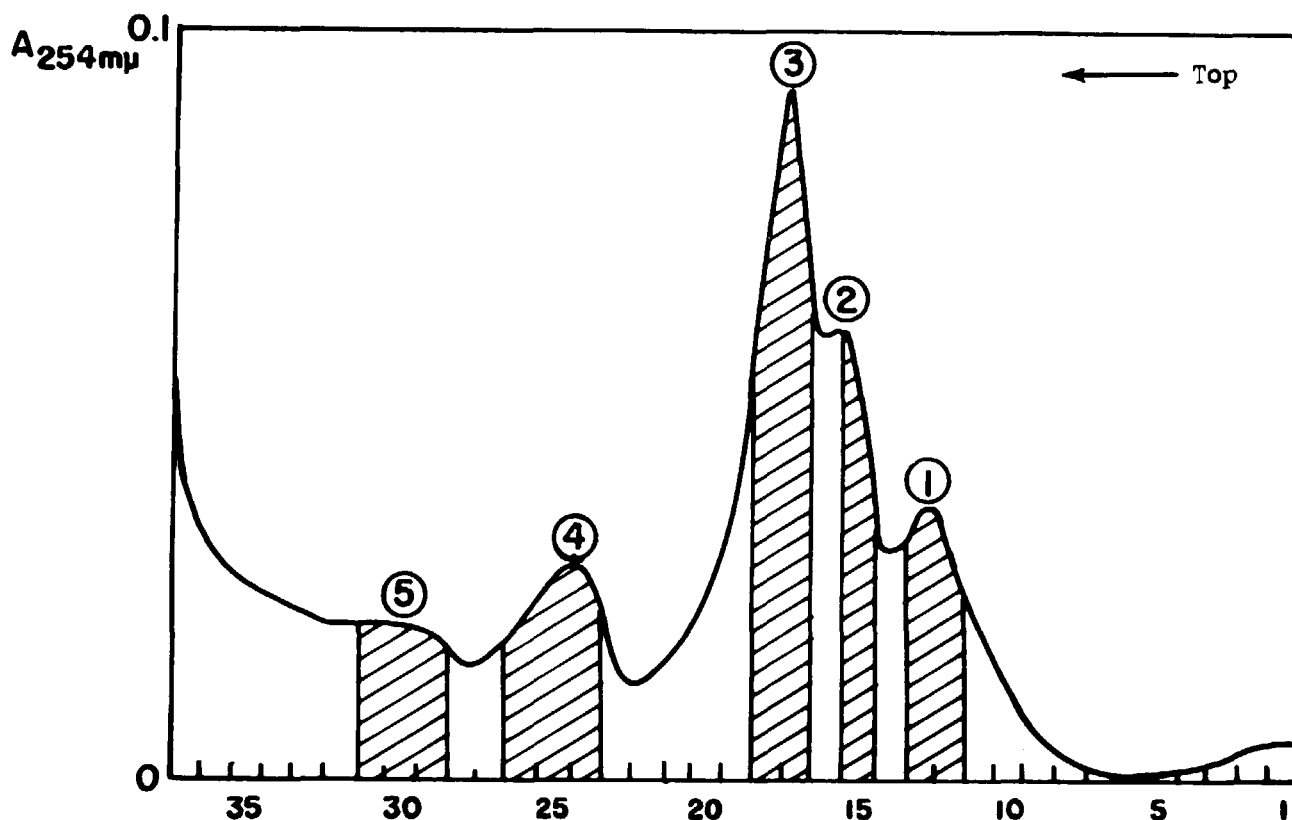


Fig. 17. Band velocity sedimentation in a neutral, linear 5-20% sucrose gradient of sonicated K-DNA. By electronmicroscopic examination, the 5 peaks were identified as follows: 1. linear fragments, 0.14 μm long; 2. linear fragments, 0.29 μm long; 3. monomeric circles; 4. catenated dimers; 5. catenated trimers. [Reprinted from *J. Mol. Biol.* (ref. 19) by permission of Academic Press, New York.]

Phylogeny of the K-DNA

The final aspect of the kinetoplast I would like to discuss is the phylogeny of the K-DNA. The work of Hoare, Wallace and others has produced a rational phylogenetic taxonomy of the Kinetoplastida (see 18). These range from free-living forms, like *Bodo*, to forms parasitic in 1 invertebrate host, like *Crithidia*, to forms parasitic in 2 hosts, like *Leishmania* and *Trypanosoma*. Corresponding changes in the structure of the K-DNA in thin sections have been noted, the more primitive forms having a large amount of K-DNA with the fibers arranged in no visible order. The more advanced forms, on the other hand, have smaller masses of DNA arranged in precise configurations as discussed previously. Perhaps an understanding of the molecular nature and informational content of the K-DNA in the different genera would lead to a general explanation of those selective forces which in the course of evolution have led to a constriction in the amount of genetic information in the mitochondria of all eukaryotic cells.

In conclusion, the kinetoplast represents a poorly understood but uniquely valuable experimental system the studies of which may lead to some basic answers about the replication, genetic role, and evolution of mitochondrial DNA in all eukaryotic cells.

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