KINETOPLAST DNA. A UNIQUE MACROMOLECULAR STRUCTURE OF CONSIDERABLE SIZE AND MECHANICAL RESISTANCE.

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SUMMARY

Highly purified k-DNA has been prepared from Crithidia luciliae by a two step procedure, involving differential sedimentation in sucrose followed by banding in a CsCl density gradient. This k-DNA is isolated as compact particles of homogeneous size and DNA content, each one corresponding to the whole DNA complement of individual kinetoplasts (3.72×10^{-14}g).

Kinetoplast DNA (k-DNA) has been described as consisting of circular duplexes of very small size, less than one micron in contour length (1-6). However, unlike all other DNAs described so far, when analysed by centrifugation in CsCl gradients, it forms a detectable band in the first two hours of the run (7). Our experience of T. mega and C. luciliae has shown that a band of k-DNA may appear during initial gradient formation, as early as 40 minutes after starting the centrifuge. Moreover, the band formed by k-DNA at equilibrium in the analytical centrifuge is exceptionally narrow (7,8), indicating, together with its fast banding property, an extremely high molecular size. Although large associations of small circular molecules with variable amounts of linear elements have been described from k-DNA preparations (2, 4,5,6), there is some discrepancy between the concept of kinetoplast DNA being made of minicircles and its particular behavior in CsCl gradients. Particles of very large size could either be produced by artificial aggregation of smaller units or be present as such in the living cell.

It will be shown here that the whole DNA content of the kinetoplast of C. luciliae forms a unique structure, highly resistant to shearing forces, which is isolated as a whole by the detergent-phenol extraction procedure. Similar observations have been made independently on Trypanosoma brucei by Williamson et al. (9). This exceptional character of k-DNA allows it to be separated from nuclear DNA by differential sedimentation in sucrose solution.

MATERIAL AND GENERAL METHODS

Crithidia luciliae are grown at 24ºC in five liter bottles, each containing four liters Boné's medium (10), adjusted to pH 7.4 and stirred by continuous air bubbling.

One ml of Antifoam B (Sigma Chemical Corporation) is added per liter. The cells are collected after two days from rich but still log-phase cultures and washed twice in NET (NaCl 0.1 M, EDTA 0.01 M, Tris 0,001 M, pH 8.0).

Ethidium bromide has been kindly provided by Boots Pure Drug Company Ltd. Ribonuclease A, 5x crist., was purchased from Sigma Biochemical Corporation and Pronase, B grade, from Calbiochem.

Whole DNA extraction. Washed cells are lysed with lauryl sulfate 1% in NET, for 1 hour at 37ºC, in the presence of 100 µg/ml RNAase. Pronase is added (2 mg/ml) and the digestion allowed to proceed for 3 hours more. Na 4-amino-salicylate and Na deoxycholate are added (5% and 1% final concentration, respectively) to the lysate which is shelved at room temperature for 1 hour. The digest is then extracted with one volume of aqueous phenol plus 1% hydroxyquinoline. This extraction is repeated twice, the aqueous phases are pooled, dialysed overnight against SSC (NaCl 0.15 M, Na citrate 0.015 M, pH 7.2), incubated a second time with RNAase (100 µg/ml) for 1 hour at 37ºC and dialysed against SSC.

Isolation of k-DNA. Ten ml of whole DNA extract (± 5 mg DNA) are deposited on top of 20% sucrose in SSC, in Spinco SW25 rotor tubes, and centrifuged for 1 hour at 21000 rpm and 4ºC. The pellet is resuspended in SSC, dialysed overnight against SSC, adjusted to a density of 1.7 g/ml with CsCl and centrifuged for 72 hours at 35000 rpm,

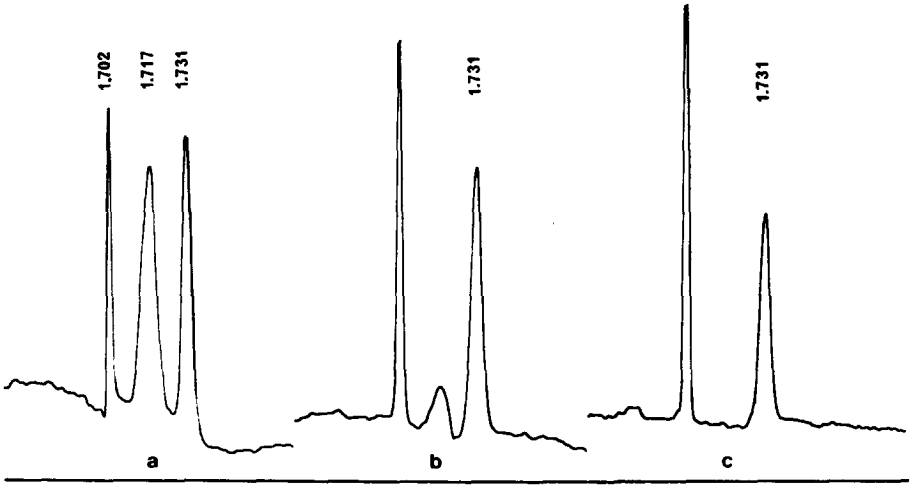

Figure 1. CsCl gradient analyses.

a- Total DNA extract. The reference at right is from Micrococcus lysodeikticus. Kinetoplast DNA (ρ = 1.702 g/cm^2) forms the hypersharp band at the left of the nuclear component (ρ = 1.717g/cm^2).

b- k-DNA from the pellet of a preparative sedimentation in20% sucrose (see General Methods). There still remains some 1.717 g/cm^2 component which is eliminated by the second step (fig.2).

c- Pure k-DNA pooled from fractions 58 to 61 of the gradient shown in fig.2.

16ºC, in a 65Ti Spinco rotor. Fractions, 3 drops each, are collected from the punctured tubes and diluted in 0.5 ml SSC for optical density readings. The k-DNA rich fractions are pooled and dialysed against SSC.

Analytical CsCl runs are performed in a Spinco Model E ultracentrifuge, for 20 h. at 20ºC and 44770 rpm. The photographs are scanned with a Joyce-Loebl microdensitometer.

RESULTS AND DISCUSSION

The efficiency of the differential sedimentation in 20% sucrose as a first step for the preparation of k-DNA is illustrated by fig.1a and b.

The viscosity of k-DNA solutions being very low, as compared to that of other undegraded DNAs, high concentrations of this particular DNA may be handled without difficulty and as much as 0.5 mg can be further processed in each of the 12 ml tubes which are used for the second step of k-DNA separation, in CsCl gradients. The concentration achieved in the k-DNA band at equilibrium is so high that it becomes opalescent and clearly visible under dark field illumination. The separation of k-DNA from the nuclear contaminant is satisfactory (fig.2) and the final product is homogeneous in the analytical centrifuge (fig.1c).

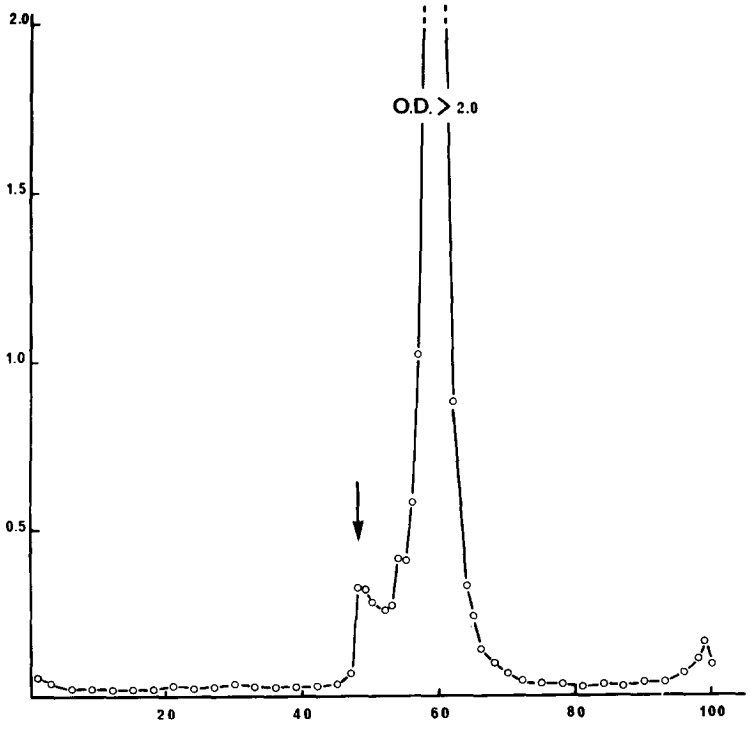

Figure 2. CsCl density gradient separation of DNAs from the 20% sucrose pellet. The optical density profile is plotted against fraction number (abscissa) and shows the nuclear DNA contaminant (arrow) at the left of the highly concentrated k-DNA band.

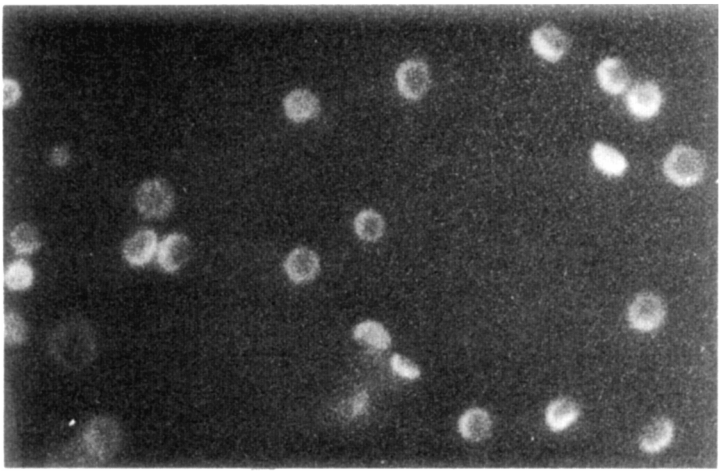

Figure 3. k-DNA solution in SSC, as seen under the fluorescence microscope in the presence of 40 µg/ml ethidium bromide. Excitation at 546 nm is obtained through a Leitz Fluoreszenzopak incident light illuminator. 2000 x.

Observations of purified k-DNA solutions under the fluorescence microscope in the presence of ethidium bromide (40 µg/ml), shows fluorescent particles of rather constant size, ± 2 µm in diameter (fig.3). The particles have a characteristic cap shape, which is however rapidly destroyed by swelling in a matter of seconds after the onset of fluorescence excitation. This alteration is supposed to be due to energy transfer through the dye.

In order to determine the amount of DNA per particle, the DNA concentration of pure k-DNA samples was measured by taking their optical density at 260 nm and, in the same samples, the particles were counted with the fluorescence microscope in the presence of ethidium bromide. The calculation was based on the assumption that 50 µg/ml DNA give an optical density of 1.0 (optical path: 1 cm). The countings were made either in a Neubauer hemocytometer or in a Petroff-Hausser bacteria counter. Assuming that all the DNA present is particulate, which seems a valid assumption owing to the isolation method used, based on the very high sedimentation velocity of k-DNA, a mean amount of $3.72 \pm .17 \times 10^{-14}$g of DNA per particle was found.

This amount is close to the amount of k-DNA which is expected to be present in each single cell. Indeed, the quantity of total DNA per C. luciliae cell was determined, using Keck's method (11), and found to be 14.3×10^{-14}g. Kinetoplast DNA represents approximately 25% of this amount, as shown by quantitative estimation of ^{3}H-thymidine intake (12) and taking into consideration the fact that k-DNA has a higher AT content than nuclear DNA.

A more direct, cytochemical estimation of the DNA content of single purified k-DNA particles and intact kinetoplasts has been performed in the following way. Purified k-DNA was spread on glass slides, using a technique adapted from the method commonly

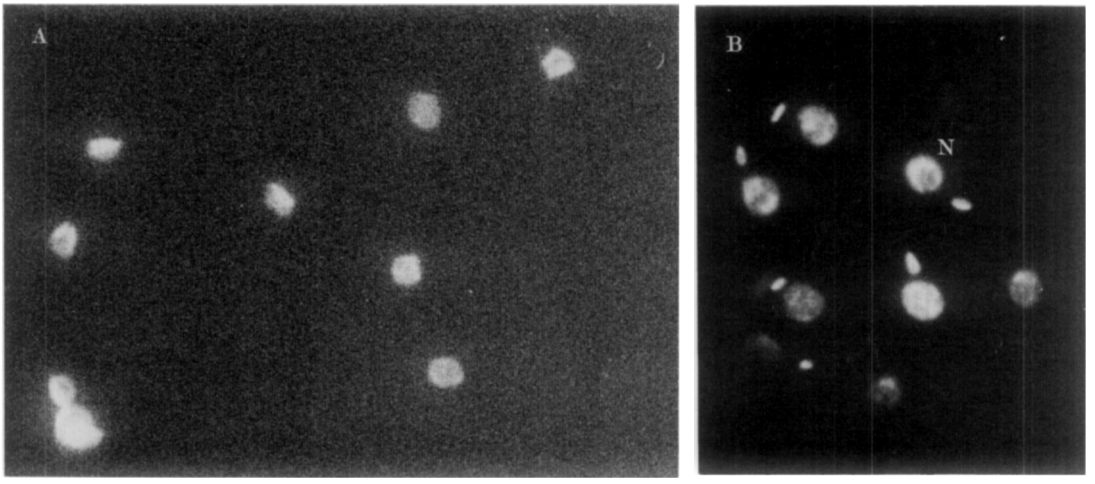

Figure 4. Purified k-DNA particles (a) and whole cells (b) stained by the Feulgen method. 2000 x.

a- A small drop of k-DNA solution (± 100 µg/ml) containing methylated albumin (1%) is deposited at the surface of distilled water spread on a glass slide. The film is dried, fixed in methanol-formaline-acetic acid (85-10-5 volumes, respectively) and stained with Schiff's reagent (1 hour) after acid hydrolysis (4 hours, at 25ºC in 1N HCl). For microscopical observation, the red fluorescence is excited by incident green light, with a Leitz Fluoreszenzopak illuminator, according to Ploem (14). The k-DNA particles show a bright fluorescence which can be quantitatively estimated (fig.5).

b- Whole C.luciliae, fixed and stained as described for fig.4 a. Only the nucleus (N) and the kinetoplast of each cell are stained and show intense red fluorescence. Smears incubated with DNAase were completely negative.

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used for preparing DNA samples for electron microscopy (13). Cytochrome c is replaced by a protein which can be more easily "fixed" by some suitable cytological fixative. We found methylated albumin to be a good substitute. More details on the method are given in legend of figure 4. Smears of whole C. luciliae cells were also prepared and preparations of both kinds were fixed and stained as described in fig.4. The kinetics of Feulgen 1 N HCl hydrolysis was studied in preliminary tests and a duration of 4 hours for this hydrolysis, at 25ºC, was found to be the most suitable as regards fluorescence intensity and reproducibility. The intensity of the Feulgen reaction was estimated fluorometrically, with a Leitz MPV cytophotometer, equipped with Ploem's incident light illuminator (14,15). The results are shown in fig.5. The distribution of purified k-DNA particles according to fluorescence intensity is similar to the corresponding distribution of kinetoplasts from intact cells, although with a significant shift to higher fluorescence values (fig. 4 a and b). This shift is, most probably, not to be attributed to a difference in DNA content, but to a different geometry of the two bodies : kinetoplasts in whole cells are more compact and thicker, so that the deepest parts of the organelle are expected to receive a reduced flux of photons,

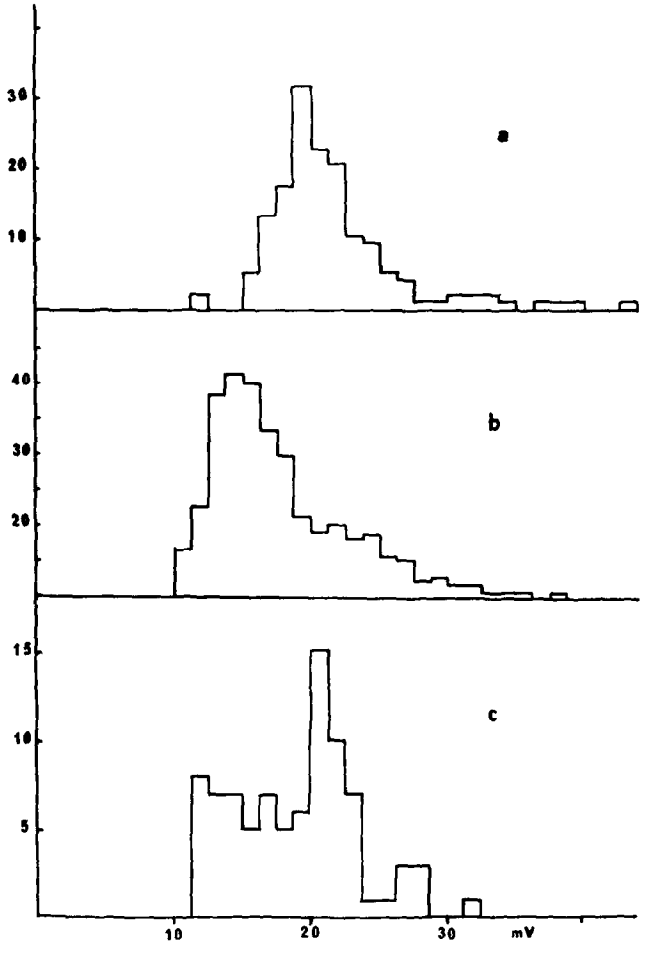

Figure 5. Histograms of the distribution of Feulgen-fluorescence intensity of purified k-DNA particles (a), kinetoplasts in whole cells of C. luciliae smears (b) and of osmotically swollen kinetoplasts from cells maintained in water before making the smear (c). The cytochemical procedure is as described in fig.4. The fluorescence intensity is expressed as the photomultiplier output, in mV, and is recorded within one second after the onset of excitation.

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due to absorption of green light by the red Feulgen stain. This has been confirmed by measuring the fluorescence of kinetoplasts in cells which had been put in distilled water immediately before making the smears (fig.5 c). The histogram shows a broader distribution, due to variable degrees of swelling of the kinetoplast. However, for these swollen organelles, the maximum frequency peak now corresponds to the one observed for purified k-DNA particles.

It is thus clear that each DNA particle from our pure k-DNA fractions represents most if not all the DNA content of an individual kinetoplast, and that this organelle

consists of a unique DNA structure, which is extracted as a whole and can be purified without being disrupted. This structure is resistant to further treatment by pronase and RNAase, so that neither proteins nor RNA are likely to be structural elements of this k-DNA particle. Its molecular weight might thus be as high as 2.2×10^{10}.

From electron microscopical work on k-DNA isolated from C. luciliae, we could see that, in keeping with what had been observed from other hemoflagellates (1-6), it comprises minicircular duplexes of constant size. Although some valuable information has been gained recently as regards the topological association of this circular DNA into larger units (2,4,5,6), the molecular fine structure of the kinetoplast remains an open question of considerable interest. The presence of numerous sequences having structural function could indeed be expected in k-DNA.

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