CIRCULAR DNA MOLECULES IN THE GENUS DROSOPHILA[1]

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ABSTRACT

The satellite DNA's from the embryos of five species of Drosophila (*D. melanogaster, D. simulans, D. nasuta, D. virilis* and *D. hydei*) have been analyzed for the presence of closed circular duplex DNA molecules, as determined by CsCl-EBr gradients. Circular DNA molecules were found in every species but *D. melanogaster*. Analyses of cell fractions from adult Drosophila and organ fractions from Drosophila larvae show that fractions containing mitochondria are highly enriched in these molecules.

WE have reported that several classes of satellite DNA's, as distinguished by their buoyant densities in CsCl, are found in embryos of various species of Drosophila (TRAVAGLINI *et al.* 1972b) but we have not been able to assign these satellite DNA's to any specific subcellular particle. Analyses of the DNA from crude nuclear and mitochondrial preparations of adult Drosophila show that the DNA from the mitochondrial preparation is highly enriched in the low density satellite DNA's, whereas the nuclear fraction contains primarily nuclear DNA (M-DNA) and has only trace amounts of the low density satellites.

When we isolate pure salivary glands, salivary gland nuclei and imaginal discs from Drosophila larvae using the techniques developed by ZWEIDLER and COHEN (1971), we find that the light density satellites are usually absent in the nuclei but are present in measurable amounts in the whole glands and the imaginal discs, both of which contain cytoplasm rich in mitochondria. However, clean preparations of subcellular particles such as mitochondria are not easily obtained from Drosophila in amounts large enough for more complete DNA analyses; therefore, we decided to approach the problem in the following manner.

The mitochondrial DNA's of most higher organisms have been shown to be closed circular duplex DNA molecules (CLAYTON and VINOGRAD 1967; BORST and KROON 1969), a type of molecule which, hitherto, has not been found in the nuclear DNA of eukaryotes. In 1967, RADLOFF, BAUER and VINOGRAD demonstrated that closed circular DNA molecules have certain physical properties which make it possible to detect and analyze them in the presence of linear DNA. They showed that circular DNA molecules have a greater buoyant density than linear DNA molecules in a neutral CsCl equilibrium gradient in the presence of an intercalating dye such as ethidium bromide, EBr. Using their method, we were

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able to determine which of the Drosophila DNA's were circular and thus, probably localized in the mitochondria.

We analyzed the DNA's from embryos of five species of Drosophila (*D. melanogaster, D. simulans, D. nasuta, D. virilis,* and *D. hydei*) and found circular DNA's in every species except *D. melanogaster*. Tentative sedimentation studies on the DNA's from three of the species have confirmed the presence of closed circular duplex molecules. The mitochondrial fractions from each species, although impure, were found to be highly enriched in these circular DNA molecules.

These results indicate that certain classes of DNA's in Drosophila are circular and suggest they are located in the mitochondria of the cell.

MATERIALS AND METHODS

Biological material: Flies were bred and embryos collected as described in the first paper of this series (TRAVAGLINI *et al.* 1972a). Third instar Drosophila larvae were raised as described by TRAVAGLINI and TARTOF (1972).

Organ fractionation: Salivary glands, salivary gland nuclei and imaginal discs were isolated from 1000 ml volumes of third instar larvae of *Drosophila virilis* using the method described by ZWEIDLER and COHEN (1971) for this species.

Cell fractionation: The method described by RITOSSA and SPIEGELMAN (1965) was used for fractionating flies into nuclear and mitochondrial components. Drosophila (approximately 15 g) were ground at 0°C in 150 ml of a buffered sucrose solution (0.35 M sucrose, 0.05 M Tris pH 7.6, 0.025 M KCl and 0.005 M $MgCl_2$) using a mortar and pestle. The homogenate was filtered through four thicknesses of gauze and centrifuged for 10′ at 500 *g* (International Model HN centrifuge) at 4°C. This pellet constituted the nuclear fraction. The supernatant fraction was centrifuged at 10,000 rpm in the angle head 50 rotor in the Spinco Model L centrifuge for 20 min in order to pellet the mitochondria. The mitochondrial fraction was then homogenized in CsCl ($\rho = 1.40$ g cm^{-3}) and the DNA extracted as described for Drosophila embryos (TRAVAGLINI *et al.* 1972a).

DNA analyses: The DNA analyses, MAK column fractionation, and CsCl equilibrium centrifugation were as described earlier (TRAVAGLINI *et al.* 1972a).

The dye-buoyant density method devised by RADLOFF, BAUER and VINOGRAD (1967) was used to determine the circularity of the Drosophila DNA's. EBr, (Boots Pure Drug Co., Ltd., Nottingham, England) was used at a concentration of 100 μg per ml CsCl, $\rho = 1.580$ g cm^{-3}. DNA samples, 50–150 μg, were centrifuged in 3 ml volumes of EBr-CsCl in cellulose nitrate tubes overlaid with mineral oil for 60 hr at 40,000 rpm at 25°C in a Spinco SW 50 rotor. The tubes containing the fluorescent gradient were photographed in the near ultraviolet using a 4″ × 5″ plate camera with a yellow filter and the negatives scanned in a densitometer. Finally, the gradients were fractionated into three drop aliquots, and the EBr removed from the DNA by shaking the pooled fractions of each band with an equal volume of isopropanol saturated with CsCl and discarding the alcohol layer. The DNA from each band was dialyzed against 1 × SSC (0.15 M NaCl, .015 M Na citrate) for 18 hr and frozen at —30°C. The density of each fraction was analyzed by CsCl equilibrium centrifugation in the analytical ultracentrifuge.

Sedimentation rate studies of the size and shape of the DNA molecules were made using the banding method devised by VINOGRAD *et al.* (1963). Two cells with 12 mm Kel F Type II gap centerpieces were run simultaneously in an Anal D rotor. Usually, 25 μl of a DNA solution ($\simeq$ 5 μg DNA) were put in the sample well and the cell filled with 0.55 ml of a salt solution. The bulk solution was 1 M NaCl at pH 8 or 12.3. Most measurements were taken at 4-min intervals after full speed was attained. Sedimentation coefficients were determined and related to molecular weight by the equations of STUDIER (1965) for native and single-stranded DNA.

RESULTS

The DNA's isolated from five species of Drosophila (*D. melanogaster, D. simu-*

TABLE 1

Classes of DNA in various fractions of Drosophila virilis *larvae*

Larval fraction	Percent DNA in each class				
	I 1.669*	II 1.683	II 1.685	M 1.699	III 1.710
Salivary glands	0.0	7.0	7.0	86.0	0.0
Salivary gland nuclei	0.0	3.0	0.0	97.0	0.0
Imaginal discs	3.0	4.0	29.0	61.0	3.0

* Buoyant density in CsCl of each class of DNA expressed as g cm^{-3}.

lans, D. nasuta, D. virilis and *D. hydei*) can be placed in four arbitrary classes as determined by their buoyant densities in CsCl: DNA I, ρ 1.661–1.669 g cm^{-3}; DNA II, ρ 1.683–1.692 g cm^{-3}; M-DNA, ρ 1.699 g cm^{-3}; and DNA III, ρ 1.711 g cm^{-3}. When the DNA's from pure salivary glands, salivary gland nuclei and imaginal discs of *D. virilis* larvae were analyzed by neutral CsCl equilibrium centrifugation, the results shown in Table 1 were obtained. The whole salivary gland DNA consists of 86% M-DNA and two types of Class II DNA: 7%, ρ 1.683 g cm^{-3} and 7%, ρ 1.685 g cm^{-3}; but the DNA from the polytene nuclei of these glands is composed of 97% M-DNA and only 3% of DNA II, ρ 1.683 g cm^{-3}. No Class I or Class III DNA was detected in either of these preparations. Whole imaginal disc DNA, however, had M-DNA, DNA I, DNA III and both varieties of DNA II. The low density DNA II represented only 4% of the total DNA as compared to 29% for the higher density DNA II. These results indicate that the types and proportions of these various DNAs are different, not only among organs, but among subcellular fractions of these organs. CsCl-EBr analyses of the DNA from these isolates indicate that circular DNA is present in the DNA from the salivary glands and the imaginal discs but not in that from the salivary gland nuclei. Unfortunately, the amounts of DNA obtained from the isolates were too small to determine which class of DNA was circular; therefore, we analyzed the DNA from 18-hr embryos of each of the species using the CsCl-EBr gradient to separate circular from linear DNA and then, after removing the EBr, classifying each type molecule by determining its density on neutral CsCl gradients.

When the DNA's from the 18-hr embryos of the various species were analyzed, the results in Figure 1 were obtained. All of the species contain circular DNA except *D. melanogaster*. The circular DNA in three of the species (*D. simulans, D. virilis* and *D. hydei*) belongs to the Class II type molecules; that of *D. nasuta* does not. However, *D. nasuta* contains three circular DNA's: two Class I DNA's and a third of the same density as M-DNA. It is remarkable that the DNA I which contains the higher moiety of apposed dA-dT sequences as determined by its low density in CsCl (ρ 1.661 g cm^{-3}) is more dense in the EBr-CsCl gradient than the DNA I whose density in CsCl is high (ρ 1.669 g cm^{-3}) because it is primarily alternating d(A·T) (FANSLER *et al.* 1970).

In *D. virilis*, the Class II DNA, ρ 1.683 g cm^{-3}, is linear while that of ρ 1.685 g cm^{-3} is circular. DNA III cannot be detected as a circular molecule in either *D. virilis* or *D. hydei*.

The lack of closed duplex circular DNA in *D. melanogaster* appears to be an

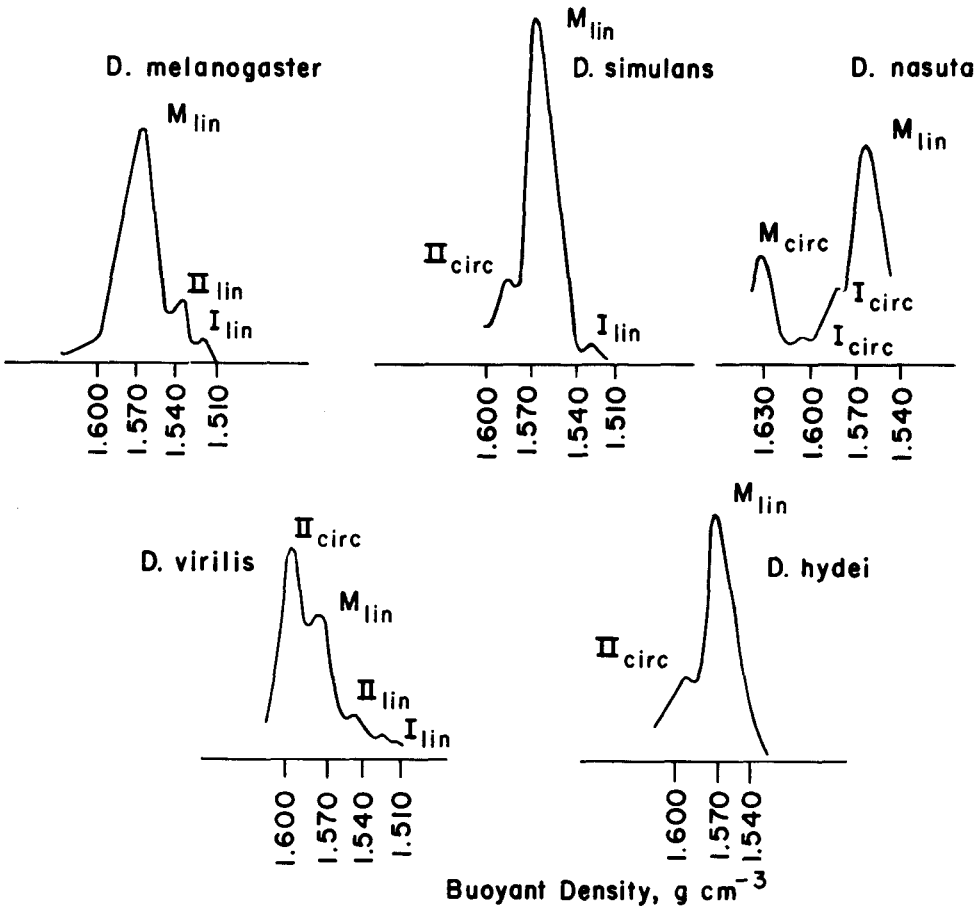

FIGURE 1.—CsCl-EBr density gradient profiles of the total DNA from the 18-hr embryos of five species of Drosophila. 50–150 μg of DNA was dissolved in 3 ml of CsCl, $\rho = 1.580$ g cm^{-3} containing 300 μg EBr and centrifuged 60 hr at 40,000 rpm at 25°C in a Spinco SW 50 rotor. Then the tube was photographed in UV light and the negatives scanned with a densitometer. The gradient was fractionated, the density of each fraction determined, the EBr removed and the DNA in each peak analyzed in the analytical centrifuge to determine its buoyant density class in CsCl. For this analysis, the DNA from each peak was dissolved in 0.7 ml of CsCl, $\rho = 1.700$ g cm^{-3}, and centrifuged to equilibrium 20 hr at 44,770 rpm in a Spinco Model E analytical centrifuge. The gradient was scanned on a densitometer and the buoyant densities of the DNAs calculated as described by VINOGRAD and HEARST (1962). The Roman numerals refer to the density classes of DNA, Class I $\rho = 1.661$–1.669 g cm^{-3}, Class II $\rho = 1.683$–1.692 g cm^{-3}, and the M-DNA $\rho = 1.699$ g cm^{-3}. The densities expressed as g cm^{-3} are those of the DNAs in the EBr-CsCl gradient. The abbreviations "circ" and "lin" after each Roman numeral designate which class of molecules is circular and which linear. The high GC DNA, DNA III, $\rho = 1.711$ g cm^{-3} which is found in *D. virilis* and *D. hydei* DNA, is not shown on these profiles for the sake of simplicity; in both cases, they band as linear molecules. The ordinate is a measure of the film density of the photographic negative for each gradient.

intrinsic characteristic of this species. The DNA's from four other wild-type strains of *D. melanogaster* (Canton S, Lausanne, Oregon R-M and Swedish C) were analyzed with the same results. If these results are an artifact of the DNA extraction procedure, it applies only to *D. melanogaster*, for when DNA is extracted from a mixture of *D. virilis* and *D. melanogaster* embryos, the circular DNA from *D. virilis* is not destroyed.

Since the only circular DNA molecules found in eukaryotes have been shown to be mitochondrial, it seemed pertinent to find whether the circular DNA's we observed were similarly localized. When the DNA's from crude nuclear and mitochondrial fractions of adult flies were brought to equilibrium in an EBr-CsCl gradient, the results in Figure 2 were obtained. Again, only *D. melanogaster* has no detectable circular DNA. In every other species, the DNA's from the mitochondrial fractions were greatly enriched in both circular DNA and poly dAT (Class I) molecules. This does not mean that the poly dAT is located in the same subcellular particle as the circular DNA. For instance, the nuclear fraction from *D. virilis* contains poly dAT, but has no circular DNA, suggesting that these molecules are contained either in the nucleus or in cell particles which co-sediment with the nuclei.

When the DNA's from nuclear and mitochondrial fractions of *D. melanogaster* are put directly on a CsCl gradient, the results in Figure 3 are obtained. The DNA from the mitochondrial fraction is highly enriched in DNA II and poly dAT; this suggests, by analogy, that the DNA II in *D. melanogaster* corresponds to the circular DNA II's found in other species.

Another method for determining which DNA's in Drosophila are closed circular molecules is that of WEIL and VINOGRAD (1963). This method depends on the fact that strand separation in solvents such as alkaline NaCl does not occur for covalently closed circular duplex DNA's, and their sedimentation coefficients, rather than being less, as is the case with linear molecules, are even greater than they are at neutral pH. We have made only tentative measurements of the sedimentation coefficients of the DNA's from three of the species: *D. melanogaster*, *D. hydei* and *D. virilis*. Since we could not obtain pure preparations of any one class of DNA, MAK column fractions enriched in particular classes of DNA were used for these determinations.

The sedimentation values for the DNA's from *D. melanogaster* were very low. The average $S_{20,w}$ value was 15 S at pH 7, which indicates a molecular weight of 2.6×10^6 daltons. When this DNA was made alkaline, it segregated into three molecular species, all with molecular weights less than half that of a single-stranded molecule, indicating that there are many single-stranded scissions already present in the duplex DNA. If these scissions occurred during isolation or MAK column fractionation, they would account for our inability to detect circular DNA in *D. melanogaster*.

The DNA's from *D. hydei* and *D. virilis* have higher S values. At pH 7, the DNA from *D. hydei* has an $S_{20,w}$ value of 18, or a molecular weight of 4.7×10^6 daltons; in alkali, the $S_{20,w}$ value of 60% of the DNA remained the same, indicating a molecular weight half of that of the linear duplex DNA. The remaining

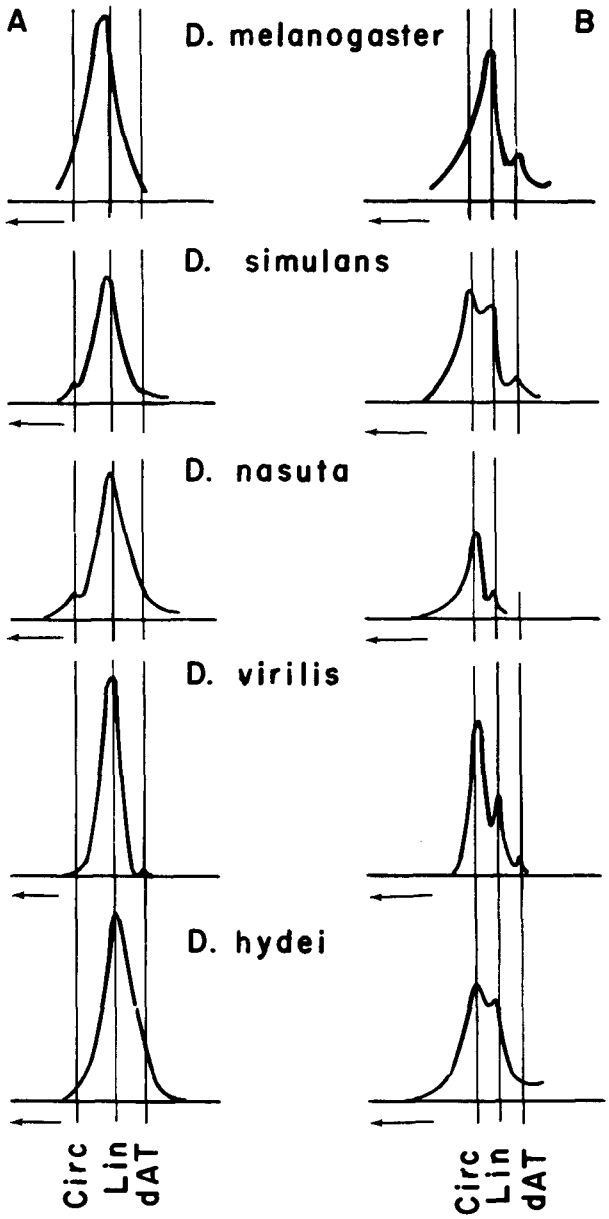

FIGURE 2.—A comparison of the density gradient profiles of the DNA from nuclear and mitochondrial fractions from adults of five species of Drosophila. A. Densitometer scans of the photographic negatives of the EBr-CsCl gradients of the DNA's from crude nuclear fractions of adult Drosophila. The method is that described in Figure 1. The arrows are in the direction of increasing CsCl density and the annotations distinguish circular DNA, linear DNA and linear poly dAT. B. The same as A, except that the DNA's are obtained from crude mitochondrial fractions of adult Drosophila.

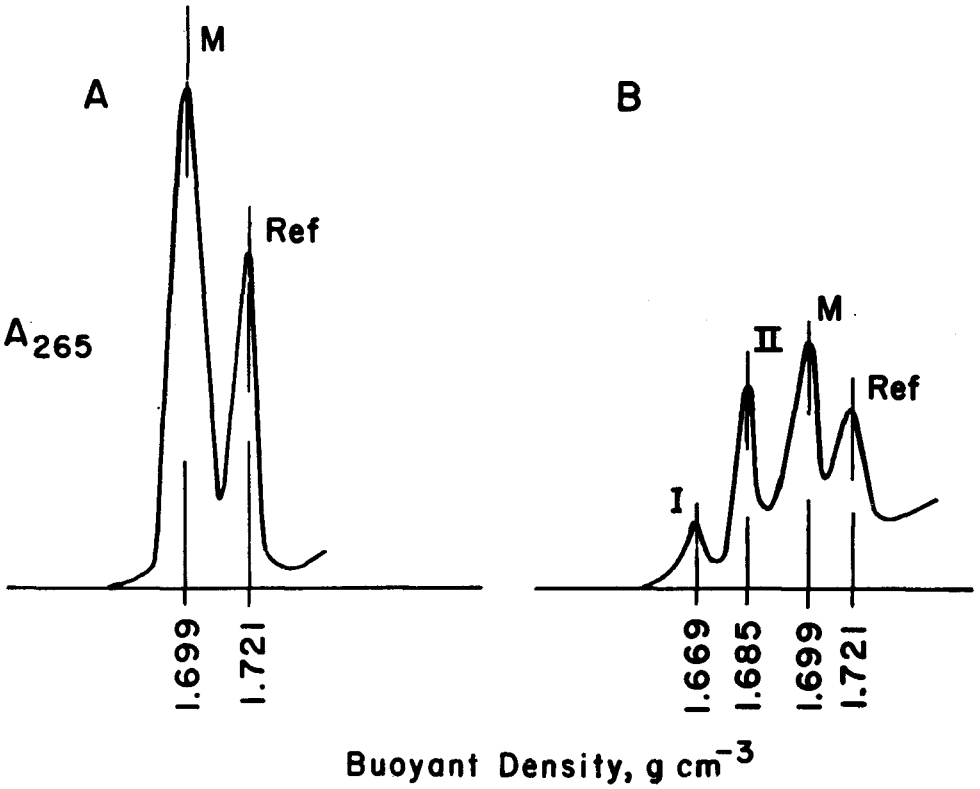

FIGURE 3.—Density gradient profiles of the DNA from nuclear and mitochondrial fractions of *D. melanogaster* adults in CsCl. The DNA's from the nuclear fraction (A) and the cytoplasmic fraction (B) of *D. melanogaster* were analyzed on a CsCl equilibrium gradient as described in Figure 1. The reference DNA is that of *P. fluorens* ρ 1.721 g cm^{-3}.

40% has an S value of 28, which would indicate the presence of relaxed circular DNA's whose strands have not separated on alkaline denaturation. A CsCl density gradient centrifugation analysis of the DNA's from *D. hydei* showed that it consisted of 50% M-DNA, 40% DNA II and 10% DNA III, with the circular DNA probably corresponding to DNA II.

A mixture of DNA's from *D. virilis* which was composed of 32% M-DNA, 45% DNA II and 23% DNA I had an $S_{20,w}$ value of 28 at pH 7, corresponding to a molecular weight of 18×10^6 daltons; when run at pH 12.8, 40% of the DNA had an increased $S_{20,w}$ value, 35 S; and the remainder a value of 29 S. The 35S component appears to consist of nicked circular molecules which are present in approximately the same proportion as the Class II DNA in the mixture.

Until pure preparations of each class of DNA can be obtained, this methodology will not yield more precise data. In every case, when the dye-buoyant density gradients have indicated the presence of circular DNA, the sedimentation data have supported the finding.

DISCUSSION

We have found circular DNA's in four of the five species of Drosophila we have studied but have not been able to detect circular DNA in any of several strains of *D. melanogaster*. These circular DNA's appear to be located in the cytoplasm, most probably in the mitochondria (BORST and KROON 1969).

With the exception of *D. nasuta*, which will be discussed below, these circular DNAs in CsCl have a density characteristic of the Class II DNA, i.e., ρ 1.683–1.692 g cm^{-3}. *D. melanogaster* possesses this class of DNA and although it appears to be located in the cytoplasm, it is a linear molecule. Still, by analogy to the other species, it, too, may be of mitochondrial origin. *D. simulans*, a sibling of *D. melanogaster* which can be interbred with it, has circular mitochondrial DNA. Also, the density of the Type II DNA in *D. simulans* is 1.692 g cm^{-3}, as compared to that of *D. melanogaster* which is 1.685 g cm^{-3}. The study of the mitochondria in the offspring of such a cross-breeding should be of extreme importance in the understanding of mitochondrial biogenesis and also the interrelationships between nuclear and cytoplasmic inheritance in Drosophila.

Three types of circular DNAs are present in *D. nasuta*, one of which has the same density as M-DNA. By simple melting-reannealing experiments (TRAVAGLINI *et al.* 1972b), we were not able to detect the presence of a highly renaturable DNA other than the two types of poly dAT in this species; only on an EBr-CsCl gradient did one become apparent. *D. nasuta* has approximately 30% of its DNA in the form of Class I DNA's, i.e., poly dAT, as in various species of crab (CHENG and SUEOKA 1964; SMITH 1964). The fact that the poly dAT molecules are circular in *D. nasuta* would indicate they are probably located in the mitochondria. If so, one could hypothesize the presence of a heterogeneous variety of DNA molecules in the mitochondria of this species. However, the poly dAT's are not necessarily located in mitochondria; of all the species studied, the nuclear fraction from *D. virilis* adults contains some poly dAT, but does not contain any circular DNA. This would suggest that the plasmids containing poly dAT fractionate differently than the mitochondria. These circular poly dAT's may also be interesting from a physical chemical point of view, for example, in learning whether base sequences affect the supercoiling of circular duplexes.

In *D. hydei* and *D. virilis*, most of the Class II DNA is circular and apparently of mitochondrial origin. This would account for the large discrepancies found by several investigators (GALL, COHEN and POLAN 1971; DICKSON, BOYD and LAIRD 1971) in the amounts of this class of DNA in diploid, as compared to polytene, tissues in *D. virilis* and *D. hydei*. Since they considered this DNA to be primarily localized in the chromosomes, specifically in the heterochromatic regions, they postulated that the low amounts found in polytene chromosomes were due to the underreplication of the heterochromatic regions. Since most of the Class II DNA is circular and of mitochondrial origin, this assumption may no longer be valid. Most probably, in *D. virilis*, only the linear Class II DNA is localized in the nuclei. To date, there have been no reports of closed circular duplex DNA isolated from nuclei.

The present results suggest that the mitochondrial DNA in four of the five species studied belongs to the Class II DNA which has a rather narrow density range, 1.685–1.692 g cm^{-3}. In three of these species, this DNA is primarily composed of closed circular duplex molecules. This does not rule out the possibility of linear DNA of Class II being of chromosomal origin; our preliminary results from organ and cell fractionation in *D. virilis* and the *in situ* hybridization data from other laboratories (GALL, COHEN and POLAN 1971; DICKSON, BOYD and LAIRD 1971; HENNIG and STEIN 1970) show this to be the case. *D. nasuta* appears to have an anomolous type of cytoplasmic DNA which may consist of three classes of circular molecules; until organ and cell fractionation studies can be made on this species, the precise intracellular location of these molecules will remain obscure.

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