A PHYSICAL CHARACTERIZATION OF MITOCHONDRIAL DNA

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ABSTRACT

A physical characterization of L. Pictus Sea Urchin mitochondrial DNA using electron microscopy and analytical ultracentrifugation is described. The mitochondrial DNA, known to be circular, is found to have a length of 4.45μ and a molecular weight of 10.1×10^6. Sedimentation analyses show two distinct components. At pH 8.0 the corrected velocities are found to be 20 S and 27 S, while at pH 12.5 the fast component has a sedimentation velocity of 70 S. Bouyant density analysis shows the mitochondrial DNA has a bouyant density of 1.703 at pH 8.0. The single strands of the DNA are shown to be of different bouyant densities, 1.758 and 1.762, at pH 12.51. The fast component in sedimentation analysis is found to be unstable at pH 12.5, degrading to single strands in about four hours.

INTRODUCTION

The presence of DNA in the mitochondria of various organisms has been known for some time,[1,2] and the fact that this DNA component is circular in some organisms has also been established.[3,4] However, up until recently,[4] a complete physical study of the mitochondrial DNA of a given organism had not been performed. Such a physical characterization could be extremely useful, since mitochondrial DNA's are four to five times larger than the circular DNA's which have been extensively studied, namely Polyoma Virus DNA and Simian Virus 40 (SV - 40) DNA. The increased length should magnify many characteristics of the DNA, perhaps making some detectable which have not been detectable before. Extensive physical studies of many different types of mitochondrial could also help to prove or disprove the theory that all mitochondria have evolved in organisms from a common extracellular precursor.

The physical characterization of L. Pictus Sea Urchin was begun in this lab and experiments reported herein are a continuation of those reported earlier.[5] New physical parameters of this DNA are determined and several interesting and possibly significant new properties are discovered.

MATERIALS AND METHODS

DNA -- Purified L. Pictus mitochondrial DNA was obtained from L. Piko,[6] and was prepared by him using two distinct methods. One fraction was isolated from whole egg homogenates prepared using a procedure similar to that of Kay[7] which is presented in a paper by Piko, Tyler and Vinograd.[5] The homogenate is extracted with sodium dodecyl sulfate, alpha amylase treated to remove contaminating polysaccharide, precipitated in alcohol, and the mitochondrial fraction finally isolated by CsCl banding. The DNA from homogenates used in these experiments

was first concentrated by pelletting the DNA in a Spinco SW - 50 rotor, using a Spinco Model L preparative centrifuge, at 44,000 rpm for 24 hours. All but approximately 0.2 ml of solution was removed from the tube and the DNA was then allowed to resuspend in the CsCl, Tris solution. The final concentrated fraction contained about 11 μg, mostly mitochondrial DNA, with a small quantity of nuclear DNA contaminent.

A second sample of DNA was obtained from Dr. Piko which had been extracted from isolated and purified L. Pictus mitochondria. The mitochondrial isolation procedure used is described elsewhere. The DNA is extracted from the isolated mitochondria using the same SDS extraction procedure used in the whole-egg homogenates.

DNA obtained using both purification methods were used in the experiments described below.

The Polyoma DNA used as a molecular weight standard was obtained from Bob Watson.[8] It had been extracted from whole virus using the phenol extraction method described be Weil.[9] The fraction used contained about 60% Polyoma II,[10] the open circular form, and 40% Polyoma I, the twisted circular form, by centrifuge analysis.

ELECTRON MICROSCOPY -- DNA was prepared for electron microscopy using the method of Kleinschmidt and Zahn[11] modified by Wetmur and Mohr.[12] The modifications consisted of making the DNA visible in the electron beam using Uranyl Acetate and Uranyl Acetate-HCl staining solutions rather that Pt or Pt-C shadowing. After picking up the cytochrome-DNA film on a parlodion-coated 200 mesh copper grid, the grid is dipped into a staining solution containing 8 mg UrAc dissolved in 100 ml EtOH (95%), for 30 seconds. The grid is then dipped in isopentane for 10 seconds, removed, dried in air, and examined in a Phillips EM 200 electron

microscope. Better results were obtained using a staining solution containing 0.1 ml UrAc-HCl stock solution and 9.9 ml 95% ethanol. The stock solution is made up of 5 X 10^{-3} M Uranyl Acetate and 5 X 10^{-2} M HCl dissolved in 95% EtOH. This solution keeps several months and gives reasonably good contrast in DNA staining.

The DNA photographs were prepared for measurement by projecting the 35mm frames on a Nikon viewer at 20 X's (calibrated) magnification and tracing molecules from the Nikon viewing screen onto vellum tracing paper. The tracings were measured using a calibrated map measurer (Charles Bruning Company #80 - 879).

The Phillips EM 200 magnification stages used in the EM portions of these experiments were calibrated at 80 KV and 40 KV accelerating voltages using a measured diffraction grating, calibrated at 2160 lines/mm.

CENTRIFUGATION -- All centrifuge runs were performed on Spinco Model E analytical ultracentrifuges using 12 mm centerpieces. The solvents used were varying densities of CsCl (Harshaw Chemical Company -- Optical Grade) buffered in either 0.01 M Tris (pH 8.0) or 0.05 M $PO_4^=$ (pH 12.5). Analysis was performed using either the ultraviolet optical system, Kodak Commercial film and a Joyce-Loeb densitometer, or an Offner photoelectric scanner and Beckman monochrometer set at 265 millemicrons.

Bouyant densities were determined using m. lyso and crab dAT as markers, with all densities calculated assuming Θ = 1.710 for E. coli DNA (neutral). Analytical velocities were performed using band centrifugation, and calculated using an Olivetti Programma computer program written by R. Bowman.

Alkali runs were performed by titrating the appropriate density CsCl solution (buffered ~~.0105~~ 0.05 M $PO_4^=$) under argon at 20 degrees C, and then filling the centrifuge cell, which already contains the appropriate quantity of DNA.

The titrating solution contained CsCl, at a density equal to the one used for the run, in 0.05 M PO_4, 1.0 M KOH. The final titrated pH was generally 12.51 ± 0.01, and was measured on a Beckman research model pH meter, a general purpose probe glass electrode and a calomel reference with a ground-glass junction.[14]

RESULTS

ELECTRON MICROSCOPY — A total of 144 circular molecules were measured to determine the length of the Sea Urchin DNA molecule. This total is the pooled result of four independent preparations of DNA-cytochrome solutions using Sea Urchin DNA prepared from whole-egg homogenates. A typical field is shown on Fig. 1. All traceable molecules on all photographs were traced and measured. Traceable was defined as those where a continuous circular contour could be followed, and molecules where there was reasonable doubt as to the contour path were discarded without tracing. Linear molecules, such as in Fig. 2, were probably contaminating nuclear DNA and were not traced. This procedure hopefully insured that experimental bias with regard to types of molecules measured was kept to a minimum.

Of the 144 molecules measured, 70 were open molecules containing no crossovers, while the remaining 74 contained one or more crossovers. The lengths measured, using calibrated magnifications for the electron microscope stages used, together with the calculated standard deviations are given in Table 1.

MATERIAL	LENGTH	S.D.
all molecules (144 mol.)	4.45 μ	± 0.25 μ
all molecules without crossovers (70 mol.)	4.46 μ	± 0.26 μ

Table 1.

A histogram, showing the distribution and scatter of all the molecules measured

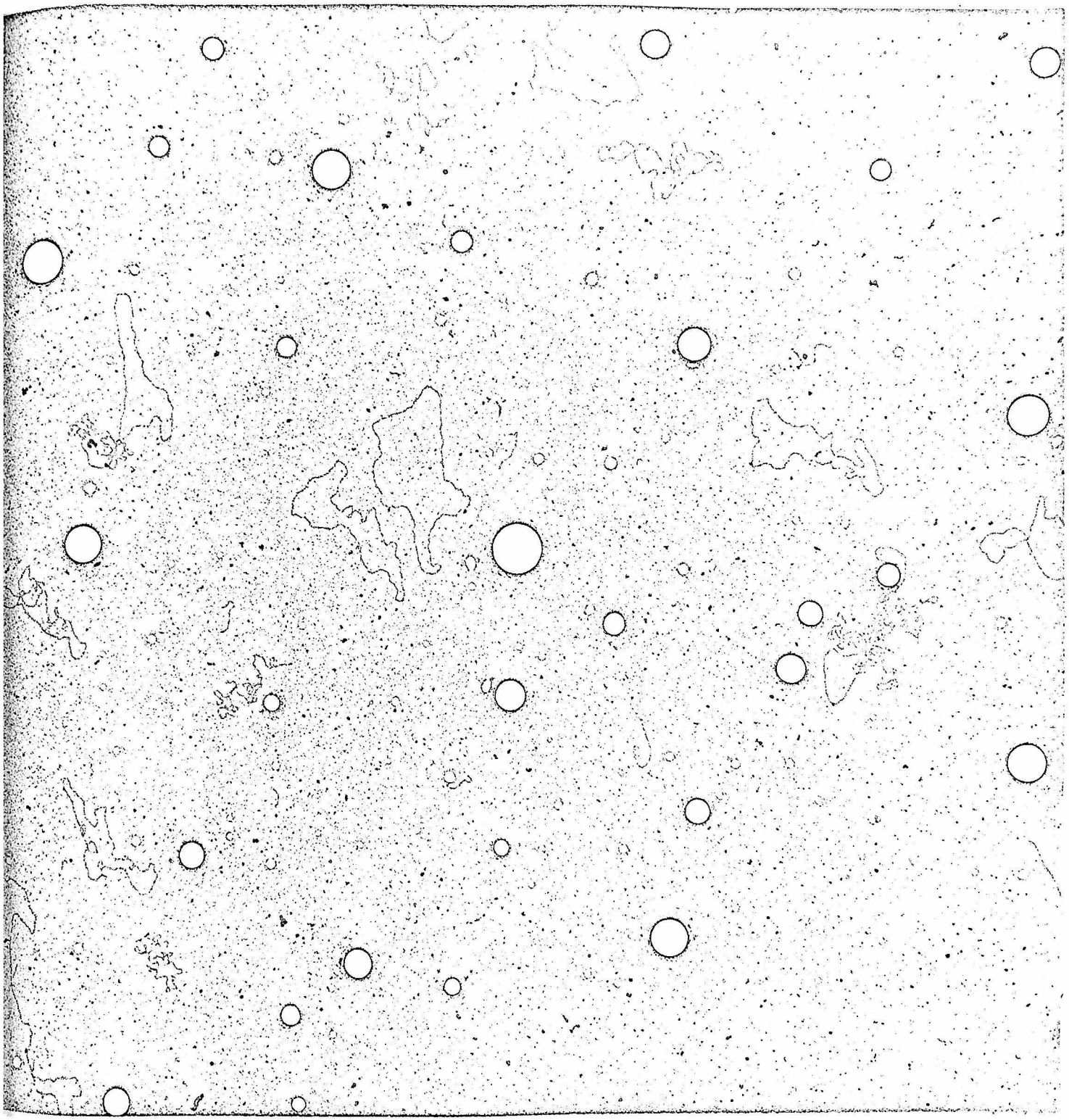


Figure 1. L. Pictus Sea Urchin Mitochondrial DNA prepared by the method of Klienschmidt and Zahn, stained with UrAc-HCl (see methods). Magnification is approximately 20,400.

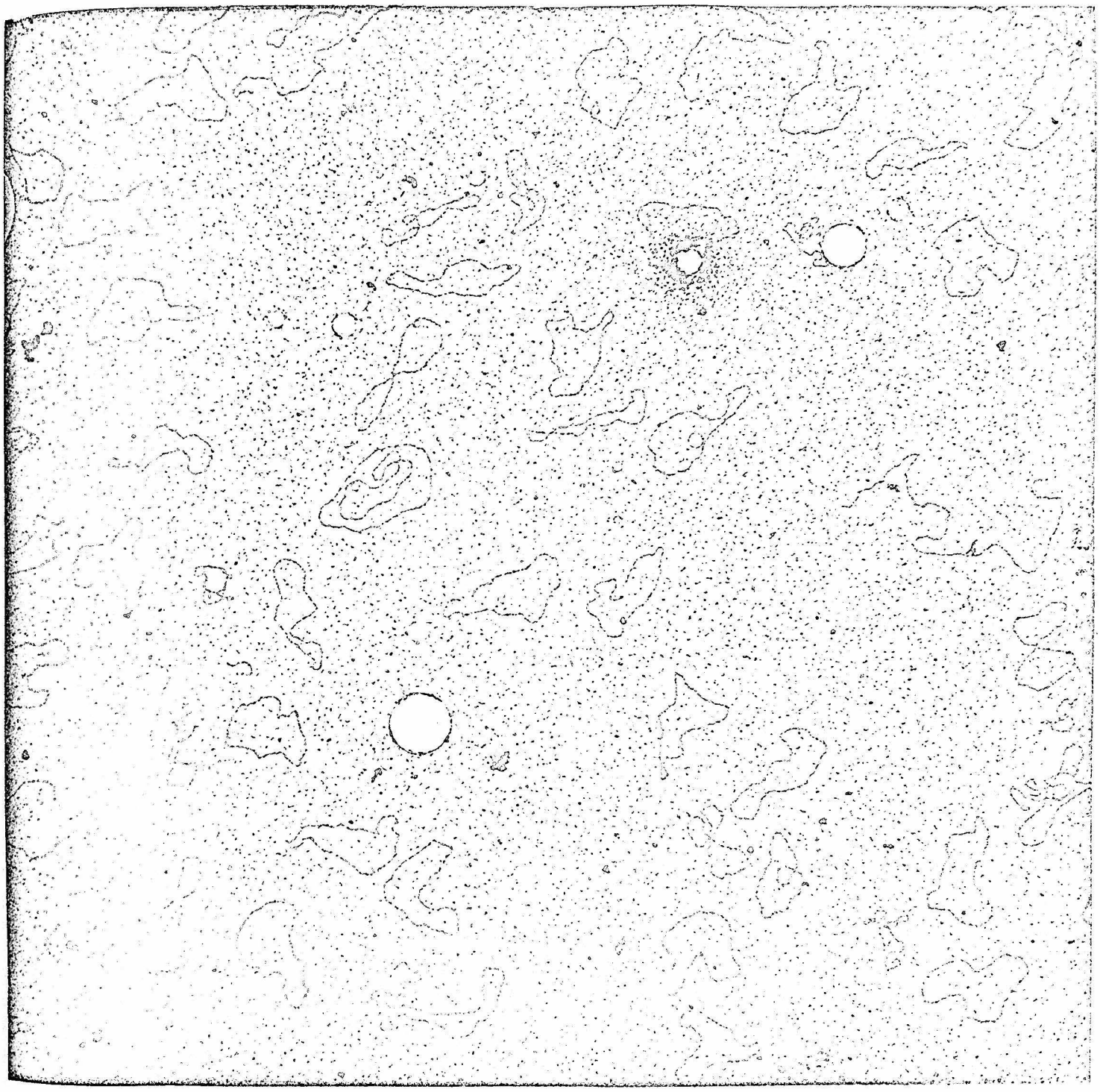


Figure 1a. Polyoma DNA stained with UrAc. Magnification is approximately 34,000.

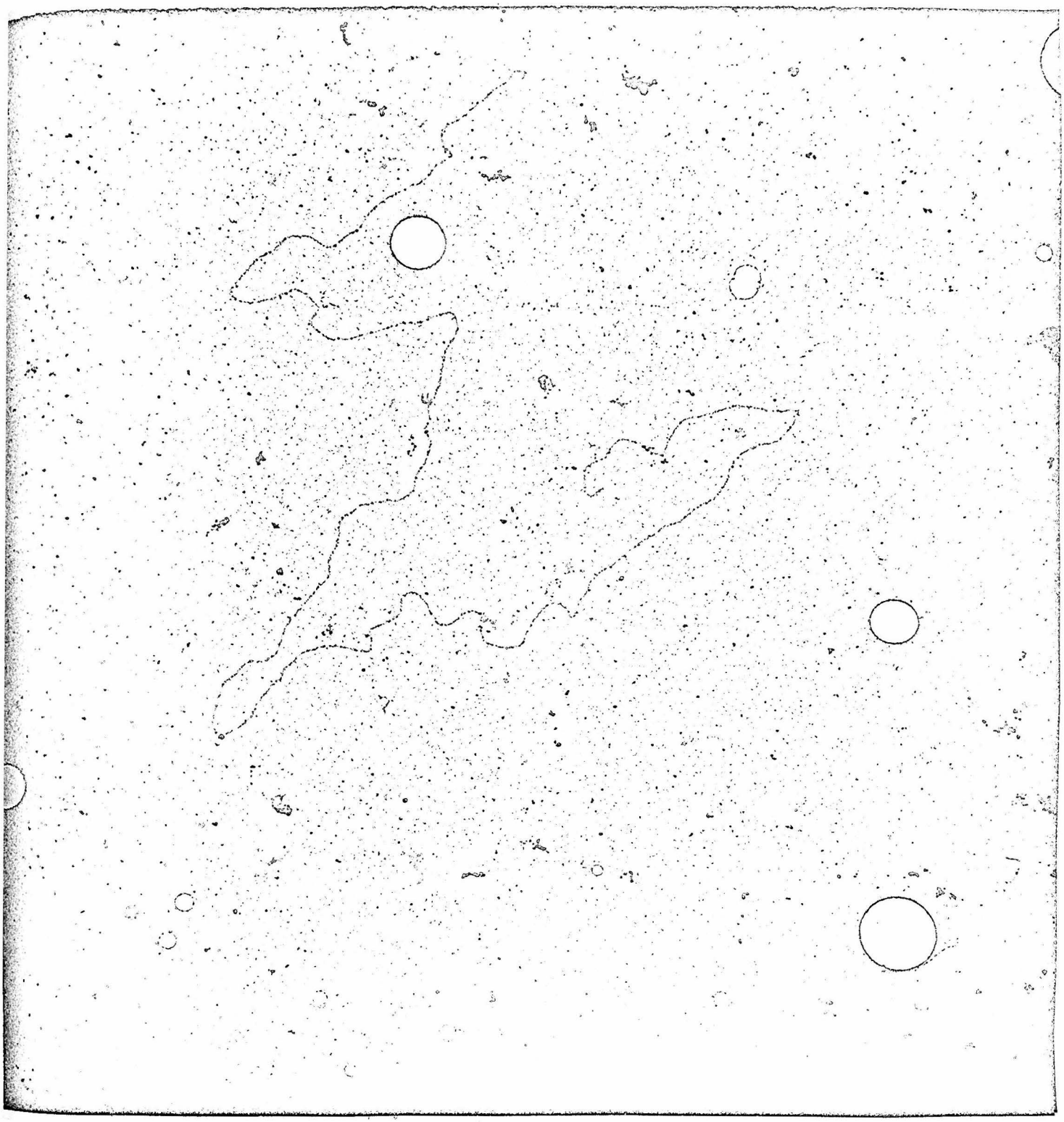


Figure 2. Nuclear DNA in preparation of L. Pictus mitochondrial DNA; stained with UrAc-HCl. Magnification is approximately 34,000.

can be found in Fig. 3.

To obtain an accurate molecular weight from this length, an internal calibration system was devised which would compensate for changes in DNA due to stretching and distortion during the Kleinschmidting process. Grids of Polyoma DNA were prepared at the same time as the Sea Urchin DNA samples measured above, and were photographed (Fig. 1a) on the same electron microscope under similar conditions. The solutions used in the preparative processed were the same in both cases. Then, assuming that structural changes which would have occurred would have affected the lengths of both molecules equally, and assuming molecular weight is directly proportional to length for DNA, the molecular weight of the L. Pictus DNA was calculated, using a value for the molecular weight of Polyoma DNA obtained from the literature. The results are given in Table 2.

MATERIAL	LENGTH	MOLECULAR WEIGHT
Polyoma II DNA (46 mol.)	$1.50\mu \pm 0.17\mu$	3.4×10^6 [15]
L. Pictus DNA (144 mol.)	$4.45\mu \pm 0.25\mu$	$10.1 \times 10^6 \pm 1.2 \times 10^6$ (12%)

Table 2.

The deviation in the L. Pictus DNA was calculated from the standard deviations of the two individual length determinations. No attempt was made to assess the possible errors in the Polyoma molecular weight.

CENTRIFUGATION -- Neutral velocity analysis in CsCl of L. Pictus DNA prepared from whole-cell homogenates showed two components present, a fast component (designated Component I) and a slower component (designated II). See Fig. 4. Alkaline velocity analyses in CsCl titrated to pH 12.5 showed that the velocity of Component I showed a large increase (Fig. 5). This suggests strongly that Component I possesses a closed circular superhelical structure, while Component II possesses an open circular structure with at least one single strand scission

No. of Molecules vs. length – Sea Urchin Mitochondrial DNA

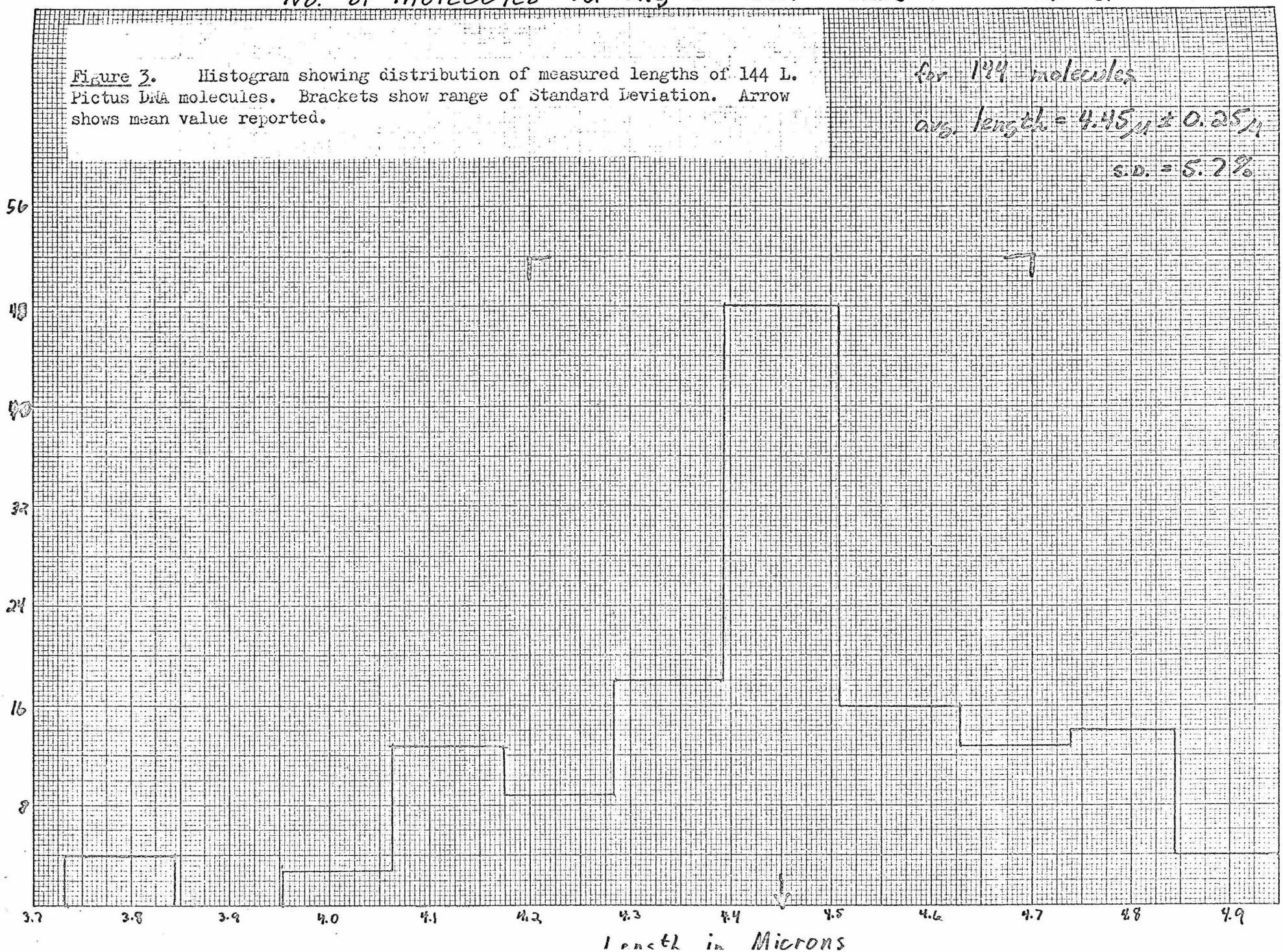


Figure 3. Histogram showing distribution of measured lengths of 144 L. Pictus DNA molecules. Brackets show range of Standard Deviation. Arrow shows mean value reported.

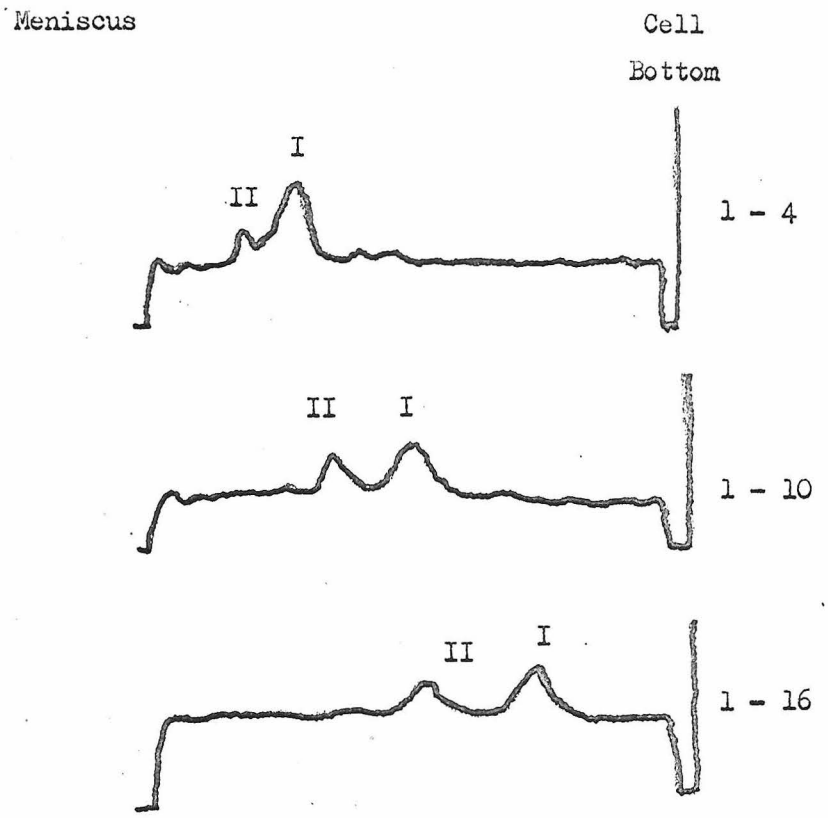


Figure 4. Tracing of Scanner record, velocity analysis of L. Pictus mitochondrial DNA. Solvent CsCl = 1.36, 0.01 M Tris; Speed 23,847 rpm; Temperature = 20 degrees C. Numbers refer to cell and scan number; interval between consecutive scans was four minutes.

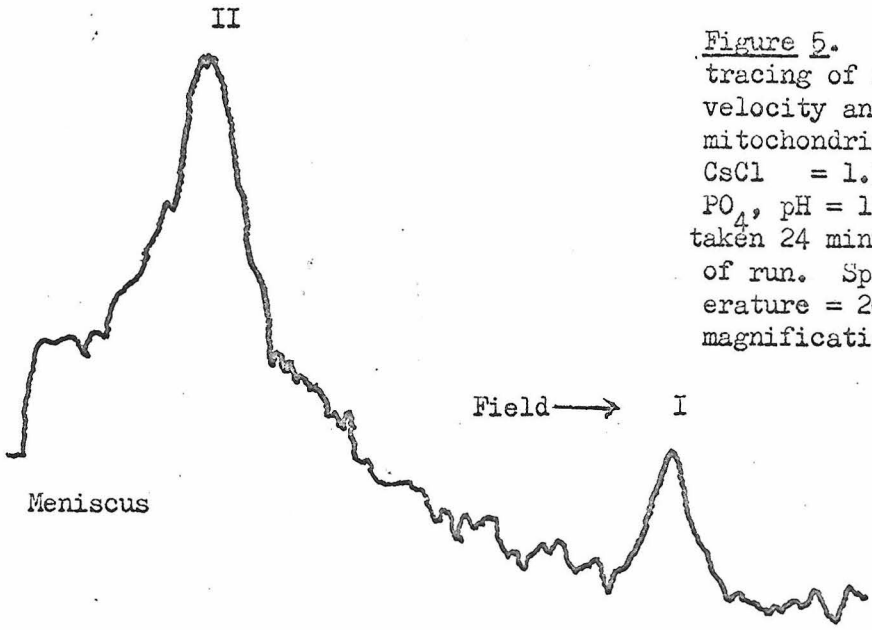


Figure 5. Densitometer tracing of film record of velocity analysis, L. Pictus mitochondrial DNA. Solvent CsCl = 1.39, = 1.39, 0.05M PO_4, pH = 12.58. This photo taken 24 minutes after start of run. Speed 29,500; Temperature = 20 degrees C; magnification = $7\frac{1}{2}$.

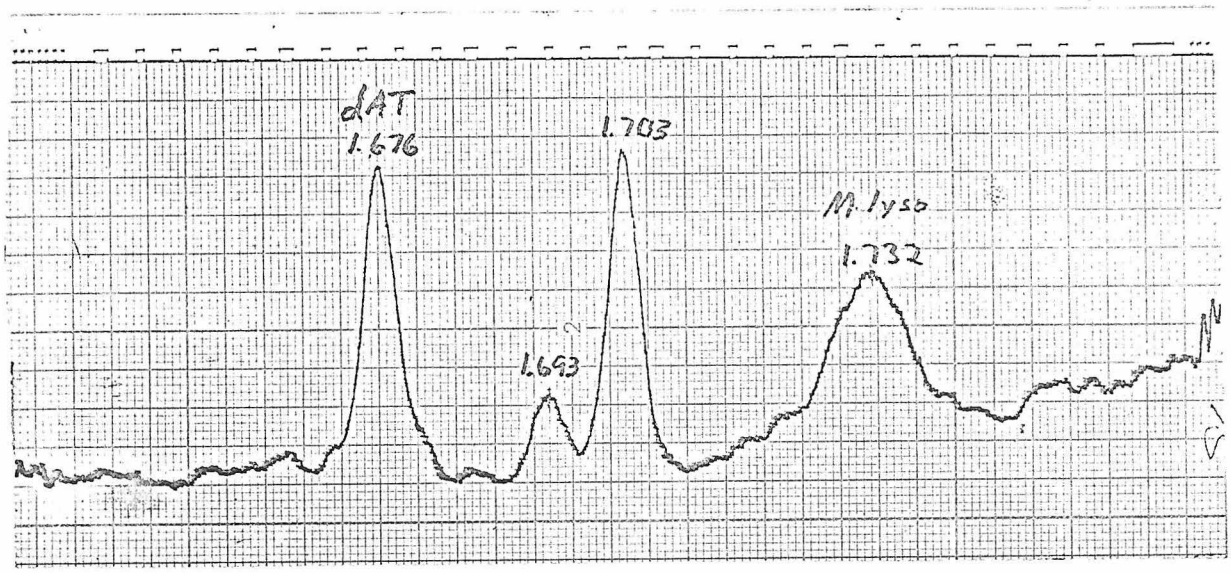
such as was postulated by Vinograd et al[10] to explain similar behavior by Polyoma and SV - 40 DNA. The results of the velocity calculations are shown in Table 3.

pH	MATERIAL	VELOCITY
8.0	Component I	$S_{20,w} = 27$
	Component II	$S_{20,w} = 20$
12.5	Component I	$S_{uncorrected} = 70$
	Component II	------

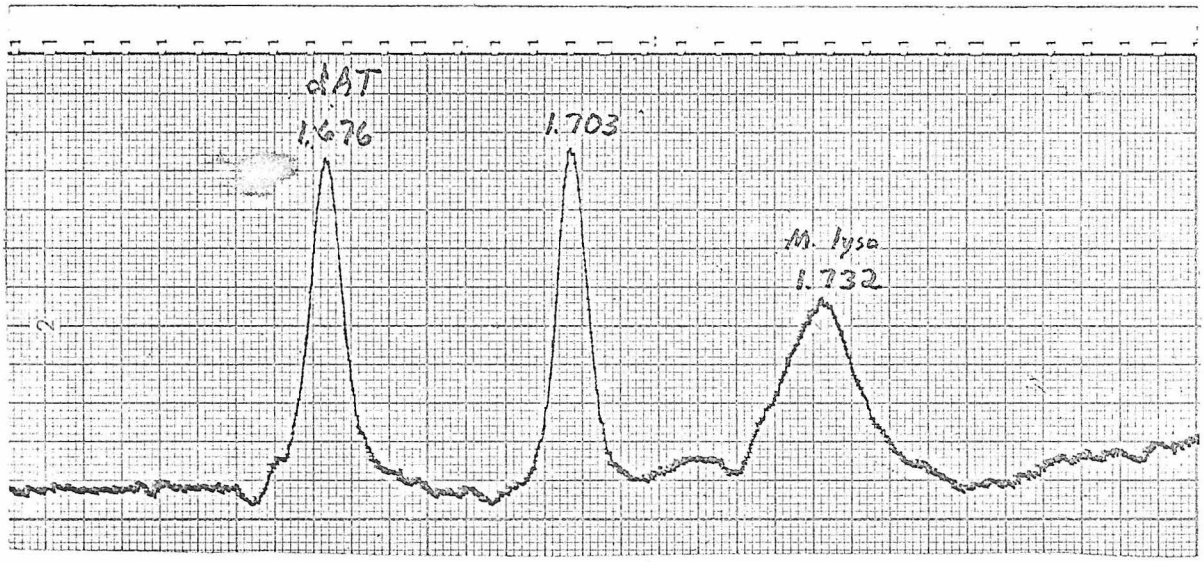
Table 3.

Neutral bouyant density analyses performed on L. Pictus DNA prepared both from whole-egg homogenates and purified mitochondria showed one principal component, with a bouyant density of 1.703 (Fig. 6). This is identified as the mitochondrial DNA. In one case, a small band of density 1.693 was observed. This was thought to be a nuclear DNA component present in the mitochondrial preparation. In no case was there evidence of a dense satellite band which had been reported in earlier investigations of L. Pictus DNA.[5]

The most interesting results were obtained, however, when an attempt was made to determine the alkaline bouyant density of the L. Pictus DNA. By analogy with work done on Polyoma DNA,[10] it was expected that two bouyant species should be present at pH 12.5. Component I, because of the restrictive nature of its closed double-stranded helical structure, would be shifted to a denser bouyant position than the denatured Component II, which at this pH should be in the form of single-stranded circular and single-stranded linear fractions. If the analogy with Polyoma DNA is accurate, and if DNA length does not affect the factors contributing to the shift, then from Polyoma results, one would expect



A. Mitochondrial Preparation



B. Whole-egg Preparation

Figure 6. Neutral bouyant density analysis of L. Pictus mitochondrial DNA. Speed 44,770; Temperature = 20 degrees C; Solvent CsCl, 0.01 M Tris ρ = 1.716 pH = 8.0; Scans taken 24 hours after start of run. Sensitivity 100 mv/cm, slow scan.

a shift of 0.018 density units between Component I and Component II.[16] Initial experiments (Fig. 7) showed what appeared to be two overlapping bands, one at Θ = 1.758 and the other at Θ = 1.762. This appeared to be too small a shift for the Component I - to - Component II change.

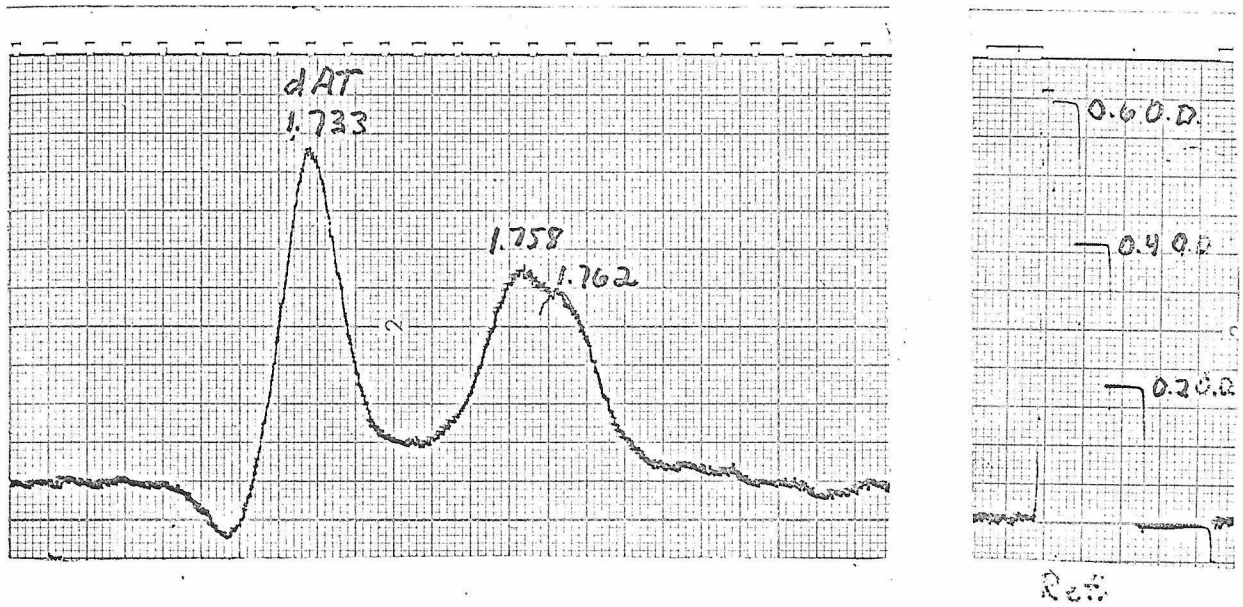


Figure 7. Alkaline bouyant density analysis of L. Pictus mitochondrial DNA. Speed 44,770; Temperature = 20 degrees C; Solvent CsCl, 0.05 M $PO_4^{=}$ ρ = 1.772 pH 12.51; Slow scan taken 24 hours after start of run. Sensitivity 100 mv/cm.

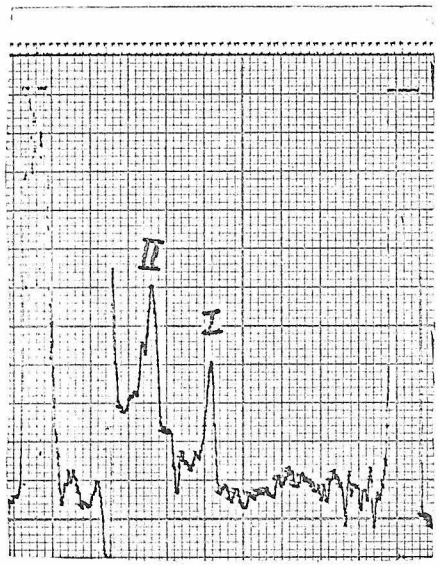
Note Movement of pen below baseline apparently due to leakage between sectors while filling.

More runs were done with only dAT as a marker, to assure that the shifted Component I was not being obscured by the M. lyso marker DNA band.

The results of this series of experiments were once again negative. The overlapping bands were seen at the same density, but no band was seen in the

area where the shifted Component I was expected.

At this point the possibility that the Component I was degrading rapidly into Component II was considered. To test this possibility a band bouyancy analysis was performed. Fig. 8 shows that a fast Component I is indeed present but that it is rapidly degraded and disappears completely in about four hours of running time. Since in a normal equilibrium run the first picture is not taken



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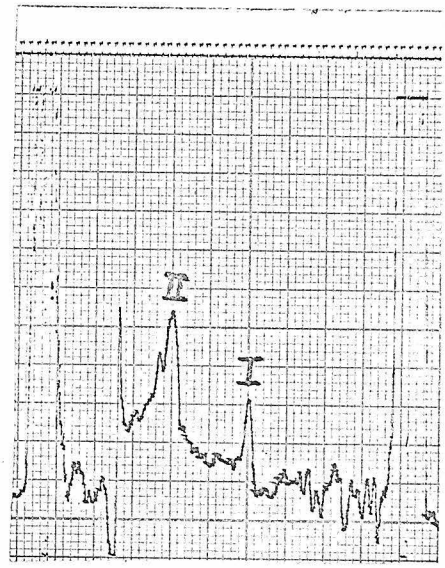

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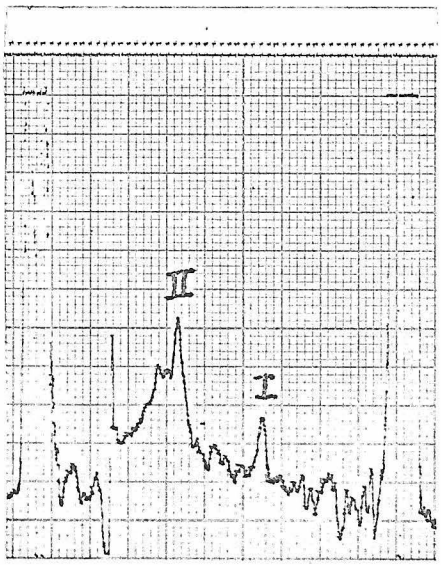

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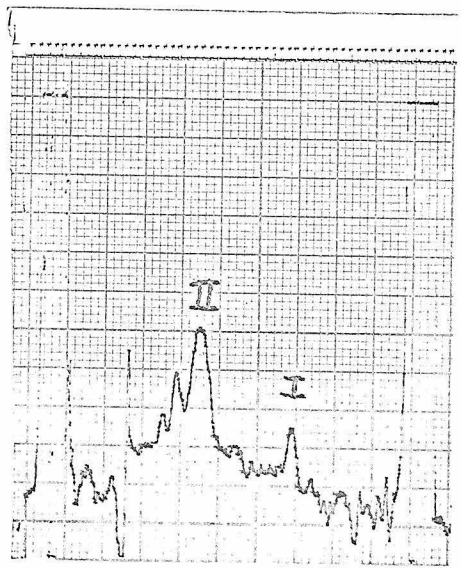

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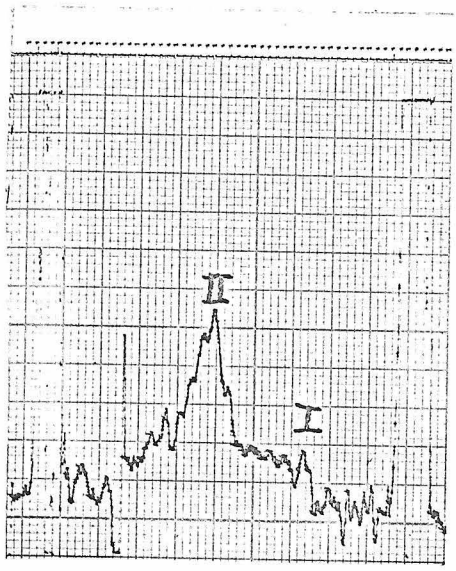
Figure 8.



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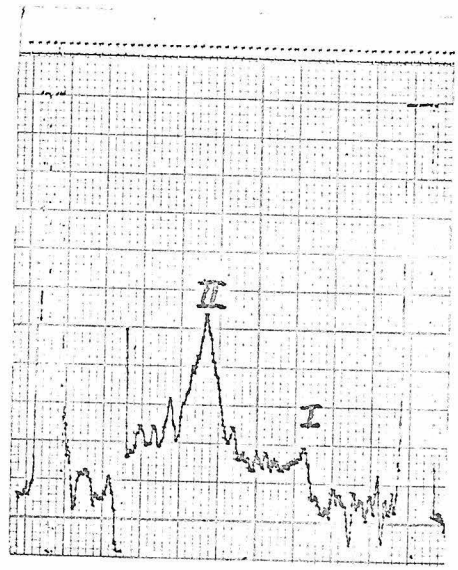

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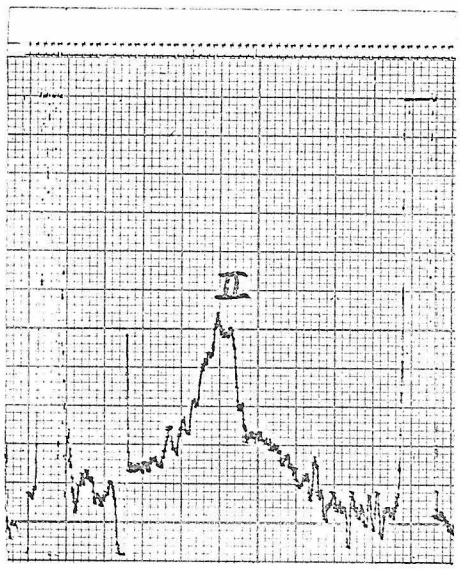

244 min.

Figure 8. Band bouyancy experiment showing instability of closed circular Component I. Solvent CsCl 0.05 M $PO_4^{\equiv}$ pH = 12.51 ρ = 1.772; Speed = 44,770; Temperature = 20 degrees C; Times refer to elapsed time from start of run. Sensitivity 100 mv/cm. Fast scan.

until about the tenth to fifteenth hour, it was now clear why the dense band had not been seen in other bouyant density runs. Fig. 9 shows that the band bouyancy run appears the same at equilibrium as the other earlier runs, namely that the two overlapping bands are present. The degree of separation and the fact that a dense, fast alkaline component is now seen to exist make it probable that

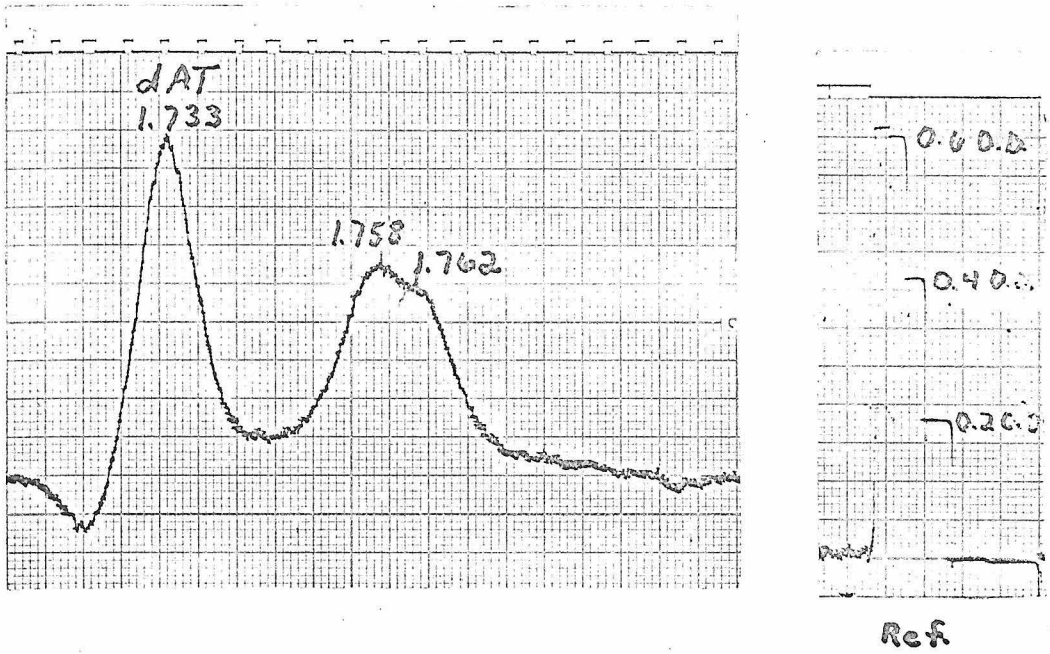

Figure 9. Band bouyancy experiment described in Figure 8 at equilibrium. Approximately 24 hours after start of run. Sensitivity 100 mv/cm. Once again leakage between sectors has produced a negative trace.

the two bands are two single strands, which are separated in density by 0.004 density units. Such behavior has been observed in thw two strands of phage lambda DNA by Hogness and Doeffler.[17] They found that, depending on the source of DNA, the separation between bands varied from 0.003 density units to 0.005 density units.

The combined results of both bouyant density analyses are shown in Table 4.

pH	Bouyant Density	
	COMPONENT I	COMPONENT II
pH 8.0	1.703	1.703
pH 12.5	-----	1.758 1.762

Table 4.

DISCUSSION AND CONCLUSIONS

ELECTRON MICROSCOPY -- The length reported here for L. Pictus mitochondrial DNA of 4.45μ is on the low side of the range of mitochondrial DNA lengths reported in the literature. For example, Nass[3] reports a value 4.74 $\pm$ 0.02μ for the length of mouse mitochondrial DNA. Kroon et al[4] have reported that sheep heart mitochondrial DNA has a length of 5.4μ, while mitochondrial DNA from various other sources (ox heart, mouse liver, chicken liver, duck liver) give lengths ranging from 4.4μ to 6.6μ. This range, and the fact that L. Pictus DNA is in the low end of that range, is probably not sufficient to either affirm or deny the possibility that all mitochondrial DNA's are the same length.[4] Effects such as ionic strength (Lang et al[18]), the use of parlodion-coated grids, and surface effects during grid drying could all combine to produce a 10% to 20% length variation. An examination of the length distribution for L. Pictus (Fig. 3) shows the range which can be obtained using just one DNA preparation, so a range of values for different mitochondrial DNA's such as the one reported above would not be completely unlikely. The variation shown in Fig. 3. is undoubtedly a result of measurement techniques and variations in experimental conditions. Though a range of DNA lengths for a single species DNA has been reported for Hela mitochondrial DNA,[19] this distribution was only truly continuous at the shorter lengths and became discrete when the region $l > 3.5\mu$ was reached. There was no evidence in L. Pictus DNA of such a range of short molecules, or of multiples of the basic length.

Turning now to the molecular weight reported above, it can only be emphasized that the value for L. Pictus is only as accurate as the value for Polyoma, which was obtained using other methods. The use of Polyoma as an internal

standard should minimize the errors introduced due to variations in solutions used, the conditions under which the grids were prepared and the conditions of the electron microscope at the time the photograph was taken. If an accurate molecular weight can be determined for an easily measured DNA such as Polyoma or SV - 40, then the whole problem of accurate determination of the molecular weight of an unknown DNA can be reduced to simple measurements. The determination of the molecular weight of a circular DNA such as Polyoma is unfortunately not that easy. Values found in the literature for the molecular weight range from 2.5×10^6 [20] up to 3.4×10^6 [15]. The value used is based on the equilibrium $S_{20,w} = 0.105\ M^{0.35}$, which is a modification of the Hershi-Burgi equation[15], which is itself based on molecular weights obtained from equilibrium band spreading. The circularity of the argument is evident, so until an accurate standard can be established, the molecular weight of L. Pictus DNA, must be only considered useful approximation.

CENTRIFUGATION — The results of the neutral bouyant density studies were essentially in agreement with what had been observed earlier.[5] Individual evaluations of the bouyant density ranged $\pm$ 0.001 density units from the mean reported value of 1.703 (Table 4). The absence of a nuclear fraction in all the preparations save one (Fig. 6a) is probably partially due to the obscuring effects of noise in the model E scanning system. It is also possible that the nuclear component of the concentrated whole-egg homogenate is too small to be detected. The nuclear component was detected only in DNA extracted from purified mitochondria.

Neutral velocity studies also gave essentially confirmation of results obtained by earlier experimenters. Two components were observed, identified as

closed circular (I) and open circular (II), (Fig. 4) with velocities calculated as 20S and 27S. This compares to values of 23S and 28S obtained by Piko et al.[5] Only one neutral determination was done in CsCl, however, so until more accurate work is done in KCl, there is no confirmation that the variations observed above are real.

The results obtained in the alkaline studies, however, are new and intriguing. The alkaline bouyant density experiments (Fig. 7) showed two overlapping bands, and failed to show a dense alkaline shifted Component I band. Two overlapping bands, most likely two single strands which appear to have different densities, have been observed in the two strands of phage lambda DNA. The observed densities of 1.758 and 1.762 bracket the expected 1.761 value for Component II, which assumes a 0.057 neutral to alkaline density shift.

Besides being a curiosity in itself, this difference in density between the two single strands of the double-stranded circular DNA raises the possibility for interesting experiments. For instance, since the two strands can be distinguished, it should be possible to tell whether or not the conversion of Component I to Component II occurs at one particular labile site, i.e. at a linker, or whether the reaction is purely random. Purified samples of the two single strands could be examined in the electron microscope for the ratio of single strands to linears. A large ratio in any one sample would imply that one strand was more sensitive to scission than the other. Such a technique could be used to study any enzyme or chemical agent which produces single-strand scissions in DNA.

The high degree of alkaline lability observed in Component I (Fig. 8) can be accounted for in a number of ways. The simplest and perhaps the most likely

is that the L. Pictus DNA has become depurinated during preparation. A fractional depurination greater than one per molecule would tend to produce a result like that observed in Figure 8. The second possibility is that L. Pictus DNA is much more alkali labile per unit length than circular molecules such as Polyoma or SV-40. This effect would have to be produced by something more than simply an enhancement of sensitivity due to an increase in length. DNA is approximately four times as long as Polyoma DNA, but no detectable I - II conversion is seen in Polyoma alkali bouyant density experiments of up to 72 hours duration, while L. Pictus DNA converts completely in about four hours. The presence of alkali sensitive loci in the molecule, or unknown structural effects of the super-coiling of large molecules could account for this rapid conversion rate.

It is much more likely, however, that the alkali lability is caused by the presence of depurinated regions in the molecule. The extensive preparative treatment[5] which the DNA received before reaching this lab provided ample opportunity for such a depurination reaction to take place. Though depurination is normally an acid catalyzed reaction, and there is no evidence that the DNA was in an acidic enviornment during its preparation, this must still be accepted as the most likely explanation until DNA prepared under more carefully-controlled conditions is available for study.

This lack of close control over the experimental material was the principal problem faced during the course of the experiments described in this report. This, coupled with the shortage of L. Pictus eggs due to the seasonal nature of the animals' reproductive cycle, often made methods of minimizing the amount of DNA used per experiment the primary experimental concern. If these problems can be overcome, however, further studies of this particular DNA molecule should certainly be attempted. The confirmation of some of the physical parameters obtained above, and a definite answer to the question of alkali lability should be obtained.

SUMMARY

A physical study of L. Pictus Sea Urchin mitochondrial DNA was undertaken and the the following results were obtained:

1. The DNA was in the form of closed circles of length $4.45 \pm .25\mu$
2. Based on Polyoma DNA, with a molecular weight of 3.4×10^6, the DNA has a molecular weight of 10.1×10^6.
3. Ultracentrifuge analysis showed two components at neutral pH, of velocity 20 S and 27 S; at pH 12.5 the fast component moved with a velocity of 70 S (uncorrected).
4. Bouyant density showed the mitochondrial DNA had a bouyant density of 1.703 at 20 degrees C, pH 8.0. What was believed to be the nuclear DNA banded at $\theta = 1.693$.
5. Alkali bouyant density analysis showed that the two single strands of the DNA had densities of 1.758 and 1.762 at pH 12.51. At this pH the fast component (closed circular) was unstable, degrading to single strands in about four hours at 20 degrees C.

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