THE PYRIMIDINE OLIGONUCLEOTIDE ANALYSIS OF MITOCHONDRIAL DNA FROM HE LA CELLS

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Submitted in partial fulfilment of the requirements for the degree of Master of Science in Chemistry.

California Institute of Technology

1972

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ABSTRACT

A pyrimidine cluster analysis of the light strand of HeLa mitochondrial DNA has been undertaken. The major difficulties in this attempt to obtain the pyrimidine oligonucleotide distribution in mitochondrial DNA are the limited amount of material available and the multistep procedure which may incur losses of DNA material as well as complications leading to irreproducible results.

To overcome the scarcity of material, the sensitivity of isotopic labelling is enhanced by using the enzyme 5'-OH-polynucleotide kinase to attach a radioactive phosphate to the end of each oligonucleotide. The fingerprinting procedure is adapted to the microgram level of DNA material by thin layer displacement chromatography.

The complications involved by multiple enzyme reactions have been solved by varying one parameter at a time. As a result of eight experiments, the conditions for a successful pyrimidine oligonucleotide analysis have been outlined.

SUMMARY

The initial phase of the research was an attempt to improve the method of strand separation of nicked duplex circular DNA. By introducing a polar solvent, dimethyl-sulfoxide, into alkaline CsCl, it was hoped that the strands of the nicked duplex circular DNA would titrate at a lower pH. It was found that at pH 11.5, which is 0.2 pH units lower than the melting point of polyoma DNA in aqueous CsCl, in the presence of 7% DMSO, PM2 Form II DNA titrated almost completely. However, due to uncertainty in the distribution of DMSO and CsCl in the tertiary solvent system, the extent of titration of PM2 Form II was not certain.

The major project of pyrimidine oligonucleotide analysis of HeLa mitochondrial DNA began with mitochondrial DNA labelled with ^{32}Pi in vivo. Fingerprinting was done with first dimension electrophoretic resolution on cellulose acetate, followed by second dimension ionophoretic resolution on DE ion exchange paper. The amount of ^{32}Pi incorporated into the HeLa mitochondrial DNA was so low that it would have required as much mitochondrial DNA as that isolated from a 10 liter culture of cells per experiment. The practical difficulties of working with such a large volume of high radioactivity (5 mC per liter) made it an infeasible project to persue.

The research reported is the pyrimidine oligonucleotide analysis of unradioactive HeLa mitochondrial DNA end-labelled with ^{32}Pi transferred from γ-labelled ATP by 5'-OH-polynucleotide kinase. The fingerprinting is more sensitive with the use of homochromatography on thin layer DE cellulose plates for second dimension resolution. A ribonucleic acid partial digest is used in the ascending elution buffer to displace the radioactive DNA oligonucleotides bound to the DE thin layer. After the oligos are resolved, they can be recovered from the thin layer plate, and the ribonucleotides contaminant are easily removed by RNase digestion. In this manner, carrier is not required, and the recovered pyrimidine oligonucleotides can be used for further analysis.

In the reported work, a pyrimidine oligonucleotide fingerprint has not yet been resolved successfully for HeLa mitochondrial DNA. Some of the difficulties encountered are reported, and solutions to the major problems have been found.

CHAPTER ONE

Relation Between DMSO Concentration and Titration of PM2 DNA in CsCl Gradient Centrifugation

Introduction:

In the pyrimidine oligonucleotide analysis of single-stranded HeLa mitochondrial DNA, it is important to get a pure and intact strand to ensure meaningful interpretation of the results. The standard procedure for separating the complements of mitochondrial DNA is alkaline CsCl gradient buoyant density centrifugation at pH 12.5 to pH 13. The theory behind it is that at pH 11.75, the G and T in each strand titrate and form the Cs salt. The attachment of many Cs ions to each strand changes its buoyant density. When there exists a bias in the G content and T content in the 2 complementary strands, they will have different buoyant densities after the formation of their Cs salts. With the introduction of a nick in either strand of the circular duplex, the two strands can be separated in a CsCl density gradient. The DNA is variably nicked at pH 12.5 and above, but the mechanism and specificity of nicking are not known. A method to effect strand separation at a lower pH is desirable. In hopes of separating the two strands of HeLa mitochondrial DNA at a pH lower than 12.5, the polar solvent DMSO is added and the effects on strand titration are studied.

Theory:

The organic solvent dimethylsulfoxide(DMSO) is known to decrease the hydrophobic aggregation of RNA and prevent RNA precipitation in Cs_2SO_4. The effects of addition of DMSO to salt distribution a Cs_2SO_4 density gradient have been studied and the behavior of single stranded RNA in an aqueous DMSO-Cs_2SO_4 system has been reported.[1] It is hoped that the solvation of single stranded DNA by DMSO might help to separate the complementary strands of HeLa mitochondrial DNA, and lower the pH of alkaline strand separation. The buoyant densities of DMSO-Cs_2SO_4 gradients however are not high enough for the separation of the complementary strands of HeLa mitochondrial DNA($\rho^H_{alk} = 1.779$, $\rho^L_{alk} = 1.738$). The possibility of running a DMSO-CsCl gradient at the desired density is therefore explored, and the effect of DMSO-CsCl on the titration of the complementary strands of PM2 Form II DNA is studied.

First, the solubility of CsCl in water in the presence of various concentrations of DMSO are found out, and the maximum DMSO concentration is chosen at which the buoyant density of the CsCl-DMSO system at mid-point of gradient will equal that of the titrated PM2 DNA, ($\rho = 1.774$) .

To study the effect of DMSO on the titration of PM2 Form II, the highest permissible DMSO concentration is used, and a mixture of PM2 Form I and Form II DNA's are run together in a CsCl gradient at various alkaline pH's. In alkali, it is known that PM2 Form I DNA bands at $\rho = 1.774$,

while PM2 Form II, in the absence of a G-T bias in the complementary strands, band at $\rho = 1.756^2$. $\Delta\theta = 0.018$. If we can find an equivalent $\Delta\theta$ between PM2 Form I and Form II at a pH lower than 12.5, then we may be able to use the DMSO-CsCl system for strand separation of HeLa mitochondrial DNA.

Experimental:

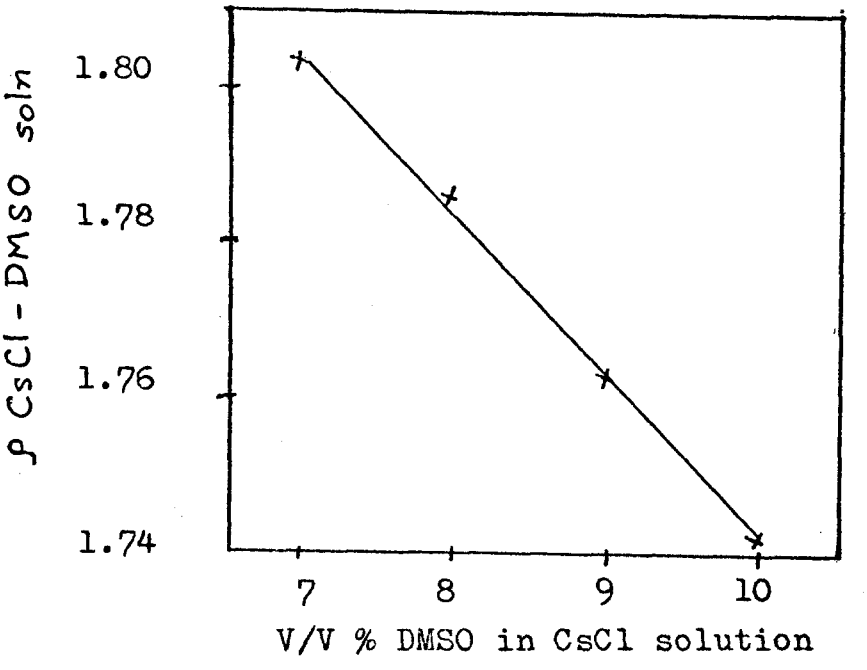
A) Effect of DMSO Concentration on Solubility of CsCl in an Aqueous CsCl-DMSO System -

The solubilities of CsCl in 0.05M phosphate buffer, pH = 12.5, in the presence of 10%, 9%, 8% and 7% DMSO, at 25°C, have been determined. Table I presents the data for the experiment, and Figure 1 is a graphical presentation of the results.

Table I

Flasks	1	2	3	4
Vol DMSO(ml)	0.50	0.45	0.40	0.35
Vol 0.5M (P) buffer(ml)	0.50	0.50	0.50	0.50
CsCl-H_2O	CsCl and H_2O added to saturation to final volume of 5.0ml at 25°C			
V/V % DMSO	10%	9%	8%	7%
ρ mixture (by weighing)	1.741	1.763	1.787	1.803
Weight CsCl (g) (back calculate)	4.873	5.028	5.192	5.308

Figure 1



B) Effect of 7% DMSO on Titration of PM2 DNA -

From the results of experiment (A), it is obvious that picking a DMSO concentration higher than 7% V/V will not provide the CsCl gradient necessary for the separation of PM2 Forms I and II in alkali. 7% DMSO is therefore used throughout experiment (B).

A Beckmen Model E ultracentrifuge is used throughout the analytical equilibrium ultracentrifugation experiments. Either a 4 or 6 cell rotar is used, with double-sector 12 mm charcoal-filled Epon centerpieces and a double-sector offset counterbalance. Table II shows the contents of each of three centrifuge cells, and Figures 2 to 5 show the U.V. spectra of the cells after 16½ hrs and 19 hrs of centrifugation at 38,000 rpm at 20°C.

Table II

Cell No.	1	5	4
Vol DMSO (ml)	0.035	0.035	0.035
Form I PM2 (γ)	0.5	0.5	0.5
Form II PM2 (γ)	0.5	0.5	0.5
pH of cell	10.5	11.5	12.5
ρ_e of cell	1.82	1.83	1.83

Figure 2 (16½ hr. scan)

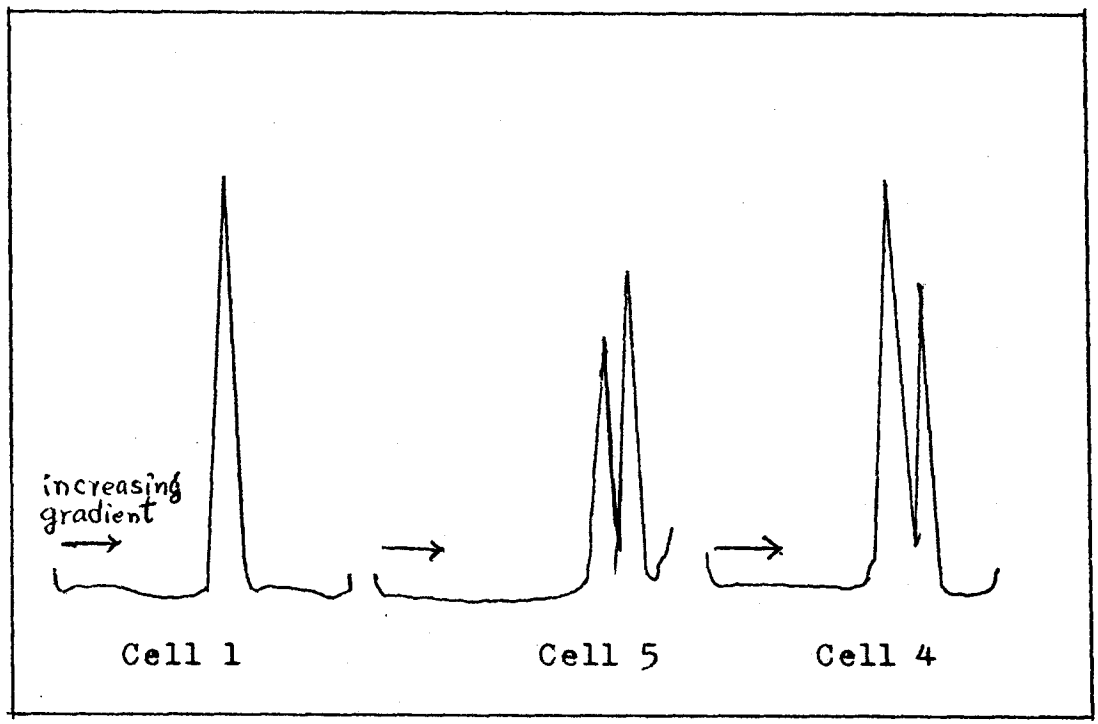


Figure 3 (Cell 1)

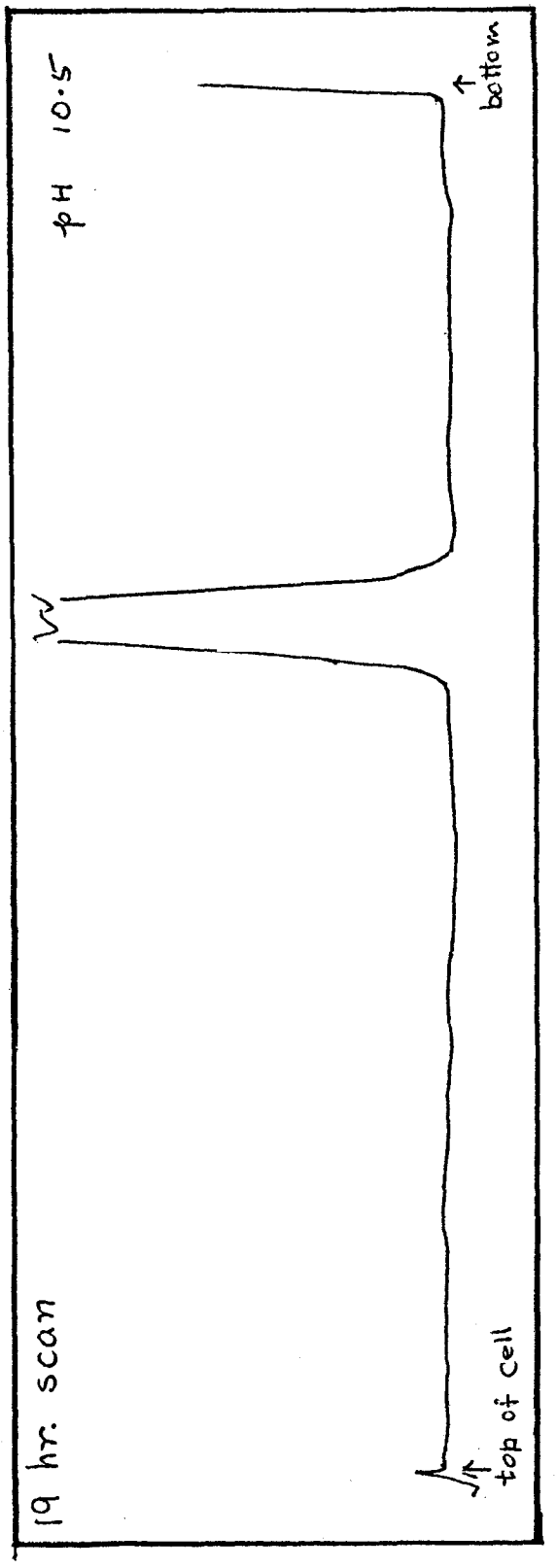
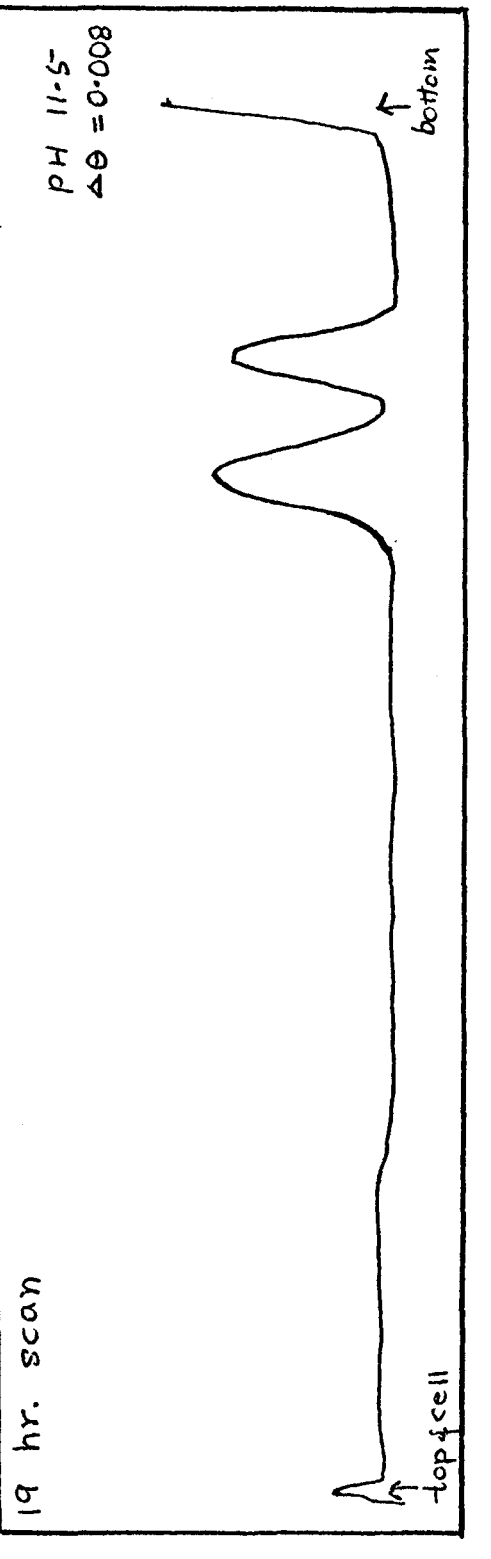


Figure 4 (Cell 5)



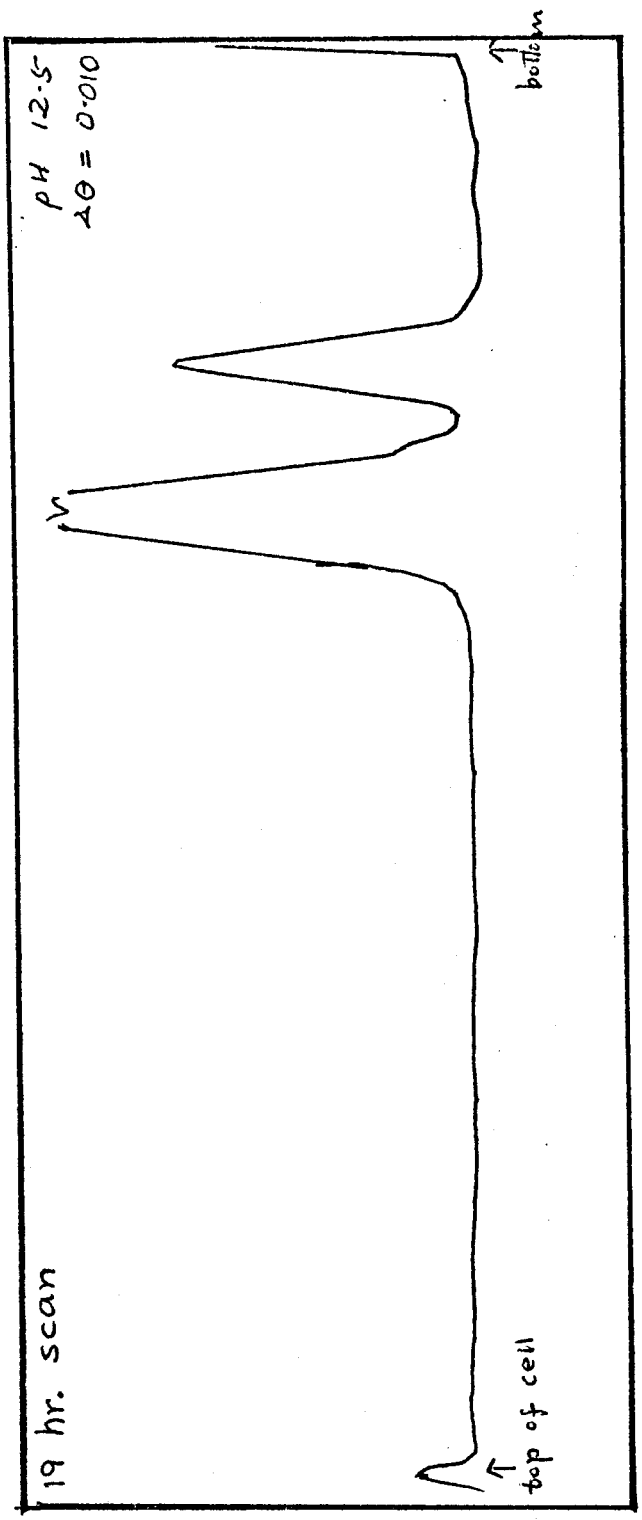


Figure 5 (Cell 4)

Results:

PM2 Form II titrates at pH 11.5, but the $\Delta\theta$ between the two peaks at pH 11.5 is 0.008 whereas at pH 12.5, $\Delta\theta$=0.010. This may indicate that at pH 11.5, titration of the DNA is incomplete. Table III summarizes the results, the calculation based on a linear CsCl gradient, and 0.1 gm is taken as the difference between top and bottom of gradient.

Table III

pH	10.5	11.5	12.5
ρ of Form I	1.832	1.858	1.857
ρ of FormII	1.832	1.850	1.847
$\Delta\theta$	0.000	0.008	0.010

Discussion:

It has been demonstrated that at pH 11.5, in the presence of 7% DMSO(V/V), singly nicked PM2 DNA titrates and separates from the intact duplex DNA in a buoyant density gradient. However, the exact degree of titration is not known. Compared to the separation of Form I and Form II at pH 12.5(at which pH titration is assumed to be complete), the titration appears to be only 80%.

A point of interest is that in the presence of 7% DMSO, for PM2 Form I and Form II, $\Delta\theta_{pH 11.5} = 0.010$, whereas in the absence of DMSO, $\Delta\theta_{PH 12.5} = 0.018$. We can explain this

observation in two ways. First, assuming a linear CsCl gradient in DMSO, the buoyant densities of titrated PM2 Form I and Form II are different from those in pure aqueous CsCl, and experimental results showed increased buoyant densities. It is possible that DMSO has increased the buoyant density of Form II DNA more than that of Form I due to preferential solvation of the open structure. Since the molecular weight of DMSO is four times that of H_2O, the preferential solvation of Form II by DMSO would increase its buoyant density.

Another explanation for the observation of decreased $\Delta\theta$ is that in the presence of DMSO, the distribution of CsCl in an ultracentrifugation gradient is no longer linear. In fact, Allan Williams and Vinograd have demonstrated that the distribution of Cs_2SO_4 in DMSO-aqueous system is non-linear, but more like a parabola. For Cs_2SO_4-DMSO, the gradient is almost doubled at mid-point in the presence of 10% DMSO. In the present case, the $\Delta\theta$'s calculated are probably all underestimations of the real values.

In the absence of further experimental data, the results cannot be applied to the HeLa mitochondrial DNA strand separation with validity.

CHAPTER TWO

Pyrimidine Cluster Analysis of Duplex HeLa Mitochondrial DNA Labelled with ^{32}Pi in vivo.

Introduction:

Theoretically, it is possible to carry out a pyrimidine cluster analysis on 5γ of mitochondrial DNA labelled with ^{32}Pi in vivo by using two dimensional ionophoresis. Yet with such a small amount of material and low efficiency of ^{32}Pi incorporation (approx. 10^5 cpm/γDNA), it remains to be seen whether or not such an analysis is feasible. To find out the feasibility of performing a pyrimidine cluster analysis on strand separated mitochondrial DNA, a pilot run with 5γ of ^{32}Pi labelled total mitochondrial DNA is made.

Theory:

DNA labelled with ^{32}Pi is degraded to apurinic acid by treatment with 3% diphenylamine in 98% formic acid[3]. The apurinic acid is hydrolyzed to yield a mixture of pyrimidine oligonucleotides. Diphenylamine in formic acid gives specific cleavage to give only Py_nP_{n+1} oligonucleotides. To make sure uniform pyrimide to phosphate ratios are obtained for the isostichs, enzymatic digestion with alkaline phosphatase is employed. A mixture of isostichs with pyrimidine to phosphate ratio of n::(n - 1) is obtained.

Electrophoresis on cellulose acetate separates the oligos on a charge basis - the highest charged oligos move the fastest in the electric field. The oligonucleotides are transferred to DEAE ion exchange paper, on which is run a second dimensional ionophoresis at pH 1.9. The negatively charged oligonucleotides are held by the ion exchange paper. The smallest nucleotides migrate the fastest from cathode to anode. Of the oligonucleotides possessing the same no. of phosphate residues, those with high cytosine content move the fastest. This process of ionophoresis achieves partial separation of the isomers.

Experimental:

A) Pyrimidine Cluster Analysis of ^{32}Pi labelled E. coli DNA using Cellulose Acetate Electrophoresis in First Dimension and DEAE Paper Ionophoresis in Second Dimension -

Materials and Methods:-

E.coli alkaline phosphatase is obtained from Worththington, and purified by heating to 90°C for 20 minutes to remove nucleases. Cellulose acetate strips were from S. & S., Carl Schleicher & Schuell Co., Keene, New-Hampshire. (N_o. 2500, 30 x 550 mm, lot 83/9). DE81 ion exchange paper was from Whatman Company, England.) All chemicals were reagent grade.

Alkaline phosphatase activity was assayed by hydrolysis of p-nitropheny phosphate. Enzymatice hydrolysis releases p-nitrophenol, a yellow chromophore with λ_{max} = 410 mμ, and molar extinction coefficient = 1.62×10^4. The heat treated enzyme retained alkaline phosphatase activity of 51.3 U/mg protein. After appropriate dilutions, 1 ml of enzyme stock solution contains 14.25 U/ml. This alkaline phosphatase solution will be used throughout the following experiments.

E. coli ^{32}P- DNA is extracted by the Marmur procedure from 200 ml of E.coli K 12* culture grown in glucose casamino acids medium[5] labelled with 1 mC of ^{32}Pi.

Pyrimidine oligonucleotides

All the DNA extracted from the E. coli (approx. 1 mg after drying in absolute ethanol and ether) was dissolved in 10 ml of freshly prepared 3% diphenylamine in 98% formic acid in a plastic capped vial, and incubated at 37°C for 17 hrs. At the end of incubation, 10 ml double distilled water was added, and the diphenylamine-formic acid was extracted with 10 volumes of anhydrous ether 8 times. Double distilled water was added occasionally to maintain the aqueous phase at a volume of 5 ml. The final aqueous phase was lyophilized. The sodium salt of the pyrimidine tracts was obtained.

*A gift from Jung Lee.

Dephosphorylation

The pyrimidine clusters were dissolved in 10 ml of 0.005M Tris buffer (pH 8.00), and 0.2 U (14%) of alkaline phosphatase was added. Incubation was at 37°C for 6 hrs. At the end of incubation, the mixture was frozen in dry ice-acetone, and lyophilized.

Fingerprinting of pyrimidine oligonucleotides[6]

First dimension electrophoresis was run on cellulose acetate in 5% acetic acid, 0.5% pyridine(pH 3.5), 7M urea, 10^{-2} M EDTA. Sample was applied 10 cm from the cathode end of the cellulose acetate strip, flanked by two spots of dye markers. The radioactivity registered 10^{4} cpm detected by the hand monitor. Electrophoresis was run at 4,500 volts for 1 hr in a High Voltage Electrophorator, Model D, from Gilson Medical Electronics, Middleton, Wisconsin. Most of the radioactivity migrated between the red and blue dye markers, as detected by hand monitor.

The cellulose acetate strip was removed from the rack, and laid on DE81 Whatman paper (46 x 57 cm^2) at 20 cm from one of the short edges of the paper. Three strips of Whatman No. 1 paper, presoaked with distilled water, were laid on top of the cellulose acetate strip and weighed down with 3 glass plates. When the water had soaked to a band width of 15 - 20 cm, the transfer was completed. Recovery was 90% or better from hand monitor detection.

Second dimension ionophoresis was carried out in

7% formic acid buffer (pH 1.9).

Two ionopherograms were run, one for 4 hrs, at 180 mA. The high resolution ionopherogram was run overnight for 16½ hrs at 180 mA. The ionopherograms were dried in a hood, and autoradiographed, using Kodak X-ray film, and developed after 24 hrs. exposure.

Figures 6 and 7 show the two autoradiograms for the pyrimidine cluster distribution of E. coli DNA.

B) Pyrimidine Cluster Analysis of ^{32}Pi labelled Duplex HeLa Mitochondrial DNA -

Materials and Methods:-

^{32}Pi HeLa Mitochondrial DNA

To 4 spinners (1 liter/spinner) of HeLa cells suspended in phosphate free Eagles medium at conc. 5×10^4 cells/ml was added a total of 20 mC ^{32}Pi, carrier phosphate buffer being added to make up to 10^{-4} M phosphate. The cells were labelled for 96 hrs until harvest.

The cells were harvested by spinning for 5 min. at 2000 rpm in an IEC centrifuge, model PR-6, at 4°C. 8.5 ml of packed cells were obtained. The cell pellet was resuspended in 2 x 40 ml of TD buffer (10^{-3} M Tris, 10^{-4} M EDTA) and pelleted at 1,600 rpm. The washing was repeated. The final cell pellet was resuspended to final volume of 30 ml in RSB (0.01M NaCl, 0.01M Tris, 0.0015M $MgCl_2$), and set in an ice bucket for 5 min to swell the cells. The swollen

Figure 6

4 hr 2-Dimensional Ionopherogram of E. coli Pyrimidine Clusters
(Autogradiogram after 24 hr exposure)

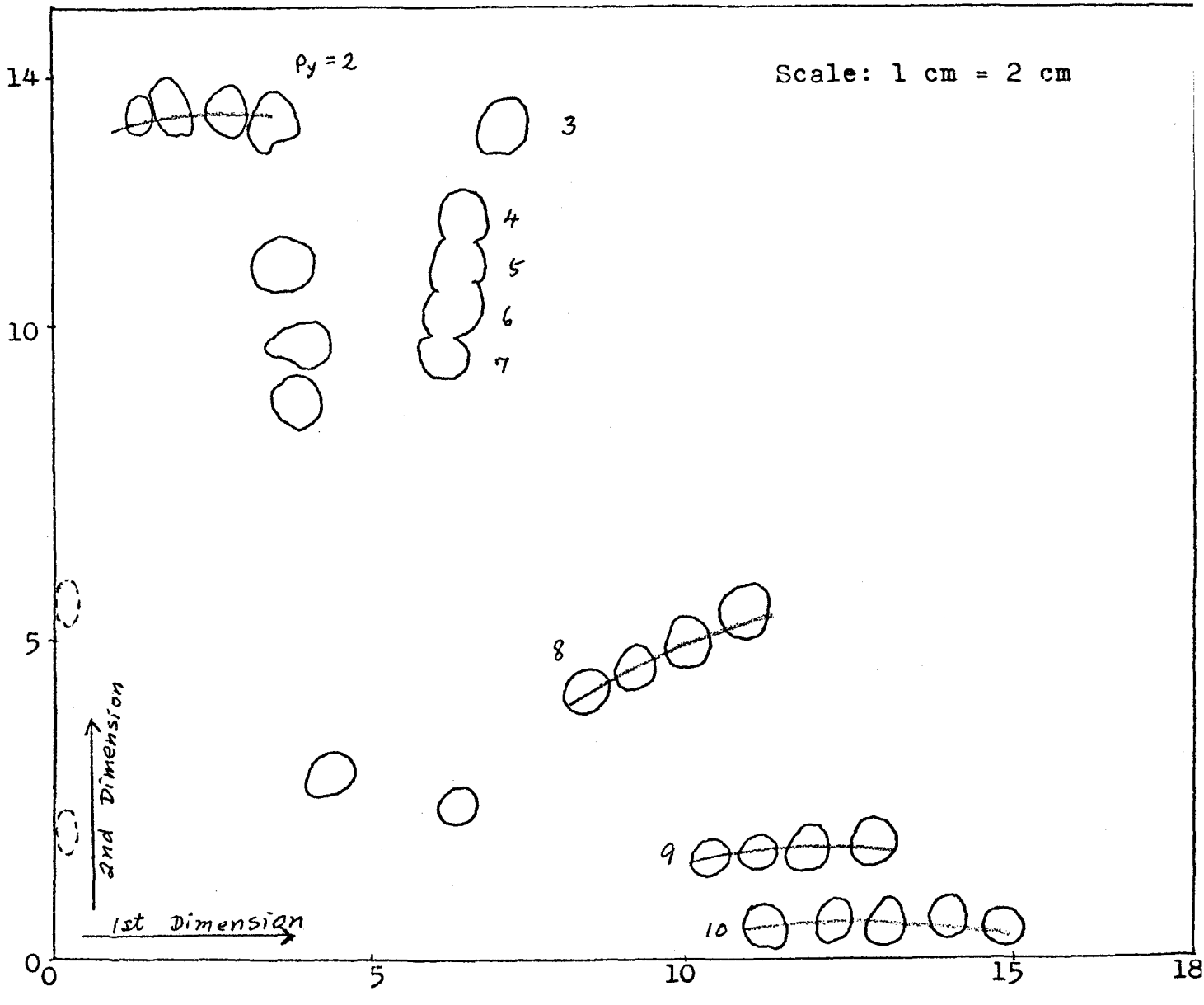
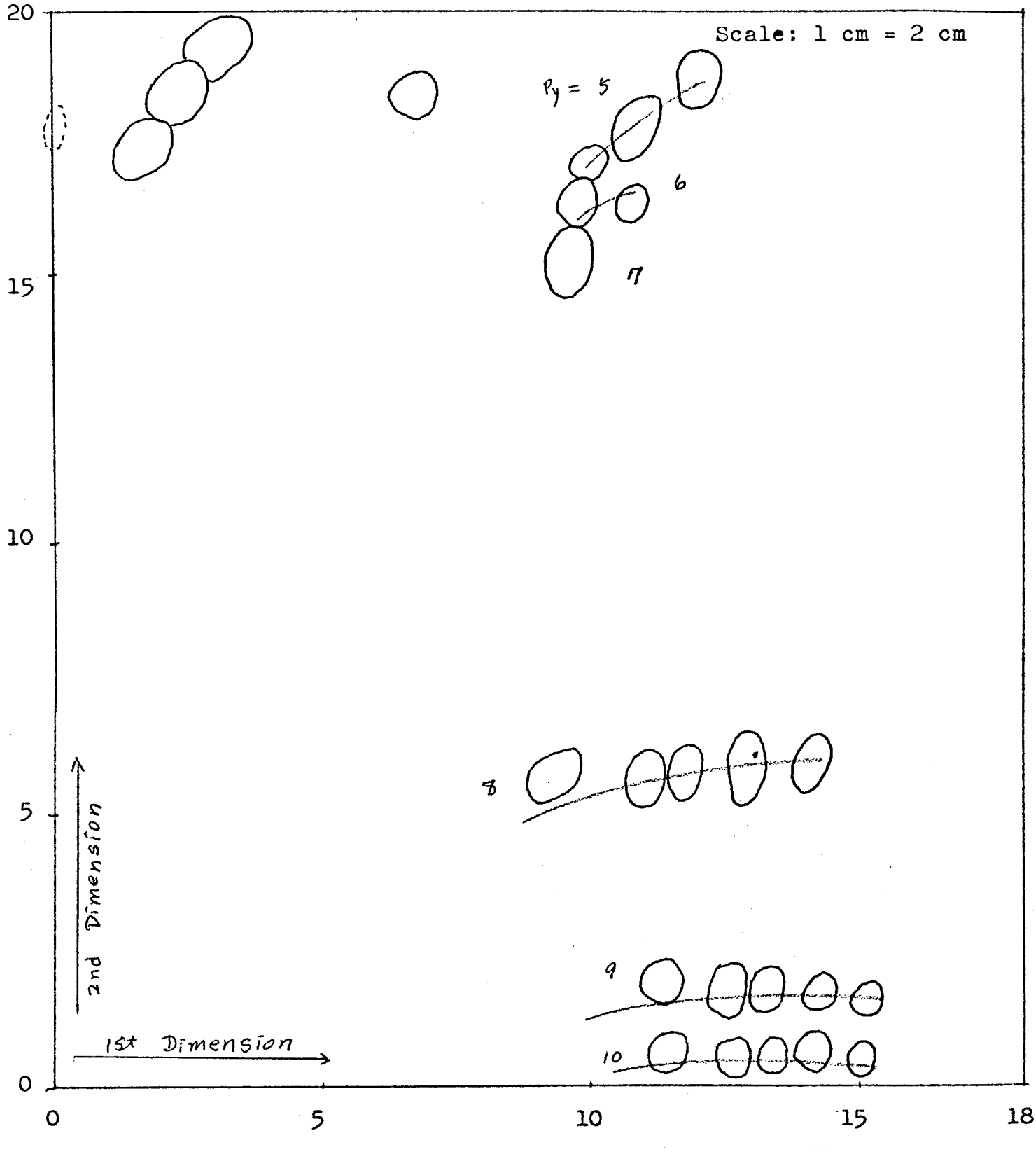


Figure 7

16½ hr 2-Dimensional Ionopherogram of E. coli Pyrimidine Clusters
(Autoradiogram after 24 hr exposure)



cells were broken with a Downs Homogenizer. Azure dye which stains only cell nuclei but not intact cells was used to check the degree of cell breakage. After 4 strokes, over 90% of the cells were broken. The homogenate was then poured into 10 ml of 1M sucrose. The final conc. was ¼M sucrose, necessary to prevent lysis of nuclei which would otherwise release nuclear DNA. Three low speed spins each for 5 min at 3,000 rpm in the IEC centrifuge (rotar No. 284, 250 ml cups) removed cell nuclei and cell debris. Final supernatant was centrifuged in the Sorvall RC2-B at 15K rpm at 4°C for 20 min to precipitate the mitochondria. In the mean time, 2 sucrose gradients were prepared by layering 20 ml of 1M sucrose (in 10^{-3} M Tris, 10^{-4} M EDTA) over 12 ml of 1.5M sucrose(in 10^{-3} M Tris, 10^{-4} M EDTA).

Mitochondrial pellet (0.5 ml) was homogenized in a small Downs homogenizer in 6 ml of 1M sucrose, layered on the sucrose gradients, and velocitized in SW 27 rotar at 25K rpm or 1 hr at 20°C. Lipid material remained on top, nuclei went to the bottom of the tube, and mitochondria were retained at the interphase. The interphase was salvaged, (approx. 10 ml), diluted with 2 vols distilled water, and spun at 14Krpm for 30 min at 4°C in a Sorvall centrifuge (SS34 rotar) to repellet the mitochondria. A final pellet of 0.2 ml was obtained.

Mitochondria were suspended by gentle pipetting in 2 ml of 2% SDS solution and left to stand at room temperature.

When the mitochondria were completely lysed (clear solution), 1/6 volume of saturated CsCl was added, and the solution mixed in a cyclomixer, then chilled in an ice bucket for 20 minutes. The SDS precipitated out, together with denatured proteins. Solid was removed by centrifugation. in the Sorvall (SS34 rotar), at 13 - 14 Krpm for 10 to 15 min at 4°C. Volume of supernatant = 2.4 ml. One seventh its weight of CsCl was added, and propidium iodide was added to give a final concentration of 200 γ/ml. Refractive index of final soln = 1.384, corresponding to a density of 1.532 g/ml.

The propidium-iodide-DNA mixture was centrifuged at 38 K rpm for 18 hrs in a Spinco centrifuge, model L, to separate the mitochondrial DNA from nuclear DNA by buoyant density equilibrium centrifugation. At the end of the run, the dye bound mitochondrial DNA formed a lower band, while the nuclear DNA formed an upper band. The tube was dripped to collect the lower band. The mitochondrial DNA was dialyzed against double distilled water for 15 minutes to remove most of the CsCl. Then the crude mitochondrial DNA was layered over a CsCl velocity gradient and run for 4½ hrs at 38 K rpm in a SW 50 rotar. (Gradient was formed by layering 3.2 ml CsCl-PI of ρ = 1.40 over 0.8 ml of CsCl-PI of ρ = 1.70). The mitochondrial DNA formed two bands, an upper band for partially open material and catenanes, and a lower band for closed monomers.

For the purpose of a pyrimidine cluster analysis, both the monomers and catenanes were retrieved, and rebanded in CsCl-PI of $\rho = 1.54$. At the end of 20 hrs, the tube was dripped to collect 100λ fractions (50 drops per fraction). 5λ aliquots were taken and Čerenkov counted for 10 minute intervals in 10 ml water, using the Beckmen scintillation counter, ^{3}H channel. A sharp band of radioactivity was obtained. The fractions under the peak were pooled to give a total of 5.25×10^{4} cpm. By scintillation counting, this would be the equivalent of 1.5×10^{5} cpm.

A second mitochondrial DNA prep from 5 spinners of HeLa cells yielded 1.18×10^{5} Čerenkov counts.

Pyrimidine oligonucleotides

The HeLa mitochondrial DNA was dialyzed against/Dowex 50 2 ml
in/10 x SSC at room temperature for 4 hrs, then another 40 ml
4 hrs after a change of Dowex. The dye free DNA was then dialyzed exhaustively against distilled water at 4°C for 2 days to remove salt.

The DNA solution was incubated with 2 volumes of 3% diphenylamine in 98% formic acid, and pyrimidine oligonucleotides of formula Py_nP_{n-1} were obtained as for E. coli DNA.

Fingerprinting of HeLa Pyrimidine Oligonucleotides

With half of the sample from the first HeLa prep, first dimensional electrophoresis on cellulose acetate indicated very low radioactivity. The hand monitor showed

20 to 120 cpm. The cellulose acetate strip was dried and autoradiographed. It took one week of exposure to give a discernible pattern. To run a second dimension ionopherogram on such low level of radioactivity would not be practicable.

The second HeLa prep had higher radioactivity, but second dimension ionophoresis gave only two or three faint spots on the autoradiogram after four days of exposure. It became obvious that to do a pyrimidine oligonucleotide analysis with HeLa mitochondrial DNA labelled in vivo would be very impractical.

Discussion:

The in vivo ^{32}Pi labelling of HeLa cells did not give the desired level of radioactivity for a pyrimidine oligonucleotide analysis on the mitochondrial DNA. Two factors make it infeasible to label the mitochondrial DNA in vivo. The extraction procedure alone takes almost one week. Mitochondrial DNA constitutes only 1% of total DNA found in the cell. With such small amounts of material and a short half life of 14 days of the isotope, it becomes uneconomical to persue the problem in this manner. An alternative method is to use the end labelling technique, as described in the following chapter.

CHAPTER THREE

Pyrimidine Cluster Analysis of the Light Strand of HeLa Mitochondrial DNA using End-labelling Technique[7] and DE Thin Layer Homochromatography[8].

Introduction:

Whereas the use of E. coli 5'OH-polynucleotide kinase to end-label polynucleotides in the fingerprinting of non-radioactive nucleic acids has been puclished by Sanger's group in 1969, it was not considered at the outset due to the disadvantage that carrier DNA cannot be used if further work is to be done on the oligonucleotides. In the previous experiment, it has been demonstrated that the in vivo labelling of HeLa mitochondrial DNA does not give enough radioactivity for a feasible pyrimidine oligonucleotide analysis. The end labelling method becomes the alternative way to study the pyrimidine cluster distribution. A method must be found to circumvent the difficulty of working with only a few micrograms of DNA without using carrier. Homochromatography procedure is used for second dimension separation.

The end labelling of cold mitochondrial DNA has two advantages over in vivo labelling. Cold pyrimidine clusters can be prepared and accumulated without regard to time. The second advantage is the efficiency of labelling. By using carrier free γ^{32} P-ATP, the efficiency of labelling per mitochondrial DNA strand is increased by at least 10^3.

As mentioned earlier, one cannot use carrier DNA if the oligonucleotides are to be retrieved for further analysis. In the absence of carrier DNA, recovery is a major problem. To improve recovery of DNA material, all reaction vessels have been coated with dimethyl-dichlorosilane. Second dimension ionophoresis on DE81 paper is replaced by thin layer homochromatography.

Theory:

The principle for thin layer homochromatography is a displacement chromatography. The oligonucleotides bound to DE cellulose on the TL plate are replaced by a migrating front of ribonucleotides in the ascending elution buffer. Depending on the size and charges of the DNA pyrimidine oligos, the rates of displacement differ. The smaller and less acidic nucleotides are displaced the fastest, and they move closest to the solvent front. The bigger and more acidic oligonucleotides stay closer to the origin of the thin layer plate.

In end labelling reaction, a slight excess of ATP is recommended, to prevent the smaller oligonucleotides from being preferentially labelled.

Excess ATP sometimes blacks out the autoradiogram, so that myosin ATP-ase is used to degrade excess ATP. The enzyme can be denatured by quick freezing in dry ice acetone. The ^{32}Pi is then removed from the oligos by ionophoresis on DE81 paper at pH 3.5. The pyrimidine oligonucleotides are eluted with triethylammonium bicarbonate,

evaporated to dryness, and fingerprinted.

Experimental:

A) Pyrimidine Cluster Analysis of HeLa Light Strand Mitochondrial DNA -

Materials and Methods:-

Preparation and Assay of Myosin ATP-ase from Rabbit Muscles

Myosin was prepared by the method of Perry[9]. Two freshly killed rabbits(beheaded and skinned) were obtained from the rabbit market, chilled in ice, and the thigh and back muscles were dissected free of ligaments in the cold room. The muscles were weighed out in 100 g portions and extra muscles were deep frozen. 100 grams of muscles were ground up in a pre-chilled waring blender for a few seconds, and 200 ml of KCl-K Ⓟ buffer (pH 6.5) were added to the blender. The mixture was blended several times, each time for a few seconds. Prolonged blending would cause the blender to heat up and denature the myosin enzyme. When an even suspension was obtained, the homogenate was poured out, and the blender was rinsed out with 100 ml of KCl-KⓅ buffer.

The combined suspension was stirred in the cold room for 1 hr. The mixture was centrifuged in the IEC PR-6 centrifuge (rotar no. 284) at 3000 rpm for 10 min at 0°C. The supernatant was filtered through a No. 2 buchler funnel lined with Whatman No. 40 paper, cushioned with paper pulp from S. & S. (No. 289 Ash Free paper pulp). Filtration was

rapid, 210 ml filtrate were obtained. The filtrate was slowly poured into 3 liters of ice cold double distilled water with stirring. The flocculent precipitate of crude myosin was left to settle overnight.

The supernatant was decanted off, and the myosin was precipated by centrifugation for 15 min at 3000 rpm in an IEC centrifuge, rotar 284. 95 ml of gel was obtained. Solid KCl was added to give ionic strength of 0.5M. The pH was adjusted to 6.6 to precipitate actomyosin. A small amount of solid $NaHCO_3$ was used, and pH was determined with Acutint pH paper from Anachemia Chemicals Ltd., Montreal. The colloidal solution was diluted to μ = 0.3, and left to sit for ½ hr in the cold room. The colloid was centrifuged at 3 Krpm in the IEC for 15 minutes. A slightly yellowish precipitate was obtained. The colloidal supernatant(300 ml) was decanted off in a 3 liter beaker. Ice cold double distilled water was slowly poured into the beaker with stirring. Final volume = 2250 ml, corresponding to μ = 0.04. The precipitate was left to settle overnight. This is the first recrystallized myosin. The myosin was again recrystallized by precipitating, adjusting to μ = 0.5 with KCl at pH between 6.5 and 7.0, and pouring into water to give final μ = 0.04.

Although three preparations of myosin with different degree of recrystallization have been made, assay for endonuclease has demonstrated that 2 x recrystallized myosin already contains below-detection limit of contamination. The latest prep yielded 100 ml of myosin soln. with 9mg/ml.

Assay for Myosin Activity

The method of assay reported by Perry[9], Kielley[10] and others is based on an estimation of inorganic phosphate released from ATP by myosin. Since ATP is very unstable, and the conditions for developing color(Allen procedure) are hydrolytic toward ATP, the background is very high and the results are not always reproducible. A new method of assay was therefore devised.

Myosin ATP-ase was reacted with γ-labelled ATP, under the conditions of the pyrimidine cluster analysis, and the mixture was electrophoreses on Whatman DE.81 paper in pH 3.5 buffer(5% acetic acid, 0.5% pyridine) for 45 minutes. It was at this point that the γ-ATP was found to be highly impure. An analysis for the γ-ATP showed only 65.8% purity. After subtracting the background the assay for myosin activity in the prep showed 97.2% degradation of ATP under the experimental conditions. For the small volume of reactants, this is within the margin of experimental errors. This would sufficiently reduce the background noise of unreacted ATP.

Assay for Endonuclease activity

The myosin ATP-ase was assayed for endonuclease activity by equilibrium centrifugation with PM2 Form I DNA. Endonuclease would nick the Form I to yield Form II. Equilibrium banding of myosin treated PM2 Form I showed the DNA band at the same position as blank PM2 Form I. Endonuclease was beyond the level of detection.(Fig.8)

Assay for Nucleic Acid Contamination

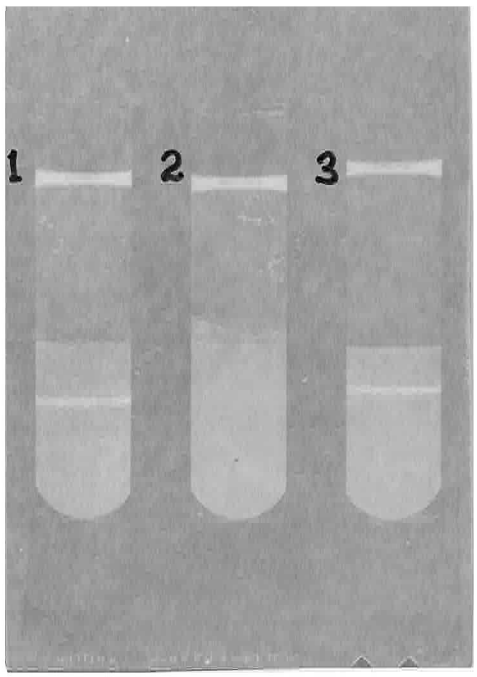
The $O.D._{280}/O.D._{260}$ ratio for a pure protein = 1.75. However, the myosin prep gave a ratio = 1.07, indicating possible nucleic acid contamination. Since the experimental conditions would require an appreciable amount of myosin (25 µg myosin per 3 µg pyrimidine clusters), a nucleic acid contamination might pose a considerable problem. In the ultracentrifugation assay for endonuclease activity, a blank myosin sample was run along with the myosin-PM2(I), and the U.V. picture of the myosin band was photographed along with the PM2 blank and the myosin treated PM2 band. A rough estimation (by comparing intensity of the bands) indicates less than 0.04% nucleic acid contamination in the myosin. Compared to mitochondrial DNA used per experiment, the nucleic acid contamination from myosin is less than 0.2%. (See Figure 8 for results)

Triethylammonium bicarbonate

70 ml of double distilled water was added to 30 ml of triethylammine in a 250 ml graduated erlenmeyer flask in an ice bath. CO_2 was bubbled through the mixture until the 2 phases merged into one. Volume decreased to 90 ml, more water was added, and CO_2 was bubbled through until a final volume of 100 ml was obtained. This reagent is stable at 4°C.

Thin Layer DE cellulose plates were prepared according to Barrell's procedure[6]. These will be available from Whatman Company with plastic backing in the near future.

Figure 8



1. Myosin + PM2 (I)
2. Myosin
3. PM2 (I)

Assay for Endonuclease and Nucleic acid contamination

Homochromatography Mixture

Homomixture "c" was used, prepared as in Barrel's method with one modification. Instead of 15 minutes hydrolysis, the RNA was hydrolysed for 30 minutes.

Kinase

5'OH-polynucleotide kinase was a gift from Paul Johnson, and prepared according to a modified Richardson procedure.

Strand Separation of Mitochondrial DNA from HeLa Cells

Mitochondrial DNA, obtained by standard procedure described previously, was dialyzed against 10^{-4} M Tris overnight after dye removal, and concentrated to ¼ ml by blowing nitrogen over the DNA solution. Solid CsCl

was added to saturation, final volume = ½ ml. To the CsCl-DNA solution was added 0.05 ml of 0.5M K_3PO_4. Then a solution of CsCl in 0.05M K_3PO_4, 0.05M KOH, (ρ = 1.75, pH = 12.98) was added. Final volume was 2.5 ml, ρ = 1.769, pH = 12.54.

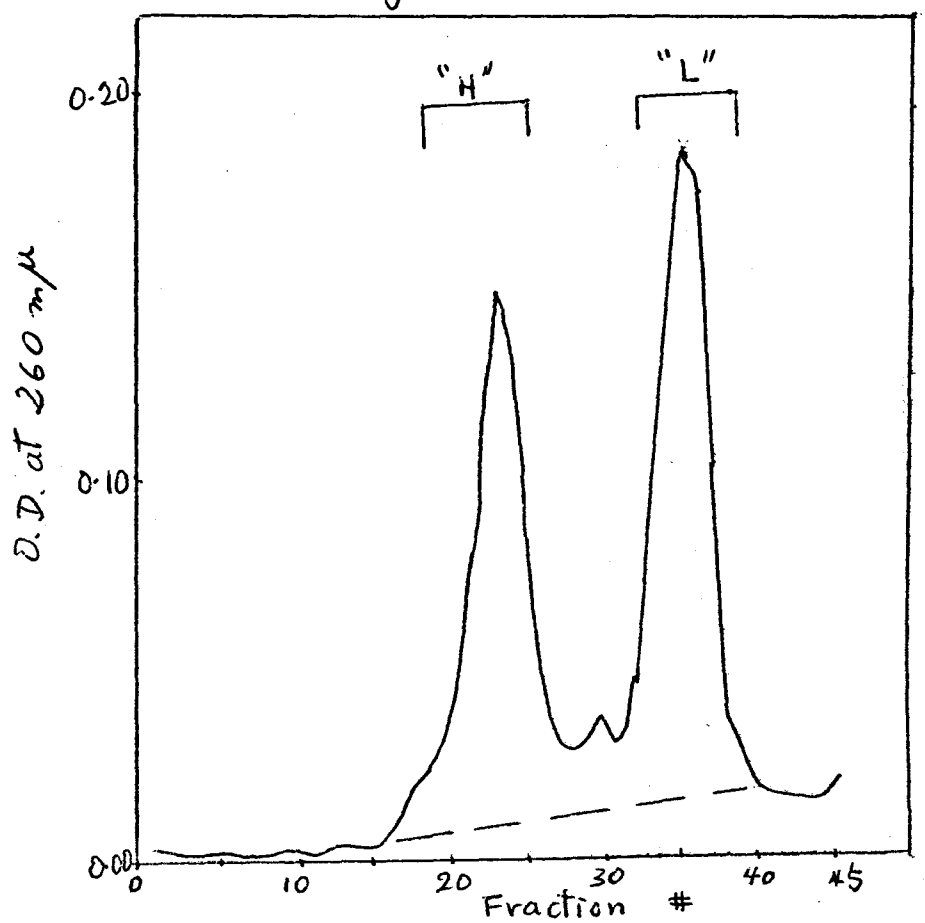
The aldaline CsCl-DNA solution was put in a polyallomer tube, and centrifuged at 31.5 K rpm for 42 hrs at 20°C in a SW 50.1 rotar. At the end of the uninterrupted centrifugation, the tube was dripped, 5 drop fractions (50λ) were collected in 1 ml plastic conical vials. To each fraction was added o.4 ml of 0.02M Tris. The fractionation profile is given in Figure 8'. Fractions 20-28 were pooled and put in a dialysis bag marked H, (4 knots), fractions 31 - 39 were pooled and put in a dialysis bag marked L (3 knots). The DNA strands were dialyzed 48 hrs exhaustively against distilled water. Based on the figure of 36γDNA/O.D. unit/c.c., 15.06γ heavy strand material and 15.60γ light strand material were obtained.

Another preparation yielded 11.3γ light strand mitochondrial DNA.

Degradation into Pyrimidine Clusters and Treatment with Alkaline Phosphatase

Procedure for these reactions were described for E.coli DNA. Amount of phosphatase used was 0.07 U per 5 μg of single stranded mitochondrial DNA, i.e. approximately 3.5 μg of pyrimidine clusters.

Figure 8'



Strand separation profile for Hela mitochondrial DNA

End-Labelling of Pyrimidine Clusters with γ^{32} P-ATP and 5'OH polynucleotide Kinase

To the dephosphorylated pyrimidine oligonucleotides were added 0.6λ to 0.8λ of 0.1M P buffer to give final concentration of 1 mM to inactivate the alkaline phosphatase left from the former reaction. In addition, 3λ of 0.2M $MgCl_2$ (final conc. 0.01M), 4λ of 0.5M mercaptoethanol (final conc of 0.02M) were added. Excess γ^{32} P-ATP was added (4λ, equivalent to 1.2 muM, almost 40% excess). At time 0 minute, 1λ of kinase (2units)/was added to the reaction mixture, and incubated for 30 minutes at 37°C. Another λ of kinase was added at 30 minutes of incubation, and reacted for the same amount of time. At the end of the reaction, the mixture was frozen in dry ice-acetone to denature the kinase. Upon thawing, 5λ of myosin ATP-ase was added, and the mixture was incubated at 37°C for 1 hr. Myosin ATP-ase was denatured again by quick freeze-thawing at the end of the reaction. The mixture was then evaporated to dryness in an evapo-mix (manufactured by the Buchler Company)

Fingerprinting of End-labelled Pyrimidine Oligonucleotides from HeLa Light Strand Mitochondrial DNA

To the end-labelled oligonucleotide mixture was added 1 ml of Calf thymus pyrimidine tracts (100 ug) in 10^{-4} M EDTA and the mixture was passed through a milipore syringe to remove denatured enzymes. The filtrate was lyophilized and chromatographed on cellulose acetate at pH 3.5 in the first dimension for 1 hr. and 15 min. at 4,500 volts.

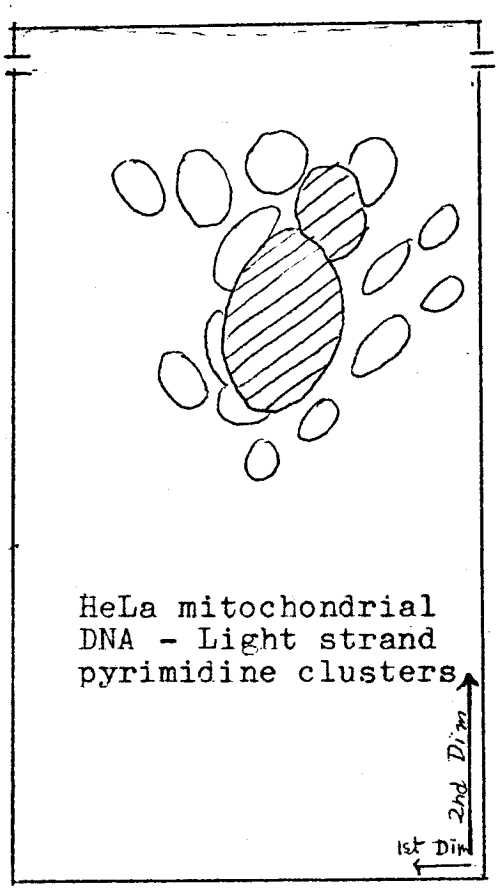
The pyrimidine tracts were transferred to a thin layer plate. Transfer was only up to 60% from hand monitor detection. Homochromatography was run at 60°C with homomixture "c" as elution buffer. Although Barrel reported that 8 hrs were sufficient to elute a long plate (20 x 40 cm^2), and 4 hrs for a short plate (20 x 20 cm^2), it was found that on an average, after 16 hr, the solvent front would reach only 3 quarters up the long plate, and most of the time the front was uneven.

Before removal of ^{32}Pi, the autoradiogram of a typical fingerprint is shown on Figure 9 . It is obvious that the level of ^{32}Pi is much higher than the 10% to 50% excess ATP applied. The reason was always attributed to the overestimation of pyrimidine oligonucleotides. It was thus necessary to remove ^{32}Pi from the oligo mixture before fingerpringing. This was done by electrophoresis of the mixture in pH 3.5 buffer on Whatman DE81 paper for 90 minutes. Figure 10 shows a separation of pyrimidine nucleotides, from unreacted γ^{32}P-ATP.

After three experiments with light strand HeLa mitochondrial DNA, two autoradiograms being shown on Figures 9 and 11, it was decided that something about the kinase enzyme system was not working, either the kinase was not labelling completely, or the phosphatase was still active, or both. This was suggested by the overwhelming amount of unreacted ATP and ^{32}Pi present. An investigation was carried out using Calf-thymus DNA.

Figure 9

Autoradiogram of a thin layer plate after 18 hrs in 2nd D.



High radioactivity

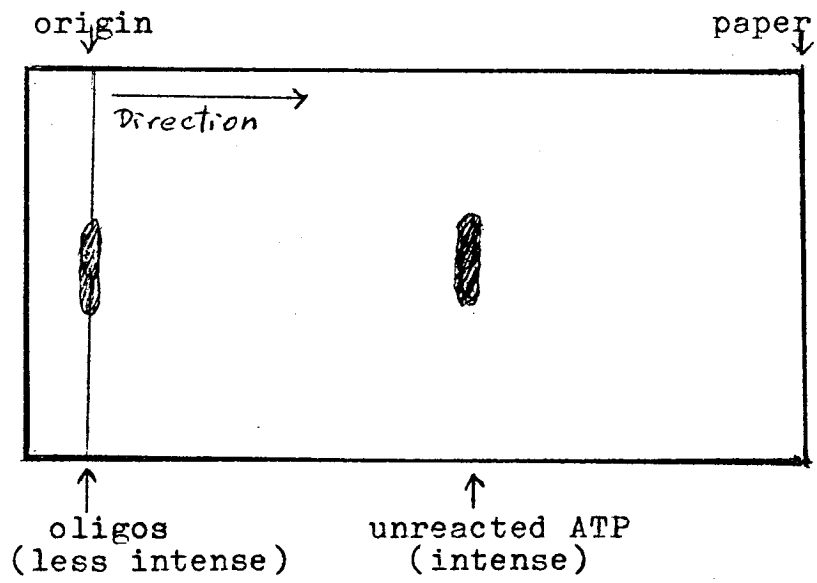
Very faint

Scale: 1 to 3.3

Exposure time = 18 hrs

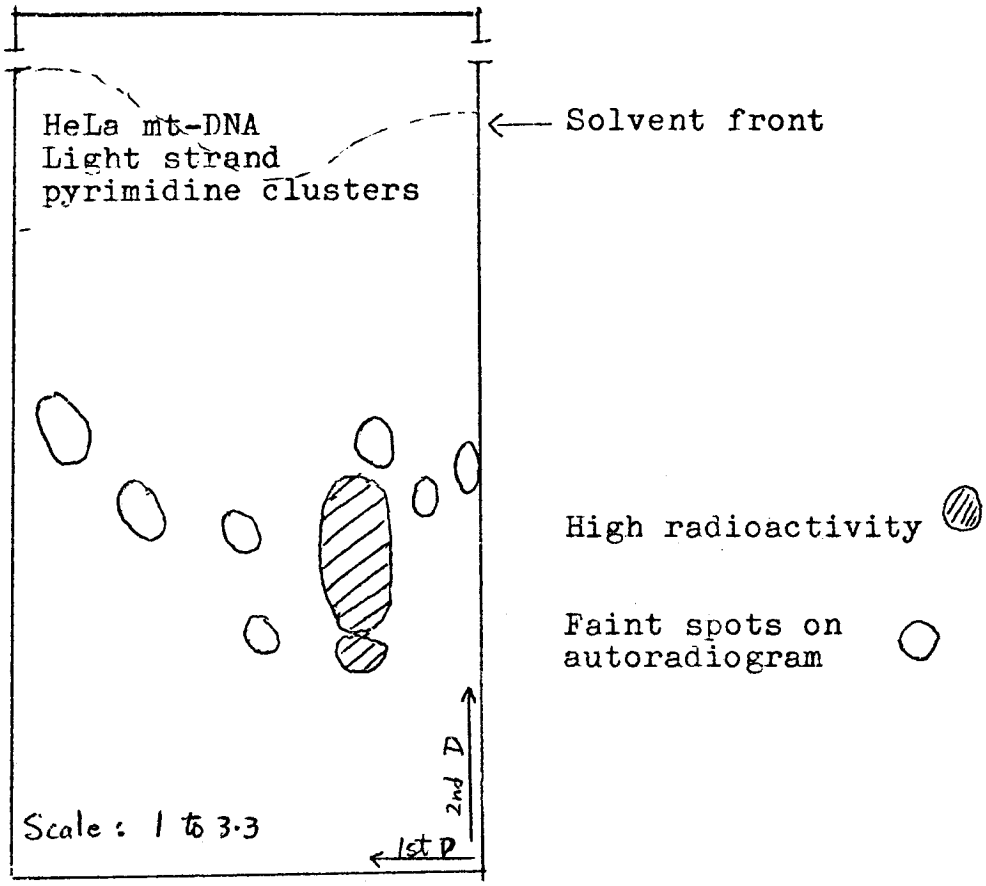
Figure 10

Ionophoretic separation of labelled oligos and unreacted ATP



Scale: 1 to 2

Figure 11
Autoradiogram of a thin layer plate after 14 hrs in 2nd D.



Experimental:

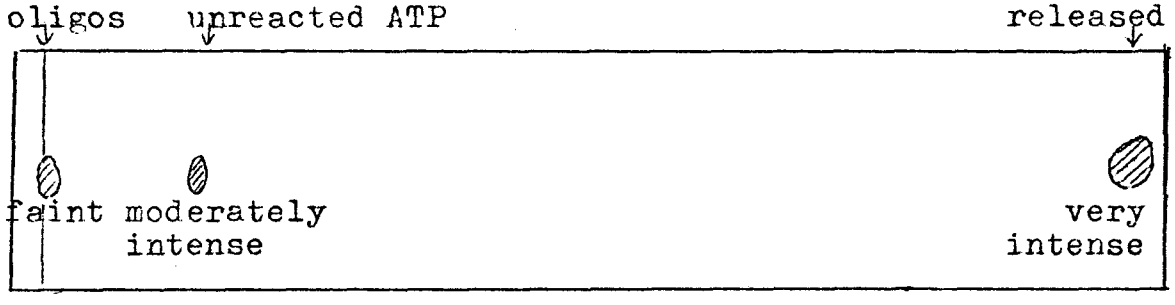
B) Pyrimidine Cluster Analysis of Calf-thymus DNA -

Materials and Methods:-

Depurinated Calf Thymus DNA (reaction as in previous experiments) was phosphatased, kinased, and the reaction mixture electrophoresed on DE81 paper, in pH 3.5 buffer (5% acetic acid, 0.5% pyridine, 10^{-2}M EDTA), at 3,500 volts for 45 minutes. The ionopherogram was autoradiographed, and ^{32}Pi was detected (Figure 12). This could have been due only to

the release of terminal phosphate from the 5'-end of the labelled oligonucleotides. According to Novogrodsky[11], and reported to work by Weiss, Live and Richardson[12], 1.5mM of inorganic phosphate should inactivate the alkaline phosphatase without affecting the kinase. However, it has not worked out in my experiments, and Sneider[13] also reported the same difficulty.

Figure 12
Detection of Pi released from kinased oligonucleotides



Discussion:

The major source of error now seems to have been resolved. In the kinase step, as long as alkaline phosphatase is still active, the net amount of ^{32}P labelled onto the nucleotides cannot be representative of the real oligonucleotide distribution. In addition to being invalid, the autoradiogram is also overshadowed by the excess Pi background.

Private communication between Theodore Live and Larry Grossman has suggested a method of inactivating the alkaline phosphatase by EGTA chelation. The principle is similar to the alkaline phosphatase inactivation reported by Sneider[13]. Future work appears promising. 28.5 mg of ^{32}P labelled E.coli DNA has just been prepared.

It will serve as marker DNA in analysing the pyrimidine nucleotide map of light strand HeLa mitochondrial DNA.

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