AN ABSTRACT OF THE THESIS OF

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Abstract approved: _____
R. W. Newburgh

The DNAs (deoxyribonucleic acids) of the thoracic tissue of the tobacco hornworm, Manducta sexta, were studied by physical and chemical means. The adult moth was considered to be fully formed two days after emergence from the pupal case, as indicated by its ability to fly and the integrity of the dorsal longitudinal flight muscle as seen in electron micrographs. The dorsal longitudinal flight muscle development was followed by assaying for total RNA (ribonucleic acid), DNA and protein during the period of time four days prior to emergence and two days after, α-glycerolphosphate dehydrogenase and cytochrome C oxidase were used as mitochondrial marker enzymes, and to follow the development of the mitochondria over a similar period of time. The enzyme assays also showed that the majority of activity was in the mitochondrial fraction.

Once it was established that the insect tissue was developing normally and that an adult moth could be considered fully developed at the end of two days the DNA from the thoracic muscles was isolated and characterized.

The material isolated from the nuclear (and debris) fraction was identified as DNA and characterized by its buoyant density in CsCl gradients, purine and pyrimidine composition, melting temperature (hyperchromicity), spectral analysis and molecular weight. The genome size was estimated from renaturation kinetics and the amount of rapidly reassociating material (repeated sequences) was determined.

DNA was isolated from the mitochondrial fraction in impure form but was identified on analytical CsCl gradients and its buoyant density determined. The number and presence of super coils was estimated by reversible denaturation experiments (snap back). The molecular weight of the DNA was determined by two different methods: 1) from its band width in CsCl gradients and 2) from contour lengths, measured on electron micrographs, of the circular and super coil forms. Circles and super coils were readily identified in the electron micrographs. The molecular weight found by band width analysis is 10.4×10^6 daltons and the molecular weight range as determined from the electron micrographic studies is 7.9×10^6 to 16.8×10^6 daltons. The molecular circumference of the mitochondrial DNA, on formvar coated grids, is 4.0 μ.

A Partial Characterization of the Deoxyribonucleic Acid Found in the Thoracic Muscles of the Tobacco Hornworm Moth (*Manducta sexta*)

by

Thomas Richard Hinds

A THESIS

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Chairman of Department of Biochemistry and Biophysics and Professor of Biochemistry
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Dean of Graduate School

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Typed by Opal Grossnicklaus for Thomas Richard Hinds

TO

Marilyn, Tommy and Robbie

My wife for her understanding, patience and hard work;

My sons for making it all worthwhile.

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INTRODUCTION

Insects in General

One of the more numerous groups of animals that man has coexisted with is the insects. Often a particular species of insects causes considerable damage to the health and well being of other animals. Often in the past, man has been powerless to stop the hoards of locusts that devoured crops, the weevils that ruined grain stores, or the insects such as flies or mosquitoes that transmit a myriad of diseases. Throughout the course of history the fate of whole civilizations has been at the mercy of insects.

On the other hand the beauty and marvel of certain insects often resulted in an admiration of particular species. The hard working beetle was thought to carry the sun through the heavens on its back by the ancient Egyptians. Even today we use such sayings as "Busy as a bee," or "Industrious as an ant" to characterize hard working people.

Motivated both by admiration and a need for control, man has studied insects, and even created a branch of science called Entomology that studies these animals. It is of interest that there are 1.5 million species of insects (Rockstein, 1964), and they live in almost

all possible niches. This is probably due in part to their adaptability which results from their high mutation rate and their motility. In the latter instance their most important means of movement is flight.

There are groups of insects that start out life, after hatching from an egg, as miniature replicas of the adult form and, through a series of successive molts, increase in size until they become full grown adults. Insects that develop this way are very primitive and are termed Ametabola; meaning, attaining the adult stage without metamorphosis. Another group of more advanced insects develop from immature nymphal stages, which do not necessarily look like the adult, through gradual molts and then to the adult form in one dramatic moult. These insects are termed Hemimetabola; meaning attaining the adult stage by incomplete metamorphosis, via three stages; egg, nymph and adult. A third group have four distinct stages, egg, larva, pupa and adult. The adult does not resemble any of the preceding stages and is usually winged. These are termed Holometabola; meaning, attaining the adult stage by complete metamorphosis.

If we examine the holometabola one important conclusion that can be stated is that the pupal stage is not a "resting stage," as it has sometimes been called, but an active period of transformation. The form of the animal that pupated does not appear like the form that emerges from the pupal case. This is not only in external physical appearance but also in internal morphology and physiology. This

suggests that during the pupal period (time in pupal case) there must be a tremendous turnover of tissue, a rebuilding of old structures (e.g., legs) and the synthesis of new structures (e.g., wings).

The flight muscles of insects are truly remarkable tissue in that they must expend more energy than any other type of muscle. Since insects are small they are more dependent upon their muscles to sustain flight than their counterparts, birds, which are capable of soaring. If we compare the net mechanical output of a good flier, such as the bee, fly or locust to that of the muscle of man, we find that the insect is about 5 times more powerful (Pringle, 1965). For example, the wind beat frequency of flies can be as high as several per second. The metabolic rate of these muscles is in the range of 220-400 Kcal/hr. per kilogram muscle (Pringle, 1965).

Flight Muscle Mitochondria

This suggests that there is some special characteristic of flight muscle which allows it to generate this tremendous amount of energy. The most outstanding feature is the number and the size of the mitochondria (sarcosomes). In the fibrillar muscle of the locust the mitochondria makes up 40% of the volume and are 2μ or greater in diameter (Wigglesworth, 1965). In addition the glycerophosphate cycle is highly active in flight muscle but almost absent in other muscles of insects (Bücher, 1965). This cycle pumps

extra-mitochondrial reducing power into the mitochondria, replacing the anaerobic mechanisms found in most mammalian muscle and other insect muscles (leg muscle of the locust (Gilmour, 1965). There is also a considerable number of tubules around the mitochondria and the fibrils in order to maintain an adequate supply of oxygen to this highly active tissue (Pringle, 1965).

Developmental studies by Bücher (1965) of the flight muscle of the locust have shown that the precursor muscle is almost devoid of mitochondria and is ill formed. Concurrent with growth of the muscle is a proliferation of the mitochondria and a corresponding increase in mitochondrial enzymes. Lennie and Birt (1967) found in the holometabolous fly, Lucilia, that there are different rates of synthesis of structural protein and respiratory enzymes of the mitochondria. This was related to the formation of various internal structures in the "immature" mitochondria.

Herold (1965) using the bee, Apis, showed that there was a correlation between the amount of cytochrome and the structure of the mitochondria during development. At early stages of muscle development, the mitochondria were found to be "vesicular" and almost devoid of "internal tubules" (cristae); at ecdysis (emergence) the increase in mitochondrial size and the number of these "tubules" was associated with the appearance of the cytochromes. It should be pointed out that during the development of the muscle not all enzymes

increase. The enzymes involved with anaerobic metabolism, such as lactic acid dehydrogenase, show a marked decrease (Bücher, 1965).

Lennie et al. (1967) showed that in dipterous muscle tissue the mitochondria contained nucleic acids. The amount varied with the developmental stage. It was noted that (Lennie, Gregory and Birt, 1967) just prior to emergence the amount of DNA associated with the mitochondria increased. This is at the time of the onset of the most rapid increase in non-enzymatic protein of the mitochondria. From these data it can be concluded that in this insect tissue there appear to be two protein synthesizing systems associated with the mitochondria and that one of them (non-enzymatic) is under the influence of mitochondrial DNA.

Mitochondria are known to be able to incorporate radioactive amino acids into proteins (Bücher, 1965; Beattie, Bosford and Koritz, 1966; Chan and Richardson, 1969). It was found that most of the radioactivity was in the structural protein. According to Bücher (1965) the rate of incorporation is dependent upon the developmental age of the mitochondria. The protein synthesizing system appears to be similar to the bacterial system from the standpoint of inhibitor sensitivity (Ashwell and Work, 1970). Thus the differential rate of protein synthesis of Lucilia (Lennie and Birt, 1967) is consistent with the idea that there are two protein synthesizing systems, one under nuclear and one under mitochondrial control.

Mitochondrial DNA

The idea of mitochondria containing DNA was not readily accepted in the late '50's and early '60's but in 1963 it was shown quite conclusively that mitochondria did contain DNA (Nass and Nass, 1963a, b). It appears that all mitochondria contain DNA, RNA and a protein synthesizing system (Ashwell and Work, 1970). This may explain the apparent confusion concerning cytoplasmic inheritance (Gibor and Granick, 1964). A petite mutant of yeast shows cytoplasmic inheritance, which is linked to a structural protein modification in the mitochondria. Similar results have been shown for the "poky" mutant of Neurospora. The later results from a mutation in the genetic machinery (DNA) involved in the synthesis of the structural protein of the mitochondria.

The mitochondria contain their own DNA which resembles the DNA of procaryotics in that it is not associated with histones, but with membranes. In all vertebrates and echinoderms studied the DNA is circular and the size is around 5 μ in circumference. The molecular weight is about 10 million daltons and is found as a Watson-Crick double helix. There are reports of circular dimers and catenanes found in certain types of mitochondria, but this is usually associated with abnormal mitochondria (Nass, 1969; Clayton and Vinograd, 1967; Hudson and Vinograd, 1969). Linear molecules have been found

in DNA preparations but this is usually due to breaking of the circular form. In the isolated state covalently closed circular DNA appears as a super coil, and nicked DNA will appear as a circle or rod, depending upon the number of nicks (Nass, 1969). At different times of development (rapid growth) the mitochondrial DNA is associated with the membrane of the mitochondria, much the same as in protozoans and bacteria (Nass, 1969). In the mitochondria of yeasts and higher plants, the size range is quite variable and most appear to be linear (Ashwell and Work, 1970). Since plant tissue extracts are more difficult to handle it is not known if the linear molecules are due to isolation techniques or if they are naturally occurring.

The protostomes to which insects belong have not been as extensively studied as the deuterostomes (vertebrates) with regard to their mitochondrial DNAs. Borst, VanBruggen and Ruttenberg (1968) have reported the mitochondrial DNA of the house fly appears as a 5.2 μ circle when studied with the electron microscope. This appears to be the only published value dealing with physical properties of insect mitochondrial DNA.

The present paper's main concern is with the physical and chemical characterization of the mitochondrial and nuclear DNA of the tobacco hornworm, Manducta sexta. This insect was chosen because of its large size, which permits the isolation of large amounts

of flight muscle from a minimum number of animals. In addition the tobacco hornworm is relatively easy to rear under laboratory conditions and a continuous supply was available through the entire year.

METHODS

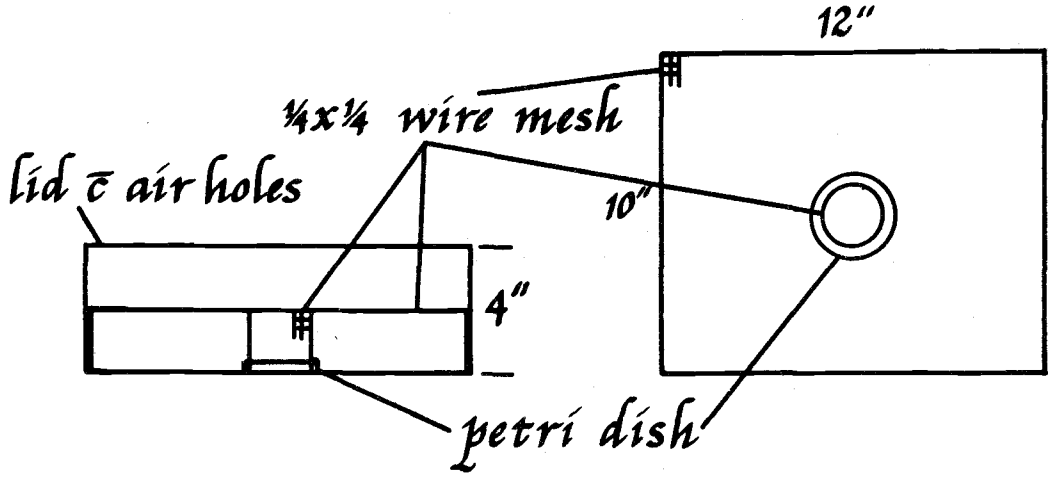
Insect Rearing

The procedure for rearing the tobacco hornworm, Manducta sexta, was essentially that of Hoffman, Lawson and Yamamoto (1966) and Yamamoto (1968).

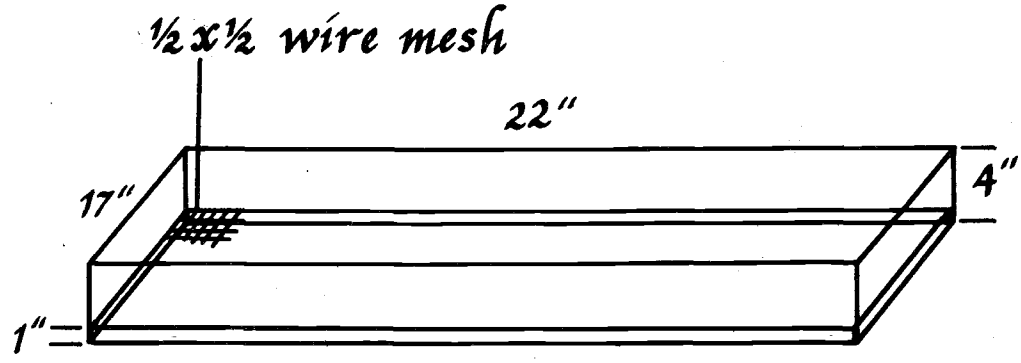
Eggs were obtained weekly from Dr. R. Yamamoto, Dept. of Entomology, North Carolina State University, Raleigh, North Carolina or from Mr. A. H. Baumhover, U.S.D.A., Oxford Research Station, Oxford, North Carolina. Each shipment contained between 200 and 600 eggs. Upon arrival the eggs were counted and placed in petri dishes lined with filter paper. The petri dishes were then placed in establishment boxes containing the artificial diet (Figure 1).

The composition of this diet is as follows:

DRY PREMIX	
Wheat germ	100.0 gm
Casein, purified	45.0 gm
Sucrose	40.0 gm
Torula yeast	30.0 gm
Wesson salt mixture	15.0 gm
Ascorbic acid	4.0 gm
Sorbic acid	1.5 gm



Establishment Box



Growth Box

Figure 1. Specifications for the establishment boxes and growth boxes used to grow the tobacco hornworm larve.

DRY PREMIX

Methyl-p-hydroxy benzoate	1.0 gm
Cholesterol	1.0 gm
Vitamin mix*	32.0 mg

Vitamin Mix*	Ratio of Ingredients
Nicotinic acid	50 mg
Calcium pantothenate	50 mg
Riboflavin	25 mg
Thiamine hydrochloride	11.5 mg
Pyridoxine hydrochloride	11.5 mg
Folic Acid	11.5 mg
Biotin	1.0 mg

The dry mix was prepared in large batches and mixed in a Waring blendor to a fine powder. This was then mixed further by hand until homogeneous and stored in a cold room until needed. The vitamin mix was made in large batches, ground in a morter and pestle to a fine powder and also stored in a cold room.

Diet Preparation

To 600 ml of water was added: 237.5 gm dry mix, 18 ml of a 10% formaldehyde solution, and 32 mg vitamin mix. This material

was then stirred until a smooth suspension was obtained. Agar, 16.7 gm, was dissolved in 400 mls of water. The two solutions were then combined and stirred until uniform. This was poured into rectangular cake pans, cooled, and stored in a cold room. Strips of solid medium were then placed in the establishment boxes and in the growth boxes. (Fig. 1.) Food was changed every other day or when consumed.

The larvae were kept in the establishment boxes for about 10 days prior to transfer to the larger growth boxes. At the end of the 5th instar (18 days) the larvae began to crawl actively and show a prominent heartbeat, at which time they were placed in modified Hoffman pupation boxes. The boxes were then placed in a humidity chamber. The pre-pupae remained in the pupation box for about 5 days. Once the pupae were fully formed they were transferred to large burlap covered boxes (2'x2'x2') and allowed to emerge. Emergence occurs 15 days after removal from the pupation box.

The adult moths were collected and either saved at 4° C for about 3 days in order to obtain larger amounts of insects[1] or were hand fed 25% sucrose with an eye dropper.[2]

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[1]Adult moths stored for 3 days at 4° C show no visible signs of deterioration. Upon warming to room temperature, they seem to be as active as non-stored 1 day old moths. Stored adults were never used for developmental or enzymatic studies; however, they were collected to provide larger amounts of tissue for DNA extractions.

[2]Without the large flight rooms the moths will not feed themselves. They will, however, begin taking the sucrose solutions placed on the coils of their sucking mouth parts one day after emergence, at the rate of 1.0 ml/day.

The developmental times of the tobacco hornworm in this laboratory were as follows: Egg--4 days, Larva--18 days, pre-pupa --5 days, pupa --15 days.

During development the larva is maintained at 28° C and a relative humidity of 72% with a 16 hour photoperiod. The pre-pupae were maintained at 28° C, at 95% relative humidity and in total darkness. The pupae and adults were maintained at 28° C with no control of the humidity and in total darkness.

Preparation of the Insects

Pharate adults were dissected from the pupal case, and the dorsal longitudinal flight muscle removed with scissors. The adults were chilled and scrubbed with a brush under cold water, and the dorsal longitudinal flight muscle was dissected, or the whole thorax was used, depending upon the type of experiment.

Protein Determination

The protein content of thoracic homogenates of mitochondria was determined by the Biuret method of Cleland and Slater (1953). The protein content of mitochondrial preparations used for the DNA isolations was determined by the Biuret method without precipitation with 10% TCA and were not washed.

Plate 1. Tobacco hornworm Manducta sexta. Larva near the end of the 5th instar. Larva on left is entering the prepupal stage, as indicated by the darkened exoskeleten. Scale is in centimeters and about 3/4 real size.

Plate 2. The two insects on the left have just entered the pupal stage. Insect on right is in the prepupal stage. Scale is in centimeters and about 3/4 real size.

Plate 3. The adult moth of the tobacco hornworm one day after emergence. Shown about 1/2 real size.

Extraction of DNA and RNA from Thoracic Tissue

The nucleic acids were extracted by the method of Schneider (1945). The tissue was homogenized in a glass Potter-Elvehjem type tissue grinder in 0.25M sucrose. The ratio of tissue to sucrose was 500 mg/10 ml. Aliquots were removed for the protein and nucleic acid assays. The nucleic acids were precipitated with an equal volume of cold 1N $HClO_4$. They were then centrifuged at 3,000 x g for 10 minutes and the supernatant discarded. The precipitate was washed with cold 0.5 N $HClO_4$ and centrifuged as before. This precipitate was washed in cold 95% ETOH and re-centrifuged. The resulting pellet was resuspended in 3.0 mls of 0.5N $HClO_4$ and heated in a water bath for 15 minutes at 95° C. The solution was chilled in an ice bath and centrifuged at 3,000 x g for 15 minutes. The supernatant was used for the determination of RNA and DNA.

DNA Determination (Developmental Study)

The diphenylamine reaction of Dische (1955) was used with some modification for the quantitative determination of DNA from the insect thoracic muscle. Calf thymus DNA was used as a standard. The diphylamine reagent contained: 0.3 gm diphenylamine, 20.0 ml glacical acetic acid, 0.3 ml conc. H_2SO_4, 0.1 ml diluted acetaldehyde (1:20). Since this reagent is light sensitive it was prepared

immediately prior to its use. The assay tubes contained 0.5 ml of the sample (0-250 mg of DNA), 0.5 ml of 0.5 N $HClO_4$ and 2.0 ml of the diphenylamine reagent. The tubes were mixed thoroughly and incubated for 18 hours at 30° C at the end of which the optical density was determined at 600 nm.

Quantitative RNA Determination

A modified Bial's orcinol (Dische, 1955) reaction was used for the total RNA determination of the insect thoracic muscle. The assay tubes contained: one ml of RNA sample in 0.5 N $HClO_4$, 1 ml of $FeCl_3 \cdot 6H_2O$ (100 mg/200 ml of conc. HCl) and 1 ml of orcinal reagent (1.42 g orcinal in 100 ml ethan 1). The above mixture was mixed well and placed in a boiling water bath for 30 minutes and chilled. The optical density as read at 660 nm. D-Ribose was used as a standard.

Preparation of Tissue for Enzyme Assays

The dorsal longitudinal flight muscle was removed from the thorax and homogenized in 0.25 M sucrose at 4° C for 20 seconds with a Potter-Elvehjem homogenizer, fitted with a loose fitting teflon plunger (0.01 inch clearance as described by Lennie and Birt (1967). This homogenate was centrifuged at 120 x g for 5 minutes to remove cell debris. The supernatant was then centrifuged at

12,000 x g for 10 minutes to pellet the "mitochondria." The specific activities of various enzymes in the supernatant, pellet and the debris were then determined as a function of age.

Cytochrome C Oxidase Assay

The development of the flight muscle was studied in part by measuring the amount of the mitochondrial enzyme, cytochrome C oxidase. This enzyme is known to be associated with the inner membrane or the matrix of the mitochondria (Schnaitman and Greenawalt, 1968). The polarographic procedure of Wharton and Griffiths (1962) was used. The assays were performed in a GME Oxygraph, which had been fitted with a Clark electrode. Assay volume was 2.2 ml and the reaction was initiated by the addition of ascorbic acid after temperature equilibration at 25°C. A mitochondria suspension was used in the assay at a final concentration of 50-150 μg/ml, so there was no need for the addition of a phospholipid solution. Rates of O_2 uptake for different cytochrome C concentrations were measured for each sample. Double reciprocal plots were made and the V_{max} was used for the determination of the specific activity of the enzyme sample as suggested by Slater (1949).

α-3-Glycerophosphate Dehydrogenase Assay

Another enzyme used for the developmental study of the mitochondrial is α-3 glycerophosphate dihydrogenase, which is associated in the inner membrane.

The assay procedure for glycerophosphate dehydrogenase is that of Dawson and Thorne (1969. It is a coupled assay that measures the reduction of DCIP (2, 6-dichlorophenolindophenol) at 600 nm. The reaction mixture contains 50 mM KH_2PO_4-NaOH buffer, pH 7.6, 25 mM α-3 glycerophosphate as the D, L mixture, and 47.5 μM of DCIP in a final volume of 0.5 ml. The reaction was started by the addition of 0.1 ml mitochondrial suspension (0.5 mg). The assay was carried out in a Beckman DB spectrophotometer with a thermostated cell holder maintained at 38° C. A linear O.D. strip chart recorder was used to measure the rate of dye reduction, which was linear for about 1 minute. The specific activity was recorded as m-Moles DCIP reduced/min./mg protein. ε_{600} was taken to be 2.1×10^4 $cm^{-1} M^{-1}$.

Isolation of Nuclear DNA

Manducta nuclear DNA was prepared by a modified method of Laird and McCarthy (1968). To 100 gm wet weight of washed and scrubbed insect thorax was added 100 ml of a mixture of 0.05 M

Tris buffer, 0.025 M KCl, 0.005 M Mg acetate, 0.35 M sucrose, pH 7.6. This was homogenized by hand in a pre-cooled mortar and pestle, and filtered through 2 layers of cheese cloth. The debris in the cheese cloth was removed and re-homogenized with 100 mls of the same buffer and again filtered and squeezed. The debris was saved and used to prepare "debris DNA." The resultant filtrate was centrifuged in the cold using a Sorval centrifuge with a swinging bucket head at 1000 x g for 15 minutes. The resulting pellet was resuspended in 50 ml of 0.015 M NaCl, 0.1 M EDTA, 2% SDS (sodium dodecyl sulfate), pH 8, to lyse the cells and nuclei. Upon lysis, as shown by a marked increase in viscosity, an equal volume of water saturated neutralized phenol was added. This mixture was placed on a shaker and allowed to shake for 20-30 minutes. The phases were separated by centrifugation in a Sorval clinical centrifuge at 500 x g for 5 minutes. Using a wide mouth pipette the aqueous phase was removed taking care not to remove the interphase. Both the aqueous and the phenol phase were re-extracted and the aqueous phases were pooled. The final aqueous solution was made to 0.1 M in acetate by the addition of 3 M Na acetate containing 0.001 M EDTA, pH 7.0. The DNA was precipitated with 0.54 volume of cold isopropanol.[3] The fibrous

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[3]The isopropanol step does not precipitate the bulk of the RNA or polysaccharides (Marmur, 1963).

DNA obtained was washed with cold 67% ETOH and dissolved in 10-15 mls of 0.1 x SSC (SSC is .15 M NaCl and .015 M Na_3 Citrate, pH 7.0). This solution was then extracted three times with an equal volume of ether. The ether was evaporated under a stream of N_2 until no smell of ether could be detected.

Any remaining traces of RNA and polysaccharide were digested with 10 μg of pancreatic RNAse[4] and 200 μg of α-amylase (Worthington) per ml of solution for 50-60 minutes at room temperature or longer in the cold. This was followed by two phenol extractions. The solution was then adjusted to 1.0 x SSC and the DNA precipitated with 2 volumes of cold ethanol. The precipitate was resuspended in 0.1 x SSC and dialysed against the appropriate buffer. The DNA concentration was estimated by its absorption at 260 nm using an extinction of 20 O.D. = 1 mg/ml. The "debris DNA" was prepared by the same method except the SDS concentration in the initial lysing solution was made about 5% by the addition of extra SDS.

Preparation of Mitochondrial DNA

The 1,000 x g supernatant from the nuclear DNA preparation was centrifuged at 10,000 x g for 10 minutes. The resulting pellet (mitochondrial) was suspended in the original homogenizing medium

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[4]Pancreatic RNAse was made 0.2% in 0.15 M NaCl containing 0.15 M trisodium citrate, pH 5.0 and heated at 80° C for 10 minutes to inactivate any contaminating DNAse.

of 0.25 M sucrose and recentrifuged. This was repeated twice and the resulting pellet used directly for preparation of mitochondrial DNA.

To approximately 50 mg of mitochondrial protein was added 1.5 mls of lysing solution. After the solution appeared clear (protein completely solubilized), an equal volume of neutralized H_2O saturated phenol was added. This solution was stirred on a magnetic stirrer for 15 minutes at room temperature. The solution was then centrifuged at 500 x g for 5 minutes and the aqueous phase removed. The phenol layer was re-extracted with 1 x SSC and centrifuged. The aqueous phases were pooled and dialyzed for 24 hours against 1 x SSC. The dialysate was then digested with pancreatic RNAse (10 μg/ml) for 20 minutes. The solution was extracted with phenol twice and dialyzed against large volumes of 1 x SSC. The resulting DNA solution yielded about 1.35 μg DNA/mg protein and gave $\frac{230}{260}$ and $\frac{280}{260}$ ratios of .442 and .437 respectively.

Preparation of E. coli DNA

E. coli strain B obtained from Miles Laboratory in paste form was prepared the same way as the N-DNA of Manducta, except that the amount of starting material was much smaller (5 gm for 50 mls of lysing solvent). The final solution was dialyzed against 1.0 x SSC for 24 hours.

CsCl Buoyant Density and GC Content

Cesium chloride gradients were used as an analytical tool to determine the purity and buoyant density of the DNA (Szybalski, 1968). Optical grade CsCl was obtained from Calbiochem, and required no further purification. CsCl runs were performed in a Spinco Model E ultracentrifuge equipped with a photoelectric scanner. An AN-D "white" rotor was used with a 12 mm carbon filled epon, 2° double sector cell. A standard quartz window was used on the bottom and a 1° negative wedge window on top. The centrifuge was run at 44,000 rpm for about 20 hours at 25° C. Each sector of the cell was filled with 0.5 mls of solution (left-hand sector, solvent and right-hand sector, DNA solution). The cells were scanned at the slowest scanner speed after 20 hours and 22 hours to insure that equilibrium had been reached. A stock solution of CsCl was made by dissolving 13 gm CsCl in 7.0 ml of 0.02 M Tris, pH 8.5. 0.84 ml of this solution was mixed with 0.18 ml H_2O, 0.01 ml of reference DNA (50 μg/ml) and 0.04 ml of the unknown DNA (50 μg/ml). A small portion of this mixture was used for the determination of refractive index on an Abbe-3L refractometer (Bausch and Lomb). Refractive index and the density of CsCl can be related by the following equation:

$$\rho^{25.0°C} = 10.8601\, \eta_D^{25.0°C} - 13.4974 \qquad (1)$$

The density was calculated to be about 1.71 g/cc. The solution was then adjusted so that the sample would band in the center of the cell by the addition of a small amount of H_2O, and the refractive index measured. A reference CsCl solution was prepared with approximately the same density for the reference sector of the cell. The density of the unknown was then calculated from its relative position to the marker DNA in the cell, as described by Mandel, Schildkrout and Marmur (1968).

For samples of DNA with a low O.D. solid CsCl was added in the proportion of .89 gm/.70 ml, and .01 ml of reference DNA added per .70 ml. The refractive index was measured and the density adjusted by addition of H_2O or solid CsCl. Buoyant densities of the reference DNA were obtained from Szybalski (1968).

Determination of DNA Base Composition by the Felsenfeld Method

Determinations of nuclear DNA base composition and concentrations by spectral analysis, were performed by the method of Felsenfeld (Hirshman and Felsenfeld, 1966). The particular method was the one derived for native spectra. The nuclear DNA purified by the phenol method as previously described was dialysed against large volumes of 0.01 M NaCl-0.001 M phosphate buffer, pH 7 in the cold for 2-3 days. The native spectra were recorded on a Cary model 15

spectrophotometer at room temperature while the temperature was measured with a thermister probe (Yellow Springs Instruments). The absorption spectra were determined between 300 nm and 220 nm. From the spectra, optical densities were measured at 5 nm intervals between 235 and 290 nm. These measurements were corrected for base line absorption and volume expansion. The corrected optical densities were then used in the formulas derived by Felsenfeld to determine the fractions of AT in the sample and its concentration in moles of nucleotide per liter. The latter can be used to calculate the molar extinction at 260 nm.

Chemical Analysis of Nuclear DNA

The chemical analysis of DNA depends upon the cleavage of the acid labile glycosidic bonds by formic acid and the separation of the purine and pyrimidine bases by paper chromatography as described by Bendich (1957). About 1.0 mg of nuclear DNA was weighed into a small pyrex test tube. To this was added 0.5 ml of 88% formic acid. The tube was sealed about 2 cm above the solution level in order to avoid charring of the sample. The sealed tubes were heated in an oven at 175° C for 30 minutes. The tubes are allowed to cool and then secured in a stand. At this point the tube is under extreme pressure due to the formation of carbon monoxide. The pressure is released by directing a pinpoint flame to the top of the tube and a

small explosion is experienced when the carbon monoxide is released from the tube. The top of the tube is then cut off and the solution is evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 25 µl of 1.0 N HCl.

The purine and pyrimidine bases in the above hydrolysate are separated on Whatman No. 1 filter paper using an HCl: isopropanol solvent system. Sheets of filter paper measuring 15 x 57 cm were divided longitudinally into four sections. A line to designate the origin was drawn 50 cm from one end and the remaining 7 cm were folded to fit into the solvent trough. In one section a mixture of standards was spotted (10 µg of each base/10 µl of 0.1N HCl). The center two sections contained 5 or 10 µg of the hydrolysate and the last section had 10 µl of 1.0 N HCl applied. After placing the spots on the paper the chromatogram was dried under a stream of filtered air until the spots were visibly dry.

The developing solvent was composed of 65 ml of peroxide-free absolute isopropanol and 16.7 ml of concentrated HCl diluted to 100 ml with water after mixing. The solvent was placed in a glass trough in a chromatography jar measuring 28 x 60 cm and a small beaker with 25 ml of solvent was placed in the bottom of the jar in order to saturate the atmosphere inside the closed jar. After 2 hours equilibration the chromatogram was placed in the trough and secured with a glass rod. When the solvent front neared the end of

the paper (approximately 20 hours at room temperature), the chromatogram was removed and the solvent front was marked. The chromatogram was hung upside down in a hood and allowed to air dry.

The dried paper was viewed under UV light in a darkened room and the spots were circled with a pencil. The bases appear as dark spots except for guanine which appears as a light blue fluorescent spot. The center of maximum absorption (or fluorescence) was estimated for calculation of R_f values.

The spots were cut out, cut into small pieces with a scissors, placed in small test tubes and eluted with 4.0 ml of 0.1 N HCl for 2-3 hours at room temperature with occasional mixing. Similar R_f positions were cut out of the solvent section (section spotted with HCl) to be used as blanks. The solutions were filtered in vacuuo into test tubes through glass fiber pads which had been washed prior to use with 5 ml of 0.1 N HCl. The pieces of paper were removed and squeezed against the glass pad, and the test tube was rinsed with 1.0 ml of 0.1 N HCl and filtered. The total volume of filtrate was 5.0 ml.

The quantity of each base (as identified by its R_f value with respect to the knowns) was determined by the differential extinction technique. This method takes into consideration the low but variable ultraviolet absorption of 0.1 N HCl extracts of the filter paper. The concentration of the bases can be determined by the difference in

extinction at the absorption maximum for each base and some other wave length. This is related to known values for standard solutions containing 10 μg of the base in 1 ml of 0.1 N HCl (Bendich, 1957). The following Δ values have been determined:

Adenine	$E_{262.5} = 0.930$	$E_{290} = 0.030$	$\Delta E = 0.900$
Guanine	$E_{249} = 0.737$	$E_{290} = 0.262$	$\Delta E = 0.475$
Uracil	$E_{259} = 0.738$	$E_{280} = 0.148$	$\Delta E = 0.590$
Thymine	$E_{265} = 0.632$	$E_{290} = 0.083$	$\Delta E = 0.549$
Cytosine	$E_{276} = 0.910$	$E_{300} = 0.047$	$\Delta E = 0.863$
5-Methyl-cytosine	$E_{283} = 0.603$	$E_{310} = 0.036$	$\Delta E = 0.567$

From these ΔE values and the ΔE value obtained for the standard base a simple proportion can be set up to give the concentration of unknown in μg per ml;

$$\text{concentration unknown} = \frac{\Delta E_{\text{unknown}} \times \text{concentration of known}}{\Delta E_{\text{known}}}$$

Melting Temperature of Nuclear DNA

Melting temperatures were determined by the method of Marmur and Doty (1962), using a Beckman D.U. fitted with a Gilford optical density converter. The temperature was measured using a copper-constantan thermopile. Both temperature and O.D.

were recorded on a Hewlett Packard X-Y recorder. All samples were melted in 1.0 x SSC and had an initial O.D. in the range of 0.5 O.D. units. In order to prevent the formation of O_2 bubbles upon heating all samples were bubbled with He for 10 minutes at room temperature. The quartz cells were purchased from Helma and were made for high temperature work. The teflon stoppers were sealed with Dow-Corning silicon rubber to prevent evaporation. The solutions were heated at a rate of 0.6° C/minute, with a Haake circulating water bath.

In all cases the unknown samples were dialyzed against 2 liters of 1.0 x SSC. Calf thymus DNA was used as a standard and was dialyzed in the same vessel (total of 20 ml in the two dialysis bags).

The GC percent of the unknown DNA was obtained from the relationship:

$$\% GC = 2.41 \ (T_m - 69.3) \qquad (2)$$

Calf thymus DNA was used as a reference standard.

Sedimentation Coefficient Determination of Nuclear DNA

The sedimentation coefficient of nuclear DNA was determined in a Spinco Model E analytical ultracentrifuge (Eigner and Doty, 1965). The instrument was equipped with a photoelectric scanner which

automatically took an absorbance scan from the outer edge of the rotor to the center. A Spinco AN-D rotor "black" equipped with a 12 mm filled epon, 2° double sector cell fitted with quartz windows was used. A scanner counter balance with reference holes was used in the second hole in the rotor. The temperature of each run was maintained constant and the temperature for cell runs was between 18-24° C. The sedimentation coefficient was determined independently at 36 K and 48 K rpm. The cells were scanned at 265 nm using the split beam mode of the scanner, and 4-6 scans were recorded for each run. The average rpm was measured by clocking the revolution counter. Sedimentation velocity was studied using the moving boundary technique.

All DNA samples were run in 1.0 x SSC as a buffer and the reference sector of the cell was filled with 1.0 x SSC. To avoid shearing of the DNA the cells were filled with the upper window removed. The actual concentration of the DNA solution placed in the centrifuge cell was measured on a Cary 15 spectrophotometer prior to filling. In all cases the O.D. at 260 nm was used as a measure of concentrations. The sedimentation coefficients were determined as described in the Results Section.

Molecular Weights of Nuclear DNA

The molecular weight was determined from the sedimentation constant ($S_{20,w}$) extrapolated to zero concentration ($S^{o}_{20,w}$) using the following formula (Eigner and Doty, 1965):

$$S^{o}_{20,w} = 0.034\, M_{w}^{0.405} \qquad (3)$$

Renaturation Kinetics

The renaturation kinetics of nuclear DNA were studied as described by Laird and McCarthy (1969). The DNA at 4° C was sheared using a Biosonik sonicator, with a microprobe, set at 60 for 4-5 minutes under a stream of N_2. The length of fragments were measured using alkaline CsCl band sedimentation velocity as developed by Vinograd (Bruner and Vinograd, 1965). From the alkaline sedimentation coefficient the molecular length of the single stranded DNA was calculated (Wetmur and Davidson, 1968). The renaturation solvent was 1.0 x SSC and the renaturation temperature was maintained at 60° C. The DNA solution was placed in quartz-cuvettes and He bubbled through the solution for 15 minutes. The cuvettes were stoppered with a teflon stopper, sealed with silicon rubber and allowed to cure for about 30 minutes. The O.D. was read at 25° C and 60° C and then the cuvettes were placed in a sand bath at 100° C for 15-20 minutes to insure complete denaturation.

After the alloted time the cleaned cuvette was transferred to the Beckman DU with a Gilford O. D. converter and the decrease in O. D. with respect to time was continuously recorded. The cell compartment was maintained at 60° C. A sample of E. coli DNA was treated in the same way and was used as a standard.

Electron Microscope Study of Flight Muscle and Mitochondrial DNA

The dorsal longitudinal flight muscle of an adult moth was removed and fixed in 4.5% glutaraldehyde in phosphate buffer, pH 7.4, for 3 hours. It was cut then into small sections and placed in 3.0 ml of phosphate buffered osmium tetraoxide (1% OsO_4 in 0.15 M NaH_2PO_4, pH 7.2) in a stoppered vial, and allowed to stand for 2 hours. The fixed muscle was dehydrated by transferring the tissue into graded concentrations of ethanol of 50, 70, 90, 95, and 100%. The latter two steps were repeated twice. After the ethanol steps were completed, the next steps consisted of treatment in 50% propylene oxide (in absolute ethanol) and finally, 100% propylene oxide. Each step in the dehydration series was allowed to proceed for 30 minutes at room temperature.

The fixed and dehydrated tissue was then embedded in araldite. The sections were made on a Porter-Blum ultra microtome. The sections were collected on 100 mesh copper grids (Ernest Fullham, Schnechtedy, N. Y.) previously coated with a film of formvar

(polyvinyl formal), stained with a water saturated solution of uranyl acetate for 2 hours at 60° C (Brody, 1959) and with a 2% aqueous solution of lead citrate for 1-10 minutes (Venable and Coggeshall, 1965).

A Phillips 300 electron microscope was used for viewing and photographing the sections. The photomicrographs were printed on Kodak F-5 or F-6 paper.

To study the size and shape of mitochondrial DNA, Kleinschmidt's spreading technique was used (Kleinschmidt, 1968). The hypophase was glass distilled water that had been filtered through a 25 μ millipore filter. The spreading solution (hyperphase) consisted of about 2 M $NH_4CO_2CH_3$ containing 10 μg DNA/ml and 0.01% cytochrome C type IV. A small (100 x 50 mm) crystallizing dish was used instead of a Langmuir trough. The dish was filled with hypophase and a clean acid washed microscope slide was used as a ramp for the DNA solution in slide down. The small dish was placed inside a larger crystallizing dish (190 x 100 mm) on a block so the opening was about 1 cm below the edge of the larger dish. The microscope slide then rested on the rim of the large and the small dishes and was slightly inclined (See Fig. 2). The slide was wetted with water and a few drops of DNA solution were allowed to flow down the ramp. The DNA protein monolayer formed was picked up with formvar coated grids. Grids were 100 mesh coated with a 0.5% formvar solution in ethylene dichloride. The DNA protein

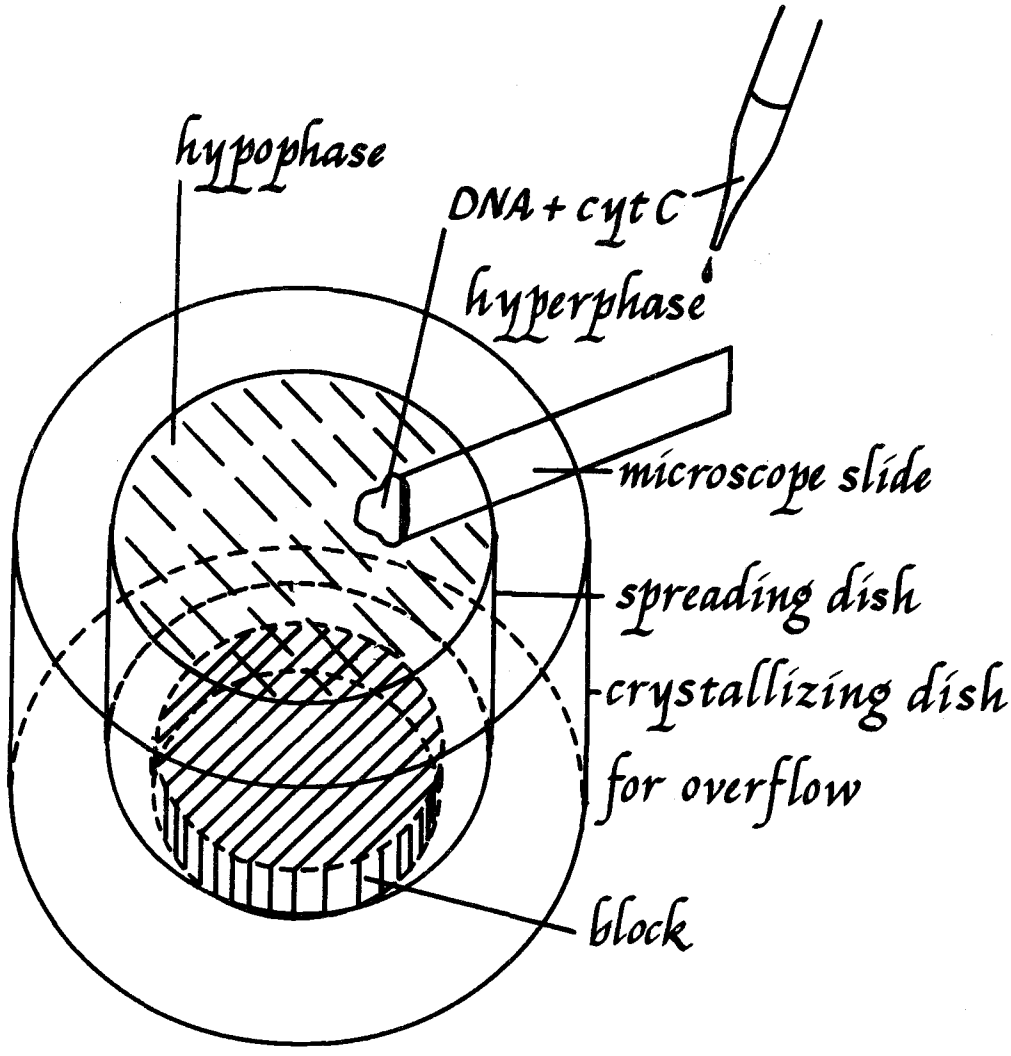

Figure 2. Apparatus used in place of a Langmuir trough for the spreading of a DNA protein film.

coated grids were stained with uranyl acetate (Wetmur, Davidson and Scaletti, 1966). The stain is a 1:100 dilution of the stock (0.005 M uranylacetate, 0.05 M HCl in 95% ETOH) in 95% ETOH. The stock solution was kept in the dark and filtered through a millipore filter (VFWP 02500 10 mμ pore size) before use. The grids were dehydrated and stained at the same time in the ethanolic uranyl acetate solvent for 30 seconds and were further dehydrated for 10 seconds in isopentane (Wetmur, Davidson and Scaletti, 1966).

The grids were scanned in a Phillips 300 electron microscope at a high voltage setting of 60 K. V. Photographs were taken at various magnification factors between 1×10^4 to 4×10^4. Negatives were printed on Agfa 4 or 5 paper (2.4 x enlargement) and contour lengths were measured with a map measuring device (K and E map measure 62030000).

Histograms were made of the contour lengths of the circular M-DNA and the same was done for T7 DNA (generous gift of Dr. M. Nesson) which was used as a standard.

EXPERIMENTAL RESULTS

Preliminary Experiments

Electron Micrographs of Dorsal Longitudinal Flight Muscle

In order to characterize the type of muscle tissue in the adult, thin sections were prepared of the dorsal longitudinal flight muscle from a two day old adult moth for electron microscopy. The muscle was considered fully developed by this time as indicated by the insect's ability to fly. After the light brown flight muscle was removed from the thorax, fixed in osmium tetroxide and dehydrated (see Methods), it was subjected to a double stain using lead citrate (Venable and Coggeshall, 1965) and uranyl acetate. This choice of stains was made on the basis that the uranyl acetate will emphasize nucleic acid rich areas and the lead citrate will emphasize lipid rich membranes. The use of both stains coupled with osmium tetraoxide provide sharp structural details.

From observations of plates 4, 5 and 6 it may be concluded that the muscle is a typically close packed tissue with fibrils about 1 μ in diameter. The mitochondria appear to be fully formed as indicated by their size and "fullness" (plate 6). In this oblique section the cristae are abundant and completely fill the internal hollow of the

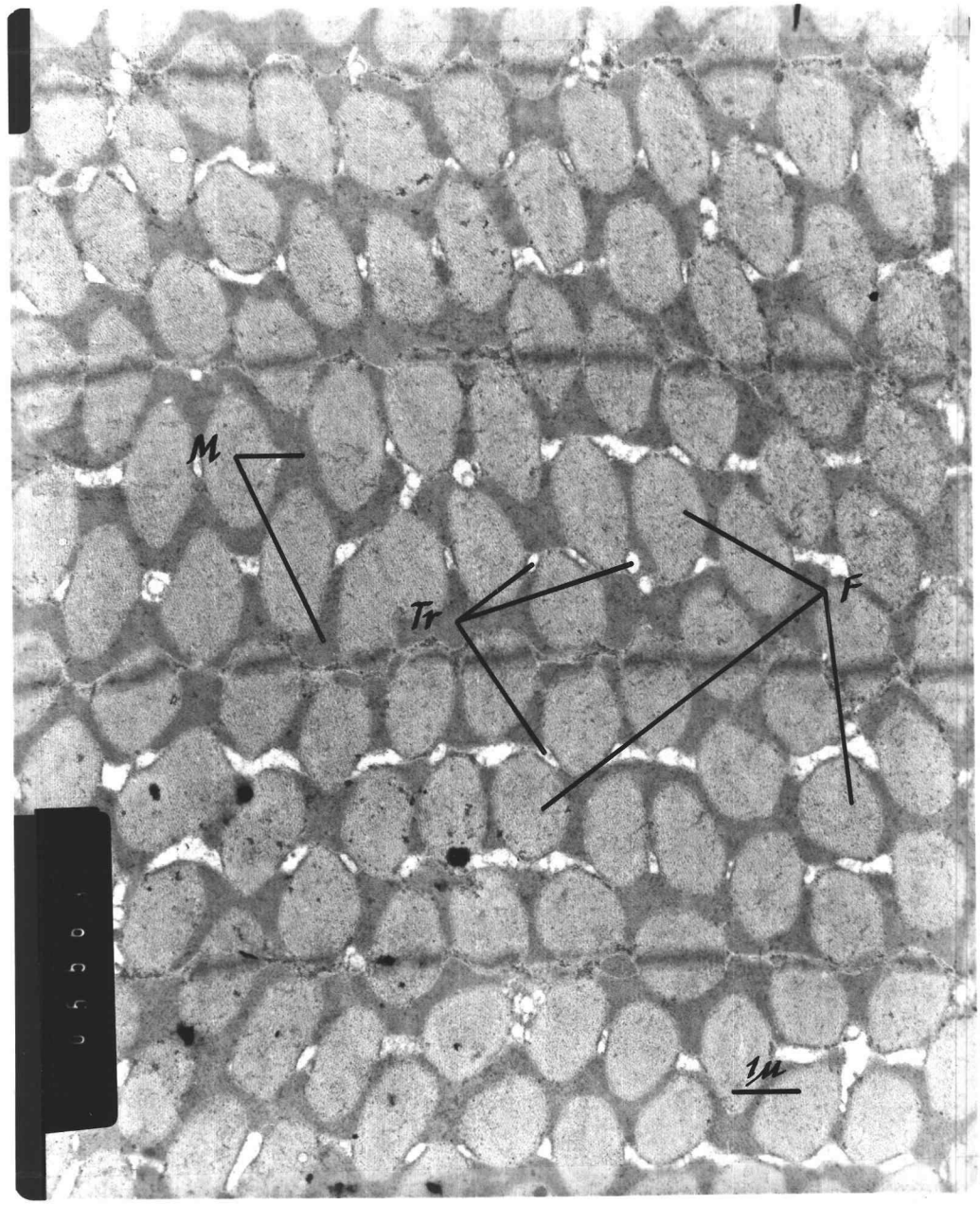

Plate 4. Cross section of the dorsolongitudinal flight muscle of a 2 day old adult showing mitochondria (M), fibrils (F) and trachea (Tr). Magnification 9.4×10^3, bar represents 1 μ.

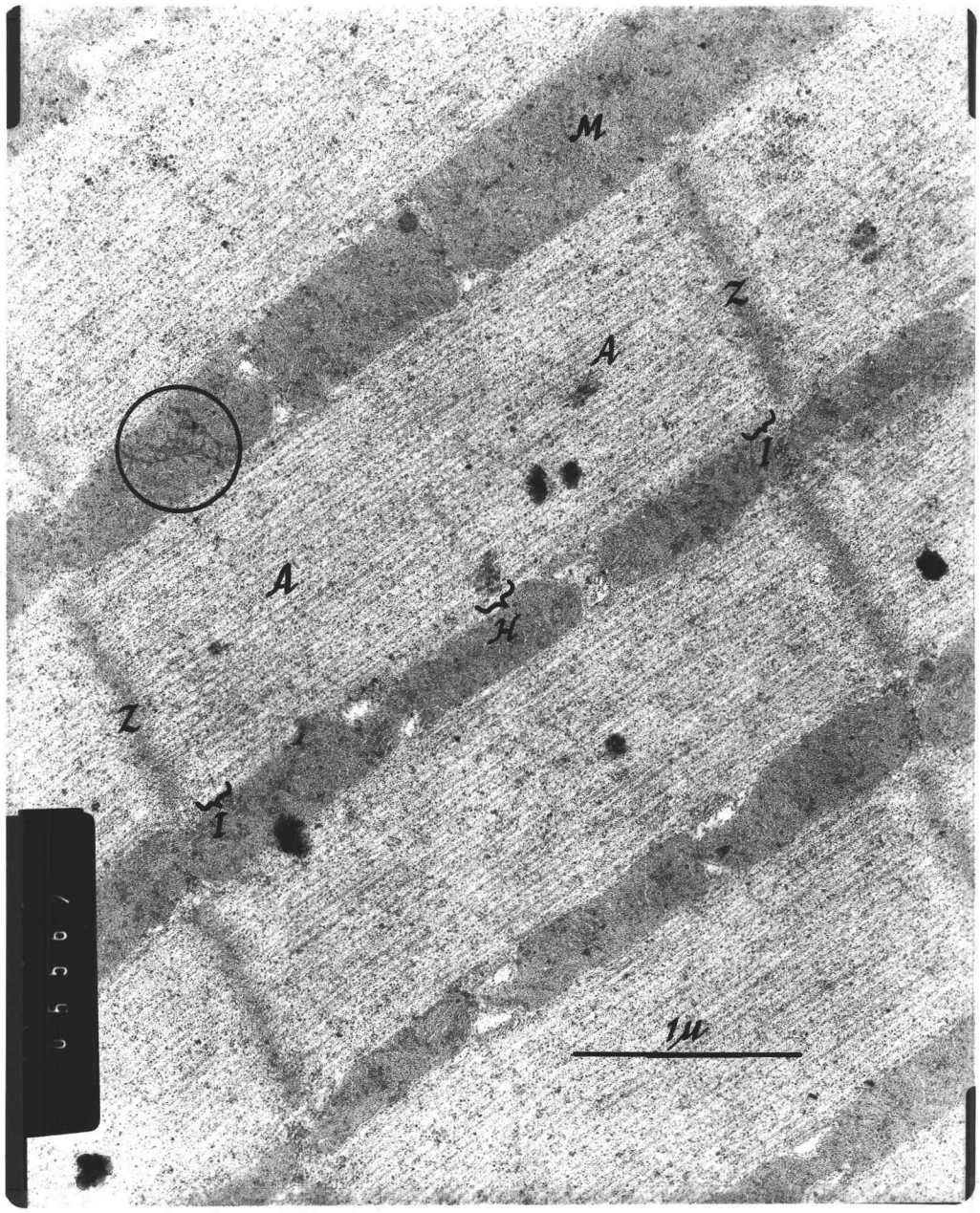

Plate 5. Longitudinal section of the dorsal longitudinal flight muscle for 2 day old adult. The areas for the bands of the sarcomers (Z, A, I and H) are indicated. The circle is around a fibre found within one of the mitchondria (M). Magnification 1.9 x 10^4.

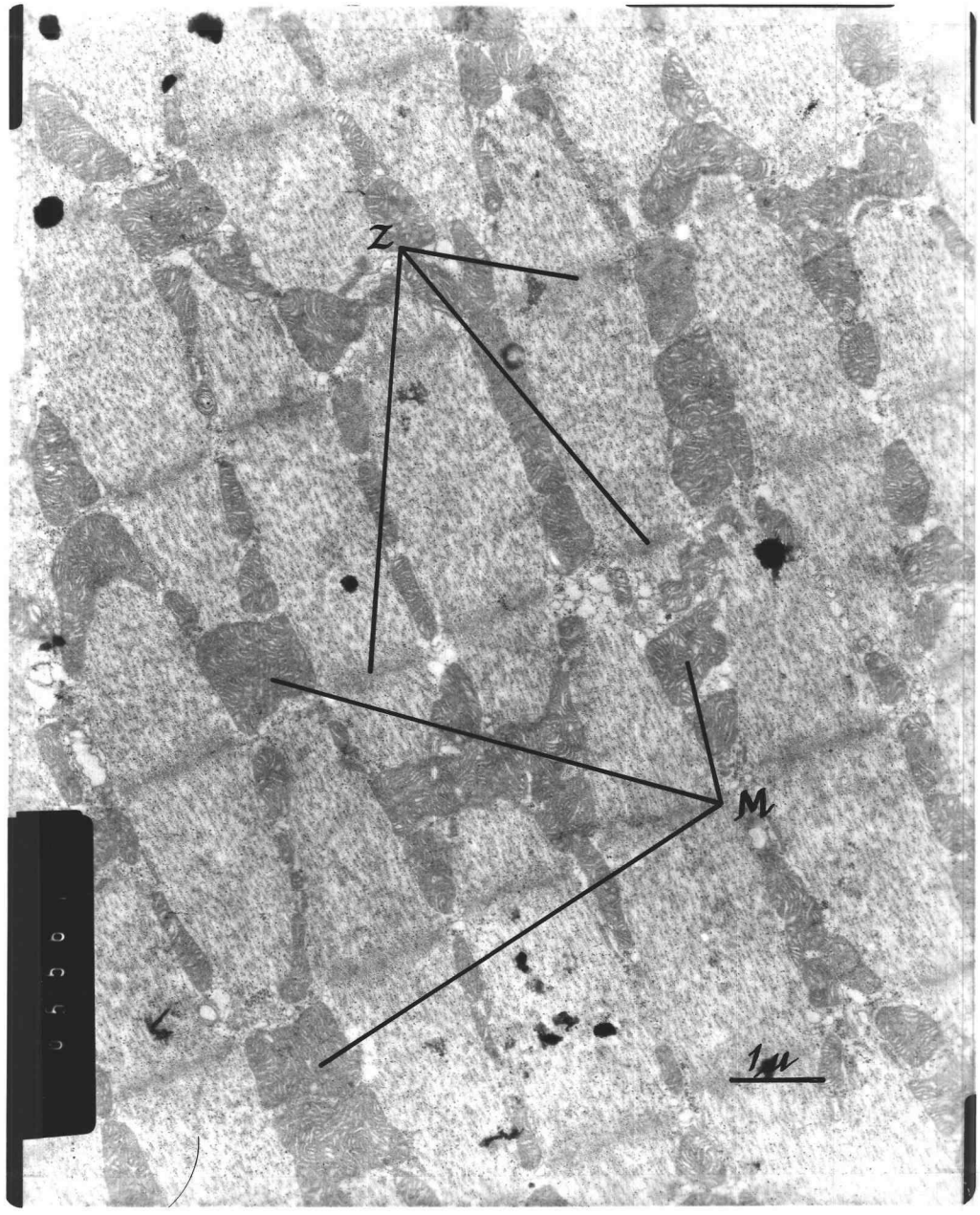

Plate 6. Oblique section of the 2 day old adult dorsal longitudinal flight muscle. Magnification 7.8×10^3. Fully formed mitochondria (M) and Z bands are clearly visible.

mitochondria. The size of this organelle appears to be variable. This result is consistent with the results from electron micrographs of locust flight muscle isolated 8 days after imaginal ecdysis (Bücher, 1965) but not of the micrographs of Smith (1968) showing the flight muscle of the wasp, Polistes sp. and the dragon fly, Sympetrum sp. It appears that in Smith's preparation the muscle may have been stretched during the fixation and dehydration steps, as evidenced from the clarity of the striations of the myofibrils. In my preparations I did not prevent the muscle from shrinking. This would have a tendency to distort the mitochondria, giving them irregular shapes and sizes.

In the cross sectional view shown in plate 4 the round fibrils are surrounded and, for the most part, in very close contact with the energy supplying mitochondria. There are large air spaces (trachea) which supply the mitochondria of this highly active tissue with oxygen. On closer observation of the fibrils in plate 4 there appears to be a lattice structure, or array of dark spots which is typical of this type of tissue (Bücher, 1965; Smith, 1968; Philpott, Szent-Györgyi, 1955; Wigglesworth, 1965). It is due to the arrangement of the actin and darker staining myosin fibers. This occurs also in vertebrate muscle except that the lattice is a little different. The micrograph (Plate 4) is not of high enough quality to determine the kind of array.

In Plates 5 and 6 we can see the regularity and the lining up of the Z band which is normal for close packed muscle. The Z band is very apparent, but the H and I bands are less obvious, if present at all. It does appear that the entire sarcomere consists of a single A band which could be due to shrinkage of the muscle or experimental artifacts. The flight muscles of the Lepidoptera are supposed to be syncronious muscle in which the I band is about 20-30% of the length of the sarcomere (Pringle, 1965).

Since the uranyl acetate should stain nucleic acid rich bodies it was felt that mitochondrial ribosomes, DNA or cytoplasmic ribosomes should be visible in one of the three plates. No definite conclusion can be reached because the micrographs appear grainy, with little black spots which make interpretation impossible. In Plate 5 the sarcosome indicated "appears" to have a dark fiber 100 Å in width which does correspond to the approximate width of mitochondrial DNA fibers as found by Nass and Nass (1963a).

Development of the Dorsal Longitudinal Flight Muscle

An idea of the growth patterns was mandatory for an understanding of the development of the muscle and the mitochondria. Fig. 3 shows the quantitative assays at different ages for the total nucleic acid content and protein content, based on a wet weight basis, for the dorsal longitudinal flight muscle.

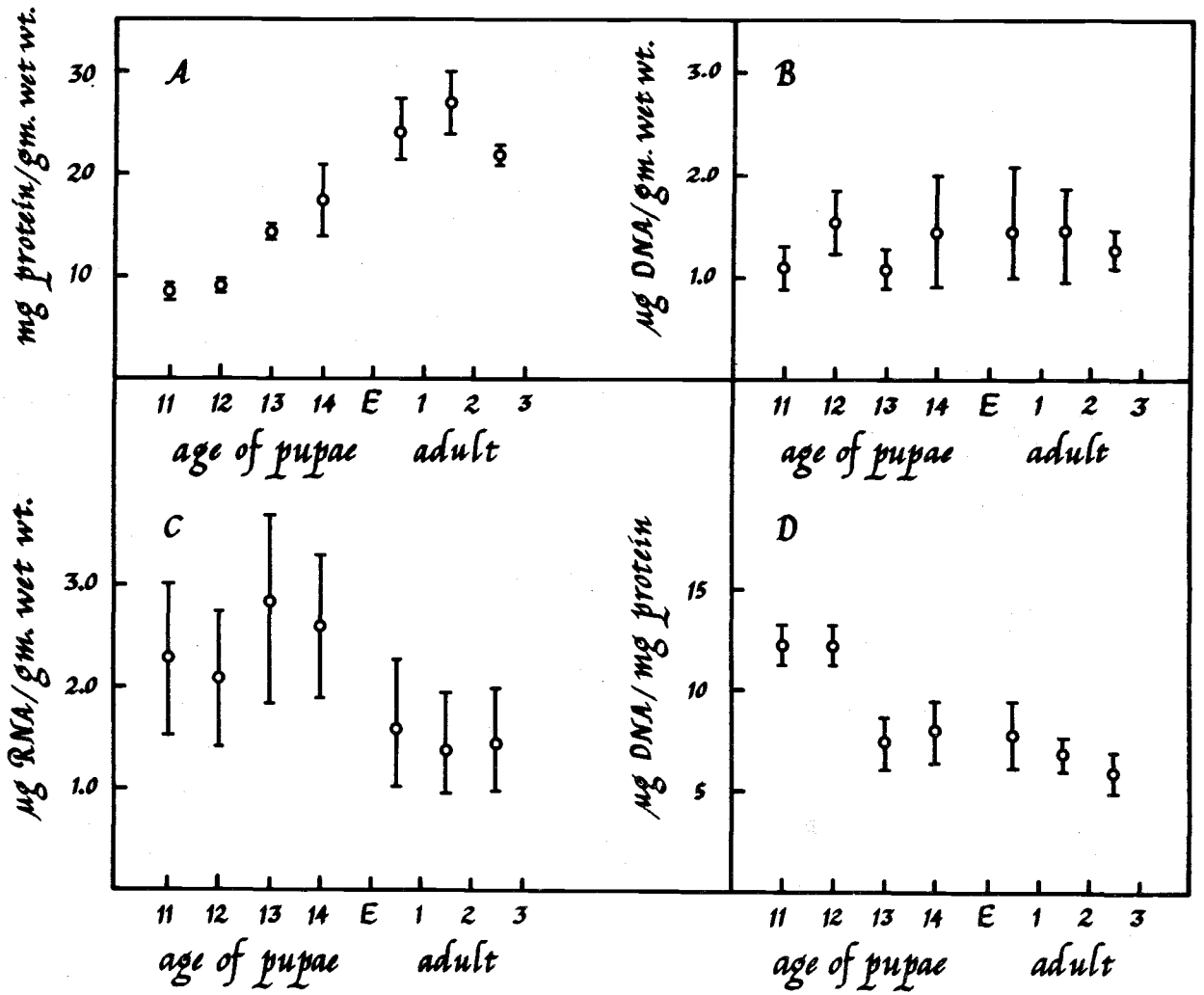

Figure 3. Developmental patterns of the dorsal longitudinal flight muscle. Age of pupa is the number of days from the formation of the pupal case, E is emergence and age of adult is given in days. The open circles (o) represent the average value and the bars represent the maximum deviation from the average.

Eleven days after the formation of the pupal case the integrity of the muscle of the developing adult was beginning to be apparent. A striated pattern emerged but the tissue was not well formed. This also was the earliest day that the muscle could be easily removed. In each experiment three healthy pupae (or adults) were used. The adults emerged in the early morning and were considered to be one-half day old by the time they were collected. The one and a half and two and a half day old adults were hand fed 25% sucrose in the mornings. In Fig. 3 the circles represent the average of the experimental results and the bar represents the spread of values. In Fig. 3a the amount of protein, measured as soluble protein per gram wet weight of muscle tissue shows an increase starting at about 12 days and increasing to approximately 3 times this starting value at maturity. The value for the 2 1/2 day adult, although lower than the 1/2 day old adult is probably within a plateau region of growth and the amount of protein remains constant with increasing age. The values are based on a wet weight basis because the amount of muscle dissected out of the thorax from individual insects was not reproducible. From Fig. 3b it can be seen that the amount of DNA per gram wet weight was essentially constant over the period examined.

The wet weight of the muscle varied due to the completeness of dissection. Therefore wet weight per insect was not a good index of development. At the early days of development the muscle was

nearly all liquid and presumably contained hemolymph. This probably caused a discrepancy between these results and those of Lennie and Birt (1967). An error in the wet weight would result in changes of the curves in Fig. 3a, 3b, and 3c but not of that in Fig. 3d.

The Enzymatic Development of the Muscle

From the preceding it is apparent that the muscle is in a stage of rapid development and it appears that the 11 day old pupa is close to the time of onset of the development of the flight muscles. The initial proliferation of myoblasts probably begins a few days earlier.

The enzymatic development of the muscle was followed by assaying for α-glycerophosphate dehydrogenase and cytochrome C oxidase (Fig. 4). The former is very active in aerobic muscle tissue of insects and is found in the inner membranes of the mitochondria (Ernster and Kuylenstierna, 1969), the latter is an inner membrane or matrix enzyme of the mitochondria and is relatively active (Dawson and Thorn, 1969). The homogenized muscle was differentially centrifuged as described in the methods. The debris fraction, F-1, is the pellet from the 1,000 rpm run (120 x g), F-2 is the supernatant of the 10,000 rpm run (12,000 x g) and F-3 is the pellet. The results were clouded by the rapid amount of tanning that took place so that some samples had to be omitted in the cytochrome C oxidase assay. Fraction F-1 still contains a few mitochondria giving a low, but

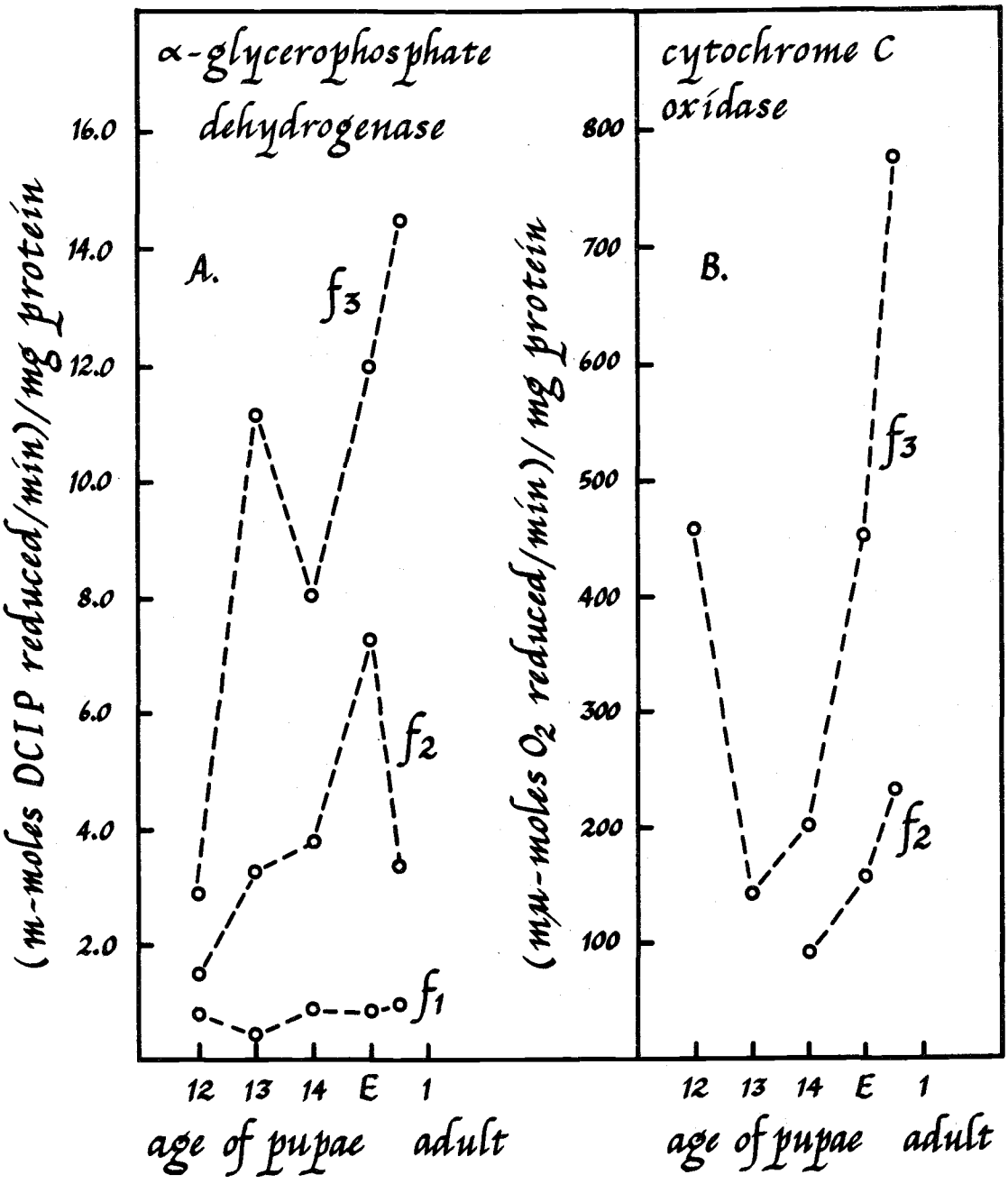

Figure 4. Enzymatic analysis of the fractionated dorsal longitudinal flight muscle homogenate. F_1 is the pellet from the differential centrifugation of the homogenate at 120xg for 5 min., F_2 is the supernatant from the centrifugation at 12, 00xg for 10 min. and F_3 is the resulting pellet.

measurable activity of α-glycerophosphate dehydrogenase (Fig. 4a). This was probably due to the mild homogenizing procedure used (Lennie and Birt, 1967). Fraction F-3 contained the most activity in both enzyme assays with a variable but lower activity in the supernatant fraction. The latter results could be due to small mitochondria which do not sediment under the conditions employed. If the two enzymes are compared it is obvious that their patterns during development are different. α-glycerophosphate dehydrogenase increases prior to cytochrome C oxidase. This could be due to the synthesis of the inner membrane prior to the synthesis of the inner membrane matrix enzymes. The F-2 fraction is indicative either of broken mitochondria or a synthesis of very small mitochondria during muscle development. The drop in the α-glycerophosphate dehydrogenase activity in the F-2 fraction after emergence could be due to the relatively small number of small mitochondria found in the adult tissue. A ready explanation is not available for the dip at 14 days in the α-glycerophosphate dehydrogenase activity in the F-3 fractions and the initially high activity of the cytochrome C oxidase in this same fraction.

We see by comparing Fig. 3 with Fig. 4, that the enzyme activity parallels the synthesis of protein, and appears to lag behind the synthesis of RNA, as would be expected.

Studies on Nuclear DNA

Tissue used in these experiments consisted of the muscle tissue of the thorax which is mainly composed of indirect flight muscles.

Isolation of Nuclear DNA

The procedure of Laird and McCarthy (1968) was readily adaptable to the muscle tissue of the tobacco hornworm. Since these preparations were also used as the source of mitochondria the tissue was not frozen prior to or during the preparation of the tissue homogenates. The nuclear fraction was considered to be the pellet remaining after low speed centrifugation (600 x g for 15 min.) of the homogenate. The fraction labeled "debris" is defined as the residue left in the cheese cloth through which the homogenate was filtered prior to the low speed centrifugation. The supernatant from this slow speed centrifugation contained the mitochondrial fraction and was used for the isolation of mitochondria as described in the methods.

The first attempts to isolate nuclear DNA gave very low yields. This was found to be due to the use of too many layers of cheese cloth to strain the homogenate. When the number of layers of cheese cloth were reduced to two the final yield increased to about 0.2 mg of DNA per 100 gm of tissue (dissected thorax). As

indicated by the diphenylamine assays of the flight muscle homogenate there should be about 50 mg of DNA per 100 gm of wet weight of dissected thoraxes, assuming that 1/3 of the wet weight of the thorax is due to the muscle.

The next procedure used to try to increase the DNA yield was to perform all centrifugations (during the phenol extractions) at room temperature. There is evidence that at colder temperatures there is a co-precipitation of SDS and DNA at the interface between the aqueous and phenol phases. This method increased the yield to 2.0 mg of DNA per 100 gm of tissue. This value was about one fifth of that obtained by McCarthy and Laird (1968) using whole bodies of Drosophila. Since it is possible that the remainder of the DNA was in the "debris fraction," the latter was treated in the same way as the nuclear fraction except that the SDS concentration was increased from 2% to 5%. The additional SDS was needed to release the DNA because of large amount of lipid and protein found in the debris. The yield from this fraction was 5.7 mg of DNA per 100 gm of tissue. Therefore the total yield from the muscle was 7.7 mg of DNA per 100 gm of tissue. It is possible that a series of "freeze-thaw" procedures would help break the sarcolemma, which is a tough membrane (Wigglesworth, 1965), and release more DNA. This method would also rupture the mitochondria which was not desirable. Using washed sea sand in the homogenizing medium did not increase the

efficiency of the release of nuclei from the muscle tissue. Since a quantitative release of DNA was not necessary for these experiments, attempts to increase the yield were not pursued further.

After the aqueous phases were pooled the first DNA precipitation was accomplished by the addition of 0.1 volume of a solution of 3.0 M sodium acetate plus 0.001 M EDTA, pH 7.0, and 0.54 volume of isopropanol at 4° C (Marmur). This method prevented the occurrence in the later procedure, of an emulsion which occurs if the DNA is precipitated with ethanol. This step did not precipitate RNA or polysaccharides. The spooled DNA was washed with cold 67% ethanol and dissolved in 0.1 x SSC. The ethanol wash causes the DNA to swell and be more easily solubilized. This DNA solution was then washed twice with equal volumes of ether to remove traces of phenol.

After the ether extraction the DNA solution was incubated with α-amylase and RNAse as described in the methods. It was found by analysis of the purity in terms of DNA, that a 1 hour digestion at room temperature or 10 hours at 4° C was sufficient to remove any contaminating material. Two phenol extractions were again performed and the pooled aqueous phases were adjusted to 1.0 x SSC before precipitation with ethanol at 4° C. Reduced yields were obtained if the ionic strength was not increased. The DNA was washed with cold 67% ethanol and dried for chemical analysis or dissolved in 0.1 x SSC and dialyzed against the appropriate buffer

as indicated elsewhere. In SSC this material gave a typical 280/260 ratio of .523, and a 230/260 ratio of .422 which are those expected from DNA.

Cesium Chloride Analysis of the Nuclear and Debris Fractions

The material isolated by the above procedure was examined to determine its purity and compostion. In addition it was of interest to determine if the DNA isolated from the debris fraction was of the same composition and purity as the DNA from the nuclear fraction. Fig. 5a shows the nuclear DNA banded isopycnically with Clostridium perfringens DNA as a marker in the Model E analytical ultracentrifuge. The fact that the isolated material bands in the 1.65-1.75 range is good evidence that both fractions were DNA. The exact buoyant density of the nuclear DNA in CsCl is 1.688 g./cc. with the marker DNA banding at 1.685 g/cc. The particular marker was chosen because it bands close to the suspected density of the unknown and this minimizes pressure and non-linearity effects in the gradient. It was identified by its position relative to the hinge point in the cell, using the initial density of the solution as the density at the hinge point. In Fig. 5b it can be seen that the material in the debris fraction has a density in CsCl of 1.689 g/cc. This is considered as not being significantly different from the density of the nuclear DNA.

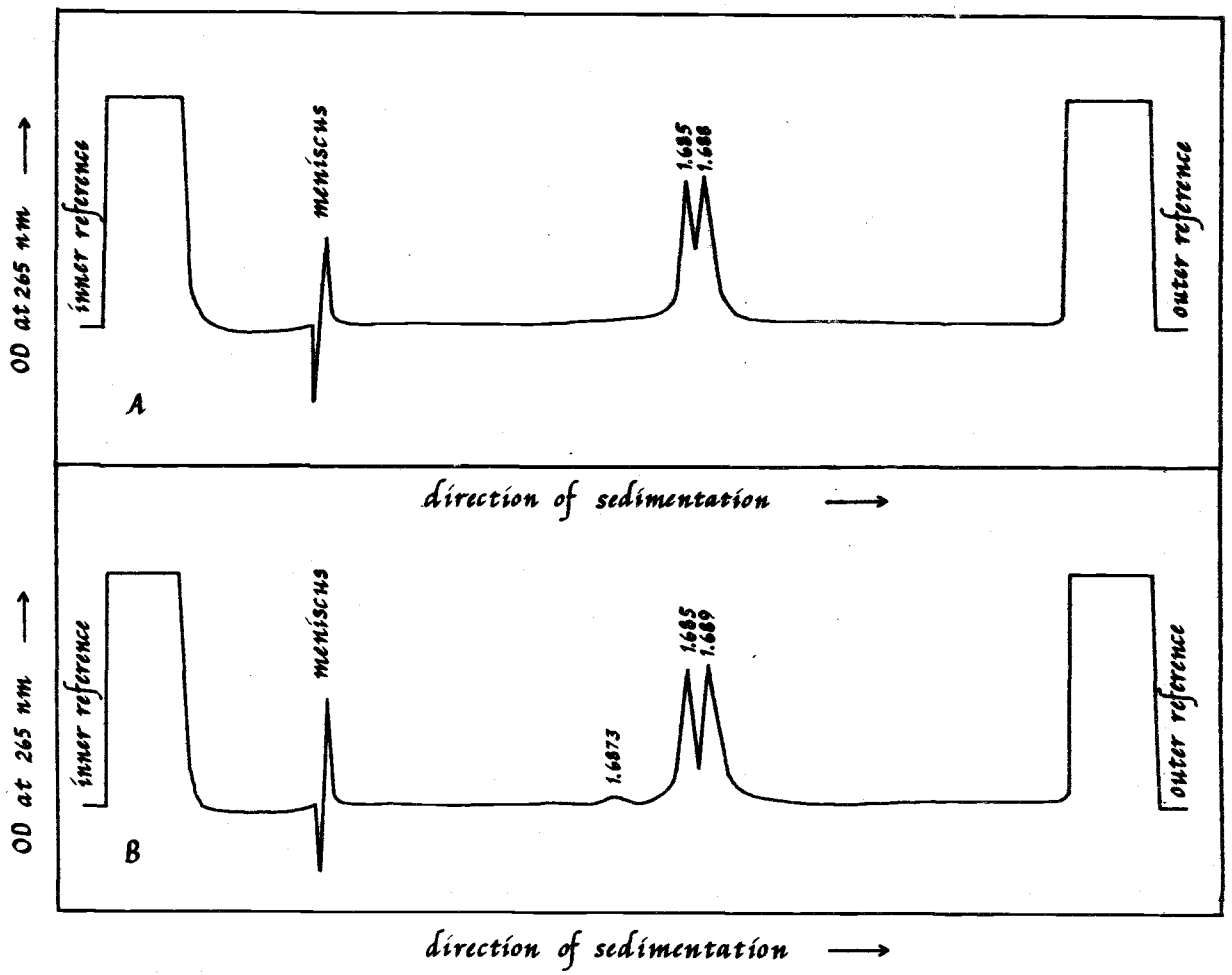

Figure 5. CsCl analytical gradients of nuclear and "debris" DNA.
A) Nuclear DNA mixed with C. perfringens DNA as a density marker of 1.685 g/cc.
B) "Debris" DNA mixed with C. perfringens DNA as a density marker. The gradients were run in the Spinco Model E at 44, 000 rpm for 20 hrs.

The purity of the nuclear fraction was considered good as shown by the lack of secondary peaks. This is not the case with the debris DNA since a very small peak was found in the very low density region. This UV absorbing material has a density of 1.673 g/cc. This material comprises about 5% of the total UV absorbing material in the sample. This peak is considered to be mitochondrial DNA as is shown later. The samples appear to be free of RNA as indicated by the lack of UV absorbing material at the bottom of the cell. They appear to be free of protein as indicated by the lack of UV absorbing material at the air-solution miniscus. Of course this is only a qualitative indication of purity and is not a conclusive test for non-DNA contamination.

Using the data compiled by Szybalski (1968) a least squares fit was made to relate the mole percent GC and the CsCl buoyant density using the value of 1.035 g/cc for E. coli DNA. The equation obtained is:

$$\text{mole \% GC} = \frac{\rho - 1.6535}{.0997} \times 100\% \qquad (4)$$

ρ is equal to the buoyant density of DNA in CsCl. From this equation the mole % GC of the nuclear and debris DNA was determined to be 34.6 and 35.6, respectively, and, the mole % GC of the minor peak was 19.6.

Absorption Spectra and Felsenfeld Analysis

The native spectra analysis of DNA requires DNA of high purity that has been extracted with phenol. The method will give erroneous values for the AT mole fraction if RNA or protein is present. Fig. 6 is a typical absorption spectrum for nuclear DNA obtained using a Cary 15 spectrophotometer. The buffer used was 0.01 M NaCl-0.01 M phosphate, pH 7.0, since the citrate in the SSC buffer absorbs in the ultraviolet range. The absorbance is corrected for the base line drift and thermal expansion from 4° C and then the data treated using the following three linear equations:

$$\mu_1 = \Sigma A_i \alpha_i = A_{235}\alpha_{235} + A_{240}\alpha_{240} + \cdots A_{290}\alpha_{290}$$

$$\mu_2 = \Sigma A_i \beta_i = A_{235}\beta_{235} + A_{240}\beta_{240} + \cdots A_{290}\beta_{290} \qquad (5)$$

$$\mu_3 = 2 A_i \gamma_i = A_{235}\gamma_{235} + A_{240}\gamma_{240} + \cdots A_{290}\gamma_{290}$$

Where A_i is the corrected absorption at wave length i; α_i, β_i and γ_i are values found empirically by Felsenfeld (1968) and are given in Table 1a. Each sum contains 12 terms. Table 2 gives the computed value of μ_1, μ_2, μ_3, where they are coefficients in a quadratic expression and are functions of wave length and concentration. Table 1b contains the S_i values used in the native DNA analyses and are the solutions to 12 linear equations, one for each wave length, for DNA's of known base compositions.

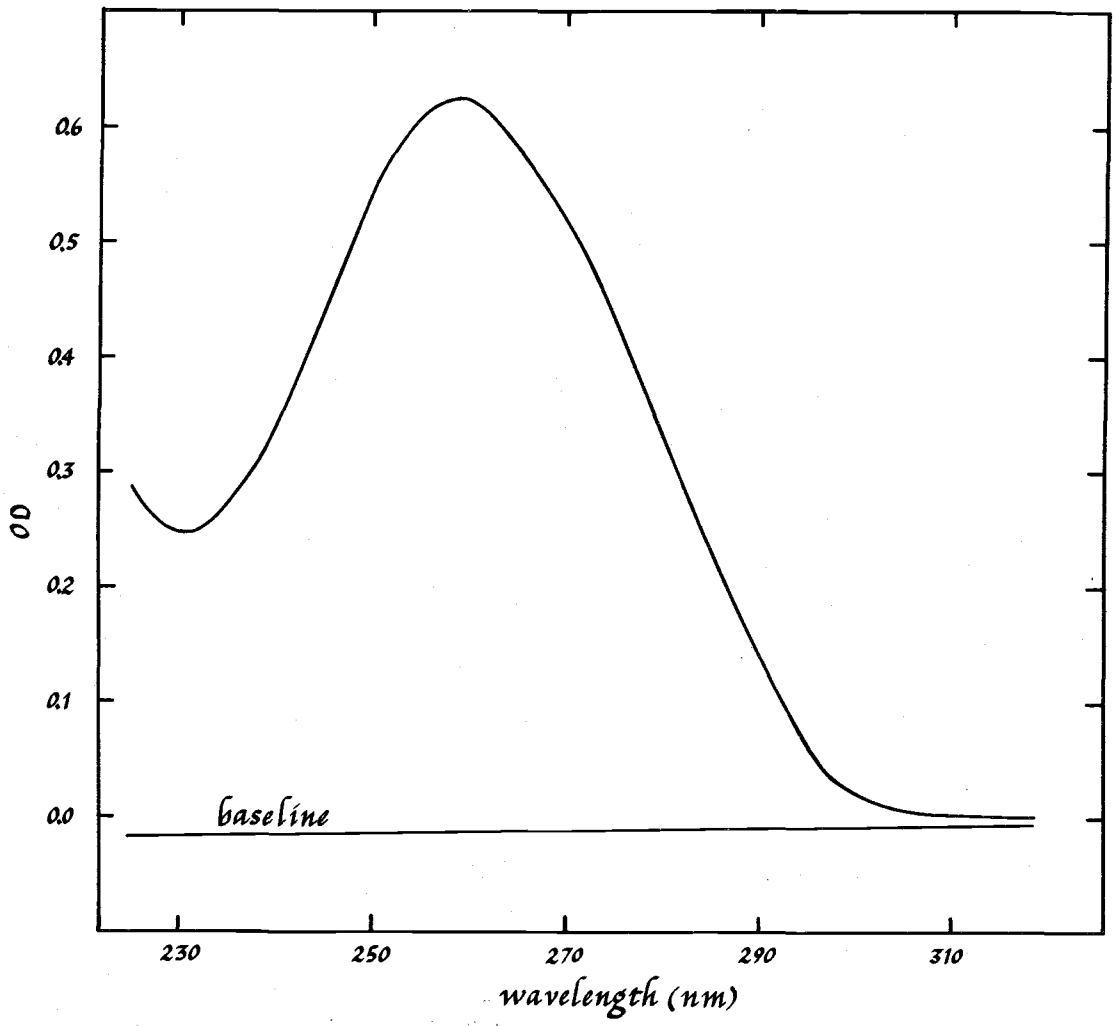

Figure 6. Ultraviolet absorption spectrum of native nuclear DNA. The absorption spectra was measured on a Cary model 15 spectrometer at room temperature in 0.01 M NaCl-0.001 M phosphate pH 7 buffer.

Table 1. Parameters for three term analysis of native DNA spectra.

(a)

λ_i mμ	α_i	β_i	γ_i
235	-2026	-656	3952
240	-1889	-1251	5031
245	-1390	-1917	6338
250	43	-2830	7480
255	-319	-1807	7616
260	-608	-1141	7307
265	2515	-3379	7052
270	871	-1409	5740
275	-386	-154	4587
280	1159	-1558	3938
285	1797	-2424	3164
290	1187	-2099	2188

Multiply each term by 10^{-7}

(b)

S_1	0.9329	S_4	0.2792
S_2	2.0631	S_5	0.2124
S_3	0.6198	S_6	0.8513

Table 2. Sample calculation of μ_1, μ_2 and μ_3 used in the spectral analysis of native nuclear DNA

Experiment @ 24.5°C

λ	A	expansion correction C.R.C. 1959	A_i	A_α	A_β	A_r
235	270	1.0028	.271	-549.0	-177.8	1071.0
240	.371	"	.372	-702.7	-465.4	1871.5
245	.468	"	.469	-651.9	-899.1	2972.5
250	.572	"	.574	24.7	-1624.4	4293.5
255	.647	"	.649	-207.0	-1172.7	4942.8
260	.650	"	.652	-396.4	-743.9	4764.2
265	.605	"	.607	1526.6	-2051.1	4280.6
270	.547	"	.549	478.2	-773.5	3151.3
275	.451	"	.452	-174.5	-69.6	2073.3
280	.356	"	.357	413.8	-556.2	1405.9
285	.247	"	.248	445.7	-601.2	784.7
290	.151	"	.151	179.2	-316.9	330.4
Σ =				386.7	-9451.8	31941.7
				μ_1	μ_2	μ_3

Using the values of μ_1, μ_2 and μ_3 and the following formula

$$C\phi = \mu_1 S_1 + \mu_2 S_2 + \mu_3 S_3 \qquad (6)$$

$$C = \mu_1 S_4 + \mu_2 S_3 + \mu_3 S_5 \qquad (7)$$

one can compute the concentration (C) as total nucleotide concentration in moles/l and ϕ, the mole fraction of AT.

Table 3 gives the values for the two experiments using this method of analysis for nuclear DNA.

Table 3. Results of the spectral analyses of native nuclear DNA

	C $\frac{\text{moles nucleotide}}{\text{l}}$	ϕ mole fraction AT	% AT	A_{260nm}	ε (P)	A/1 mg/mL
Ex 1	1.034×10^{-4}	.637	63.7	.650	6290	19.05
Ex 2	$.915 \times 10^{-4}$	.653	65.3	.581	6350	19.35
Avg.			64.5±.2		6320	19.2

The results for the mole % AT can be used to estimate the mole % GC by subtracting from 100%. This gives a value of 35.5% which agrees with the previous value (buoyant density). In addition to the base composition, it is possible to determine the molar extinction coefficient, which is necessary to equate absorption to concentration when working with very dilute solutions.

Chemical Analysis of Nuclear DNA

From the chemical analysis of the nuclear DNA it is possible to obtain the exact mole fraction of all the individual bases, to detect the presence of any unusual base, and to determine if there is any RNA contamination.

After the hydrolysis of the DNA and chromatography of the free bases the identity of the individual bases was established by comparing the R_f values of the unknown bases to that of standards. The hydrolysates from three different DNA preparations were chromatographed and did not show any unusual bases or uracil. Table 4

is a summary of the values obtained from the three experiments.

Table 4. Base composition of nuclear DNA.

Exp. no.	mole fraction	A	T	G	C	% GC	A/T	G/C	Pu/Py
1		.333	.323	.171	.172	34.4			
2		.321	.382	.169	.178	34.4			
3		.326	.330	.173	.172	34.7			
average		.327	.328	.171	.174	34.5	.997	.983	.992

As indicated by this table the reproducibility in the three experiments was reasonable in terms of the values obtained for the mole fractions present for the different bases. The mole % GC is 34.5 and is to be used to compare with the results from other analytical tests performed on the nuclear DNA, such as buoyant density, spectral analysis and melting temperature. The ratio of purine/pyrimidine indicates that the DNA is double stranded, since in double stranded DNA the mole fraction of guanine equals that of cytosine. The results are considered to be within experimental error close to the expected values of 1.0. The chemical analysis is also further proof that the material isolated from the thorax is indeed DNA because no other known biological material contains the above bases paired in the manner indicated.

Melting Temperature of Nuclear and Debris DNA

When double stranded DNA is denatured by heating an increase in absorption at 260 nm is obtained due to the separation of the two strands and the unstacking of the bases. The midpoint between the fully denatured state and the native state is called the melting point and the corresponding temperature the melting temperature, T_m. The value of the T_m is related to the mole percent of guanine and cytosine in the double stranded helix and can be used as a measure of the amount of these base pairs present. The shape of the melting curve also is indicative of the inter-molecular hetrogeneity. purity of the sample.

Fig. 7 shows the normalized melting curves for nuclear, debris and calf thymus DNA. Calf thymus was chosen as a reference standard to check the procedure and the equipment. The absorption at a given temperature is divided by the absorption at 25° C. This facilitates comparison of samples of differing DNA concentrations. The T_m for the calf thymus sample was 85.3° C which corresponds to 39.0% GC which is in good agreement with published values (Szybalski, 1968). Its hyperchromicity is about 0.41 which is equal to that reported by Mahler and Cordes (1966). The slope of the curve is relatively steep and monophasic indicating that the sample was pure and entirely double stranded. The T_m for nuclear and debris DNA

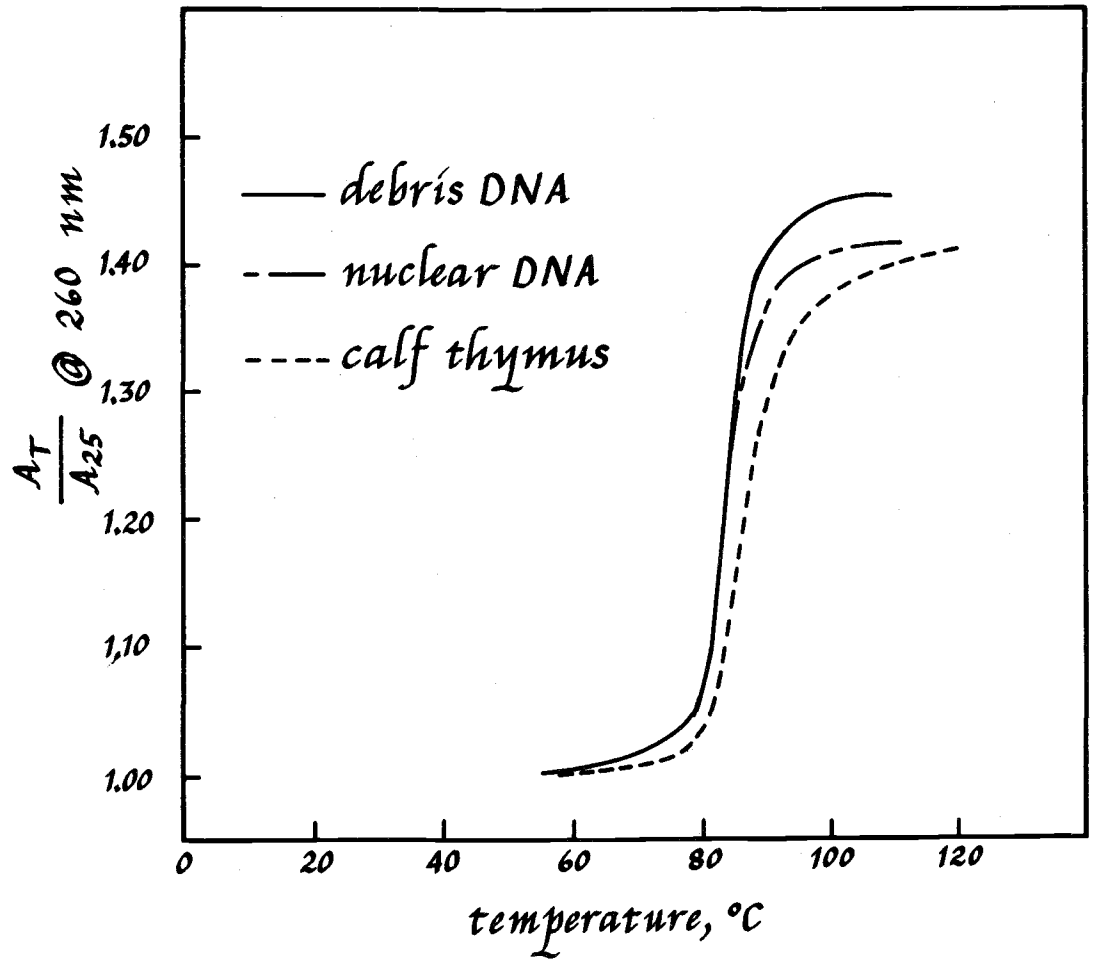

Figure 7. Melting curves of nuclear, "debris" and calf thymus DNA. A plot of the relative absorbance (AT/A25°C) at 260 nm as a function of temperature in 1.0 x SSC buffer.

was 83.6 and 83.8° C respectively which corresponds to 34.9 mole % GC for the nuclear DNA and 35.4 mole % GC for the "debris DNA." The hyperchromicity for the nuclear DNA is .41 and for the debris DNA is .44 which is in accord with the low GC content (Mahler and Cordes). The insect DNA has more intra-molecular hetrogeneity than does the calf thymus DNA as shown by the relative steepness of their melting curves.

Sedimentation Velocity of Nuclear DNA and Molecular Weight

It was of interest to determine the sedimentation coefficient of the nuclear DNA in order to calculate a molecular weight. The molecular weight will give an indication of the severity of the isolation procedure in terms of shearing and will indicate if high molecular weight material was obtained. Of course, the goal is to isolate biologically active DNA but since there is no test to determine that at present, the molecular weight is used as an index of "intactness" of the DNA. It is considered that the higher the molecular weight the more the isolated DNA may be like the *in vivo* molecule.

Sedimentation coefficients were obtained from moving boundary sedimentation velocity experiments run on the Model E analytical ultracentrifuge as described in the Methods. Fig. 8 shows the composite of such sedimentation velocity experiment, with each

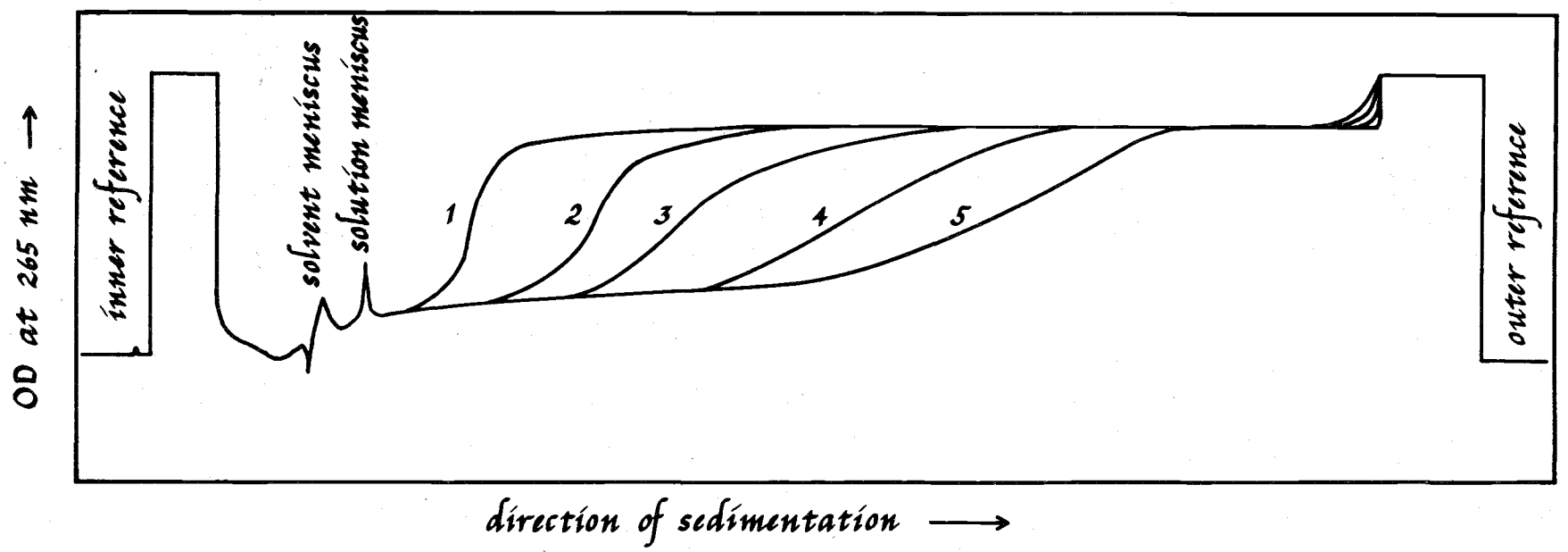

Figure 8. Moving boundary sedimentation velocity patterns of nuclear DNA. The nuclear DNA sample was placed in one side of a double sector cell and 1.0 x SSC buffer as a blank in the other sector. The Spinco Model E ultracentrifuge was run at 36, 000 or 48, 000 rpm at about 20°C. 1-5 indicate the sedimentating boundaries of the DNA molecules as it moved toward the bottom of the cell.

boundary measured every 4 minutes, with a correction for the position of the boundary during the scan. In the analysis of the results the mid-point of the boundary was considered the absorption mid-point (Eigner and Doty, 1965). The relative position vs. time can be related to the observed sedimentation coefficient S_{obs} by;

$$S_{obs} = \frac{2.303\ (\Delta \mathrm{Log}_{10} r)}{\omega^2\ t} \qquad (8)$$

where r is the true radius of the boundary from the center of rotation at time, t, and ω is the speed in radians per second. The actual dimensions of the rotor and cell are known and the measured values obtained from the scanner chart paper can be scaled down and adjusted so that the true radius can be obtained. The distance between reference edges in the reference cell is 1.60 cm and the inner reference is 5.7 cm from the center of rotation. The S_{obs} value was obtained from a least squares fit of equation 8. S_{obs} values were determined for DNA solutions in 1.0 x SSC for various concentrations in the range of 0.1-1.0 O.D. units.

Having determined the observed value from the data, it can then be converted to a sedimentation coefficient at standard conditions. $S_{20,w}$ is the sedimentation coefficient in water at 20° C.

$$S_{20,w} = S_{obs} \cdot \frac{\eta_t}{\eta_{20}} \frac{\eta}{n_o} \frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_t} \qquad (9)$$

where S_{obs} is the observed sedimentation value as defined above, η_t is the viscosity of water at the temperature the run was made, η_{20} is the viscosity of water at 20° C, η/η_b is the viscosity of the solvent relative to water, $\rho_{20,w}$ is the density of water at 20° C, ρ_t the density of the solvent at temperature, t, and $\bar{v}$ is the partial specific volume of the DNA molecule.

The following values were used:

S_{obs}	Varied with each run
η_1/η_{20}	Varied with each run and (C.R.C., 1959)
$\eta/\eta_b = 1.0517$ $\rho_1 = 1.0111$	(Eigner and Doty, 1965)
$\rho_{20,w} = 0.9982$	(C.R.C., 1959)
$\bar{v} = 0.55$	(Mandelkern and Flory, 1952)

Equation 9 then reduces to

$$S_{20,w} = S_{obs} \cdot \frac{\eta_t}{\eta_{20}} \quad 1.0685 \qquad (10)$$

Since $S_{20,w}$ for DNA is concentration dependent (Tanford, 1961), it must be corrected to zero concentration. Fig. 8 shows a graphical extrapolation of $S_{20,w}$ to zero concentration using the linear relation for non-symmetrical particles.

$$\frac{1}{S_{20,w}} = \frac{1}{S^{o}_{20,w}} + kC \qquad (11)$$

where $S^{o}_{20,w}$ is the standard sedimentation coefficient at zero concentration, k is some empirical constant and C is concentration, expressed in terms of optical density. Fig. 9 shows three experiments, two at 48,000 rpm and one at 36,000 rpm. All three experiments were from different DNA preparations. The two speeds were chosen to see if there was any dependence of $S_{20,w}$ upon rotor speed as reported by Eigner, Schildkraut and Doty (1962). The least squares fit of the two plots was extrapolated to zero concentration and the bars represent the error in each value. The $S^{o}_{20,w}$ values obtained are 45.3 ± .8 for 18,000 rpm and 46.5 ± 1.8 for 36,000 rpm.

The values are in good agreement for a large molecular weight double stranded DNA. The disturbing fact that the positions of the two reciprocal plots in Fig. 9 is reversed cannot be answered. The dependence upon concentration should show a decrease in $S_{20,w}$ at the lower speeds for a given size of DNA (Eigner and Doty, 1962). The only rational explanation is that the DNA used for the 36,000 rpm run was not of the same molecular weight as the DNA's in the two 48,000 rpm experiments.

Since it is the molecular weight that is to be determined the following equation was used to convert $S^{o}_{20,w}$ to M_w as determined by Eigner and Doty (1965).

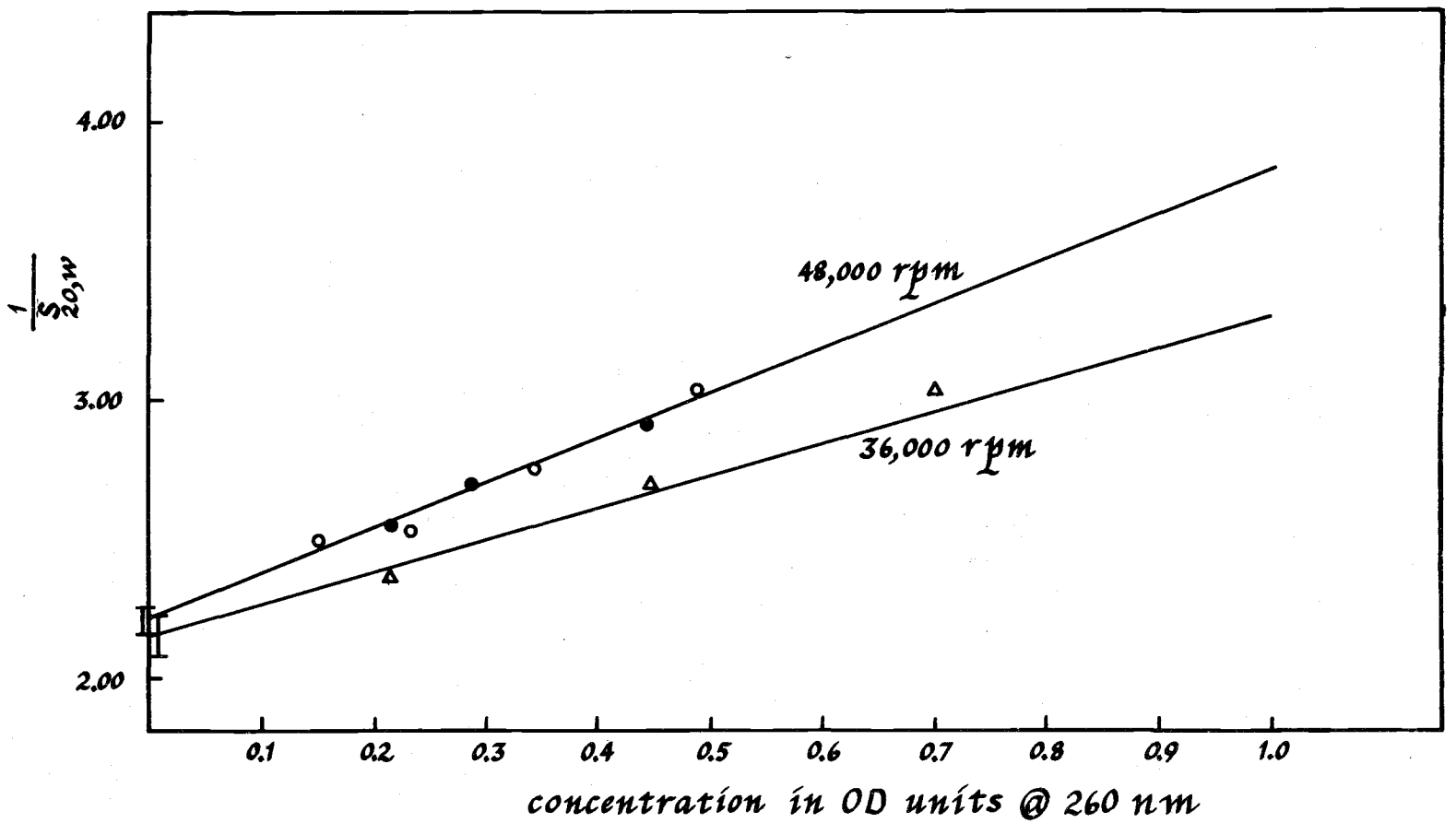

Figure 9. Plot of sedimentation coefficients of nuclear DNA ($S_{20,w}$) versus concentration. Solid circles (•) and open circles (o) represent two independent nuclear DNA preparations run at 48,000 rpm and the triangles (△) represent a single independent nuclear DNA preparation run at 36,000 rpm.

$$S^{o}_{20,w} = 0.034\ M_{w}^{0.405} \qquad (12)$$

The molecular weight from the 36,000 rpm run was $55.0 \pm 2.0 \times 10^6$ daltons and from the 48,000 rpm run was $52.0 \pm 2.0 \times 10^6$ daltons. These show that the isolation procedure is relatively mild in that high molecular weight material is obtained.

Molecular Characterization of the Nuclear DNA Genome

In the studies of DNA from different sources it is evident that many non-related organisms have comparable GC contents, which makes this a non-unique label for a particular DNA. The molecular weight of DNA from higher organisms is largely a measure of the isolation technique which is usually not relatable to any intrinsic property of the DNA. What is needed is a quantity that will give information about the identity and individuality of the DNA. This can be obtained from renaturation kinetics. The property that makes calf DNA, calf DNA, and not some other type is the genetic makeup which is a consequence of the sequence of the four bases. This is true for all organisms including the tobacco hornworm. The renaturation kinetics is in effect a measure of these base sequences, and a measure of the genome size of an organism (Britten and Kohne, 1968). In the lower forms of life such as bacteria, the genome size estimated from such data is the same as the molecular length of the

DNA. In such studies second order kinetics are assumed and the results are a measure of two complementary sequences pairing with each other. In lower cell types the amount of variation in base sequence should be small because of the limited amount of genetic material and in renaturation kinetic experiments there would be a large population of complementary strands. In higher cell types there is much more DNA and more information carried by the DNA. In a given population of DNA, individual repeated sequences would be relatively small and would take a longer time to renature. From renaturation experiments a rather loose phylogenetic relationship can be derived.

Britten and Kohen (1968) have described the kinetics of renaturation as second order. In second order kinetics the reaction involves the collision of two reactants: in this case, two complementary strands. The concentration of each strand is potentially rate limiting. The rate of disappearance of denatured DNA can be described by:

$$-\frac{dc}{dt} = k_2 C^2 \qquad (13)$$

where C = the concentration of denatured DNA, k_2 = second order rate constant, and t = time of renaturation.

If equation (13) is integrated over all times and evaluated at t = 0, when C = Co (initial concentration) we obtain:

$$\frac{C}{C_o} = \frac{1}{1 + k_2(Cot)} \tag{14}$$

at half renaturation:

$$\frac{C}{C_o} = \frac{1}{2} = \frac{1}{1 + k_2(Cot)} \text{ or } Cot_{\frac{1}{2}} = \frac{1}{k_2} \tag{15}$$

$Cot_{\frac{1}{2}}$ is directly proportional to the number of different genes (Wetmur and Davidson, 1968), and k_2 is inversely proportional to the sequence diversity. In other words, the larger the number of different sequences the longer for the reaction to become 50% complete. $Cot_{\frac{1}{2}}$ from now on will represent the 50% renaturation point, and its units are moles nucleotide/liter x seconds. In Fig. 10 is shown an ideal second order reaction, used to illustrate the features of the log Cot plots. Since Cots can vary about 10^8 fold from lower organism to higher organisms a logarithmic plot makes analysis easier. The value for 100% denatured DNA is equal to the initial concentration, in terms of absorption times the hyperchromicity (h). Zero denaturation is equal to the absorption of native sheared DNA at 60° C (renaturation temperature). This is shown by the following:

$$\% \text{ Denatured} = \frac{A - A_o}{A_o h} \tag{16}$$

where A_o is the initial absorption at 260_{nm}, h is the hyperchromicity and A is the absorption at time t. $Cot_{\frac{1}{2}}$ is measured as absorption and corrected to concentration using the values 20 O. D. = 1 mg/ml

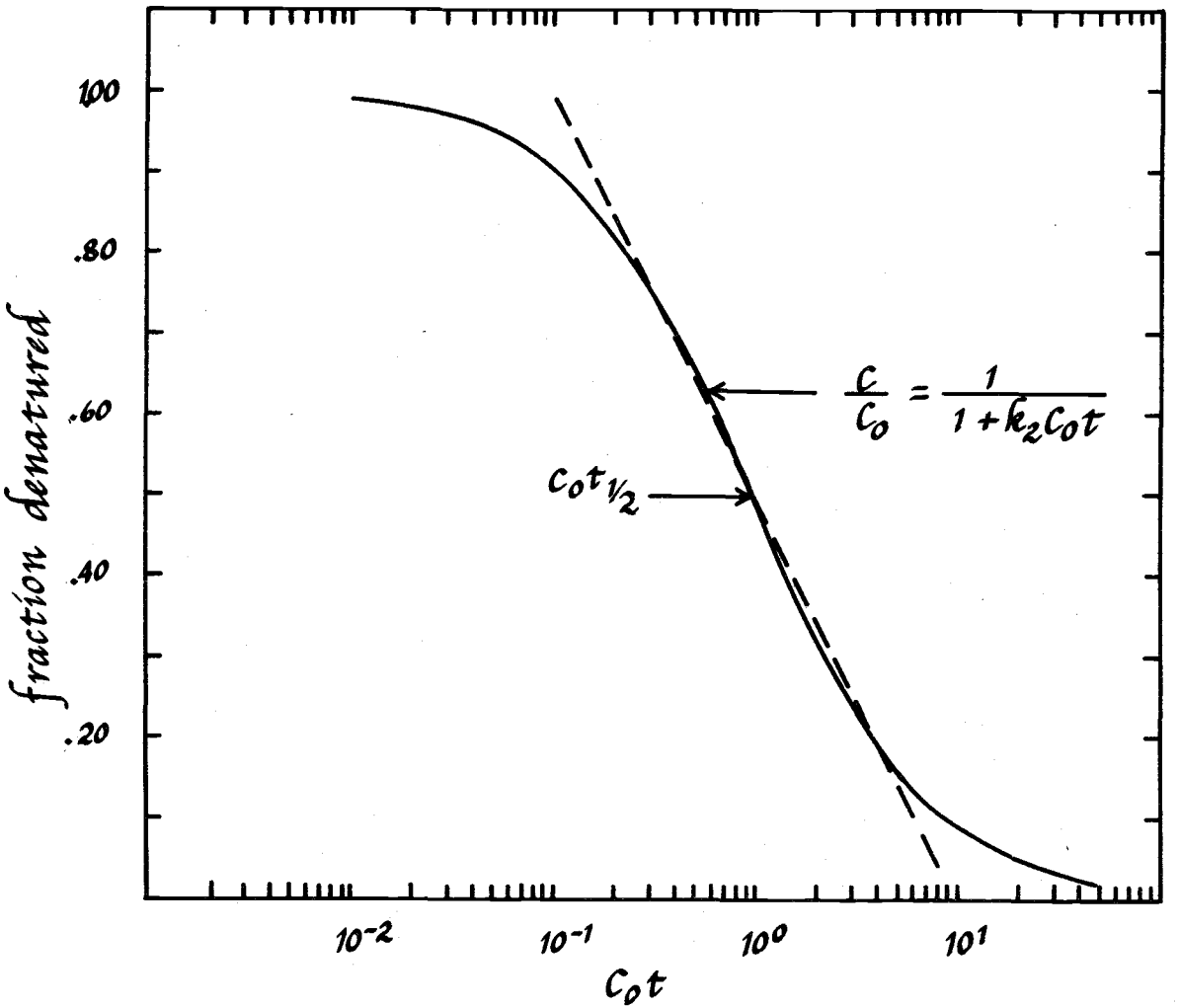

Figure 10. Time course of an ideal second order reaction. Cot is expressed as initial concentration in moles nucleotides/liter x time in seconds. $Cot_{\frac{1}{2}}$ is the value when half of the single stranded DNA has renatured. In this example $k_2 = 1$.

and 1 mole nucleotide = 331 gm.

Wetmur and Davidson (1968) have shown that the rate of renaturation is partially dependent upon the molecular length of the single stranded DNA. In order that the results would be consistent with others (Laird and McCarthy, 1969; Brittin and Kohn, 1965) the DNA was sheared to small fragments so that the average molecular length was about 1,000 nucleotides. This is a little longer than the lengths used by Brittin and Kohn (1968) or Laird and McCarthy (1969) which are 400-600 nucleotides long. However, this did not appear to have any large effect on the $Cot_{\frac{1}{2}}$ as shown in Fig. 11 which shows the Cot plot for E. coli. There is a slight deviation from the expected second order kinetics but the $Cot_{\frac{1}{2}}$ is 4 which is the same obtained by Laird and McCarthy (1969). From this it was assumed that the renaturation conditions were similar to those of others (Wetmur and Davidson, 1968; Brittin and Kohn, 1969; Laird and McCarthy, 1969). Fig. 12 shows the renaturation of single stranded tobacco hornworm nuclear DNA that was sheared to about 1,000 nucleotides long. The curve deviates greatly from ideal second order kinetics and has a $Cot_{\frac{1}{2}}$ of 1.90×10^{2}. The vertical lines represent relative errors at each measurement. It is obvious that there is a small fraction, 20% of the total DNA, that renatures quite rapidly. The remainder of the DNA takes much longer to renature.

Fig. 13 shows the relationship of tobacco hornworm DNA to

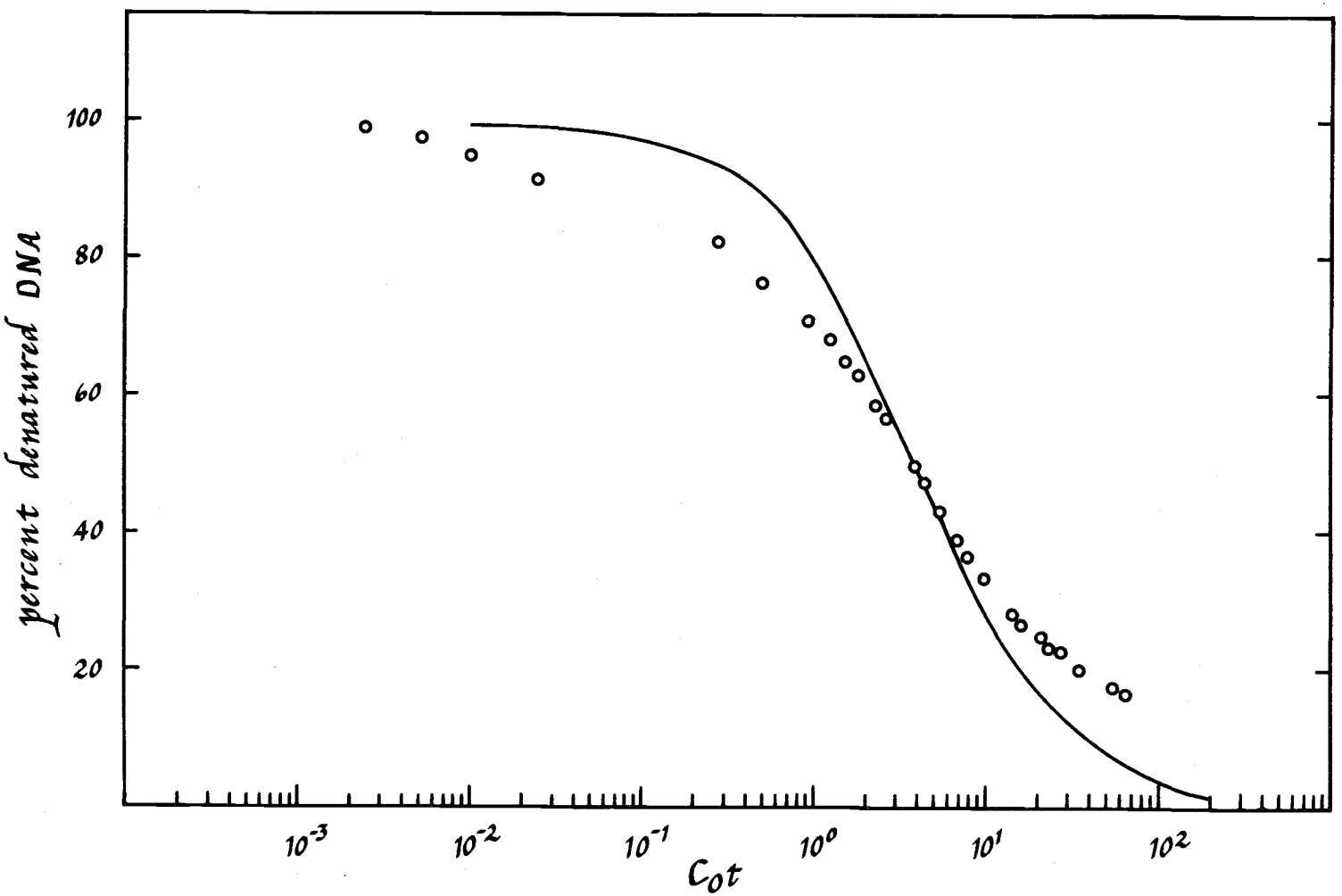

Figure 11. Renaturation of E. coli DNA. E. coli DNA was renatured at 60°C in 1.0 x SSC buffer at an initial concentration of 30 μg/ml. Hyperchromicity at 260 nm was used to follow the extent of renaturation as a function of time. The open circles (o) are the experimental points and the solid line is the theoretical second order reaction curve drawn through the 50% renaturation point, $Cot_{\frac{1}{2}}$.

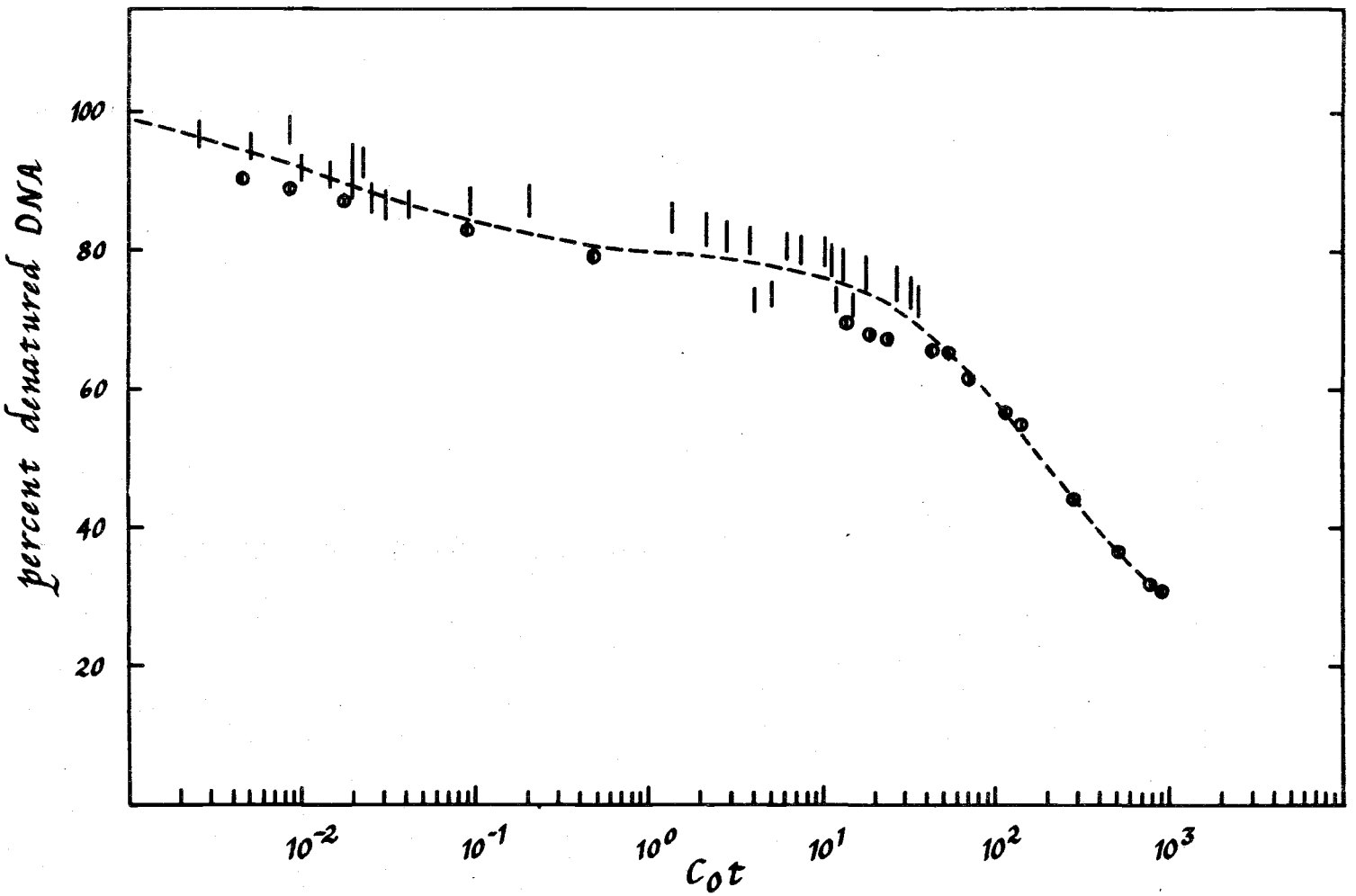

Figure 12. Renaturation of tobacco hornworm nuclear DNA. The bars represent experimental points with associated errors. The circled bars (Ⓘ) represent DNA at a concentration of 98 μg/ml the remaining bars are for DNA at concentrations of 27 and 42 μg/ml. The samples were renatured at 60°C in 1.0 x SSC buffer.

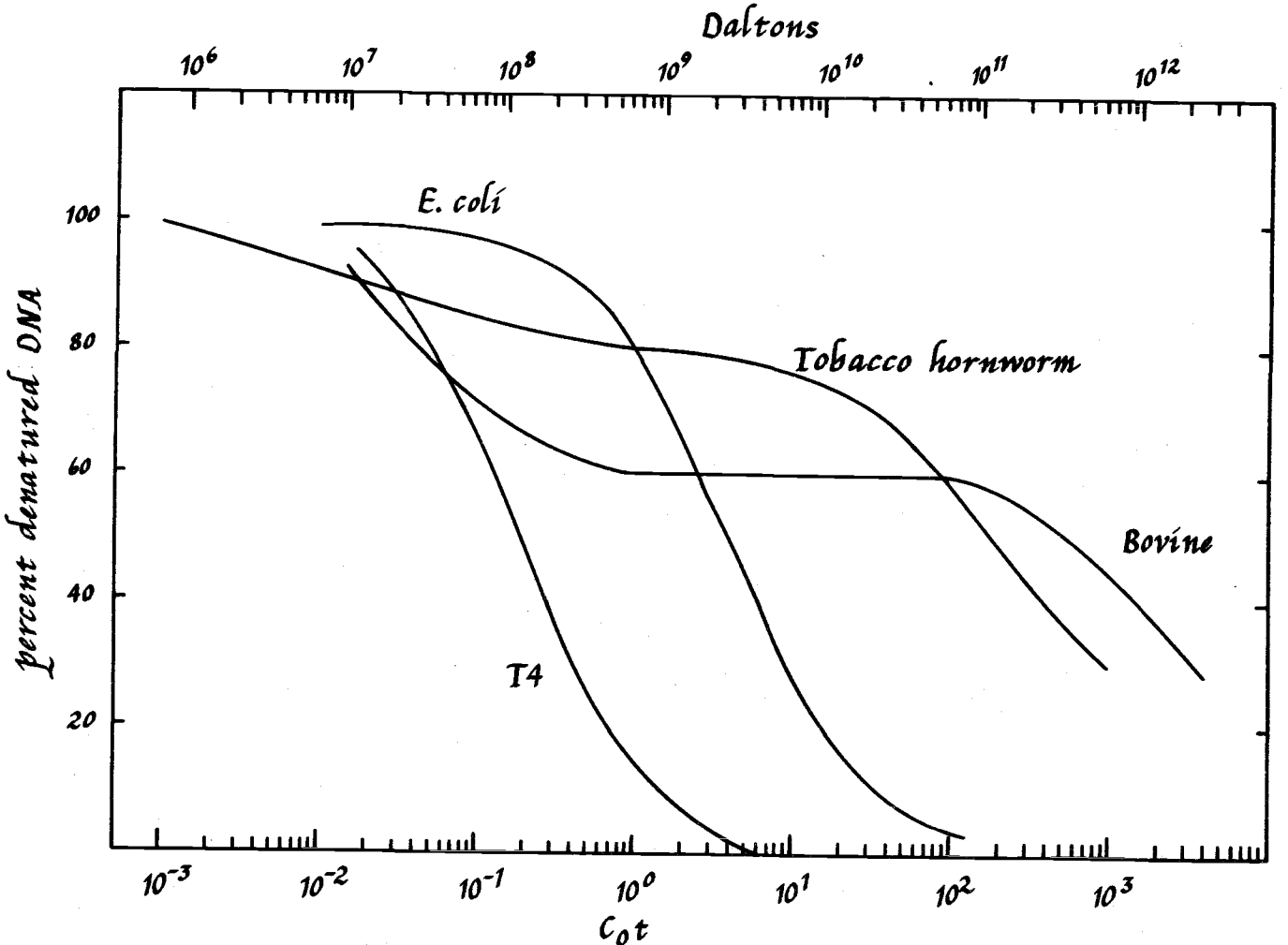

Figure 13. Renaturation of E. coli and tobacco horn worm DNA compared with T4 and bovine DNA. Experimental conditions are as described in Figure 11. Bovine and T4 DNA data are shown as examples of complex and simple genomes, respectively (Britten and Kohne, 1968). Upper scale is a measure of genome size, in daltons, T4 and E. coli have $Cot\frac{1}{2}$ values of .2 and 4.0 and genome sizes of 1.3×10^8 and 2.7×10^9 daltons, respectively. These values are used to calibrate the upper logarithmic scale.

that of some other organisms. E. coli and T4 are used to calibrate the upper scale which relates $Cot_{\frac{1}{2}}$ to the number of nucleotide pairs in daltons per genome. T4 has a $Cot_{\frac{1}{2}}$ value of 0.2 and a genome size of 1.3×10^8 daltons and E. coli has a $Cot_{\frac{1}{2}}$ of 4.0 and a genome size of 2.7×10^9 daltons (Laird and McCarthy, 1969). These DNA's are used to determine and calibrate the upper scale, so that a genome size can be determined from any $Cot_{\frac{1}{2}}$ value.

If it is true that the renaturation of the DNA from tobacco hornworm is biphasic, then these two phases can be separated into their individual modes. Since the data seemed to indicate that 20% of the DNA renatured rapidly and 80% slowly, this was considered the relative quantity of DNA in each fraction. Fig. 14 shows a plot of the two separated fractions. The rapid fraction has a $Cot_{\frac{1}{2}}$ of 3.6×10^{-3} when corrected for total concentration and the unique fraction has a $Cot_{\frac{1}{2}}$ of 2.73×10^2 when corrected for total concentration. The rapidly renaturing fraction has an indicated genome size of 2.43×10^6 daltons and the unique fraction has a genome size of 1.84×10^{11} daltons. Laird and McCarthy (1969) do not believe that the concentration should be corrected, because this does not give a measure of the genome. In order to make the values for tobacco hornworm comparable to theirs, the concentrations must be re-evaluated, by multiplying the rapid $Cot_{\frac{1}{2}}$ value by 5 and the slower $Cot_{\frac{1}{2}}$ value by 1.25.

It has also been shown that GC content affects renaturation

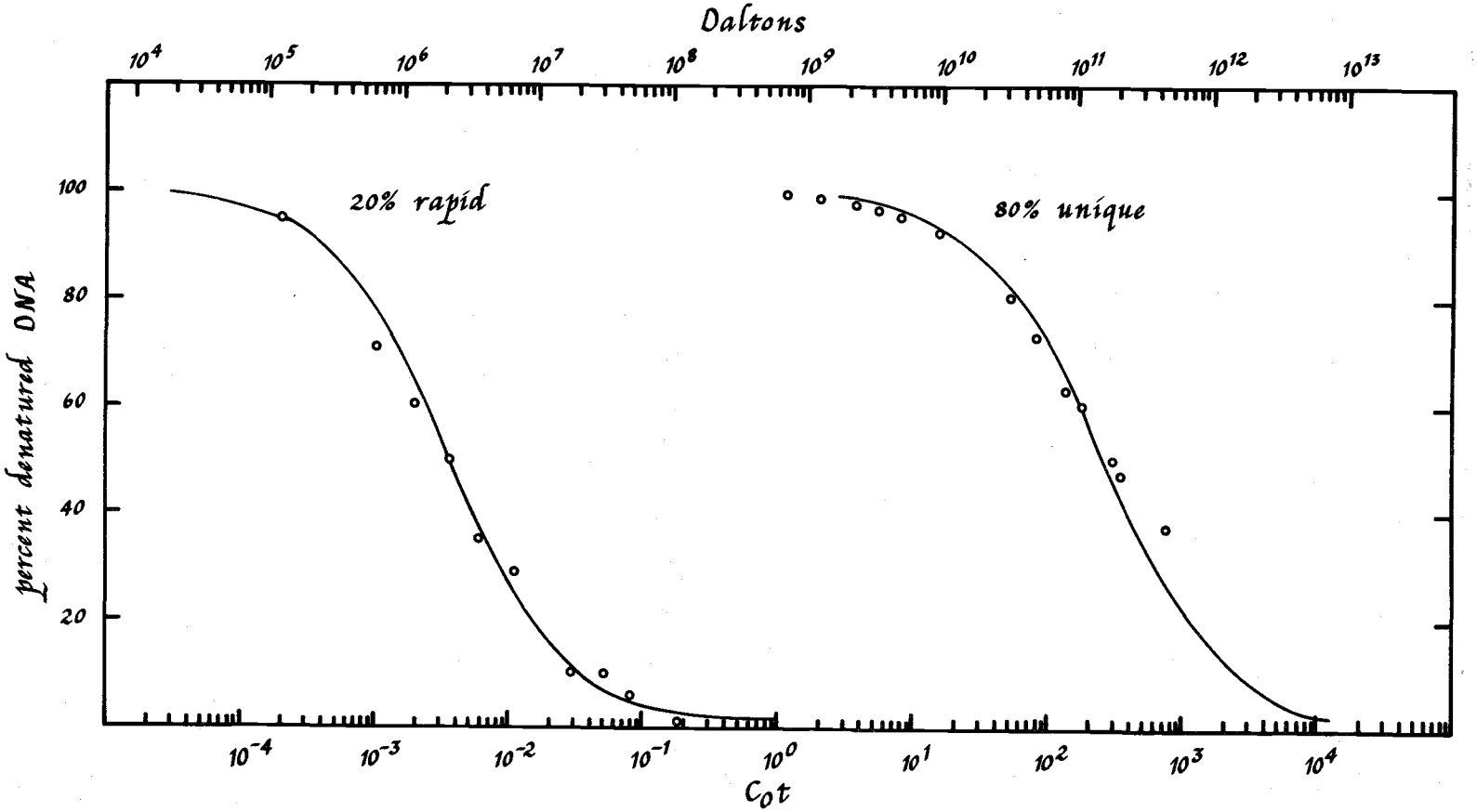

Figure 14. Renaturation of tobacco hornworm nuclear DNA, separated into a rapid and a unique fraction. The renaturation curve in Figure 12 is analyzed assuming 20% of the total DNA renatures rapidly and independently from the remaining 80% unique DNA. The open circles (o) represent the two normalized renaturation curves and the solid lines (—) are the theoretical second order reaction curves drawn through the $Cot\frac{1}{2}$ points.

kinetics and a simple correction has been derived by Seidler and Mandel (1971).

$$RC = 1 - (\Delta GC \cdot 0.018) \qquad (17)$$

where RC is the rate constant correction and ΔGC is equal to %GC in E. coli - %GC in the unknown sample. RC is used as a multiplier to correct genome size for the unique fraction. Using a value of 35% GC for tobacco horn worm a RC of 0.71 is obtained. If this is multiplied by the unique fraction genome size, 1.84×10^{11} daltons, a corrected value of 1.31×10^{11} daltons is obtained. This is only the relative size of this fraction and is an underestimate by 12.5% if all the genetic material is considered.[5] The size of the rapid fraction corrected in the same manner is 1.73×10^{6} daltons.

The unique fraction is usually considered as one complete fraction, i.e., one series of sequences repeated once and the rapid fraction is considered to be a group of small sequences repeated many times. An estimate of how many times this unit is repeated, family size, can be made by looking at the relative $Cot_{\frac{1}{2}}$'s of the two fractions, and their relative concentrations. The $Cot_{\frac{1}{2}}$ for the rapid fraction is 2.56×10^{-3} and the $Cot_{\frac{1}{2}}$ for the slower unique fraction is 1.94×10^{2} which means that the rapid fraction renatures 7.6×10^{4} faster than the unique fraction. Thus the number of DNA sequences

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[5]Genome is defined as the haploid DNA content of a cell or virus particle (Britten and Kohne, 1968).

which reassociate rapidly is 7.6×10^4 times the number of those sequences which reassociate slowly; or on the average the rapid sequences are repeated 7.6×10^4 times as much as the unique sequences.

Studies on Mitochondrial DNA

Isolation of Mitochondrial DNA

One of the goals of this research was to isolate and characterize, intact mitochondrial DNA and to compare this to the nuclear DNA and the mitochondrial DNA from other organisms.

The supernatant fractions from the nuclear preparations were centrifuged at 10,000 x g for 10 minutes, then washed with 0.25 M sucrose and re-centrifuged twice. The pellet was considered to be predominately mitochondria and was similar to fraction F-3 used in the enzymatic assays (see Methods).

The first method for the isolation of mitochondrial DNA to be tried was that of Kalf and Gréce (1967). The mitochondria was first incubated with DNAse in order to remove any contamination nuclear DNA and then sedimented in a 30-65% sucrose gradient at 23,000 rpm for 1 hour in a Spinco SW25.1 rotor. In this procedure the mitochondria are found in a band that corresponds to about 43% sucrose. This procedure was designed to yield pure mitochondria prior to the actual DNA isolation. The DNA is then isolated by a modified phenol extraction.

The above procedure was not adaptable to the isolation of insect mitochondrial DNA because it requires relatively large amounts of mitochondrial tissue. Because of losses in each one of the steps in the procedure, attempts to scale down the procedure were not successful.

The second method tried was developed by Vinograd (Hudson and Vinograd, 1967), and is based upon the fact that ethidium bromide will intercalate less with covalently closed circles than with nicked and/or linear molecules. The DNA can then be separated by centrifugation in CsCl gradients. This method will quantitatively separate circular DNA from linear DNA and is adaptable to small quantities. The DNA-dye complex fluoresces and thus is detectable at low levels. The mitochondria were lysed using 0.5% sarcosyl for 2 hours and then placed in a CsCl solution made to a final density of 1.55 gm/cc containing ethidium bromide at a concentration of 100 μg/ml. This solution was centrifuged and the fluorescent bands located. For some reason on one occasion two bands were found. In most preparations only one band, which also contained nuclear DNA, was found. The DNA-dye complex is sensitive to shear forces and this is probably why all the DNA was found in a band corresponding to nicked DNA. Since the correct conditions for separation of circular DNA from linear could not be reproduced, this method was not considered satisfactory.

The best procedure proved to be a modification of the one used

to isolate nuclear DNA. This method is easily scaled down to small quantities of tissue and yields pure DNA. A dialysis step was used in place of the ethanol precipitation since the latter is a point where large quantities of DNA may be lost. Some DNA is lost on the walls of the dialysis bag, but this is a relatively small amount. Since there are no polysaccharides found in the mitochondria the digestion with α-amylase was omitted and the digestion with RNAse was performed after the first dialysis. All volumes were kept as small as possible and the proportion of DNA to the solvents used were the same as that used in the nuclear DNA procedure. Occasionally more dilute solutions were obtained. If this occurred the solution in the dialysis bag was dehydrated before the final dialysis by placing the bag in solid granular sucrose. When an appropriate volume was reached the DNA solution was dialyzed for about 24 hours against 200 volumes (changed 4 times) of 1.0 x SSC. The final product has an absorption spectrum characteristic of DNA (Fig. 15). The average yield of DNA was 1.35 μg/mg of mitochondrial protein with 230/260 and 280/260 ratios of .442 and .432 respectively.

CsCl Buoyant Density of Mitochondrial DNA

The solutions of DNA obtained by the above procedure were very dilute (5-10 μg DNA/ml). With dilute DNA solutions CsCl density gradient centrifugation is considered the most desirable

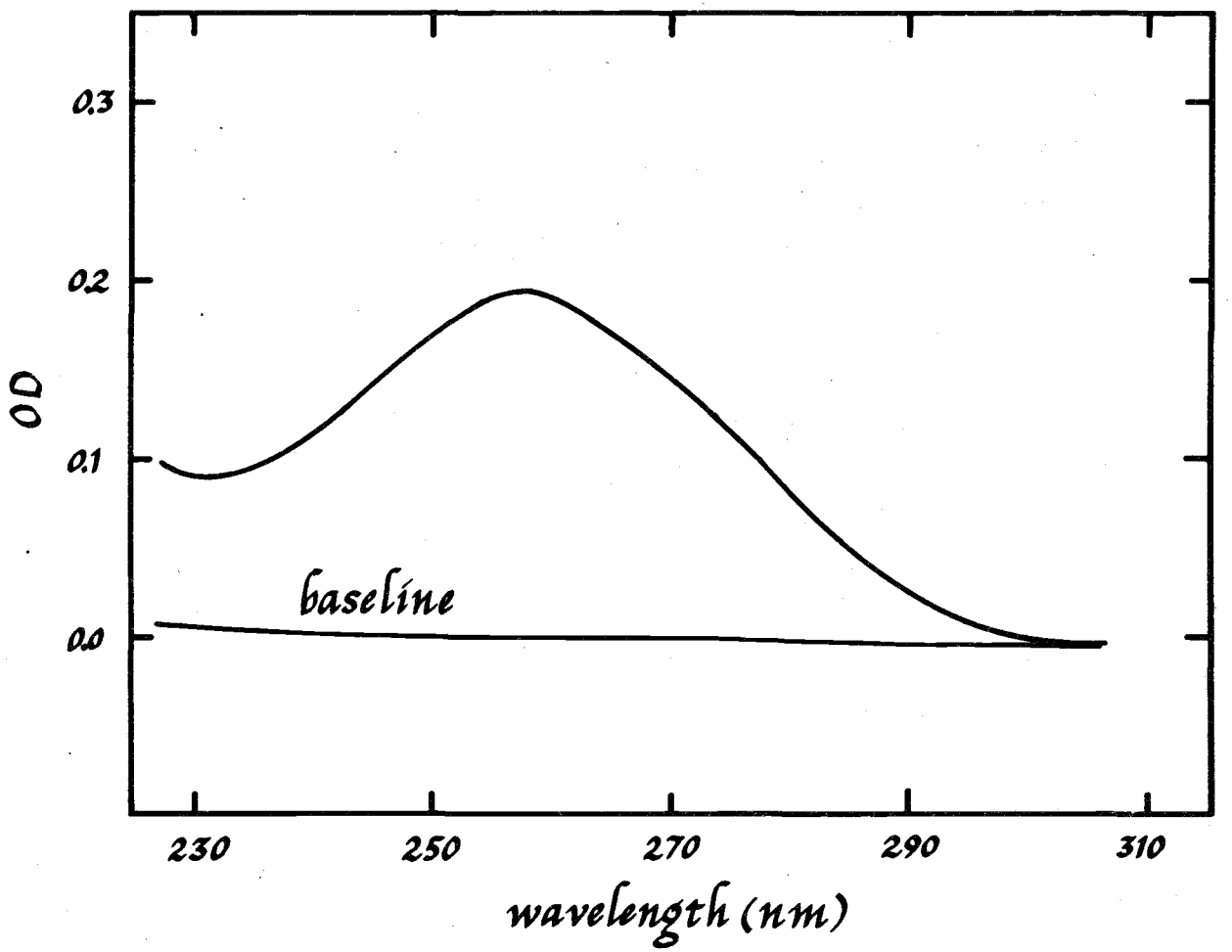

Figure 15. Ultraviolet absorption spectrum of DNA isolated from the mitochondrial fraction. The absorption spectra was measured on a Cary model 15 at room temperature. DNA was in 1.0 x SSC buffer.

method for further analysis of the nature of the DNA and its purity. Since the solutions were dilute, solid CsCl was added in a ratio of .89 gm of CsCl/7 ml of solution. M. lysodeikticus DNA was used as a marker. Fig. 16 shows a typical scan of a CsCl buoyant density experiment. Peak C is the marker with a density of 1.724 g/cc, peak B has a density of 1.689 and corresponds to that of nuclear DNA. Peak A is tentatively identified as mitochondrial DNA and has a density of 1.674 g/cc which corresponds to a GC content of 20.1%. From the areas of peak A and peak B, it was determined that peak A is 20% of the total DNA added to the centrifuge cell. This value varied slightly between preparations. The amount of mitochondrial DNA per mg protein was estimated from the relative area under the peak. The total amount of DNA obtained from each DNA preparation was corrected for the fraction that was considered truly mitochondrial in origin as determined by peak area. This value was divided by the total amount of mitochondrial protein used in the nucleic acid preparation and the average from the two assays is 0.27 ± 0.02 μg DNA/mg mitochondrial protein.

If peak A is mitochondrial and if it is composed of covalently closed circles then there should be no change in buoyant density when it is denatured and rapidly renatured (Dawid and Wolstenholme, 1967). The DNA from the mitochondrial preparation was denatured with 0.1 N NaOH, allowed to stand for 4 minutes at 4° C and then rapidly

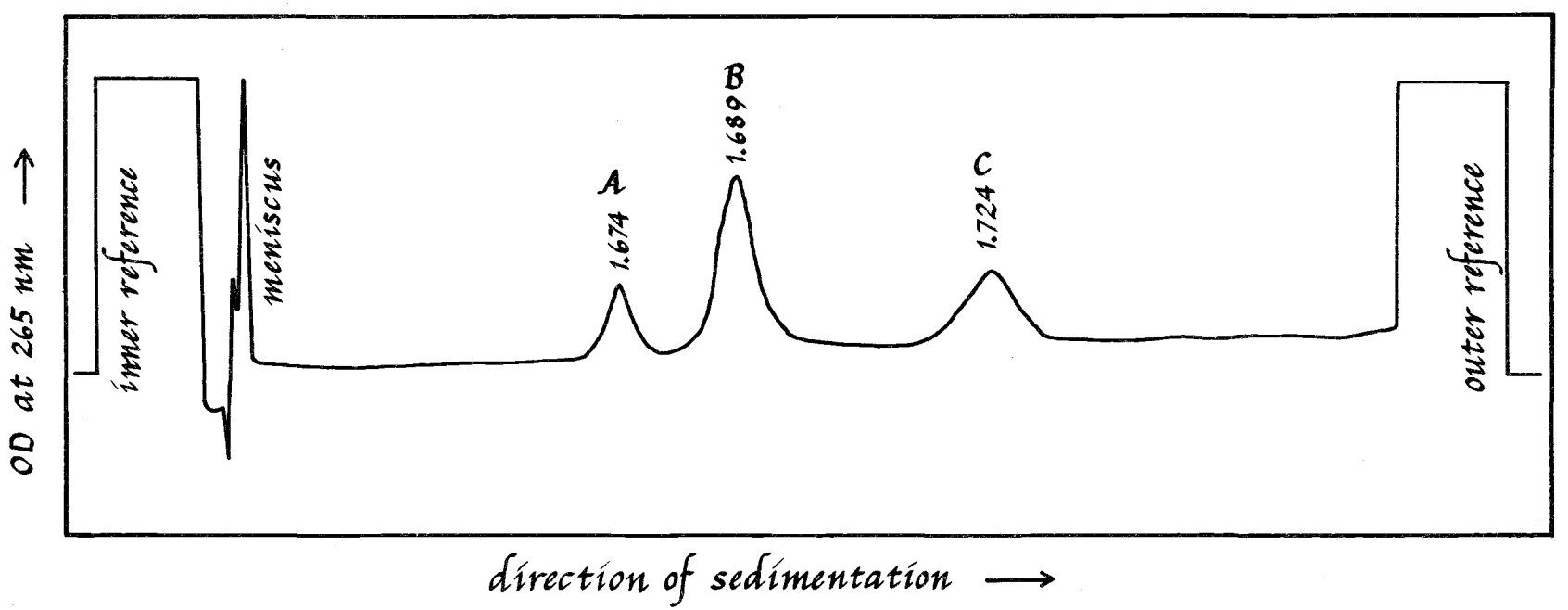

Figure 16. CsCl analytical gradient scan of DNA isolated from the mitochondrial fraction. The DNA sample was mixed with M. lysodeikticus DNA, used as a density marker. Peak A and B are from the mitochondrial fraction, peak B has the same buoyant density as nuclear DNA. Peak C M. lysodeikticus marker DNA. The centrifuge conditions are as described in Figure 5.

neutralized with HCL to a pH of 7.0. Solid CsCl was added to this solution together with marker DNA and the mixture was centrifuged. Fig. 17 shows the results of such an experiment. Peak D is the marker, M. lysodeikticus DNA. Peak C is nuclear DNA which has been denatured, which causes a shift in the buoyant density of 0.017 g/cc to 1.705 g/cc. Peak B is mitochondrial DNA which was not composed of covalently closed circles and it has increased in density to 1.690 g/cc, approximately 0.016 g/cc shift. Peak A is covalently closed circular DNA which has "snapped back" to its original structure. Strand separation has occurred in the DNA in peaks B and C but not in the DNA of peak A and therefore it remains at its original buoyant density. The relative amounts of peak A and B varied due to the amount of handling. Peak B comprised 60-85% of the total mitochondrial fraction, and there was more DNA under peak B when the DNA solution had been concentrated against sucrose. In Fig. 17 there is no peak corresponding to native nuclear DNA indicating that the denaturation process was complete.

It can be assumed that the DNA in peak A is comprised of all super coils (covalently closed circles), and they should all have the same molecular weight and diffusion coefficient. If these assumptions are correct, an approximate molecular weight can be calculated (Chervenka, 1970). Fig. 18 gives an idealized band in a buoyant density experiment as recorded by the scanner; h is the maximum

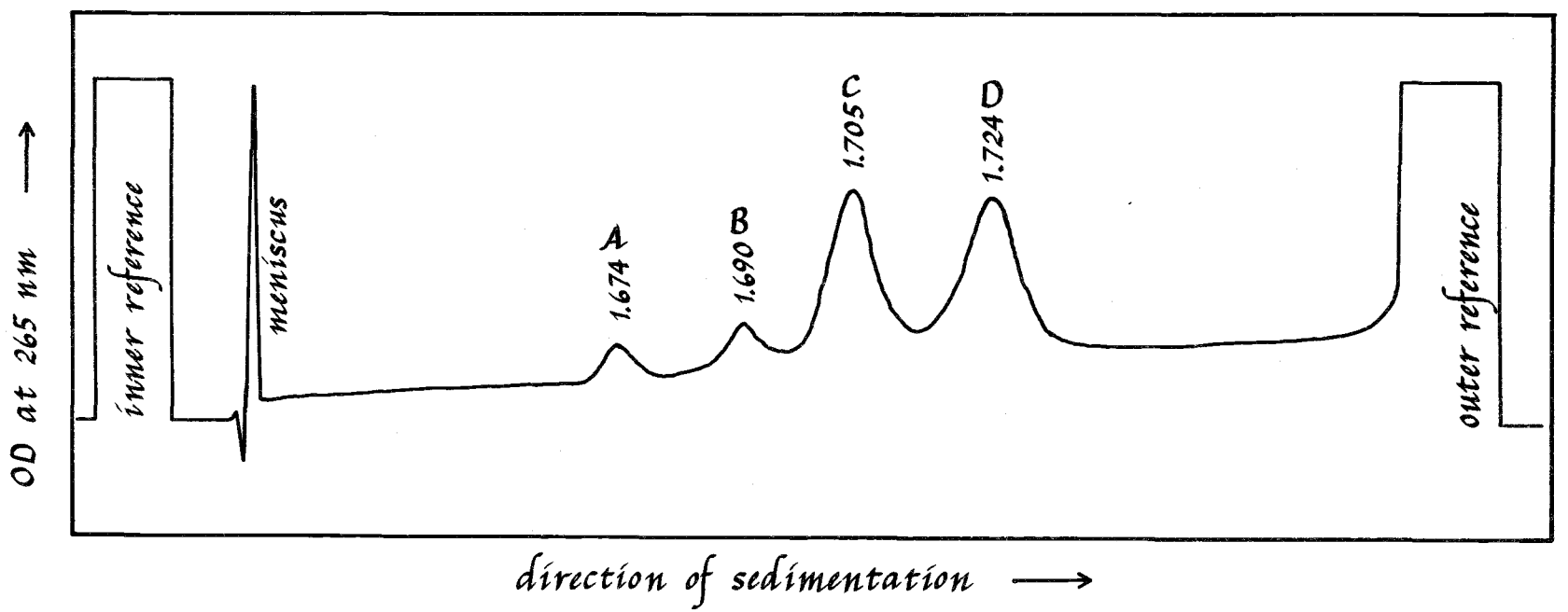

Figure 17. Reversible denaturation "snap back" of mitochondrial DNA. Mitochondrial DNA was denatured in 0.1 N NaOH for 3 min. at 4°C and then reneutralized, mixed with M. lysodeikticus marker DNA, and centrifuged in a CsCl analytical gradient. The conditions of centrifugation are as described in Figure 5. Peak A is mitochondrial DNA which "snapped back," peak B is denatured mitochondrial DNA, peak C is denatured nuclear DNA, and peak D is M. lysodeikticus marker DNA.

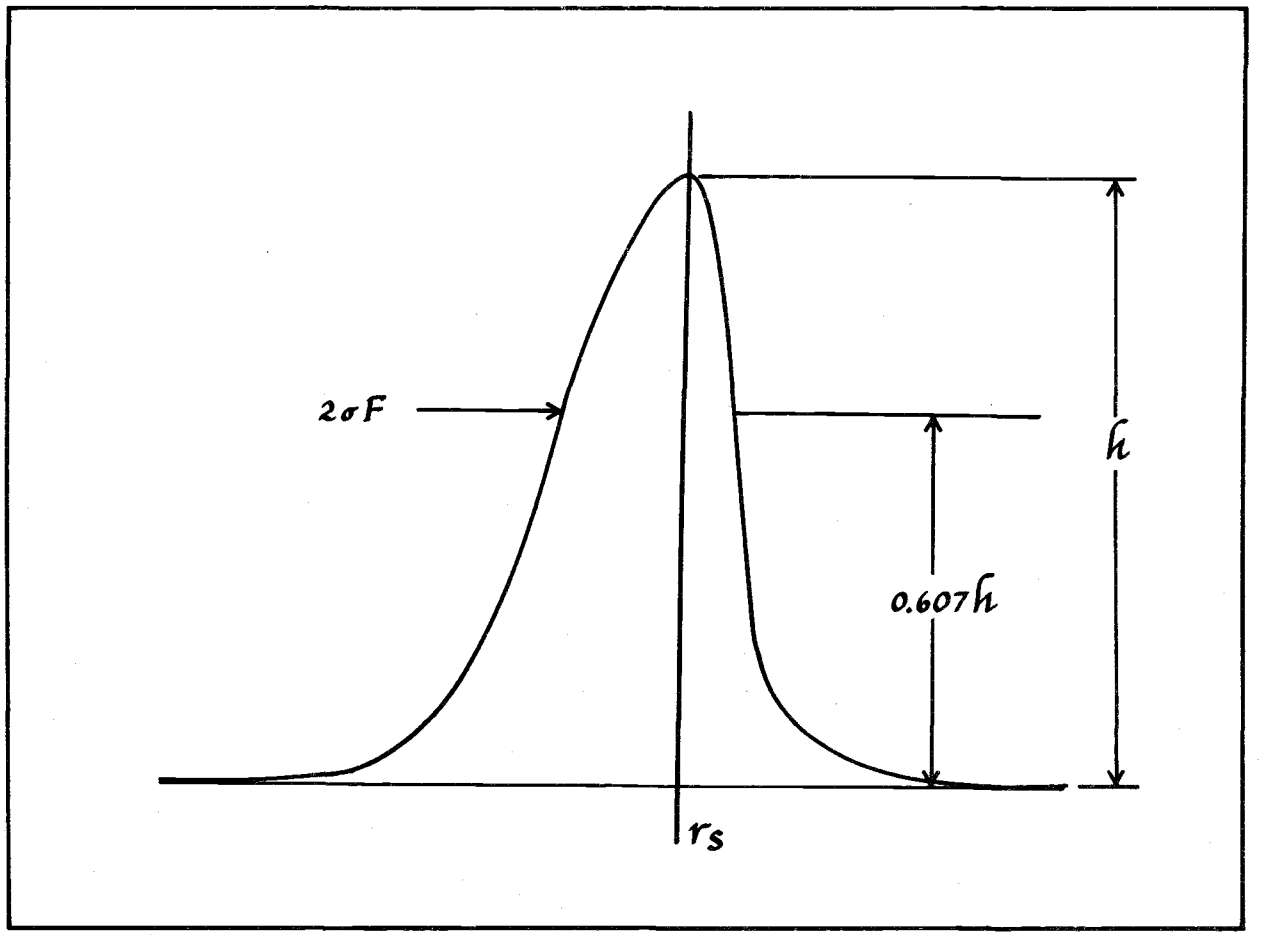

Figure 18. Graphic determination of σ, for the estimation of the molecular weight of mitochondrial DNA (an idealized scanner trace). Where r_s is the distance from the center of rotation to the middle of the peak (band), h is the height of the peak from the base line, F is the magnification factor of the scanner, and σ is the standard deviation of the gaussian distribution of molecules.

height of the peak at r_s, the distance of the peak from the center of rotation, F is the magnification factor and 2σ is the real width of the band in the centrifuge cell. σ is equal to the standard deviation of the distribution of the molecules. Equation 18 can be used to calculate an apparent molecular weight, in CsCl

$$M_{C_s} = \frac{RT\rho_o}{\sigma^2\omega^2 r_s (\frac{\partial\rho}{\partial r})_{r_{s_{eff}}}} \qquad (18)$$

where R is the universal gas constant, T is the absolute temperature in degrees Kelvin, ρ_o is the initial density of the solution, or the density at the hinge point of the cell, ω is the angular velocity in radians/sec., r_s is the true distance of the peak from the center of rotation $(\frac{\partial\rho}{\partial r})_{r_{s_{eff}}}$ is the effective density gradient. Equation (19) evaluates the density gradient in terms of angular velocity (Vinograd, 1963).

$$(\frac{\partial\rho}{\partial r})_{r_{s_{eff}}} = (\frac{1}{\beta} + \psi\rho)(1-\alpha)\omega^2 r_s \qquad (19)$$

β is an empirical factor derived initially to relate rotor speed to the density gradient and has a value of 1.19×10^9, $\psi\rho$ equals 0.66×10^{-10} and corrects for pressure effects in the cell, and α equals 0.24 and relates water activity with buoyant density and solution density (Vinograd, 1963). Equation (19) will give the anhydrous molecular

weight in CsCl. Daniel (1969) has been able to determine a correction factor, G, that will convert this to the anhydrous molecular weight of Na DNA. The factor G = 1.05 is a function of solvation of the two different DNA salts and electronic gradient effects in the centrifuge cell, and its value seems to be independent of the type of DNA.

$$M_{Na} = G M_{Cs} \tag{20}$$

The molecular weight value obtained from equation (20) should then be extrapolated to zero DNA concentration, as determined by the maximum optical density of the DNA band. The optical density value in Fig. 17 indicated a concentration of 5.0×10^{-6} g/ml at the mid-point of the peak, which is considered small enough to be considered zero concentration. The value thus obtained for mitochondrial DNA, anhydrous sodium form, is 10.4×10^{6} daltons $\pm .6 \times 10^{6}$. The error is the maximum deviation from the average of three estimates and does not contain any error analysis of the correction factors. Also since the value obtained is slightly above zero concentration it would tend to be slightly on the large size.

Electron Micrographic Studies on Mitochondrial DNA

The electron microscope was employed to further elucidate the size and shape of mitochondrial DNA. The mitochondrial DNA was treated as described in the methods section. Plates 7, 8 and 9

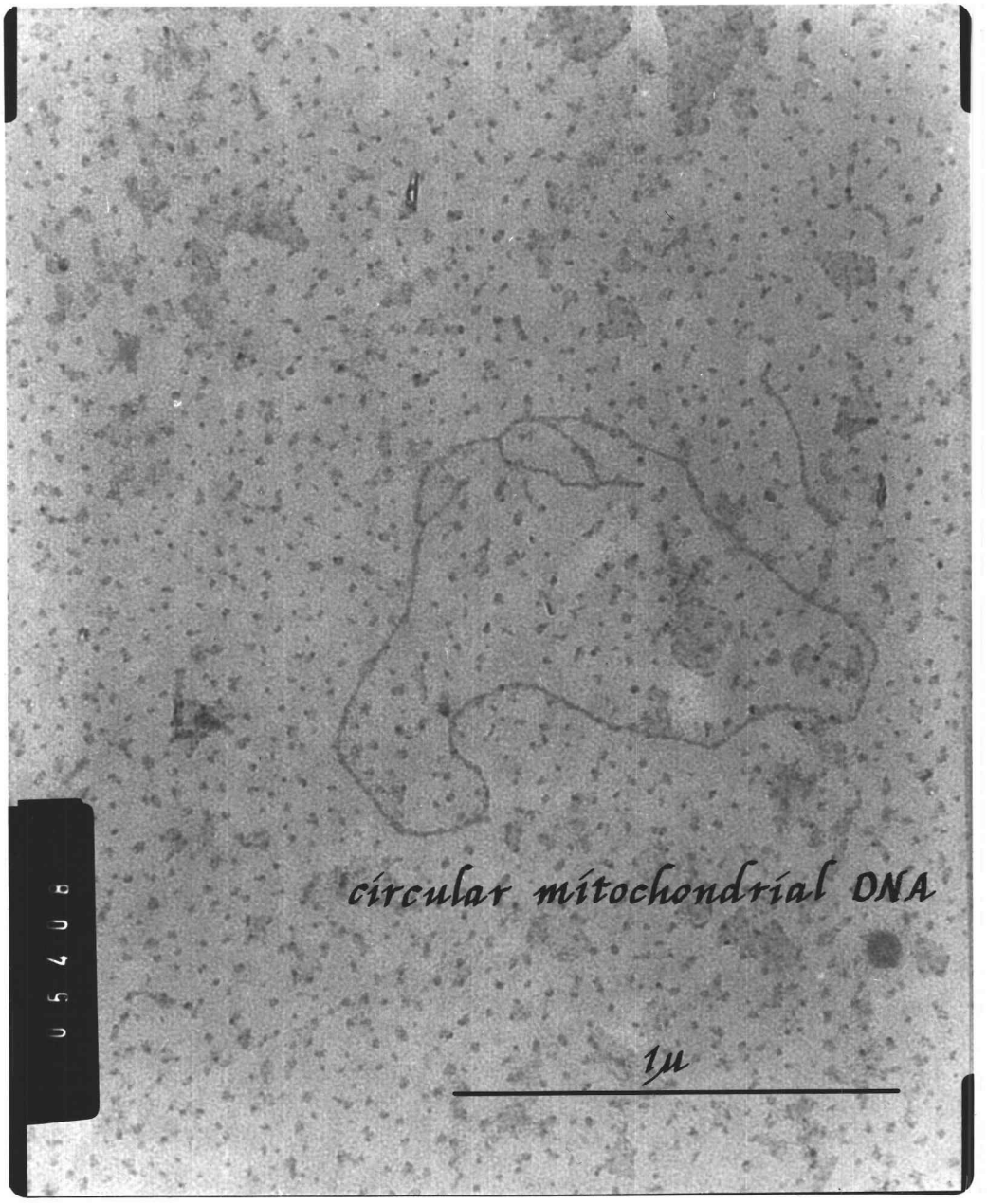

Plate 7. Mitochondrial DNA: one open circle

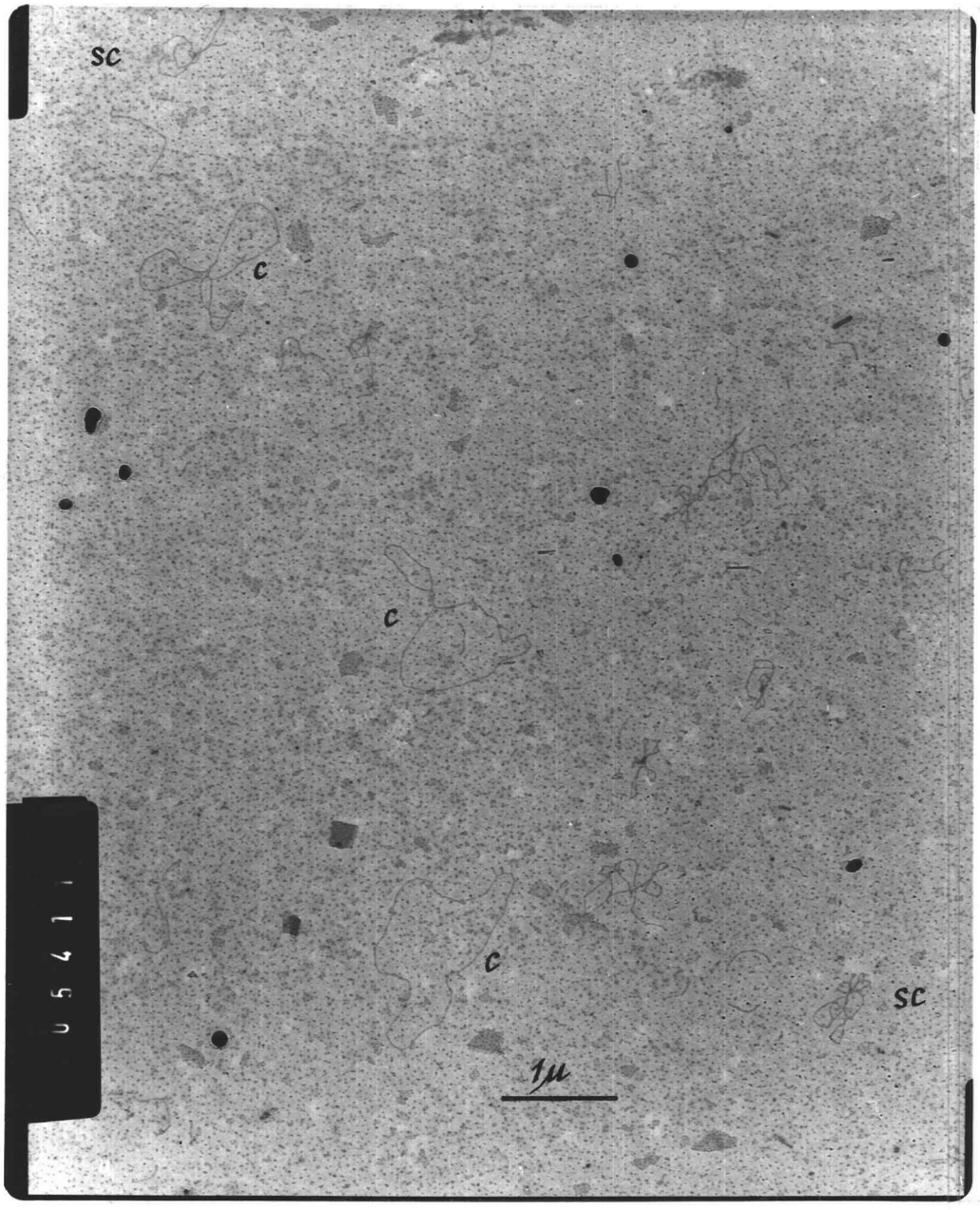

Plate 8. Mitochondrial DNA: open circles (C) and super coils (SC).

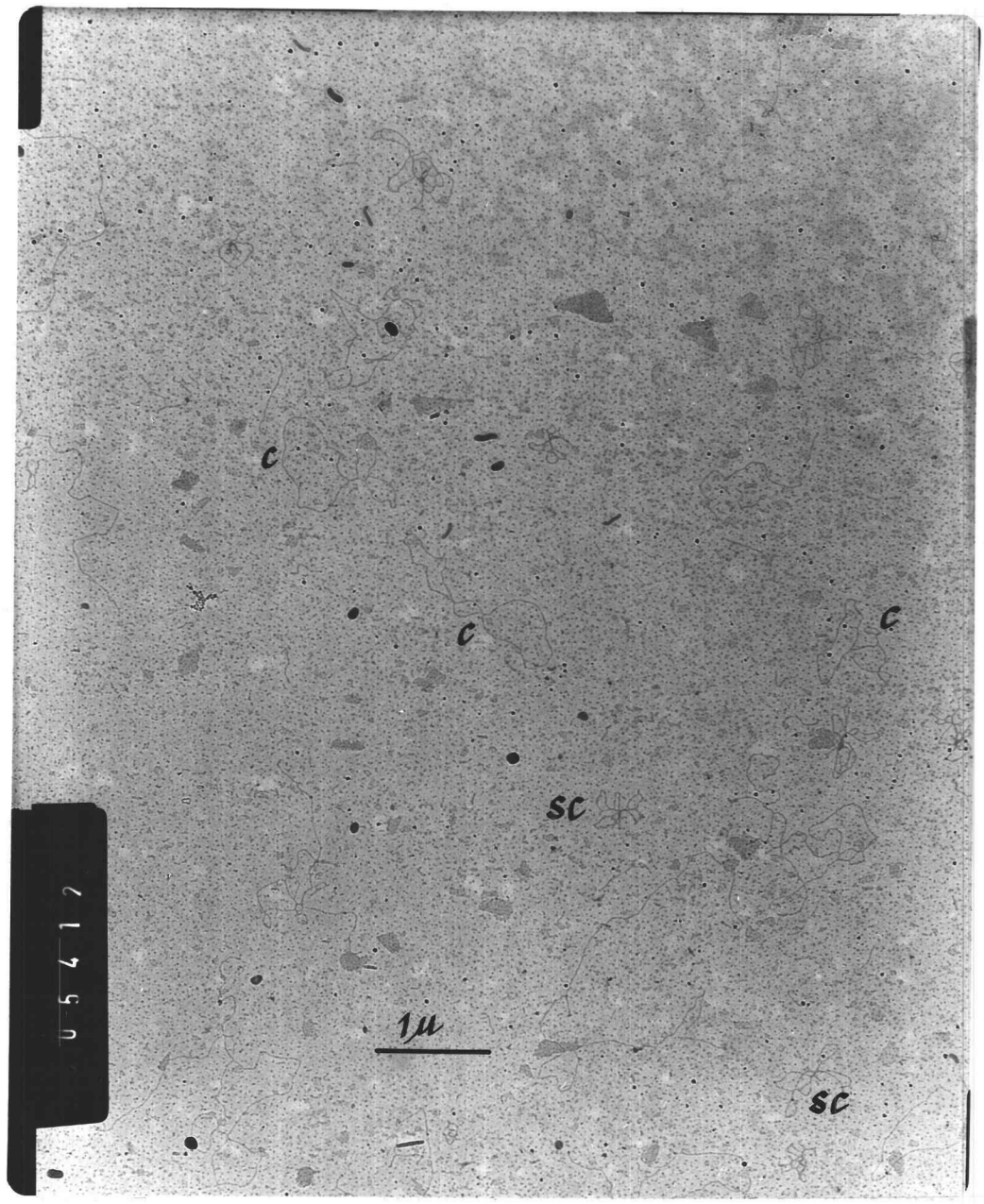

Plate 9. Mitochondrial DNA: open circles (C) and super coils (SC).

are photomicrographs of mitochondrial DNA at various magnifications. The grainy background is due to small crystals of uranyl acetate that were not filtered out. Plate 7 is a close up of a single circular DNA molecule. It was from molecules like this that contour lengths were measured. The molecule appears to be an open circle, as indicated by the lack of twisting. Plates 8 and 9 are photographs at lower magnifications which allowed one to obtain more molecules to measure per plate. As can be seen there seems to be a sizeable amount of non-circular material in the photograph. This is because only 20% of the total DNA is mitochondrial as indicated in the CsCl buoyant density centrifugation (Fig. 16). Only molecules that did not have free ends and whose contour could be followed were measured. Thus any linear mitochondrial DNA was not measured. Only circular and super coil DNA molecules were used in the contour determination. Super coils are covalently closed circular DNA and appear as highly twisted circular molecules. Molecules believed to be super coils are indicated in plates 8 and 9. A total of 26 molecules were measured and their true lengths were determined by dividing the contour lengths by the magnification factor of the electron microscope and the enlarger. The magnification at each tap setting for the electron microscope was checked, using a grating replica, 2,160 lines/mm (Ernest F. Fullham, Inc.), before the contour study and at the end and there was no appreciable drift. Fig. 19a is a histogram of the

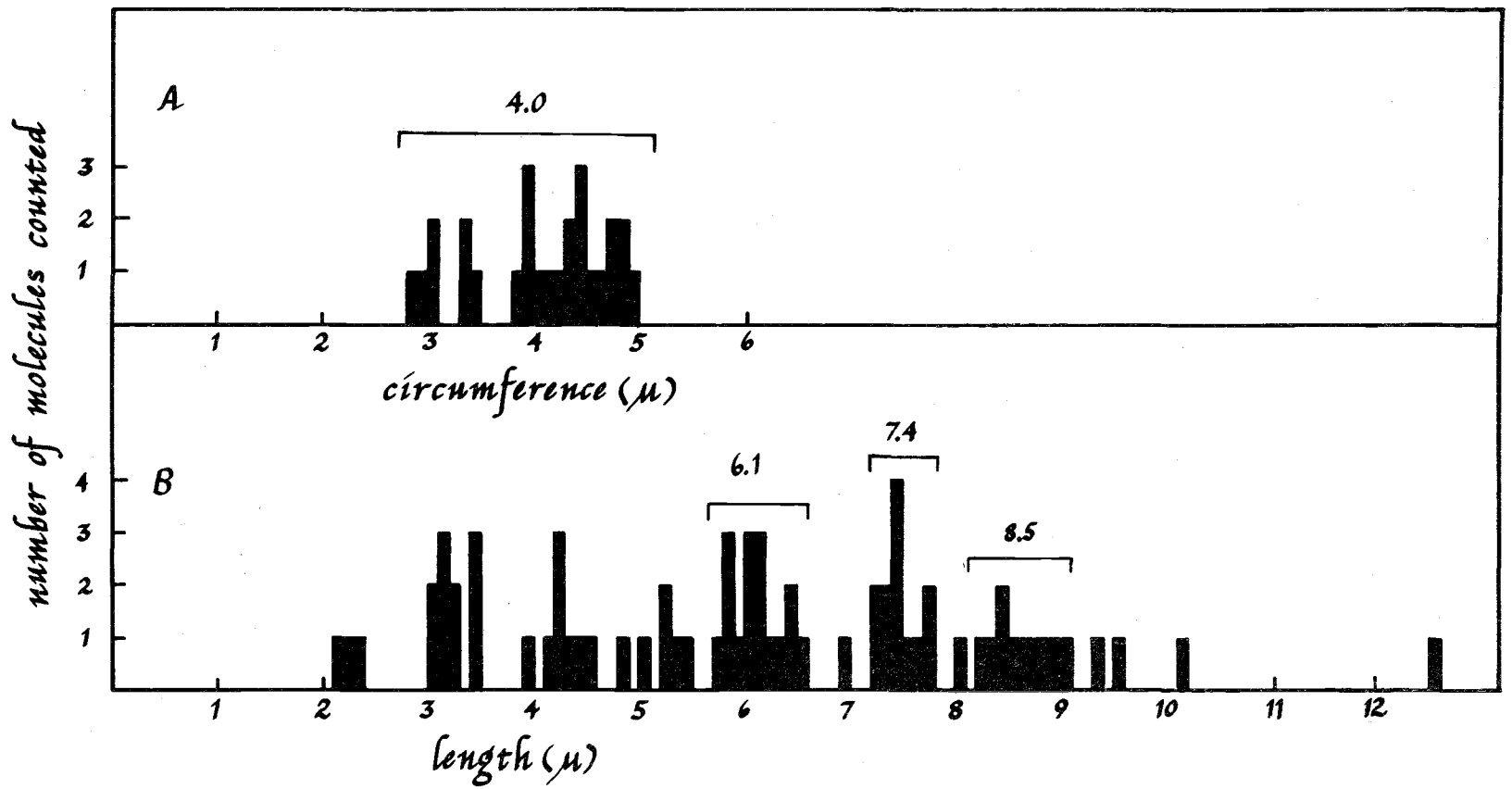

Figure 19. Histograms of mitochondrial circular DNA and T7 phage DNA. A) The distribution of the circumference lengths of mitochondrial DNA. B) The distribution of lengths of T7 phage DNA. Both DNA samples were picked up on formvar coated grids. T7 DNA was a generous gift from Dr. M. Nesson.

contour lengths of mitochondrial DNA, with the circumference reported to the nearest tenth of a unit. An average circumference was determined to be 4.0 μ.

Plate 10 shows a typical micrograph of T7 DNA which was used as a length standard. Since the molecular weight of T7 has been accurately determined (Lang, 1970) to be 25.55×10^6, a value for weight per length can be obtained by measuring the contour length. The contour length for 71 molecules of T7 is shown in the histogram in Fig. 19b. As can be seen the population of T7 molecules is very heterogeneous, probably due to shearing. In the histogram there appeared to be three regions of lengths. The smaller fragments were much too small to be considered anything but sheared pieces.[6]

The three regions indicated in the histogram give weight to length ratios of 4.2×10^6 daltons/μ, 3.44×10^6 daltons/μ and 3.00×10^6 daltons/μ respectively. From these values three possible molecular weights from the mitochondrial DNA were determined: 16.8×10^6 daltons, 13.7×10^6 daltons, and 12.0×10^6 daltons. If the average of the three regions is used, a weight to length value of 3.45×10^6 daltons/μ is attained. Using this figure the mitochondrial DNA has a molecular weight of 13.8×10^6 daltons.

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[6]When carbon coated grids are used, a weight to length value of about 2×10^6 daltons/μ is obtained. Formvar tends to shorten the measured length so a value slightly greater should be obtained.

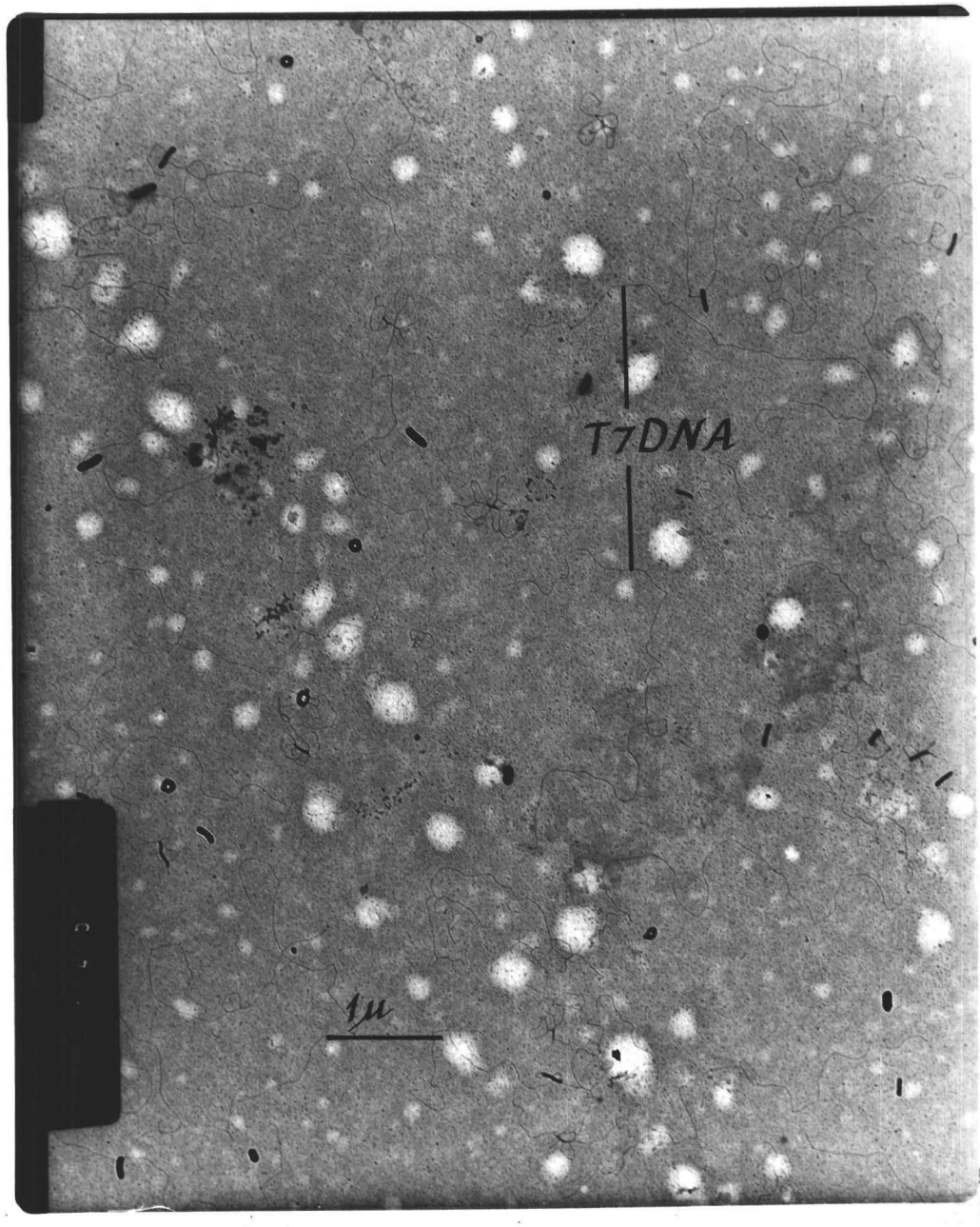

Plate 10. T7 DNA.

In Plate 10 it is obvious that there is a good number of molecules that cannot be measured because they are mixed with other molecules and there is also a large number of very small fragments. This makes the above computations very tenuous and therefore the molecular weight value should only be considered an order of magnitude value. If we assume that the amount of shrinkage on formvar is negligible and using 1.94×10^6 daltons/μ (Lang, 1970) as the weight to length value we can compute 7.8×10^6 daltons as a molecular weight. This value can be thought of as the lower limit and 16.8×10^6 daltons as the upper limit.

DISCUSSION

Nuclear DNA

It was of interest to compare the DNA isolated from the nucleus or mitochondria of the tobacco hornworm and compare the physical properties of these macromolecules with DNA from other organisms. In the study of the nuclear DNA an attempt was made to devise a procedure in which relatively high yields of DNA could be obtained. It was found that the majority of the thoracic muscle DNA was in a debris fraction. This was probably due to the fact that the nuclei are still bound by the sarcolemma. Table 5 gives a summary of the physical and chemical properties of the nuclear DNA.

The debris fraction contained 5% mitochondrial DNA and 95% nuclear DNA. The values for mole % GC for the debris DNA were slightly higher than the mole % GC of the nuclear DNA, but this is not considered a significant difference. The data for the nuclear fraction does not indicate the presence of any unusual bases since the mole % GC and the mole % AT total approximate 100%. The molecular weight of the DNA is quite large considering that it was isolated from eukaryotic tissue. This was probably due to the fact that during the isolation procedure the DNA solutions were kept as dilute solutions, about 10-50 μg/ml and the exposure to shearing forces was minimized (Eigner and Doty, 1965).

Table 5. Summary of data obtained on the nuclear and debris DNA from the tobacco hornworm.

	Nuclear	Debris
1. yield/100 gm tissue	2.0 mg	5.7 mg
2. chemical analysis for mole % GC	34.5%	--
3. CsCl buoyant density (% GC)	1.685 g/cc (34.6%)	1.673 g/cc (19.6%) minor 1.689 g/cc (35.6%) major
4. Spectral analysis - mole % AT	64.5%	--
5. T_m (% GC)	83.6°C (34.9%)	83.8°C (35.4%)
6. Hyperchromicity	.41	.44
7. ε (P)	6320	--
8. M_w	52-55 x 10^6 daltons	--
9. $Cot_{1/2}$	1.90 x 10^2	--
	rapid	unique
10. $Cot_{1/2}$ (corrected for mole % GC and separated into rapid and unique fractions)	2.56 x 10^{-3}	1.94 x 10^2
11. size of fractions	(20%) 1.73 x 10^6 daltons	(80%) 1.31 x 10^{11} daltons
12. size of total genome (based on total DNA)		1.64 x 10^{11} daltons
13. size of family	7.6 x 10^4 members	

$$\text{hyperchromicity} = \left[\left(\frac{A_{max} \text{ after heating}}{A \text{ at room temp}}\right) - 1\right] @ 260 \text{ nm}$$

ε(P) = molar extinction coefficient (on a nucleotide basis) of the room temperature form.

1 dalton = 1.67 x 10^{-24} gm.

In Table 6 the mole % GC for some insects is listed. The insects are grouped in Orders and the Orders are arranged in increasing taxonomic developmental order (Storer, 1951). As indicated, there does not appear to be any taxonomic correlation between the Order and the mole % GC. It is of interest to note that the tobacco hornworm has a very low mole % GC. The bee and Sarcophaga are the only insects with a lower mole % GC. The range of 30 to 45 mole % GC does not appear to be characteristic of insects as this spread can be found in most other classes of animals (C.R.C., 1968).

The hyperchromicity of thermally denatured DNA is inversely related to the mole % GC and the tobacco hornworm DNA appears to follow this generalization. Also the extinction of all DNA tends to be similar regardless of their base composition, as is also borne out by Table 7. Normal double stranded DNA in neutral 0.2 M Na solutions have molar extinction coefficients $\varepsilon(P)$ in the range of 6,100 to 6,900 (Mahler and Cordes, 1966).

Renaturation kinetics were used as another method of characterizing the nuclear DNA of the tobacco hornworm. The data obtained also can be related to the minimum amount of DNA in the nucleus, and may be employed to estimate the genetic relationship between closely related species. The kinetics of renaturation indicate a fraction of DNA (20%) which renatures very rapidly, and

Table 6. Mole % GC for some insects[a]

		Mole % GC	
Order Orthoptera			
1.	cricket nymphs	41.6	
2.	Locusta migratoria	41.2	.2 me Cyt.
Lepadoptera			
1.	Bombyx mori-whole	38.3	
2.	Gypsy moth- eggs	37.9	
3.	Oak moth - whole	36.2	
4.	Ceropia, silk moth	35.2	
5.	tobacco hornworm[b]	34.5	
Diptera			
1.	Drosophila[a] - imagos	45.0	
2.	Drosophila - whole	39.8	
3.	Chironomus plumosos	39.3	.2 ml Cyt.
4.	House fly - whole	39.2	
5.	Mosquito - larva	37.9	
6.	Sarcophaga bullata[c]	33.0	
Coleoptera			
1.	meal worm larve	41.1	
2.	Tenebrio molitor	40.8	
Hymenoptera			
1.	ant - pupa	42.7	
2.	bee - pupa larva	32.4	

[a] All values except b and c are from C. R. C. (1968)

[b] this work

[c] Laird and McCarthy, 1969

Table 7. Optical properties of representative deoxypolynucleotides

Deoxypolynucleotide	%GC	h	ε (P)	A/1 mg/ml
dG:dC in DNA (calc)[a]	100.0	0.18	7,000	
Sarcina lutea DNA[a]	63.0	0.29	6,690	20.2
Escherichia coli DNA[a]	50.0	0.39	6,740	20.3
Bacillus subtilis DNA[b]	43.5	0.32		
Drosophila melanogaster DNA[b]	43.0	0 38		
Calf Thymus DNA[a]	41.4	0.41	6,600	19.9
Bacillus megaterium DNA[a]	38.0	0.42	6,860	20.7
dA:dT in DNA (calc)[a]	0.0	0.56	6,650	
Tobacco hornworm[c]	34.5	0.41-.44	6,320	19.2

h = maximum thermal hyperchromicity at 260 nm

ε (P) = molar extinction coefficient

A/1mg/ml = the absorption of a solution at a concentration of 1 mg/1 ml @ 260 nm

[a]Mahler Cordes, 1966

[b]Laird and McCarthy, 1969

[c]This study

has a family size of 7.6×10^4. Most DNAs obtained from higher organisms possess a fraction of rapidly renaturing material (Britten and Kohn, 1968). Examples of this can be seen in the dipterans; Drosophila melanogaster, D. simulans, D. funebris and Sarcophaga bullata which contain 5%, 5%, 12% and 8% repetitious DNA respectively (Laird and McCarthy, 1969). The average family size in these insects is about 60 copies (Laird and McCarthy, 1969). In mammalian DNA such as bovine or mouse, 100,000 and 1,000,000 copies of repetitious DNA are present respectively (Britten and Kohn, 1968). The DNA from the tobacco hornworm is not like that of other insects studied, but is more like mammalian DNA in this respect. Recently mouse satellite DNA has been identified in the centromeric hetrochromatin of metaphase chromosomes (Pardue, 1970), and has been identified as a rapidly reassociating fraction of the mouse genome (Waring and Britten, 1966). Botchan et al. (1971) have shown that the highly repeated (rapidly renaturing) sequences found in D. melanogaster nuclear DNA are also associated in the centromeric regions of the chromosomes. They propose that these sequences might be involved in recognition and pairing of chromosomes during cell division, and in chromosomal folding.

From the above it would appear that the family size of the repeated sequence is related to the number of chromosomes, and the number of repeats per chromosome. If this is the case then

the size of the repetitious fraction could be an indirect measure of the number of chromosomes in the genome.

Correcting the $Cot_{\frac{1}{2}}$ values for repetitious fractions and base composition, minimum haploid genome sizes can be estimated. This is considered the minimum amount of DNA found within a given cell (nucleus), and does not give any estimate for polyploidy of cells. The minimum haploid genome size for the tobacco hornworm DNA is 1.64×10^{11} daltons which is 2.69 times as large as the D. melanogaster and 0.59 times the size of Sarcophaga bullata when Laird and McCarthy's (1969) results are corrected for mole % GC.

Using Laird and McCarthy's (1969) value for the genome size of the D. melanogaster a value of 1.0×10^{-13} gm. of DNA/haploid nucleus was obtained. This is in good agreement with Kurnick and Herskowitz's (1952) estimate of 1.7×10^{-13} gm. of DNA/haploid nucleus and Laird's (1971) value of 1.5×10^{-13} gm. of DNA/haploid nucleus. If the same analysis is used on the tobacco hornworm DNA a value of 2.7×10^{-13} gm. of DNA/haploid nucleus is found. As indicated by Laird (1971) this method might be an extremely sensitive method of estimating DNA in the cell.

Table 8. Comparison of $Cot_{1/2}$ values for some selected DNAs.

T4	0.2	Laird and McCarthy, 1969
B. subtilis	3.0	Laird and McCarthy, 1969
E. coli	4.0	Laird and McCarthy, 1969
D. melanogaster	80.0	Laird and McCarthy, 1969
D. fundebris	160.0	Laird and McCarthy, 1969
Sarcophagus bullata	480.0	Laird and McCarthy, 1969
Tobacco hornworm	190.0	This work
Bovine	1600.0	Laird and McCarthy, 1969

The $Cot_{\frac{1}{2}}$ values found in Table 8 are not corrected for base composition or repetitious fractions. The $Cot_{\frac{1}{2}}$ value for the tobacco hornworm DNA is about the same as the other insects indicated. The $Cot_{\frac{1}{2}}$ values for insects are higher than those of bacteria or phages, as would be expected from what we know about genome size. The $Cot_{\frac{1}{2}}$ values for insects are also smaller than the bovine $Cot_{\frac{1}{2}}$ value. Since mammals are deuterostomes and insects are protosomes there is not much value in comparing degrees of development between the two groups by using $Cot_{\frac{1}{2}}$ values since they are parallel branches in the phylogenetic tree. Unicellular organisms are at the base of the "tree" and can be considered to be relative to both branches.

Mitochondrial DNA

The DNA isolated from the mitochondria was never obtained in pure form. It contained a sizable amount of nuclear DNA contamination (about 80% of the total). From calculations based upon

a nuclear genome size of 1.64×10^{11} daltons per nucleus, a probable minimum of two mitochondrial DNA per mitochondrial entity (Nass, 1969) and a molecular weight of 1×10^{7} daltons for mitochondrial DNA, it can be estimated that the 80% contamination arises from one nucleus per 10,000 mitochondria. For this reason CsCl buoyant density gradients and electron microscopy were chosen as analytical tools to characterize the mitochondrial DNA.

From the buoyant density experiment it was found that the DNA from the mitochondria had a buoyant density of 1.674 g/cc which corresponds to a mole % GC of 20.1. The nuclear DNA had a buoyant density of 1.689 g/cc (35% GC). Thus it would be feasible to design a preparative scheme based upon the buoyant density difference of the two DNAs. This would eliminate the need to incubate the mitochondria with DNAse or wash the mitochondria prior to isolation of the DNA. These alterations in method would probably increase the yield of DNA from the flight muscle mitochondria.

The yield of mitochondrial DNA was determined to be 0.27 μg of DNA/mg. of mitochondrial protein. This is in good agreement with the value obtained from adult beef heart (0.25 μg of DNA/mg. of mitochondrial protein). This value is probably low since it is based upon the amount of DNA isolated by a phenol extraction method which does not give a quantitative yield. However, this is about half

the amount found in the livers of adult rats, hamsters, mice and chickens (Nass, 1969), which is about 0.5 μg of DNA/mg. of mitochondiral protein. If one examines rapidly dividing cells such as embryonic or cancer cells the amount of DNA/mg protein may increase to a level as high as 5.3 μg/mg protein (Nass, 1969). From this, it may be assumed that slower growing adult cells tend to have smaller DNA/protein ratios than rapidly dividing cells.

Table 9 is a compilation of the buoyant densities and mole % GC of the nuclear and mitochondrial DNAs from a variety of higher organisms. In this list only the insects belong to the protostomes, while the rest belong to the deuterostomes. After examining this list it is apparent that the mole % GC of the tobacco hornworm is lower than any other organism by at least 13%. In general the mole % GC in the insect's mitochondrial DNA seems lower than the mole % GC in the mitochondrial DNA of other animals. Another outstanding feature is that in the deuterostomes most nuclear DNA has a lower mole % GC than does the mitochondrial DNA. In the protostomes the opposite is true. The mitochondrial DNA is much lower in its mole % GC than the nuclear DNA. In addition there does not appear to be any correlation between base composition of the nuclear DNAs and that of the mitochondrial DNAs. The base composition of mitochondrial DNA appears to be quite variable, with the mole % GC varying between 20% and 58%. It is of interest to note that the base compositions of

Table 9. Mole % GC of mitochondrial and nuclear DNA's of some animals.

Animal		Mitochondrial		Nuclear		
		Density	% GC	Density	% GC	Ref.
Insecta	D. melanogaster	1.6919	33[a, b]	1.7038	45[a]	1
	D. simians	1.6927	34[a, b]	1.7041	45[a]	1
	D. funebris	1.6922	33[a, b]	1.7002	41[a]	1
		1.6860	27[a, b]			
	M. sexta (tobacco hornworm)	1.6735	20.1[c]	1.685	34.6[c]	2
Echinoidea	sea urchin	1.703	49.6[c]	1.694	40.6[c]	3
Amphibia	R. pipins	1.702	48.6[c]	1.702	48.6[c]	3
	X. laevis	1.704	50.7[c]	1.702	48.6[c]	3
Aves	chick	1.707	53.7[c]	1.698	44.6[c]	3
		1.708	54.7[c]	1.701	47.6[c]	3
	pigeon	1.707	53.7[c]	1.700	46.6[c]	3
	duck	1.711	57.7[c]	1.700	46.6[c]	3
Mammalia	mouse L cell	1.698	44.6[c]	1.703	49.6[c]	3
	mouse liver	1.699	45.6[c]	1.699	45.6[c]	3
	mouse brain	1.701	47.6[c]	1.702	48.6[c]	3
	rat liver	1.699	45.6[c]	1 699	45.6[c]	3
	guinea pig liver	1.702	48.6[c]	1.700	46.6[c]	3
		1.700	46.6[c]	1.700	46.6[c]	3
	sheep heart	1.703	49.6[c]	1.704	50.7[c]	3
	rabbit	1.703	49.6[c]	1.701	47.6[c]	3
	He La cells	1.707	53.7[c]	1.700	46.6[c]	3
	Chang liver cells	1.706	52.7[c]	1.700	46.6[c]	3
	Human leukaemic leukocytes	1.700	46.6[c]	1.689	35.6[c]	3
	Ox	1.703	49.6[c]	1.704	50.7[c]	4

[a] mole % GC taken directly from McCarthy and Laird (1968) data using E. coli = 1.7100 g/cc

[b] McCarthy and Laird (1968) report this DNA as non nuclear satellite DNA found in whole body preparations, and are considered mitochondrial in origin.

[c] mole % GC calculated from the formula; $\% GC = \frac{\rho - 1.6535}{.0997}$ as described in the methods, using E. coli = 1.7035 g/cc

Ref.

1. McCarthy and Laird, 1968
2. this work
3. Ashwell and Work, 1970
4. Borst, van Bruggen, and Ruttenberg, 1968

bacterial DNAs also vary a great deal, due to their high degree of mutability, and show a wider range of base compositions than the range found in nucleated cells. Mounolou, Jakob and Slonimski (1968) have shown that mitochondrial DNA is capable of mutating, giving rise to different types of cytoplasmic mutants in yeasts.

The mitochondrial DNA of all animals studied is found in a circular form (Ashwell and Work, 1970; Borst, vanBruggen and Ruttenberg, 1968). The size and shape of mitochondrial DNA from plants and protista tend to be more variable and larger in length than that found in animal cells. The circumference of the circular DNA from animals seems to be about 4.7-5.5 μ which corresponds to a molecular weight of 9.0×10^6 to 10×10^6 daltons (Nass, 1969). Borst, vanBruggen and Ruttenberg (1968) have reported that M. domestica (house fly) has a mitochondrial DNA that is 5.2 μ in circumference, as determined by electron microscopy using carbon coated grids. The circumference of the tobacco hornworm mitochondrial DNA appears to be 4.0 μ when prepared on formvar coated grids. The electron microscopic studies reported in this paper show that they can be found as circles and super coils. Super coils are covalently closed circles which appear as highly twisted circles. The discrepancy in circumference between the value determined for the tobacco hornworm and those reported above are more than likely due to the support material on the electron microscope grids. From

the molecular weight of 10.4×10^6 daltons as determined by band width in the ultracentrifuge and the daltons/μ (Lang, 1970) we can estimate what the contour length should be on a carbon coated grid. This is 5.36 μ, which is in good agreement with the cited values.

Even though molecules which appeared as highly twisted coils were present in the electron micrographs this was not considered conclusive proof that they were covalently closed circles, since the number of tertiary turns in the super coils were not counted. It has been shown that all DNA's found as super coils contain 3.7 tertiary turns per 1.0×10^6 daltons (Nass, 1969). The presence of super coils was demonstrated more conclusively in the "snap back" experiment (Figure 17). According to Dawid and Wolstenholm (1967) only covalently closed circles would be capable of retaining their original density, in a CsCl gradient, after being denatured with alkali. From this experiment it was shown that a portion of the mitochondrial DNA did indeed consist of covalently closed circles. Complete denaturation of DNA that is not in a super coil under these conditions is indicated by the complete shift of the nuclear DNA peak.

Since the mitochondrial DNA was never free of a nuclear contamination the sedimentation coefficients of the different forms were not determined. Vinograd et al. (1965) observed changes in the sedimentation coefficients for polyoma virus DNA as a function of pH, from which they were able to elucidate the different forms of

the DNA. This has been done on frog mitochondrial DNA by Dawid and Wolstenholm (1967), on chick liver mitochondrial DNA by Borst, vanBruggen and Ruttenberg (1968) and on sheep heart mitochondrial DNA by Kroon et al. (1966). The different forms are shown in Figure 20. The s values are those found using chicken liver and are included because they are representative of the values found for other mitochondrial DNAs. Form I is the super coil, Form II is what is considered circular DNA, Form III is the rod and the rest are forms in different states of denaturation. In the course of this study I believe that forms I and II have been observed and identified but form III could not be found because of contaminating nuclear DNA.

In the "snap back" experiment it was found that the amount of super coils, form I, depended in part upon the amount of handling of the DNA. The maximum amount of super coils found was 40% with the remaining 60% composed of a mixture of the other forms. Borst, vanBruggen and Ruttenberg (1968) report that Form I of mitochondrial DNA from chick liver occurs at a level as high as 80%, and is also sensitive to handling. Carnegie (1970) has found circular DNA in a nuclear polyhedric virus which contained almost 100% super coils when isolated on ethidium bromide gradients but reverted to the other forms when dripped from a centrifuge tube. It appears that the mitochondrial DNA of mature tissue (slowly dividing) is found in situ as covalently closed circles, and forms II and III might be

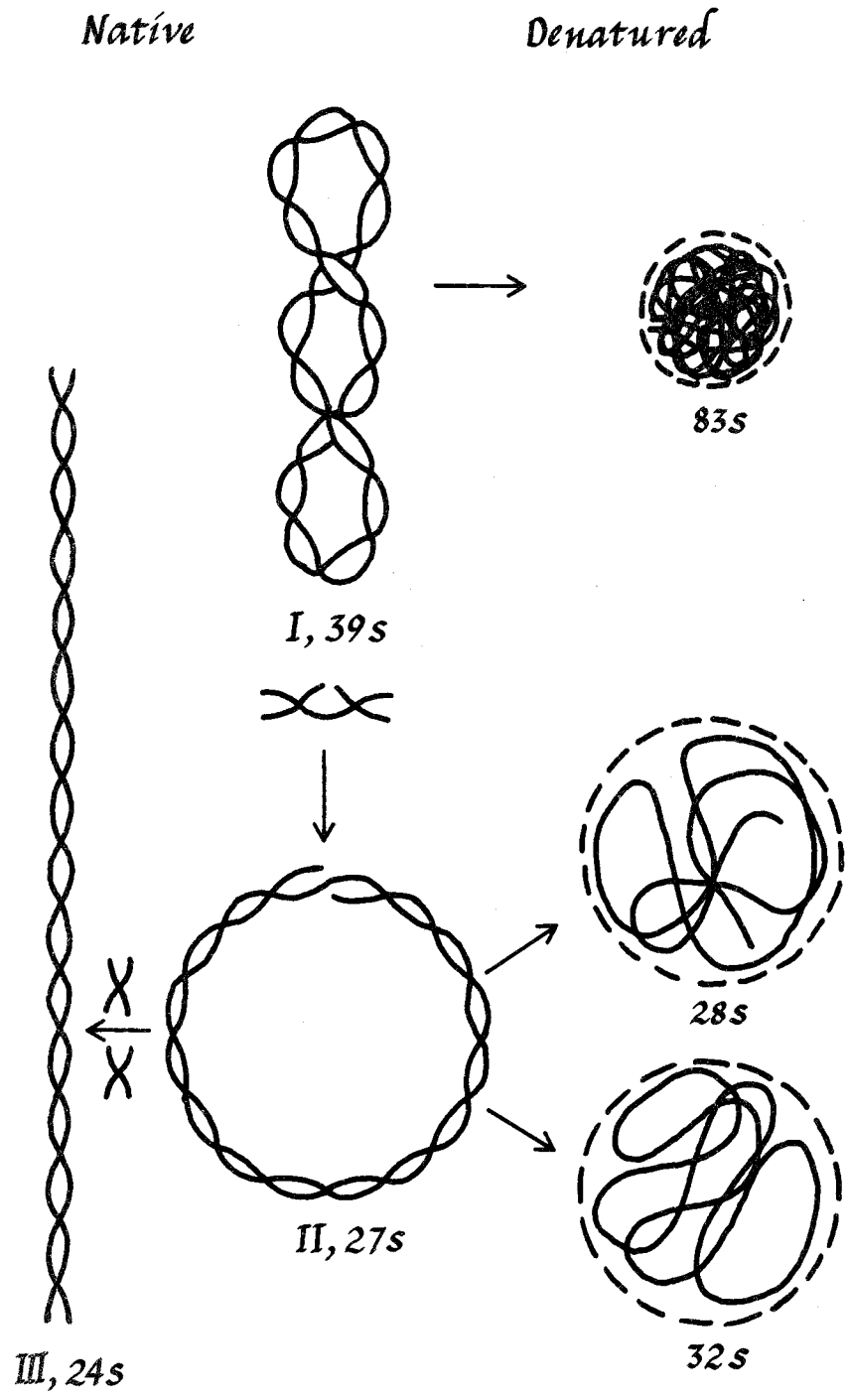

Figure 20. Diagramatic representation of the various forms of mitochondrial DNA. This diagram is taken from work done by Kroon, Borst, van Bruggen and Ruttenberg (1966). Component I is the twisted circular form, component II is the open circular form, and component III is the linear (rod) form. The denatured forms are enclosed by their relative hydrodynamic diameters. 83s is the alkali form of component I. The 28s form is the linear single stranded fragment from component II and the 32s form is the single stranded circular fragment from component II.

artifacts brought about by isolation, though a control mechanism for replication involving one of the other forms could still be present in dividing tissue. Nass (1969) has shown that in rapidly growing L cells and ascites tumor cells there was a very small percent of mitochondrial DNA in the super coil form and the majority of that DNA was open or loosely coiled. DNA liberated from adult liver cells was found almost entirely as super coils.

It appears now that all animal mitochondrial DNA is found in a circular form with a circumference of about a 5.0 μ. The base composition seems to be quite variable, indicating that 5.0 μ is the minimum size allowable to function genetically in animal cells. The larger mitochondrial DNAs from plants and protesta may contain more information than necessary or an abundance of redundant sequences (Ashwell and Work, 1970). Though dimers and catenans have been found these are usually associated with abnormal mitochondria (Nass, 1969; Clayton and Vinograd, 1967; Hudson and Vinograd, 1969).

From simple calculations it can be estimated that the informational content of the mitochondrial DNA is small and could only code for 5,000 amino acids or 30 proteins of molecular weight of 20,000. Kadenbach (1968) has shown that not only is cytochrome C coded for by the nucleus but that there is an actual transfer of microsomal proteins into the mitochondria. Also chromosomal "petites" in

yeast have been identified, and are different from mitochondrial "petites."

The evidence seems to indicate that the mitochondria are capable of synthesizing their own specific RNAs such as ribosomal and transfer RNA (Nass, 1971). Also some of the structural proteins associated in the inner membrane have been shown to be synthesized by isolated mitochondria (Beattie, Basford and Koritz, 1967). On the other hand, it seems likely that the majority of the proteins and enzymes associated with the mitochondria are coded by the nuclear chromosomes. When one compares the capability of the E. coli genome, which does not contain many (if any) redundant sequences, and has a molecular weight (genome size) of 2.7×10^9 daltons (Laird and McCarthy, 1969) to that of mitochondrial DNA of 1×10^7 daltons we can see why it would be impossible for the mitochondria to be autonomous. It has been estimated (Attardi and Attardi, 1970) that just to synthesize the machinery necessary for protein synthesis would require a genome six times larger than the mitochondrial genome. So, what seems to be the case is that the mitochondrial and nuclear DNA both are involved in the biogenesis of the mitochondria, and the mitochondria can only be considered semiautonomous.

The mitochondrial DNA from the tobacco hornworm appears to be similar in size and shape to the mitochondrial DNA from other animals. The base composition of all mitochondrial DNA varies but

none have as low a GC content as that of the tobacco hornworm. It would be worth investigating to learn if this is a general trend in the insects and to see what significance it has in relation to the protein synthesizing capabilities of the mitochondria. It would also be interesting to determine if all mitochondrial DNA from insects is lower in mole % GC than respective nuclear DNA, and if this pertains only to insects or to protosomes in general.

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