STUDIES OF THE REPLICATIVE INTERMEDIATES AND OF THE STRUCTURE OF ANIMAL CELL MITOCHONDRIAL DNA

Thesis by

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Abstract

Studies on the replication and structure of animal cell mitDNA are reported in the four sections of this thesis.

I. Density Labeling of HeLa mitDNA with 5-Bromodeoxyuridine

Bromouracil labeling of the mitochondrial DNA in exponentailly growing HeLa cells produces two hybrid mitochondrial DNA species, with density shifts of 41.9 and 54.0 mg/ml relative to unlabeled mitochondrial DNA, as well as heavy mitochondrial DNA, with a shift of 95.3 mg/ml. The two hybrid species result from the difference in thymine composition of the complementary strands of mitochondrial DNA. In addition, mitochondrial DNA with a density intermediate between the hybrid and unlabeled species was found. This quarter heavy mitochondrial DNA represents 25% (w/w) of the total DNA after eight hours of labeling, and forms two peaks with shifts of 20.6 and 27.0 mg/ml relative to unlabeled mitochondrial DNA. 70% (w/w) of the quarter heavy mitochondrial DNA is in catenated forms, while 30% (w/w) is monomeric. Degradation of the catenanes by shearing of purified quarter heavy mitochondrial DNA results in the appearance of hybrid and unlabeled mitochondrial DNA bands, demonstrating that the quarter heavy catenanes contain both hybrid and unlabeled submolecules. The implications of the structure of the quarter heavy catenanes on the mechanism of formation of catenanes are discussed.

II. The Isolation of Complementary Strands of mitDNA and the Mapping of the rDNA and tRNA's on the L Strand Relative to the Origin of Replication

The alkali lability of mitDNA makes the preparation of intact separated strands for electron microscope mapping studies difficult. Even at pH 11.6 (the minimum pH required for strand separation of singly nicked HeLa mitDNA) the strands suffered an average of one additional nick per strand during the alkaline buoyant CsCl banding. Covalently closed mitDNA did not nick under these conditions. The rapid nicking of the BrUra-labeled strands enabled preparations of L strands containing 30 wt% single stranded circles to be obtained from covalently closed BrUra hybrid mitDNA.

The relative positions of the ribosomal RNA gene complement (rDNA) and the tRNA sites on the L strand relative to the origin of replication (7S DNA) were determined by hybrid mapping in the electron microscope using the above L strand preparation. The center of the rDNA is located almost directly opposite (0.5 G) the 7S DNA, and the L3 tRNA site is very near to one end of the 7S DNA.

III. MitDNA Replicative Intermediates: Isolation by Benzoylated DEAE-Cellulose Chromatography and Enzymatic Analysis of Structure

Replicative intermediates of LA9 mitDNA were partially purified from clean duplex DNA by virtue of the binding of their single stranded regions to benzoylated DEAE-cellulose. The purification was incomplete because the clean duplex DNA elutes as though a portion of the molecules

contain denatured regions (or regions which denature in response to the column environment). Similar results were obtained with closed and nicked PM2 viral DNA. SV40 viral DNA eluted normally.

These enriched preparations of replicative intermediates and the clean duplex DNA free of replicating forms were subjected to enzymatic analysis with T4 DNA polymerase. The results show that: (1) the nicks in the upper band clean duplex DNA are not preferentially located in either strand; (2) the gapped molecules in the upper band have a greater overall deficiency of L than H strand DNA by a ratio of 2.5 to 1 (implying that these molecules probably represent displaced H strands with incomplete complement synthesis); and (3) the 7S DNA in the lower band D-loop forms is not measurably extended by the enzyme, although label was incorporated (suggesting that a superhelix restriction on the further extension of the D-loop exists in isolated mitDNA). Attempts to covalently close the upper band clean duplex DNA with combinations of E. coli exonuclease III, polymerase I, and ligase, as well as T4 polymerase, were unsuccessful.

IV. A Method for the Detection of Ribonucleotides in mitDNA

A method for the detection of ribonucleotides in mitDNA has been partially developed. The procedure involves alkaline hydrolysis to expose ribonucleotides at the 3' ends of mitDNA fragments, removal of terminal phosphates with alkaline phosphatase, labeling by oxidation-reduction with $NaIO_4$-^3H-$NaBH_4$, removal of unincorporated ^3H counts by extensive dialysis, spleen acid DNase II and spleen exonuclease digestion to ribonucleoside trialcohols and deoxynucleoside-3'-mono-

phosphates, removal of the latter on DEAE-cellulose, and identification of labeled ribonucleoside trialcohols by thin layer chromatography and fluorography. The feasibility of the analysis is demonstrated, but attempts to label mitDNA were unsuccessful due to the unreliability of commercial preparations of ^{3}H-$NaBH_4$ of the high specific activity required for these experiments. The method is reported here in the event that high specific activity ^{3}H-$NaBH_4$ becomes available.

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5-Bromodeoxyuridine Labeling of Monomeric and Catenated Circular Mitochondrial DNA in HeLa Cells

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Bromouracil labeling of the mitochondrial DNA in exponentially growing HeLa cells produces two hybrid mitochondrial DNA species, with density shifts of 41·9 and 54·0 mg/ml relative to unlabeled mitochondrial DNA, as well as heavy mitochondrial DNA, with a shift of 95·3 mg/ml. The two hybrid species result from the difference in thymine composition of the complementary strands of mitochondrial DNA. In addition, mitochondrial DNA with a density intermediate between the hybrid and unlabeled species was found. This *quarter heavy* mitochondrial DNA represents 25% (w/w) of the total DNA after eight hours of labeling, and forms two peaks with shifts of 20·6 and 27·0 mg/ml relative to unlabeled mitochondrial DNA. 70% (w/w) of the quarter heavy mitochondrial DNA is in catenated forms, while 30% (w/w) is monomeric. Degradation of the catenanes by shearing of purified quarter heavy mitochondrial DNA results in the appearance of hybrid and unlabeled mitochondrial DNA bands, demonstrating that the quarter heavy catenanes contain both hybrid and unlabeled submolecules. The implications of the structure of the quarter heavy catenanes on the mechanism of formation of catenanes are discussed.

1. Introduction

Density labeling has been used to observe the replication of mitochondrial DNA in Neurospora (Reich & Luck, 1966), using ^{15}N as the density label, and in rat liver (Gross & Rabinowitz, 1969), with bromodeoxyuridine. In both cases, the results are consistent with a semiconservative mode of replication, but the buoyant density shifts are too small (15 mg/ml or less) to permit the separation of the different labeled species. The purposes of this study initially was to examine the buoyant densities of duplexes and single strands of mitDNA† from HeLa cells grown in the presence of BrdUrd. One heavy and two hybrid duplex species were formed as expected for the semiconservative replication of a duplex DNA having strands of different thymine composition. In addition, an unexpected species with a buoyant density between hybrid and light mitDNA was formed. These *quarter heavy* molecules were found to be predominantly catenanes, and were shown to contain both hybrid and light monomer submolecules topologically bonded to each other. The implications of this new structure on the possible modes of formation of catenanes are discussed.

A study of the mitDNA in mouse L cells (Kasamatsu *et al.*, 1971) has shown that intermediates containing a replicating displacement loop (D-loop) occur at a high

† Abbreviations used: mitDNA, mitochondrial DNA; EthBr, ethidium bromide; nDNA, nuclear DNA.

frequency. Similar forms are present in HeLa cells. The buoyant densities of light molecules containing a small D-loop would not be significantly affected (approximately 2 mg/ml) if the short 7 S displacing strand were density labeled. The other replicating intermediates are present at too low a frequency to interfere with the examination of the mature density labeled molecules.

2. Materials and Methods

(a) *Isolation and purification of total mitochondrial DNA*

Suspension cultures of HeLa cells were grown in the dark in Eagle's phosphate medium (Grand Island Biological Co., Berkeley, Calif.) containing 5% calf serum, 20 μg (BrdUrd/ml (7×10^{-5} M) and (in initial experiments) 0·1 to 0·01 μg FUrd/ml (0·03 to $0{\cdot}003\times10^{-5}$ M). The cells were harvested by centrifugation at 1000 *g* for 3 min and kept at 0 to 4°C thereafter. After two washes with 0·14 M-NaCl, 0·005 M-KCl, 0·0007 M-Na_2HPO_4, 0·025 M-Tris, pH 7·4 (TD buffer), the cells were suspended in a 10-fold vol. of 0·01 M-NaCl, 0·0015 M-$MgCl_2$, 0·01 M-Tris, pH 7·4 (RSB buffer), and allowed to swell for 10 min before disrupting them with 2 to 4 strokes in a Dounce homogenizer. Sucrose was immediately added to a concentration of 0·25 M and nuclei were removed by pelleting at 1000 *g* for 3 to 5 min. The crude mitochondria were pelleted at 27,000 *g* for 10 to 15 min, taken up in MS buffer (0·21 M-mannitol, 0·07 M-sucrose, 0·001 M-EDTA, 0·01 M-Tris, pH 7·4), and sedimented through 1·0 M-sucrose to an interface with 1·5 M-sucrose in a Spinco SW27 rotor at 27,000 revs/min at 4°C for 45 min. After a wash with MS buffer, the mitochondria were taken up in 0·25 M-sucrose, 0·025 M-KCl, 0·0025 M-$MgCl_2$, 0·05 M-Tris (pH 6·7), and treated with 50 to 100 μg DNase I/ml and 100 μg RNaseA/ml for 30 min at 37°C. Digestion was stopped by chilling and adding EDTA to 0·04 M. The mitochondria were washed three times with MS buffer and lysed by adding sodium dodecyl sulfate to 1% for 5 to 10 min at 25°C. Sodium dodecyl sulfate was precipitated by adding CsCl to 1 M and chilling; the precipitate was removed by centrifugation at 27,000 *g* for 5 to 10 min. Ethidium bromide was added to 300 μg/ml, the density was adjusted to 1·65 g/ml with CsCl, and the solution was centrifuged at 35,000 to 40,000 revs/min for 24 h in an SW50.1 rotor. The entire DNA-containing region of each tube was recovered by drop-collection in ultraviolet light. EthBr was removed with a Dowex-50 column (0·5 cm × 2 to 5 cm); CsCl was removed by dialysis against 0·01 M-Tris (pH 7·5), 0·001 M-EDTA. In later experiments, small nuclear DNA fragments remaining after the DNase digestion were removed by velocity sedimentation. Samples of 0·3 to 0·6 ml were layered onto 4·0 ml of CsCl (density 1·40 g/ml), 100 μg EthBr/ml, 0·01 M-Tris (pH 7·5), 0·001 M-EDTA, and centrifuged in an SW50.1 rotor for 3 h at 38,000 revs/min at 20°C. The tubes were fractionated by drop-collection; the lower part of the gradient was consolidated and treated as described above to remove the EthBr and CsCl.

(b) *Preparation of nuclear DNA*

A small amount of the nuclei pelleted after the homogenization step above was lysed by adding sodium dodecyl sulfate to 1%. After 5 to 10 min at 25°C, sodium dodecyl sulfate was removed by chilling, adding CsCl to 1 M, and centrifuging at 27,000 *g* for 5 to 10 min. EthBr was added to 300 μg/ml, the density of the solution was adjusted to 1·65 g/ml with CsCl, and the solution was centrifuged as above. The DNA region was collected, EthBr was removed, and the sample was dialyzed as above. DNA obtained by phenol extraction gave the same buoyant density patterns.

(c) *Analytical centrifugation*

Buoyant density experiments were performed at 25°C for 24 h at either 44,000 or 44,770 revs/min in a Beckman model E ultracentrifuge equipped with a photoelectric scanner. Alkaline buoyant densities were measured in 0·05 M-K_3PO_4, 0·05 M-KOH (pH 12·5). The buoyant density gradient (Vinograd & Hearst, 1962) was used in the calculation of the results in both the neutral and alkaline experiments. The relative amounts of

DNA in different peaks were determined by ordinate summation, either enlarging the trace from the Offner recorder in the scanner or measuring directly from a scan recorded on a Moseley 7001 AM recorder. Correction was made for the sector shape of the centrifuge cell.

(d) *Analysis of DNA in CsCl gradients by fluorescence*

Density-labeled mitDNA samples were centrifuged in CsCl with a density of 1·68 g/ml at 35,000 revs/min for 48 h in an SW50.1 rotor. The gradients were collected into tubes containing 0·5 ml of 10 μg EthBr/ml, 0·1 M-NaCl, 0·01 M-Tris (pH 7·5), 0·001 M-EDTA. The fluorescence of each fraction was measured in a Hitachi Perkin–Elmer model MPF2A fluorescence spectrophotometer using a microcell. The excitation and emission wavelengths were 380 and 586 nm, respectively.

(e) *Hydrodynamic shearing of DNA*

Shearing of mitDNA was performed in a capillary shear apparatus (Yew & Davidson, 1968). The DNA samples were at a concentration of 0·2 to 0·8 μg/ml in 2 μg EthBr/ml, 0·01 M-Tris (pH 7·5), 0·001 M-EDTA. Each sample passed through the capillary 400 to 500 times under 150 lb/in^2 of nitrogen at 25°C.

(f) *Electron microscopy and fluorescence photography*

DNA was mounted for electron microscopy by a modified aqueous or formamide Kleinschmidt procedure followed by rotary shadowing with platinum/palladium. Grids were scored by the procedure described by Clayton *et al.* (1968), using a Philips EM300 microscope. Length distributions of the sheared samples were obtained by photography, enlarging and tracing the molecules with a Nikon 6F projection microcomparator, and measuring the contour length with a map measurer.

The camera system described by Watson *et al.* (1971) was used to photograph EthBr/CsCl gradients.

(g) *Reagents, enzymes and substrate DNA*

5-Bromodeoxyuridine was obtained from Schwarz/Mann, Inc., Orangeburg, N. Y. Fluorouridine was a gift from Hoffman-La Roche, Inc., Nutley, N. J.; ethidium bromide was a gift from Boots Pure Drug Co. Ltd, Nottingham, England. Optical grade cesium chloride was obtained from the Harshaw Chemical Company, Solon, Ohio. DNase I and RNase A were obtained from Sigma Chemical Co., St. Louis, Mo. DNase II was supplied by Worthington Biochemical Corp., Freehold, N. J. Closed PM2 viral DNA was a gift from Drs B. M. J. Révet and M. Schmir in our laboratory. All other chemicals were reagent grade.

3. Results

(a) *Assay for nuclear DNA contamination of total mitochondrial DNA preparations*

In the absence of density label, closed mitDNA is readily freed of nDNA as a lower band in an EthBr/CsCl gradient. This procedure is not applicable to BrUra-labeled mitDNA preparations because the density shift due to BrUra substitution is approximately the same as the EthBr-induced shift in closed, unlabeled mitDNA (Plate I(b) and (c)). We therefore adopted a procedure in which contaminating nDNA was digested to small fragments by DNase treatment of the mitochondria and removed in a later velocity sedimentation (Plate II) of the mitDNA sample. The amount of contaminating nDNA remaining after this treatment was assayed by examining the buoyant profiles of the total mitDNA from unlabeled cells (Plate III(a) and (b)). A small amount of high molecular weight nDNA appears at neutral pH as a slight skewing of the peak to the light side. The nDNA contamination estimated by this criterion is less than 5%(w/w). The nDNA bands between the two mitDNA

strands in alkaline CsCl and again accounts for 5% of the optical density. The flat baseline in both cases indicates that the sample is free of small nDNA fragments. When the sedimentation velocity step is omitted, fragments of nDNA give rise to broad, curved baselines (Plate III(c)).

(b) *Neutral buoyant densities of bromodeoxyuridine-labeled DNA species*

Buoyant density scans of BrUra-labeled mitDNA samples are presented in Plate IV. In each case, the band at the left is unlabeled mitDNA, which was used as an internal marker. We checked its buoyant density by adding *Micrococcus lysodeikticus* DNA to several preparations and obtained the value 1·6998±0·0005 g/ml (nine determinations), in good agreement with the value of 1·6996 g/ml found for human leukocyte mitDNA (Clayton *et al.*, 1970). The buoyant density of *M. lysodeikticus* DNA was taken to be 1·7257 g/ml (Bauer *et al.*, 1971). Similarly, unlabeled nDNA was used as an internal marker for density-labeled nDNA. Its buoyant density, also referred to *M. lysodeikticus* DNA present as a marker, is 1·6912±0·0009 g/ml (four determinations), slightly higher than the value of 1·6896 g/ml reported for human leukocyte nDNA (Clayton *et al.*, 1970). When the amount of unlabeled nDNA was insufficient (Plate V), hybrid nDNA was used as the reference.

Three major and two minor BrUra-labeled mitDNA species appear to the right of the unlabeled mitDNA in Plate IV. The band furthest to the right, with a separation of 95·3 mg/ml (Table 1), contains the heavy mitDNA and consists of molecules with both strands density labeled. A semiconservative mechanism for replication predicts two hybrid species for HeLa mitDNA because of the difference in thymine content of the two strands. The BrUra content and, therefore, the density of the hybrid molecule will depend on which strand has been labeled. The two major bands in the center have an average separation of 48·0±2·2 mg/ml, which is 0·50±0·02 of the heavy mitDNA separation. These bands, therefore, contain the two hybrid species.

The two minor species between the hybrid and unlabeled bands are called quarter heavy because their average separation, 23·8±1·3 mg/ml (Table 1), is 0·25±0·01 of the heavy mitDNA separation. They are also half way between hybrid and unlabeled

TABLE 1

Buoyant separations of bromouracil-labeled mitochondrial DNA species in neutral cesium chloride

Band	mitDNA (mg/ml)	mitDNA (FUrd)† (mg/ml)	Nuclear DNA (mg/ml)
Quarter heavy	20·6±0·8 (15)	not determined	not applicable
	27·0±1·0 (16)	not determined	not applicable
Hybrid light	41·9±1·5 (18)	44·2±1·8 (6)	52·7±1·2 (5)
Hybrid heavy	54·0±1·7 (18)	57·1±2·2 (6)	
Heavy	95·3±1·8 (8)	100·8±3·0 (5)	104·8±1·9 (3)

Separations for mitDNA are given relative to unlabeled mitDNA at 1·6998±0·0005 g/ml (nine determinations). Those for nuclear DNA are relative to unlabeled nuclear DNA at 1·6912±0·0009 g/ml (4 determinations). The numbers in parentheses after each value are the number of determinations which were averaged together.

† From cells labeled with BrdUrd in the presence of 0·01 μg of FUrd/ml.

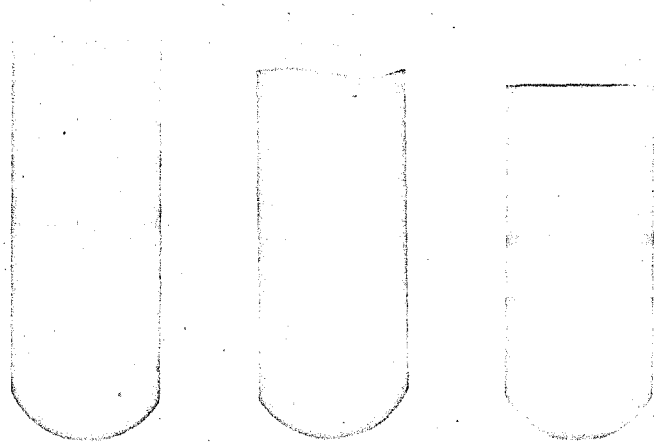

PLATE I. Fluorescent bands formed in EthBr/CsCl buoyant density gradients. (a) mitDNA from cells labeled with BrdUrd for 44 h; (b) nuclear DNA from cells labeled for 44 h; (c) mitDNA from untreated cells. Centrifugation conditions are described in Materials and Methods, section (a). The tubes were photographed in ultraviolet light as described in Watson *et al.* (1971).

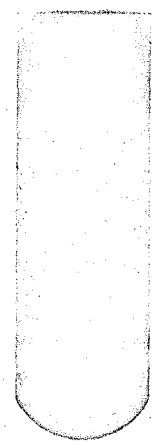

PLATE II. The purification of bromouracil-labeled mitDNA (lower bands) by velocity sedimentation to remove nuclear DNA fragments. The diffuse band of nuclear DNA near the meniscus at the top of the photograph was discarded. Centrifugation conditions are described in Materials and Methods, section (a). The tube was photographed as in Plate I.

[*facing p. 84*

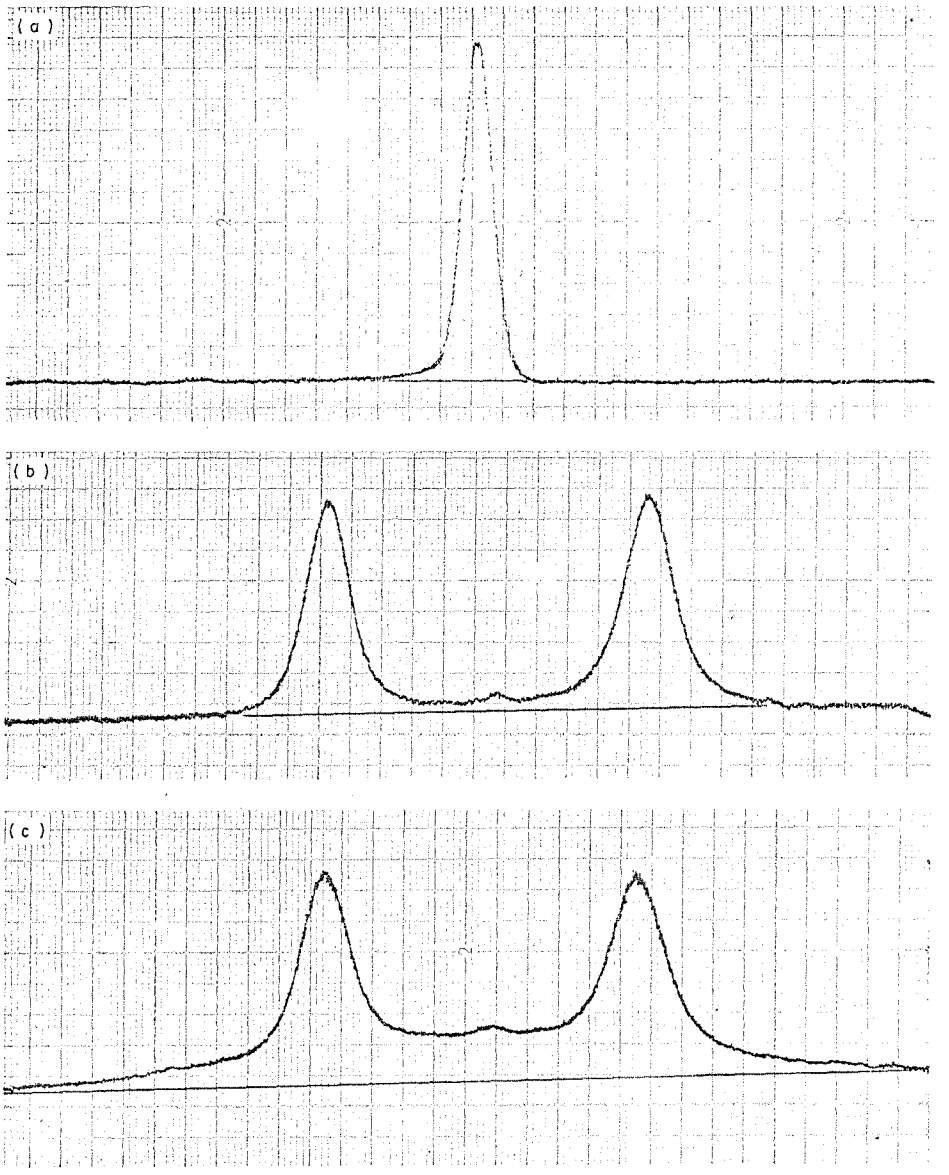

PLATE III. Photoelectric scans of (a) unlabeled mitDNA in buoyant neutral CsCl after velocity sedimentation to remove small nuclear DNA fragments; (b) the same sample; (a), in buoyant CsCl (pH 12·5), 0·05 M-K_3PO_4; (c) unlabeled mitDNA which had not been purified by velocity sedimentation, in alkaline buoyant CsCl, at 44,000 revs/min, 25°C. The field is directed to the right. The two bands in (b) and (c) contain the complementary strands of mitDNA. The entire scan was used in each case for drawing in the baseline.

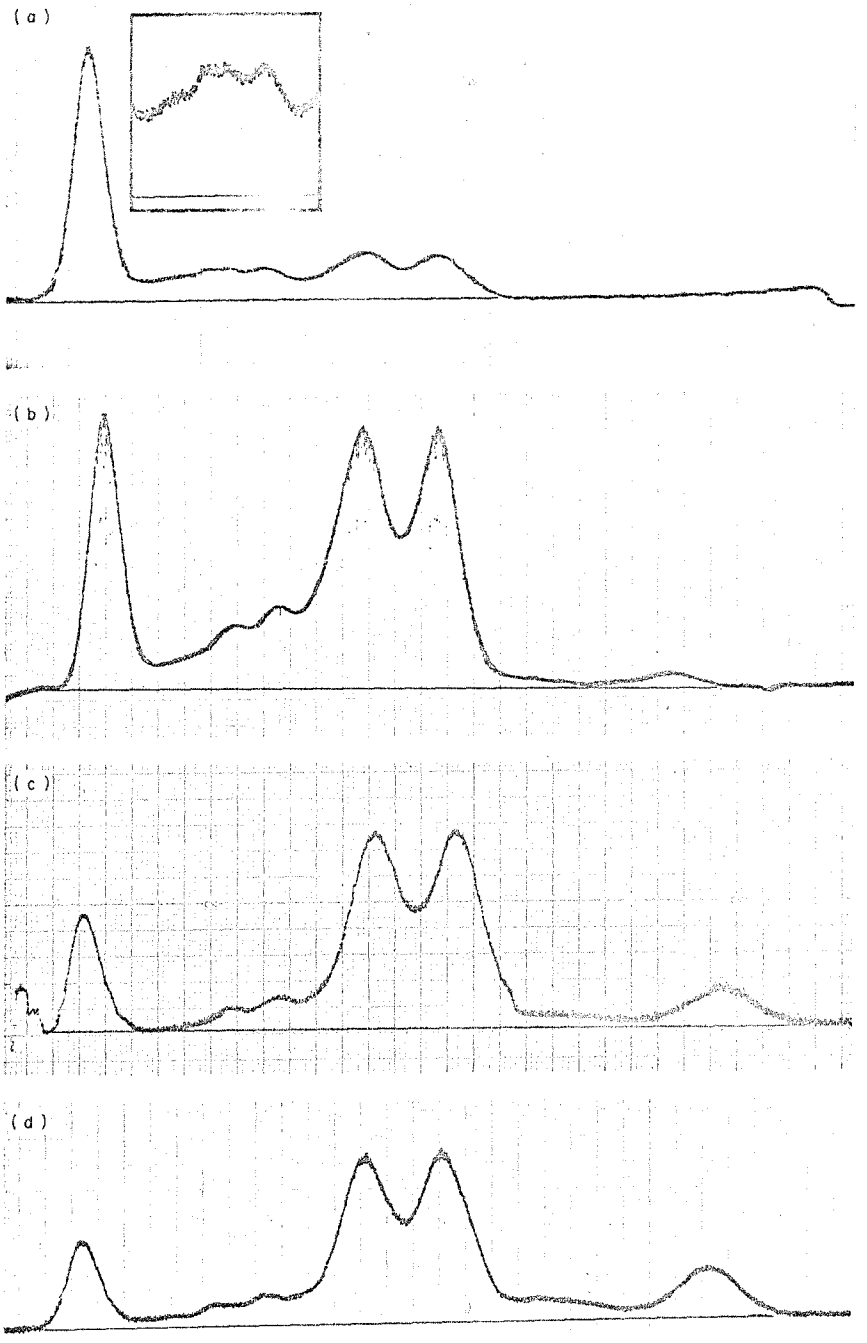

PLATE IV. Photoelectric scans of mitDNA from cells labeled with BrdUrd for (a) 8 h; (b) 16 h; (c) 24 h; (d) 44 h. The samples are in neutral buoyant CsCl at 25°C and 44,000 revs/min, except (b), at 44,770 revs/min. The field is directed to the right. In each case, the band at the left is the unlabeled mitDNA, the two peaks in the middle are the hybrid mitDNA and the band at the right (when present) is the heavy mitDNA. The quarter heavy bands are between the hybrid and the unlabeled mitDNA bands. The inset in (a) corresponds to the region immediately below it, scanned at a higher sensitivity.

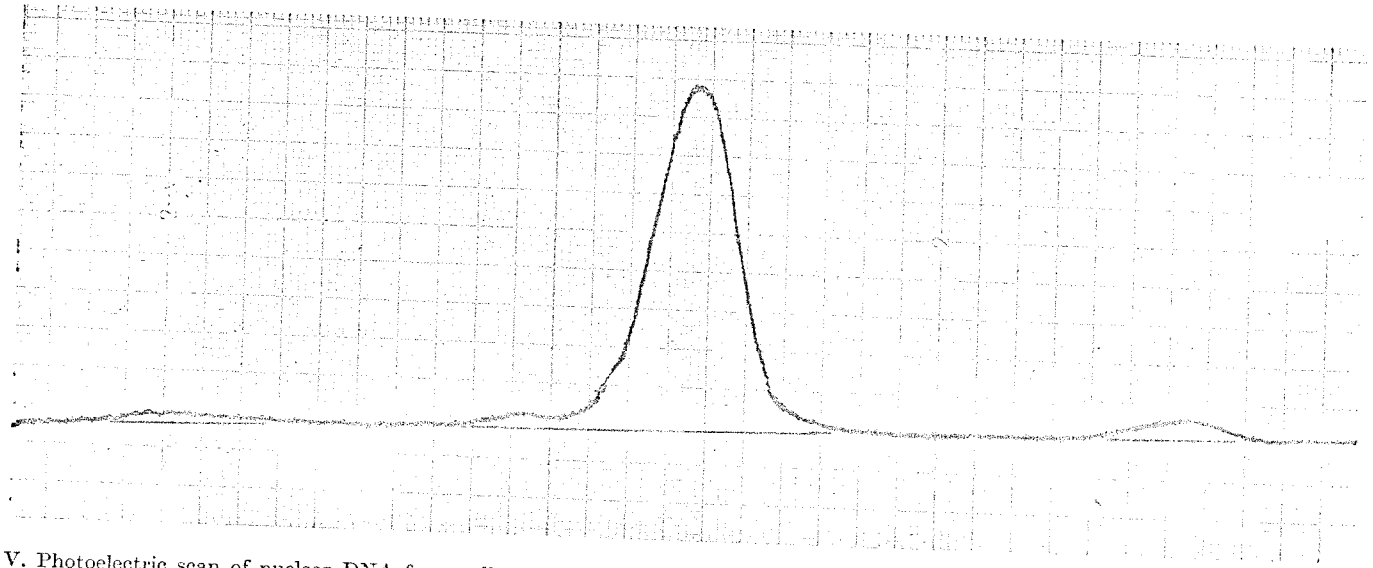

PLATE V. Photoelectric scan of nuclear DNA from cells labeled for 44 h with BrdUrd. The sample is in buoyant neutral CsCl at 44,000 revs/min, 25°C. The field is directed to the right. The large band at the center is hybrid nuclear DNA. A very small amount of unlabeled nuclear DNA appears at the left.

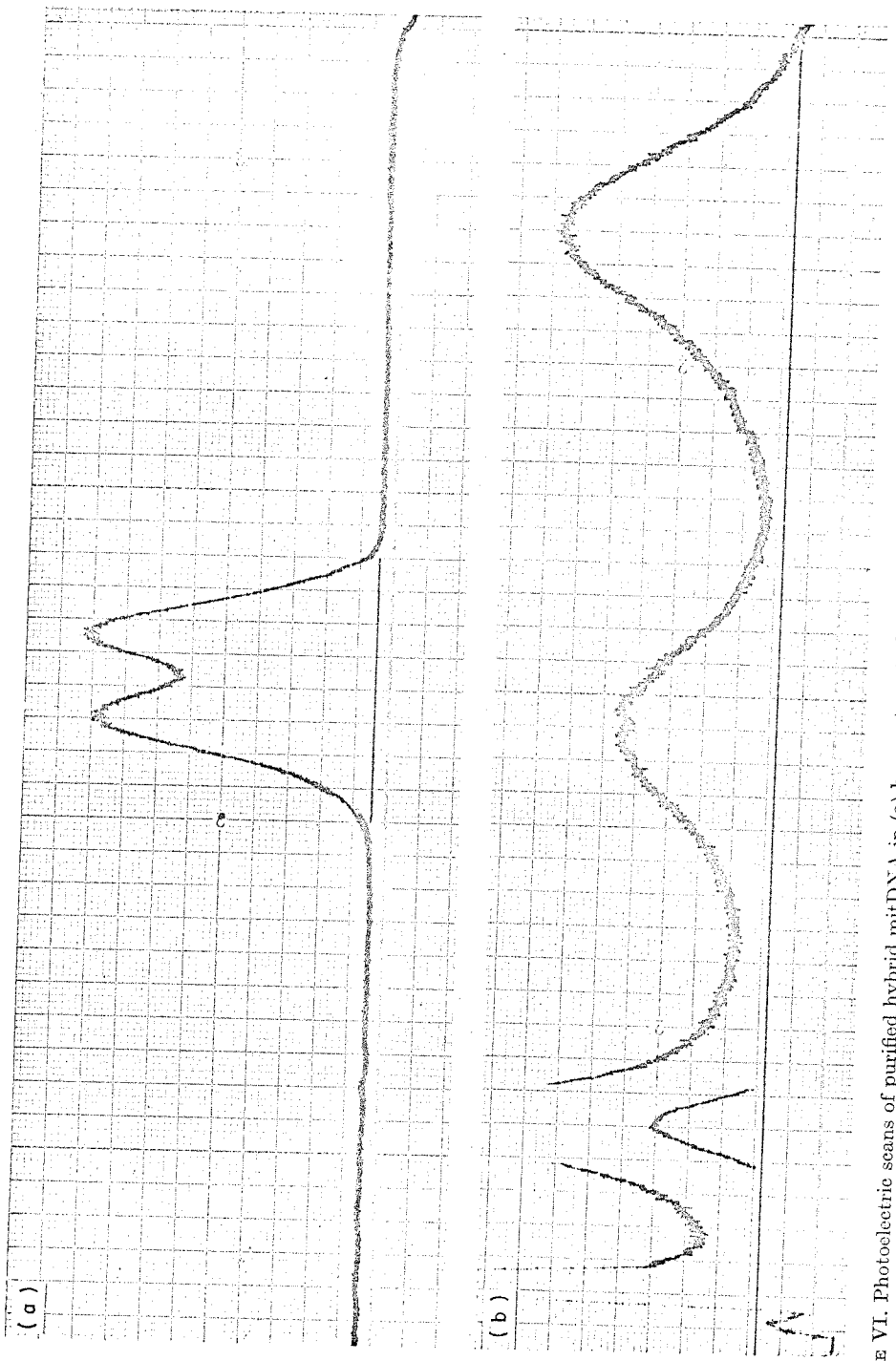

PLATE VI. Photoelectric scans of purified hybrid mitDNA in (a) buoyant neutral CsCl and (b) buoyant alkaline CsCl (pH 12·5), 0·05 M-K_3PO_4, at 44,000 revs/min, 25°C. The field is directed to the right. The two bands at the right in (b) are the BrUra-labeled strands. The heavy unlabeled strand is at the far left, while the light unlabeled strand is at the meniscus. The scale was changed during the scan so as to include the maximum of the band at the left.

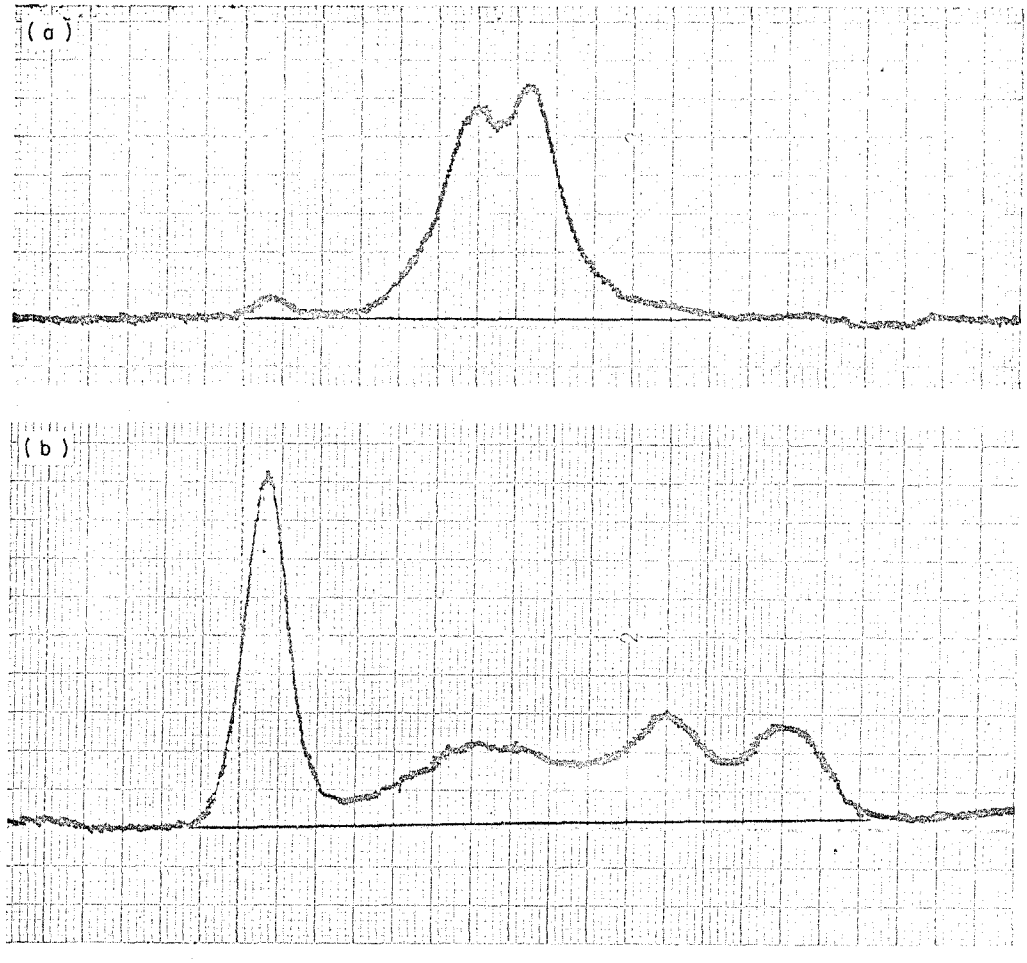

PLATE VII. Photoelectric scans of (a) the quarter heavy fraction isolated by combining the fractions shown in Fig. 1, and (b) the same fraction after shearing in buoyant neutral CsCl at 44,000 revs/min, 25°C. The field is directed to the right.

mitDNA. The separation between the two quarter heavy bands, 6·4 mg/ml, is approximately half the separation between the two hybrid bands, 12·1 mg/ml. The structure of these species will be considered in later sections.

The magnitudes of the buoyant separations can be used to estimate the extent of substitution of BrUra for thymine. The buoyant separation between poly[d(A-T)] and poly[d(A-BrUra)] is 200 mg/ml (Wake & Baldwin, 1962). Since the mitDNA contains 28% thymine (Wesley M. Brown, personal communication) and poly[d(A-T)] contains 50% thymine, the separation for heavy mitDNA would be 112 mg/ml if the substitution were complete. The observed separation of 95·3 mg/ml indicates that 85% of the thymine residues were replaced by BrUra. A similar calculation for the nDNA, with 30% thymine (Kin *et al.*, 1966) gives a value of 87% substitution, based on an observed separation of 104·8 mg/ml. Preparations of mitDNA from cells treated with FUrd gave a separation of 100·8 mg/ml indicating 90% substitution. The small difference observed on addition of FUrd led us to abandon its use.

The separations of the two hybrids from unlabeled mitDNA can be used to estimate the thymine bias between the complementary strands. Since the average of the separations between the hybrids and the unlabeled mitDNA is exactly half the separation between heavy and unlabeled mitDNA, the buoyant shift is proportional to BrUra content:

$$\Delta\theta = kB, \tag{1}$$

where $\Delta\theta$ is the buoyant density shift on BrUra incorporation, k is a constant, and B is the mole fraction of bases which are BrUra. The density shift for a hybrid in which the fraction of thymine residues on the labeled strand replaced by BrUra is a is:

$$\Delta\theta = akT, \tag{2}$$

where T is the thymine content of the strand before labeling. If we assume that the fractional extent of BrUra labeling, a, is the same for the labeled strands of the two hybrids, the ratio of the buoyant separations for the two hybrid species becomes

$$\Delta\theta_2/\Delta\theta_1 = T_2/T_1.$$

The thymine bias, T_2/T_1, obtained in this way is independent of the extent of labeling, a. The value of $1{\cdot}29 \pm 0{\cdot}05$ is in agreement with the value of 1·26 determined by base analysis (31·2 and 24·9% thymine in heavy and light strands (Wesley M. Brown, personal communication)). The agreement between these numbers indicates that the hybrids contain one labeled and one unlabeled strand and are, therefore, products of semiconservative replication.

(c) *Alkaline buoyant densities of density-labeled mitochondrial DNA*

Alkaline buoyant separations were calculated with the value of $1/\beta_b$ used for the calculations at neutral pH. Recent work by Schmid & Hearst (1971) indicates that this assumption is valid with a maximum error of 5%. The value for the separation between the two unlabeled strands is $42{\cdot}3 \pm 0{\cdot}6$ g/ml (Table 2; Plate III) which agrees fairly well with the value 41·0 mg/ml reported by Clayton *et al.* (1970) for human leukocyte mitDNA. When hybrid mitDNA was fractionated from a preparative CsCl gradient and banded in alkaline CsCl, four bands were formed. The separation between the lightest and heaviest bands was so large (0·149 mg/ml) that they could not all be banded in one analytical centrifuge cell at 44,000 revs/min. The three most

TABLE 2

Buoyant densities and separations between unlabeled and bromouracil-labeled mitochondrial DNA strands in alkaline cesium chloride

Strand	ρ (g/ml)	Separation (mg/ml)
Light, unlabeled	1·737	
Heavy, unlabeled	1·779†	42·3±0·6 (4)
Light, BrUra-labeled	1·825	45·8±0·4 (3)
Heavy, BrUra-labeled	1·886	60·8±1·0 (3)

† Marker value from Clayton *et al.* (1970).

dense species are shown in Plate VI(b): from left to right, they are the unlabeled heavy strand, the BrUra light strand, and the BrUra heavy strand. The densities of the alkaline BrUra-labeled strands are 1·825 and 1·886 g/ml, referred to the unlabeled heavy strand at 1·779 g/ml (Clayton *et al.*, 1970). The ratio of the shift of the light strand on BrUra labeling to that of the heavy strand on BrUra labeling provides an estimate of the thymine bias of 1·20±0·02. This value agrees fairly well with the value of 1·26 determined from base composition analysis.

(d) *Kinetics of labeling of mitochondrial and nuclear DNA*

HeLa cells were labeled for increasing lengths of time with BrdUrd, 20 μg/ml. The mitDNA was extracted and examined in neutral buoyant CsCl (Plate IV, Table 3). In this concentration of BrdUrd, the cells undergo only 1 to 1·5 doublings before growth ceases due to toxic effects of the analogue (Simon, 1961). It is clear that the mitDNA is labeled rapidly for the first 24 hours (1·2 generations for normal cells) but that the incorporation is greatly reduced during the next 20 hours. Similar results were obtained with nDNA after 44 hours (Plate V). Almost all of the nDNA is at hybrid density, with only 5% at the heavy position and less than 2% still unlabeled. In contrast, 10% of the mitDNA is still unlabeled after 44 hours, and another 10% is heavy. The unlabeled mitDNA could not have come from cells which did not grow in BrdUrd since such cells would have contained unlabeled nDNA. A mitDNA

TABLE 3

Buoyant density distributions of HeLa mitochondrial DNA as a function of labeling time in the presence of 5-bromodeoxyuridine

Time (h)	% of total mitDNA (w/w)				
	Unlabeled	Quarter heavy†	Hybrid	Hybrid-heavy†	Heavy
8	45	25	30	—	—
16	19	19	57	2	2
24	11	10	68	4	7
44	9	8	66	6	11

These density distributions were determined by summation of ordinates on enlargements of the photoelectric scans, as described in Materials and Methods, section (c).

† These categories include all material banding between the major species.

molecule therefore does not necessarily replicate once during each cell cycle; some molecules replicate twice and others not at all. Similar results have been found by Rownd (1969) for the replication of the R-factor in *Proteus mirabilis.*

The scans in Plate IV also show that the quarter heavy species appear as a major fraction of the mitDNA after eight hours (0·4 generation in normal cells) and then decrease in amount at later times. The kinetic behavior of these species corresponds to that of an intermediate in the labeling process. After 16 hours, a possibly equivalent material between the hybrid and heavy positions appears in small amounts.

(e) *Purification and properties of the quarter heavy mitochondrial DNA*

In view of the unusual buoyant density and labeling properties of the quarter heavy material, we decided to purify it and examine its properties in greater detail. Mitochondrial DNA from cells labeled for eight hours with BrdUrd was banded in a preparative CsCl gradient, and the fractions analyzed fluorometrically (Fig. 1). The fractions containing quarter heavy DNA were pooled as shown and banded in neutral buoyant CsCl in the analytical ultracentrifuge to assay for the presence of hybrid and unlabeled mitDNA (Plate VII(a)). In general, the amount of this contamination was 10% or less; if more, the amount was determined and taken into account in the evaluation of later results.

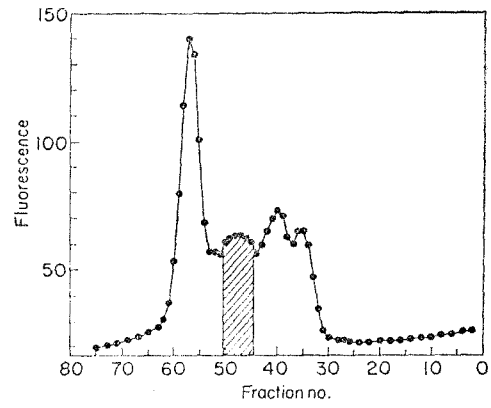

FIG. 1. Isolation of the quarter heavy mitDNA fraction.
Total mitDNA from cells labeled for 8 h with BrdUrd was banded in neutral CsCl for 48 h at 35,000 revs/min, 25°C, in an SW50.1 rotor. The tube was fractionated in 3-drop fractions into 0·5 ml of 10 μg EthBr/ml, 0·1 M-NaCl, 0·01 M-Tris (pH 7·5), 0·001 M-EDTA. The fluorescence of each fraction was determined using excitation at 380 nm and emission at 586 nm in a Hitachi Perkin–Elmer MPF2A fluorescence spectrophotometer. Fluorescence (arbitrary units) is plotted *versus* fraction number, with the field directed to the right. A baseline has been included for reference. The shaded area represents the fractions which were pooled to make the quarter heavy fraction. An analysis of this material in neutral CsCl is presented in Plate VII(a).

Electron microscopy showed that an unusually large portion of the DNA in this fraction is in the form of catenated molecules (Table 4). The several preparations of quarter heavy mitDNA contained about 70% (w/w) catenanes of all types, as compared with 37% (w/w) in the total mitDNA from cells labeled for eight hours and with 38% (w/w) in an unlabeled mitDNA preparation. Enrichment of catenanes in

TABLE 4

Electron microscope analysis of mitochondrial DNA from HeLa cells

Sample	% of mitDNA in the sample (w/w) Linear†	Monomer	Catenated dimer	Catenated trimer and larger forms	Molecules scored
Total mitDNA, unlabeled‡	n. s.§	62	22	16	1200
Total mitDNA, BrdUrd-labeled (8 h)	n. s.	63	22	15	1200
(a) Hybrid (8 h)	n. s.	78	15	7	2710
(b) Unlabeled (8 h)	n. s.	80	15	6	1320
(c) Quarter heavy, (8 h), purity‖					
90%	n. s.	35	35	31	1350
90%	n. s.	31	40	29	590
95% (Expt 1)¶	n. s.	27	46	27	1320
91% (Expt 2)	n. s.	26	45	26	760
80% (Expt 3)	n. s.	50	31	13	920
(d) Quarter heavy, sheared	54	44	2·1	—	450
(Expt 2)¶	50	47	2·1	0·5	850
(Expt 3)	38	60	2·4	0·3	1620

† All the linear molecules were assumed to be half the monomer length in determining the % (w/w). The value for experiment 3 was corrected for the presence of small linear fragments.

‡ These data were obtained from C. J. B. Tibbetts, this laboratory.

§ n. s., Not scored. Linear molecules of up to monomer length were seen, but did not constitute more than 10% (w/w) of the sample.

‖ Purity was determined by measuring the fraction of the quarter heavy mitDNA in buoyant CsCl in the analytical ultracentrifuge. Hybrid and unlabeled mitDNA were present as impurities.

¶ The experiment numbers correspond to the experiments listed in Table 5.

the quarter heavy region is at the expense of catenanes in the hybrid and unlabeled regions. The isolated hybrid and unlabeled peaks contained about 20% (w/w) catenanes (Table 4). A rough estimate of the fraction of the catenanes in the quarter heavy region can be obtained by weighting the catenane level in each band by the fraction of the total mitDNA in that band (Tables 3 and 4). About 50% (w/w) of the catenanes in the total sample are in the quarter heavy fraction; also, about 80% (w/w) of the density-labeled catenanes are in this region.

Analytical band sedimentation experiments were performed on the quarter heavy fraction in an attempt to confirm the electron microscope results. The measured sedimentation velocities agree well enough with the published values (Brown & Vinograd, 1971) to allow identification of the peaks. An accurate determination of the amount of catenanes in the sample was not possible because of the poor resolution between the doubly nicked catenated dimer and the closed monomer. The results indicated that between 50 and 80% (w/w) of the mitDNA in the quarter heavy fraction was in the form of catenated molecules.

(f) *Structure of the quarter heavy mitochondrial DNA*

In view of both the buoyant properties and the high catenane content of the quarter heavy fraction, it appeared likely that this fraction contained catenanes composed of hybrid and unlabeled submolecules. The dimers of this type would be

expected to form two bands midway between the hybrid and unlabeled positions, since the hybrid submolecule could have either of the two densities. Catenated trimers and higher forms containing both hybrid and unlabeled forms would also band in this region.

The most direct way to test the proposed structure for the quarter heavy mitDNA is to degrade the catenanes with double-strand scissions so that the submolecules can separate. Banding in neutral buoyant CsCl would then show peaks at the hybrid and unlabeled positions. We first tried enzymic degradation using the cleaving nuclease, DNase II. Examination of the digestion products of closed PM2 DNA by sedimentation velocity showed that the enzyme introduces many single-stranded nicks for each cleavage. Careful calibration of the enzyme was necessary to minimize fragmentation of the DNA due to this nicking. Despite these precautions, a sedimentation analysis showed that the quarter heavy mitDNA was fragmented to small linear molecules. In neutral buoyant CsCl the bands were broader than usual; nevertheless, it was clear that a conversion of quarter heavy material to hybrid and light DNA had occurred (Table 5, experiment 1).

TABLE 5

Density distributions of degraded quarter heavy mitochondrial DNA fractions

Sample	% of the mitDNA fraction (w/w)			
	Unlabeled	Quarter heavy	Hybrid 1	Hybrid 2
Expt 1 (DNase II)	31	38	17	14
Expt 2 (shearing)	33	32	17	19
Expt 3 (shearing)	29	38	16	18
Average	31	36	17†	17‡

Distributions were determined by ordinate summation as in Table 3, and were corrected where necessary for the presence of contamination in unlabeled and hybrid mitDNA.

† Average separation from the unlabeled mitDNA peak was $40{\cdot}9 \pm 1{\cdot}5$ mg/ml (3 determinations).

‡ Average separation from the unlabeled mitDNA peak was $54{\cdot}1 \pm 0{\cdot}9$ mg/ml (3 determinations).

Mechanical shearing was then used to degrade the catenanes. The DNA solution was driven back and forth through a fine capillary by nitrogen gas at high pressure (Yew & Davidson, 1968). This method has several distinct advantages over enzymic digestion. It is simple and reproducible, since the shear breakage depends only on temperature, DNA concentration, number of passes through the capillary, and shear stress (proportional to nitrogen pressure). For a given shear stress, the size of the product approaches an asymptote as the number of passes increases. Product size can, therefore, be controlled by using a constant (large) number of passes and varying the shear stress. The shear stress necessary to break a catenated dimer should be roughly the same as that required to break a 10×10^6 dalton linear DNA in half, since the effective lengths in the shear gradient are probably similar. The linear mitDNA molecules resulting from the first rupture of the catenanes in the shear gradient should also be broken only once, to 5×10^6 daltons. Monomer mitDNA circles should not be broken under these conditions.

Three preparations of quarter heavy mitDNA were sheared under conditions appropriate to shearing $\lambda b_2 b_5 c$ DNA (mol. wt, 25×10^6) into quarters. EthBr at a concentration of 2·0 μg/ml was added to relax the closed molecules. Electron microscope examination of the sheared DNA showed that only 2% of the molecules were still catenanes; most of the molecules were either monomer circles or linear (Table 4). A histogram of the lengths of the linear molecules was constructed using the monomer circles as an internal standard (Fig. 2). The peak in the distribution is at 0·5 monomer length, as expected. The peak at 0·1 length (0·5 μm) is variable in amount in different preparations. These smaller linear fragments accounted for less than 2% (w/w) of the sheared quarter heavy mitDNA preparation. In all other preparations the amount was still less. The small fragments probably represent residual nDNA. The electron microscope analysis, therefore, demonstrates that the shearing process degraded the catenanes without extensive fragmentation of the mitDNA.

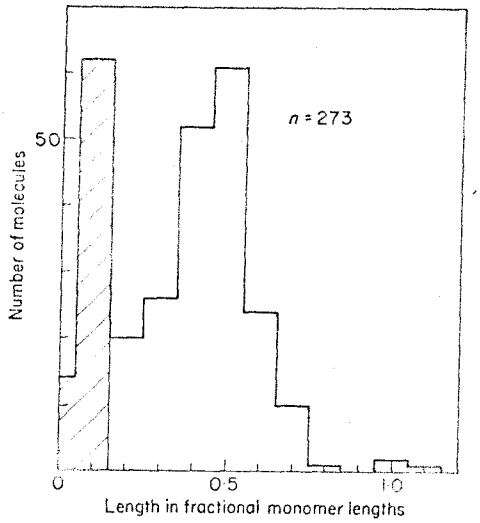

FIG. 2. Length distribution of the linear molecules in the quarter heavy fraction after shearing. The sheared quarter heavy fraction was mounted for electron microscopy by a modified Klenschmidt procedure. Linear molecules were photographed randomly, traced and measured. Monomer circles were also measured as an internal standard. The number of molecules seen is plotted against their length in fractions of the monomer circle length. The shaded area represents small fragments which account for less than 2% of the mass of DNA in the fraction.

The sheared samples, when banded in neutral buoyant CsCl, gave profiles like that in Plate VII(b). Clearly, shearing released hybrid and unlabeled DNA from the quarter heavy region. There was still some DNA at the quarter heavy position, despite the fact that there were no catenanes left. Measurements of the areas (Table 5) show that about 35% of the DNA was not shifted by shearing. This number is quite close to the percentage of monomers in the unsheared material, suggesting that part of the quarter heavy peak consists of monomers with one strand partly BrdUrd-labeled. The quarter heavy mitDNA, therefore, consists mainly of catenanes having hybrid and unlabeled submolecules, but contains incompletely labeled hybrid monomers as well.

4. Discussion

(a) *Extent of substitution*

Several factors indicate that the substitution of BrUra for thymine is almost complete under our conditions. The density shifts for both mitDNA and nDNA are very large. The mitDNA shifts are five times those previously seen in BrUra-labeled animal cell mitDNA (Karol & Simpson, 1968; Gross & Rabinowitz, 1969). The extent of substitution was estimated using the base compositions and the density shift of BrUra substitution in poly[d(A-T)] (Wake & Baldwin, 1962). The values of about 85% obtained in this way are only approximate for several reasons. The separation between poly[d(A-T)] and poly[d(A-BrUra)] in CsCl is so large that it is difficult to measure accurately. Moreover, synthetic polynucleotides may not be appropriate as standards inasmuch as the buoyant density of poly[d(A-T)] does not correspond to its base composition (Schildkraut *et al.*, 1962). Nevertheless, this calculation does indicate that the substitution is extensive. The addition of FUrd to the cultures in an attempt to block endogenous thymidine synthesis increased the density shifts only slightly to a value of 90% substitution as calculated by the above method. This observation, together with the fact that the densities do not increase at longer labeling times, suggests that the substitution may be virtually complete under our conditions.

(b) *Semiconservative replication and thymine bias*

The buoyant density data are in excellent agreement with the semiconservative model. The separation for heavy mitDNA is exactly twice the average of the separations for the two hybrids, which is consistent with the idea that the heavy species has two labeled strands and the hybrid only one. The two hybrids predicted by the difference in thymine content of the two strands are found. The hybrid and heavy peaks appear relatively homogeneous and do not shift with time, indicating that the mature molecules formed have discrete buoyant densities.

The thymine bias determined from the ratio of the separations of the two hybrids, 1·29, is very close to that determined from the base compositions of the separated strands, 1·26. If there were any BrUra on the "unlabeled" strand, the thymine bias determined from the buoyant shifts would be low. Hybrid catenanes containing submolecules of both hybrid densities would also tend to *reduce* the separation between the two hybrid peaks and give a low estimate of the thymine bias. These calculations are not affected by the extent of substitution, provided that it remains constant for the two strands.

The hybrid doublet gives rise to four bands in buoyant alkaline cesium chloride. Two of these bands appear to be completely unlabeled, since they have the same separation as that obtained from unlabeled mitDNA. These band widths also appear to be normal. The other two bands are the density-labeled strands. The thymine bias determined from the separations between the density-labeled strands and the corresponding unlabeled strands is 1·20, in fair agreement with the value determined by base analysis. The band widths of both density-labeled strands are larger than those of the unlabeled strands from the hybrid mitDNA. The increased band widths could result from faster alkaline hydrolysis of the density-labeled strands than the unlabeled ones. Alternatively, hydrolysis of the density-labeled strands may result in fragments of heterogeneous buoyant density due to differences in BrUra content.

(c) *Composition and structure of the quarter heavy mitochondrial DNA*

Electron microscope analyses show that the quarter heavy mitDNA fraction contains 30% (w/w) monomers and 70% (w/w) catenanes. The quarter heavy catenanes consist of hybrid submolecules catenated to unlabeled ones. The evidence for this structure comes from buoyant density and electron microscope analysis before and after shearing. The buoyant density analyses show that the quarter heavy mitDNA has an average buoyant density exactly half way between the hybrid and unlabeled species (Plates IV(a) and VII(a)). The quarter heavy region was enriched in catenanes as compared with the 40% (w/w) in the total sample. Examination of the other fractions confirms that catenanes were concentrated in the quarter heavy region.

The buoyant analyses revealed two peaks at the quarter heavy position with a separation exactly half that between the two hybrid peaks. Catenated dimers containing one hybrid and one unlabeled submolecule form two peaks because the hybrid submolecule can have either of two densities. The separation between these two peaks is expected to be half that between the hybrid species due to the contribution of the unlabeled submolecule. Most of the quarter heavy catenanes are dimers. Electron microscope analyses show that the quarter heavy catenanes are 63% dimers, 22% trimers, 7% tetramers, and 9% higher forms (w/w). The effect of the larger forms will be simply to broaden the bimodal distribution of the quarter heavy dimers. For example, the trimers containing either one or two hybrid submolecules will band in this region because they will be one-sixth and one-third heavy. The two buoyant densities for the hybrid submolecules will cause further splitting into bands too close to appear as separate peaks.

The strongest evidence for the structure of the quarter heavy catenanes comes from the shearing experiments. After degradation of the quarter heavy fraction into monomer circles and half-length linear molecules, 70% (w/w) of this mitDNA bands in the hybrid and unlabeled positions. Furthermore, approximately equal amounts of mitDNA appear at the hybrid and unlabeled positions. The hybrid material is evenly distributed between the two hybrid positions. Since 70% (w/w) of the quarter heavy fraction was catenated before shearing and only 2% (w/w) remains in this form after shearing, the hybrid and unlabeled mitDNA have apparently been released from catenanes composed of approximately equal numbers of unlabeled and hybrid monomer submolecules. The existence of discrete, sharp hybrid and unlabeled peaks after shearing excludes the possibility that the quarter heavy catenanes are composed of partially hybrid submolecules containing strands which are density labeled along part of their length. The quarter heavy catenanes are predominantly composed of hybrid and unlabeled submolecules.

Approximately 30% (w/w) of the quarter heavy fraction are monomers. They are probably not degraded by the shearing process, and so represent the 30% (w/w) of the quarter heavy fraction remaining at the quarter heavy position after shearing (Plate VII(b)). The broadness of this peak suggests that these monomers are a heterogeneous population containing one strand which has been partially density labeled. These probably represent molecules which had undergone various extents of replication when the BrUra incorporation began. Such molecules would be expected to have heterogeneous buoyant densities intermediate between the unlabeled and hybrid densities. They would form a peak at the quarter heavy position in the

sheared sample because the quarter heavy fraction contains a selected population of these molecules.

(d) *Implications of the structure of the quarter heavy catenanes*

HeLa cell mitDNA contains about 60% (w/w) monomeric circular duplexes and about 40% catenanes, of which 24% are dimers, 9% are trimers and 7% are tetramers and higher forms. Both the fraction of the mitDNA in this catenated form and the relative amounts of the various catenated oligomers have remained constant for over 500 cell generations. There must, therefore, be mechanisms which generate catenanes as the cells multiply and maintain the frequencies of the different catenated forms. New catenanes could be formed either from monomers or from pre-existing catenanes. Formation from monomers would entail either an aberrant form of replication in which the progeny molecules fail to separate or a linking process in which monomers become interlocked. A double recombination event is an example of such a linking step; two monomers recombine to form a circular dimer which can then form a catenated dimer *via* a second, internal, recombination event. Broker & Lehman (1971) have developed a double recombination model which avoids duplex cleavage. A double recombination event could also occur between two submolecules in a catenane, resulting in unlinking (Hudson *et al.*, 1968). An equilibrium condition might be established in which the frequency of the larger catenated forms is small because the proximity of the submolecules renders them more susceptible to unlinking.

Alternatively, new catenanes could be formed from pre-existing catenanes by replication of submolecules. This possibility must be considered seriously because catenanes containing submolecules with D-loop structures have been seen frequently during the course of this study. If linearization does not occur during replication, both of the progeny submolecules necessarily remain directly interlocked with the unreplicated submolecule(s). For example, replication of one submolecule in a catenated dimer would lead to a linear trimer. A subsequent unlinking process would be necessary, both to prevent the conversion of all the catenanes into large and complex forms and to produce new catenated dimers. The formation of new catenanes from pre-existing ones therefore requires replication of submolecules to form tetramers or higher oligomers followed by unlinking.

The quarter heavy catenanes could not have been formed by an aberrant type of monomer replication in which the progeny become linked into a catenated dimer, since this process would result in hybrid rather than quarter heavy dimers. This result is in disagreement with the replicative mechanism proposed for the formation of catenated dimers of SV40 DNA (Jaenisch & Levine, 1972) and is consistent with the recombinational mode of formation of ϕX RF-DNA (Benbow *et al.*, 1972).

If catenanes are formed from monomers by a linking process, the rate must be high in order to form 60% "new" (quarter heavy plus hybrid) dimers after eight hours. If the process is a random one, the frequencies of the different density-labeled dimer forms should approximately reflect the frequencies of the different density-labeled monomer units. After eight hours, 54% of the monomer units (including those in catenanes) are unlabeled, 39% are hybrid and 8% are intermediate in density. Linking of a hybrid and an unlabeled monomer would be more probable (0·49) than linking of two unlabeled (0·34) or two hybrid (0·18) monomers. We have calculated from the electron micrograph analysis and buoyant density distributions that about 40% of the dimers are quarter heavy, 38% are unlabeled and 21% are hybrid at

eight hours, in good agreement with the above calculations. Finally, if the linking process is a result of double recombination, the two events must occur very close together on the mitDNA molecule. Otherwise, a section of the hybrid monomer would be transferred to the unlabeled one (and *vice versa*) so that shearing would not have resulted in clean hybrid and unlabeled mitDNA. The transfer of as little as 4% of the monomer would have caused a measurable reduction in the buoyant separations, and no such change was observed.

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Appendix

Since the publication of this work, studies have appeared on catenated circular DNA in SV40 (Jaenisch and Levine, 1973), E.coli resistance transfer factor R6K (Kupersztich and Helinski, 1973), and several S.aureus plasmids (Novick et al., 1973). In each case the basic finding that catenanes are rapidly and transiently labeled with radioactive thymidine is interpreted as indicating that catenanes are formed from monomers by an aberrant replication process. The main weakness of such an interpretation is that replication of submolecules within pre-existing catenanes should also cause labeling of catenated DNA. D-loop (Plate 1) and expanding D-loop (Plate 2) structures are often seen in catenanes, so the submolecules within a catenane do replicate. Because catenanes are aggregations of replication initiation sites, labeling may occur rapidly by this process.

Furthermore, the above studies have not measured the kinetics of labeling of catenanes relative to monomers. Jaenisch and Levine (1973) used an ingeneous limited nicking procedure to determine the proportion of catenated dimers in a mixture of catenated and circular dimers. Unfortunately the calculation contains the implicit assumption that the specific activities of the two species are the same, and this assumption is clearly invalid. A better method would have been to prelabel the DNA with a different label and to use this prelabel to determine (1) a "specific activity" for the total DNA (the ratio of pulse to prelabel), (2) a "specific activity" for catenanes (the ratio of pulse to prelabel in the middle band resulting from limited nicking),

and (3) the fraction of the prelabel which is in catenated dimers (the limited nicking procedure would be valid here because the specific activities of the different species would be the same for the prelabel). The "specific activity" of the circular dimers could be calculated, and the relative specific activities of monomers, catenated dimers, and circular dimers could then be compared. In the two studies on plasmid DNA, prelabels were included, so that determination of the relative specific activities of monomers and catenanes could have been performed. In both cases, however, the specific activity of catenanes found in the singly nicked form on isolation of plasmid DNA was simply compared with the specific activity of the total lower band DNA (closed monomers and catenanes). The proportion of catenanes which are in the rapidly labeled singly nicked form was not determined. These catenanes may be molecules which are singly nicked in the cell as a result of the replication of one of their submolecules, and may represent a small proportion of the total catenated plasmid DNA. In this case, the pulse label should be located in the nicked submolecule. Similarly, the finding of Kupersztoch and Helinski (1973) that the pulse label chases into closed DNA does not differentiate between closed monomers and catenanes. These studies therefore do not demonstrate that catenanes are labeled more rapidly than monomers, and certainly do not show that catenanes are formed from monomers by an aberrant replication process.

A major consequence of the replication of submolecules within catenanes which was not stressed in Flory and Vinograd (1973) is that there must exist a linking-unlinking process within the mito-

chondrion. This process forms or breaks topological bonds between DNA molecules in catenanes. It could be part of the replication process (as,for example,if replication results in linearization of one of the daughter molecules followed by cyclization),or it could be an independent process such as double recombination. It need not involve actual physical linearization of any circular molecule, but it can be conceptualized as a linearization followed by a recyclization in a different topological bonding arrangement. As pointed out previously, replication of submolecules in catenanes in the absence of such a mechanism would result in large, highly interlocked catenated forms. If such molecules were destroyed, they would pose no problem, and a linking-unlinking process would not be necessary. The observation of "linear" catenated tetramer (Plate 3) and higher forms (Plate 4) requires the existence of a linking-unlinking process, for they would not be formed otherwise. Replication of submolecules in a catenated dimer would result in linear catenated trimers, which would replicate to form nonlinear catenated tetramers (Figure 1 and Plate 5). Therefore there must be a linking-unlinking process of some type within the mitochondrion.

At present, there is little basis for choice between double recombination and replication with linearization as the linking-unlinking mechanism. Replication with linearization of one of the progeny would result in formation of a catenated dimer if the linear progeny cyclized while looped through a circular molecule (Hudson, Clayton, and Vinograd, 1968). Replication of one of the submolecules in a catenane via this mechanism would not in general increase the size

of the catenane, since the linear progeny would not be likely to cyclize through one of the submolecules of the catenane. This process would explain both the rapid labeling of catenanes and the formation of quarter heavy dimers described above. Enlargement of catenanes would occur at a low frequency, resulting in predominantly "linear" or "branched" structures because cyclization of a linear through two circles would be relatively improbable.

As discussed previously, double recombination also provides a simple explanation for both the distribution of catenated forms and the existence of quarter heavy catenated dimers. It would allow the reduction in complexity of the multiple topological bonding expected from replication of a submolecule without linearization of one of the progeny. Rapid labeling of catenanes would be expected both from replication of submolecules and from a possibly enhanced probability of recombination of newly replicated molecules. It is not yet possible to ascertain which mechanism is used for the similar process which must exist in animal cell mitochondria.

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Figure 1: The Formation of Catenated Tetramers by a Replication Process in Which Unlinking Does Not Occur

Replication of one submolecule of a catenated dimer leads to a linear catenated trimer in the absence of an unlinking process. Replication of either terminal submolecule in this linear trimer leads to a branched tetramer of the type shown in Plate 5. Replication of the central submolecule in the linear trimer results in a complex tetramer form.

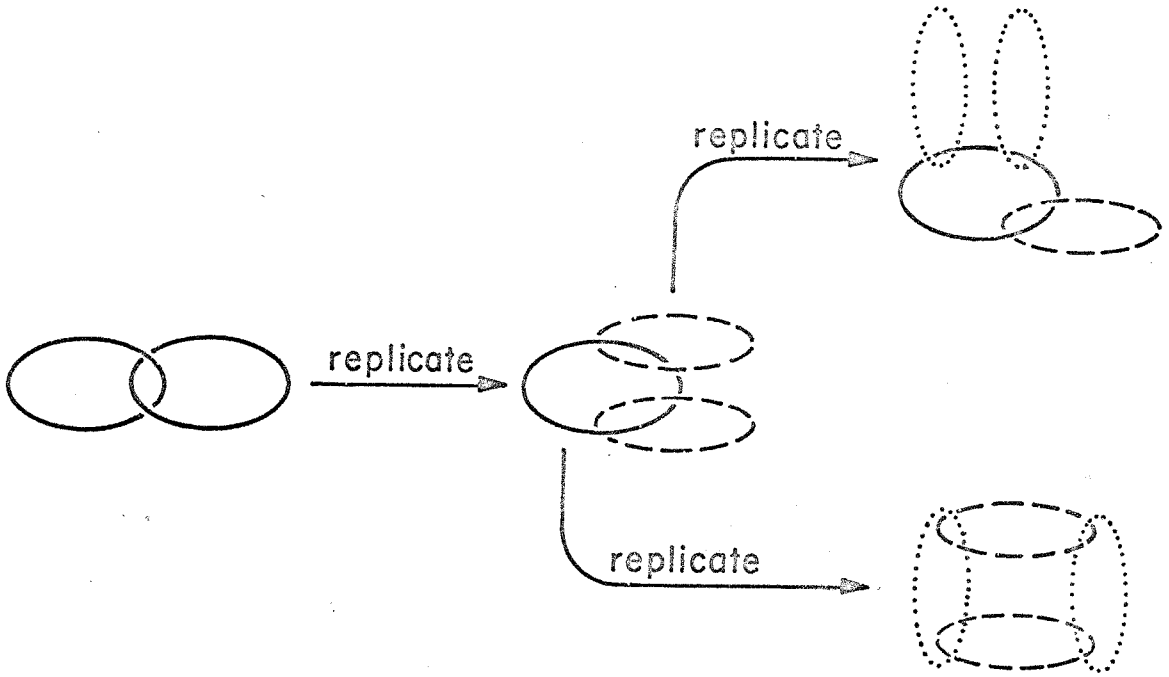


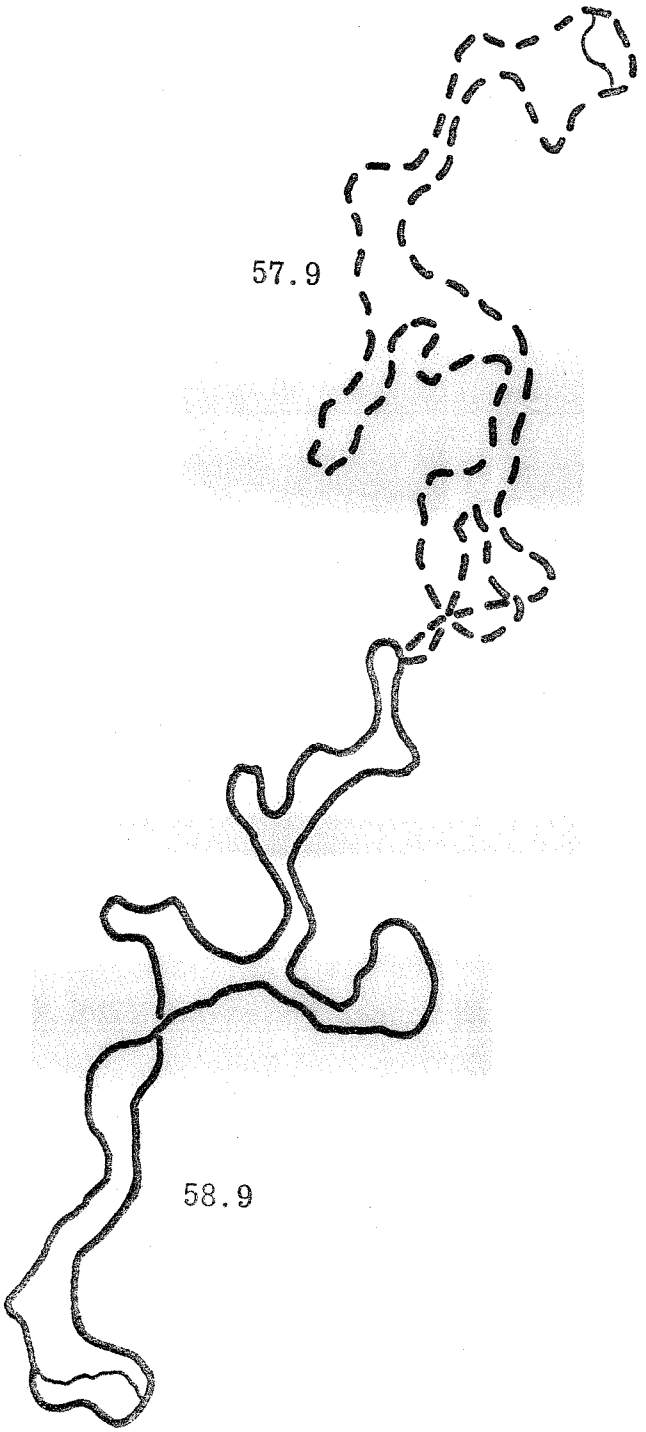
Figure 1

Plate Legends

HeLa mitDNA from the intermediate region between the upper and lower bands in EB-CsCl (250 μg/ml Ethidium Bromide, $\rho = 1.55$ CsCl) gradients was mounted for electron microscopy by the formamide Kleinschmidt technique described in Chapter 2 (Materials and Methods (5)). Selected molecules were photographed and traced on a Hewlett-Packard digitizing tablet. An interpretation of each molecule is included on a translucent overlay. Alternate submolecules are indicated by dashed and solid lines; single stranded regions are indicated by thinner lines (Plates 1 and 2). Traced lengths (in cm.) are included adjacent to each submolecule or portion thereof, as indicated. Since different molecules were photographed at different magnifications in the electron microscope, the traced length of a monomer subunit is not necessarily the same in different plates.

Plate Number

1. A catenated dimer, with a D-loop on both submolecules.
2. A catenated dimer, with an expanding D-loop on one submolecule. The submolecule at the upper is an expanding D-loop form of about 0.5 G, with complement synthesis visible as a duplex region on the displaced single strand.
3. A linear catenated tetramer.
4. A linear catenated octamer.
5. A branched catenated tetramer.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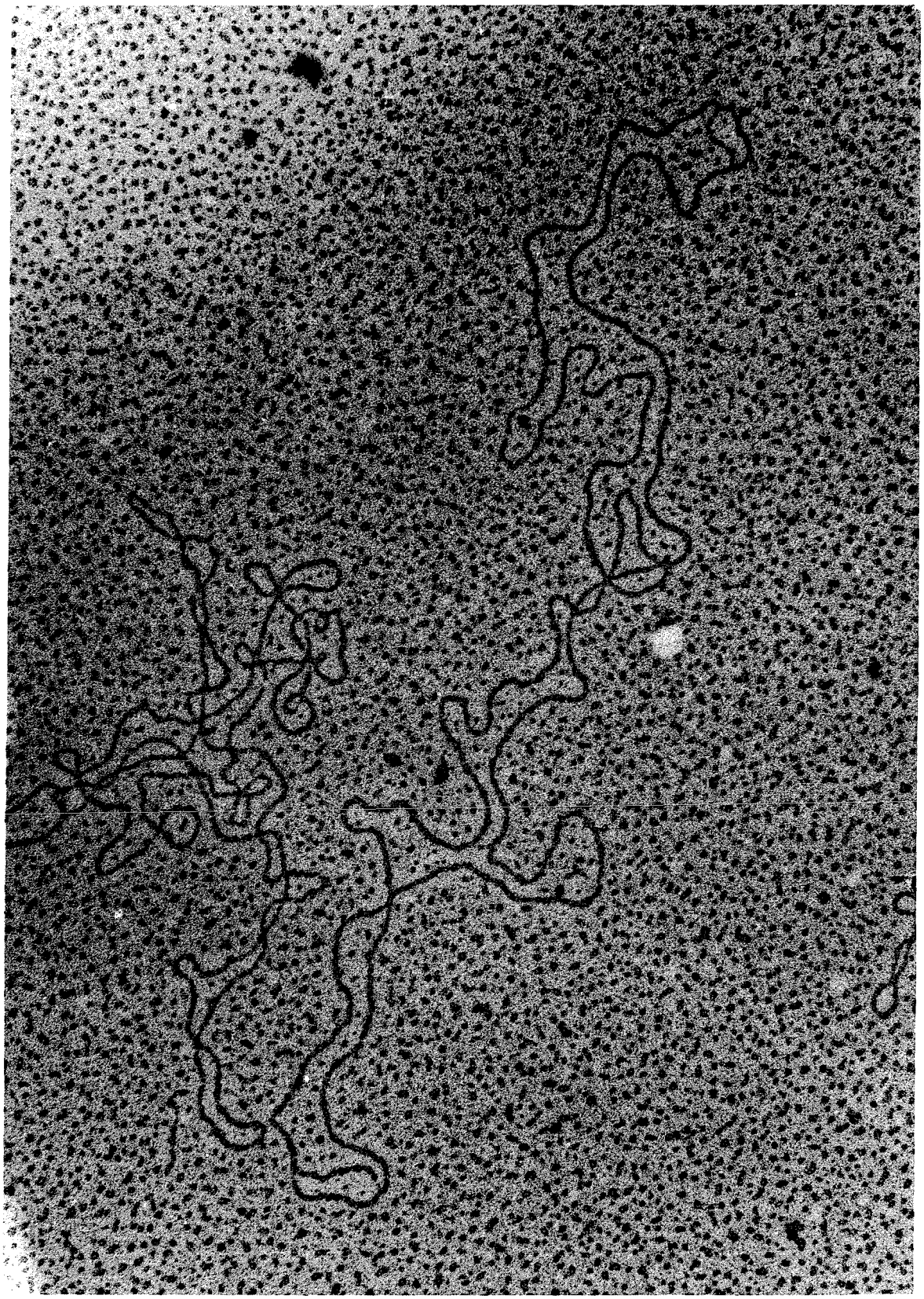
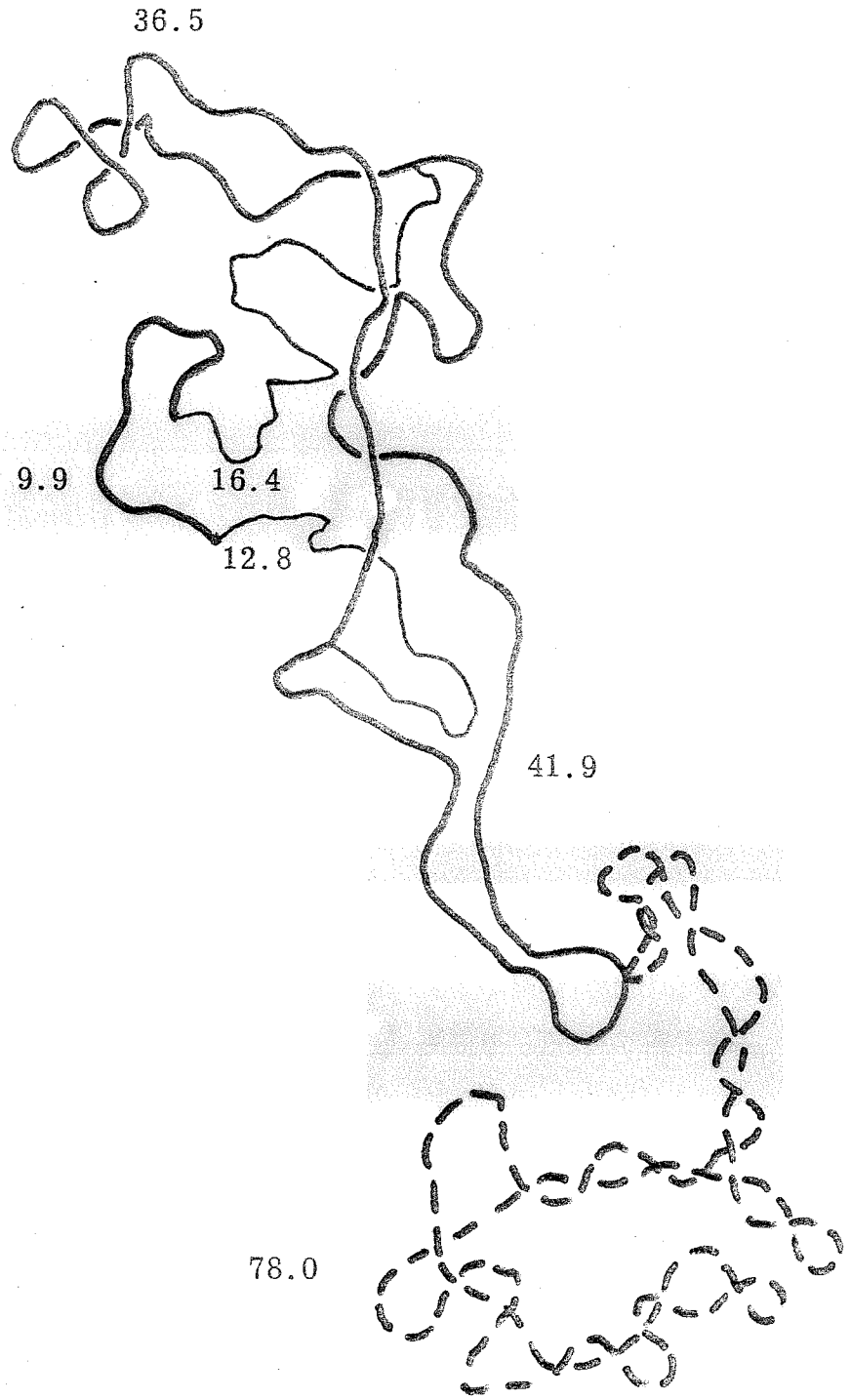


Plate 1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16.4
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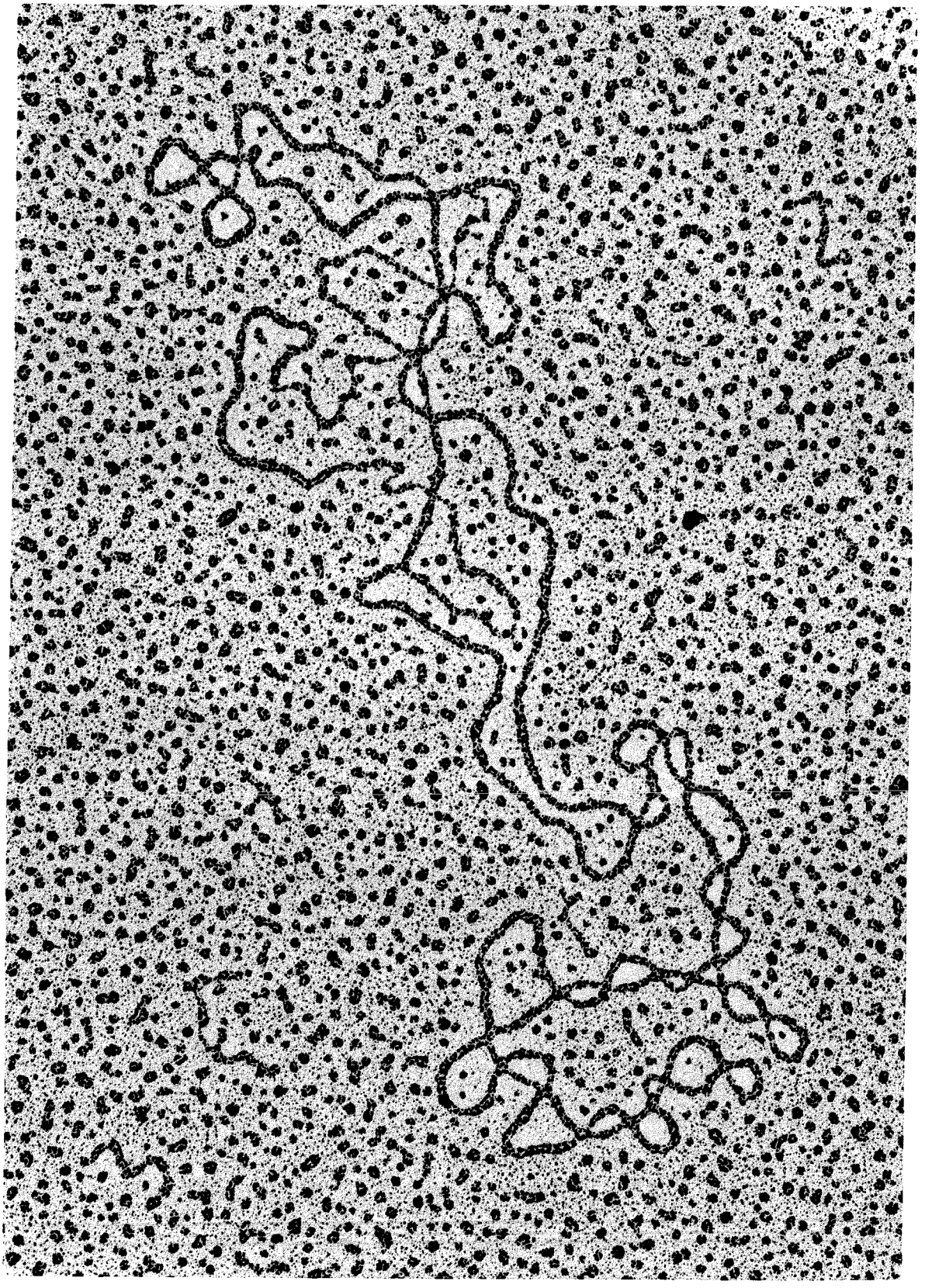
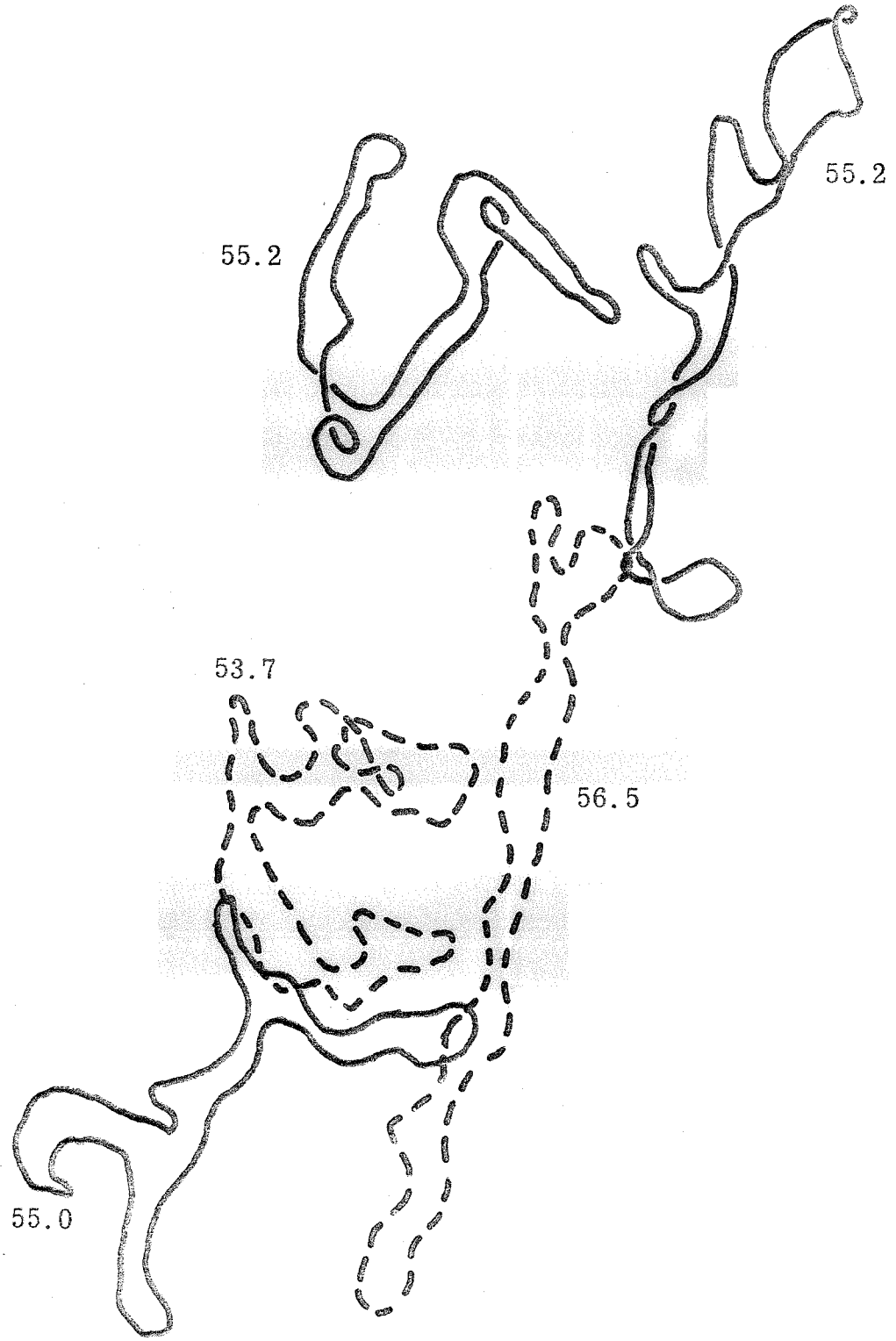


Plate 2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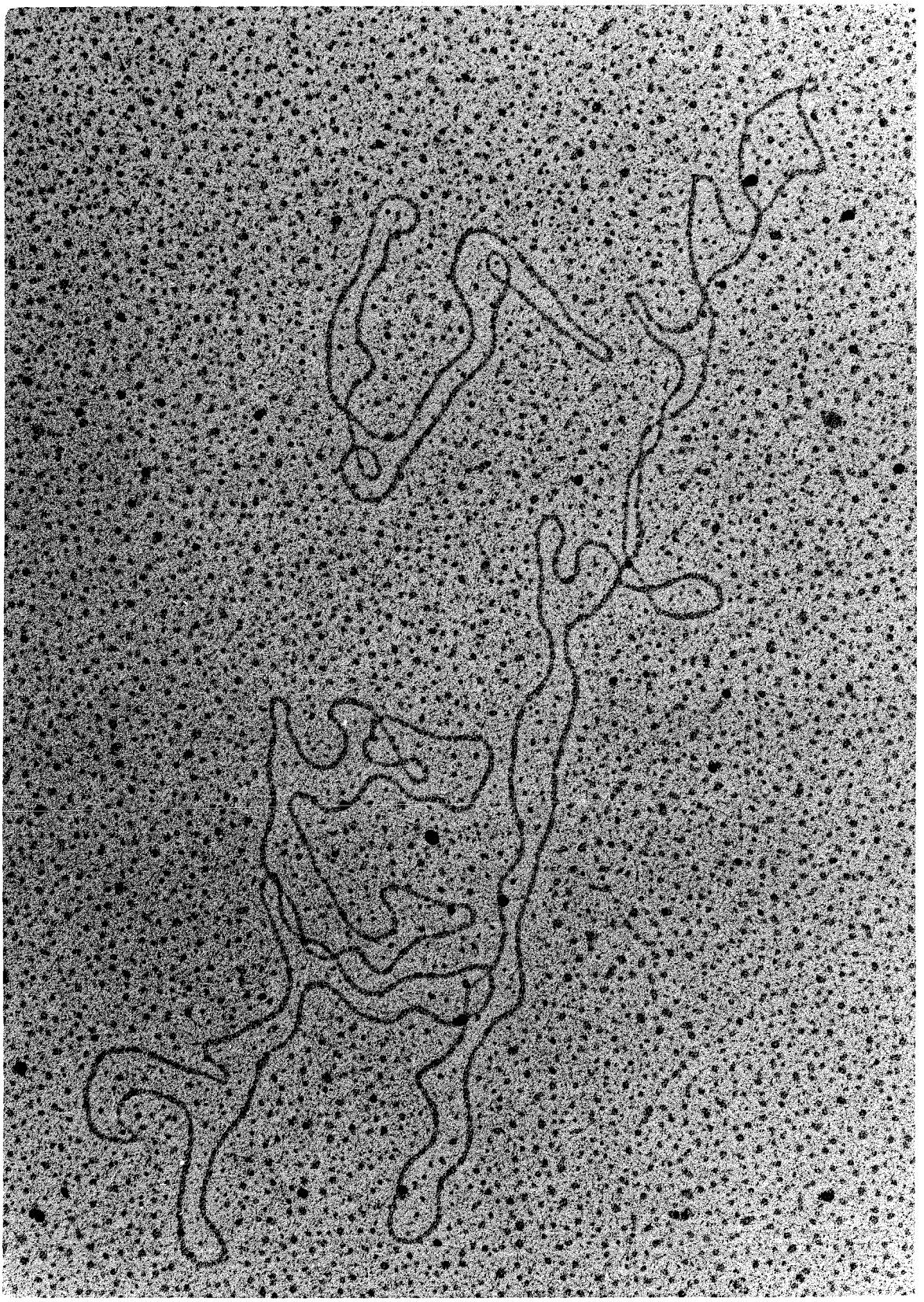
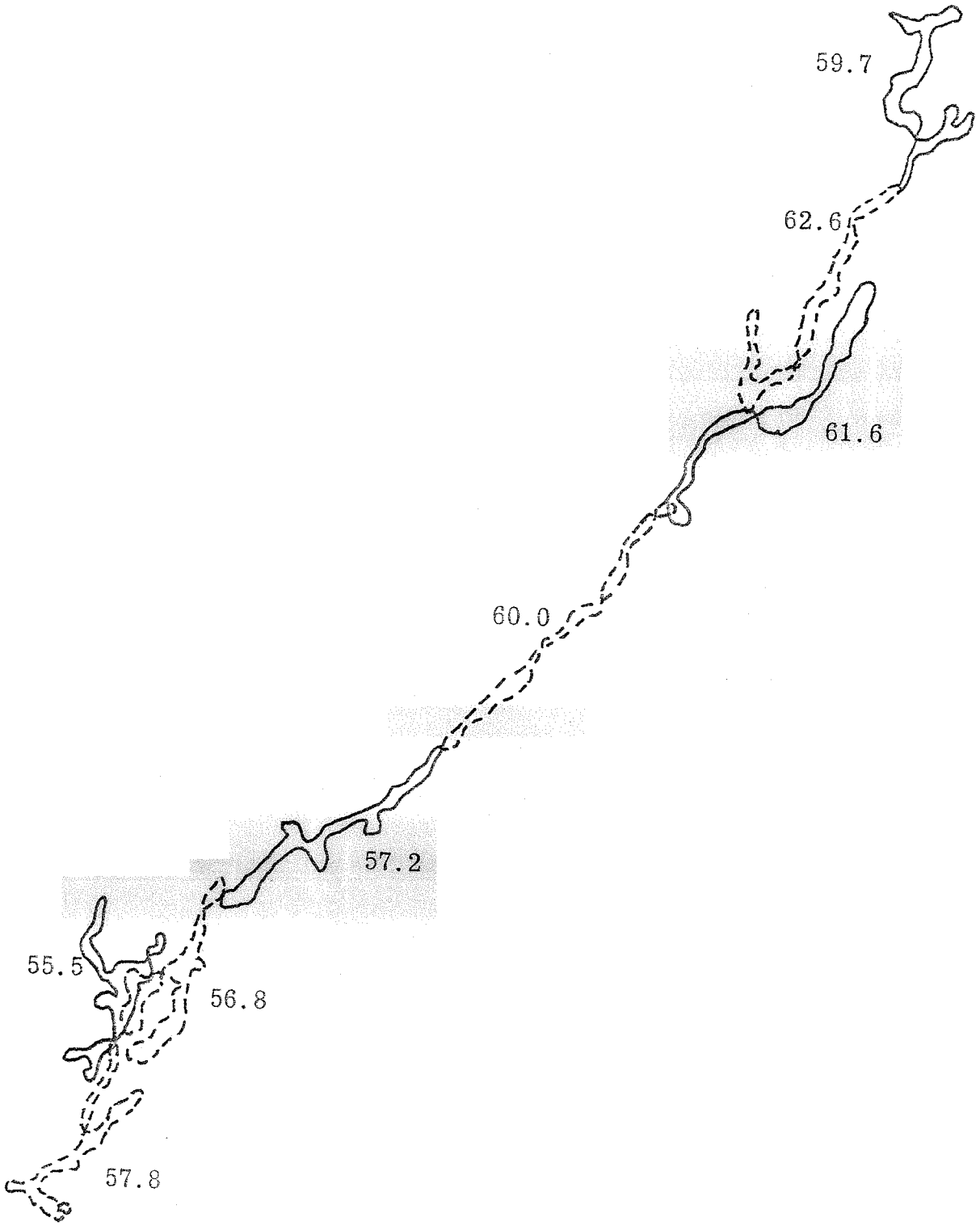


Plate 3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55.5
56.8
57.8

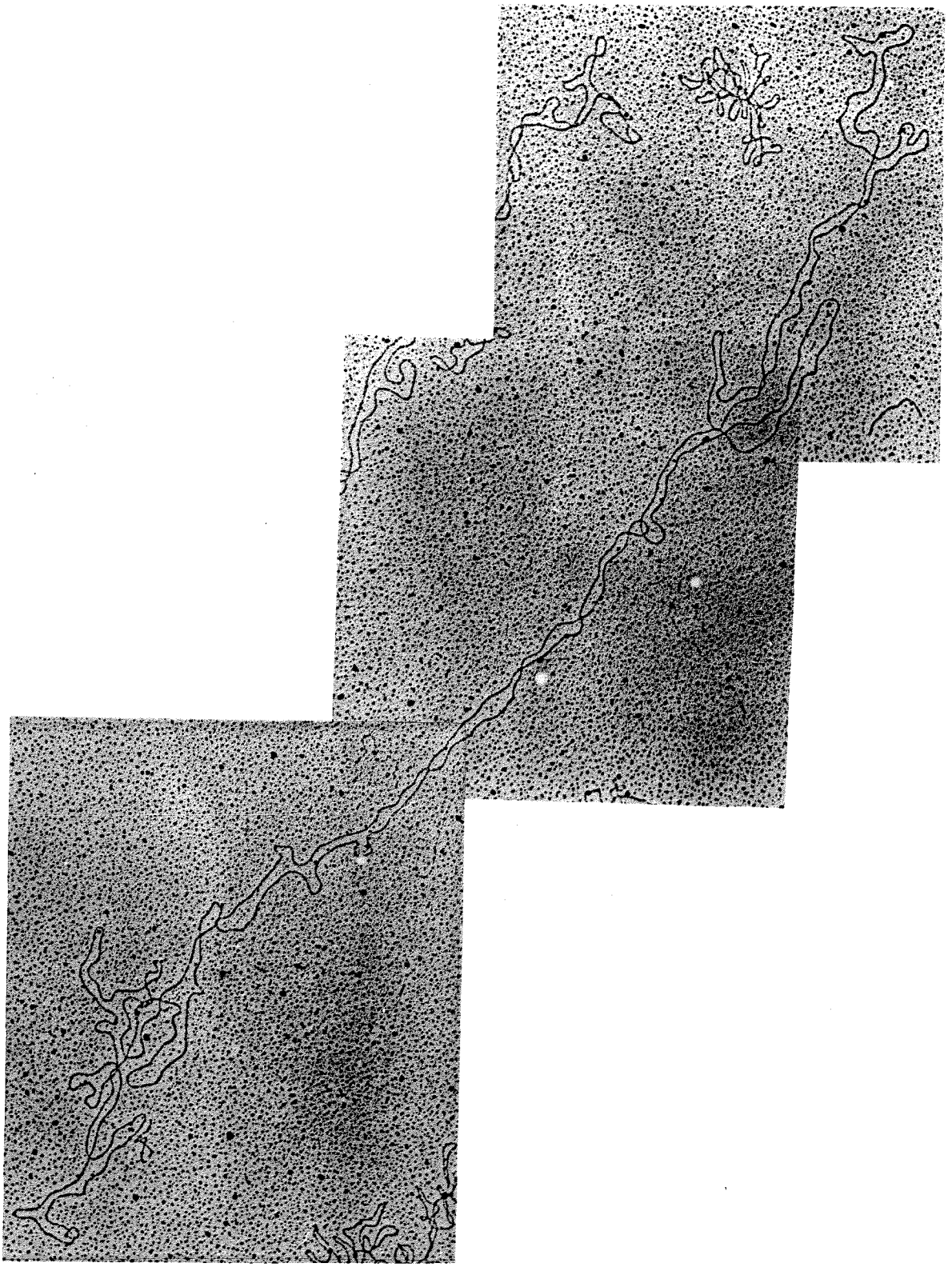
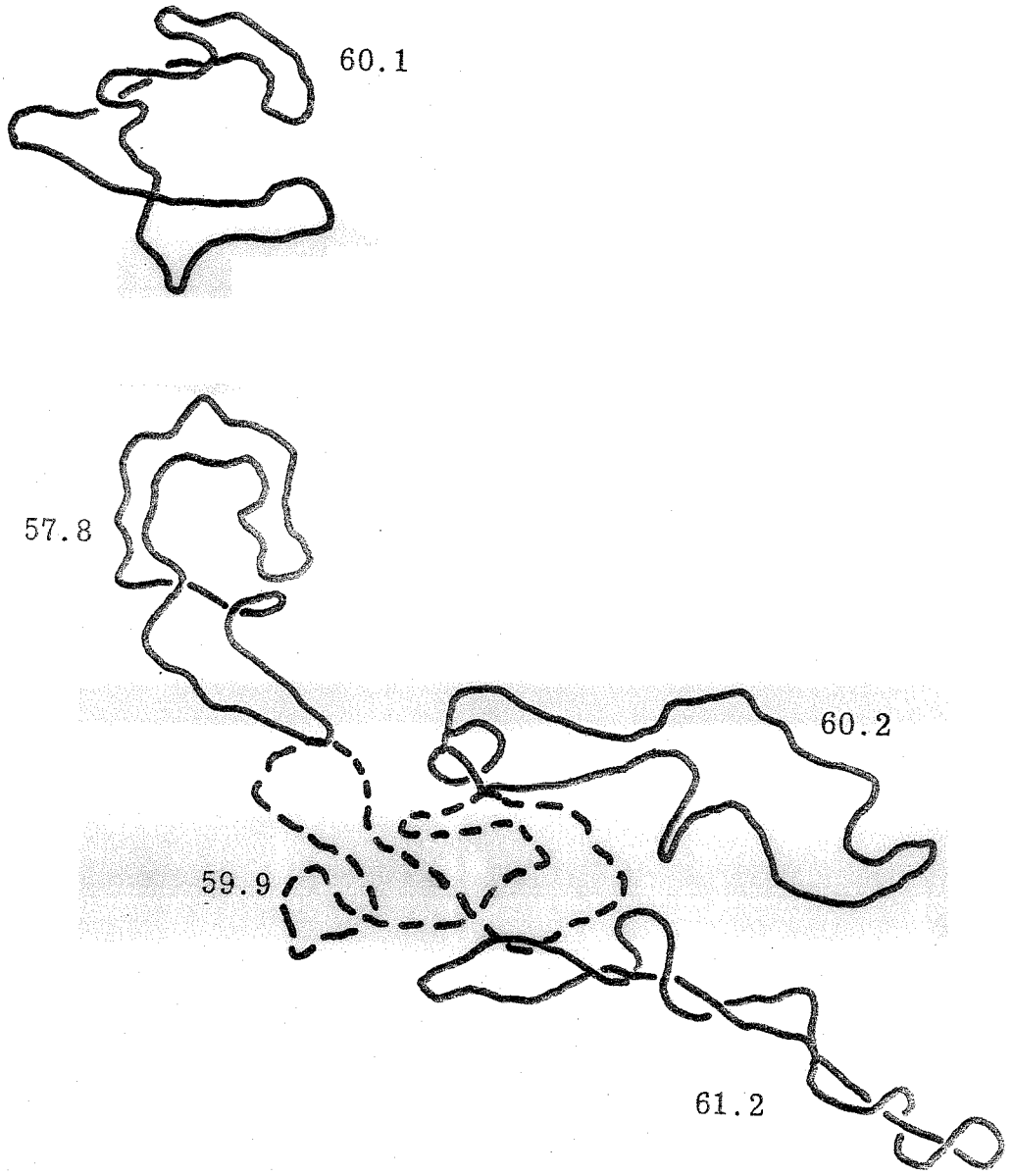


Plate 4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60.2
59.9
61.2

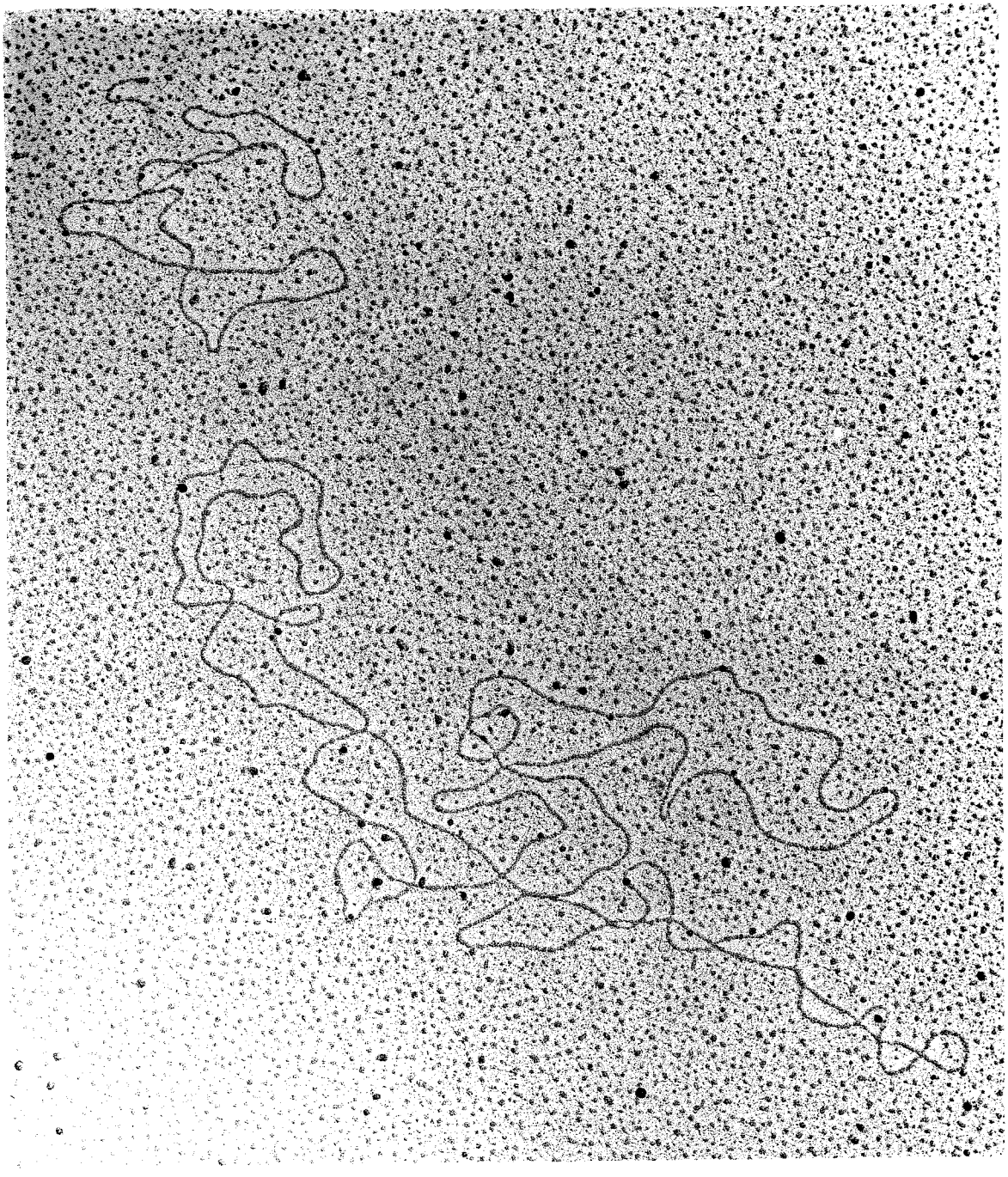


Plate 5

Chapter 2

The Isolation of Intact Complementary Strands of mitDNA and the Mapping of the rDNA and tRNA's on the L Strand Relative to the Origin of Replication

Introduction

The gene products of mitDNA are still largely unknown. Hybridization studies in yeast, Xenopus, and HeLa cells (Wintersberger and Viehhauser, 1968; Dawid and Chase, 1972; Aloni and Attardi, 1971(b)) have clearly established that mitDNA contains one copy of each of the two rRNA genes. Mitochondrial DNA also codes for mitochondrial specific tRNA's. Hybridization saturation studies have shown a level of about 20-30 tRNA genes in yeast (Reijnders and Borst, 1972), 15 in Xenopus (Dawid and Chase, 1972), and 11 in HeLa cell mitDNA, of which about 8 are on the H and 3 on the L strand (Aloni and Attardi, 1971(b)). Mitochondrial tRNA's aminoacylated with labeled amino acids have been used in hybridization experiments demonstrating seryl- and tyrosyl-tRNA genes on the L strand, and leucyl- and phenylalanyl-tRNA genes on the H strand of rat liver mitDNA (Nass and Buck, 1970). Similar studies indicate that yeast mitDNA codes for tRNA's for at least 14 amino acids, including formyl methionine (Cohen and Rabinowitz, 1972; Casey et al., 1974).

Ribosomal and transfer RNA genes occupy at most 25% of the genome of animal cell mitDNA. The function of 75% of the genome is still unspecified. The entire genome is transcribed, since mitRNA complementary to 85-100% of the H strand is found in HeLa cells (Aloni and Attardi, 1971(a)). The L strand is also transcribed, but the resulting RNA is very rapidly degraded (Aloni and Attardi, 1972). Several mitochondrial proteins are synthesized on mitochondrial polysomes in yeast, but whether the mRNA involved is transcribed

from mitDNA is not known. In vitro labeling studies have shown that the three larger polypeptides in yeast cytochrome c oxidase are synthesized on mitochondrial polysomes (Mason and Schatz, 1973; Werner, 1974), since the labeling is sensitive to chloramphenicol but not to cycloheximide. Similar studies suggest that both the component of mitochondrial ATPase which binds it to the membrane and cytochrome b are synthesized on mitochondrial polysomes (Tzagoloff and Meagher, 1971; Weiss and Ziganke, 1974). These methods do not prove that these proteins are specified by mitDNA.

Physical mapping studies of the tRNA and rRNA genes in HeLa mitDNA were begun in order to determine the organization of the known functions within the genome (Wu et al., 1972). Electron microscope analysis of DNA-RNA hybrids was used to map the tRNA and rRNA sites on the H strand and the tRNA sites on the L strand. Ferritin was covalently linked to the tRNA's in order to improve their visibility in the electron microscope. The two rRNA genes are close together, separated by a small spacer of about 160 nucleotides. The spacer contains a tRNA site, and there is one tRNA site at each end of the rRNA region. The other five tRNA sites on the H strand and the three on the L strand are scattered around the molecule, with one cluster of two tRNA's on the H strand.

The present work describes an extension of the map of the L strand to include the rRNA gene complements and the DNA replication initiation region (Kasamatsu, Robberson, and Vinograd, 1971). In order to perform all of the mapping on circular L strands, a method for preparing relatively intact separated strands of HeLa mitDNA

was developed. The usual method of strand separation in buoyant alkaline CsCl results in fragments too small ($\overline{M}_n \sim 10^6$) to be suitable for mapping experiments. Wu et al. (1972) avoided this difficulty by using a mixture of H and L strands formed by brief exposure of singly nicked mitDNA to alkali, but this procedure has the disadvantage of extensive renaturation of duplex DNA under the hybridization conditions. The method described here takes advantage of the enhanced alkali sensitivity of BrUra labeled DNA strands to produce unlabeled H and L strand preparations containing 15 and 30 wt% single stranded circles respectively, using hybrid BrUra labeled mitDNA as the starting material. The H strands were used in the preparation of 'rDNA', the portion of the H strand complementary to rDNA. Mapping experiments with the L strand preparation show that the rRNA genes are located approximately 180° from the 7s DNA replication initiation region on the circular map, and permit the positioning of the three L strand tRNA's relative to these markers.

This work was performed in collaboration with Madeline Wu and Norman Davidson (preparation of ferritin-tRNA, hybridization, and electron microscopy), Giuseppe Attardi (preparation of rDNA), and Harumi Kasamatsu (preparation of 7sDNA). While the author's contributions were limited to the preparation of HeLa mitDNA strands and collation and calculation of data, the entire project is presented here for the first time.

Materials and Methods

1. Preparation of intact L and H strands

a. Closed mitDNA

Suspension cultures of HeLa cells were grown in Eagle's phosphate medium (Grand Island Biological Co., Berkeley, Calif.) to a density of at least 1 x 10^5 cells/ml and then labeled for 24 hours with 0.5μci/ml ^{3}H-thymidine (New England Nuclear Corp., Boston, Mass.). Cells were then harvested by centrifugation at 1000g for 3 minutes and kept at 0° to 4°C thereafter. After two washed with TD buffer (0.14 M NaCl, 0.005 M KCl, 0.0007 M Na_2HPO_4, 0.025 M Tris, pH 7.4), the cells were suspended in a 10-fold volume of RSB± buffer (0.01 M NaCl, 0.001 M EDTA, 0.01 M Tris pH 7.4) and allowed to swell for 10 minutes before disrupting them with 2 to 4 strokes of a Dounce homogenizer. Sucrose was immediately added to a concentration of 0.25 M and nuclei were removed by pelleting at 1000g for 3 minutes. The homogenate was then layered in 20 ml. aliquots onto 10 ml aliquots of 1.5 M sucrose, 0.001 M EDTA, 0.01 M Tris, pH 8.0 in SW27 tubes, and centrifuged at 25,000 revs/min for 45 minutes at 4°C in the Spinco SW27 rotor. The material at the interface was collected, diluted with MS buffer (0.21 M mannitol, 0.07 M sucrose, 0.001 M EDTA, 0.01 M Tris, pH 8.0), and pelleted at 27,000g for 15 minutes. After one or two washes with MS buffer, the mitochondria were lysed by adding 1%w/v SDS, 0.5 M NaCl, 0.01 M EDTA, 0.01 M Tris, pH 8.0. SDS was precipitated by adding CsCl to 1 M and chilling; the precipitate was removed by centrifugation at

27,000g for 5 minutes. Ethidium bromide was added to 300 μg/ml, the density was adjusted to 1.55 g/ml with CsCl, and the solution was centrifuged at 36,000 revs/min for 24-36 hours in a Spinco SW50.1 rotor. The lower band was removed and rebanded under the above conditions. After the second centrifugation the ethidium bromide was removed with a 0.5 x 6 cm Dowex AG-50 X8 column (Bio-Rad, Richmond, Calif.) and the CsCl was removed by dialysis against NTE buffer (0.01 M NaCl, 0.001 M EDTA, 0.01 M Tris, pH 8.0).

b. Singly nicked mitDNA

Closed mitDNA was nicked with pancreatic DNase (Sigma Chemical Co., St. Louis, Mo.) to an average of less than one hit per molecule. In each case the enzyme solution was calibrated by digesting small aliquots of the mitDNA sample and then banding them in EB-CsCl. Conditions giving 37% to 60% of the input radioactivity at the closed position were determined in these assays, and were used to digest the rest of the mitDNA sample. In a typical experiment, 57% of the mitDNA was still closed after incubation of 20 μg of mitDNA with 14 μg of pancreatic DNase for 15 minutes at 37 °C in a total volume of 6 ml containing 15 μg/ml bovine serum albumin (Pentex, Miles Laboratories, Kankakee, Ill.), 0.005 M $MgCl_2$, 0.03 M Tris, pH 8.0. The singly nicked mitDNA was then isolated from the digest as an upper band in a EB-CsCl gradient.

c. BrUra labeled mitDNA

Suspension cultures of HeLa cells were labeled with 20 μg/ml BrdUrd (Schwarz-Mann, Orangeburg, N. Y.) for 24 hours, and the

mitDNA was isolated according to Flory and Vinograd (1973). Nonradioactive BrUra-labeled mitDNA preparations were pooled with two radioactive preparations: one from cells prelabeled with 0.2 μci/ml ^{3}H-Thymidine and then labeled with 0.25 μci/ml ^{3}H-deoxycytidine (New England Nuclear, Boston, Mass.) during the BrdUrd labeling, and the other from cells treated only with ^{3}H-deoxycytidine. These radioactive labels were included solely as markers for the densities of the strands in alkaline buoyant CsCl and do not reflect the distribution of mass between the different species.

d. Conditions of alkaline buoyant CsCl centrifugation

The alkaline cesium chloride solutions contained 0.05 M KH_2PO_4, 0.05 M glycine, and 0.005 M EDTA. An appropriate volume of this buffer containing cesium chloride at the desired density was titrated in a polyallomer centrifuge tube to the desired pH with a solution of 5.8 M CsCl containing 2M KOH (Tamm, Inc., Altuna, Sweden), using the apparatus described in Grossman, Watson and Vinograd (1973) to measure the pH at 20°C under argon. The DNA was then added in a relatively small volume and the pH was measured again (there was usually no change). The tube was sealed with Bayoil and the refractive index of the contents was checked. The tube was centrifuged 25 to 56 hours at 34,000 revs/min at 20.0 ± 0.5°C in an SW 50.1 rotor. The temperature control system of the Beckman L2-55B ultracentrifuge used was calibrated by measuring the temperature of a blank tube immediately after the run using a precision thermometer. The tubes were dropped in 5-or 6-drop

fractions (approx. 8μl/drop), either directly onto Whatman GF/A filters or into plastic tubes (Croan Plastics, Huntington Beach, Calif.) each containing 10μl of 1 M Tris, pH 8.0.

The solutes other than CsCl in these samples make direct correlation of refractive index with density impossible. Unlabeled mitDNA banded near the center of the gradient if the initial refractive index was about 1.4030 (2.92 g CsCl in a final volume of 3.0 mls). For BrUra-labeled mitDNA, an initial refractive index of 1.4076 (3.20 g CsCl per 3.0 ml) was used because the density-labeled H strand has a density near 1.89 g/ml (Flory and Vinograd, 1973). This solution myst be prepared with care because CsCl is saturated near ρ = 1.9 g/ml.

2. Preparation of 7s HeLa mitDNA

Closed ^{3}H-thymidine labeled HeLa mitDNA was prepared by the method of Kasamatsu, Robberson, and Vinograd (1971). The 7s was released by heat denaturation at 90°C for 80 sec followed by quenching in ice. It was then isolated on a 5-20% sucrose gradient, containing 0.5 M NaCl, 0.001 M EDTA, 0.01 M Tris, pH 8.0, centrifuged at 36,000 revs/min for 130 minutes at 20°C in an SW50.1 rotor (Figure 1(a)). The fractions indicated were pooled and applied to another sucrose gradient (Figure 1(b)). The final yield after the fractions indicated were pooled was about 0.6μg of purified 7s mitDNA.

3. Preparation of rDNA

The procedure involves hybridization of H strands to mitochondrial rRNA, digestion of nonhybridized regions of the DNA with S_1

Figure 1: Purification of 7S mitDNA from HeLa Cells

Heat-denatured lower band HeLa mitDNA was sedimented through neutral 5-20% sucrose gradients for 130 minutes at 36,000 revs/min in an SW50.1 rotor at 20°C, as described in Materials and Methods (2). The field is directed to the left.

(a) Sedimentation of denatured lower band mitDNA

The ordinate has been expanded (solid points) at the right to show the 7S peak more clearly.

(b) Resedimentation of pooled fractions from (a)

The fractions indicated by the arrows in (a) were resedimented under the same conditions. The fractions indicated by arrows were pooled.

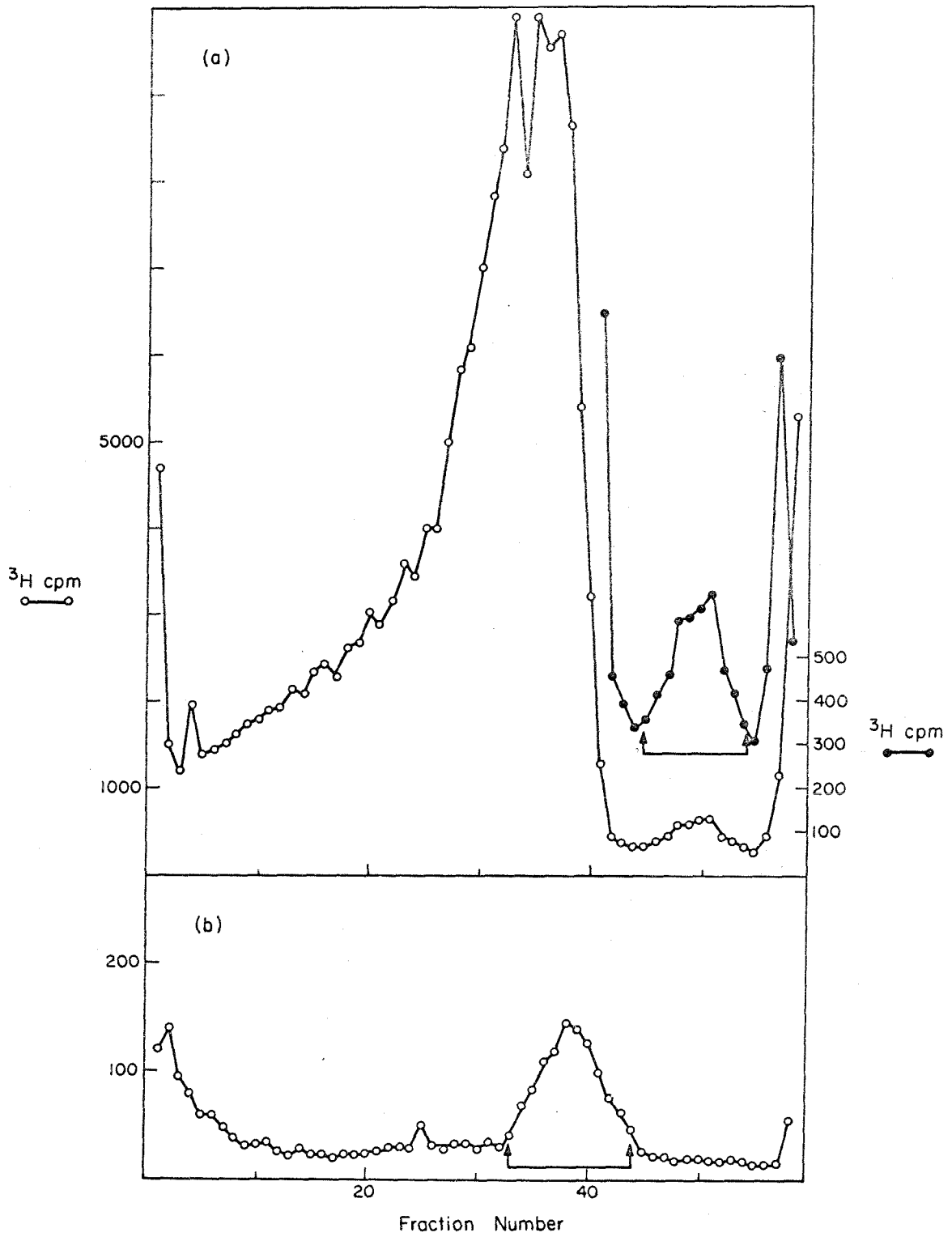

(a)
(b)
^{3}H cpm
5000
1000
500
400
300
200
100
200
100
20
40
Fraction Number

Figure 1

endonuclease, digestion of excess rRNA with pancreatic RNase, and isolation of hybrids. For the hybridization, a 0.3 ml mixture containing ^{3}H-labeled H strands (from BrUra labeled hybrids) at 6 μg/ml, ^{3}H-uridine labeled rRNA (an equimolar mixture of 12s and 16s) at 39 μg/ml, 0.3 M NaCl, 0.001M EDTA, and 0.01 M Tris, pH 8.0 (25°C) was incubated at 60°C for 40 minutes. After rapid chilling, the nucleic acid was precipitated by adding two volumes of ethanol and then pelleted. For the S_1 digestion, the pellet was dissolved in 0.3 ml of 0.15 M NaCl, 5×10^{-4} M $ZnCl_2$, and 0.03 M sodium acetate, pH 4.6. After adding 100 μg of denatured HeLa nuclear DNA in 0.13 ml 0.001 M NaCl, 0.001 M EDTA, the hybrids were incubated for 60 minutes at 37°C with 3 μl of S_1 endonuclease (a gift from Dr. Paul Berg). The reaction was terminated by adding EDTA to 0.01 M and ethanol precipitating the hybrids as before. The pellet was then dissolved in 2.0 ml 0.4 M CsCl, 0.001 M EDTA, 0.01 M Tris, pH 7.4, and excess rRNA was digested with 10 μg/ml pancreatic RNase for 30 minutes at 25°C. The reaction was terminated by adding 40 μg/ml Pronase and incubating for an additional 15 minutes. The mixture was made 1% in SDS and extracted with an equal volume of cold phenol for 15 minutes. The phases were separated by centrifugation, and the aqueous phase was extracted with ether to remove traces of phenol. Nitrogen was then bubbled through the aqueous phase to remove ether, CsCl was added to a density of 1.74 g/ml, and the rDNA:rRNA hybrid was centrifuged at 41,500 revs/min for 120 hours in a polyallomer tube in the SW65 rotor. The gradient was dripped, and the fractions corresponding to the hybrids were pooled and precipitated with ethanol as above.

The rRNA was denatured from the rDNA and degraded, presumably by a brief exposure to alkali.

4. Preparation of ferritin-tRNA

The methods of covalently coupling tRNA to ferritin have been described in Wu et al. (1972).

5. Electron microscopy

Length distributions of separated strands of mitDNA were determined by spreading the DNA by a formamide Kleinschmidt technique. 50 μl of a solution containing DNA at 0.5 to 0.3 μg/ml, 50% formamide, 50 μg/ml cytochrome c, 0.01 M EDTA, 0.1 M Tris, pH 8.5 was spread onto 17% formamide, 0.001 M EDTA, 0.01 M Tris, pH 8.5. Grids were stained with uranyl actetate and rotary shadowed with platinum-palladium. They were examined and photographed in a Philips EM300 electron microscope. Molecules were traced on a Nikon 6F projection microcomparator and measured with a map measurer. In general the intact circular strands of mitDNA were used as an internal length standard in determining fractional lengths. However, ØX174 viral DNA was added to the spreadings of the strands from BrUra labeled hybrid mitDNA as a standard. The ØX174 DNA was also spread separately and found to contain 15% (number) linear strands. These fragments were subtracted from the mitDNA length distributions.

Spreadings of the 7s, rDNA, and hybrids were performed according to Wu et al. (1972).

Results

1. Preparation of HeLa mitDNA strands

a. Measurement of the alkaline gradient in the preparative ultracentrifuge

If the alkaline gradient is very large, the pH experienced by a given species would be affected by its equilibrium position in the tube, which would be a function of the initial CsCl density. It was therefore necessary to measure the pH gradient under conditions appropriate to separating the strands of mitDNA. Two blank tubes, each containing 3.2 ml of the standard buffered CsCl solution at a pH near the pH_m for strand separation, were centrifuged at 34,000 revs/min for 26 hours. They were dripped into five portions (stored under Bayoil), and the pH of each portion was measured under argon (Figure 2). The temperature of the run was 20.8°C as measured in two other blank tubes from the same rotor. The pH difference across the entire tube was 0.32 pH units, The separation between the strands on HeLa mtDNA in other identical density gradients can be used to estimate the density gradient, since the density difference between the strands is known (Clayton et al., 1970; Flory and Vinograd, 1973). The alkaline gradient is about 0.0013 pH per mg/ml in density, so the heavy strand experiences a pH about 0.05 pH units higher than the L strand in these gradients.

b. Strand separation of singly nicked mitDNA

A series of experiments was performed in order to determine whether the amount of alkali nicking of mitDNA during strand separa-

Figure 2: Measurement of the pH Gradient in Alkaline Buoyant CsCl Gradients

Two polyallomer SW50 tubes containing the glycine-phosphate buffered alkaline CsCl solution described in Materials and Methods (1d) were centrifuged to equilibrium, and each was fractionated dropwise into five portions. The pH of each portion is plotted versus mean drop number of that portion from the bottom of the tube. A least-squares line has been drawn for each set of points. The slope in both cases is 0.0095 pH/drop.

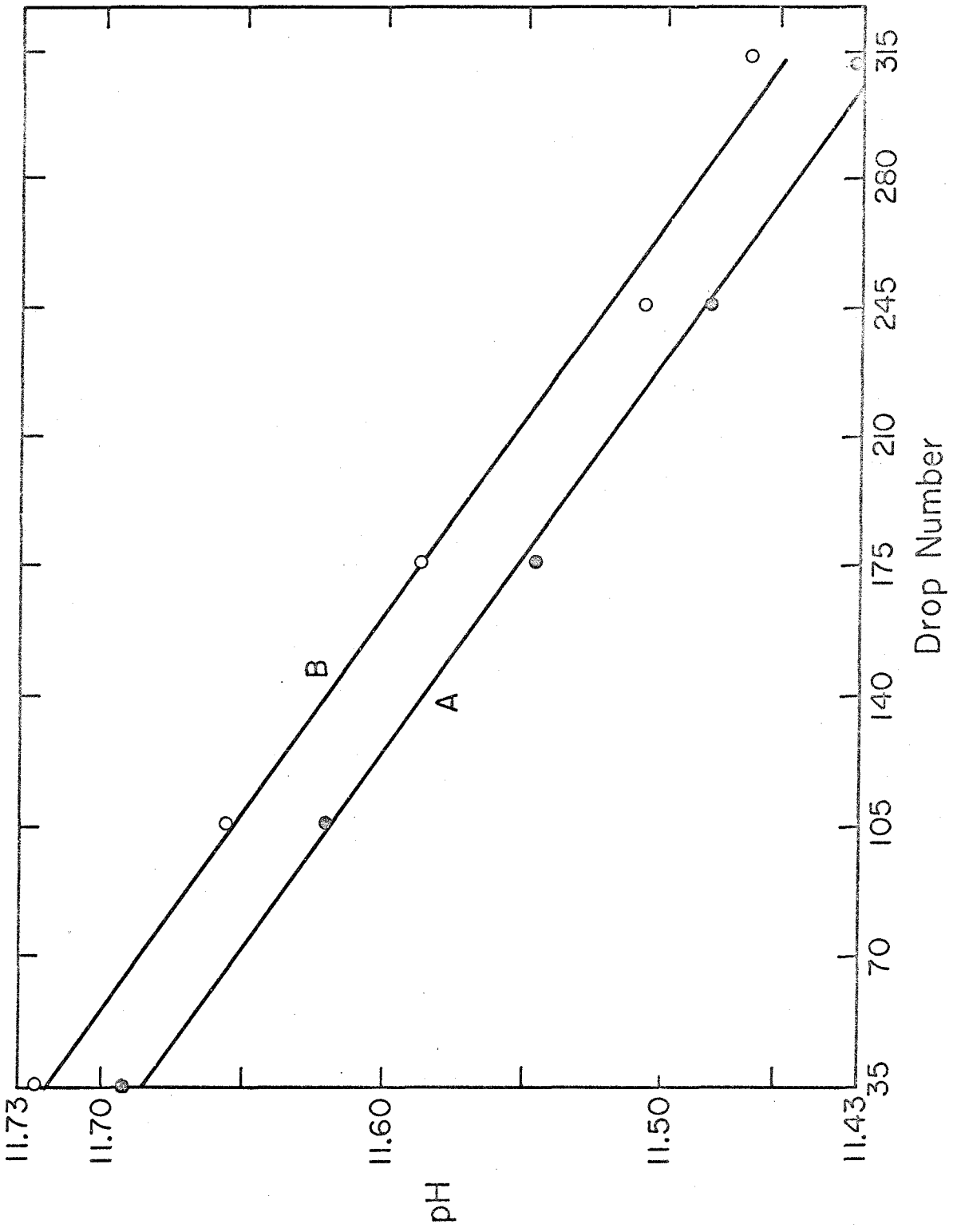

11.73
11.70
11.60
11.50
11.43
pH
35
70
105
140
175
210
245
280
315
Drop Number
A
B

Figure 2

tion in alkaline CsCl gradients could be reduced to an acceptably low level simply by reducing the pH to the minimum required for strand separation. The pH_m of strand separation was first determined by banding aliquots of singly nicked ^{3}H-thymidine labeled mitDNA (prepared as described in Materials and Methods, 1(b)) in alkaline buoyant CsCl at different pH's. The gradients were run for 48 hours at 34,000 revs/min at 20°C in polyallomer tubes in the SW50.1 rotor, dripped in 5 or 6 drop fractions onto Whatman GF/C filters, and counted. Typical profiles (Figures 3a-3d) show the separated strands as two peaks near the center of the gradient and the nondenatured duplex as a single peak further to the right. The percentage of counts present as separated strands is plotted versus the initial pH in Figure 4 (solid points). The results clearly show that the strands separate in a sharp transition near pH 11.55 in this system.

A larger quantity of singly nicked mitDNA was now banded in alkaline buoyant CsCl at pH 11.63, the minimum required for complete strand separation, for 48 hours at 20°C. The profile (Figure 5(b)) shows that 95% of the mitDNA was denatured in this gradient. The fractions containing the strands were pooled as shown and examined in the electron microscope to determine how much additional nicking had occured during the centrifugation. The original material was isolated from a pancreatic DNase digest in which 57% of the DNA was not nicked, corresponding to an average of 0.56 hits per molecule (Radloff and Vinograd, 1971). If no additional nicking has occured, each strand preparation should contain 43wt% single stranded circles, 49wt% full length linears, and 8wt% smaller linear fragments. The

Figure 3: Banding of Singly Nicked HeLa mitDNA in Alkaline Buoyant CsCl

Samples of singly nicked ^{3}H-labeled HeLa mitDNA were added to buffered alkaline CsCl solutions, resulting in the pH values given at the upper right in each case. In (c) and (d), the pH was reduced to the second value by adding 1 M Tris, pH 8.0. The solutions were then centrifuged to equilibrium, fractionated dropwise onto filters, and counted as described in Materials and Methods (1d). In each plot, the two bands at the left represent the H and L strands of mitDNA and the band at the right is the duplex DNA. The field is directed to the left.

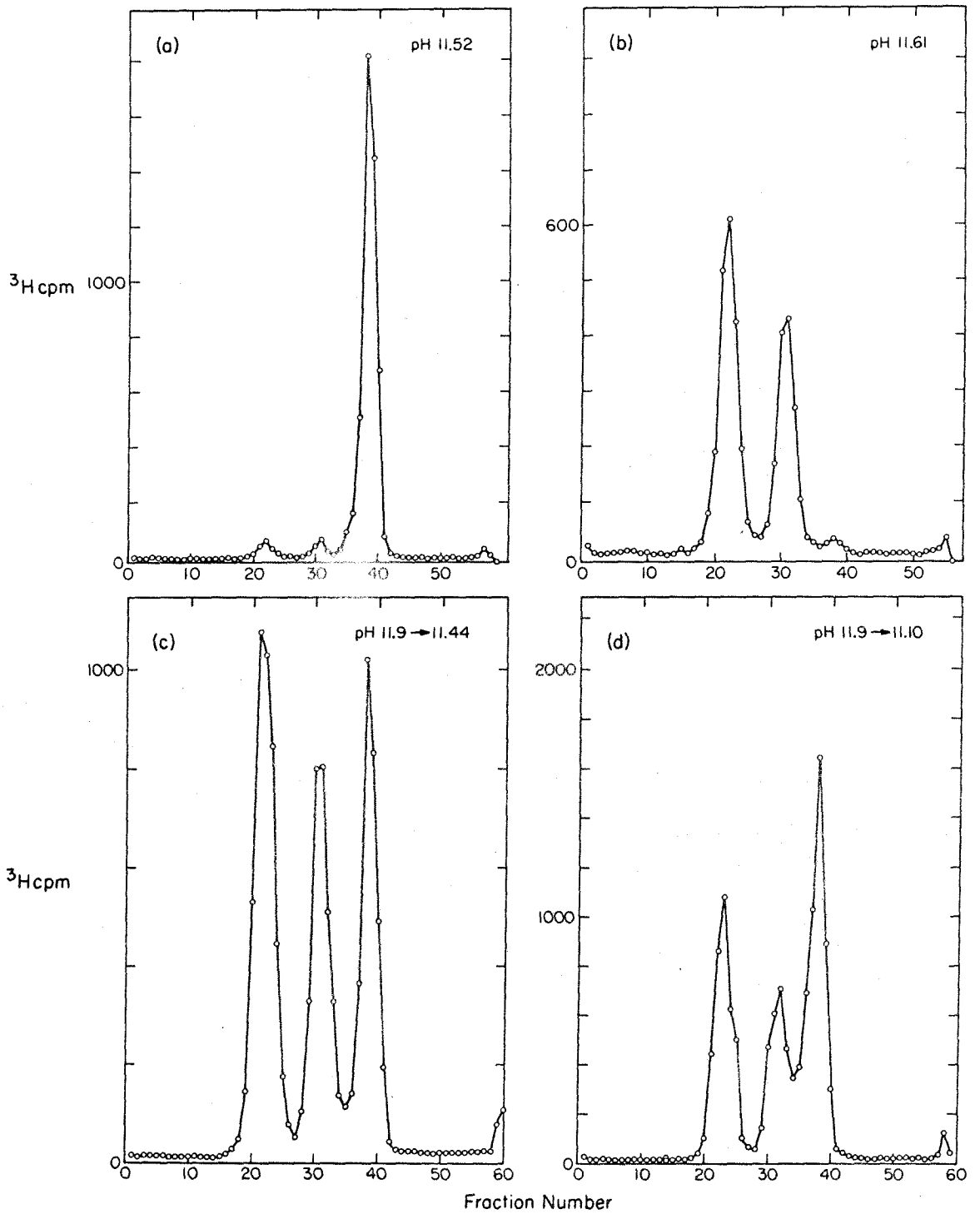

(a)
pH 11.52
(b)
pH 11.61
(c)
pH 11.9→11.44
(d)
pH 11.9→11.10
^{3}H cpm
^{3}H cpm
Fraction Number

Figure 3

Figure 4: Titration of Singly Nicked HeLa mitDNA in Alkaline Buoyant CsCl

The fraction of the total ^{3}H label which bands as separated H and L strands is plotted versus pH for a series of experiments of the type shown in Figure 3. The solid line and open points describe the behavior of singly nicked mitDNA when added to buoyant CsCl at the indicated pH (using the conditions described in Materials and Methods (1d)). The dashed line and solid points summarize the results when the mitDNA was first added to pH 11.9 buoyant CsCl and then titrated to the indicated pH with 1 M Tris, pH 8.0.

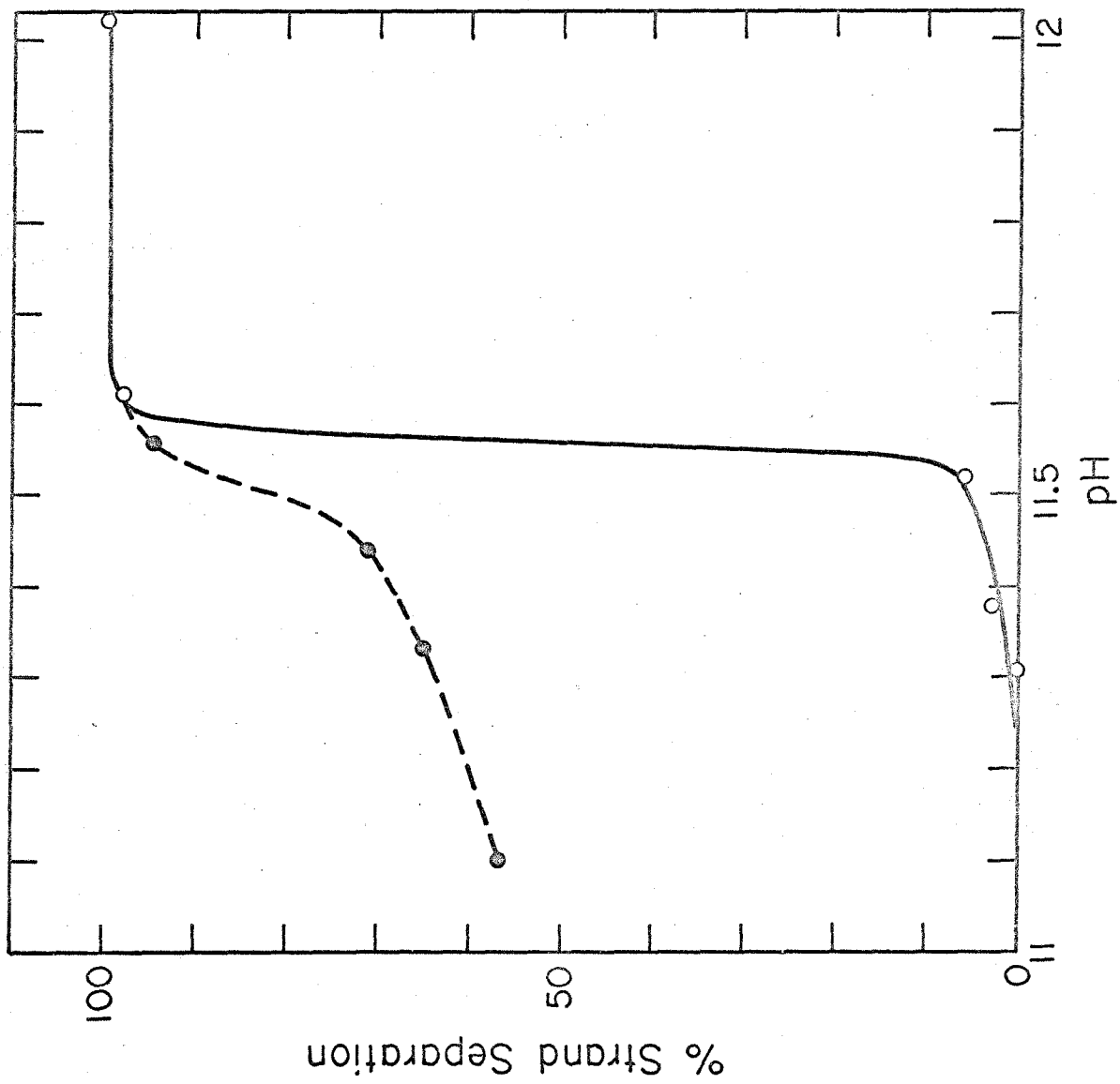


Figure 4

Figure 5: Preparation of H and L Strands from Singly Nicked HeLa mitDNA

(a) EB-CsCl gradient of DNase I-nicked mitDNA

Covalently closed HeLa mitDNA was lightly nicked with pancreatic DNase under the conditions described in Materials and Methods (1b) and then banded in EB-CsCl (250 μg/ml Ethidium Bromide, ρ = 1.55 CsCl). The profile shows that 57% of the DNA was still closed after this treatment, indicating an average of 0.57 nicks per molecule. The upper band fractions indicated by the arrows were pooled.

(b) Alkaline buoyant CsCl banding of singly nicked mitDNA

The pooled fractions from (a) were banded at pH 11.63 in buoyant CsCl (as described in Materials and Methods (1d)). The fractions indicated by arrows were pooled and used for electron microscopy.

In both cases the field is directed to the left.

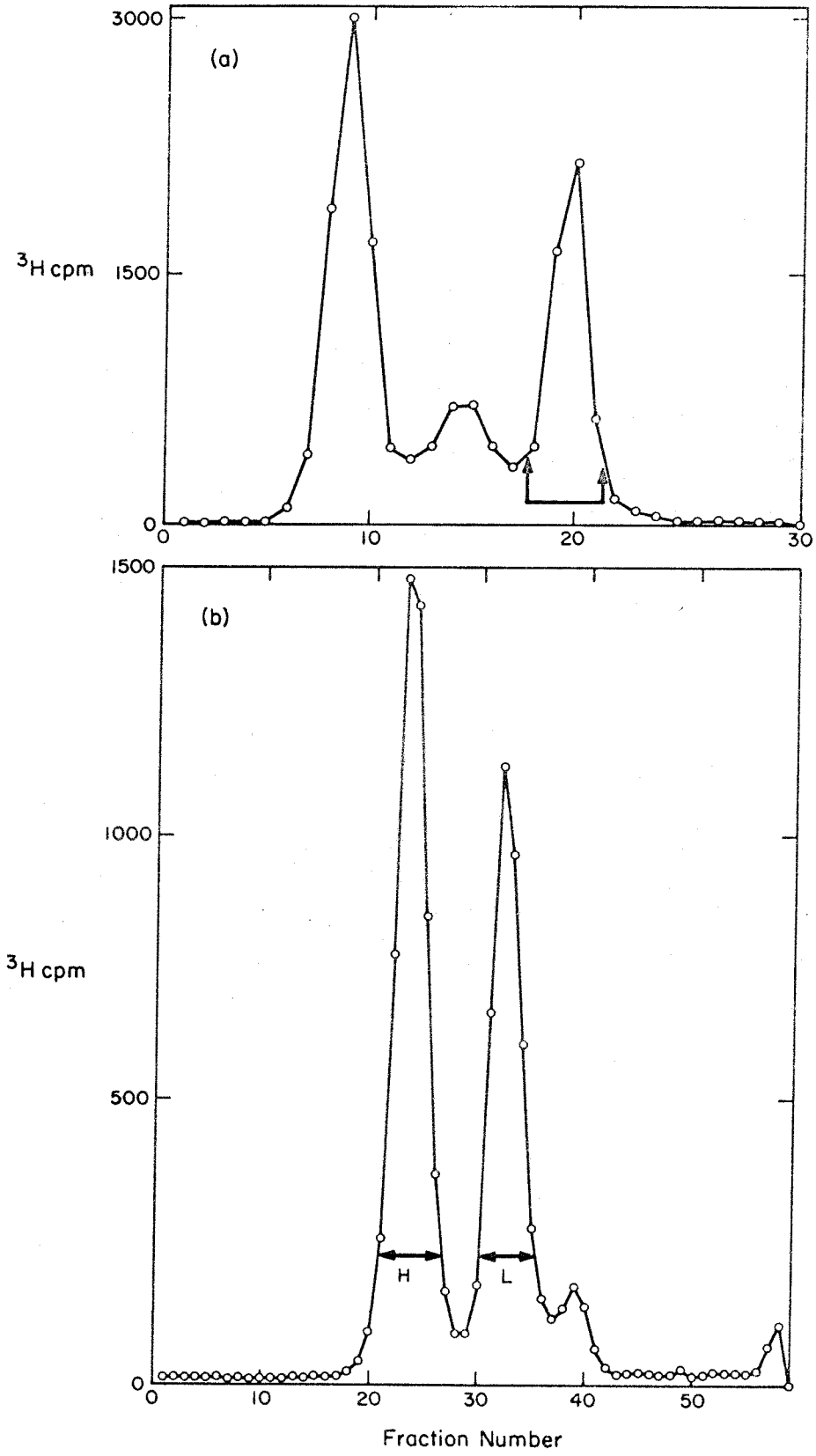

3000
(a)
^{3}H cpm
1500
0
0
10
20
30
1500
(b)
1000
^{3}H cpm
500
H
L
0
0
10
20
30
40
50
Fraction Number

Figure 5

observed length distributions (Figures 6(a), 6(b)) show that additional nicking has occurred. The equations of Radloff and Vinograd (1971) indicate that each strand has suffered about one additional nick during the centrifugation. This additional nicking does not occur during dialysis to remove CsCl after the banding, since grids (of poor contrast due to the high salt concentration) prepared directly show a similar length distribution on a smaller sample size. Hence the alkaline nicking rate even at minimum pH for strand separation is too high to permit the preparation of separated strands containing high levels of single stranded circles.

A series of experiments was performed to determine whether singly nicked mitDNA could be briefly exposed to high pH and then banded at a lower pH without extensive renaturation. Singly nicked mitDNA was added to alkaline CsCl solutions at pH 12.0 and then titrated to a lower pH before centrifugation. Banding profiles of about 0.5μg of singly nicked mitDNA under these conditions are shown in Figures 3(c) and 3(d), and the results are summarized in Figure 4 (open circles). At pH 11.1 (0.5 pH below pH_m for denaturation), 43% of the material has renatured. This loss was felt to be too great a penalty to pay for the reduction in nicking rate to be expected from this reduction in pH, especially as an even greater loss would be expected with the larger amounts of DNA involved in a preparative gradient.

An experiment was also performed on closed mitDNA in the hope that a higher yield of single stranded circles could be achieved in this way, since some of the nicks incurred during the centrifugation

Figure 6: Length Distribution of H and L Strands Prepared from Singly Nicked HeLa mitDNA

The pooled fractions indicated in Figure 5(b) were mounted for electron microscopy by the formamide Kleinschmidt technique described in Materials and Methods (5). The weight fraction of each size class is plotted, with the circular strands indicated by shading. About 17% of the L strands are circular, and another 41% are full length linears. The H strand preparation contains 13% circles and 35% full length linears.

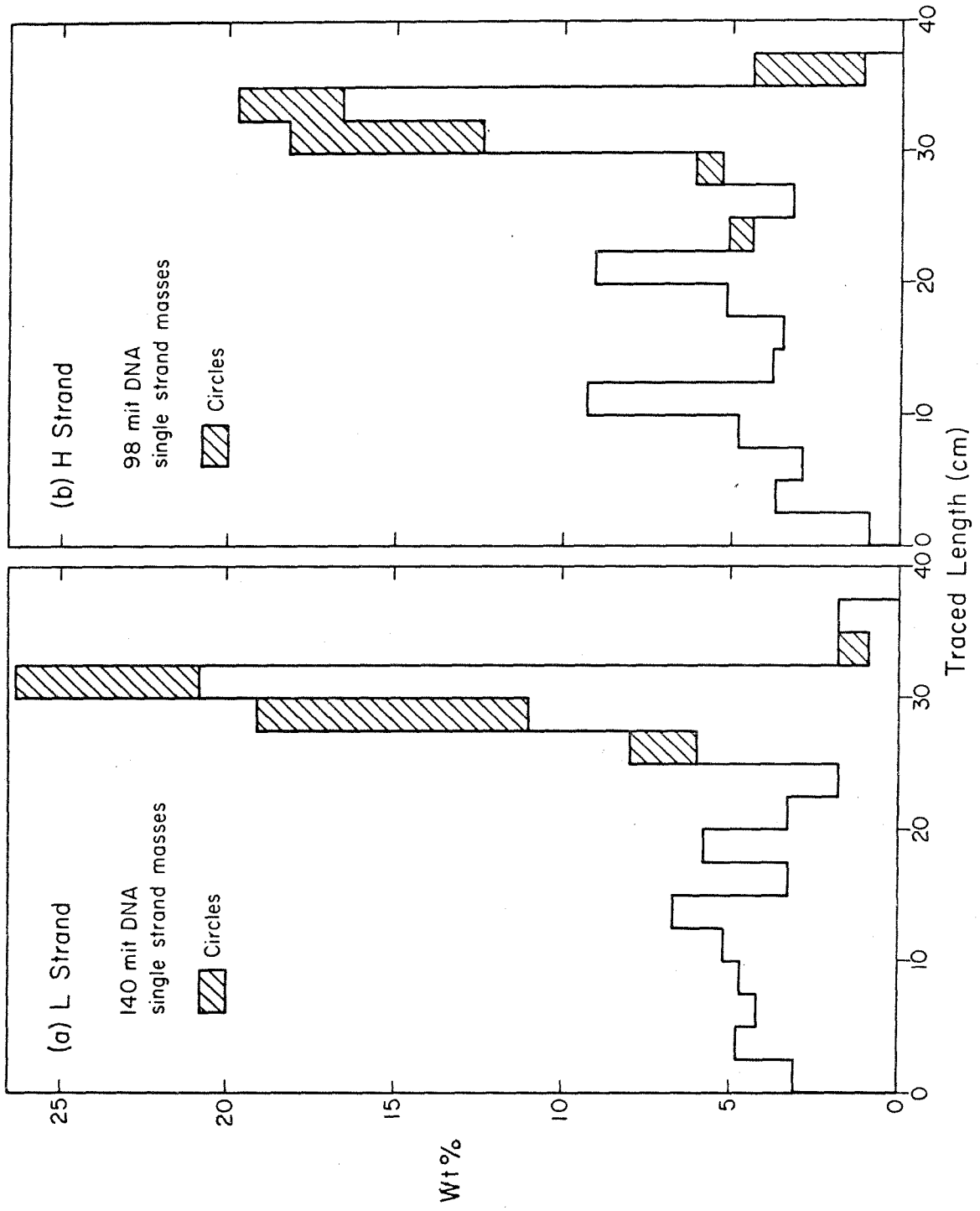

(a) L Strand
140 mit DNA
single strand masses
Circles
(b) H Strand
98 mit DNA
single strand masses
Circles
Wt %
Traced Length (cm)
0
5
10
15
20
25
0
10
20
30
40

Figure 6

would be the nicks required for strand separation. No strand separation was observed at pH 11.64 (Figure 7), even though the results above suggest that an average of 2 nicks per duplex would occur at this pH during the banding. Evidently closed duplex mitDNA is much less sensitive to alkali than the separated strands are. This effect has also been observed during measurements of the rate of alkali nicking of closed mitDNA above pH 12.0 (see Appendix).

c. Strand separation of BrUra labeled HeLa mitDNA

When the total mitDNA from HeLa cells labeled for 24 hours with BrdUrd was banded in a CsCl gradient at pH 11.81, five bands resulted (Figure 8). Fractions were pooled as shown, dialyzed versus 1 M NaCl, 0.001 M EDTA, 0.01 M Tris, pH 7.5, and examined in the electron microscope by the formamide technique. Except for a few duplex circles in the third peak from the left, all of the DNA in the four left peaks was single stranded. Most of the material in the far right peak, however, was duplex circles. A scoring showed 145 monomer duplex mitDNA circles, 8 catenated duplex forms, and 98 single stranded linear molecules, of which 93 were small fragments less than 1/3 of the full mtDNA length. This peak therefore contains unlabeled closed mitDNA, which would nick only slowly at pH 11.81. The two broader peaks at the far left in Figure 9 are the two BrUra labeled strands, and the two middle peaks contain the H and L strands. A large number of molecules from these four peaks were traced and measured, and length distributions were constructed (Figures 9(a) - 9(d)). Clearly the two BrUra strands are highly fragmented, but the two unlabeled strands show high proportions (15wt% of the H and

Figure 7: Banding of Covalently Closed HeLa mitDNA in Alkaline Buoyant CsCl

^{3}H-labeled covalently closed HeLa mitDNA was added to buoyant CsCl at pH 11.62 and centrifuged to equilibrium as described in Materials and Methods (1d). The gradient was fractionated by dripping onto filters and counted in toluene-PPO-PPOP scintillation fluid. Less than 3% of the DNA nicked and separated into H and L strands under these conditions. The field is directed to the left.

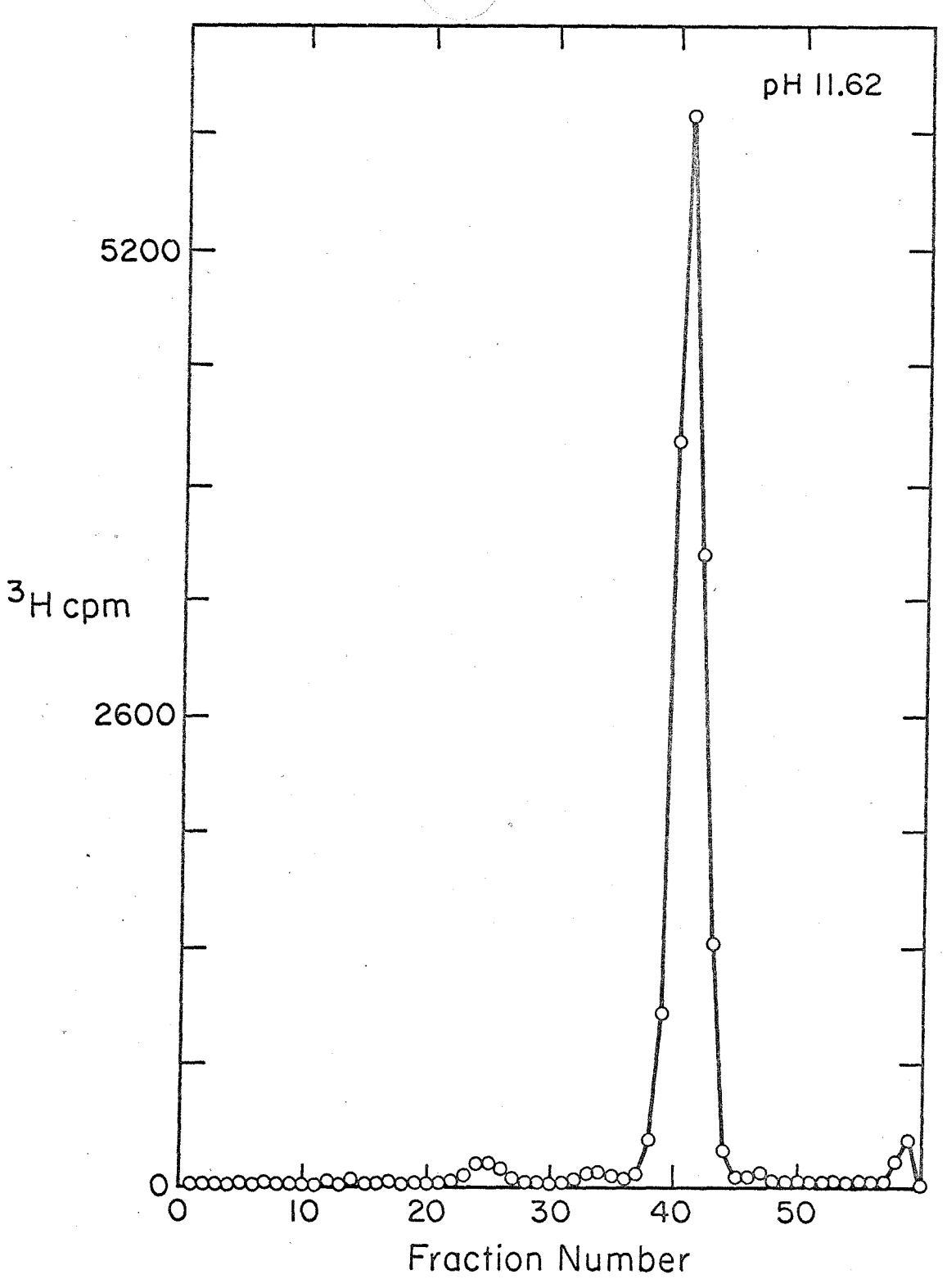


Figure 7

Figure 8: Banding of BrUra-Labeled HeLa mitDNA

Total BrUra-labeled HeLa mitDNA (also labeled with ^{3}H-deoxycytidine and ^{3}H-thymidine) was added to buoyant CsCl at pH 11.81 and centrifuged to equilibrium as described in Materials and Methods (1d). Fractions indicated by arrows were pooled. The field is directed to the left. H BU, BrUra-labeled H strand; L BU, BrUra-labeled L strand; H, H strand; L, L strand; HL, duplex DNA not nicked at pH 11.81.

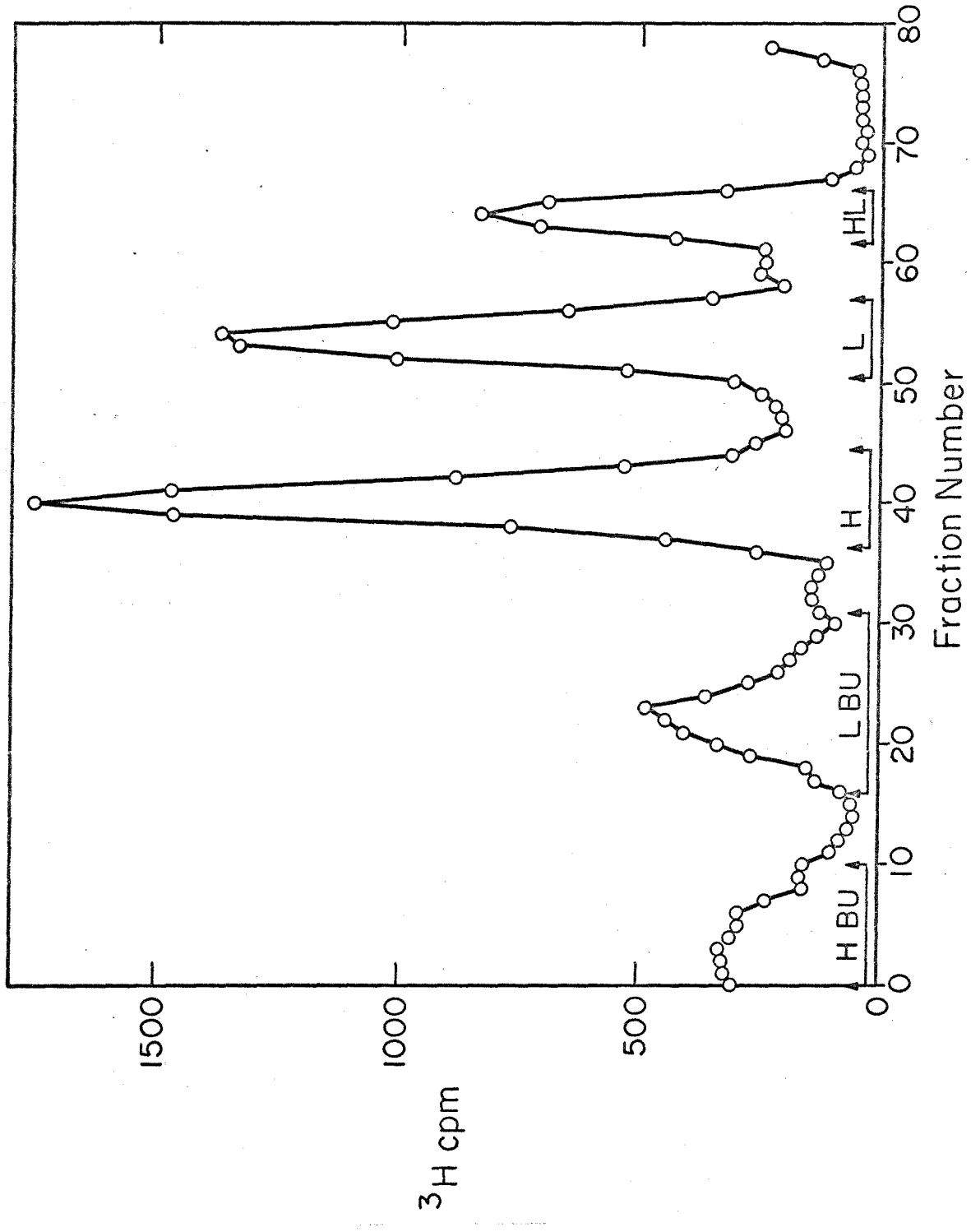

1500
1000
500
0
^{3}H cpm
0
10
20
30
40
50
60
70
80
Fraction Number
H BU
L BU
H
L
HL

Figure 8

Figure 9: Length Distributions of Strands Prepared from BrUra-Labeled mitDNA

The pooled fractions shown in Figure 8 were mounted for electron microscopy by the formamide Kleinschmidt technique described in Materials and Methods (5). Molecules were traced and measured, and the weight fraction of each size class is plotted. Circular strands are indicated by shaded areas. The average length of ØX174 viral DNA spread on the same grid is indicated by an arrow in each case. The weight fractions of circles and full length linears are:

	weight %	
	circles	linears
L	29	30
H	15	31
L BU	7	29
H BU	2	15

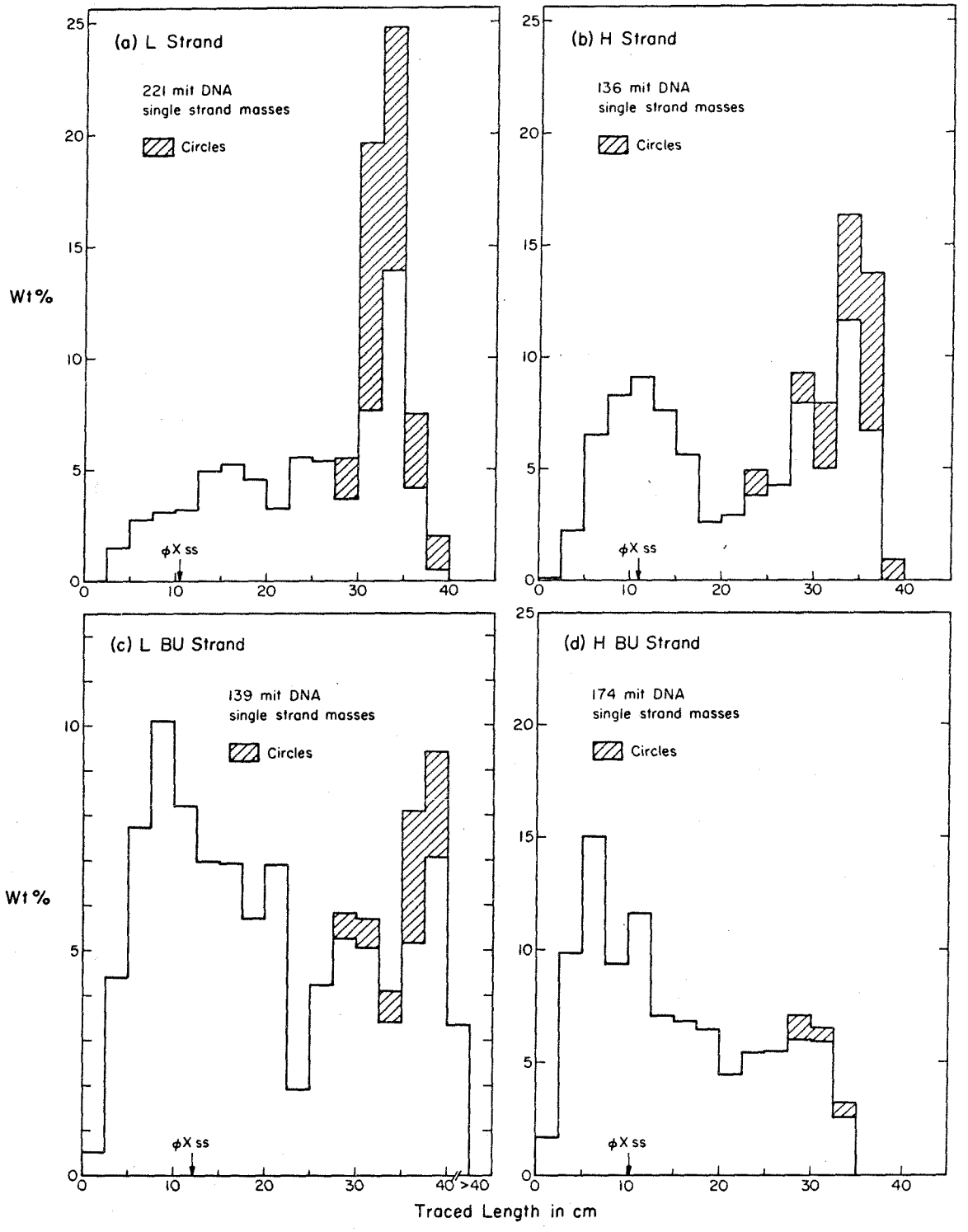

(a) L Strand
221 mit DNA
single strand masses
Circles
φX ss
(b) H Strand
136 mit DNA
single strand masses
Circles
φX ss
(c) L BU Strand
139 mit DNA
single strand masses
Circles
φX ss
(d) H BU Strand
174 mit DNA
single strand masses
Circles
φX ss
Wt%
Wt%
Traced Length in cm
>40

Figure 9

30wt% of the L strands) of single stranded circles. The H strand preparation was used as the starting material for the rDNA preparation, and the L strands were used in the mapping experiments.

2. Single strand lengths of 7s and rDNA

Figures 10(a) and 11(a) depict the single strand length distributions of 7s and rDNA when examined by formamide spreadings. About 75% of the 7s DNA is between 0.1 and 0.25 μm in length, corresponding to roughly 2 to 5% of the full mtDNA length (0.02 to 0.05 G). Since the displacement loop is approximately 0.030 - 0.035 G (Kasamatsu, Robberson, and Vinograd, 1971) in both La9 and HeLa cells, this material is apparently intact. The length distribution of the rDNA shows that some fragmentation occurred during the preparation. Most of the rDNA is less than 0.3 μm in length, whereas the 12s and 16s rDNA genes are 0.26 and 0.46 μm respectively (Wu et al., 1972). The causes of this fragmentation are discussed later.

3. Individual hybridizations of 7s and rDNA to L strands

It was first necessary to find hybridization conditions giving reasonable yields of hybrids without too much nonspecific interaction. In initial experiments multiple duplex regions were observed in hybridizations of either 7s or rDNA to L strands, so the hybridization conditions were modified (mainly by reducing the length of the incubation) until most of the hybrid molecules had only one duplex region. The final procedure involved incubation of 0.01 μg of L strand with either 0.005 μg 7s DNA or 0.04 μg rDNA in a total volume of 100 μl in

Figure 10: Length Distributions of 7sDNA

(a) Single strand length distribution

7S HeLa mitDNA prepared as shown in Figure 1 was mounted for electron microscopy by the formamide Kleinschmidt technique (Materials and Methods (1d)), and molecules were traced and measured. The number of molecules in each size class is plotted.

(b) Length distribution of hybridized 7S

7S DNA was hybridized to L strands as described in Results (3) and examined by electron microscopy as above. The number of duplex regions in each size class is plotted.

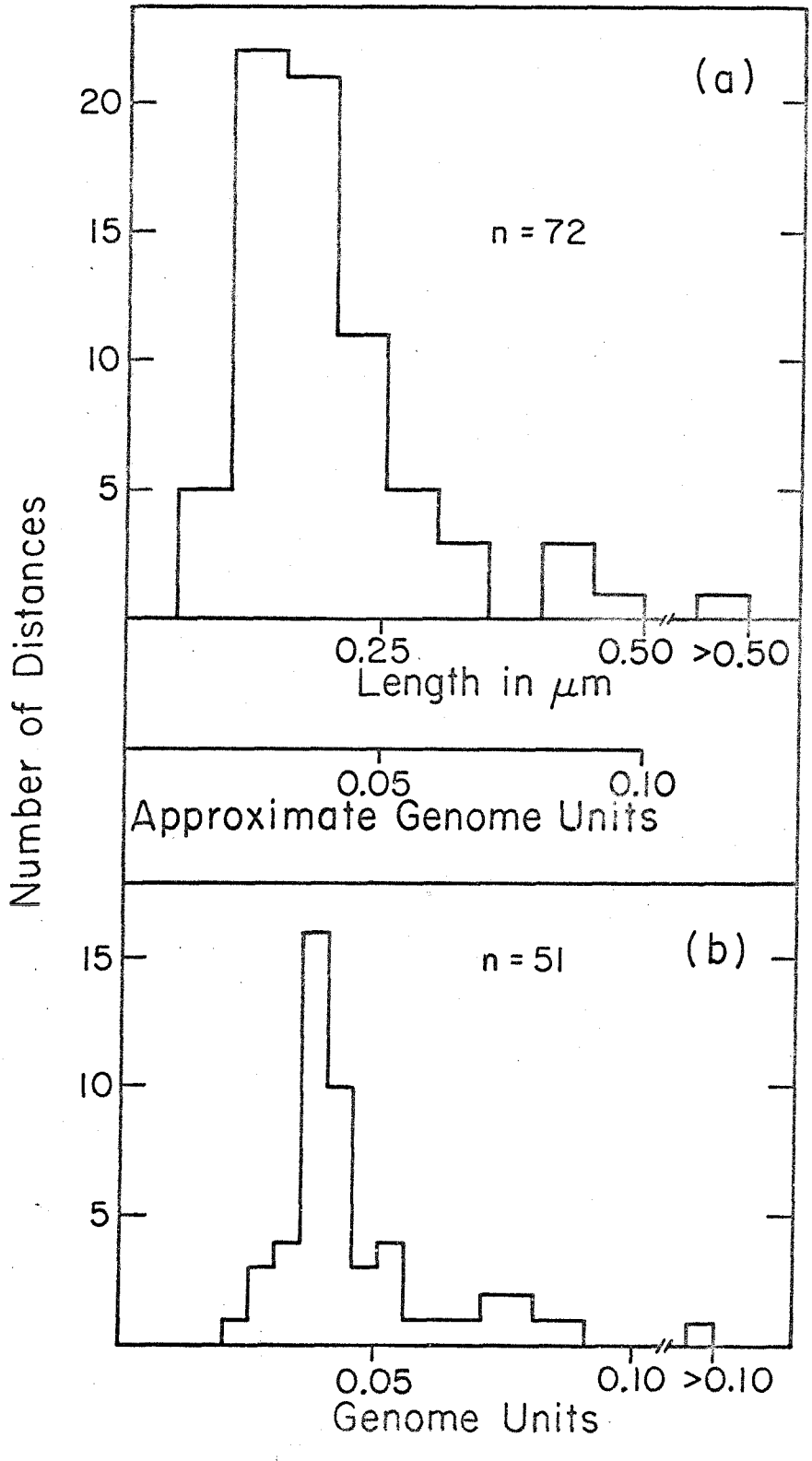

(a)
n = 72
20
15
10
5
0.25
0.50
>0.50
Length in μm
0.05
0.10
Approximate Genome Units
Number of Distances
(b)
n = 51
15
10
5
0.05
0.10
>0.10
Genome Units

Figure 10

Figure 11: Length Distributions of rDNA

Length distributions of rDNA before and after hybridization to L strands were constructed as in Figure 10.

(a) Single strand length distribution

(b) Length distribution of hybridized rDNA

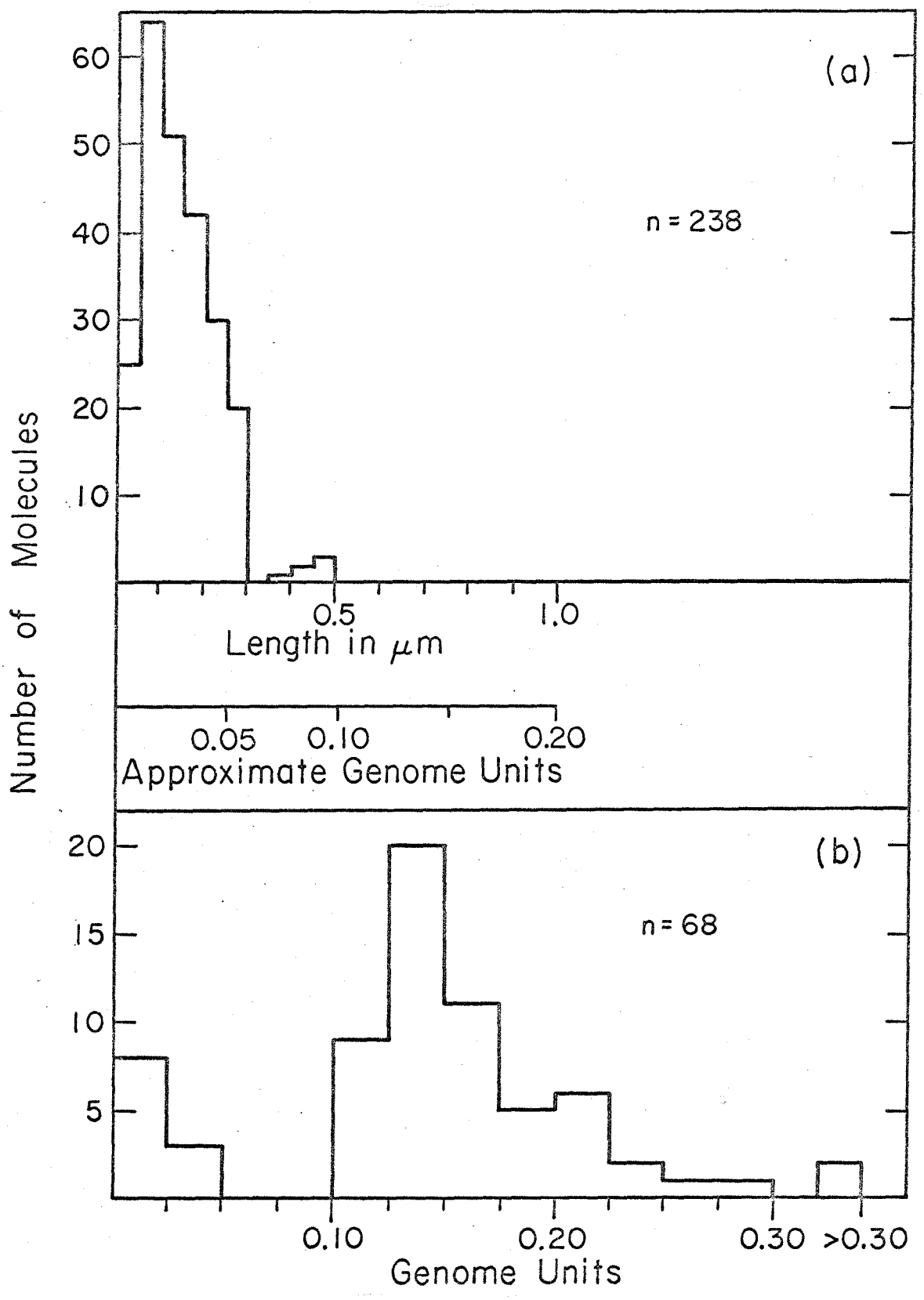

(a)
n = 238
Number of Molecules
60
50
40
30
20
10
0.5
1.0
Length in μm
0.05
0.10
0.20
Approximate Genome Units
(b)
n = 68
20
15
10
5
0.10
0.20
0.30
>0.30
Genome Units

Figure 11

40% formamide and 0.3 M NaCl for 1 hour at 40°C. Under these conditions approximately 5-10% of the 7s hybrids appeared to have more than one duplex region. However, a duplex region this small (450 nucleotides) is difficult to see, so that some of these apparent duplex regions could have been simply regions of uneven contrast. The lengths of the 7s DNA duplex regions are plotted in Figure 10(b), using the length of the circular single strand as the standard in each case. Most of the duplex regions cover about 0.04 G, as expected.

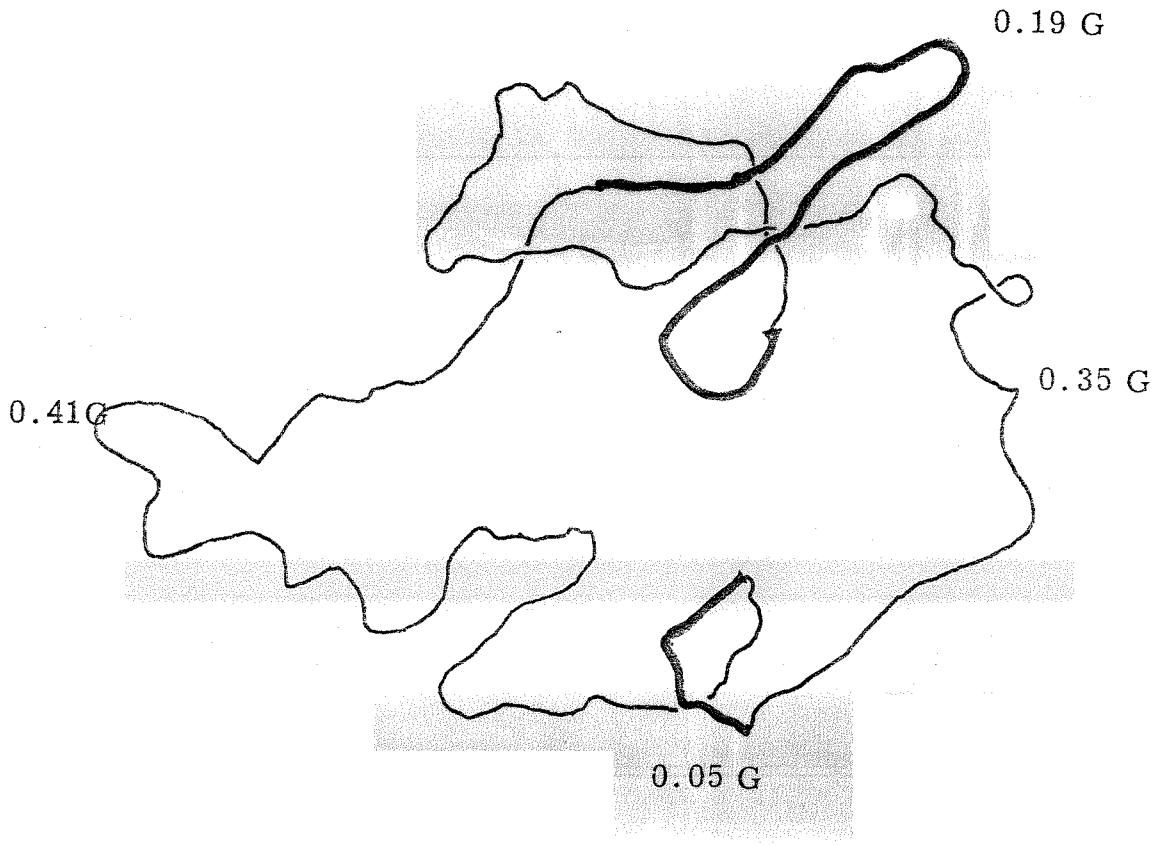
The lengths of the rDNA hybrid regions (Figure 11(b)) are more variable, at least partly because the rDNA was fragmented. Each hybrid would not cover all of the rRNA region unless all of the fragments had hybridized. The peak of the length distribution is at 0.14 G, slightly below the expected value of 0.16 G (Wu et al., 1972). In a few additional cases small duplex regions were also observed, and are included in the distribution.

4. Hybridization of mixtures of 7s and rDNA to L strands

The above conditions were used to hybridize mixtures of 7s and rDNA to L strands using 0.04 μg rDNA and either 0.000, 0.002, or 0.004 μg 7s DNA. The hybrids were then examined in the electron microscope by the formamide technique (Plate 1). The results are presented in two ways. In the first, the single stranded inter-duplex lengths are plotted as three histograms (Figure 12). No peak is seen when the 7s is omitted, but the background of apparent duplex regions (which may be artifacts as mentioned above) gives a few inter-duplex distances. With increasing amounts of 7s DNA a broad peak in the distribution appears between 0.3 and 0.5 G (Figures 12(b), 12(c)).

Plate 1: A Heteroduplex of L Strand HeLa mitDNA with 7S and rDNA

7S and rDNA were hybridized to L strand mitDNA as described in Results, and mounted for electron microscopy by the formamide Kleinschmidt technique. This molecule is the second one in Figure 13, and the first interpretation shown there is given here on the translucent overlay. Duplex regions are indicated by a thicker line, and the lengths of the different segments are indicated in genome units (G) using the total contour length as the standard.


0.19 G
0.35 G
0.41G
0.05 G

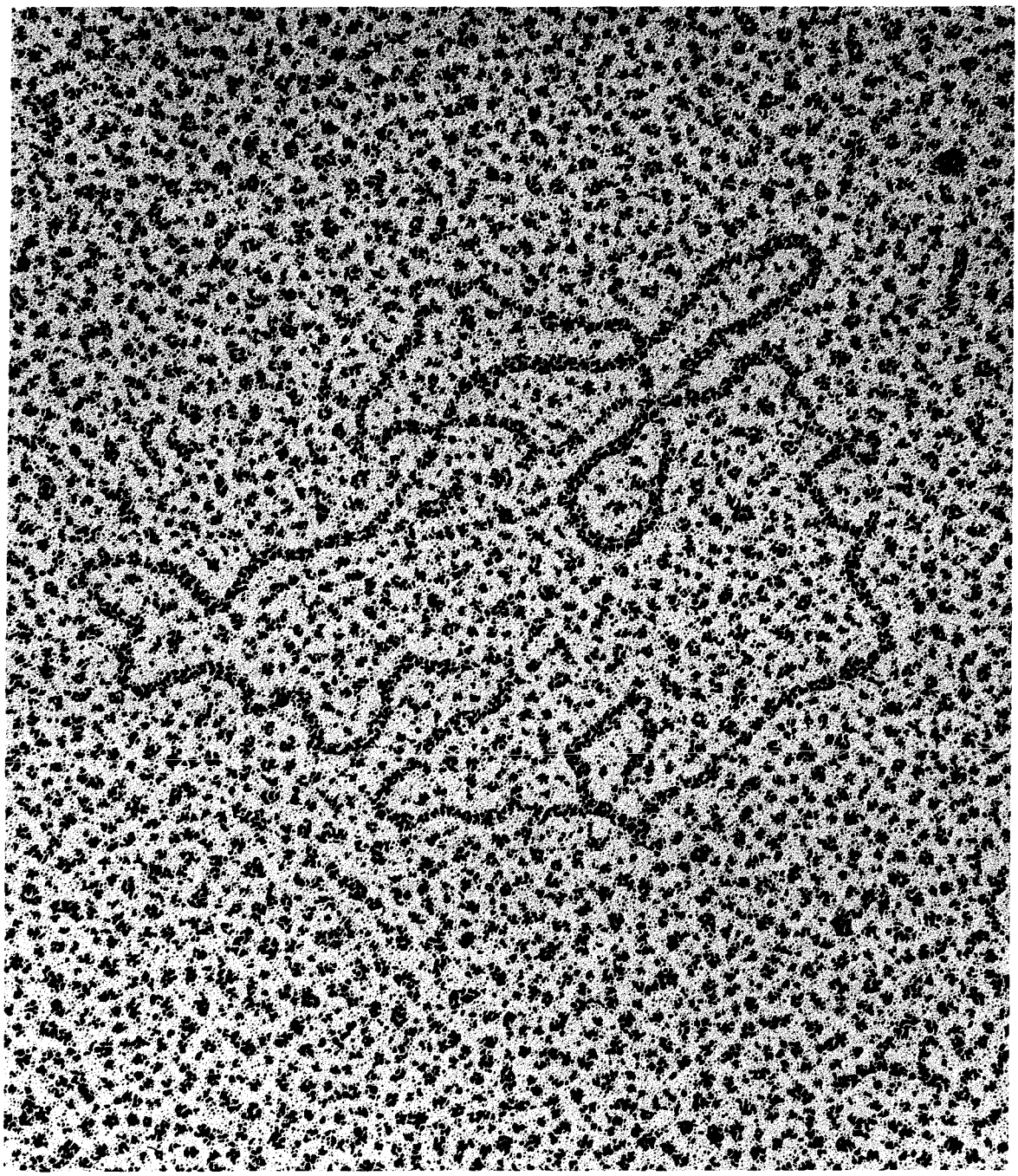


Plate 1

Figure 12: Interduplex Distance Distributions in Hybrids of L Strands with rDNA and Variable Amounts of 7S DNA

L strands (0.01 μg) were hybridized with 0.04 μg rDNA and a variable amount (indicated in the upper left of each panel) of 7S DNA, as described in Results (4). The single stranded distances between duplex regions on circular hybrids were measured on hybrids containing two apparently duplex regions, and the number of interduplex distances in each size class is plotted here. Note that each hybrid contributes <u>two</u> interduplex distances to the distribution.

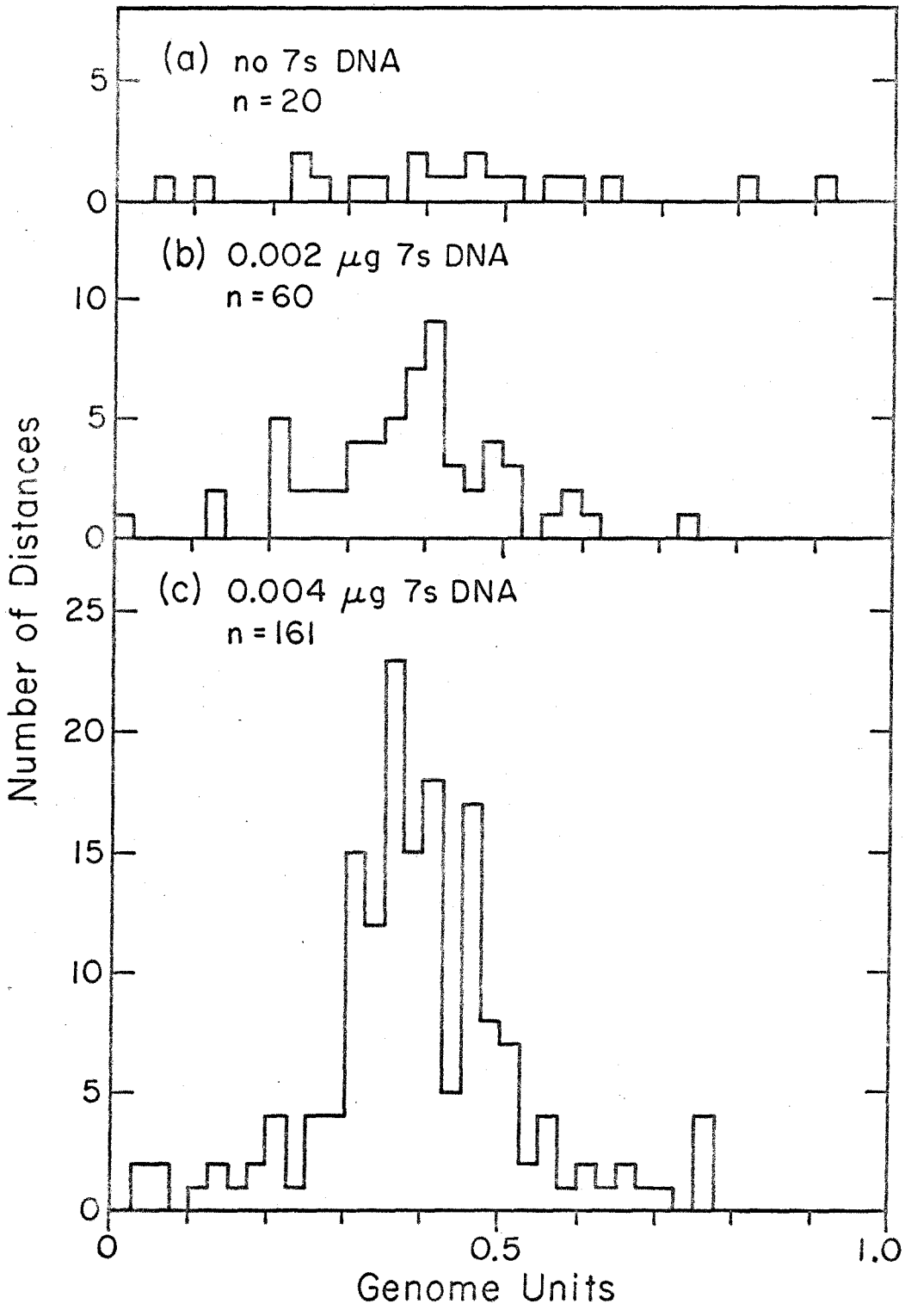

(a) no 7s DNA
n = 20
(b) 0.002 μg 7s DNA
n = 60
(c) 0.004 μg 7s DNA
n = 161
Number of Distances
Genome Units
0
0.5
1.0
5
10
15
20
25

Figure 12

This result suggests that the 7s DNA is almost directly opposite the rDNA. Since the duplex regions should account for 0.19 G, a peak at 0.40 G is expected if the center of the 7s is directly opposite the center of rDNA. Broadening of the peak is expected both if the 7s is not precisely opposite the rDNA and if the rDNA duplex region is variable in length. If it is sufficiently asymmetrically located, two peaks were expected.

In order to eliminate any bias in the interpretation of the photographs, fourteen molecules were independently traced by Drs. E. Ohtsubo and M.-T. Hsu, without knowledge of how many duplex regions to expect. Their interpretations of these molecules are presented in Figure 13, together with the original interpretations. In each case the hybrid circle has been cut at what was originally designated as the end of the rDNA region nearest the 7s duplex, and the resulting linears have been aligned with this point at the left edge of the figure. The agreement between the different investigators is quite good, with the two independent ones tending to see a few additional duplex regions. The variability in the length of the rDNA region affects the apparent position of the 7s DNA considerably, but is difficult to average out becuase the rDNA can lack fragments from either end. These results are also presented as inter-duplex distance distributions in Figure 14. Although the sample size is small, clearly all three agree that most of the inter-duplex distances are between 0.30 and 0.50 G. Therefore the 7s DNA region is located approximately directly opposite the rDNA region on the L strand.

Figure 13: Comparison of 7S-rDNA-L Strand Circular Hybrids

7S and rDNA were hybridized to L strands as described in Results (4), and the DNA was mounted for electron microscopy by the formamide Kleinschmidt technique described in Materials and Methods (5). Fourteen circular molecules each of which contained apparent 7S and rDNA duplex regions were independently traced by two additional investigators experienced in distinguishing single stranded from duplex DNA. They were not told how many or what size duplex regions were expected. The results are presented here, with the three interpretations of each molecule aligned with respect to each other. Each circular hybrid has been cleaved at the end of the rDNA duplex region nearest to the 7S DNA region in order to linearize the maps. "n.t." denotes "not traced" for cases where interpretation could not be made with confidence.

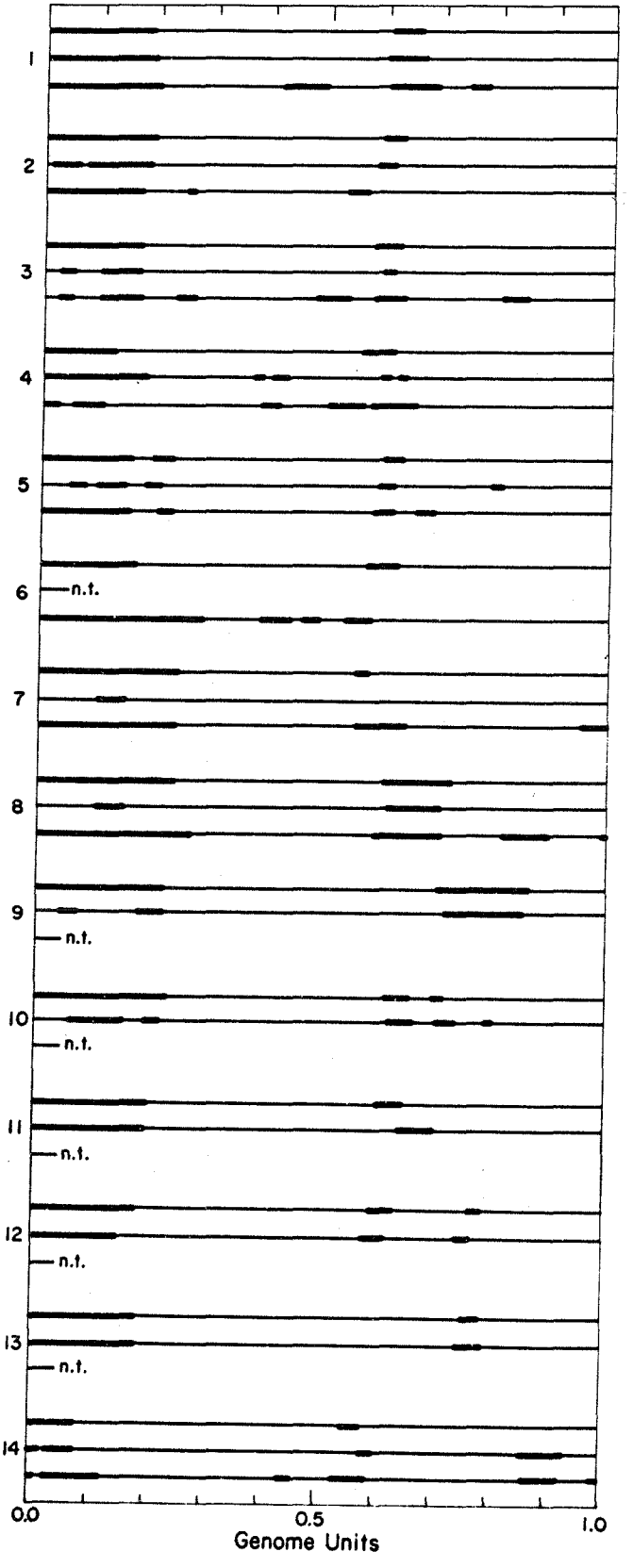


Figure 13

Figure 14: Interduplex Distance Distributions in Independently Traced Hybrids

The interduplex distances were measured for the molecules shown in Figure 13, using the procedure described in Figure 12. In cases where more than one apparent 7S region was seen, interduplex distances to the ends of the rDNA region were included for each possibility. The three distributions resulting from the three sets of interpretations as in Figure 13 are presented in the same order.

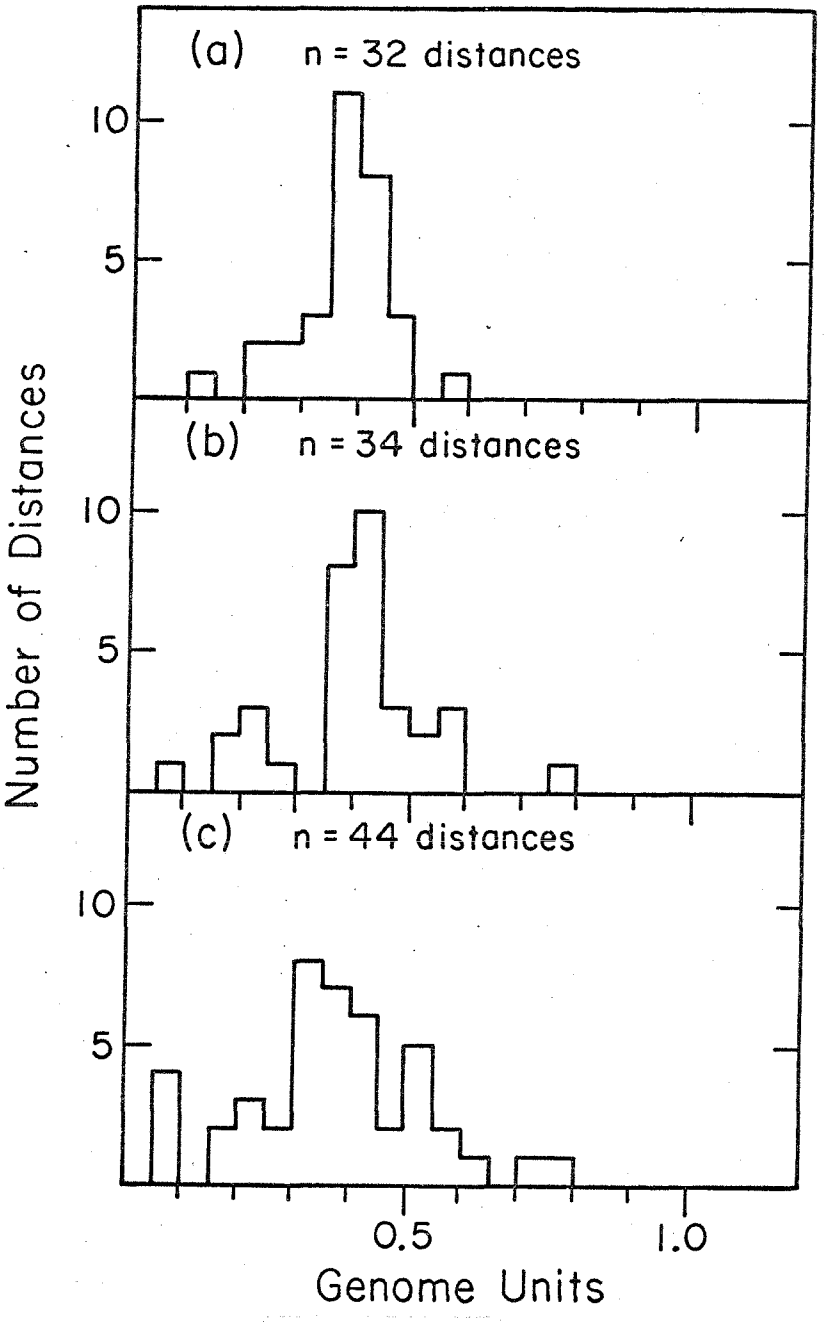

(a) n = 32 distances
(b) n = 34 distances
(c) n = 44 distances
10
5
10
5
10
5
Number of Distances
0.5
1.0
Genome Units

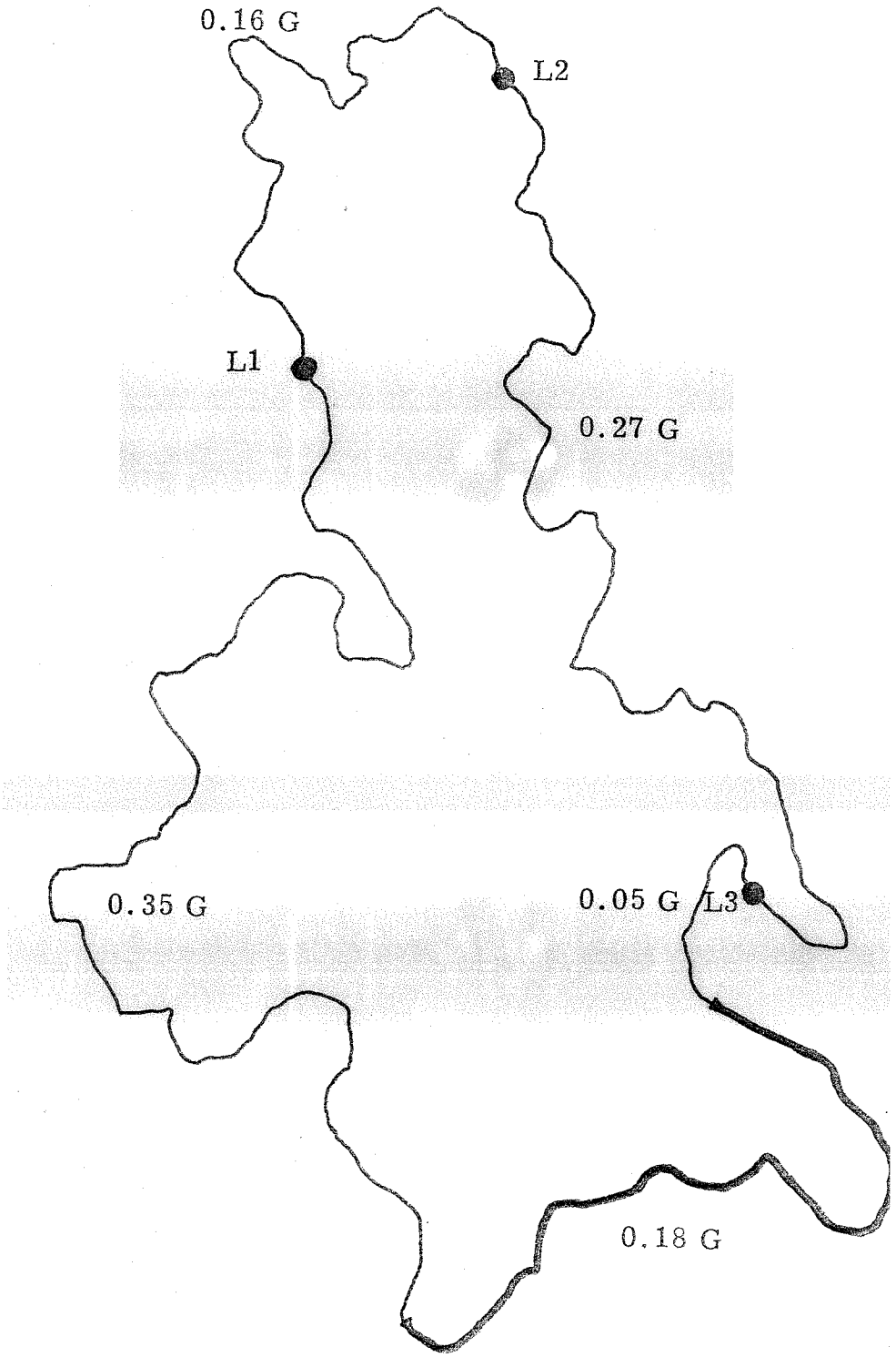
Figure 14

5. Hybridization of mixtures of rDNA and ferritin-tRNA to L strands

Ferritin-tRNA was added to hybridization mixtures of L strands and rDNA, using the methods described in Wu et al. (1972), and the hybrids were examined by the formamide technique (Plate 2). Three sites for the ferritin-tRNA were found, in agreement with the previous results. Their positions relative to one end of the rDNA region were measured and are presented in Figure 15. Although only 34 distances were measured, a tentative map can be constructed (Figure 16). Two tRNA sites are located in one of the spaces between the rDNA and the 7s DNA regions. The third is very near the 7s DNA region, probably in the other space between the 7s and rDNA.

Plate 2: A Heteroduplex of L Strand HeLa mitDNA with rDNA and Ferritin-tRNA

Ferritin-tRNA and rDNA were hybridized to L strand mitDNA as described in Results, and mounted for electron microscopy by the formamide Kleinschmidt technique. An interpretation of the molecule is given on the translucent overlay, with the duplex rDNA region indicated by a thicker line. Traced lengths in genome units (G) of portions of the molecule corresponding to those in Figure 16 are given. The total contour length is used as the standard.


0.16 G
L2
L1
0.27 G
0.35 G
0.05 G
L3
0.18 G

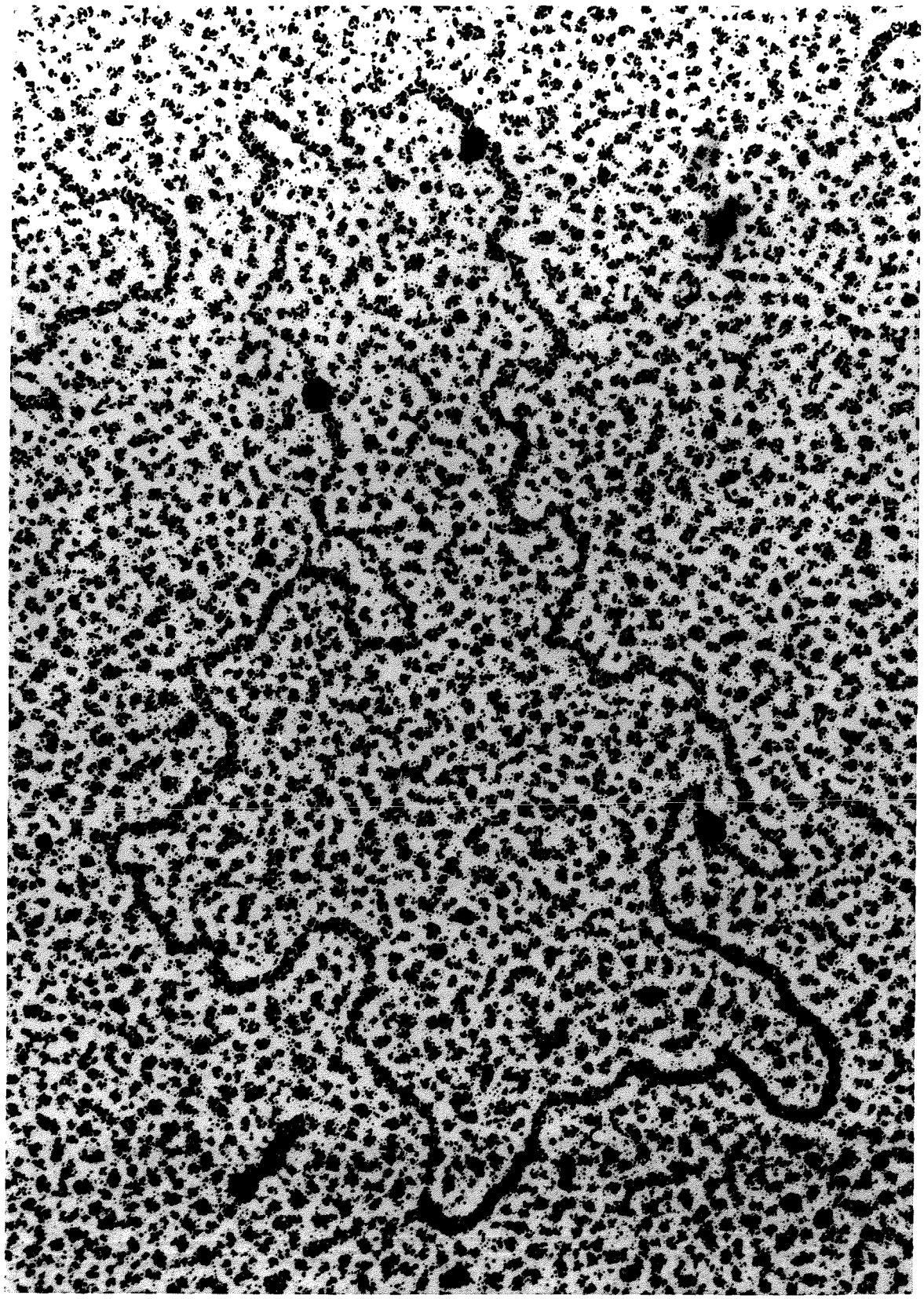


Plate 2

Figure 15: Distribution of Ferritin t-RNA to rDNA Distances

Ferritin-tRNA and rDNA were hybridized to L strands as described in Results (5), and the hybrids were examined by electron microscopy using the formamide Kleinschmidt technique (Materials and Methods (1d)). The distances between one end of the rDNA region (the end nearest to ferritin L3) and the ferritins were measured, and are plotted here.

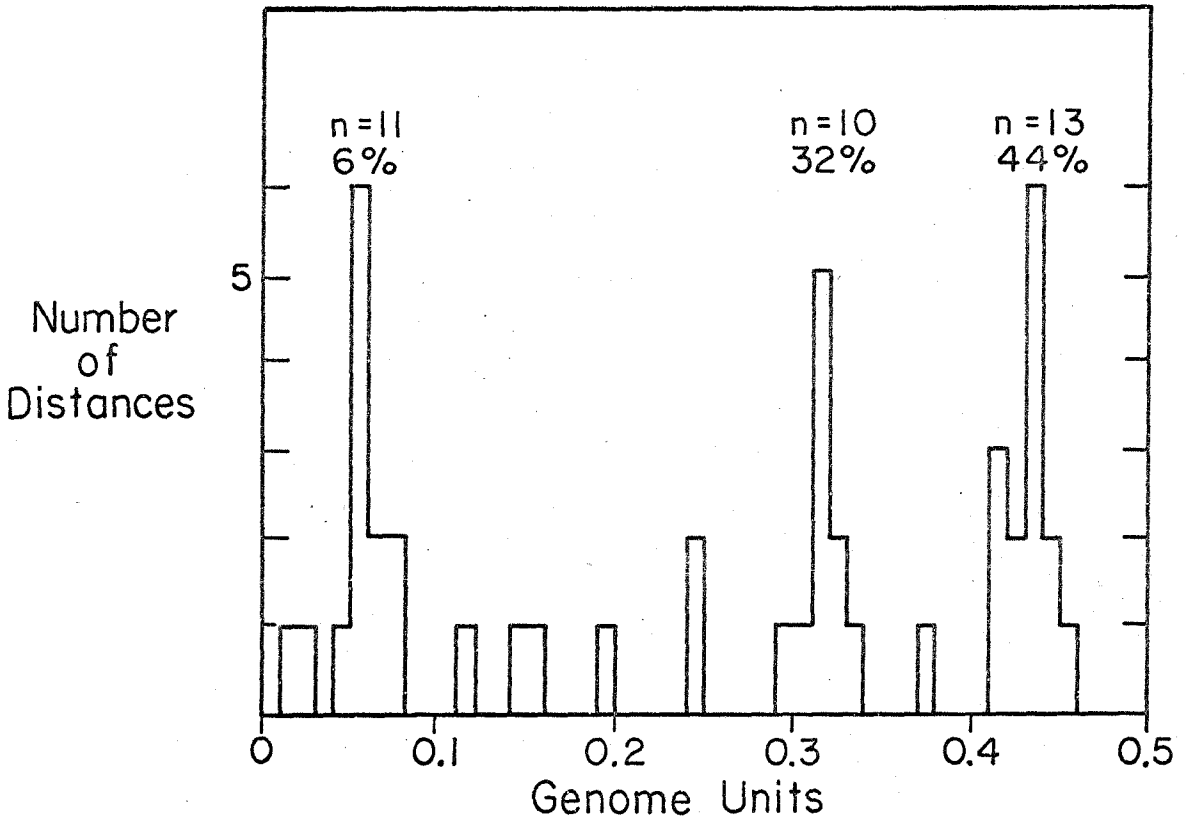

n = 11
6%
n = 10
32%
n = 13
44%
5
Number
of
Distances
0
0.1
0.2
0.3
0.4
0.5
Genome Units

Figure 15

Figure 16: Map of the L Strand of HeLa mitDNA

The ferritin-tRNA sites are labeled as in Wu et al. (1972). The distances between markers are given in the inner circle in genome units (G). The uncertainty in the positions of the 7S and rDNA is sufficiently large that L1 could be on either side of the 7S region.

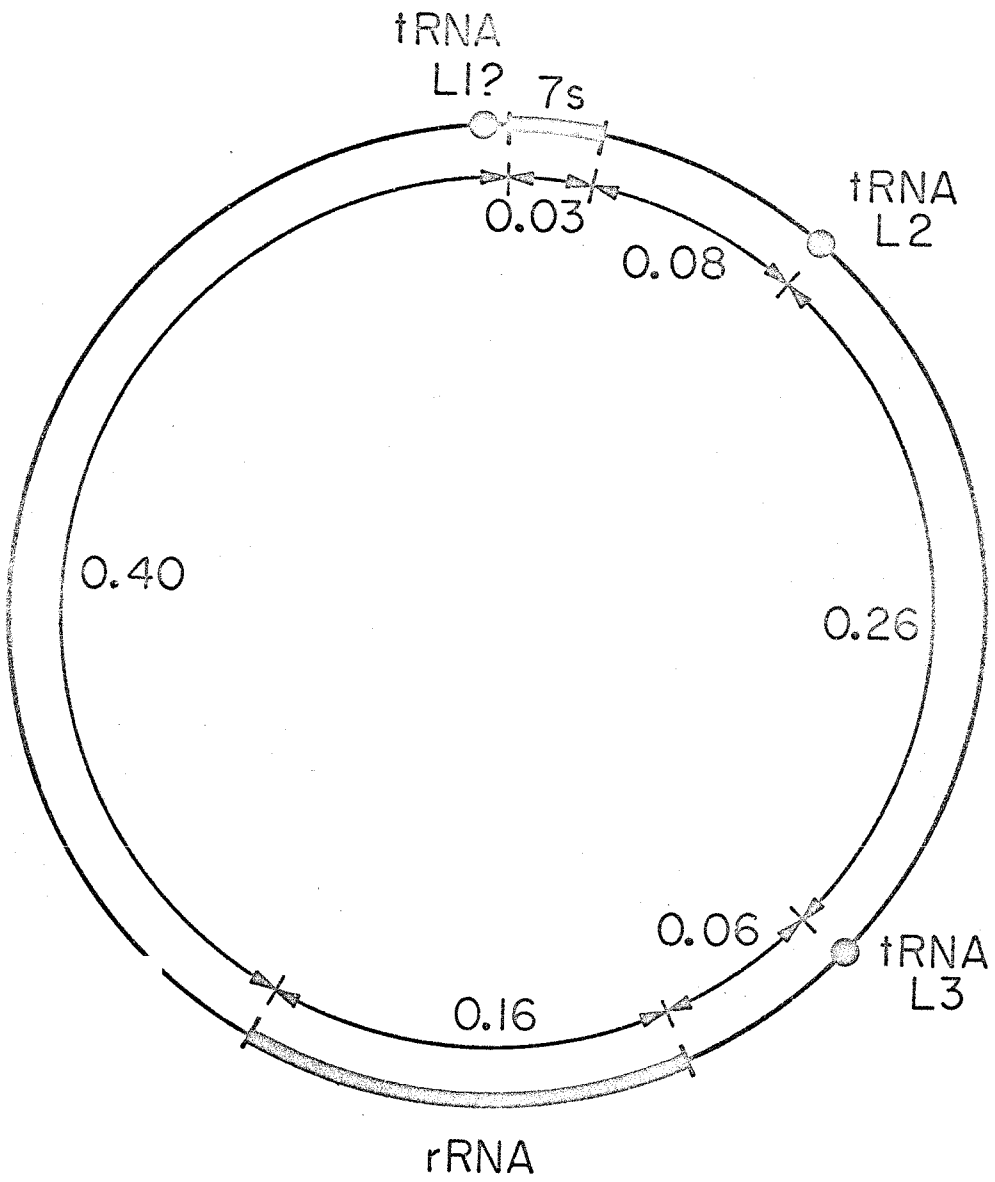


Figure 16

Discussion

1. Isolation of intact mitDNA strands

In an electron microscope hybridization mapping experiment the single strands to which the markers are hybridized must be at least as long as the separation between the markers. Furthermore, mapping studies with circular DNA are facilitated if single stranded circles are used because each circular strand acts as an internal standard for the full genome length. These requirements are difficult to satisfy with animal cell mitDNA due to its alkali lability, presumably due to the incorporation of ribonucleotides into the closed mitDNA (Grossman, Watson, and Vinograd, 1973). A mixture of heavy and light strands consisting of approximately equal amounts of single stranded circles and full length linears can be prepared by brief exposure of singly nicked mitDNA to alkali (Wu et al., 1972). Isolated strands are preferable for mapping studies because renaturation between strands is eliminated. The only presently available technique for isolating heavy and light mitDNA strands, alkaline buoyant CsCl centrifugation, involves prolonged exposure to alkaline pH with concomitant fragmentation of the mitDNA. We have explored the possibility of minimizing this degradation by using the minimum pH (11.6) which still gives complete strand separation of singly nicked mitDNA. Even under these conditions, each strand suffers about one additional hit in 48 hours. Since the material had been singly nicked prior to alkaline banding in order to allow the strands to separate, it already contained 0.5 hits per strand. The resulting preparations contained

too low a level of single stranded circles to be useful for the mapping experiments. The singly nicked mitDNA cannot be briefly exposed to high pH and then banded at pH 11.1 without suffering a 50% loss due to renaturation even if less than 0.5μg of DNA is run in each tube. Attempts were also made to avoid the 0.5 hits per strand introduced to allow the strands to separate by using closed mitDNA and allowing the alkali nicking to provide these nicks. These experiments were unsuccessful because covalently closed mitDNA appears to be resistant to alkali nicking at low pH. No strand separation was observed after 48 hours at pH 11.6, in agreement with observations made by Dr. L. I. Grossman above pH 12.0 (see Appendix).

Even if alkali nicking could be eliminated, all of the above methods would result in preparations of H or L strands with an average of 0.5 hits per strand. Therefore a theoretical maximum of 50% of the strands would be circular, and a higher level would be possible only if linear strands were subsequently removed. We have observed that the BrUra labeled strands of mitDNA are highly fragmented in buoyant alkaline CsCl gradients at pH 12.5 (Flory and Vinograd, 1973). This fragmentation is probably due to an increased sensitivity of this DNA to either alkali or light. The density labeled fragments are so much denser than the unlabeled strands that they are easily removed in the density gradient. The unlabeled strands, however, are nicked only by the alkali, so that a level of 100% single stranded circles is theoretically possible. The preparation described here resulted in an L strand preparation containing 30wt% single stranded circles and 30wt% full length linears. The H strand preparation was probably

contaminated by fragments of the density labeled L strand, and therefore had a lower apparent level of single stranded circles.

The yield of single stranded circles might be improved by reducing the pH of the gradient. BrUra titrates at a lower pH than T, so that hybrid BrUra labeled DNA denatures with a pH_m about 0.7 units lower than unlabeled DNA (Baldwin and Shooter, 1963). The transition is very broad, however, so it may not be possible to reduce the pH of the gradient by this amount without encountering incomplete strand separation. Also, closed hybrid BrUra labeled mitDNA may be resistant to alkaline nicking in the same way that closed unlabeled mitDNA is (see Appendix). In fact, some closed duplex hybrid BrUra labeled DNA was seen in the H strand preparation. Either a brief exposure to high pH or a light enzymatic nicking might be necessary if the pH were reduced much below the pH 11.81 used in the preparation described here. These possibilities were not pursued because the levels of circular single strands obtained at this pH were adequate for the mapping experiments.

2. Relative positions of 7s and rDNA

Both the inter-duplex length distributions (Figures 12 and 14) and the parallel maps of individual molecules (Figure 13) clearly show that the 7s DNA hybridizes to a region approximately directly opposite to the rDNA on the circular map. For several reasons, however, the uncertainty in its position is of the order of ±5% of the genome. First, there is the basic difficulty of seeing a 450 nucleotide duplex region on a longer single strand on an electron microscope negative. Variations in contrast due to fluctuations in the amount of cytochrome c or

platinum can mimic short duplex regions, giving rise to a background of non-significant apparent duplex regions as shown in Figure 12(a). In Figure 13, measurements of individual molecules by different microscopists are in good agreement on the presence of these apparent duplex regions.

In addition, the rDNA was fragmented to an average size of about 0.03 G, so that about 5 pieces would be required to cover the entire rDNA region. This fragmentation may have occurred during S1 endonuclease digestion, since the nicking activity of this enzyme towards duplex DNA (St. John, Johnson, and Bonner, 1974) may also apply towards the DNA in DNA-RNA hybrids. Alternatively, the RNase or pronase used in the preparation may have contained low levels of DNA nicking activity. In any case, if pieces were missing from either end of the rDNA region in a hybrid molecule, the apparent position of the 7s DNA would be shifted accordingly.

Preliminary experiments showed that multiple duplex regions could be obtained in hybridizing either 7s or rDNA to L strands. Whether this additional hybridization was due to contamination of these preparations by small amounts of fragments of other regions of the H strand, or whether it was due to a nonspecific interaction between noncomplementary regions is unclear. Both the 7s and rDNA should hybridize to only one region of the L strand, since the rRNA is complementary to only one region of the H strand (Wu et al., 1972) and the 7s DNA is uniquely located on the mitDNA molecule (Kasamatsu, Robberson,

and Vinograd, 1971).* The hybridization conditions were modified (mainly by shortening the length of incubation) to minimize this problem, but the residual level of multiple hybridization would contribute to the uncertainty in the location of the 7s rDNA.

Finally, a statistical effect contributes to this uncertainty. The measured inter-duplex lengths are fairly accurate, since all of the mapping was performed on hybrids with circular strands, so each molecule has an internal standard. The relative orientation of the hybrids, however, is not known, so the distributions contain both distances between the 7s and rDNA. If the orientation were known (by addition of an asymmetric marker such as ferritin-tRNA), the inter-duplex distances could be separated into two distributions and the 7s DNA located more precisely. If the 7s is nearly but not exactly opposite the rDNA, the inter-duplex length distribution would be broadened unless this separation were done.

3. Position of the tRNA sites relative to rDNA

Previous work by Wu et al. (1972) has shown that there are three sites for hybridization of ferritin-tRNA to the L strand. They are located 0.73μm (0.15 G), 1.25μm (0.25 G), and 3.02μm (0.60 G) from each other. In the present work the same pattern has been duplicated, although the number of molecules measured is small. As can be seen from Figure 15, the separations of the ferritin-tRNA's were

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* The 7s DNA is known to be uniquely located on molecules isolated from mitochondria, but the sequences could conceivably be partially repeated elsewhere in the molecule.

0.12 G, 0.26 G, and 0.62 of the genome. In addition, the rDNA region has now been located relative to these markers, as shown in Figure 15 and Plate 2. Although the sample size is small, the rDNA is clearly in the 0.60 G gap between L1 and L3 of Wu et al. (1972), and ends about 0.06 G from L3. The accuracy of these experiments is primarily affected by the variability of the position of the end of the rDNA duplex region caused by the extensive fragmentation of the rDNA. As indicated on the map in Figure 16, L1 is very near the 7s DNA region and may be on either side.

4. Further experiments

There are several possibilities for the refinement of this map. First, the 7s DNA site could be more precisely placed using the ferritin-tRNA system, since these markers are more precise and easily visible than the rDNA region. Ferritin-labeled 7s DNA would be useful, but simply measuring a large number of molecules would also suffice to accurately locate the 7s DNA. Next, the relative orientation of the H and L strands could be determined by preparing 12s and 16s rDNA separately and doing separate hybridizations. Again the ferritin-tRNA system would be necessary as the other marker. The variability of the rDNA region would require a large number of molecules here as well. Finally the direction of replication and the location of restriction enzyme cuts could be included on this map. The locations of the three Hin dIII and three E.coli RI restriction enzyme cuts have been determined relative to the 7s site by fixing the D-loop with glyoxyal prior to restriction enzyme incubation (W. M. Brown, personal communication). The direction of replication relative to

these cuts was determined by examining expanding D-loops in this system. The relative orientation of these two maps could be determined by measuring the hybridization of tRNA to the separated strands of isolated fragments. The two maps could then be combined using the 7s region as a common point.

Appendix: pH Dependence of the Rate of Nicking of Closed LA9 mitDNA

(Unpublished experiments by Dr. L. I. Grossman and R. W. Watson)

A series of kinetic experiments of the type described by Grossman, Watson, and Vinograd (1973) were performed at different pH's in order to measure the pH dependence of the rate of alkali nicking of closed mitDNA. In each experiment closed LA9 mitDNA was incubated at 20 °C in a solution buffered at a given pH. Aliquots withdrawn at various times were banded in CsCl-ethidium bromide in order to determine the proportion of the mitDNA which had been nicked (exact conditions are given in the above reference). A plot of log % closed mitDNA remaining versus time was made for each pH, resulting in a biphasic curve in every case. The slope of the slower decay was measured and has been plotted versus pH in Figure 17 (solid points and solid line). There is a clear inflection at pH 12.4 in the dependence of the rate of nicking on pH. If the mitDNA was exposed to pH 12.8 for one minute before incubation at the indicated pH (open circles), the rates of nicking fall on an extrapolation (broken line) of the nicking rate versus pH curve above pH 12.4. A simple explanation of these results is that pH 12.4 is the denaturation pH for closed duplex mitDNA under these conditions, and that duplex mitDNA is much less susceptible to alkaline nicking than single stranded mitDNA. The samples titrated to pH 12.8 are evidently in a closed denatured form which is more sensitive to alkali than the native form. Closed HeLa mitDNA has the same nicking rate at pH 13.0 as LA9 mitDNA (Grossman, Watson, and Vinograd, 1973),

so these conclusions probably apply to HeLa mitDNA as well.

Figure 17: pH Dependence of the Rate of Nicking of Covalently Closed mitDNA in Alkaline Buoyant CsCl

(See Appendix.)

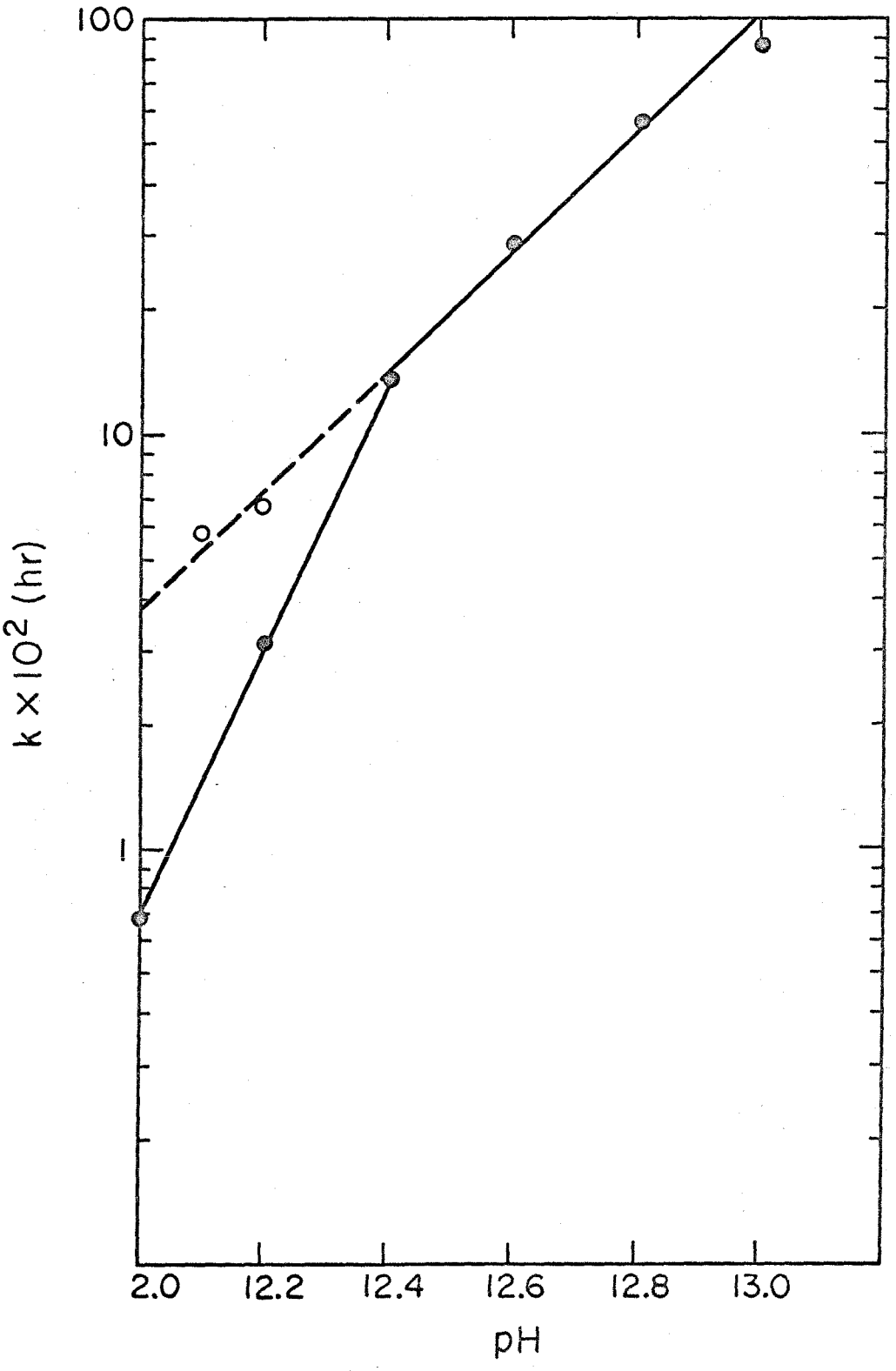

Figure 17

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Chapter 3

MitDNA Replicative Intermediates: Isolation by Benzoylated DEAE-Cellulose Chromatography and Enzymatic Analysis of Structure

Introduction

Electron microscope studies on mitDNA have established the existence and frequency of D-loop, expanding D-loop, and gapped replicating molecules, and have provided important information about their structure (Kasamatsu, Robberson, and Vinograd, 1971; Arneberg et al., 1971; Robberson, Kasamatsu, and Vinograd, 1972; Robberson and Clayton, 1972; Kasamatsu and Vinograd, 1973). D-loop replicating forms comprise 25% (HeLa) to 30-60% (LA9) of the lower band mitDNA, while expanding D-loop and gapped molecules comprise roughly 8-12% and 16-22% respectively of LA9 upper band mitDNA (Robberson, Kasamatsu, and Vinograd, 1972; Kasamatsu et al., 1973). The D-loop is located in a unique position on the molecule, and contains a fragment of the H strand with a sedimentation coefficient of about 7s in neutral sucrose (Kasamatsu, Robberson, and Vinograd, 1971). The D-loop expands unidirectionally (Kasamatsu and Vinograd, 1973) by extension of the H strand fragment to form expanding D-loop molecules (Figure 1). In some cases the displaced H strand is partially duplex due to complement synthesis of progeny L strand, which may initiate at specific sites (Robberson, Kasamatsu, and Vinograd, 1972). Simple circular molecules containing gaps of different sizes have been observed (Robberson, Kasamatsu, and Vinograd, 1972), probably representing the daughter molecules which have separated prior to completion of complement synthesis (Figure 1).

The work described here is an attempt to further characterize

Figure 1: The Replication of mitDNA (taken from Robberson, Kasamatsu, and Vinograd, 1972, and reproduced with the permission of the authors).

Replicative intermediates of mitDNA are depicted in order of increasing replication. H strands are represented by the dark, external lines; L strands are represented by light, internal lines. Dashed lines are newly replicated strands, while solid lines are parental strands. Exp D and Gpc refer to expanding D-loop and gapped molecules, respectively. Complement synthesis of light strand on an Exp D molecule is shown in (D) and (E). The α and β Gpc molecules represent progeny containing newly synthesized H and L strands, respectively, and are shown as having separated when H strand synthesis was complete.

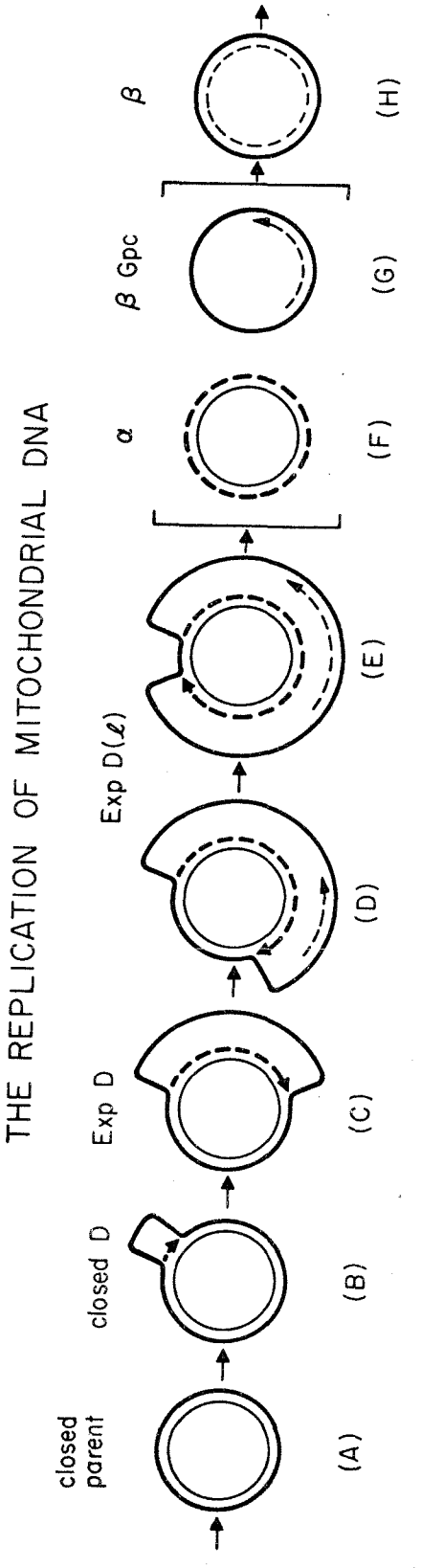
THE REPLICATION OF MITOCHONDRIAL DNA
closed parent
(A)
closed D
(B)
Exp D
(C)
Exp D(ℓ)
(D)
α
(E)
(F)
β Gpc
(G)
β
(H)
Nicks and small gaps are omitted.

Figure 1

these structures by enzymatic analyses with T4 and E. coli DNA polymerases, E. coli DNA ligase, and E. coli exonuclease III. Several specific hypotheses were tested. If most gapped molecules represent β daughters lacking portions of the L strand due to incomplete complement synthesis (Robberson, Kasamatsu, and Vinograd, 1972), repair of the gaps by T4 DNA polymerase should result in more L than H strand synthesis. If further extension of the 7S H strand fragment in D-loop DNA is not terminated by superhelix density considerations (Berk and Clayton, 1974), it should be possible to extend the 7S fragment with T4 DNA polymerase without prior nicking of the molecule. Finally, the nicked clean duplex DNA in the upper band was analyzed to determine (a) whether the nicks are strand specific and (b) which activities are necessary for their closure.

Since clean duplex circles would not be a template for T4 DNA polymerase, it was not absolutely necessary to separate them from the replicating forms. Nevertheless, attempts were made to purify replicating forms, both to simplify interpretation of the results and to permit other types of experiments. All of the replicating molecules reported to date contain single stranded regions, so this feature was chosen as the basis for their separation from mature clean duplex molecules. Benzoylated DEAE cellulose columns (Sedat, Kelly, and Sinsheimer, 1967; Gilliam et al., 1967) separate DNA containing small single stranded regions from purely duplex DNA. Both single stranded and duplex DNA are bound to the column by ionic interactions between the DNA phosphates and the DEAE

residues, but single stranded DNA is also bound by aromatic interactions between the bases and the benzoyl groups. In practice, both types of DNA are bound to the column at low ionic strength; the ionic strength is raised to elute the duplex DNA (salt eluate), and caffeine is then added to release single stranded DNA (caffeine eluate). This system has been used to isolate replicating intermediates from ØX174 (Sinsheimer, Knippers, and Komano, 1968; Knippers et al., 1969), λ (Kiger and Sinsheimer, 1969), polyoma (Bourgaux, Bourgaux-Ramoisy, and Seiler, 1971), and SV40 (Levine, Kang, and Billheimer, 1970). In the studies described here, PM2 viral DNA and ØX174 viral DNA were used as control duplex and single stranded DNA's to test the system before the separation of mitDNA replicating forms was attempted.

The enzymatic analyses were performed in collaboration with Dr. Lawrence I. Grossman.

Materials and Methods

1. Nucleic acids and enzymes

^{3}H labeled PM2 I DNA and ^{35}P labeled ØX174 viral DNA were gifts from Robert Watson and Lloyd Smith, respectively. Singly nicked PM2 II DNA was prepared by limited pancreatic DNase digestion as described in Chapter 2, Materials and Methods (1b). HeLa and LA9 mitDNA were isolated from cells labeled for 24 hours with either 10 nCi/ml ^{14}C-thymidine (Schwarz-Mann, Orangeburg, N.Y.) or 300 nCi/ml ^{3}H-thymidine (Schwarz-Mann, Orangeburg, N.Y.), by the method described in Kasamatsu, Robberson, and Vinograd (1971). Lower and upper band mitDNA were separated in EB-CsCl as shown in Figure 2. In one isolation of LA9 mitDNA, the DNase-RNase treatment of isolated mitochondria was omitted. 4S and 18S RNA markers were prepared from HeLa cells by Dr. Larry Grossman.

T4 DNA polymerase, E. coli DNA polymerase I, E. coli DNA ligase, and E. coli exonuclease III were gifts from Wai-Mun Huang, Douglas Brutlag, I. R. Lehman, and Paul Modrich (respectively) of the Stanford Medical School. Pancreatic DNase I was purchased from Sigma Chemical Co. (St. Louis, Mo.).

2. BD-cellulose chromatography

BD-cellulose (50-100 mesh) was purchased from Schwarz-Mann (Orangeburg, N.Y.) and contained approximately 3 moles benzoyl per mole anhydroglucose. It was washed extensively with a solution contaning 2 M NaCl, 0.01 M Tris, 0.001 M EDTA, 0.02%

Figure 2: Separation of Upper and Lower Band LA9 mitDNA

^{14}C-labeled LA9 mitDNA prepared as described in Materials and Methods was banded in EB-CsCl (250 μg/ml Ethidium Bromide, ρ = 1.55 CsCl) for 36 hours at 36,000 revs/min. Fractions of 5 drops were collected, and aliquots were spotted onto GF/A filters and counted in toluene-PPO-PPOP scintillation fluid. Fractions corresponding to upper band (U) and lower (L) bands were pooled.

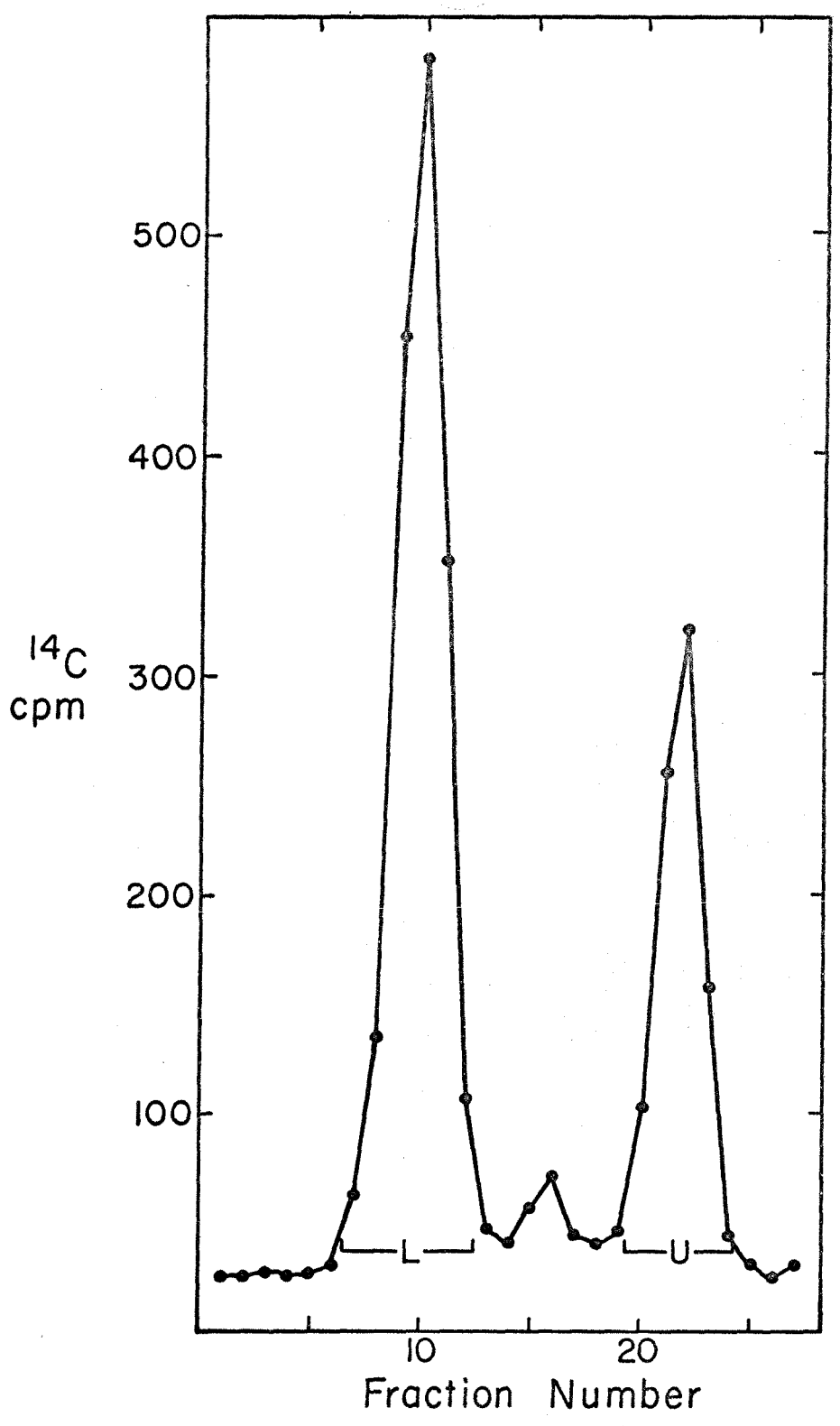

Figure 2

NaN_3, pH 8.0; and then stored at 2°C in the same solution with the NaCl concentration reduced to 0.3 M. Columns approximately 0.9 x 2.5 cm were prepared from this material and extensively washed with 0.3 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 8.0 (0.3 NTE buffer) to remove the NaN_3. Samples of DNA in 1-2 ml of 0.3 NTE buffer were applied to the columns and eluted by either of two procedures:

(a) gradient elution

A NaCl gradient formed by 15 ml of 0.3NTE buffer in a mixing vessel and 15 ml of 1.0 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 8.0 (1.0NTE buffer) in a reservoir was applied to the column, followed by 8 ml of 1.0NTE buffer. A caffeine gradient of 25 ml 1.0NTE buffer and 1 or 2% caffeine in 1.0NTE buffer was then applied, followed by 8 ml of 2% caffeine in 1.0NTE buffer.

(b) step elution

The column was rinsed with approximately 10 ml 0.3NTE buffer, and the DNA was eluted by 20 ml 1.0NTE buffer (NaCl eluate) followed by 20 ml 2% caffeine in 1.0NTE buffer (caffeine eluate).

The flow rate was controlled to 1 ml/min, and 2 ml fractions were taken. An aliquot of each fraction was then precipitated by adding trichloracetic acid to 10% and 200 μg bovine serum albumin (Pentex, Miles Laboratories, Kankakee, Ill.) at 0°C. The precipitates were collected on 0.45 μmembrane filters (Matheson-Higgins, Woburn, Mass.) before counting in toluene-PPO-PPOP scintillation

fluid. An aliquot of the sample applied to the column was also acid precipitated to monitor recovery of DNA from the column. In general, 90-95% of the counts applied were recovered. BD-cellulose has a high capacity for DNA (Sedat, Kelly and Sinsheimer, 1967). At least 10 μg of DNA was usually applied in order to reduce losses in handling. Caffeine was removed from fractions where necessary by extraction with chloroform, followed by extensive dialysis.

3. Enzyme reaction conditions

a. T4 DNA polymerase labeling of LA9 mitDNA

Reactions were performed in a volume of 0.4 ml containing: 0.16 units of T4 polymerase, LA9 mitDNA at 3-10 μg/ml, 46 μM ^{3}H-TTP (1460 cpm/nmole; Schwarz-Mann, Orangeburg, N.Y.), 49 μM ^{3}H-dCTP (1310 cpm/nmole; Schwarz-Mann, Orangeburg, N.Y.), 50 μM dATP, 50 μM dGTP, 67 mM Tris (pH 8.5), 6.7 μM EDTA, 6.7 nM $MgCl_2$, 17 mM ammonium sulfate, 10 mM 2-mercaptoethanol, and 167 μg/ml bovine serum albumin. Incubations were performed at 37°C for 60 minutes except in the kinetic studies and in the case of the NaCl eluate of the upper band mitDNA. In the latter instance a 70 minute preincubation in the absence of triphosphates was performed. The reaction was terminated by adding pyrophosphate to 0.1 M and EDTA to 0.25 M. In the preparative reactions, the DNA was then chromatographed on a porous glass bead column (Sigma G 240-50, 0.9 x 30 cm column) to remove unincorporated counts. The DNA eluted at about 10 ml, and was then ready for further analysis.

b. Covalent closure of upper band LA9 mitDNA

The basic reaction mixture of 0.3 ml contained upper band LA9 mitDNA at 2 μg/ml, 6.2 units of E. coli DNA ligase, 20 mM KPO_4 buffer (pH 7.5), 5 mM $MgCl_2$, 1mM EDTA, 17 μM DPN, 10 mM 2-mercaptoethanol, and 50 μg/ml bovine serum albumin. If E. coli DNA polymerase I (1.5 units) was present, all four deoxynucleotide triphosphates were added at 66 μM each. If T4 DNA polymerase (1.6 units) was present, 20 mM Tris (pH 8.0) was substituted for the phosphate buffer, 10 mM ammonium sulfate was added, and all four deoxynucleotide triphosphates were added at 66 μM each. E. coli exonuclease III (0.08 units) was present in one case. The reactions were stopped after 30 minutes at 30°C by adding EDTA to a concentration of 10 mM. Bovine serum albumin (250 μg per sample), Ethidium Bromide (300 μg/ml), and CsCl (p = 1.55) were added and the samples were centrifuged at 37,000 revs/min for 24 hours. The gradients were then fractionated dropwise onto GF/A filters which were counted in toluene-PPO-PPOP scintillation fluid.

4. Miscellaneous

Specimens were prepared for electron microscopy by the formamide Kleinschmidt technique described in Chapter 2, Materials and Methods (5).

Results

1. Chromatography of single stranded DNA on BD-cellulose

The elution of viral ØX174 DNA was examined to determine whether single stranded DNA elutes at a specific caffeine concentration. If so, it might be possible to fractionate replicating forms by the size of the single stranded regions they contain. The sample was bound to the column and eluted by the gradient elution procedure described in Materials and Methods (2) using a 0-1% caffeine gradient. As expected, none of the DNA eluted during the NaCl gradient (Figure 3). The elution profile resulting from the caffeine gradient is broad, however, indicating that clean fractionation of replicating forms by degree of single strandedness probably is not possible on BD-cellulose. In subsequent experiments, therefore, simple step elutions with 1.0NTE buffer and 2% caffeine in 1.0NTE buffer were performed.

2. Assay for nicking activity of BD-cellulose on duplex DNA

^{32}P-PM2 I DNA was chromatographed on BD-cellulose (Figure 4), and the NaCl and caffeine eluates were separately banded in EB-CsCl (Figure 5). In both cases 98% of the DNA was still covalently closed, indicating that neither the BD-cellulose itself nor the chloroform extraction procedure used to remove caffeine nicks any significant fraction of the DNA.

3. Chromatography of PM2 I and II DNA on BD-cellulose

When PM2 I DNA was chromatographed on BD-cellulose, a large

Figure 3: BD-cellulose Chromatography of ^{3}H-labeled ØX174 Viral DNA

A sample containing 10 μg ØX174 viral DNA in a total volume of 1.0 ml was applied to a BD-cellulose column and eluted by the gradient elution procedure described in Materials and Methods (2a). The points where the different eluents were applied are indicated. Fractions of 2.0 ml were taken, and the DNA was acid precipitated onto membrane filters and counted.

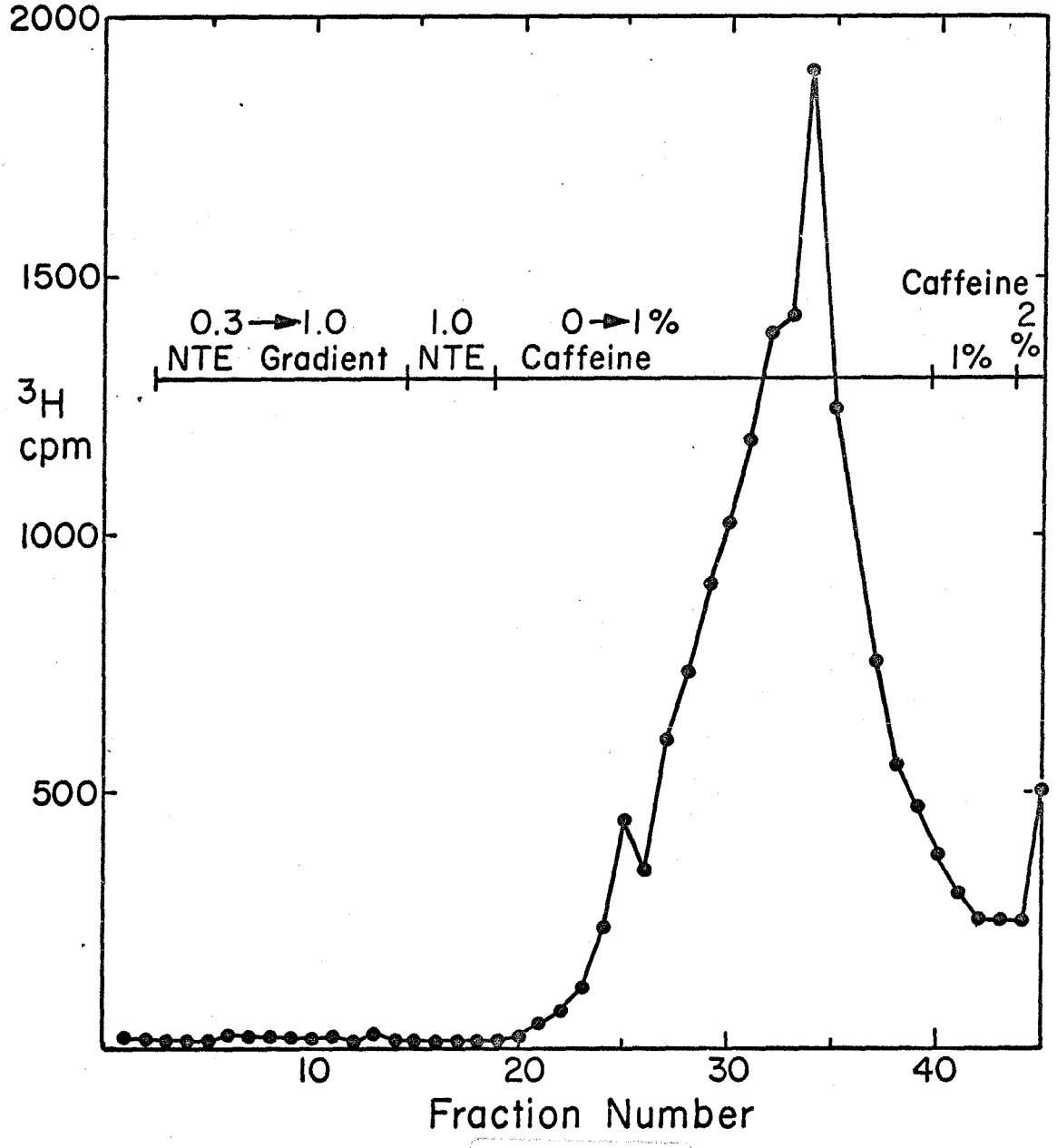
2000
1500
1000
500
^{3}H cpm
0.3→1.0 NTE Gradient
1.0 NTE
0→1% Caffeine
Caffeine 2 %
1%
10
20
30
40
Fraction Number

Figure 3

Figure 4: BD-cellulose Chromatography of ^{32}P-labeled PM2 I Viral DNA

A sample containing 5.8 μg of ^{32}P-labeled PM2 I DNA in a total volume of 1.0 ml was applied to a BD-cellulose column and eluted by the step elution procedure described in Materials and Methods (2b). The points where the different eluents were applied are indicated. Fractions of 2.0 ml were taken, and aliquots were acid precipitated onto membrane filters and counted. Fractions indicated by "N" and "C" were pooled, and caffeine was removed from the "C" fraction by extraction with chloroform as described in Materials and Methods. Both fractions were banded in EB-CsCl, giving the results shown in Figure 5.

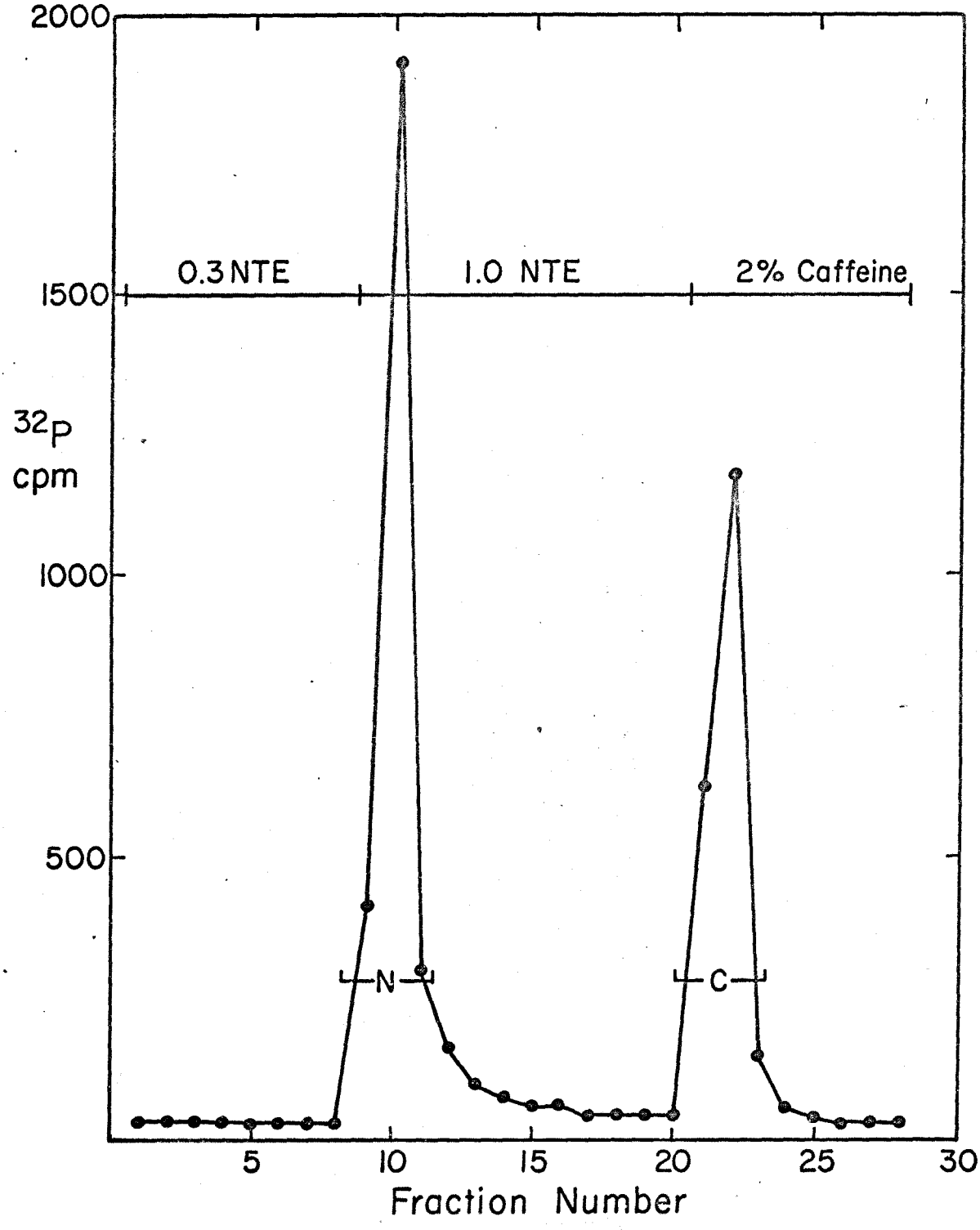
2000
1500
1000
500
0.3 NTE
1.0 NTE
2% Caffeine
32P
cpm
N
C
5
10
15
20
25
30
Fraction Number

Figure 4

Figure 5: EB-CsCl Banding of NaCl and Caffeine Fractions of PM2 I Viral DNA

The pooled fractions indicated in Figure 4 were separately banded in EB-CsCl (250 μg/ml Ethidium Bromide, ρ = 1.55 CsCl) for 20 hours at 36,000 revs/min in an SW-50 rotor, fractionated dropwise in 6 drop fractions onto GF/A glass fiber filters, and counted in toluene-PPO-PPOP scintillation fluid. The upper band position in each is indicated by an arrow.

(a) "N", or NaCl eluate

(b) "C", or caffeine eluate

The field is directed to the left.

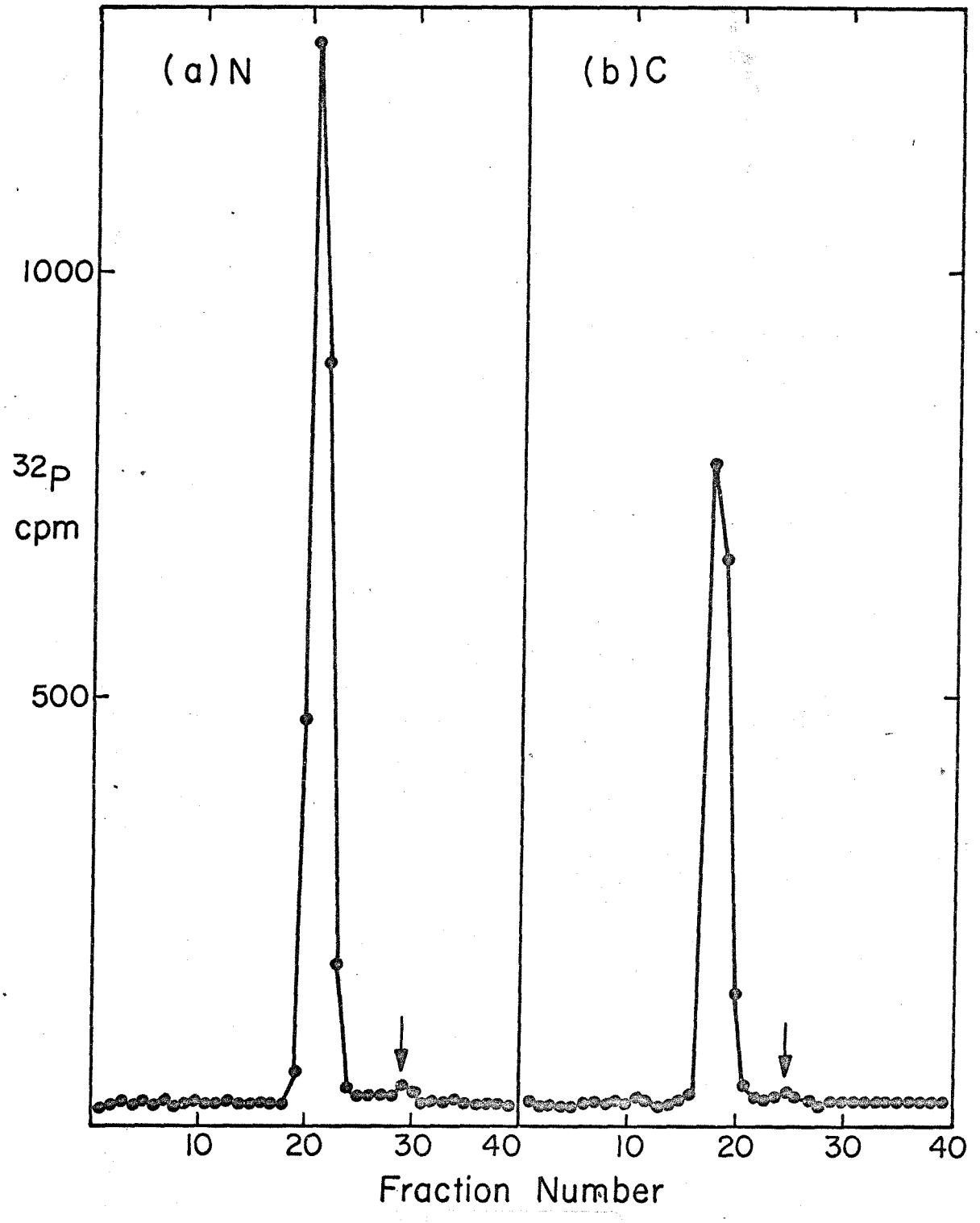

Figure 5

proportion (40%) of the DNA was retained by the column until the caffeine elution step (Figure 3 and Table 1). Similar results were obtained with singly nicked PM2 II DNA. In order to determine whether these separations reflect heterogeneity in the PM2 DNA, the NaCl and caffeine eluates were separately reapplied to BD-cellulose columns. The majority (85%) of the NaCl eluate of PM2 I DNA was eluted by 1.0NTE buffer in the rechromatography, while 57% of the caffeine eluate was eluted by caffeine (Table 1). Again, similar results were obtained with singly nicked PM2 II DNA. Therefore, both covalently closed and singly nicked PM2 DNA can be separated into two portions on BD-cellulose columns, and most of each portion elutes in the same position on rechromatography. In contrast, only 3.8% of SV40 I viral DNA from cells infected at low multiplicity is eluted by caffeine (Table 1).

The proportion of PM2 DNA retained until the caffeine elution step could be reduced by several modifications in the chromatographic procedure. When PM2 I DNA was applied to the column in 1.0NTE buffer, only 15% of the DNA bound to the column until the caffeine elution (Table 1). Viral ØX174 DNA applied at the same time was almost quantitatively (> 90%) bound and eluted with caffeine. Reduction of the temperature of the column from 24°C to 3°C also reduced the proportion of PM2 I DNA in the caffeine eluate to about 15% (Table 1).

In an attempt to determine whether the unexpected binding behavior of PM2 DNA might be due to a D-loop too small to be visible in the electron microscope, a sample was denatured with

Table 1

Chromatography of Duplex Viral DNA's on BD-cellulose

	% of counts recovered in caffeine eluate	
1. PM2 I		
a. native - exp. 1	36	
- exp. 2	40	
b. rerun - NaCl eluate	15	
- Caffeine eluate	57	
c. application in 1.0NTE	15	
(ØX174 viral under same conditions	90)	
d. reduced T	13	(3°C)
e. native	53	(27°C)
denatured-renatured	54	(27°C)
2. PM2 II, singly nicked		
a. native - exp. 1	39	
- exp. 2	41	
b. rerun - NaCl eluate	35	
- Caffeine eluate	76	
3. SV40 I (low multiplicity of infection)	3.8	

Samples were chromatographed on BD-cellulose as described in Materials and Methods, using the step elution procedure. Temperature = 23-25°C except as noted.

90% formamide at 37°C in 0.1NTE buffer, renatured in 50% formamide at 3°C in 0.1NTE buffer, and applied to a BD-cellulose column. The fraction of this material eluted by caffeine (54%) was not significantly different from that for untreated PM2 I DNA run at the same time (53%, Table 1). Both values are somewhat higher than those obtained previously, probably due to an elevated room temperature when this experiment was performed.

4. Chromatography of HeLa and LA9 mitDNA on BD-cellulose

When HeLa and LA9 mitDNA's were chromatographed on BD-cellulose, a large and variable proportion of the DNA eluted with caffeine (Table 2). Electron microscope examination of the NaCl eluate showed a complete absence of replicating forms of any type -- only clean duplex molecules were seen. Both replicating forms and apparently clean duplex molecules were found in variable proportions in the caffeine eluate (Table 2).

The apparent clean duplex molecules in the caffeine eluate could be replicative intermediates with single stranded regions too small (< 100 bases) to be visible by electron microscopy. Alternatively, some clean duplex mitDNA may be retained until the caffeine elution in the same way that some PM2 DNA is retained. In order to test whether the apparent clean duplex molecules in the caffeine eluate of the lower band LA9 mitDNA actually contain a small D-loop, a sample of the DNA was incubated at T_m-25° using the conditions for D-loop removal described by Kasamatsu, Robberson, and Vinograd (1971). Electron microscopy before and after denaturation

Table 2

BD-Cellulose Chromatography of mitDNA's

	% of counts recovered in caffeine eluate	% of D-loop monomers in caffeine eluate
1. HeLa		
(total mitDNA from DNase-treated mitochondria) - exp. 1	59	18 - 28
- exp. 2	68	-
2. LA9		
a. from DNase-treated mitochondria		
lower band DNA - exp. 1	15	-
- exp. 2	72	73
- exp. 3	35	55
upper band DNA - exp. 1	40	0
- exp. 2	71	0
- exp. 3	68	0
- exp. 4	60	0
b. from mitochondria purified without DNase treatment		
lower band	79	71
upper band	47	0

Samples were chromatographed on BD-cellulose as described in Materials and Methods, using the step elution procedure.

showed that initially approximately 50% of the molecules had D-loops, all of which were removed by the treatment. This sample and an untreated control were separately applied to BD-cellulose columns in 1.0NTE buffer at 3°C (conditions minimizing the fraction of PM2 DNA eluting in the caffeine fraction). Fifteen percent of the treated sample and 43% of the control DNA eluted with caffeine. Therefore, covalently closed mitDNA exhibits the same unexpected behavior as PM2 I DNA on BD-cellulose columns: some clean duplex DNA appears in the caffeine eluate. The caffeine-eluted mitDNA is an enriched preparation of replicating forms, since a large proportion (about 50%) of the clean duplex DNA has been removed in the NaCl elution step.

There were several indications of partial fractionation of replicating forms by degree of single strandedness in these experiments. In chromatograms with elution by caffeine gradients, the mitDNA always eluted slightly before the ØX174 viral DNA in the caffeine gradient when the two were co-chromatographed. In one experiment with HeLa mitDNA, a higher proportion of expanding D-loop forms and molecules with large gaps were found in the trailing portion of the peak of DNA eluted by caffeine than in the leading portion. Similar results have been reported for pulse-labeled ØX174 replicating molecules (Sinsheimer, Knippers, and Komano, 1968). These experiments were not pursued further because the broad elution profile of ØX174 viral DNA indicated that clean separations of different types of replicating forms could not be expected from this technique.

5. Enzymatic studies on LA9 mitDNA fractionated on BD-cellulose

^{14}C labeled LA9 mitDNA was isolated from DNase treated mitochondria in order to avoid nuclear DNA contamination, and upper and lower band preparations were fractionated on BD-cellulose. The resulting NaCl and caffeine eluates were used as substrates for E. coli DNA ligase, polymerase I, and exonuclease III; and T4 DNA polymerase.

It was first necessary to establish conditions under which T4 DNA polymerase would not exhast the available supply of nucleoside triphosphates. This enzyme has a 3' → 5' exonucleolytic activity in the absence of triphosphates. It will repair a gap in duplex DNA by adding nucleotides to the 3' end until the template is completely duplex and then alternately removes and replaces the 3' terminal nucleotide (Lehman, 1974). When the supply of nucleoside triphosphates has been exhausted in this manner, digestion occurs in the 3' → 5' direction. The kinetics of labeling of the caffeine eluate of the lower band mitDNA show that maximum incorporation under the conditions to be used for the experiments occured at 60 minutes, and that net exonucleolytic digestion began shortly thereafter (Figure 6(a)). A standard labeling time of 60 minutes was therefore chosen.

a. Studies on lower band mitDNA

Essentially no synthesis occured when the NaCl eluate of the lower band mitDNA was treated with T4 DNA polymerase (Table 3), but extensive incorporation of ^{3}H-dCTP and ^{3}H-TTP was obtained with the caffeine eluate. About 73% of the monomers in the latter

Figure 6: Kinetics of T4 DNA Polymerase Labeling of LA9 mitDNA

(a) Kinetics of labeling of the caffeine fraction of the lower band

A portion of the caffeine fraction of the lower band LA9 mitDNA was incubated with T4 DNA polymerase and ^{3}H-labeled deoxynucleoside triphosphates under the conditions described in Materials and Methods. At the indicated times, aliquots were withdrawn and synthesis stopped by adding pyrophosphate to 0.1 M and EDTA to 0.25 M. The samples were then acid precipitated onto membrane filters and counted.

(b) Kinetics of 3' → 5' exonuclease action of T4 DNA polymerase in the absence of triphosphates

Portions of the NaCl (solid line and points) and caffeine (dashed line and open points) eluates of the upper band LA9 mitDNA were incubated with T4 DNA polymerase under the conditions described in Materials and Methods, with the omission of triphosphates. Aliquots were withdrawn at the indicated times, ^{3}H-labeled deoxynucleoside triphosphates were added, and each was incubated for an additional 60 minutes. The reactions were terminated and the DNA acid precipitated as above.

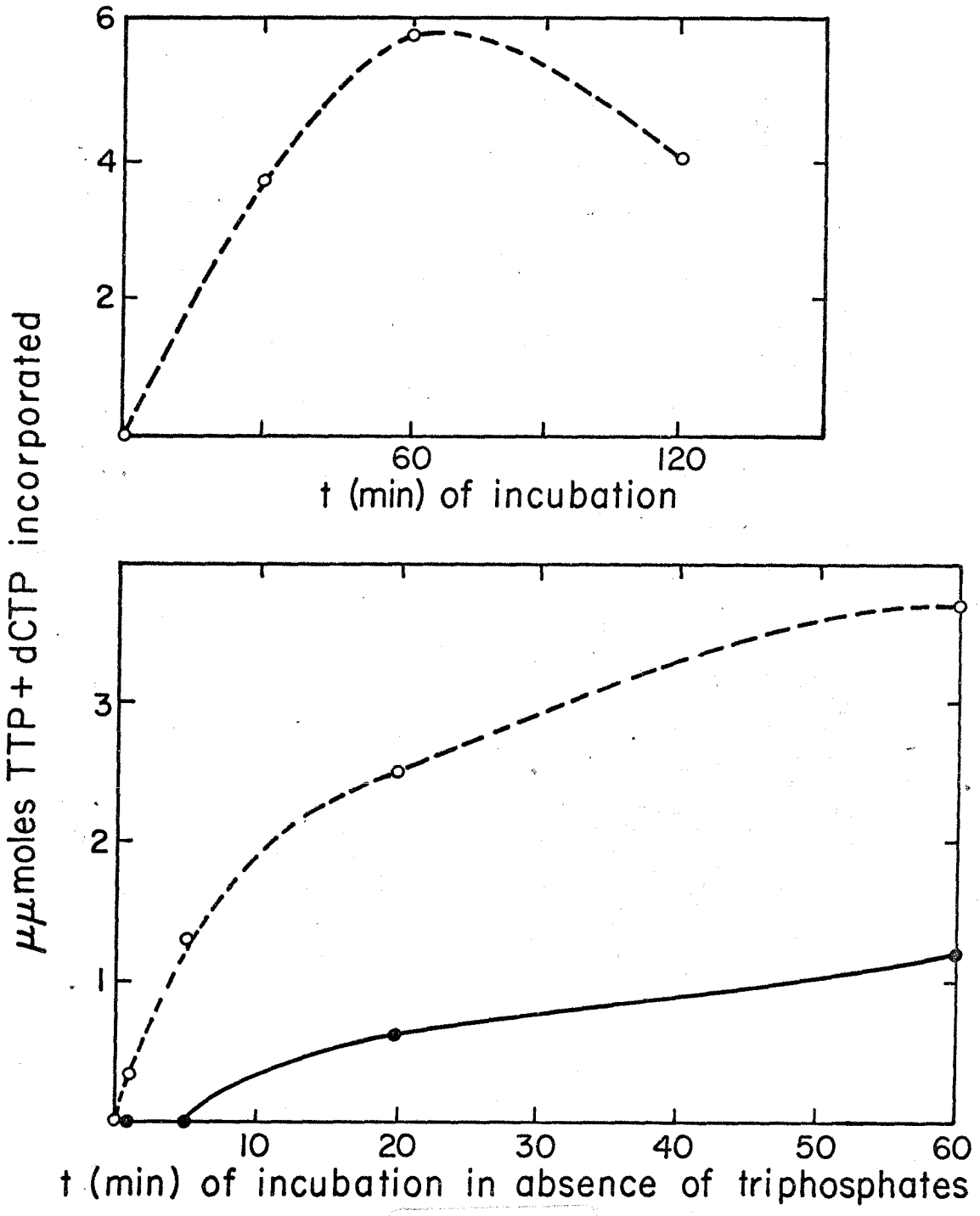
6
4
2
60
120
t (min) of incubation
μμmoles TTP + dCTP incorporated
3
2
1
10
20
30
40
50
60
t (min) of incubation in absence of triphosphates

Figure 6

Table 3

Incorporation of ^{3}H-TTP and ^{3}HdCTP into LA9 mitDNA by T4 DNA Polymerase

Sample	cpm Incorporated
Upper band DNA - NaCl eluate	1170
- Caffeine eluate	4518
Lower band DNA - NaCl eluate	942
- Caffeine eluate	6354
Blank (no mitDNA)	1750

Incubation was at 37°, 30 minutes as described in Materials and Methods. DNA was acid precipitated onto membrane filters and counted as described in Figure 6.

preparation had D-loops prior to treatment with this enzyme, and electron microscopy after treatment showed that at least 50% of the monomers still had D-loops. Banding in EB-CsCl showed that the mitDNA was not extensively nicked during the incubation, and that label was incorporated into the lower band (Figure 7). Some of the ^{3}H counts in the upper band may represent 7S DNA labeled by T4 polymerase which subsequently branch migrated off the replicating molecule, since there is a broad background of ^{3}H counts under the upper band. This interpretation is confirmed by sedimentation of the DNA through a neutral sucrose gradient, for 27% of the ^{3}H counts sedimented in a slow moving peak (Figure 8).

The T4 polymerase treated DNA was also treated with formamide to branch migrate any displacing strands off the parental template, and then sedimented through neutral sucrose (Figure 9(a)). Most (74%) of the ^{3}H label sedimented very slowly, but a small proportion sedimented almost as fast as the parental ^{14}C label. This faster peak may represent a small number of nicked D-loop molecules on which extensive synthesis has occured. In order to measure the sedimentation coefficient of the slow sedimenting material, another aliquot was cosedimented with ^{14}C-labeled 4S and 18S HeLa RNA (Figure 9(b)). The majority of the ^{3}H label sedimented at 7.7S. This result indicates that label was incorporated with little or no net synthesis, since "7S" DNA sediments at about 7.7S in this system.

b. Studies on upper band LA9 mitDNA

Enzymatic analyses of the upper band mitDNA were directed at determining: (1) what enzymatic activities are required to close the

Figure 7: EB-CsCl Banding of the Caffeine Eluate of the Lower Band LA9 mitDNA after Incubation with T4DNA Polymerase

An aliquot of the caffeine eluate of the lower band LA9 mitDNA which had been incubated with T4 DNA polymerase was banded in EB-CsCl (250 μg/ml Ethidium Bromide, ρ = 1.55 CsCl) for 36 hours at 36,000 revs/min, fractionated dropwise onto GF/A filters, and counted in toluene-PPO-PPOP scintillation fluid. The field is directed to the left.

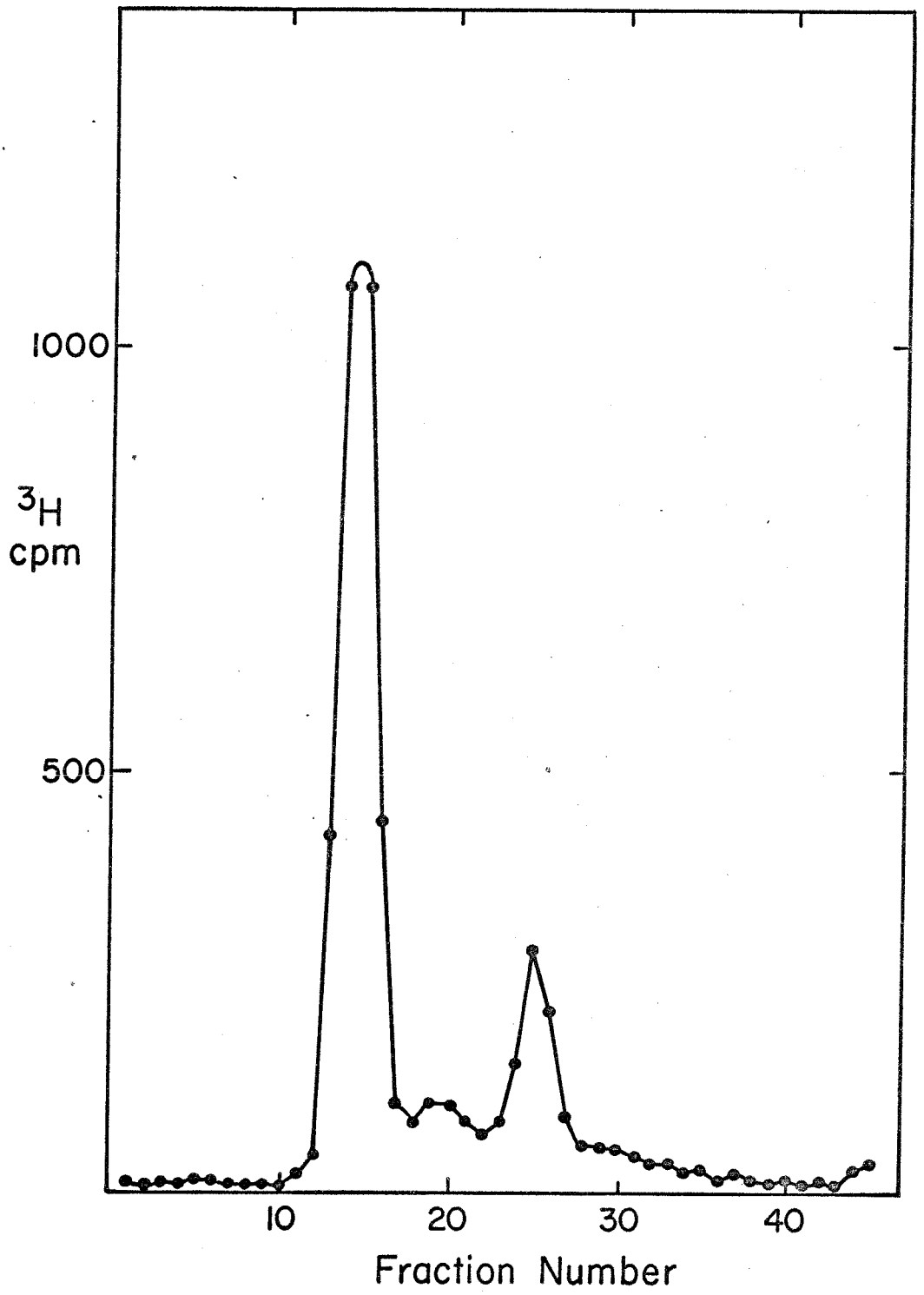

Figure 7

Figure 8: Sedimentation Analysis of Caffeine Eluate of the Lower Band LA9 mitDNA after Incubation with T4 DNA Polymerase

An aliquot of the caffeine eluate of the lower band ^{14}C-labeled LA9 mitDNA which had been incubated with T4 DNA polymerase and ^{3}H-labeled deoxynucleoside triphosphates was layered onto a 5-20% sucrose gradient containing 0.5 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 8.0 and centrifuged at 36,000 revs/min for 140 minutes at 20°C in an SW50 rotor. Fractions of 7 drops were collected on GF/A filters and counted in toluene-PPO-PPOP scintillation fluid. The arrow indicates the position of untreated lower band mitDNA in a parallel run. ^{3}H counts, solid lines and points; ^{14}C counts, dashed lines and open points. The field is directed to the left.

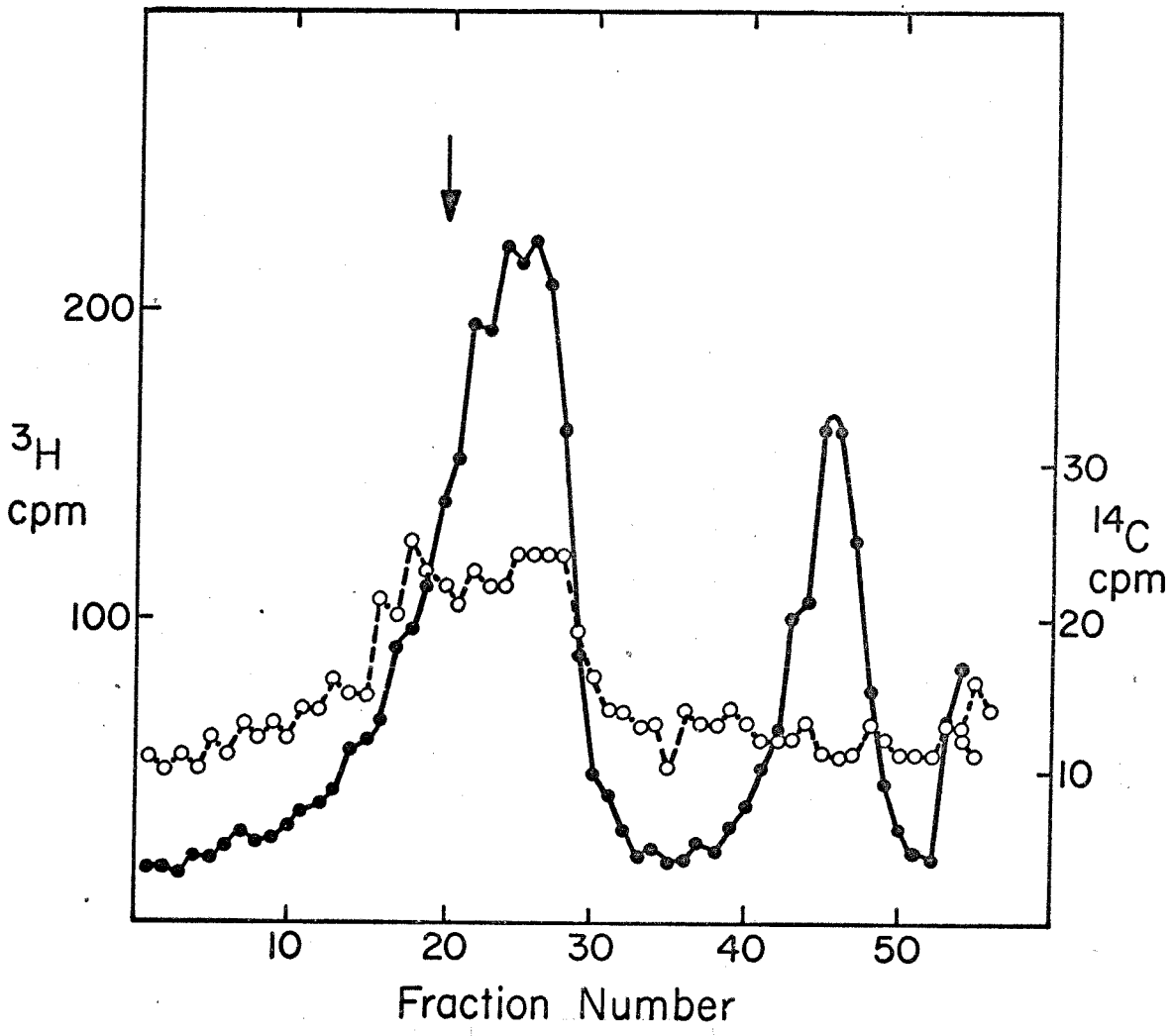

Figure 8

Figure 9: Sedimentation Analysis of Caffeine Eluate of the Lower Band LA9 mitDNA after Incubation with T4 DNA Polymerase and Denaturation in Formamide

An aliquot of the caffeine eluate of the lower band ^{14}C-labeled LA9 mitDNA which had been incubated with T4 DNA polymerase and ^{3}H-labeled deoxynucleoside triphosphates was dialyzed into 10 mM Tris, 1 mM EDTA, pH 7.5. It was then denatured by dialysis against formamide plus 1 mM Tris, 1 mM EDTA, pH 7.5 for 50 minutes; and finally dialyzed into 1 mM Tris, 1 mM EDTA for another 30 minutes.

(a) A portion of this material was layered onto a 5-20% sucrose gradient containing 0.5 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 8.0, and centrifuged and counted as in Figure 8. The arrow indicates the position of untreated lower band mitDNA in a parallel run.

(b) ^{14}C-labeled 4S and 18S RNA markers were added to another portion of this material, and it was layered onto another sucrose gradient as above. This gradient was centrifuged at 41,000 revs/min for 330 minutes at 20°C before counting as in Figure 8. ^{3}H counts, solid line and points; ^{14}C counts, dashed lines and open points. The field is directed to the left.

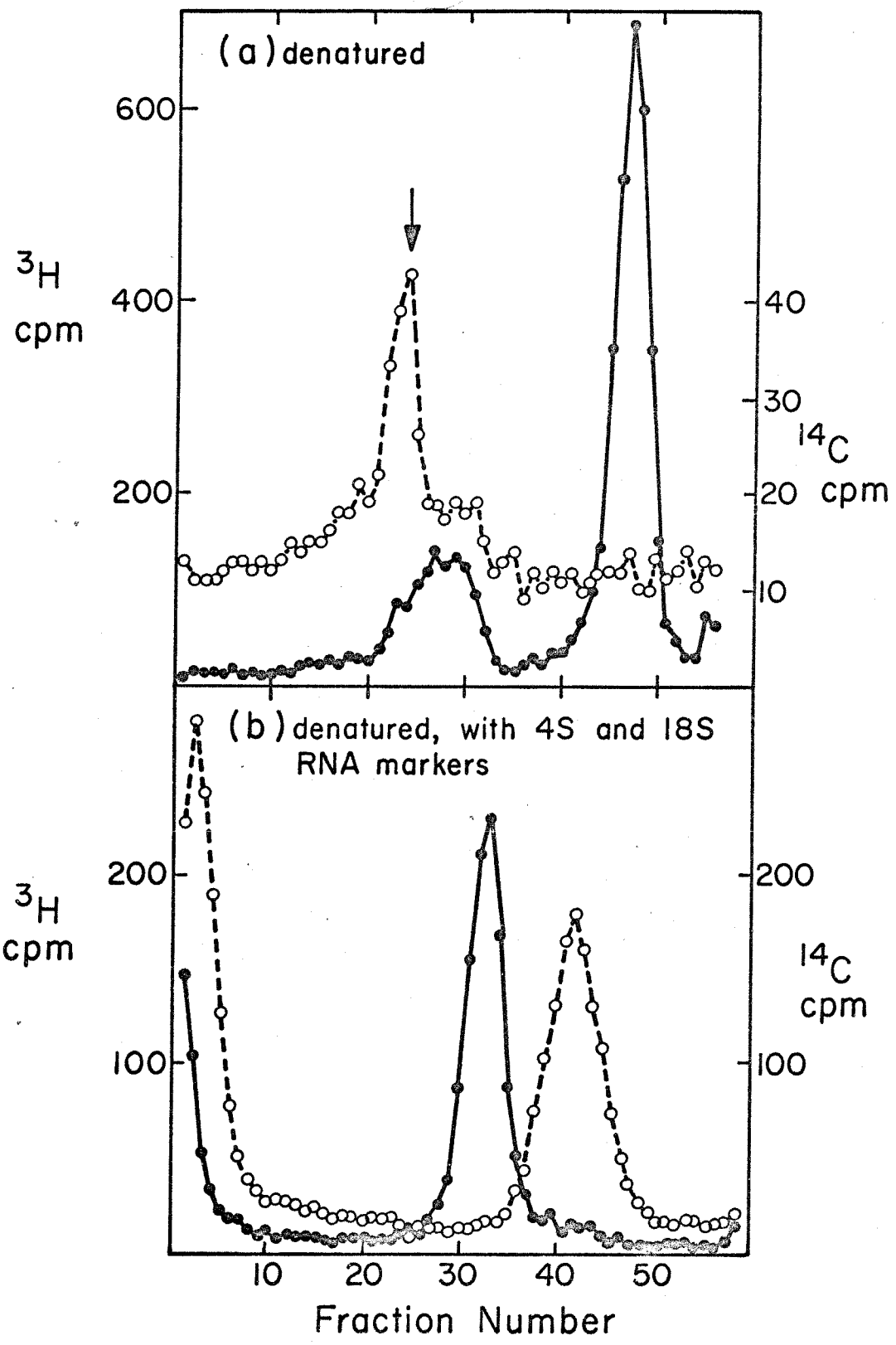
(a) denatured
600
400
200
^{3}H cpm
40
30
20
10
^{14}C cpm
(b) denatured, with 4S and 18S RNA markers
200
100
^{3}H cpm
200
100
^{14}C cpm
10
20
30
40
50
Fraction Number

Figure 9

nicks or small gaps in the molecules eluted by 1.0NTE; (2) whether these nicks are strand specific; and (3) whether the gaps in the gapped molecules in the caffeine eluate are strand specific. Incubation of the NaCl eluate with ligase alone did not result in the covalent closure of a measurable fraction of the DNA. Addition of E. coli DNA polymerase I or T4 polymerase resulted in closure of 2.9% and 4.2%,re spectively. Even with a mixture of exonuclease III (to remove any 3' phosphate ends which would block polymerase action), E. coli DNA polymerase I, and ligase, only 6.3% of the DNA was closed. Evidently either at least one terminus at the nick or gap is in a form which could not be replaced by the 3'-OH, 5'-P required for ligase action, or the incubation conditions used were incorrect.

The analysis for strand specificity of the nicks in the NaCl eluate of the upper band mitDNA was performed by labeling the nicked strands and then examining the distribution of label in alkaline buoyant CsCl. Since no label was incorporated into this DNA by T4 DNA polymerase (Table 3), the nicks were enlarged by utilizing the 3' → 5' exonuclease activity of T4 polymerase in the absence of nucleoside triphosphates. These gaps were then repaired by the enzyme on addition of labeled triphosphates. The amount of label incorporated depended on the length of the incorporation without triphosphates (Figure 6(b)). The alkaline buoyant CsCl gradient (Figure 10) shows a symmetric distribution of label in the two strands, indicating that equal numbers of nicks were located in both strands.

Electron microscope examination of the caffeine eluate of the

Figure 10: Alkaline Buoyant CsCl Banding of the NaCl Eluate of the Upper Band LA9 mitDNA after Incubation with T4 DNA Polymerase

A portion of the NaCl eluate of the upper band ^{14}C-labeled LA9 mitDNA was incubated with T4 DNA polymerase for 70 minutes at 37 °C in the absence of deoxynucleoside triphosphates. ^{3}H-labeled deoxynucleotides were then added, and the incubation was continued for an additional 60 minutes at 37 °C. The sample was banded in 2.5 ml of alkaline buoyant CsCl ($\rho \simeq 1.75$) containing 0.05 M K_3PO_4, pH 12.4, and 10 μg calf thymus DNA carrier. After centrifugation at 32,000 revs/min for 40 hours in an SW50.1 rotor, the tube was fractionated dropwise in 6-drop fractions on GF/A filters, which were counted in toluene-PPO-PPOP scintillation fluid. The arrows indicate the positions of H and L strands of mitDNA in a parallel gradient. ^{3}H counts, solid line and points; ^{14}C counts, dashed line and open points. The field is directed to the left.

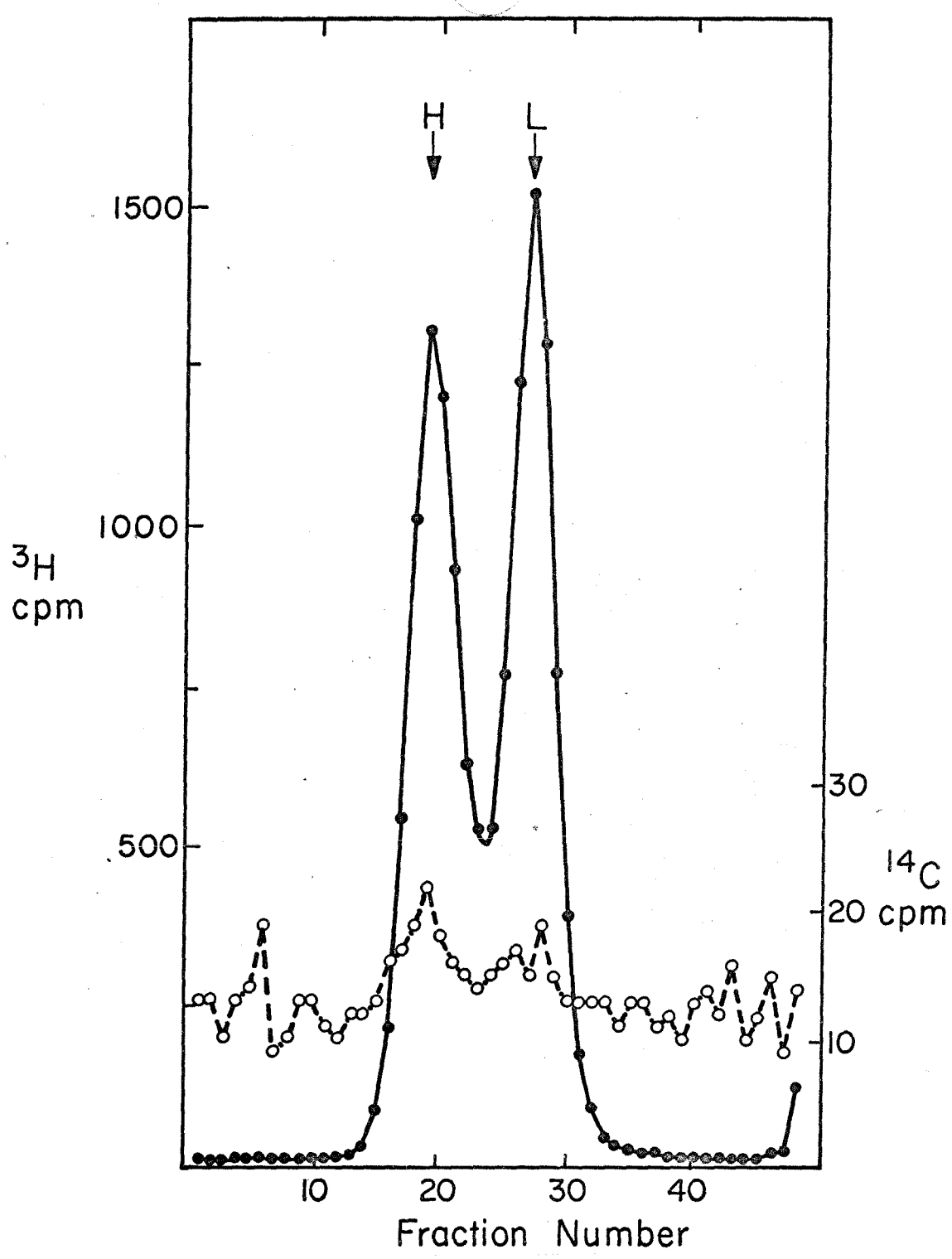
H
L
1500
1000
500
^{3}H
cpm
30
20 cpm
10
^{14}C
10
20
30
40
Fraction Number

Figure 10

upper band mitDNA showed very few expanding D-loop forms, indicating that most of this DNA is either gapped or clean duplex molecules. Closed expanding D-loop forms were probably lost in the isolation of upper and lower band mitDNA (Figure 2), since they band in the intermediate region (Kasamatsu et al., 1973). At the time these experiments were performed, LA9 mitDNA banding at an intermediate position in EB-CsCl was believed to be partially nicked catenanes and was therefore not subjected to enzymatic analysis. The strand specificity of the gaps was determined by repairing the gaps with T4 polymerase, and banding the product in alkaline buoyant CsCl (Figure 11). Although the distribution is clearly skewed towards the L strand, a large amount of material bands at a position intermediate between the H and L strands. When another aliquot was subjected to a neutral sucrose gradient prior to banding in alkaline buoyant CsCl, the ^{3}H label separated into two peaks (Figure 12). These two peaks were separately banded in alkaline buoyant CsCl. The slower sedimenting material banded in an intermediate position between the H and L strands and probably represents incorporation of label into small nuclear DNA fragments contaminating the preparation (Figure 13(b)). Most of the ^{3}H counts in the faster sedimenting material, however, were in the L strand (Figure 13(a)). When corrected for the different base compositions of the two strands and for the differences in the specific activities of the ^{3}H-dCTP and -TTP used in the T4 polymerase incubation, the ratio of synthesis of L strand to that of H strand is 2.51.

Figure 11: Alkaline Buoyant CsCl Banding of the Caffeine Eluate of the Upper Band LA9 mitDNA after Incubation with T4 DNA Polymerase

The caffeine eluate of the upper band ^{14}C-labeled LA9 mitDNA was incubated with T4 DNA polymerase and ^{3}H-labeled deoxynucleoside triphosphates for 60 minutes at 37 °C under the conditions described in Materials and Methods. A portion of the mitDNA was then banded in alkaline buoyant CsCl as described in Figure 10. The arrows indicate the positions of the H and L strands of mitDNA in a parallel gradient. ^{3}H counts, solid line and points; ^{14}C counts, dashed line and open points. The field is directed to the left.

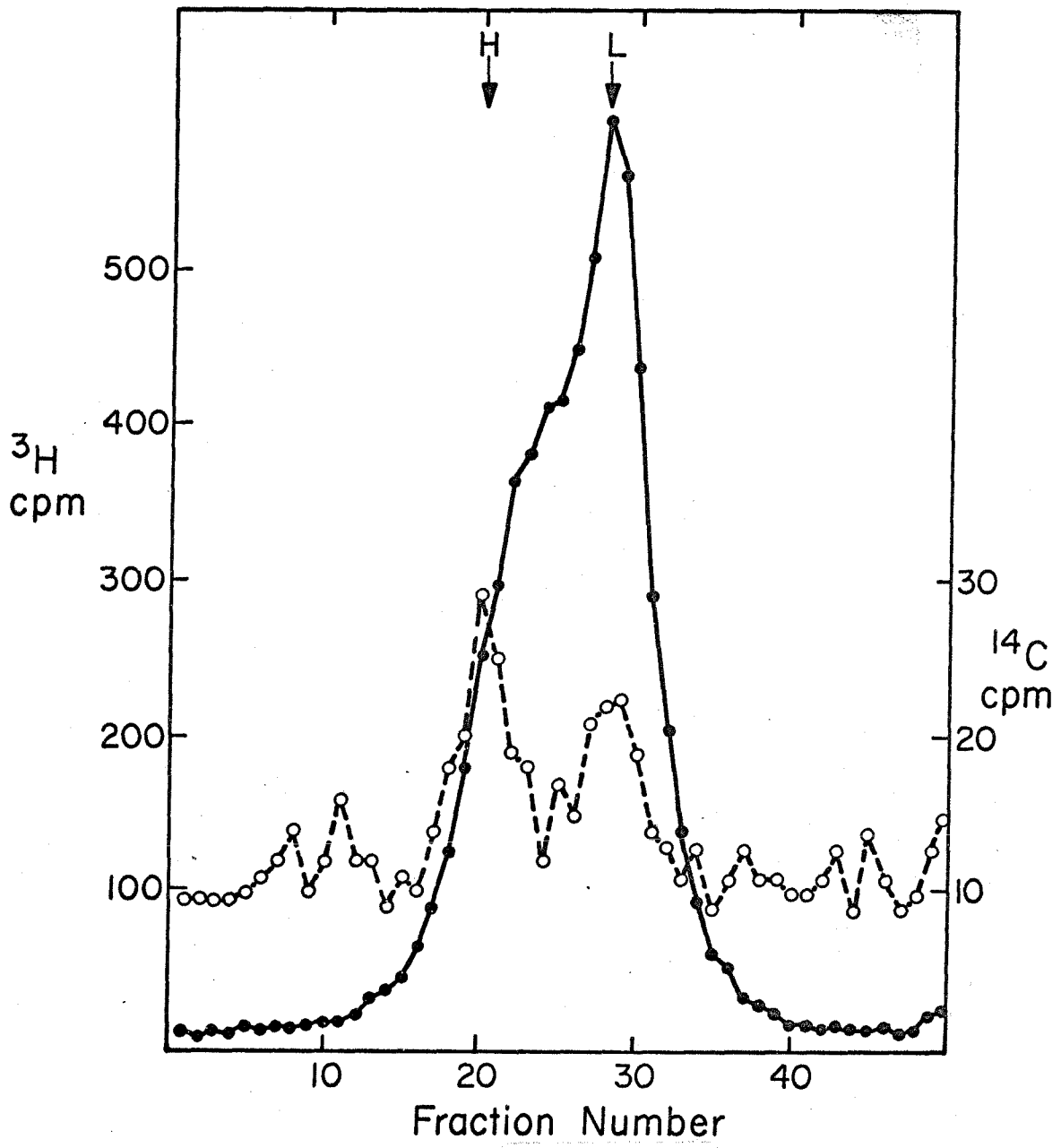
H
L
500
400
300
200
100
^{3}H
cpm
30
20
10
^{14}C
cpm
10
20
30
40
Fraction Number

Figure 11

Figure 12: Preparative Velocity Sedimentation of the Caffeine Eluate of the Upper Band LA9 mitDNA after Incubation with T4 DNA Polymerase

The remainder of the caffeine eluate of the upper band mitDNA which had been treated with T4 DNA polymerase was layered onto a 5-20% sucrose gradient containing 0.5 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 8.0, and centrifuged for 135 minutes at 36,000 revs/min at 20°C in an SW50 rotor. The gradient was fractionated into 8 drop fractions, and aliquots were spotted onto GF/A filters and counted in toluene-PPO-PPOP scintillation fluid. The arrow marks the position of lower band mitDNA. The indicated fractions were pooled and banded in alkaline CsCl as described in Figure 13. The field is directed to the left.

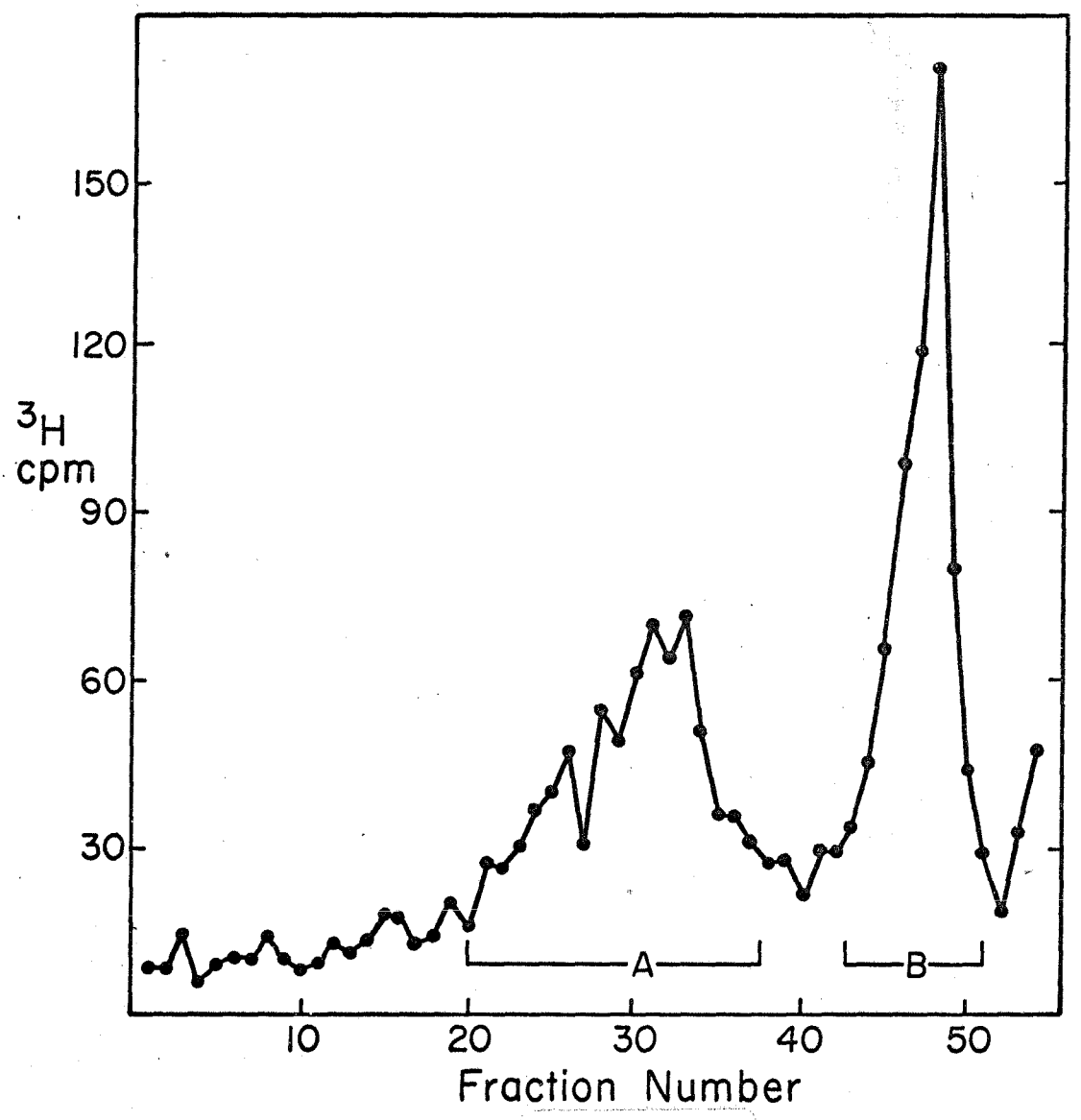

Figure 12

Figure 13: Alkaline Buoyant CsCl Banding of Fast and Slow Sedimenting Portions of the Caffeine Eluate of the Upper Band LA9 mitDNA after Incubation with T4 DNA Polymerase

The pooled fractions indicated in Figure 12 were banded in alkaline buoyant CsCl as described in Figure 10. ^{32}P-labeled LA9 mitDNA was included as a marker (dashed line and open points).

(a) Fast sedimenting material.

(b) Slow sedimenting material.

The field is directed to the left.

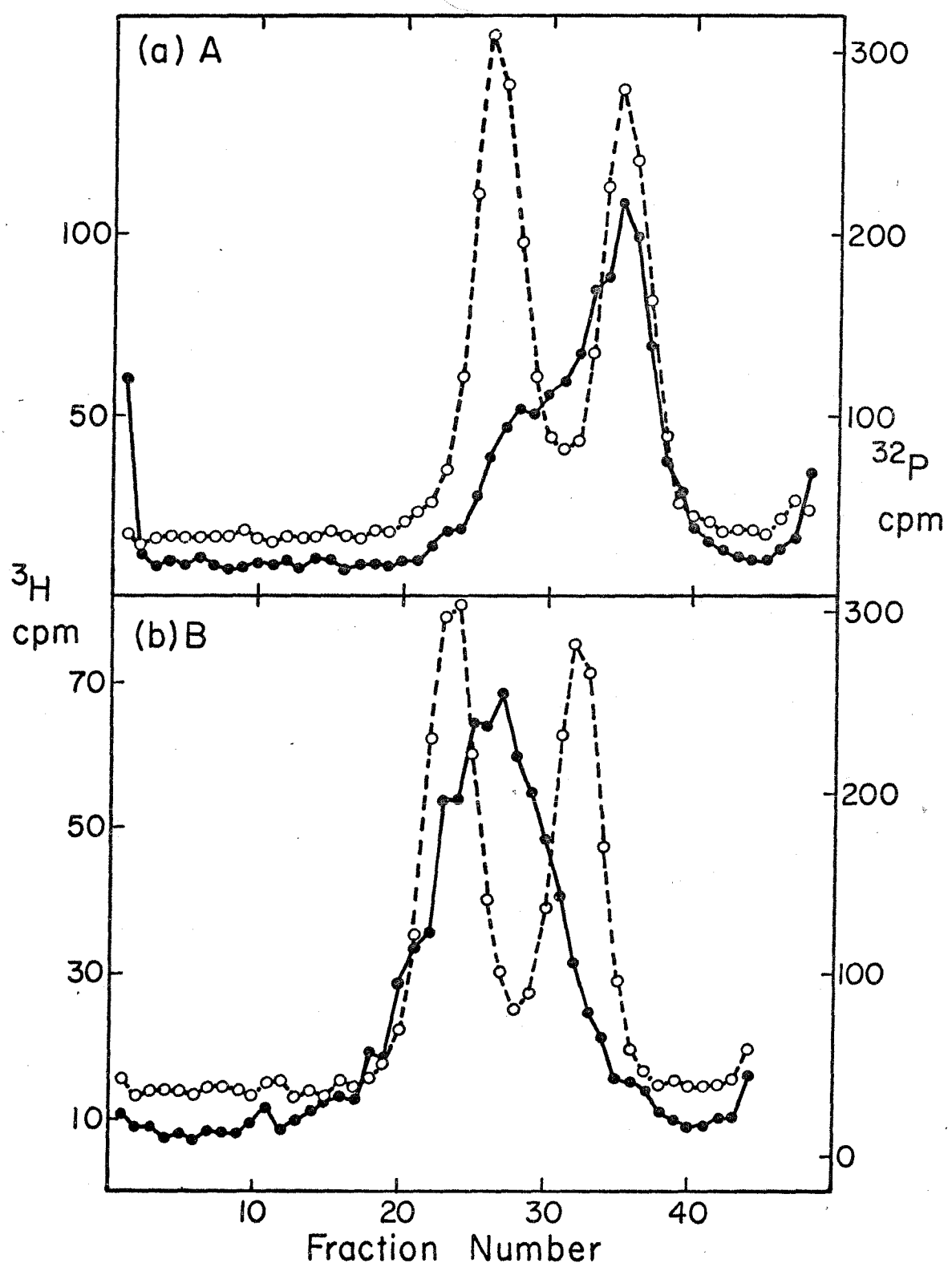
(a) A
(b) B
^{3}H cpm
^{32}P cpm
100
50
300
200
100
70
50
30
10
300
200
100
0
10
20
30
40
Fraction Number

Figure 13

Discussion

1. Chromatography of single stranded and duplex DNA on BD-cellulose

As discussed earlier, BD-cellulose separates single stranded and duplex nucleic acids by the difference in the ability of their bases to interact with the benzoyl groups of the column. The BD-cellulose used in these experiments was first tested with single stranded (ØX174 viral) and duplex (PM2) DNA to determine whether the separation is complete. As expected, none of the ØX 174 viral DNA was eluted by 1.0NTE. Both PM2 I and II (singly nicked) DNA exhibited an unexpected behavior: a large proportion of these DNA's was retained until the caffeine elution step, indicating an interaction with the benzoyl residues of the column. Other duplex DNA's such as ØX174 RF I DNA (Knippers et al., 1969), λ viral DNA (Sedat, Kelly, and Sinsheimer, 1967), and SV40 I viral DNA (Table 1) elute quantitatively with NaCl. The bases in these duplex DNA's are therefore inaccessible to the benzoyl groups, but PM2 DNA behaves as though it contains denatured regions. These regions may exist in solution, or they may be induced by the column environment. A single stranded region of more than 12 nucleotides is required for binding, since λ viral DNA with 12 nucleotide single stranded ends elutes with NaCl (Sedat, Kelly, and Sinsheimer, 1967). Single stranded regions of about 300 base pairs have been observed in PM2 I DNA at a low frequency by formamide electron microscopy (H. Kasamatsu, personal communication), and their existence in solution has been inferred from the reaction of PM2 I with formaldehyde (Jacob, Lebowitz, and Kleinschmidt, 1974). The

denaturation map of PM2 II DNA clearly shows that the molecule has 3-4 AT rich regions in which denatured segments might occur. The dependence of the fraction of PM2 DNA eluted by caffeine on both the NaCl concentration in which the sample was applied to the column and the temperature of the column could reflect either an increased stability of the duplex structure under these conditions or a reduced affinity of the benzoyl residues for denatured regions (although ØX174 viral DNA chromatographs normally under these conditions).

These results raise several difficult problems. If the denatured regions in PM2 I DNA are a result of the torsional strain in a superhelical molecule (Jacob, Lebowitz, and Kleinschmidt, 1974), PM2 II DNA would not be expected to have denatured regions. The identical behavior of PM2 I and II DNA on BD-cellulose suggests that the denatured regions responsible for binding to this material are primarily a a result of base sequence rather than superhelical free energy.

The results showing that both the NaCl and the caffeine eluates of the upper and the lower band PM2 DNA tend to elute in the same positions on reapplication to the column could be explained in two ways: either molecules with different base sequences are present in the PM2 DNA, or the denatured regions are "locked in" in PM2 II DNA and do not renature very quickly. The latter possibility could be tested by a denaturation-renaturation step before rechromatography.

Finally, both ØX174 RF I and SV40 I DNA are sensitive to single stranded specific endonucleases (Kato et al., 1973; Beard, Morrow, and Berg, 1973), and a single stranded region has been observed in the latter (H. Kasamatsu, personal communication). Yet both DNA's are

eluted from BD-cellulose by NaCl (Sedat, Kelly, and Sinsheimer, 1967; Levine, Kang, and Billheimer, 1970; Table 2). One possible explanation is that the binding of a small denatured region to the benzoyl groups is weak, and that several such regions in each molecule are required for retention. PM2 DNA may have enough denatured regions for partial retention, but the smaller number in SV40 and ØX174 RF may not be adequate for their binding.

2. Chromatography of LA9 and HeLa mitDNA on BD-cellulose

The possibility that a similar phenomenon might occur with mitDNA was suggested by both the large proportion of mitDNA not in the caffeine eluate in relation to the D-loop levels in the starting material, and the observation of clean duplex molecules in the caffeine eluate. This interpretation was confirmed by comparing the chromatographic behavior of lower band LA9 mitDNA before and after removal of D-loops by branch-migration. A substantial proportion of the mitDNA (15%) still appeared in the caffeine eluate after D-loop removal, even when applied in 1.0NTE at low temperature. The denaturation loops observed at a low frequency in electron microscope examinations of sea urchin (Matsumoto et al., 1974) and LA9 (H. Kasamatsu, personal communication) covalently closed mitDNA may be responsible for this binding, as discussed in the previous section.

No partially single stranded forms were ever seen in the NaCl eluate, suggesting that they were all in the caffeine eluate. The frequencies of D-loop forms before and after BD-cellulose chromatography support this interpretation. For example, 53% of the lower band LA9

mitDNA monomers contained a D-loop. If all of these molecules were in the caffeine eluate, a frequency of 67% would be predicted, in good agreement with the observed value of 71%. The caffeine eluate contains all of the replicating forms identifiable by electron microscopy, and therefore represents a preparation of replicating forms which has been enriched by the removal of part of the clean duplex DNA.

3. Enzymatic studies of LA9 mitDNA replicating forms

The presence of clean duplex mitDNA in the preparations of replicating forms used in the enzymatic analyses should not have affected the results. Neither upper nor lower band clean duplex DNA should be a substrate for T4 DNA polymerase, since the enzyme cannot initiate at a nick (Lehman, 1974) and is inactive on covalently closed DNA. This expectation was conformed by the failure of the enzyme to incorporate labeled triphosphates into the NaCl eluates of the upper and lower band mitDNA (Table 3). The use of the other enzymes was confined to the analysis of the NaCl eluate of the upper band mitDNA, which consists only of clean duplex molecules. Results obtained from other types of experiments utilizing BD-cellulose chromatography should be examined carefully to determine whether the results could be affected by the incomplete separation of mature molecules from replicating forms.

There are four principal results from these studies: (1) the nicks in the upper band clean duplex mitDNA could not be closed under the conditions used, even when E. coli exonuclease III and polymerase I were included in the incubation mixture; (2) these nicks were not

located preferentially in either strand; (3) the "7S" fragment in the D-loop replicating forms is labeled by T4 polymerase without any increase in size as determined by sedimentation velocity; and (4) the caffeine eluate of the upper band mitDNA is missing more of the L than of the H strand.

The studies on the nicked clean duplex DNA were facilitated by the absence of replicating forms in the NaCl eluate, since the presence of molecules with large gaps could complicate interpretation of the results. Under no conditions was a large proportion of molecules closed by DNA ligase. Addition of T4 DNA polymerase, or E. coli DNA polymerase I with or without exonuclease III, resulted in the closure of a small proportion of the molecules. The polymerases should have filled in any small gaps preventing closure, and E. coli polymerase I can also "nick translate" to provide the 3'-OH, 5'-P ends required for ligase action. The phosphatase activity of exonuclease III removes 3'-P ends, leaving the 3'-OH end for ligase action. Either most of these molecules contain nicks with termini which are resistant to these enzymes, or the conditions used for closure were not correct.

Labeled nucleotide triphosphates were incorporated into the NaCl eluate of the upper band mitDNA by utilizing the 3' → 5' exonucleolytic activity of T4 DNA polymerase in the absence of triphosphates to enlarge the nicks into gaps, which were then repaired with labeled nucleotides. The label was approximately equally distributed between the strands in an alkaline buoyant CsCl gradient, indicating an equal frequency of nicks in the two strands. Any specific nicks occuring in

one strand of clean duplex DNA during replication were not detected in this experiment, but molecules containing adventitious nicks suffered during isolation of the DNA could have masked a small number of molecules containing specific nicks.

The experiments with D-loop molecules from the lower band clearly show that T4 DNA polymerase can incorporate label into "7S" DNA, but that the size of the fragment is not increased. Calculations based on the specific activities of the DNA and labeled nucleoside triphosphates used indicate that about 260 nucleotides were incorporated per "7S" DNA molecule. If these had simply been added onto the 3' end of the "7S" DNA, an increase in the sedimentation coefficient of 1.2 to 1.6S would have been expected. Within experimental error, no increase was observed: both in vivo and in vitro labeled "7S" DNA sediment in neutral sucrose at 7.7S using 4S and 18S RNA as markers. The simplest interpretation is that the ends of the "7S" fragments branch migrate free from the template under the low ionic strength conditions required for the enzyme incubations. The 3' → 5' exonucleolytic activity of T4 DNA polymerase would then excise the 3' end, and the polymerase activity would resynthesize it using labeled nucleotides, stopping when the superhelix free energy becomes sufficiently large. The sedimentation coefficient is relatively insensitive to molecular weight, so that a 10-20% increase in the size of the fragment would not have been detected. If the base composition of the region near the "7S" replication initiation site is particularly rich in C and/or T, the number of nucleotides added could have been much smaller than 260. Nevertheless, these results appear to contradict the finding of Berk and Clayton (1974) that the

parental strands are nicked and re-closed after synthesis of the "7S" fragment. If this were true, T4 polymerase should have approximately doubled the size of the "7S" fragment.

Because of the method of isolation, most of the single stranded forms observed in the caffeine eluate of the upper band mitDNA were gapped circles rather than D-loop or expanding D-loop molecules. The labeling of this fraction by T4 polymerase therefore reflects the filling in of gaps rather than complement synthesis on D-loop forms. The actual frequency of gapped molecules may have been higher than that measured in the electron microscope because of the difficulty of seeing small gaps. After contaminating small nuclear DNA fragments have been removed,a clear asymmetry in the labeling of the two strands is apparent (Figure 13(a)). Seventy-five percent of the label is incorporated into L strand mitDNA, showing that the upper band mitDNA has a greater overall deficiency of L than of H strand regions. This evidence is consistent with the hybridization experiments of Robberson, Kasamatsu, and Vinograd (1972) which demonstrated that most of the large gapped molecules lack portions of the L strand. This result was interpreted to mean that gapped molecules result from incomplete complement synthesis at separation, leaving one daughter (the α) nearly completely duplex and the other (the β) lacking a portion of the L strand (Figure 1). The average gap size cannot be calculated accurately due to the labeling of the small nuclear DNA contaminant, but it is roughly 120 nucleotides. These results are also in agreement with the report of asynchronous replication of strands by Berk and Clayton (1974), since the upper band replicating forms are in the

later stages of DNA synthesis.

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Chapter 4

A Method for the Detection of Ribonucleotides in mitDNA

Introduction

At present, two lines of evidence indicate that ribonucleotides are incorporated in covalently closed mitDNA: its rapid degradation in alkali and its sensitivity towards ribonucleases. The alkali lability has been ascribed to depurination of the mitDNA by free radicals generated during the isolation procedure (Borst, 1972). Depurinated sites may be responsible for the "fast" alkaline nicking reported by Wong-Staal, Mendelsohn, and Goulian (1973) and Grossman, Watson, and Vinograd (1973). In both cases, however, a slower nicking rate was observed and measured. If the alkali sensitive sites are ribonucleotides, this slower rate corresponds to about 10 ribonucleotides per HeLa mitDNA molecule, using the hydrolysis rate of RNA under the same conditions as a standard. As will be demonstrated later, limit alkali digests of HeLa mitDNA have number average molecular weights corresponding to about 11 alkali-sensitive sites per molecule, in good agreement with the above results. This agreement does not prove that the alkali sensitive sites are ribonucleotides, however, since the cleavage rates for other types of alkali labile sites have not been measured under these conditions.

The complete nicking of covalently closed HeLa mitDNA by chick embryo RNase H under conditions which do not nick other covalently closed DNA's in the same incubations is strong evidence for the existence of at least one ribonucleotide in all HeLa mitDNA molecules (Grossman, Watson, and Vinograd, 1973). RNase A, T1, and E. coli H all nick only a portion of the mitDNA even at saturating enzyme levels, suggesting

heterogeneity in the distribution of RNA sites among mitDNA molecules (Miyaki, Koide, and Ono, 1973; Wong-Staal, Mendelsohn, and Goulian, 1973). Chick embryo RNase H may nick at a single ribonucleotide whereas the others may require longer sequences (Grossman, Watson, and Vinograd, 1973). In any case, these studies imply that all HeLa mitDNA molecules contain at least one ribonucleotide, and that some molecules may contain ribonucleotide sequences. The number of chick embryo RNase H sites per HeLa mitDNA molecule has not yet been determined, so it is not known whether the number of RNase H sites corresponds to the number of alkali labile sites.

In order both to demonstrate the existence of ribonucleotides in mitDNA and to determine the base composition of these ribonucleotides, development of a method for the detection of ribonucleotides in DNA was begun. The strategy adopted involves specific fragmentation of the mitDNA to expose ribonucleotide ends, labeling, digestion, and identification of the labeled products. In vitro labeling must be performed because the specific activities resulting from in vivo labeling are not adequate for the detection of such a low level of ribonucleotides (at most 0.2% of the total nucleotides, according to the alkaline nicking studies). Ribonucleotide ends are exposed for labeling by alkali rather than RNase H digestion, since it is not known which bond is broken in the latter case. Alkali digestion also removes all but the 5'-terminal ribonucleotide in any sequence of ribonucleotides, so that the base composition determined in this way includes only the 5'-terminal ribonucleotides in any sequences. Next, the (2',3')-terminal

phosphates are removed by alkaline phosphatase treatment. In vitro labeling is performed by the $NaIO_4$-^{3}H-$NaBH_4$ oxidation-reduction method (RajBhandary, 1968; Randerath and Randerath, 1971) rather than by treatment with terminal transferase, since this enzyme would add one or two ^{32}P-ribonucleotides to deoxynucleotide ends as well as one to ribonucleotide ends (Roychoudhury and Kössel, 1971).

The fragments are then digested with spleen acid DNase II and spleen exonuclease, releasing ribonucleotide termini as nucleoside trialcohols and the remainder of the DNA as deoxynucleoside-3'-monophosphates (which can be removed on small DEAE cellulose columns (Bernardi et al., 1968)). The ^{3}H-ribonucleoside trialcohols are identified by thin layer chromatography on cellulose plates (Randerath and Randerath, 1971).

The feasibility of and optimum conditions for all portions of this procedure except the labeling step were first determined with readily available DNA's. The labeling step was not tested because (1) ^{3}H-$NaBH_4$ of high specific activity is packaged only in large lots and is unstable in solution (it should be used immediately upon dissolving), and (2) appropriate conditions for ^{3}H-$NaBH_4$ of lower specific activity have been established in numerous previous studies (RajBhandary, 1968; De Wachter and Fiers, 1967; Lewandowski, Content, and Leppla, 1971; Lewandowski and Millward, 1971; Randerath and Randerath, 1971; Stephenson, Scott, and Zamecnik, 1973). When the procedure was applied to mitDNA it became apparent that the activity of commercially available ^{3}H-$NaBH_4$ of high specific activity is unreliable. The method is described here and would be applicable when ^{3}H-$NaBH_4$ of the high

specific activity required for this experiment becomes available.

Materials and Methods

Unlabeled HeLa mitDNA was used in this study because ^{3}H- and ^{14}C- labeled deoxynucleotide precursors could confuse the fluorographic analysis and ^{32}P decays rapidly with concomitant nicking of the DNA. Covalently closed HeLa mitDNA was prepared as described in Kasamatsu, Robberson, and Vinograd (1971), omitting the DNase-RNase treatment of isolated mitochondria and lysing in 1% SDS, 0.5 M NaCl, 0.01 M Tris, 0.01 M EDTA, pH 8.0. After EB-CsCl banding, preparative velocity sedimentation, and another EB-CsCl banding (as described in the above reference), EB was removed from the lower band mitDNA on a Dowex 50 column (0.9 x 6 cm, Dowex AG50W-X8, 100-200 mesh, Bio-Rad, Richmond, Calif.). The DNA was then dialyzed into 0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 8.0.

Viral ØX174 DNA was a gift from Lloyd Smith and Drs. Paul Johnson and Lois Miller. Denatured ^{32}P-labeled E. coli DNA was a gift from Dr. Larry Grossman. Calf thymus DNA was purchased from Worthington (Freehold, N.J.).

Alkaline phosphatase (BAPF, "RNase free"), spleen acid DNase II (HDAC), and spleen exonuclease (SPH) were obtained from Worthington (Freehold, N.J.). Alkaline phosphatase was assayed by the method of Garen and Levinthal (1960), spleen acid DNase II by that of Bernardi (1966a), and spleen exonuclease by that of Bernardi (1966b).

DEAE cellulose manufactured by Whatman (DE52 microgranular precycled, supplied by Reeve-Angel, Clifton, N.J.) was recycled with 0.5 M HCl and 0.5 M NaOH and degassed in 0.05 M acetic acid

before use. Thin layer cellulose plates were obtained from Eastman (#6064, 20 x 20 cm; Rochester, N.Y.) and were prerun in the second dimension with solvent B (0.1:1:2:2 formic acid, sp. gr. 1.2: water : methyl ethyl ketone : t-amyl alcohol). Ribo- and deoxynucleosides were tested for purity by one-dimensional thin layer chromatography of 3 nmole of each on cellulose plates with development with solvent A (2:3:3 7.5 $\underline{N}$ NH_4OH : isopropanol : n-butanol). Ribonucleoside trialcohol markers were prepared as described in Randerath and Randerath (1971). Deoxynucleoside-3'-monophosphate markers were purchased from P-L Biochemicals (Milwaukee, Wis.). ^3H-$NaBH_4$ was obtained from Amersham/Searle (Arlington Heights, Ill.) and ICN (Cleveland, Ohio). RPR/54 Medical X-Ray film was purchased from Kodak (Rochester, N.Y.) and is the equivalent of RB-54 Royal Blue Medical X-Ray film.

Electron microscopy was performed by the formamide Kleinschmidt technique described in Chapter 2 (Materials and Methods, 5). Molecules were measured by projection and tracing with a Hewlett-Packard digitizing tablet.

Results

1. Alkali digestion of mitDNA and strand separation

The conditions used for the alkaline CsCl banding of HeLa mitDNA were modified in order to hydrolyze and isolate the H and L strands. The mitDNA was exposed to pH 12.82 at 20°C in the standard alkaline buoyant CsCl solution (Chapter 2, Materials and Methods, 1d) for 60 hours, or about 100 times the half time for the first nick in covalently closed HeLa mitDNA (Chapter 2, Appendix). The result should be a limit alkaline digest of mitDNA. The pooled fractions (Figure 1) were examined by electron microscopy in order to determine a number-average molecular weight. The length distributions (Figures 2 and 3) show a heterogeneous population of molecules of all lengths up to 1.0G, including a few single stranded circles (which were used as length standards). The H strand (number average length = 0.16G equivalent to 0.9×10^6 daltons) is clearly more degraded than the L strand (0.21G or 1.1×10^6 daltons). These values correspond to an average of 6 alkali labile sites on the H and 5 on the L strand.

2. Alkaline phosphatase treatment of mitDNA digests

Alkaline hydrolysis between pH 12 and 13 does not result in 2',3' cyclic phosphates (Bock, 1967), so that no treatment to open this ring is necessary. In order to ensure the removal of the (2',3')-phosphates, alkaline phosphatase treatment was performed with approximately a 300-fold excess of enzyme. Fifty μg of mitDNA strands were treated with 1 unit of enzyme in 200 mM Tris, 20 mM $MgCl_2$, 10 mM EDTA, pH 8.0 for 30 minutes at 25°C in the dark. The reaction was terminated

Figure 1: Separation of H and L Strands of HeLa mitDNA in Alkaline Buoyant CsCl

7.5 ml. of a solution containing 260 μg covalently closed HeLa mitDNA, $\rho = 1.73$ CsCl, 0.05 M KH_2PO_4, 0.05 M glycine, 0.005 M EDTA, pH 12.82, in a polyallomer tube was centrifuged in a Ti50 rotor for 63 hours at 40,000 revs/min at 20°C. The gradient was fractionated dropwise into 14 drop (130 μl) fractions. Each fraction was diluted with 60 μl of 0.3 M Tris, pH 8.0, and the optical density at 260 nm was read in a 0.1 cm pathlength cell. The optical density (not corrected for the short path length) is plotted versus fraction number, with the field to the left. The fractions indicated by arrows were pooled.

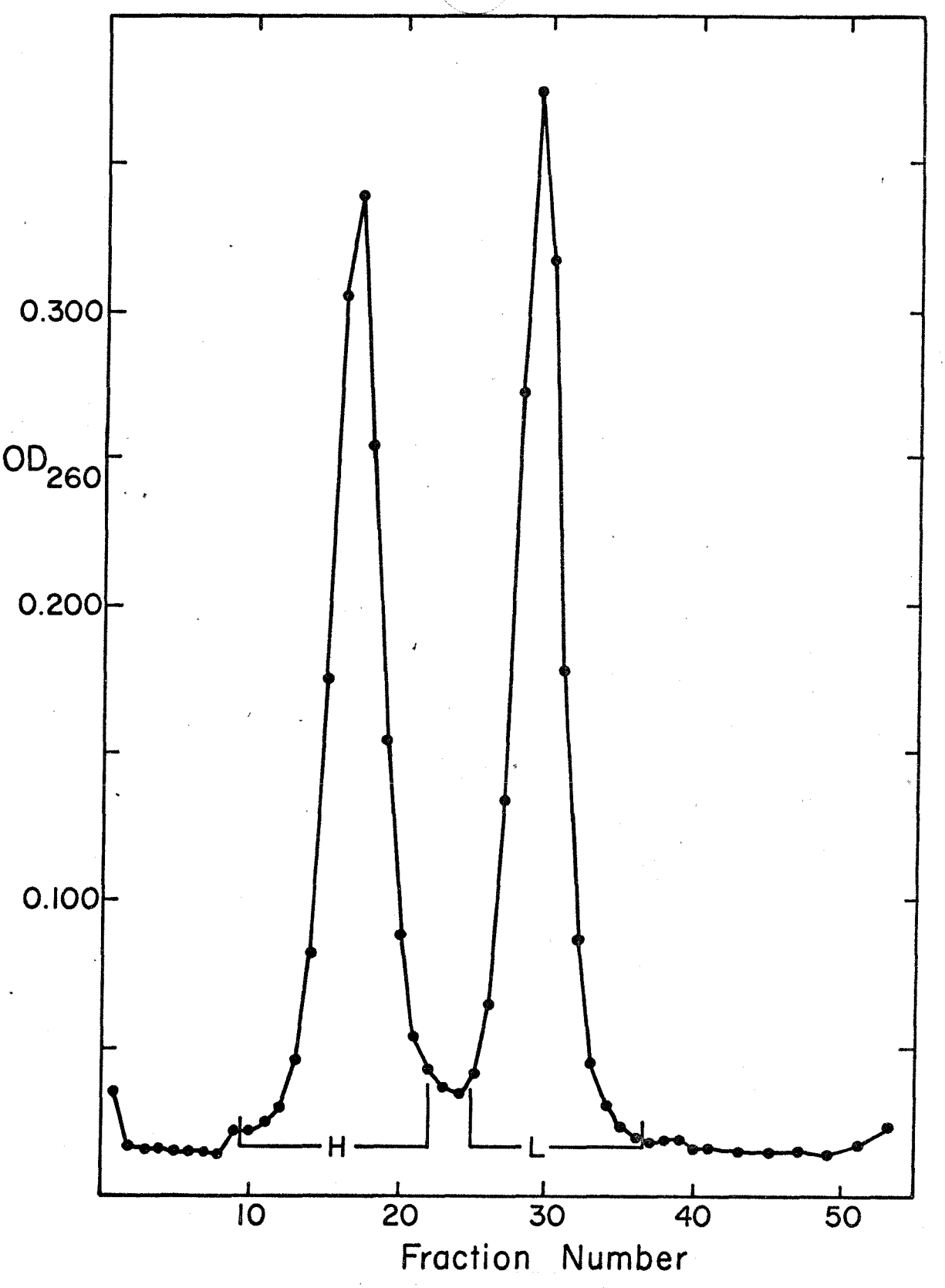


Figure 1

Figure 2: Number Distribution of Lengths of HeLa mitDNA Fragments

The H and L strand mitDNA prepared in Figure 1 were mounted for electron microscopy by the formamide Kleinschmidt technique described in Chapter 2, Materials and Methods (5). A series of photographs covering one grid hole was taken in each case, and the molecules were measured on a Hewlett-Packard digitizing tablet. The number of molecules in each size class is plotted for (a) the H and (b) the L strands, using the lengths of circular strands from the same grid hole as the standard. The number average length is indicated by an arrow in each panel.

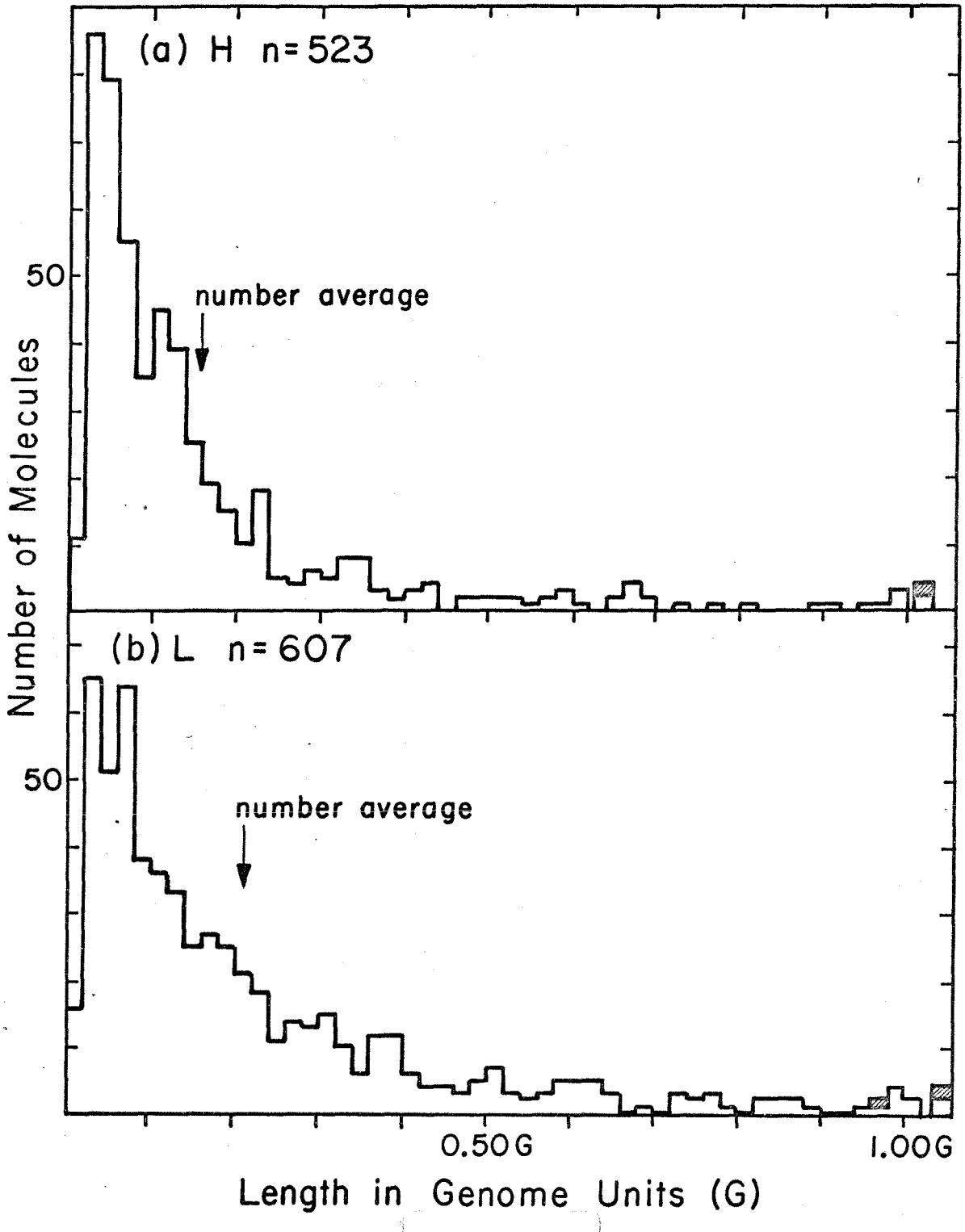

(a) H n=523
number average
50
(b) L n=607
number average
50
Number of Molecules
0.50G
1.00G
Length in Genome Units (G)

Figure 2

Figure 3: Mass Distribution of Lengths of HeLa mitDNA Strands

The distributions shown in Figure 2 were converted to mass distributions, which are included here both for comparison with the earlier mass distributions (Chapter 2, Figures 6 and 9) and for prediction of alkaline sedimentation patterns. The weight averages are indicated by arrows.

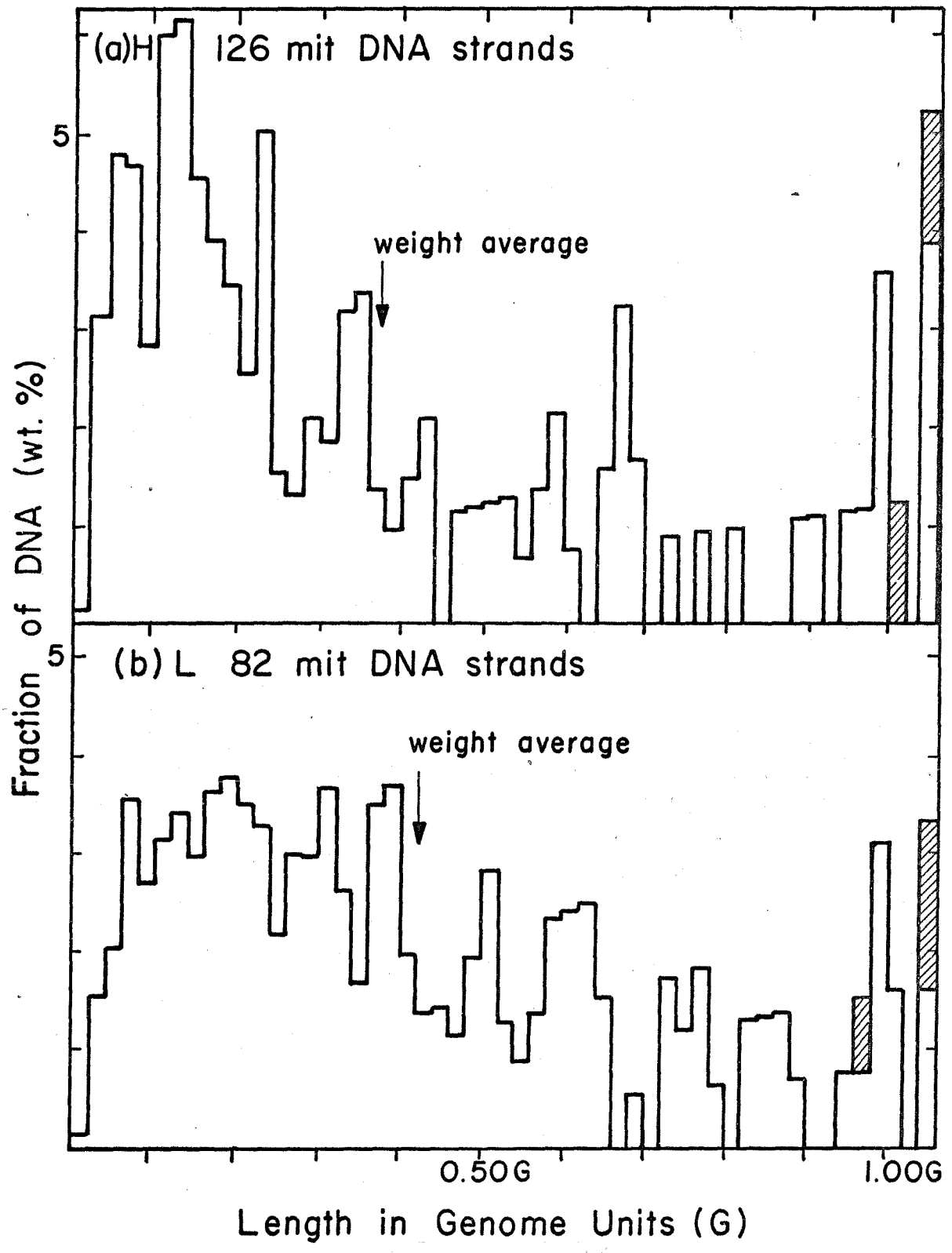

(a)H 126 mit DNA strands
5
weight average
(b) L 82 mit DNA strands
5
weight average
Fraction of DNA (wt. %)
0.50G
1.00G
Length in Genome Units (G)

Figure 3

(and the protein removed) by emulsification with 5:1 chloroform:isoamyl alcohol, followed by dialysis into 0.2 M acetate, pH 5.5.

3. Oxidation and reduction of HeLa mitDNA

The number average molecular weights determined by electron microscopy indicate that 50 μg of either strand corresponds to at most 50 pmoles of ribonucleotides. The anticipated concentration of ribonucleotide ends in reaction mixtures containing this amount of mitDNA fragments is therefore quite low, about 0.1 μM. The concentrations of $NaIO_4$ and ^3H-$NaBH_4$ were therefore elevated somewhat from those reported previously, and the reaction volumes were as small as possible (100-400 μl) without risking large losses of mitDNA in handling. Oxidations were performed in 50 mM $NaIO_4$, 0.2 M acetate, pH 5.5 at 25°C for 2 hours in the dark. (The dialdehyde formed is sensitive to photoreduction as well as amine catalyzed elimination (Rammler, 1971)). The samples were then dialyzed into 0.5 M phosphate, pH 7.9, and reduced with 50-100 mCi of ^3H-$NaBH_4$ (10-40 Ci/mMole, freshly dissolved in 1 M NaOH) for 2 hours at 25°C in the dark in a hood. The reduction was terminated by adding glacial acetic acid to 0.2-1.0 M, resulting in a rapid evolution of $^3H_2$ gas if ^3H-$NaBH_4$ were present at this point. The evolution of gas was frequently not observed, apparently because the preparations of ^3H-$NaBH_4$ were inactive. After 20 minutes, the samples were exhaustively dialyzed against 1.0 M ammonium acetate, pH 5.5, at 2°C. When the amount of radioactivity released into the dialysate had fallen to a low level, the samples were dialyzed against 0.05 M ammonium acetate pH 5.5. The removal of dialyzable

^{3}H counts could be accelerated by alternately dialyzing against these two buffers.

4. Analysis of oxidized-reduced DNA

a. Digestion to nucleoside trialcohols and 3'-monophosphates

The conditions for the complete digestion of 50 μg of single stranded DNA to mononucleotides with spleen acid DNase II and spleen exonuclease were first determined in a series of experiments with denatured calf thymus DNA, under the same conditions to be used for mitDNA strands. As shown in Figure 4, 0.07 units of spleen acid DNase II and 0.4 units of spleen exonuclease will digest 50 μg of single stranded DNA in 0.4 ml of 0.05 M ammonium acetate, pH 5.5, in 60 minutes at 37°C. These amounts of enzyme were approximately doubled in the final conditions used (described below) to ensure complete digestion to mononucleotides.

Although (as will be shown later) the thin layer chromatography separates deoxynucleosides from ribonucleoside trialcohols, the phosphomonoesterase activity of these enzyme preparations was measured in order to make certain that the amount of deoxynucleosides generated by this contaminating activity is not large enough to interfere with the chromatography of the ribonucleoside trialcohols. ^{32}P-labeled denatured E. coli DNA was mixed with denatured calf thymus DNA and digested under the conditions to be used for mitDNA. Electrophoresis of aliquots withdrawn at different points during the digestion shows that 0.7% of the deoxynucleotides are phosphatased during the spleen acid DNase digestion, while 6% and 11% are degraded during spleen exonu-

Figure 4: Digestion of Single Stranded DNA by Spleen Acid DNase II and Spleen Exonuclease

(a) Spleen acid DNase II digestion

A 1.2 ml. sample of 125 μg/ml denatured calf thymus DNA (Worthington, denatured by heating to 100°C and quenching in ice) in 0.05 M ammonium acetate, pH 5.5, was incubated with 0.21 units of spleen acid DNase II (0.07 units per 50 μg DNA) at 37°C. At intervals, aliquots of 200 μl were withdrawn, mixed with 200 μl distilled water and 400 μl of 12% $HClO_4$, and chilled on ice for 10 minutes. After centrifugation at 5,000 revs/min for 5 minutes, the optical densities of the supernatants were read at 260 nm.

(b) Spleen exonuclease

A 4.8 ml. sample of the above denatured calf thymus DNA was digested with 0.84 units of spleen acid DNase II (0.07 units/ 50 μg DNA) for 60 minutes at 37°C. Digestion was confirmed by the same assay used above. Spleen exonuclease (4.4 units, or 0.4 units per 50 μg DNA) was added, and incubation at 37°C was continued. At intervals, aliquots of 0.6 ml were withdrawn, mixed with 0.6 ml distilled water and 1.2 ml 2.5% $HClO_4$, 0.25% uranyl acetate, and chilled on ice for 10 minutes. After centrifugation at 10,000 revs/min for 10 minutes, the optical densities of the supernatants were read at 260 nm. The dashed line indicates the theoretical maximum optical density at complete digestion.

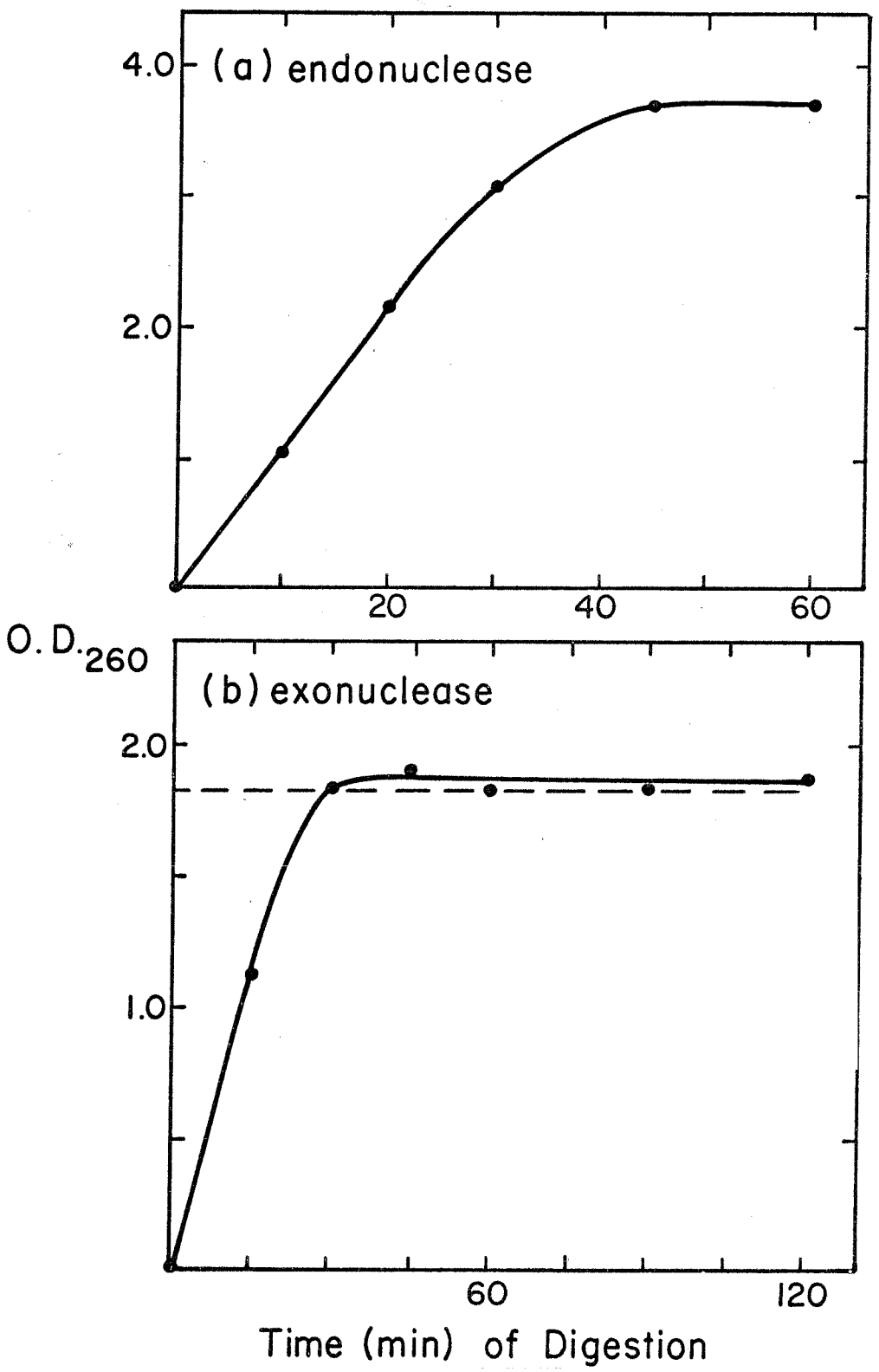

4.0
(a) endonuclease
2.0
20
40
60
O.D.260
(b) exonuclease
2.0
1.0
60
120
Time (min) of Digestion

Figure 4

clease digestion for 60 and 120 minutes respectively (Figure 5). The exonuclease preparation therefore contains a small amount of phosphomonoesterase activity. This contaminant could be removed by phosphocellulose chromatography (Richardson and Kornberg, 1964) if desired.

The final conditions used for mitDNA digestion involved incubation of 50 μg of mitDNA in 400 μl of 0.05 M ammonium acetate, pH 5.5 with 0.1 units of spleen acid DNase II for 60 minutes at 37°C, followed by addition of 0.8 units of spleen exonuclease and incubation at 37°C for an additional 60 minutes. Ten nmole of each ribonucleoside trialcohol was then added (both as a carrier and as a marker) and the digestion was terminated by applying the sample to a DEAE-cellulose column. These conditions should ensure complete digestion to deoxynucleoside-3'-monophosphates and ribonucleoside trialcohols, with dephosphorylation of about 6% of the former.

b. Removel of deoxynucleoside-3'-monophosphates and enzymes

Tests with nonradioactive ribonucleoside trialcohols and deoxynucleoside-3'-monophosphates confirmed that the former elute from DEAE-cellulose in 0.05 M ammonium acetate, pH 5.5, whereas the latter are firmly bound (and elute in 1.0 M ammonium acetate pH 5.5). Furthermore, both enzymes should be firmly bound to DEAE cellulose at this pH and low ionic strength. DEAE-cellulose also completely destroys spleen exonuclease activity (Razzell and Khorana, 1961).

MitDNA digests were applied to 0.9 x 5 cm DEAE-cellulose columns which had been extensively washed with 0.05 M ammonium acetate, pH 5.5. The columns were washed with 5 ml of the same

Figure 5: Assay for Phosphatase Activity in Spleen Acid DNase II and Spleen Exonuclease

A small amount of ^{32}P-labeled denatured E. coli DNA was mixed with 1.6 ml of 125 μg/ml denatured calf thymus DNA in 0.05 M ammonium acetate, pH 5.5. A 0.4 ml aliquot was chilled on ice (sample (a)) and the remainder was incubated with 0.21 units of spleen acid DNase II (0.07 units per 50 μg DNA) for 60 minutes at 37°C. Another 0.4 ml aliquot was withdrawn (sample (b)), and the digestion was terminated in this aliquot by emulsifying with 5:1 chloroform:isoamyl alcohol and chilling on ice. The remainder was incubated at 37°C with 1.6 units of spleen exonuclease (0.8 units per 50 μg DNA), and 0.4 ml aliquots were withdrawn at 60 (sample (c)), and 120 (sample (d)), and treated as above. The above samples were dried under vacuum, dissolved in 20 μl distilled water and applied to 7.5 x 60 cm Whatman 3MM paper strips. Electrophoresis in 5% acetic acid, 0.5% pyridine, 2 mM EDTA, pH 3.5 was performed for 125 minutes at 2,000 volts. After drying in streaming steam, the strips were cut into 1 cm strips and counted in toluene-PPO-PPOP scintillation fluid. $^{32}P_i$ appears in each panel as the small peak near fraction 40.

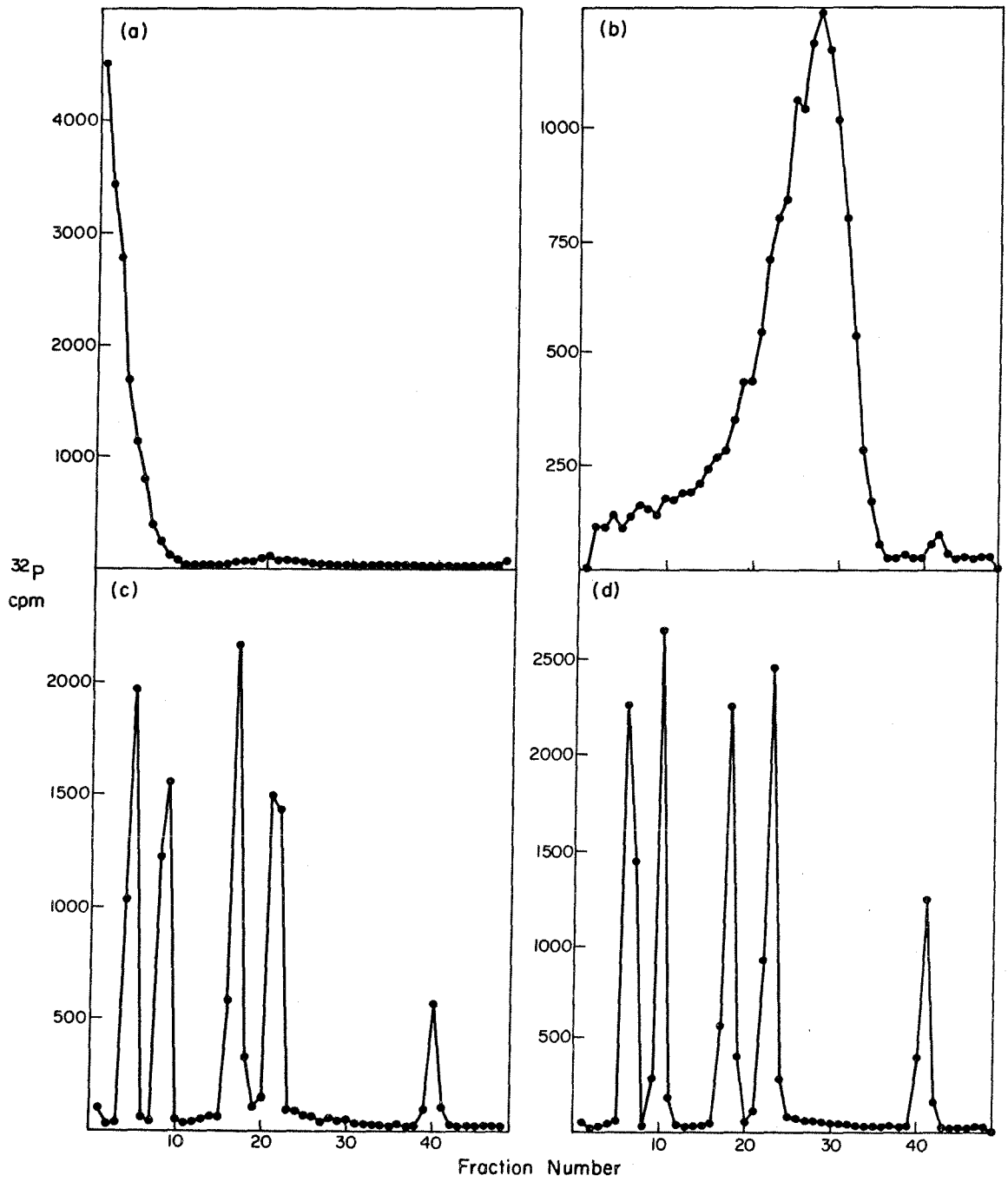

(a)
(b)
(c)
(d)
4000
3000
2000
1000
1000
750
500
250
^{32}P
cpm
2000
1500
1000
500
2500
2000
1500
1000
500
10
20
30
40
Fraction Number

Figure 5

buffer, followed by 5 ml of 1.0 M ammonium acetate, pH 5.5. Fractions containing ribonucleoside trialcohols were identified by optical density at 260 nm, pooled, and repeatedly dried under vacuum at 35°C in siliconized tubes.

c. Thin layer chromatography and fluorography

The positions of all four ribonucleoside trialcohols in the two dimensional thin layer chromatographic system of Randerath and Randerath (1971) were determined. The plates were developed with solvent A (2:3:3 7.5 N NH_4OH : isopropanol : n-butanol) in the first dimension and solvent B (0.1:1:2:2 formic acid, sp. gr. 1.2 : water : methyl ethyl ketone : t-amyl alcohol) in the second. Even though no reaction of deoxynucleosides with $NaIO_4$ and $NaBH_4$ should occur due to the absence of a cis diol, all four deoxyribonucleosides were treated with these reagents as above and chromatographed with the ribonucleoside trialcohols on thin layer plates (Figure 6). The treated deoxynucleosides separate cleanly from the trialcohols in this system. All four deoxynucleosides were also visible in the same positions in thin layer chromatograms of DNA digests due to the phosphomonoesterase activity in the spleen enzymes.

The tendency of cellulose thin layer chromatograms to streak if salt is present in the sample made the rigorous exclusion of non-volatile salts from the DNA digests imperative. It was also important to remove all traces of ammonium acetate from the sample by repeated drying under vacuum from small volumes of glass distilled water. Test samples of ØX174 viral DNA carried through the entire analysis procedure gave normal patterns of the ribonucleoside trialcohol

Figure 6: Thin Layer Chromatography of Ribonucleoside Trialcohols and Deoxynucleosides

A mixture containing 10 nmole of each ribonucleoside trialcohol and each deoxynucleoside was chromatographed on a cellulose thin layer plate as described in Results (4c). The directions of development with the two solvents are indicated. Separate runs allowed the identification of the four deoxynucleoside spots (shaded) and the indicated assignments of the ribonucleoside trialcohol spots.

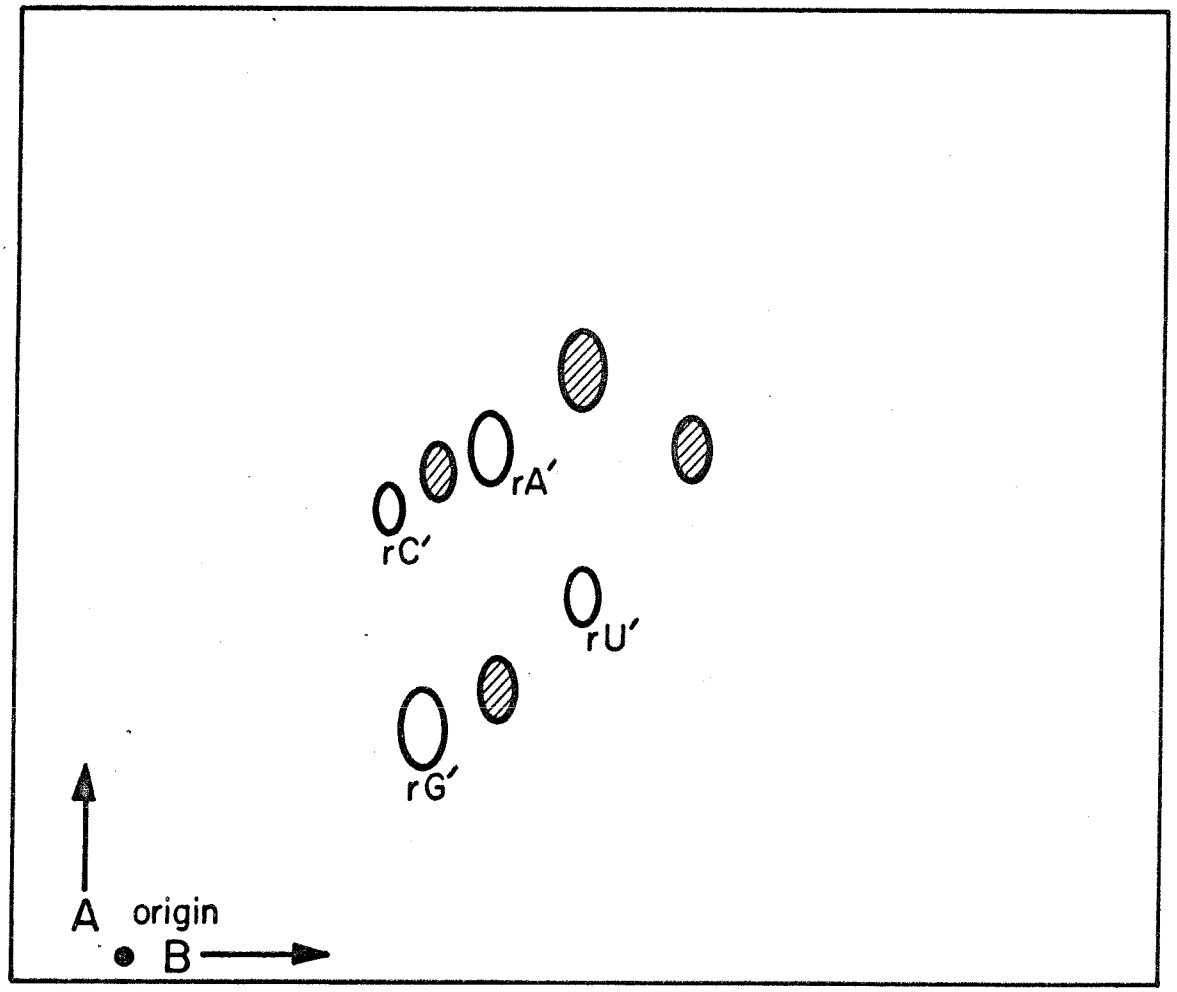


Figure 6

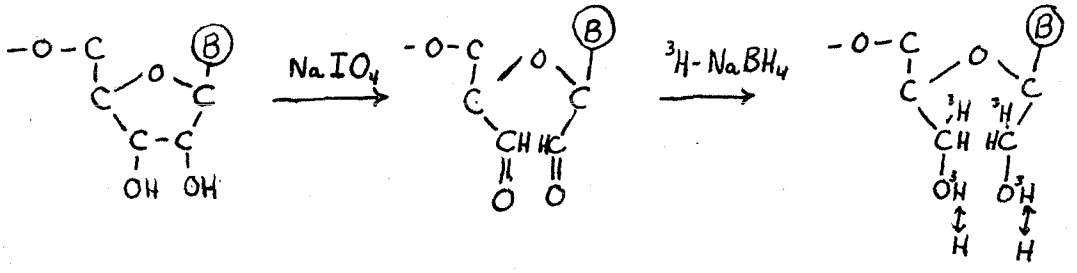
markers added after the digestion.

The mitDNA digests were dissolved in 50-100 μl of glass distilled water, applied to cellulose plates, and chromatographed as above. After location of UV-absorbing spots, 15 ml of a 7% solution of PPO in ether was quickly spread over each plate and dried (Randerath, 1970). ^{32}P ink marker spots were applied (to assist in aligning the plate and film after development of the film). Each plate was then squeezed against a sheet of RPR/54 Medical X-Ray Film (Kodak, Rochester, N.Y.) at -80°C for 24 hours between two glass plates. The film was then developed. Two types of results were obtained: either the film was heavily exposed in an irregular pattern, or the radioactivity was confined to a small region near the origin without any exposure in the regions corresponding to ribonucleoside trialcohols. The former was undoubtedly due to insufficient dialysis to remove unincorporated ^{3}H counts from the sample. The latter could reflect either the failure to label ribonucleosides (due to an inactive preparation of ^{3}H-NaBH) or the absence of detectable amounts of ribonucleotides in the mitDNA.

Discussion

1. Size of limit alkaline digests of H and L strand HeLa mitDNA

The number average molecular weight of the fragments produced by alkaline digestion of mitDNA was measured by electron microscopy mainly as an aid in estimating the specific activity of ^{3}H-$NaBH_4$ required in the labeling studies. If the alkali sensitivity of mitDNA is due to the incorporation of ribonucleotides, each alkali fragment should have a ribonucleotide at its 3' end. Fifty μg of such fragments with a number average molecular weight of 10^6 daltons would contain 50 pmoles of ribonucleotides. Labeling these ribonucleotides with 20 Ci/mMole ^{3}H-$NaBH_4$ should produce at most 500 nCi of labeled ribonucleoside trialcohols (half of the label is introduced into exchangeable positions):



As little as 5 nCi can be visualized by fluorography (Randerath, 1970), so that this amount of label should be adequate even if it is distributed among four spots.

The length distributions of these alkaline digests are also useful in considering the distribution of alkali sensitive sites in the molecule and the average number of ribonucleotides in each. If all mitDNA molecules have the same 11 alkali labile sites, a limit digest should

have showed specific fragments. Furthermore, no large (> 0.5 G) fragments would be expected unless the sites are clustered. In these limit digests (about 100 times the halftime for the first alkaline nick) a heterogeneous population of fragments was observed, many of which were quite large. This result suggests that either different molecules have different numbers of sites (with an average of 11 per molecule) or that every molecule has 11 sites which are heterogeneously located.

The number of alkali sensitive sites when combined with the estimates of 10-30 ribonucleotides per molecule obtained from the nicking kinetics (Wong-Staal, Mendelsohn, and Goulian, 1973; Grossman, Watson, and Vinograd, 1973) indicates that there are an average of 1-3 ribonucleotides per site. If the sites contain different numbers of ribonucleotides (as suggested by the differences in the sensitivity of HeLa mitDNA towards different RNases described in the above references), of course, some sites could contain as many as 20 ribonucleotides. Furthermore, the possibility that depurinated sites may contribute to these results will not be settled until the number of RNase H sensitive sites is determined by examination of a limit enzymatic digest.

2. Labeling of mitDNA fragments by $NaIO_4$ oxidation and ^3H-$NaBH_4$ reduction

The major difficulties encountered in this study were the instability and unreliability of ^3H-$NaBH_4$ of high specific activity. Although preparations of lower specific activity may be stored in 1 M NaOH at -80°C (Randerath and Randerath, 1971), the high specific activity preparations should be used immediately after opening a vial. At the time this study

was performed, no rapid assay for the activity of such a small amount of $NaBH_4$ was available, so that experiments had to be performed assuming that the material was active. In view of the fact that many of the preparations of ^3H-$NaBH_4$ did not evolve gas on acidification at the end of the incubation, it appears that the activity of the material presently available is unreliable, possibly due to radiolysis.

A good assay for the activity of ^3H-$NaBH_4$ might be developed by measuring the reduction of an aldehyde (such as acetaldehyde) on a gas chromatograph. Provision would have to be made for disposal of the 10-50 mCi of radioactive product which would be required if a radioactivity detector were not available. A simpler assay reported by Randerath (personal communication) measuring the alkali soluble counts is unreliable because the radiolysis products may not be volatile, unlike the hydrolysis product.

The conditions chosen for the oxidation and reduction of mitDNA fragments were based on those used successfully by previous investigators. The concentrations of both $NaIO_4$ and ^3H-$NaBH_4$ were raised to compensate for the low concentration of termini, and the reactions were performed in as small a volume as possible. The pH's required for oxidation (5.0-6.0) and reduction (7.5-8.0) are critical. In the former case, too low a pH will result in extensive depurination of the DNA. In the latter, reduction occurs too slowly at a higher pH but the ^3H-$NaBH_4$ hydrolyzes too rapidly at a lower pH. The conditions necessary for alkaline phosphatase treatment, oxidation, and reduction could be refined by preparation of ribosubstituted DNA with either E. coli DNA

polymerase I (van de Sande, Loewen, and Khorana, 1972; Salser et al., 1973) or RNA polymerase (Wickner et al., 1972; Hurwitz, Yarbrough, and Wickner, 1972) to use as a test material for these reactions.

3. Analysis of ^{3}H-labeled mitDNA

The procedure which has been developed for the analysis for ribonucleoside trialcohol termini involves digestion of the DNA, isolation of nucleosides, and identification on thin layer chromatography. Digestion with spleen acid DNase II and spleen exonuclease has two distinct advantages: (1) the 3' termini are released as nucleoside derivatives, which are easily separated from the deoxynucleoside-3'-monophosphates resulting from the digestion of the rest of the fragment, and (2) both enzymes have the same pH optimum (pH 5.5) without any requirement for nonvolatile ions (which would streak the thin layer chromatograms). The minimum amount of these enzymes required to completely digest a given amount of single stranded DNA was determined, in order to minimize contaminating phosphatase activities. Quantitative recovery of ribonucleoside trialcohols and removal of deoxynucleotides on DEAE-cellulose was confirmed. The thin layer chromatographic system was shown to separate deoxynucleosides from ribonucleoside trialcohols, so that even if the deoxynucleosides are labeled at a low level by ^{3}H-$NaBH_4$ the label will not appear in the ribonucleoside trialcohol positions. Phosphomonoesterase activity can therefore be tolerated in the enzymes used for digestion as long as the amount of deoxynucleosides generated is not large enough to

affect the mobilities of the ribonucleoside trialcohols. In addition, deoxynucleoside-3'-monophosphates do not chromatograph in this system, so that contamination by small amounts of these species is also allowable.

In summary, a method for the analysis of $NaIO_4$-^{3}H-$NaBH_4$ labeled mitDNA fragments for the presence of ribonucleoside trialcohol termini has been developed and tested. When active ^{3}H-$NaBH_4$ preparations of high specific activity become available, this procedure will permit the detection of low levels of ribonucleotides in mitDNA and the determination of the base composition of these ribonucleotides.

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