I. OCCURRENCE AND STRUCTURE OF COMPLEX MITOCHONDRIAL DNA IN HUMAN LEUKEMIC LEUKOCYTES AND NORMAL MAMMALIAN TISSUES

II. USE OF ALKALI METAL SALTS OF TRICHLOROACETIC ACID AS BUOYANT DENATURING SOLVENTS FOR DNA

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ABSTRACT

This thesis is divided into two parts. Part I is concerned with the occurrence and structure of complex forms of mitochondrial DNA isolated from human leukemic leukocytes and normal mammalian tissues. Complex mitochondrial DNA (M DNA) occurs in two forms. The covalently closed double-sized circle (10 μ) is called the circular dimer. Oligomers composed of interlocked 5 μ monomer submolecules are called catenanes. The catenane form is ubiquitous in nature. It has been observed in M DNA preparations from every mammalian source examined to date. These sources include various organs from rabbits, guinea pigs, and mice. The frequency of catenated dimers in normal and leukemic tissues varies from 5 to 11 percent, and the frequency of catenated higher oligomers from 0.1 to 8.0 percent.

In contrast, the circular dimer has not been observed in normal tissues. M DNA's from the peripheral blood of 14 patients with myelogenous leukemia contained a circular dimer form. No such structure could be found in M DNA's from three patients with nonmalignant proliferations of myeloid cells. The frequency of the circular dimer form is reduced upon treatment with antileukemic drugs. This result suggests that a significant relation exists between the formation and presence of the circular dimer M DNA form and myelogenous leukemia in man. Additional data presented in this section demonstrate that a sixteen-hour labeling of leukocyte M DNA results in a uniform labeling pattern of the circular dimer and monomer form.

DNA hybrids between the circular dimer and monomer were analyzed by electron microscopy and analytical centrifugation. The results indicate that the circular dimer and monomer are at least 90 percent homologous and that heterologous regions, insertions, or deletions exceeding 50 to 100 nucleotides in length do not occur. The electron microscope studies also show that monomer genomes in the dimer are connected in a head-to-tail structure, rather than in a head-to-head structure. In addition, the results show that leukemic leukocyte M DNA is substantially homogeneous in base sequence.

Part II contains a preliminary study of a denaturing buoyant system for DNA. It is shown that rubidium trichloroacetate is a potentially useful buoyant solvent for DNA. It is likely that most DNA's can be banded at neutral pH and room temperature in both the native and denatured states without introducing single-strand scissions. It is also concluded that Li, Na, and K trichloroacetate are potentially useful denaturing solvents for sedimentation velocity studies of DNA.

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Part I

OCCURRENCE AND STRUCTURE OF COMPLEX MITOCHONDRIAL DNA IN HUMAN LEUKEMIC LEUKOCYTES AND NORMAL MAMMALIAN TISSUES

A. General Introduction

The existence of a distinct DNA component in certain cytoplasmic organelles is now well established. In particular, the DNA of the mitochondrion has received extensive attention in the last three years and is the subject of a number of review articles (1, 2, 3). Mitochondrial DNA (M DNA) from plants and unicellular organisms has been isolated as a linear species. The size of these M DNA's varies from approximately 7 μ in the bean (Phaseolus vulgaris) (3) to 35 μ in the voodoo lily (Sauromatum venosum) (4). In such M DNA populations a broad distribution of lengths has been reported. The greatest confusion lies in the yeast M DNA system. Yeast M DNA has been reported to be linear (5), circular (6), and a combination of both forms (7). The latest work on this subject contends that yeast M DNA is a 25 μ circular species easily subject to degradation in isolation (8). In contrast, M DNA from a variety of organisms in the animal kingdom has been shown to be in the form of an approximately 5 μ covalently closed circle (1). This consistency has allowed direct application of many of the experimental techniques used in isolation and characterization of closed circular viral DNA's. As a result, the following general properties of animal M DNA have been reported. (a) Animal M DNA can be isolated as a closed circular duplex DNA homogeneous in base composition. (b) The buoyant density of M DNA from different organisms varies from 1.693 (mouse L cells, 9) to 1.703 (chick, 10), as related to crab dAT at 1.670 g/ml (11). The buoyant density of the M DNA from related organisms is similar and no variation in buoyant density of M DNA from different organs of the same animal has been reported. (c) There have been no

reports of unusual bases in M DNA. Mouse 3T3 M DNA in known to be immune to treatment with RNase (12). (d) There is no obvious relationship between the buoyant densities of nuclear and M DNA from the same organism. The M DNA may be lighter (9) or heavier (this thesis) than the nuclear DNA. (e) Rat and human M DNA form a bimodal distribution when centrifuged to equilibrium in buoyant alkaline cesium chloride (13, 14). Each band contains one of the two complementary M DNA strands and the buoyant density difference is thought to be principally the result of a guanine plus thymine bias between the strands. This finding has allowed investigators to utilize the separate strands in hybridization experiments involving transcription (13) and homology among M DNA forms (this thesis).

The genetic function of M DNA has not yet been elucidated. If one accepts the data that support the homogeneity in base composition of a given M DNA population, it is obvious that M DNA cannot be coding for the components of an entire mitochondrion. Indeed, it is well known that in yeast the structural gene for cytochrome C is of nuclear origin. M DNA is synthesized and replicated within the mitochondrion and serves as an active template for RNA production (1, 2, 3). Hybridization experiments have shown that M DNA is the most likely template of: (a) certain tRNA's in rat liver mitochondria (15); (b) an exported mRNA in HeLa cell mitochondria (16); (c) ribosomal-like RNA in Tetrahymena (17) and toad (Xenopus laevis) (18). Also, isolated mitochondria are capable of incorporating amino acids into protein (1, 2, 3). These experiments demonstrate that M DNA is capable of supporting

both RNA and protein synthesis in the cell. The interrelationship of this potential protein factory and the main cytoplasmic system in the cell is still obscure, but a few experiments have implicated a functional role for M DNA. Certain mitochondrial phenotypes have been transferred in Neurospora by microinjection of the whole organelle (19). There is now a collection of respiratory-deficient cytoplasmic mutants in yeast which contain M DNA with greatly altered base compositions (20, 21, 22) and drug-resistant mutants which seem to be under cytoplasmic genetic control (23, 24). It has been reported that a membrane structural protein is altered in a cytoplasmic mutant of Neurospora (25). This raises the possibility that M DNA in animal cells may similarly be coding for a vital part of the cell structure.

I have stated that mammalian M DNA occurs in the form of an approximately 5 μ closed circular duplex. This was shown to be an incomplete result in 1967, when two new forms of circular DNA (in this case, M DNA) were discovered in this laboratory. The first form is a double-length covalently closed circle, called the circular dimer. The other species is an oligomer composed of monomeric submolecules interlocked as links in a chain, called catenanes. The principal parts of this thesis will be concerned with the discovery, occurrence, and physical properties of the circular dimer and the occurrence of the catenanes.

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B. Occurrence of Catenated Oligomer Mitochondrial DNA in Normal Tissues

INTRODUCTION

The first indication that mitochondrial DNA isolated from mammalian cells was not homogeneous in form was provided by the work of Radloff, Bauer, and Vinograd (1). They found that HeLa cells contain DNA molecules which are multiples of the basic mitochondrial length, 4.8 microns. It was not known whether these multiples represented new forms of mitochondrial DNA or were simply an artifact of preparation, such as adhesion of monomeric M DNA molecules. Since such molecules were observed at low molecular density on electron microscope specimen grids, it seemed likely that they were physically connected. Nass (2) had earlier reported measurements of multiple mitochondrial molecules liberated from mitochondria by osmotic shock during specimen preparation, but she attributed them to superposition of monomeric molecules. The first conclusive evidence for the existence of multiple-length forms was the observation of circular M DNA molecules free of crossovers and twice the mitochondrial monomer length in electron micrographs of leukemic leukocyte M DNA (3). This form, called the circular dimer, will be discussed in the next two sections.

Further work with HeLa cell mitochondrial DNA led to the discovery of the second complex form, the catenated oligomer. Dimers and higher multiples of HeLa M DNA were found to be exclusively catenanes, monomer submolecules connected as links in a chain. The catenated structure was established by electron microscope and centrifugal methods (4). At about the same time, the presence of catenated

dimers in preparations of leukemic leukocyte M DNA was demonstrated (5). These initial observations showed that catenanes were an integral part of the mitochondrial DNA population of a tissue culture cell line and one fresh malignant tissue. These findings prompted a study of a variety of normal tissues to determine whether any or all complex mitochondrial DNA forms were some function of malignancy or other general cellular state. The results of an analysis of these mitochondrial DNA forms in tissues from normal rabbits, guinea pigs, mice, and humans is given in the following publication.

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Occurrence of Complex Mitochondrial DNA in Normal Tissues

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Mitochondrial DNA isolated from several mammalian organs contains from 6 to 9 per cent catenated dimers and 0·5 to 2 per cent catenated higher oligomers. Multiple length circular molecules, such as occur in leukaemic leucocytes, were not detected.

THE DNA in the mitochondria of many higher organisms is now known to occur in the form of closed circular duplex molecules with contour lengths of approximately 5μ and a molecular weight of about 10 million daltons[1]. We have recently discovered that human mitochondrial DNA (M DNA) occurs in two larger circular forms which we refer to as complex forms. The catenated oligomers are molecules that consist of two or more interlocked circular duplexes connected to each other like links in a chain (Fig. 1). The circular oligomers are circular duplexes with a contour length that is a multiple of the monomer length. White blood cells from patients with chronic granulocytic leukaemia contained catenated oligomers and circular dimers[2]. HeLa cells, in contrast, contained catenanes but no circular dimers were seen[3]. This disparity among human cells prompted us to examine a number of normal tissues for the occurrence of the two types of complex mitochondrial DNA, and to develop procedures for reliably estimating the frequency of each type in an M DNA preparation.

We have found that the mitochondrial DNAs obtained from rabbit brain, kidney, liver and bone marrow, from guinea-pig brain and liver, from normal human leucocytes, and from 13 day old mouse embryos contain 2–9 per cent catenated dimers but do not contain detectable circular dimers. These results are similar to those obtained in previous studies of HeLa cell M DNA (ref. 3) and M DNA from the unfertilized sea urchin egg[4], but are in marked contrast to the previously reported observations of the presence of circular dimers at a frequency of 26 per cent in leucocytes of a patient with chronic granulocytic leukaemia[2]. All the M DNAs studied in this work con-

tained higher catenated oligomers—trimers, tetramers and so on—at a frequency that varied between 0·5 and 1·8 per cent.

Scoring the Frequency of Complex Mitochondrial DNA

Mitochondrial DNA usually represents only a minor constituent of the total DNA in the cell. A very efficient separation process for the removal of the overwhelming amount of nuclear DNA is therefore necessary before analysis for the various complex forms can be undertaken. In our earlier work we removed most of the nuclear DNA with a cell fractionation procedure which yielded partially purified mitochondria. The DNA from such mitochondrial preparations was isolated and purified in a single step with the ethidium bromide–caesium chloride density gradient method[5]. The lower band, containing only closed circular M DNA that was free of both nuclear DNA and nicked M DNA, was recovered for further study. The use of lower band DNA introduced an uncertainty in the result for the frequency of complex mitochondrial DNA molecules, because these latter molecules are larger targets than the monomers for any process which introduces single-strand scissions into DNA. We have now improved our procedure for the purification of mitochondria so that the ratio of the closed M DNA in the lower band to the nicked M DNA plus linear nuclear DNA in the upper band is reduced from approximately 10 to 0·2–1·0 as judged by a visual estimate of the fluorescence intensity of the bands (Fig. 2). Both bands and the intermediate caesium chloride solution between them were collected together to provide an unfractionated M DNA sample for electron microscope analysis. The remaining linear DNA does not interfere in the analysis. Occasional preparations in which the amount of DNA in the upper band exceeded the amount in the lower band were rejected.

To obtain quantitative estimates of the frequency of complex forms in a sample, we have examined a large population of molecules and distinguish the categories: monomer, circular dimer, catenated dimer and catenated higher oligomer. The required size of the sample was estimated with the relation[6]

$$\alpha = \pm 1{\cdot}96\sqrt{(1-f)/nf}$$

where f represents the mean obtained in a survey of n molecules and $f(1 \pm \alpha)$ represents the interval which contains the true mean at a level of confidence of 95 per cent. In the present work we have classified about 2,000 molecules in each M DNA preparation so as to estimate the frequencies of complex forms which vary, for example, from 2 to 10 per cent. The corresponding ranges are $\pm 0{\cdot}64$ and $\pm 1{\cdot}2$ per cent if 2,000 molecules are classified, and $\pm 1{\cdot}2$ and $\pm 2{\cdot}4$ per cent if 500 molecules are classified.

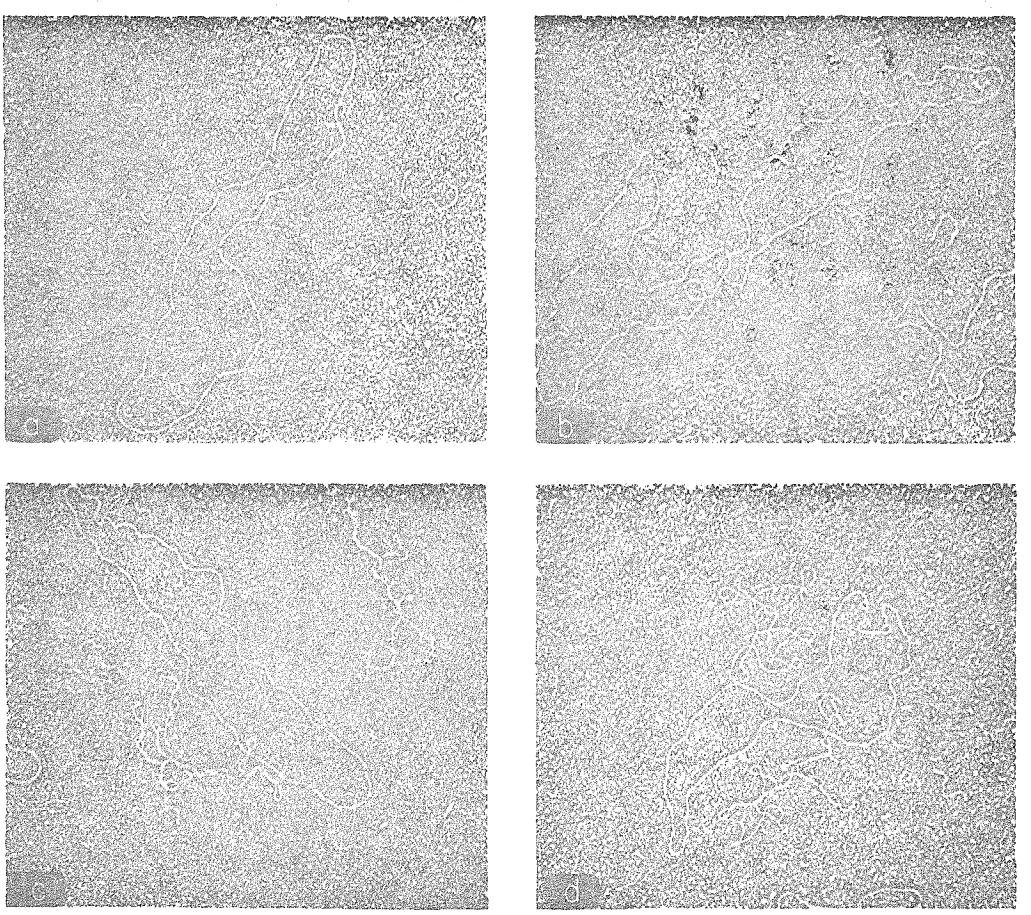

Fig. 1. Representative electron micrographs of mitochondrial DNA isolated from rabbit bone marrow. Grids were prepared as described earlier and examined in a Philips *EM*300 electron microscope[5]. *a*, Monomer length mitochondrial DNA; *b*, catenated dimer, fully relaxed; *c*, catenated dimer with one relaxed submolecule; *d*, catenated trimer with one relaxed submolecule. The contour length of the monomeric molecules and submolecules is approximately 5μ.

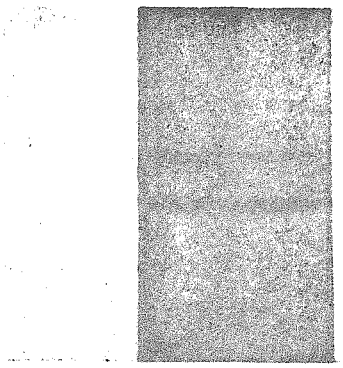

Fig. 2. A caesium chloride–ethidium bromide density gradient showing two bands of mitochondrial DNA from rabbit brain. The centrifugation and photographic conditions were described previously[4,5]. The contents of the middle one-third of the tube were collected, diluted to 5 ml., and concentrated into a pellet in an *SW* 50 rotor, 42,000 r.p.m., 10 h, 15° C. The total DNA from such preparations contained less than 5 per cent nuclear DNA as indicated by electron microscopy and analytical buoyant density centrifugation after removal of ethidium bromide.

Photographic methods are too slow to be practical for classifying such large numbers of molecules. We have therefore classified the molecules as they appear imaged on the fluorescent screen in the electron microscope. A general requirement for the scoring procedure is that circular molecules be free of extensive twisting. Highly twisted catenated dimers cannot be distinguished from twisted circular dimers even though it is possible to distinguish twisted monomers from twisted dimers. Our previously described procedure[2,3] for preparing specimen grids usually results in only a small fraction of highly twisted molecules. Specimen grids which contained more than 10 per cent of such molecules were rejected. The surface concentration of molecules must be low enough, less than about 400 per 300 mesh grid hole, for the eye readily to follow the contour of the molecule. The contrast must be adequate, for the examination of the image on the fluorescent screen and "flower patterns" should be infrequent. The linear DNA mass concentration should not exceed 50 per cent of the total DNA.

The specimen grid was examined at a magnification of 5,400 beginning at a corner grid hole. The first grid hole was scanned completely in a systematic manner and was followed by similar scans of the laterally adjacent grid hole. Scanning was continued until an adequate number of molecules had been classified or until all holes on the grid had been scanned. The results were tabulated on an eight unit blood cell counter as monomer, circular dimer, catenated dimer, catenated higher oligomer and as ambiguous molecules.

Decision making was in two stages. A decision was made between monomer, dimer and higher oligomer, and then between circular oligomer and catenated oligomer. The investigator readily learns, from the results of contour length measurements of photographed molecules with different shapes and differing extents of convolution, to distinguish monomers, dimers and higher forms. A basic problem is to ensure that overlapping monomers are not scored as dimers. We have reported earlier that molecules on a surface layer often appear to repel each other[5] and do not overlap on specimen grids unless very high DNA concentrations are used. Forms such as those illustrated in Fig. 3*a*, in which there is clearly no contact between two approximately concentric molecules, were not uncommon. If there was adventitious overlapping, the frequency of catenanes should be expected to be a steep function of the surface density of molecules. A preparation of rabbit kidney M DNA was examined on specimen grids with three different surface densities: approximately 500, 150 and 75 molecules per 300 mesh grid holes. The frequencies of total catenated oligomers in the three samples of approximately 1,000 molecules were $7{\cdot}2 \pm 1{\cdot}6$ per cent, $7{\cdot}1 \pm 1{\cdot}5$ per cent and $6{\cdot}2 \pm 2{\cdot}0$ per cent. The individual results do not differ significantly from the mean $6{\cdot}8 \pm 1{\cdot}1$ per cent, so we conclude that the extent of adventitious overlapping is small. Specimen grids with surface densities of more than 300 molecules per grid hole are difficult to score and were normally not used.

Fig. 3 (*b*, *c* and *d*) represents dimeric molecules and illustrates the problems encountered in distinguishing catenated dimers and circular dimers. Fig. 3 (b_1–b_3) represents unambiguous circular dimers because no crossover exists that could divide the dimer into two monomers. Dimers which contain a crossover (arrow) as in Fig. 3 (d_2–d_4) are regarded as catenanes and not as circular dimers, because we would have expected to have seen some unambiguous forms of circular dimers had they been present. These were not seen. Similarly, if circular dimers appeared in forms such as those illustrated in Fig. 3 (d_2–d_4) we would have expected to find examples of such kinds of molecules of monomer length. Because these are only infrequently seen, circular dimers of this form can represent only a small fraction of circular dimers present in the sample. Dimers with the pulled out form illustrated in Fig. 3 (d_2–d_4) were reported in preparations of HeLa M DNA (ref. 5) and were later shown to be catenated dimers[6]. An M DNA dimer of the type shown by Suyama and Miura[7] from monkey liver would be scored as a catenane in this laboratory.

Molecules such as in Fig. 3d_1 are scored as catenated dimers even if the detailed nature of the overlapping is not discernible. In order for a circular dimer to appear in this conformation it would have to fold as in Fig. 3b_2. We think this unlikely because tangential segments (arrow) are rarely seen in preparations known to contain circular dimers and circular monomers are never seen folded in such a manner as to appear to be a catenated dimer with a total contour length of 5μ.

Dimeric molecules in the form represented in Fig. 3*c* are classified as ambiguous because of extensive twisting. Such forms, if they do not exceed 10 per cent, are apportioned according to the frequencies obtained with unambiguous dimers. No unambiguous circular dimers were seen in this study, so ambiguous dimers were scored as catenanes.

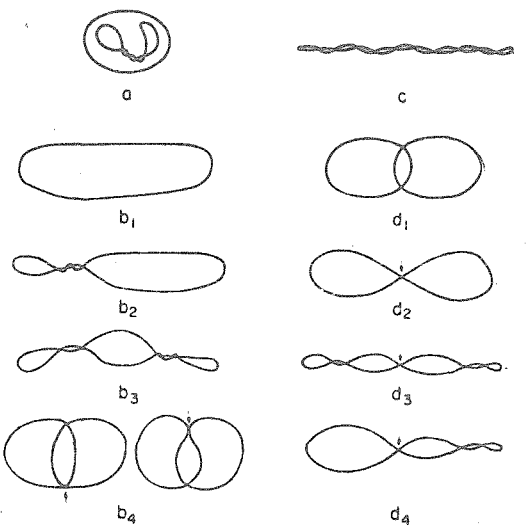

Fig. 3. Representations of circular DNA conformations. *a*, Monomers. *b*, circular dimers; *c*, ambiguous dimer; *d*, catenated dimers.

Table 1. CLASSIFICATION OF COMPLEX M DNA FORMS IN THE SAME POPULATION OF RABBIT BONE MARROW M DNA BY FOUR INVESTIGATORS

Investigator	Monomers	Circular dimers	Catenated dimers	Catenated trimers
D.A.C.	175	0	19	6
J.M.J.	175	0	18	7
C.A.S.	175	0	19	6
M.R.T.	175	0	19	6

The variation of the results between four of the authors of this study was checked in a set of measurements with a given population of rabbit brain M DNA on one grid hole (Table 1). The results demonstrate that individual investigators classify the DNA molecules in essentially the same way.

The mitochondrial DNA prepared in this study was examined in buoyant CsCl in the analytical ultracentrifuge in order to verify the purity of the DNA and to determine the buoyant densities of these DNAs. The buoyant densities are presented in Table 2. The details of the experiments are given later in this report.

Table 2. BUOYANT DENSITIES OF MITOCHONDRIAL AND NUCLEAR DNAS ISOLATED IN THIS INVESTIGATION

Source	Buoyant density*, θ (g/ml.) Mitochondrial	Nuclear
Rabbit: brain, kidney, liver, bone marrow	1·696	1·692
Guinea-pig: liver	1·695	1·694
Human leucocyte: normal and leukaemic[5]	1·700	1·690

* Referred to *E. coli* DNA, 1·704 g/ml. and calculated with the buoyant density gradient[5] in experiments with crab dAT DNA, $\theta = 1·670$, or *M. lysodeikticus* DNA, $\theta = 1·725$ g/ml.

The results of our study of the frequency of complex M DNA in normal tissues are given in Table 3. The frequency of catenated dimers varies between 5·9 and 9·1 per cent among the various organs from rabbit and guinea-pig. The frequency of the higher catenanes varies from 0·6 to 1·8 per cent. These results may be restated as the percentage of the 5μ circles that are catenated to form dimers or higher oligomers. In these units the catenated dimers vary from 10·9 per cent for rabbit kidney to 16·6 per cent for rabbit brain M DNA. Higher catenanes were assumed to be trimers in the latter calculations. We conclude from the quantitative analyses of the M DNA prepared from the normal tissues listed in Table 3 that catenated dimers and catenated higher oligomers are normal constituents of M DNA. Data obtained with rabbit liver and normal human leucocyte M DNA isolated from lower bands in the EB–CsCl gradients are also included in Table 3. These results, which must be regarded as minimum values because some selection against higher molecular weight forms may have occurred, also show that catenanes are normal constituents of M DNA.

Table 3. FREQUENCY OF COMPLEX MITOCHONDRIAL DNA FORMS IN VARIOUS TISSUES

Tissue	Catenated dimers %	Catenated higher oligomers %	Circular dimers %	Molecules scored
Rabbit				
Brain*	9·1 ± 1·3 ‡	1·2 ± 0·5	0·0	1,762
Marrow*	8·1 ± 1·2	1·5 ± 0·6	0·0	1,845
Kidney*	5·9 ± 0·9	0·6 ± 0·3	0·0	2,817
Liver†	(4·8 ± 0·8)	(0·4 ± 0·2)	0·0	2,865
Guinea-pig				
Brain*	7·6 ± 0·9	0·8 ± 0·3	0·0	3,287
Liver*	7·7 ± 1·1	1·2 ± 0·5	0·0	2,210
Human				
Leucocytes†	(1·7 ± 0·5)	(0·0)	0·0	2,707
Mouse				
Embryo*	5·6 ± 0·9	1·8 ± 0·5	0·0	2,300

* Total DNA from purified mitochondria.
† Closed DNA from purified mitochondria. These results in parentheses are regarded as minimum values for reasons explained in the text.
‡ Range of 95 per cent confidence interval calculated as described in text.

The circular dimer form was not detected in the entire course of this survey of the complexity of M DNA from normal tissue (Table 3). We estimate that our limit of detection is of the order of one to two molecules per 1,000 molecules classified. This result is in marked contrast with our previous observations[3] that the circular dimer form occurs at a high frequency in M DNA from leukaemic leucocytes. It should be noted that the circular dimers were absent from the M DNA from the rapidly growing tissues—rabbit bone marrow and mouse embryo (Table 2). An examination of 400 molecules of M DNA from sea urchin gastrulae (*S. purpuratus*) also failed to reveal the circular dimer form.

The frequency of catenanes does not seem to be especially enhanced in rapidly dividing tissue. The frequencies in rabbit bone marrow, 8·1 per cent, and 13 day old mouse embryos, 5·6 per cent, are in the same range as in rabbit brain and kidney and guinea-pig brain and liver. These comparisons must be regarded as tentative, because we have not as yet investigated the variability of the frequency of catenanes among individual animals.

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Preparation of Mitochondria and Mitochondrial DNA

Brains from four rabbits were minced in cold 0·25 M sucrose, 0·01 M *tris*, *p*H 7·5, 0·01 M KCl, 0·005 M EDTA and washed twice. The tissue was suspended in 0·21 M mannitol, 0·07 M sucrose, 0·001 M *tris*, *p*H 7·5, 0·0001 M EDTA and homogenized with a loose fitting 'Teflon' homogenizer at 4° C until the homogenate appeared to be free of particulate material. The homogenate, filtered twice through cheese cloth, was sedimented at 1,000*g* for 10 min. The supernatant was sedimented a second time to remove remaining nuclei and cell debris. A pellet containing mitochondria was obtained in a Sorvall *SS* 34 rotor, 10,000 r.p.m., 10 min. The pellet was resuspended in 5 ml. of mannitol–sucrose medium and layered on to a step gradient of equal volumes of 0·75 M, 1·0 M, 1·30 M, and 1·75 M sucrose, each containing 0·1 M *tris*, *p*H 7·5, 0·01 M KCl, 0·005 M EDTA, and centrifuged for 1 h at 50,000*g*, 4° C, in a swinging bucket or a fixed angle rotor. The material at middle interface was collected with a pipette, diluted with mannitol–sucrose media and resedimented at 10,000 r.p.m. as described. The pellet was washed once and resuspended in 10 ml. 0·25 M sucrose, 0·01 *tris*, *p*H 6·7, and 0·005 M $MgCl_2$. DNase I, 100 μg, Sigma Chemical Company, was added and the mixture incubated at 25° C for 30 min. The digestion was stopped by addition of 0·2 ml. 0·5 M EDTA and chilling to 0° C. The mitochondria were sedimented to form a pellet which was washed once and the DNA extracted with SDS and banded in CsCl–ethidium bromide gradients as described by Radloff *et al.*[5].

These procedures were also used for rabbit kidney and bone marrow, guinea-pig brain and liver, and the 10–12 day mouse embryos except as noted below. The mouse embryos were freed of the embryonic sacs and heads and limbs discarded before mincing. The homogenization and wash solution was 0·25 M sucrose, 0·01 M *tris*, 0·001 M EDTA, *p*H 7·2. This solution was also used in the preparation of total mitochondrial DNA from guinea-pig liver and brain.

In the preparation of the rabbit liver mitochondrial DNA, the first mitochondrial pellet was subjected to nine cycles of suspension in MS buffer with a Thomas loose fitting homogenizer, followed by a 1,000*g* spin (pellet discarded) and another 10,000*g* spin. No sucrose gradients or DNase treatments were used. The closed band in an ethidium bromide-CsCl gradient was used for scoring. The upper band was examined and shown to contain less than 10 per cent circular material. Mitochondrial DNA from normal human leucocytes was isolated as described previously[5].

Examination of Preparations in the Analytical Ultracentrifuge

The rabbit liver M DNA was isolated from a lower band in an ethidium CsCl gradient, freed of dye by chromatography through a small Dowex 50 column and centrifuged as previously described[5]. A single symmetrical band was obtained. A comparable experiment with rabbit liver nuclear DNA formed a band skewed toward the dense side with a maximum 4 mg/ml. less dense than M DNA. A synthetic mixture of equal parts of nuclear and M DNA gave a single broad band. The combined upper and lower bands from purified mitochondria from rabbit brain, kidney, and bone marrow formed single bands in dye-free CsCl that were skewed to the light side in rough agreement with the known 10–20 per cent contamination by nuclear DNA seen in the electron microscope. The buoyant densities corresponding to the band maxima were the same as for rabbit liver M DNA. Comparable results were obtained for the nuclear DNAs from all rabbit organs. The guinea-pig liver M DNA isolated from the bottom band in EB-CsCl gradients formed a symmetrical band 1 mg/ml. more dense than the corresponding nuclear DNA. The normal human leucocyte M DNA obtained from a bottom band formed a symmetrical band 10 mg/ml. denser than the nuclear DNA, with a buoyant density in agreement with those previously reported for the corresponding DNAs from leukaemic leucocytes.

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ADDITIONAL DISCUSSION

These results demonstrate that catenated dimers and catenated higher oligomers are normal constituents of M DNA populations isolated from mammals. To obtain the frequency statistics, it was necessary to develop a reliable method of analysis for complex forms. This was accomplished by an electron microscope scoring procedure which assigned various molecular configurations to a particular complex form. In effect, the electron microscope is being utilized to perform a chemical analysis at the 2,000 to 3,000 molecule level.

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C. Occurrence of Circular Dimer and Catenated Oligomer Mitochondrial DNA in Human Leukemic Leukocytes

INTRODUCTION

Human leukemia is a malignant condition of unknown etiology which is characterized by a proliferation of leukocytes and their precursors in the body tissues. Virchow, in 1845, was the first to recognize leukemia as a clinical entity (1). As blood staining and cell typing methods were developed it became clear that a variety of distinct disease processes had been classified under the general term of leukemia. These differences involve the type of cell affected and the clinical severity of the malignancy. The majority of all leukemias are of two types: myelogenous and lymphogenous. Myelogenous leukemia is a malignant involvement of the myeloid cell series in the bone marrow. The myeloid cell may be neutrophilic, eosinophilic, or basophilic. These three cell types are of myeloid origin and are distinguished on the basis of staining affinities of cytoplasmic granules and different morphology and physiology in the fully differentiated state. Lymphogenous leukemia is a malignant involvement of the lymphatic tissues and manifests itself by the invasive properties of the malignant lymphocyte. Both types of leukemia may present themselves in two general forms: acute and chronic. These terms describe two states of the disease with different clinical and diagnostic properties. In general, the acute form of either myelogenous or lymphogenous leukemia is more difficult to control and leads to a terminal state more rapidly than the chronic form.

There are a number of subclassifications of these two diseases and a variety of somewhat rare types of leukemia which will not be

discussed here. Since all but one of the leukemias in the following study were of the neutrophilic myelogenous variety, the remainder of this introduction will be directed to that specific condition.

The etiology of human leukemia is unknown, although many factors, including lightning (2) and bee stings (3), have been considered as possible causes. The incidence of myelogenous leukemia was clearly increased in Japan near the site of the atomic bombings during World War II (4). This finding, along with data on the increased incidence of leukemia in persons exposed to radiation in their daily life (5) would seem to indicate a definite correlation between the two phenomena. This knowledge has not led to any fruitful investigation of either cause or treatment of the disease.

In contrast, the descriptive pathology of leukemia has been extensive. Normal bone marrow contains myeloid cells in every state of maturation, but normal peripheral blood contains predominantly the fully mature neutrophilic myeloid cell. A few nonsegmented myeloid cells may be present, especially in response to infection, trauma, or other physiological state inducing a leukocytosis. In myelogenous leukemia the whole series may be present in the peripheral blood system ranging from the most immature myeloblast through promyelocyte, myelocyte, metamyelocyte, and mature forms. In cases of acute myelogenous leukemia or the terminal phase of chronic myelogenous leukemia, the entire cell population may consist of myeloblasts. It is with such mixed cell populations that the following studies on M DNA were undertaken.

A variety of methods have been employed in the treatment of leukemia. Those considered successful enough to be in extensive use at the present time fall into two catagories: irradiation and chemotherapy. Most of the drugs used have been designed to alter existing nucleic acids or block their biosynthesis. Polyfunctional alkylating agents are capable of cross-linking DNA and fall into three classes: (a) the β-chloroethyl amines, such as nitrogen mustard and chloroambucil; (b) the ethylene imines, such as triethylene melamine and thio-phosphoramide; and (c) busulfan, which is a sulfonoxy ester. Antimetabolites which have proven successful are: (a) folic acid antagonists, such as amethopterin; (b) purine analogs, such as 6-mercaptopurine; and (c) pyrimidine analogs, such as 5-fluorouracil. In chronic myelogenous leukemia busulfan has proven to be the most effective chemotherapeutic agent, while in acute myelogenous leukemia 6-mercaptopurine is generally the agent of choice.

The decision to investigate the properties of human leukemic leukocyte M DNA was the result of several factors. As discussed earlier, Radloff et al. (6) had discovered possible multiple DNA forms in HeLa cell mitochondria. This finding posed the interesting question of whether multiple-sized mitochondrial DNA molecules are a function of tissue culture cells and/or malignancy. It was felt that the leukemia system might provide at least a preliminary answer, because leukemic cells are, by definition, malignant, and because normal human leukocytes would be available as a control. Another advantage of the system is the opportunity to analyze multiple samples from the same individual,

for both continuing information and control purposes.

With these objectives, an investigation of the M DNA populations from three patients with leukemia and a pooled sampling of normal leukocyte M DNA was carried out. One of these patients, M. C., had chronic myelogenous leukemia and was treated by leukophoresis, a process by which whole blood is removed from the patient and red cells and plasma returned. This meant that a continuous supply of leukemic leukocytes was available. It was possible to accrue enough M DNA to allow characterization of the various DNA forms by centrifugation and electron microscopy. The results of this initial investigation are given in the first of the two following publications. In this paper the occurrence of both circular and catenated oligomeric forms of M DNA in human leukemic leukocytes is established.

At the start of a later examination of a variety of patients with myelogenous leukemia, it was known that the catenane form of oligomer was ubiquitous in nature (7). All tissues examined had catenanes in their M DNA populations whether they were malignant, normal, slowly or rapidly dividing, or were of tissue culture origin. The circular dimer was conspicuously absent in normal tissues. It was, therefore, important to establish whether its occurrence in human leukemia was indeed general. The second of the two following publications demonstrates that the circular dimer mitochondrial DNA form is found in every myelogenous leukemia studied in this investigation. Furthermore, the frequency of this form is a function of both the specific disease and the type of medical treatment administered to the patient.

Circular dimers are absent in the appropriate controls for leukemic cell populations, namely, leukocytes from patients with non-leukemic leukopoietic disorders. The earlier result that peripheral leukocytes from normal individuals contain a low level of oligomers does not constitute a valid comparison with leukemic cells, since in only rare instances does an untreated myelogenous leukemic display a cell population identical to a normal individual. Patients with diseases such as myeloid metaplasia, a condition in which organs other than marrow tissue assume the hemopoietic functions when the marrow becomes dysfunctional, often display blood differentials identical to that of myelogenous leukemia.

It should be noted that the method of obtaining leukocyte mitochondrial DNA is more extensive in the second paper. This method should be considered the optimal procedure for isolating DNA samples free of contaminating nuclear DNA. This procedure is the result of varying several parameters, such as composition of homogenizing medium, sucrose gradients, and centrifuge conditions. The published procedure has proven routinely successful in obtaining enough mitochondrial DNA for an electron microscope analysis with as few as 10^8 leukocytes. Additional data not included in these publications are presented prior to the general discussion.

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Circular Dimer and Catenate Forms of Mitochondrial DNA in Human Leukaemic Leucocytes

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Oligomer forms of circular mitochondrial DNA have been identified in mitochondrial extracts of human leukaemic leucocytes. The oligomers occur as circular dimers with a contour length of 10μ and catenanes made up of interlocked 5μ monomers.

MITOCHONDRIAL DNA from a variety of organisms[1-3] has been reported to occur in the form of covalently closed circular duplex molecules approximately 5μ in contour length. These cytoplasmic DNAs resemble polyoma viral DNA[4] in several physical chemical properties that are shared[5] by all closed circular DNAs. One such property, the restricted uptake[6] of the intercalating dye ethidium bromide (EB), forms the basis for a convenient centrifuge method for isolating substantially pure mitochondrial DNA[7]. The finding in this laboratory that HeLa cell mitochondrial DNA preparations obtained by the ethidium bromide method contain 10 per cent multiple length molecules[7] or oligomers prompted us to examine the structure and size distribution of mitochondrial DNA in preparations from animal cells in a variety of physiological states. This report presents the result that mitochondrial DNA from human leukaemic leucocytes contains circular dimers, closed circular duplex DNA molecules

Fig. 1. A caesium chloride–ethidium bromide density gradient showing the two bands obtained with mitochondrial DNA preparations from leucocytes of the chronic granulocytic leukaemic patient, M. C., from March 8 to May 20, 1967. The 3 ml. solution of DNA in caesium chloride at a density of 1·55 g/ml. and 100 μg/ml. of ethidium bromide, pH = 8·0, was centrifuged 36 h at 43 K r.p.m. and 20° C in an *SW* 50 rotor in a Beckman *L2–65B* ultracentrifuge. The preparative tube was photographed in near ultraviolet light[7]. In the usual procedure a 500 ml. unit of blood was withdrawn into ACD. The inverted bottle was stored for 3 h in the refrigerator and the red cells were re-infused. The leucocytes and the remaining plasma were then chilled to 4° C. The leucocytes were spun down and resuspended in the homogenizing medium, 0·01 M KCl, 0·01 M *tris* chloride, 0·005 M Na EDTA, pH 7·5. After homogenization with a tight fitting 'Teflon' pestle, sucrose was added to 0·25 M, and the nuclei and cell debris removed by centrifugation at 3 K r.p.m. in an *SS*34 Sorvall rotor for 5 min. Resuspension and centrifugation were repeated. A greenish material, presumed to contain myeloperoxidase, was then pelleted at 5 K r.p.m. for 5 min. The mitochondria were isolated and the DNA extracted as described by Radloff, Bauer, and Vinograd[7]. The upper band contains a mixture of nicked mitochondrial DNA and linear nuclear DNA. The lower band contains closed circular mitochondrial DNA. We usually obtained about 5 μg of closed mitochondrial DNA, the threshold level of detection in visible light of a DNA–EB band, from 5×10^9 leucocytes. The recovered material corresponds to 600 mitochondrial DNA monomers per cell, a value approximately equal to the estimate for the number of mitochondria in a liver cell[8]. Estimates of the number of DNA molecules in a mitochondrion vary between two and six in different organisms[9]. On the basis of these results we calculate that the lower bands contain 15–50 per cent of the mitochondrial DNA in the cell population.

approximately 10μ contour length. The preparations also contain catenated dimers, interlocked pairs of 5μ closed circular DNAs, such as have been shown to occur in HeLa cell mitochondrial preparations (preceding article).

Leucocyte samples were obtained at approximately weekly intervals from the chronic leukaemic (M. C.). Samples from the lower band (Fig. 1) obtained at the beginning of this study were pooled and recentrifuged. The lower band contains the intact mitochondrial DNA molecules which exhibit restricted dye uptake. Examination of the DNA molecules from the lower band in the electron microscope revealed that 26 per cent of the molecules were circular dimers (Table 1). Fig. 2 presents electron micrographs of the types of molecules found. Only 3 per cent catenated dimers and 2 per cent trimers were observed. In addition to the molecules which were scored (Table 1) from prints of fields with high contrast and showing molecules with few overlaps, several thousand molecules were viewed under the electron microscope and scored for the fraction of total dimers present. This percentage of circular dimers was confirmed by the sedimentation velocity analysis described later. The histograms (Fig. 3) demonstrate that the dimers have twice the contour length of the monomers.

We note that 49 per cent by weight of the mitochondrial DNA in the lower band obtained from this patient (M. C.) is in the form of oligomers, and for the most part in the form of circular dimers. It is not yet possible to relate this number—probably a minimum – firmly to the distribution in the leucocytes of the donor. The frequency of oligomers, f, will be affected by any process of selection for or against closed oligomers over closed monomers. The following processes lead to selection if they occur to a significant degree. (*a*) An incomplete recovery of mitochondria from the different cell types biases the results if the cell types contain different amounts of the various oligomer species. Inspection in the light microscope of homogenates stained with azure *C* revealed that all cell types were effectively disrupted. This effect is therefore not likely to be significant. (*b*) Hirt[11] has shown that very high molecular weight nuclear DNA is spun down with the sodium dodecylsulphate (SDS) precipitate in the isolation procedure. It is conceivable that some very high oligomers may have been lost, and that f was reduced. (*c*) In the approximately 4 h period between withdrawal of whole blood and the disruption of the mitochondria, nuclease may have been active within the mitochondria. We have no way of assessing this effect, except to note that we have recovered approximately 15–50 per cent of the mitochondrial DNA as closed DNA. (*d*) After the SDS treatment all the mitochondrial molecules are in a common environment and subject to nicking by reducing agents and active endonucleases that may be present. Because the oligomers are the larger targets, f may have been reduced. Barring an enhanced stability of the oligomers over the monomers in the mitochondria, after withdrawal of blood, the estimates of the frequency of oligomers f (Table 1) are considered to be minimal.

The physical chemical properties of this new form of mitochondrial DNA, the circular dimer, were studied by means of ultracentrifugation. The material from the lower band diluted with an equal volume of twice distilled water and freed of dye by chromatography through a 50 μl. bed volume of 'Dowex-50' cation exchange resin was examined by the band sedimentation velocity procedure[12] in 2·85 molar caesium chloride (Fig. 4). The standard sedimentation coefficients calculated for sodium DNA for the three components are $51 \pm 1{\cdot}4S$, $36{\cdot}5 \pm 0{\cdot}8S$, and $26{\cdot}9 \pm 1{\cdot}7S$. Similar experiments in alkaline 2·85 molar caesium chloride showed two discrete components with uncorrected sedimentation coefficients of 112*S* and 80*S* (Fig. 5). Figure 6 presents a double logarithmic plot of sedimentation coefficient against molecular weight for closed circular DNA with additional data obtained from the literature. The 80*S* and 112*S* alkaline components fall on the line for closed circular DNA in alkali. The slow 27*S* species at neutral pH falls on the line II for nicked circular DNA. The fast 51*S* component falls above the line I for *in vivo* closed circular viral DNA at neutral pH, as do all other mitochondrial DNAs so far reported. We conclude that the neutral 51*S* component represents the compact superhelical form of the circular dimer. The 80*S* and 112*S* components in alkali represent the fully titrated forms[6] of the closed monomer and dimer respectively. The intermediate 37*S* component is apparently a mixture of nicked dimers and intact monomers. Figure 6 indicates that the closed monomer should sediment slightly faster than the nicked dimer.

We have calculated from the fractions of intact dimer and nicked monomer observed in Fig. 4 that the original

Table 1. DISTRIBUTION OF MITOCHONDRIAL DNA FORMS ISOLATED AS INTACT CLOSED CIRCULAR MOLECULES FROM NORMAL AND LEUKAEMIC LEUCOCYTES

	Leukaemic									Normal		
	Patient M. C.[a]			Patient S. T.			Patient S. B.					
	No.	%	Wt. %	No.	%	Wt. %	No.	%	Wt. %	No.	%	Wt. %
Monomers	205	68	51	639	89	78	1,146	95	91	1,926	98·6	97
Oligomers	95	32	49	80	11	22	57	5	9	27	1·4	3
Circular dimers	79	26	39	27	4	7	22	2	3·5	27	1·4	3
Catenated dimers	10	3	5	39	5	10	32	3	5			
Trimers and higher oligomers	6	2	4·5	14	2	6	3	0·2	0·7	—	—	—
Total molecules scored		300			719			1,203			1,953	
Catenane index†		0·11			0·59			0·59			—	

[a] Pooled lower bands from leucocytes obtained from March 8 to June 7, 1967.
†Catenane index, catenated dimers/total dimers.

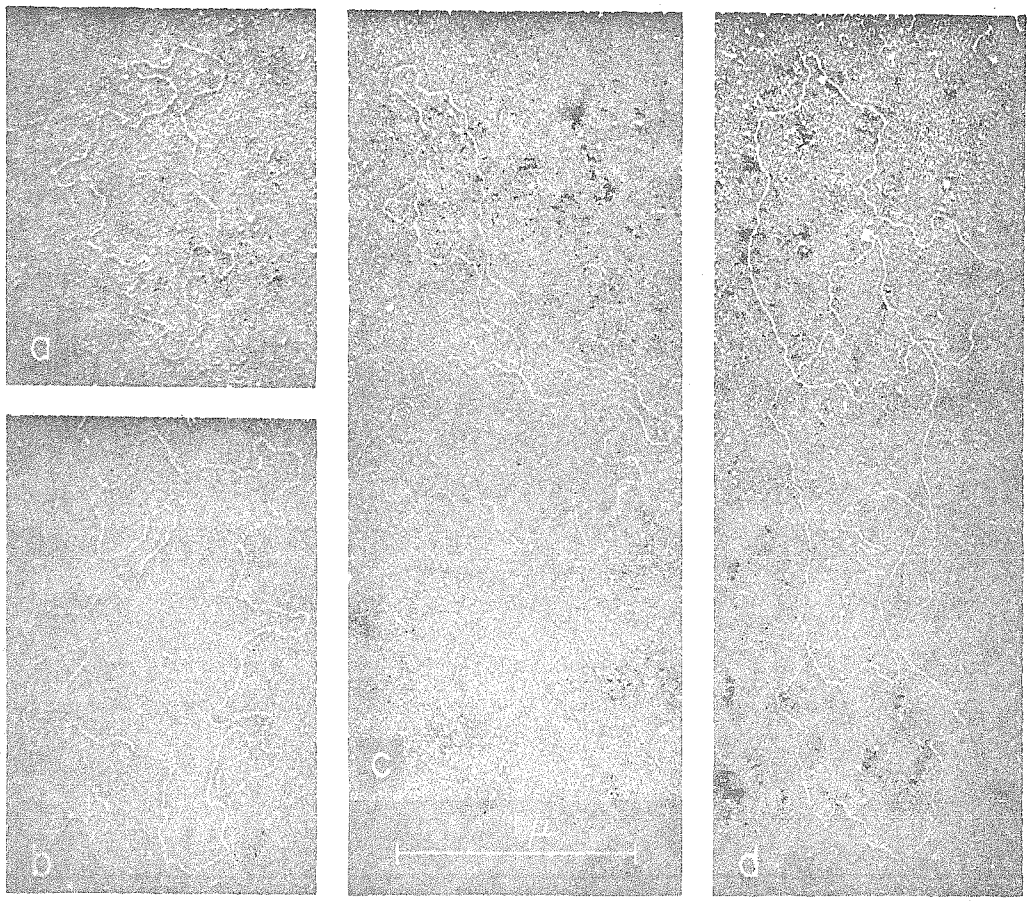

Fig. 2. Electron micrographs of mitochondrial DNA from leukaemic leucocytes. The density gradient shown in Fig. 1 was fractionated into approximately 50 μl. fractions and specimen grids were prepared with material found ten to twelve fractions below the centre of of the upper band. A 25 μl. sample of the fraction was diluted with 30 μl. of 1 mg/ml. of cytochrome *c* and 40 μl. of 0·01 M EDTA, *p*H 8·0. The specimen grids were prepared by the procedure of Kleinschmidt and Zahn[16] with 0·15 M NH_4Ac, *p*H 7·4, as the hypophase. The grids were shadowed while rotating with platinum–palladium and examined in a Philips *EM* 200 electron microscope. *a*, Single length mitochondrial DNA; *b*, circular dimer form; *c*, catenated dimer; *d*, catenated trimer. The catenation was established by focusing at the microscope and by careful examination of the negatives as described by Hudson and Vinograd (preceding article).

lower buoyant band contained 33 per cent dimers. The calculation was carried out with the assumption that statistical nicking occurred after the band was isolated. The agreement between this result and that obtained by electron microscopy (29 per cent, Table 1) suggests that there was no significant selection for or against dimers in the specimen grid preparation or in the electron microscope examination.

Further evidence that the 51*S* component represents a closed circular dimer form was obtained by analytical ultracentrifugation. Mitochondrial DNA recovered from the experiment described in Fig. 4 was centrifuged to equilibrium in buoyant caesium chloride containing ethidium bromide. Conditions were selected so that the closed DNA was separated from the open DNA in the caesium chloride–ethidium bromide gradient[6]. The ratio of amounts of closed to open DNA in the buoyant gradients was 0·5, compared with 0·75 expected for this material in the absence of further nicking. If, on the other hand, the leading component had consisted of nicked dimer and closed monomer, the middle component of nicked monomer, and the slow component of linear DNA, the ratio would have been 0·15.

The buoyant densities of the dye-free mitochondrial and nuclear DNA differed by 0·010 g/ml. (Table 2) in separate experiments with a crab dAT marker. The increment was

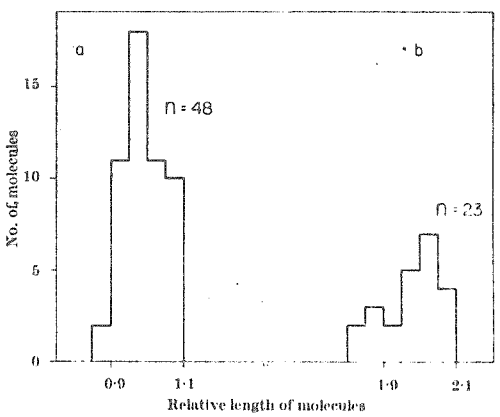

Fig. 3. Normalized contour lengths of mitochondrial DNA from the lower band in a caesium chloride–ethidium bromide density gradient. The DNA was obtained from leucocytes from the chronic granulocytic leukaemic patient, M. C. *a*, Single length molecules. The approximately 5μ lengths were normalized by the mean length. The standard deviation was ±0·05 dimensionless units. *b*, Double length molecules which were predominantly circular dimers. The approximate 10μ lengths were normalized by the mean monomer length. The standard deviation was ±0·06 dimensionless units.

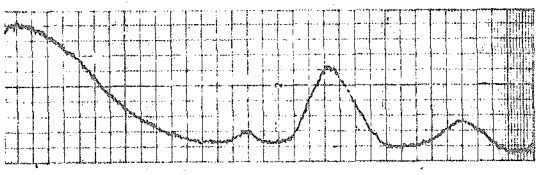

Fig. 4. Band sedimentation velocity pattern of originally closed mitochondrial DNA from M. C. in 2·85 M caesium chloride, *p*H 8·0, 20° C. Photoelectric scan of the liquid column 40 min after reaching 30 K r.p.m. The field is directed to the right. The leading band contains the 51*S* closed circular dimer. The middle band contains the 37*S* unresolved mixture of nicked circular dimer and closed circular monomer. The slowest band contains the 27*S* nicked circular monomer. The absorbing material between the slowest band and the meniscus is EDTA. No material sediments from this region after longer times at higher speed.

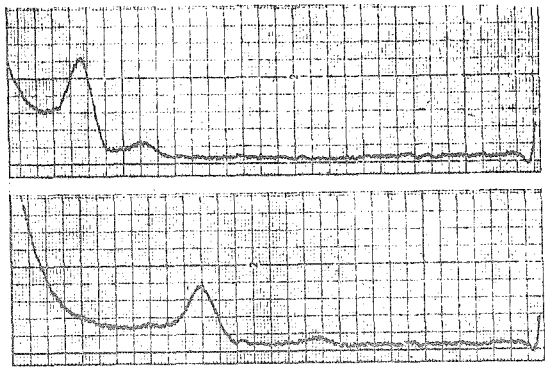

Fig. 5. Band sedimentation velocity pattern of originally closed mitochondrial DNA from M. C. in 2·85 M caesium chloride, *p*H 12·3, 20° C. Photoelectric scans of the liquid column at 8 min intervals at 28 K r.p.m. The fast band contains the 112*S* fully titrated closed circular dimer. The slow band contains the 80*S* monomer. At higher speeds a broad 26*S* band sediments out of the region obscured by the absorption of light by EDTA.

also observed in a synthetic mixture of mitochondrial and nuclear DNA (Fig. 7). The buoyant densities of nuclear DNA from leukaemic and normal leucocytes were indistinguishable. The mitochondrial DNA, containing 39 per cent by weight circular dimers, formed a symmetrical buoyant band in caesium chloride in the analytical ultracentrifuge. This result proves that the circular dimer has about the same base composition as the circular monomer and suggests that circular dimers consist of two connected monomer genomes. The clinical and diagnostic data for M.C. and the other two patients in this study are shown in Table 3.

The mitochondrial DNA from leucocytes from the subacute granulocytic leukaemic (S.T.) formed three fluorescent bands (Fig. 8). The lower band contained 9 per cent dimers, of which approximately 60 per cent were catenated dimers (Table 1). A histogram of 277 molecules is presented in Fig. 9. We do not regard the difference in oligomer content between M. C. and S. T. as significant

Table 2. BUOYANT DENSITIES OF MITOCHONDRIAL AND NUCLEAR DNA FROM NORMAL AND LEUKAEMIC HUMAN LEUCOCYTES ON CAESIUM CHLORIDE AT 25° C.

Source	Nuclear	Mitochondrial	No. of experiments
M. C.	$1{\cdot}689_4 \pm 0{\cdot}0002$	$1{\cdot}700_0 \pm 0{\cdot}0003$	3
S. T.	$1{\cdot}689_0$	$1{\cdot}700_0$	1
S. B.	$1{\cdot}689_0$	$1{\cdot}700_0$	1
Normal donors	$1{\cdot}689_4$	—	1

Buoyant densities were calculated from the distances between the above DNAs and a crab dAT marker. In these calculations the buoyant density of the marker was taken to be $\rho = 1{\cdot}669_0$, a value determined by the absolute method. The buoyant density gradient, Vinograd and Hearst[19], was used in the calculations. If these buoyant densities are calculated with an assumed value of 1·710 g/ml. for an *E. coli* DNA marker and with a gradient uncorrected for the effects of pressure, the buoyant densities of the nuclear and mitochondrial DNAs are $1{\cdot}695_4$ and $1{\cdot}705_0$, respectively. These values correspond to a G–C content of 36 mole per cent for nuclear DNA and 46 mole per cent for mitochondrial DNA[20].

because the time between the withdrawal of blood and homogenization was 10 h for S. T. compared with 4 h for M. C., and extensive nicking may have occurred. The middle band was midway between the closed and open DNA bands. This position corresponds to the expected

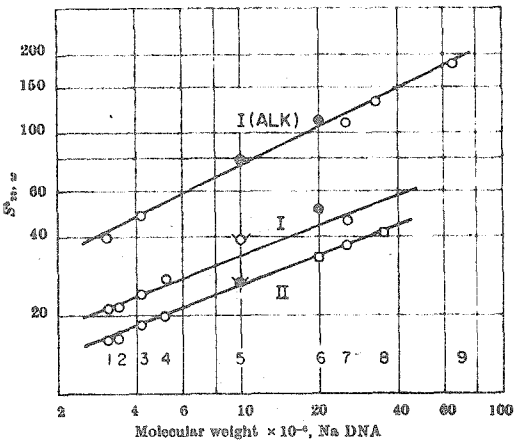

Fig. 6. Sedimentation coefficients of three forms of circular DNA as a function of molecular weight. The results for the fully titrated mitochondrial circular monomer and dimer and for the neutral nicked monomer fall on the best least squares lines for data previously published or in the press. The alkaline sedimentation coefficients represent partially corrected values obtained from experiments at 20° C in approximately 2·85 M caesium chloride, 1·35 g/ml. The data were corrected only for the small effects of solution density when the concentration of caesium chloride departed from the above value. The least squares slope of this line is 0·49. The neutral standard sedimentation coefficients were measured at 20° in caesium chloride or sodium chloride and when necessary have been corrected by the method of Bruner and Vinograd[18]. The slopes for the closed circular DNA, I, and the nicked circular DNA, II, as determined by a least squares method are 0·38 and 0·39. 1, Polyoma[4]; 2, RF φ*X*174 (ref. 14); 3, RF *M*13 (ref. 15); 4, human papilloma[16]; 5, mitochondrial monomer (ref. 2 and unpublished work of Vinograd, Pikó and Blair); 6, mitochondrial dimer; 7, *in vivo* closed λ^{+}_{pbac} (λ_{ALK}; I, II, unpublished results of Kiger, Young and Sinsheimer); 8,9, λdC_{mon} monomer and dimer[17]. ●, This work; □, 6, value calculated from *S* versus *M* relation given by Studier[18] for linear DNA. The value was raised by a factor of 1·14 for the effect of circularization; □, 3, λdg DNA[19]. The value was raised by the factor 1·14.

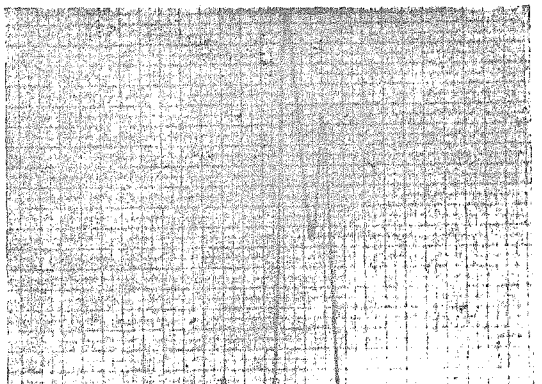

Fig. 7. Photoelectric scan of a synthetic mixture of nuclear and mitochondrial leucocyte DNA from the patient M. C. in neutral buoyant caesium chloride; 25° C, 44 K r.p.m., Moseley *X–Y* parallel recorder. The field is directed to the right. The light band contains crab dAT, the middle and dense bands contain nuclear and mitochondrial DNA, respectively.

position for a catenane containing one open and one closed molecule as described for HeLa cell mitochondrial DNA.

The catenane index, the ratio of catenated dimers to total dimers, varies by a factor of six between the preparations from M. C. and S. T. This index for the DNA in the lower band is representative of the index at the time of dissolution of the mitochondria by SDS, and is insensitive to the extent of statistical nicking that may have occurred.

In extreme cases it was easy to discriminate between circular dimers and catenated dimers, but molecules that contained several crossovers were often not scored. Completely open molecules or molecules in which the crossovers occurred only near the ends of the molecules were scored as circular dimers. Dimers consisting of an open monomer and a twisted monomer, dimers with only one crossover which divide it into statistically valid halves, and dimers in which the three dimensional character of the catenation could be seen in the electron microscope or the prints were scored as catenanes. We have made the assumption that highly twisted catenanes and circular dimers were rejected with the same probability. The catenane index is the best available basis for comparison of the different DNA preparations that we have investigated.

The mitochondrial DNA from the case of chronic lymphocytic leukaemia (S. B.) also formed three fluorescent bands in the caesium chloride–ethidium bromide gradient. The DNA in the lower band contained 5 per cent oligomers, of which approximately half were catenated molecules (Table 1, Fig. 9). There was 24 h between the withdrawal of blood and homogenization in this patient, and so the oligomer content may be artificially low.

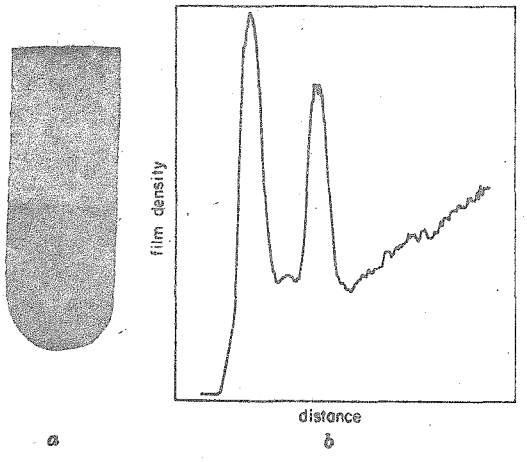

Fig. 8. *a*, Fluorescence photograph of a caesium chloride–ethidium bromide density gradient as in Fig. 1. *b*, A microdensitometer tracing showing band positions. The left hand side of the tracing corresponds to the upper part of the tube. The middle band indicates the presence of catenated dimers as shown by Hudson and Vinograd in the preceding article.

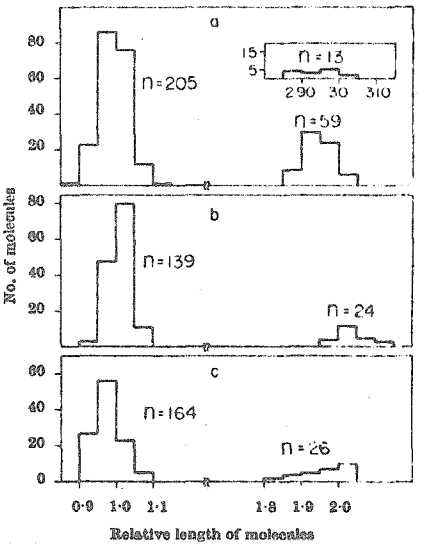

Fig. 9. Histogram of normalized contour lengths of mitochondrial DNA from the lower band in a caesium chloride–ethidium bromide density gradient as in Fig. 8. *a*, DNA from S. T.; *b*, DNA from S. B.; *c*, DNA from normal donors.

The closed mitochondrial DNA from leucocytes of normal donors was examined with material prepared from five samples of fresh blood. Only two fluorescent bands were found. Analyses of the lower band showed that 1·5 per cent of the mitochondrial DNA molecules were dimers (Table 1). A catenane index was not obtained because of the small number of dimers present in the electron micrographs. The period between blood withdrawal and dissolution of the mitochondria by SDS varied between 5 and 12 h.

The results so far can be summarized as follows. (*a*) Oligomer forms of mitochondrial DNA occur in leucocytes from normal and leukaemic human donors. The oligomers are principally circular and catenated dimers. Catenated trimers and a very small percentage of catenated higher oligomers also occur. (*b*) There are differences in the distribution of types of oligomers in the DNA preparations from the three leukaemic patients studied. The preparations from the two patients with clinically more advanced leukaemia (S. T. and S. B.) had a higher catenane index. (*c*) The circular dimers and catenated dimers represent new forms of mitochondrial DNA. The catenated forms have also been found in HeLa cells (preceding article).

Mitochondrial DNA isolated from M. C. during April to May, 1967, showed only two bands (Fig. 1) while pooled DNA preparations obtained during June to July, 1967, yielded three fluorescent DNA bands. This indicates that

Table 3. CLINICAL DATA FOR THE THREE DONORS OF LEUKAEMIC LEUCOCYTES*

	Patient M. C.			Patient S. T.	Patient S. B.	Normal†
	22-3-67	12-6-67	10-7-67	6-7, 22-7-67‡	24-7, 4-8-67‡	
Myeloblasts	—	3·3%	13·7%	6·0%	—	—
Promyelocytes	—	8·3	10·2	28·0	—	—
Myelocytes	4·0%	17·3	22·0	28·0	—	—
Metamyelocytes	10·0	22·0	27·3	14·0	—	3·0–5·0%
Band and segmented cells	69·0	44·8	16·0	26·0	—	54·0–62·0
Eosinophiles	—	—	0·3	0·2	—	1·0–3·0
Lymphocytes	18·0	1·5	4·5	0·5	99%	25·0–33·0
Monocytes	—	1·0	—	0·5	—	3·0–7·0
Nucleated red cells	—	1·0	6·0	2·0	—	—

Patient M. C. had chronic granulocytic leukaemia confirmed by bone marrow aspiration on December 1, 1966. Patient felt well. Patient S. T. had subacute granulocytic leukaemia confirmed by bone marrow aspiration on June 23, 1967. Patient was 70 per cent functional. Patient S. B. had chronic lymphocytic leukaemia confirmed by bone marrow aspiration on May 11, 1967. Patients M. C. and S. T. had received no chemotherapy before the leucocytes were sampled. Patient S. B. had received 'Prednisone' and 'Cytoxan'.

* Wright's stain smears of buffy coats.
† Range of leucocyte counts in normal adults. Normal blood also contains a low percentage of myelocytes[21].
‡ Values listed are means.

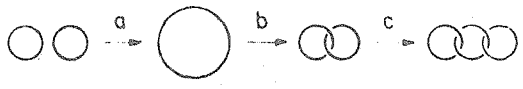

Fig. 10. Formation of oligomers by a postulated breakage and reunion mechanism. *a*, The circular dimer is formed in a single recombination event between two monomer molecules. *b*, The catenated dimer could result from a double recombination event between two monomers or a single recombination event within a circular dimer. *c*, A double recombination event between a catenated dimer and a monomer results in the formation of a catenated dimer. This scheme can be continued to form higher oligomers.

there was an increase in the fraction of catenated dimers in the preparations. Electron microscope analysis showed that the fraction of oligomers had remained essentially constant and that the catenane index approximately doubled. The change paralleled a significant shift in the leucocyte population: myeloblasts and promyelocytes increased and there was a corresponding decrease in mature forms.

A genetic recombination pathway based on the breakage and reunion model has been proposed as a possible scheme for the formation of the oligomers described here (Fig. 10) and in HeLa cell mitochondrial DNA (preceding article). Changes in the equilibrium distribution of oligomers in the leukaemics studied may have resulted either from changes in the proportion of the various cell types or from changes within a given cell type. In either case it seems that there are various equilibrium positions in the monomer–oligomer equilibria.

Other investigations in this laboratory have shown that there are interlocked mitochondrial molecules in a variety of other systems. Mitochondrial DNA of unfertilized sea urchin eggs, for example, contains catenated dimers and a small proportion of higher catenated oligomers (unpublished results of Blair, Pikó and Vinograd). A middle band containing catenated oligomers has been obtained from the mitochondrial DNA of *3T3* mouse cells transformed by *SV* 40 virus. These results suggest the possibility that oligomers were present but not reported in the mitochondrial DNA preparations earlier described[1–3]. The elucidation of the relationship between the occurrence of the oligomers and the physiological state of a cell will require further quantitative investigation of a variety of cell systems. The dye–buoyant density method used in this work provides highly purified closed mitochondrial DNA, but as discussed the method selects against open DNA that may be present. In our continuing study of the composition and structure of mitochondrial DNA in normal individuals and in leukaemic patients, non-selective methods are being used to isolate the total complement of DNA in the mitochondria.

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*COMPLEX MITOCHONDRIAL DNA IN LEUKEMIC AND NORMAL HUMAN MYELOID CELLS**

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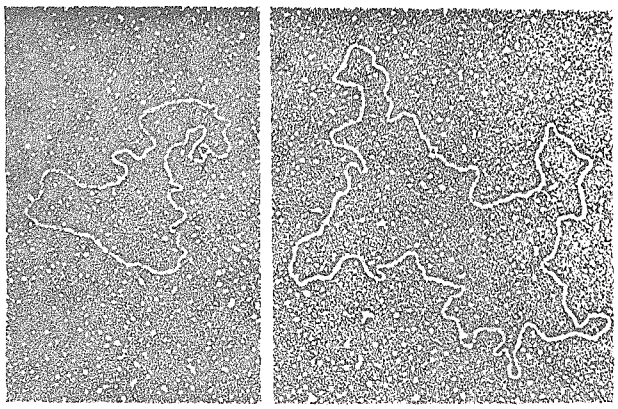
Abstract.—Mitochondrial DNA's from the peripheral blood of 14 patients with granulocytic leukemia contained a circular dimer form. No such structure could be found in M DNA's from three patients with nonmalignant proliferations of granulocytes. The frequency of the circular dimer form is reduced upon treatment with antileukemic drugs. The above results suggest that a significant relation exists between the formation and presence of the circular dimer M DNA form and granulocytic leukemia in man.

We have previously reported that animal mitochondrial DNA (M DNA) occurs in two *complex* forms[1–3] in addition to the *simple* circular form with a contour length of about 5 μ. The circular dimer form—a double-length circular molecule[1, 2]—was found in human leukemic white cells along with the catenane form,[3] in which the single-length submolecules are connected to each other like links in a chain. The presence of the circular dimer in the M DNA from the three leukemic patients studied,[2] and the absence of this form in the M DNA from normal mature human leukocytes,[2, 5] HeLa cells,[3] sea urchin eggs,[4] and a variety of normal mammalian tissues,[5] suggested to us that there might be a correlation between the occurrence of the circular dimer and human leukemia. We therefore carried out a more extensive study of the frequency of complex M DNA forms in circulating leukocytes from patients with granulocytic leukemia and, for comparison, from patients with nonleukemic leukopoietic disorders. The M DNA's from 14 leukemic patients all contained circular dimers (Fig. 1), whereas the M DNA's from three patients with normal, immature myeloid cells contained none. In the course of this study we also found that treatment with cytotoxic drugs substantially lowered the frequency of the circular dimer. The above results together suggest that a significant relation exists between the formation and presence of the abnormal M DNA form and granulocytic leukemia in man.

Materials and Methods.—Chemicals: Optical grade CsCl was obtained from the Harshaw Chemical Co. Ethidium bromide was a gift from Boots Pure Drug Co., Ltd., Nottingham, England. Sodium dodecylsulfate was obtained from the Matheson Co. All other chemicals were of reagent grade.

Preparation of leukocyte M DNA: Whole-blood samples, 10–100 ml, were allowed to settle at 4° for 2 to 20 hr. Buffy coats were drawn off and red cells were removed by hemolysis for 20 sec with distilled water. The leukocytes were spun down at 1000 × *g*, for 5 min in an International PR-1 centrifuge and resuspended in a tenfold volume of homogenizing medium: 0.21 *M* mannitol; 0.07 *M* sucrose; 0.001 *M* Tris HCl, pH 7.5; and 0.0001 *M* ethylenediaminetetraacetate (EDTA). The cells were homogenized with a tight-fitting Teflon pestle until approximately 80% of the nuclei were liberated, as observed in the light microscope. The homogenate was centrifuged at 1000 × *g* for 5



(a) (b)

FIG. 1.—Electron micrographs of M DNA isolated from leukemic leukocytes. (*a*) Monomeric M DNA. (*b*) Circular dimer M DNA.

min. The supernatant was centrifuged at 15,000 × *g* for 15 min in a Sorvall SS-34 rotor to pellet the mitochondria. The pellet was resuspended in 5 ml of homogenizing medium and placed on a step gradient containing equal volumes of 1.00 *M*, 1.50 *M*, and 1.75 *M* sucrose. The sucrose solutions contained 0.001 *M* Tris HCl, pH 7.5, and 0.0001 *M* EDTA. The tubes were centrifuged for 30 min at 20 krpm in a Spinco SW-25.1 or SW-27 rotor. The buoyant material at the 1.00 *M*–1.50 *M* interface was removed with a pipet, diluted fourfold with homogenizing medium, and pelleted. The mitochondrial pellet was resuspended in 10 ml of 0.21 *M* mannitol, 0.07 *M* sucrose, 0.05 *M* Tris HCl, pH 6.7, 0.025 *M* KCl, and 0.0025 *M* $MgCl_2$. All of the foregoing operations were at 4°.

DNase I and RNase A (Sigma Chemical Co.), 100 μg each, were added and the mixture incubated at 25° for 30 min. The reaction was quenched by the addition of EDTA, and the temperature was lowered to 0°. The mitochondria were pelleted and the DNA was isolated after the addition of sodium dodecylsulfate, CsCl, and ethidium bromide (EB), as described earlier.[1, 2, 5] In most cases, both the upper and lower fluorescent bands and the CsCl solution between them was collected from the buoyant CsCl-EB gradient. The solution was diluted with 0.01 *M* Tris HCl, pH 7.5, 0.001 *M* EDTA; and the DNA was pelleted at 45 krpm for 12 hr in a Spinco SW-50 rotor. The M DNA pellet was allowed to resuspend in 0.25–0.5 ml of supernatant and was stored at −20°.

Specimens for electron microscopy were prepared as described earlier[2] and were examined in a Philips EM-300 electron microscope. Classification of M DNA forms was performed at the microscope by direct observation on the fluorescent screen or on an auxiliary Plumbicon TV monitor system. The procedures and criteria for classifying the M DNA forms have been described.[5]

Results.—The results obtained in this study are presented graphically in a series of panels which show the M DNA and the leukocyte distributions in each blood sample. The medical diagnoses and pertinent clinical data supplied to us by our colleagues are given in Table 1, along with a summary of the M DNA distributions.

Untreated granulocytic leukemia: Figure 2*a* presents the M DNA distribution in the circulating leukocytes from M. C., a patient with chronic granulocytic leukemia. The circular dimer frequency (31%) did not change significantly in four analyses during an 18-month period. The frequency of catenated dimers (7%) is within the range found in normal mammalian tissues (6–9%).[5] The frequency of catenated higher oligomers (trimers and larger catenanes) is significantly higher (8%) than in normal tissues in which the highest frequency

TABLE 1. *Number frequency of complex mitochondrial DNA in leukemic and nonleukemic patients.*

Patient*	Diagnosis†	Chemotherapy	Circular dimer (%)	Catenated dimer (%)	Catenated higher oligomer (%)
M. C.[a]	CGL	None‡	31 ± 4	7 ± 2	8 ± 2
C. H.[b]	AGL	None	48 ± 4§	4 ± 1§	5 ± 2§
P.J.[b]	"	"	29 ± 6	6 ± 3	1.0 ± 0.9
M. W.[c]	CGL	"	21 ± 3	6 ± 2	3 ± 1
"	"	Myleran, 3 wk	7 ± 2	7 ± 2	2 ± 1
M. R.[b]	"	None	14 ± 2	7 ± 2	1 ± 0.7
"	"	Myleran, 1 wk	7 ± 2	11 ± 2	3 ± 1
"	"	" 3 wk	5 ± 1	11 ± 2	2 ± 1
M. Y.[d]	"	Myleran, 2 yr	2 ± 1	4 ± 2	2 ± 1
L. G.[b]	"	" 3 yr	2 ± 1	4 ± 1	0.1
L. S.[a]	"	" 2 yr	3 ± 1	5 ± 1	0.3
A. M.[c]	"	" 2 wk	6 ± 2	5 ± 2	1 ± 0.9
A. P.[b]	AGL	6-MP, 8 months**	1 ± 0.8	5 ± 2	0.1
L. J.[b]	"	" 5 days	2 ± 2	5 ± 2	0.6
R. B.[c]	"	" 2 wk	2 ± 1§	4 ± 2§	0.6§
E. F.[b]	At CGL	None	12 ± 2	6 ± 2	0.6
J. W.[b]	Ea CGL	"	1 ± 0.9§	3 ± 2§	0.9§
M. L.[b]	MM	"	0	6 ± 1	0.5 ± 0.3
T. D.[b]	MM	"	0	4 ± 1	0.8 ± 0.4
M. D.[c]	LR	"	0	5 ± 1	0.6 ± 0.4

* The diagnoses for the patients were supplied by: (*a*) H. R. Bierman; (*b*) R. L. Teplitz; (*c*) W. C. Moloney; (*d*) R. D. Lewis; and (*e*) R. Boardman.

† *CGL, AGL, At CGL,* and *Ea CGL* signify chronic, acute, atypical chronic, and early chronic granulocytic leukemia, respectively. *MM* and *LR* signify myeloid metaplasia and leukomoid reaction, respectively.

‡ This patient was treated periodically by leukophoresis, a procedure in which the patient's red cells and plasma are infused after removal of the buffy coat.

§ Data obtained from a lower-band M DNA in a CsCl-EB density gradient.

** This patient also received Vincristine and Methotrexate.

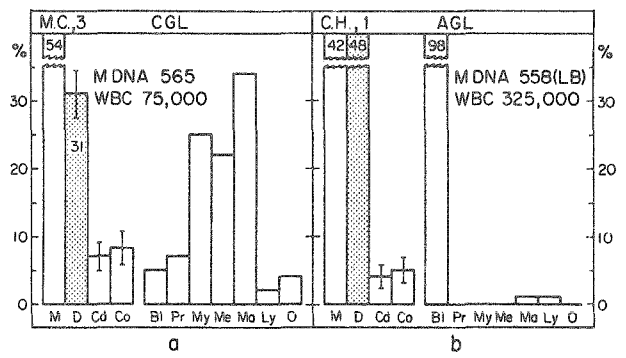


FIG. 2.—The distribution of complex forms in the M DNA obtained from two granulocytic leukemic patients who had not received cytotoxic drugs. The M DNA distribution is given in per cent at the left of each panel: Monomers, *M;* circular dimers, *D;* catenated dimers, *Cd;* and catenated higher oligomers, *Co.* The leukocyte distribution in per cent is given at the right of each panel: Myeloblasts, *Bl;* promyelocytes, *Pr;* myelocytes, *My;* metamyelocytes, *Me;* mature forms, *Ma;* lymphocytes, *Ly;* and other types, *O.*

The numbers after M DNA and WBC represent the number of DNA molecules classified and the leukocyte count in cells/mm.[3] CGL and AGL stand for chronic and acute granulocytic leukemia, respectively. The number after the patient's initials is our code number for the blood sample analyzed. The error bar indicates the interval which contains the true mean at a level of confidence of 95%. The calculation takes into account only the uncertainty associated with the finite sample. The symbol *LB* stands for lower band in the CsCl-EB gradient. An analysis of a lower band rather than the combination of the upper and lower bands and the intermediate solution provides a *minimum* frequency for higher molecular weight M DNA forms.[2]

was 2 per cent.[5] We calculate that 40 per cent of the M DNA mass is in the form of circular dimers and that an additional 25 per cent is in the form of catenated DNA molecules. The catenated higher oligomers were assumed to be trimers in the foregoing calculation.

The M DNA from C. H., a patient with severe acute granulocytic leukemia, contained the highest frequency of circular dimers (48%) that we have so far encountered (Fig. 2*b*). The catenated higher oligomer frequency (5%) is again high. The circular dimer accounts for 58 per cent of the mass of the M DNA, and the catenated form for 17 per cent. The symbol *LB* in Figure 2*b* refers to a lower band in a CsCl-EB density gradient. An analysis of a lower band yields a minimum estimate of the frequency of higher molecular weight M DNA, as discussed previously.[2] The leukocytes in this blood sample consisted almost entirely of myeloblasts; we may conclude, therefore, that these blast cells contained the circular dimer and the simple M DNA forms.

The circular dimer frequency in P. J., another untreated patient with the acute form of the disease, was 29 per cent (Table 1). The per cent leukocyte distribution was as follows: myeloblasts, 81; promyelocytes, 10; mature forms, 4; lymphocytes, 4; and other types, 1.

Two additional cases of untreated granulocytic leukemia are presented below in the section on the effects of chemotherapy.

Nonleukemic leukopoietic conditions: Leukocyte distributions similar to those observed in patients with granulocytic leukemia occur in patients with myeloid metaplasia and in patients with leukomoid reactions to a variety of stimuli. Such circulating nonleukemic myeloid cells provide the appropriate controls for the proposed correlation of the circular dimer M DNA and granulocytic leukemia. The M DNA's from three such patients are presented in Figure 3. No circular dimers were detected, even though 1300–2300 molecules were examined in each sample. The circular dimer level, if not zero, is certainly less than 0.2 per cent. The catenated dimer and higher oligomer frequencies are also in the normal range at 4–6 and <1 per cent, respectively. The leukocyte distributions (Fig. 3*a* and *b*) of the two patients with myeloid metaplasia

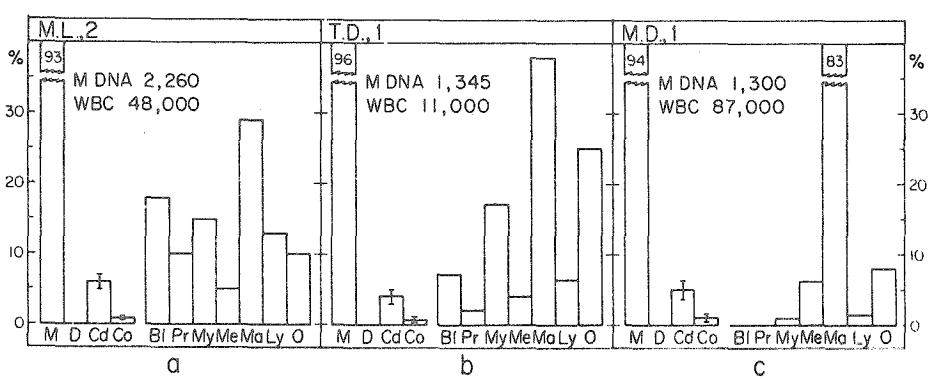


FIG. 3.—The distribution of complex forms in the M DNA obtained from two patients with myeloid metaplasia (*a* and *b*) and one patient with a leukomoid reaction (*c*). The symbols are defined in the legend for Fig. 2.

are similar to those observed in the blood of patients with chronic granulocytic leukemia (Figs. 2*a*, 4, and 5*a*, and *c*). We conclude from the above results that the circular dimer M DNA is not a constituent of normal immature and mature myeloid cells in man.

The effect of chemotherapy on the M DNA distribution in granulocytic leukemia: The M DNA from the peripheral blood of a patient with the chronic form of the disease was examined before and after therapy was initiated with the alkylating agent, Myleran—1,4 bis[methane sulfonoxyl]butane. The circular dimer frequency, which was 21 per cent before treatment began, dropped to 7 per cent after three weeks of chemotherapy (Fig. 4). A similar effect of chemotherapy was observed in a serial study of the M DNA from patient M. R. (Table 1).

The effect of treatment is illustrated further by the low levels of the circular dimer in blood samples taken from six granulocytic leukemic patients who had received cytotoxic drugs for various periods of time (Fig. 5 and Table 1). The circular dimer frequency in these samples[6] ranged from 1 to 3 per cent, in contrast to the five untreated leukemics (Table 1) in which the circular dimer frequency ranged from 14 to 48 per cent. The above effects of chemotherapy were observed consistently, although the patients had widely different leukocyte distributions.

L. J., a patient with acute granulocytic leukemia, had received the base analogue 6-mercaptopurine (6-MP) daily for only five days when the blood sample was taken for analysis (Fig. 5*c*). The white-cell distribution, which consisted almost entirely of myeloblasts, had not yet changed in response to therapy. The circular dimer level (2%) was, however, much lower than that in C. H., an untreated patient with the same form of the disease (Fig. 2*b*) whose leukocyte distribution was similar. These results raise the interesting possibility that the drug had caused selective removal of the circular dimer from the peripheral leukocytes; alternatively, a new population of blast cells with a low level of circular dimers had replaced the original population.

Atypical and early granulocytic leukemia: Patient E. F. (Fig. 6*a*) was diagnosed as an atypical granulocytic leukemic because the patient did not respond to Myleran in 1963, and bone marrow metaphase spreads did not contain the partially deleted chromosome 21 (the Philadelphia chromosome) usually present in chronic granulocytic leukemia.[7] On the other hand, the low level of

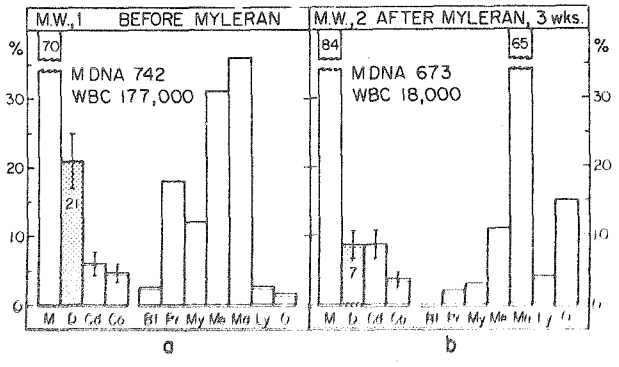


FIG. 4.—The distribution of complex forms in the M DNA obtained from a patient with chronic granulocytic leukemia before and after treatment with Myleran for three weeks. The symbols are defined in the legend for Fig. 2.

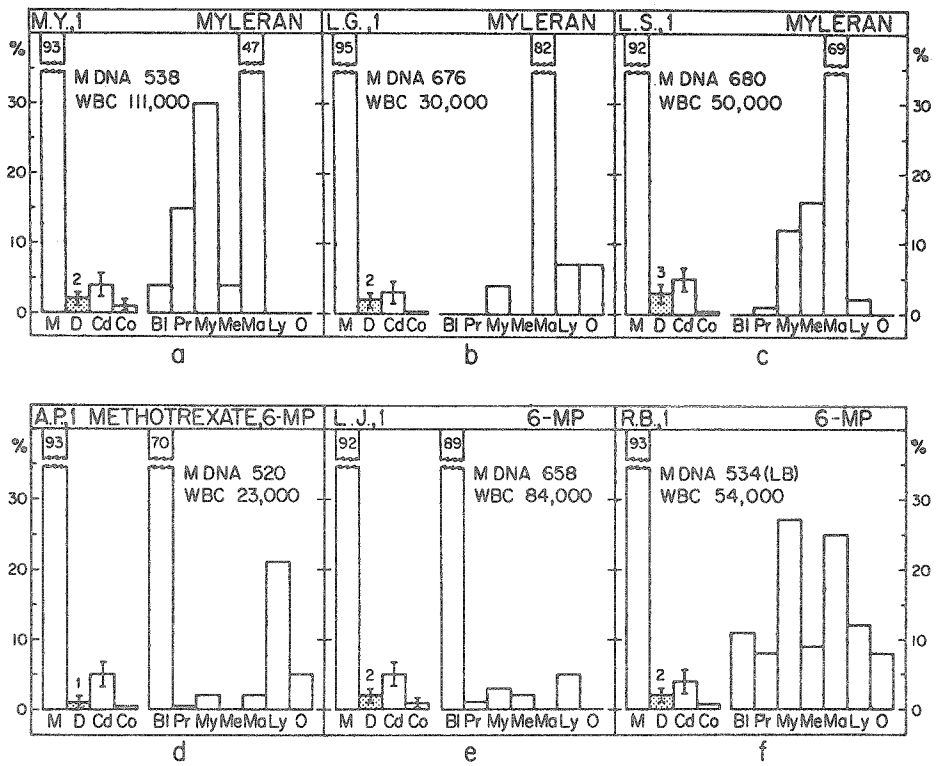


FIG. 5.—The distribution of complex forms in the M DNA obtained from three patients with chronic granulocytic leukemia (*a*, *b*, and *c*) and three patients with the acute form of the disease (*d*, *e*, and *f*) after chemotherapy. The symbols are defined in the legend for Fig. 2.

leukocyte alkaline phosphatase that is normally seen in this form of the disease was reported. The circular dimer frequency was 12 per cent. The Philadelphia (Ph') chromosome was found in about half the bone marrow metaphase spreads of another chronic leukemic patient (J. W.) diagnosed as an early granulocytic leukemic. The M DNA in the peripheral leukocytes contained 1 per cent circular dimers.

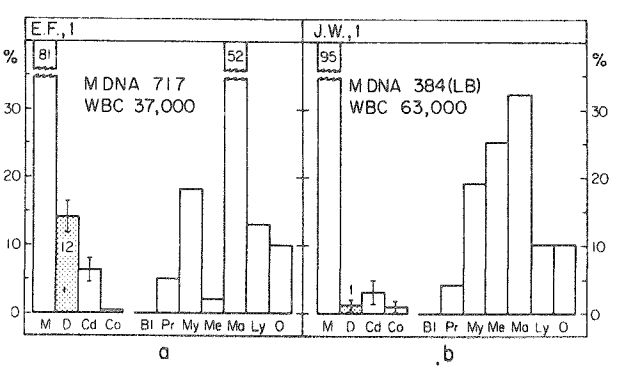


FIG. 6.—The distribution of complex forms in the M DNA obtained from untreated patients with (*a*) atypical and (*b*) early chronic granulocytic leukemia. The symbols are defined in the legend for Fig. 2.

Discussion.—Circular dimer M DNA is present in all the granulocytic leukemics that we have studied to date (Table 1). The highest frequency of circular dimers (48%) was found in a case of untreated acute granulocytic leukemia with leukocytes that contained 98 per cent myeloblasts. High frequencies of circular dimers (31 and 21%) were also observed in two cases of untreated chronic granulocytic leukemia with moderately immature leukocyte distributions. In the chronic leukemic patients with moderately mature leukocyte distributions, the frequency of circular dimers was lower: 14 per cent in an untreated case, 12 per cent in an atypical case, and 1 per cent in an early case. The above preliminary correlation of the circular dimer frequency with the immaturity of the leukocyte distribution suggests that the circular dimer frequency is related to the severity of the disease.

The effect of chemotherapy upon the circular dimer level in acute granulocytic leukemia may be dramatic: The level in two patients who had been under treatment for two weeks or less was 2 per cent, and in another, under chemotherapy for eight months, the level was 1 per cent. A drastic reduction in the circular dimer level must have occurred if it is assumed that the circular dimer levels in these patients were initially as high as in patient C. H. Serial studies are required to substantiate this reasoning.

The effect of chemotherapy upon the level of circular dimers in cases of chronic granulocytic leukemia was less pronounced. The circular dimer level was 2–3 per cent in three patients under long-term treatment with Myleran. It was 5–7 per cent in the M DNA from three patients who had received Myleran for periods of two to three weeks.

While the gene products of the M DNA in animal cells have not as yet been established, the DNA itself is known to be metabolically active. In HeLa cells it serves as a template for a rapidly labeled heterogeneous RNA found in the mitochondria and associated with the rough endoplasmic reticulum.[8, 9] The above observations, together with the report by Woodward and Munkres that a membrane structural protein is altered in a cytoplasmic mutant of *Neurospora*,[11] raise the possibility that membrane structural proteins are among the gene products of M DNA in animal cells. A defect in the regulation or the structure of such proteins in cells containing the circular dimer could contribute to the physiological properties characteristic of the neoplastic cells. A review of the evidence for a membrane theory of cancer has appeared recently.[11] At the present time we do not know whether the M DNA abnormality is an early event in the disease or a consequence of other events which change the physiology of the cell so as to permit the proliferation of the dimer M DNA form.

We noted earlier[12] that the formation of catenanes or circular dimers from circular monomers necessarily involves the cell's machinery for opening and closing the backbone chains of DNA. The various enzyme systems—enzymes, regulators, and enzyme sites—involved in DNA synthesis, recombination, and repair are thus candidates for the specific aberrations which result in the formation of the abnormal circular dimer M DNA.

Closed circular dimers and higher circular oligomers also occur as intracellular viral forms in infected bacteria[13] and animal cells,[14] and as plasmids and episomes

in bacteria.[15, 16] These simpler experimental systems may be expected to be useful in elucidating the detailed mechanism of the formation of dimers from monomers.[17, 18]

Abnormal M DNA represents the second kind of aberration to have been detected in the genetic apparatus of leukemic myeloid cells. A partially deleted chromosome, Ph′, is usually present in metaphase spreads of bone marrow cells from patients with chronic granulocytic leukemia. Aneuploidy often occurs in the cells of chronic granulocytic leukemic patients after the acute transformation of the disease has taken place,[19] and in the cells of patients with acute granulocytic leukemia. The relationship between the above chromosomal anomalies and the mitochondrial DNA anomaly is one of many problems raised by the results described in this communication.

It is a pleasure to thank Dr. R. L. Teplitz, City of Hope Medical Center, Duarte, California, for advice and several helpful discussions. We also thank Dr. Teplitz; Dr. H. R. Bierman, Beverly Hills, California; Professor W. C. Moloney, Peter Bent Brigham Hospital, Boston, Massachusetts; Dr. R. D. Lewis, Huntington Hospital, Pasadena, California; and Dr. R. Boardman, San Marino, California, for blood samples and clinical diagnoses. We are grateful to Marlyn Teplitz for expert technical assistance and to Rebecca Burress for aid in preparation of the manuscript.

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ADDITIONAL RESULTS

(a) M. C. after chemotherapy

One patient with chronic myelogenous leukemia, M. C., was the main source of leukocytes for the physical studies of leukocyte M DNA reported in the first of the two preceding papers and in the next section of this thesis. The patient had received no chemotherapy prior to the isolation of the M DNA used in these experiments. Myleran was recently instituted in this patient's therapy and the following correlation of treatment and circular dimer frequency was observed (Table 1). The patient received a total of 388 mg of Myleran from May 17, 1969, to June 18, 1969. Before chemotherapy this patient consistently displayed approximately 30% circular dimers in her leukocyte M DNA populations. The drop in the frequency of the circular dimer species from 30% to 2% after chemotherapy is consistent with the results for other chronic myelogenous leukemia patients, as reported in the preceding publication.

(b) ^{3}[H]thymidine pulse labeling of leukemic leukocyte M DNA

Human leukemic leukocytes from the patient, M. C., with untreated chronic myelogenous leukemia were incubated with ^{3}[H]thymidine to determine the relative specific activities of the circular dimer and monomer form. Approximately 5×10^{9} leukocytes were incubated without stirring at 37°C with 2 μc of ^{3}[H]thymidine (specific activity, 15 C/mM) in R. P. M. I. 1640 medium plus nonessential amino acids and 10% fetal calf sera. After 16 hours the cells were harvested and the M DNA isolated in the standard manner. The M DNA was nicked by

TABLE 1

M DNA and Cell Distributions in M. C. after Chemotherapy

	Date of leukocyte sample		
	5/28/69	6/11/69	7/02/69
Cell count:			
white blood cells/mm^3	57, 800	32, 000	7, 200
Cell differential (%):			
blasts, promyelocytes, myelocytes	25	12	4
metamyelocytes	21	19	3
mature forms	47	60	73
lymphocytes	5	7	19
other	2	2	1
M DNA:			
monomers	78	81	86
circular dimers	7 ± 2*	9 ± 2	2 ± 1
catenated dimers	11 ± 2	7 ± 2	11 ± 2
catenated oligomers	3 ± 1	3 ± 1	0.5

* The error indicates the interval which contains the true mean at a level of confidence of 95%.

exposure to visible light (100W incandescent light bulb at 8 inches) for 48 hours while remaining in a preparative tube containing 4.5 M-CsCl, 0.01 M-Tris, pH 7.5, and 300 μg/ml ethidium bromide. Upon recentrifugation, the ratio of upper band (nicked circular DNA) to lower band (covalently closed circular DNA) was 3:1. The nicked circular M DNA was isolated, diluted with 0.01 M-Tris, pH 7.5, and passed through Dowex-50 cation exchange resin to remove ethidium bromide, and then concentrated by pelleting the DNA at 45 Krpm for 12 hours, 20°C, in an SW50.1 rotor.

An aliquot of this M DNA sample was removed for electron microscopy and the remaining material was subjected to an analytical and preparative sedimentation velocity analysis. The analytical velocity pattern (Figure 1) shows complete resolution of the nicked circular dimer, $\underline{s}_{20,w} = 33.1$, and nicked monomer forms, $\underline{s}_{20,w} = 25.6$. After correcting the band areas for radial dilution and nonlinear pen excursion on the Offner recording system, the relative mass amounts of circular dimer and monomer species are 31% and 69%, respectively. An analysis of the distribution of radioactivity (Figure 2) after preparative sedimentation velocity reveals that 32% of the ^{3}[H] travels with the 33 S component and 68% of the ^{3}[H] with the 26 S component. The electron microscope frequency analysis of the original aliquot gives 76% monomers, 20% circular dimers, and 4% catenated forms. This corresponds to 32% circular dimers by mass, in excellent agreement with the sedimentation velocity data. It is concluded that a sixteen-hour pulse labeling of leukemic leukocyte M DNA results in

Figure 1. Photoelectric scan of nicked circular dimer and nicked monomer M DNA. This band sedimentation velocity experiment was performed in $\rho = 1.35$, pH 8.0, CsCl at 35,600 rpm, 20°C, in a Beckman model E ultracentrifuge. The field is directed to the right. The leading component is the 33 S nicked circular dimer and the trailing component is the 26 S nicked monomer. The DNA has traversed approximately two-thirds of the radial distance in the centrifuge cell.

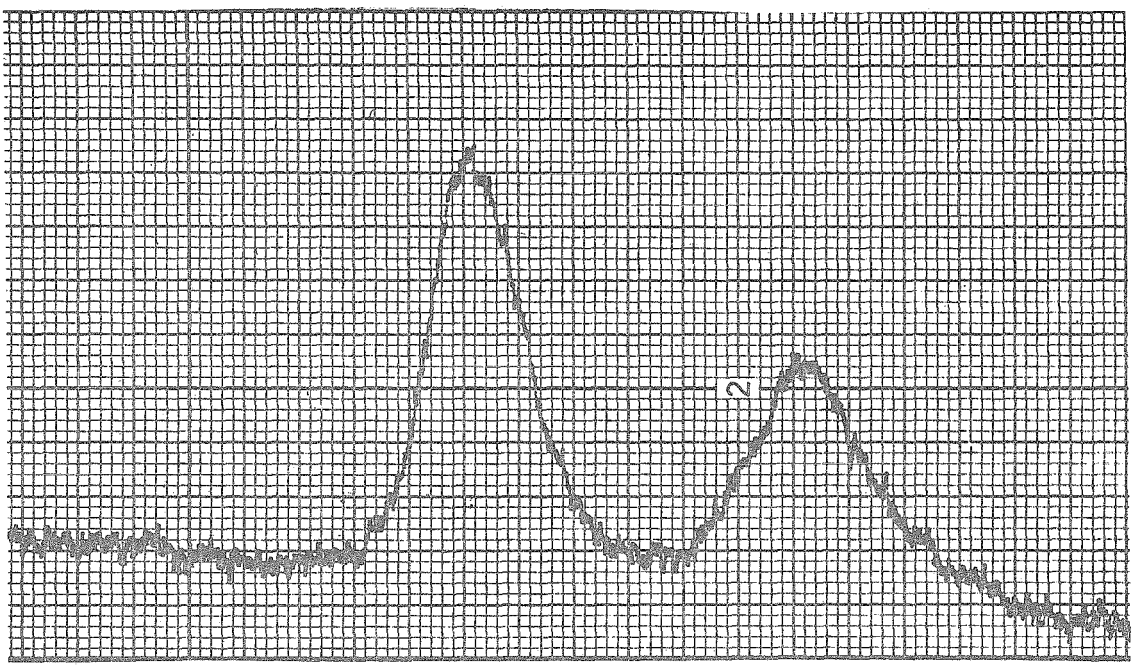

Figure 1

Figure 2. Distribution of $^{3}[H]$ versus fraction number in the preparative sedimentation velocity pattern of nicked circular dimer and nicked monomer M DNA. An aliquot of nicked M DNA (0.2 ml) was placed on 4.5 ml of CsCl, $\rho = 1.40$ g/ml, 0.01 Tris, pH 7.5, 150 μg/ml ethidium bromide and centrifuged for $3\frac{1}{4}$ hours at 45 Krpm in an SW50.1 rotor. The 33 S nicked circular dimers and 26 S nicked monomers were detected by fluorescence and were separated by approximately 4 mm in the centrifuge tube. The tube was dripped in 45 μl fractions on Whatman 3 mm filter papers and counted in a Packard Model 527 scintillation counter. One can estimate from the original fluorescence of the DNA bands that more than 0.2 μg of monomer DNA was counted. This corresponds to a specific activity of less than 3000 cpm/μg DNA.

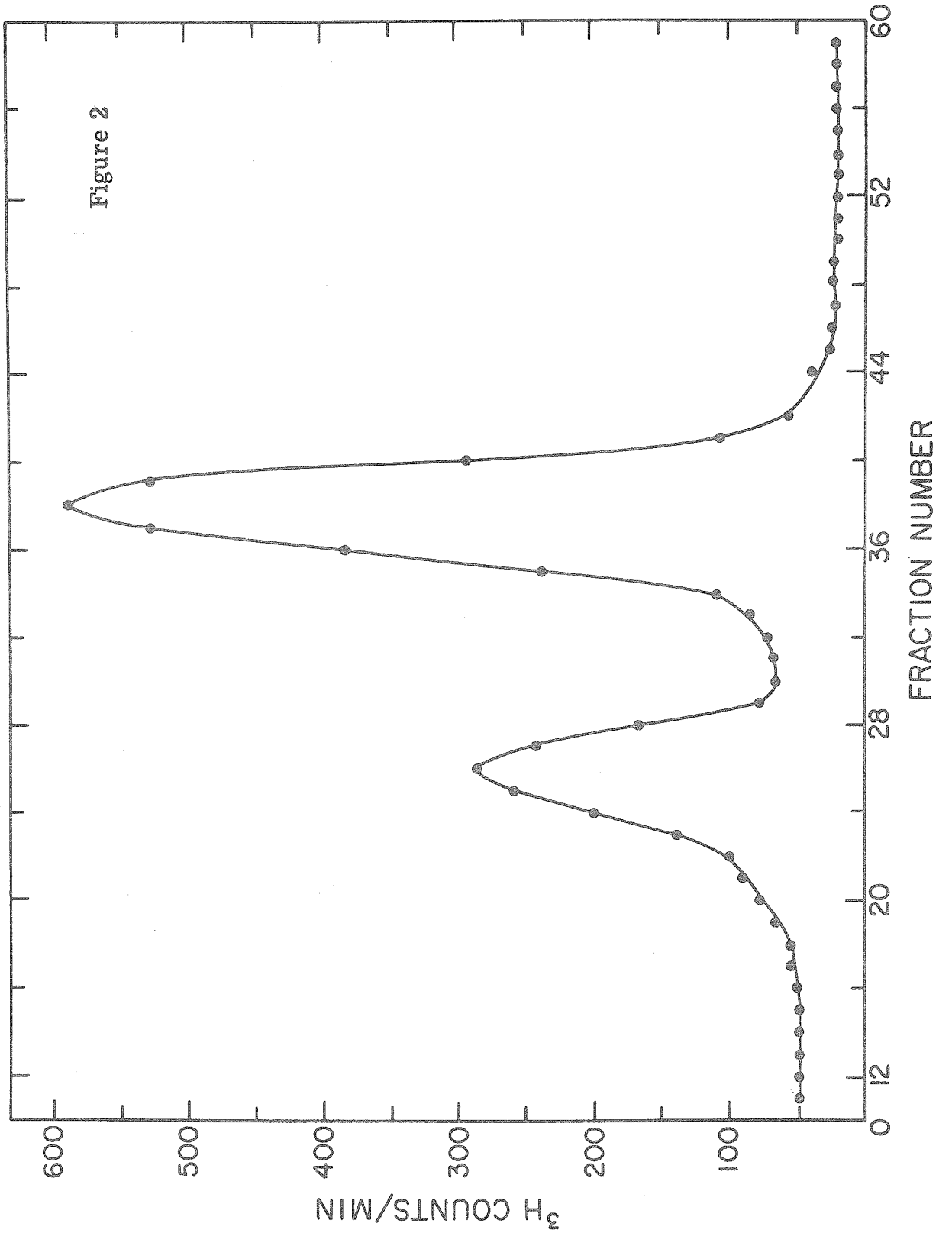
600
500
400
300
200
100
0
12
20
28
36
44
52
60
^3H COUNTS/MIN
FRACTION NUMBER

Figure 2

a uniform labeling of the circular dimer and monomer species. A similar experiment with a fourteen-hour ^{3}[H]thymidine exposure also revealed an essentially uniform labeling of the two species.

ADDITIONAL DISCUSSION

Evidence for the occurrence of the circular dimer M DNA species in leukemic myeloid cells has been presented and discussed. The interrelationship of this observation and the physiology of the leukemic cell is unknown. The Ph′ chromosome defect in chronic myelogenous leukemia is thought to be related to varying levels of the enzyme leukocyte alkaline phosphatase (8). This M DNA anomaly is one of form rather than of lost genetic information. In the next section the circular dimer is shown to be a double copy of the basic monomeric genome. Thus, the circular dimer may be discussed as a gene duplicate if the possibility of point mutational differences between the circular dimer and monomer is excluded. We have no data on the sequence homology between "normal" human M DNA and leukemic leukocyte M DNA. Such a study is now in progress to determine if the M DNA's have the same information.

A basic question concerns the mode of formation of the circular dimer species. The most obvious possibilities are: (a) altered replication of a monomer; (b) single recombinational event between monomers; (c) a cyclization phenomenon (9). Until there is evidence for sticky-ended linear M DNA molecules from mammalian mitochondria, (c) cannot be discussed. The rate and mechanism of recombination of mammalian M DNA are unknown due to a lack of any genetic markers. This problem is, therefore, difficult to approach at this time. It might be possible to distinguish between (a) and (b) with the following experiment which does not require a genetic marker system. One isolates

mitochondria from a cell that contains no circular dimers. These mitochondria are then introduced into a cell having M DNA of a different base composition and the ability to produce circular dimers. Assuming the mitochondria can pool resources the following observations can be made. If circular dimer formation is purely a replicative phenomenon, then the integrity of the dual population will be observed. If any recombination has taken place one should find circular dimers of intermediate base composition.

It is also conceivable that circular dimers represent an intermediate form in M DNA replication. This question could be approached by (a) blocking DNA replication and determining the effect on the frequency of circular dimers; (b) determining if circular dimer M DNA is localized in specific mitochondria or distributed when mixed M DNA populations are observed; (c) inducing (or discovering) a cell system in which all the M DNA is of circular dimer form. It is important to know if the circular dimer can perpetuate itself by replication. If one could establish that a particular cell line or particular mitochondrion had exclusively circular dimers, then it could be inferred that the dimers probably replicate. There is no proof that the total population of mitochondria are not constantly joining and breaking apart. This would imply that data from a single mitochondrion would have limited meaning. A more conclusive test would be the isolation of a replicating form of a circular dimer, perhaps similar to those isolated of monomer length (10).

A second question concerns the functional significance of the circular dimer. Mitochondrial RNA in HeLa cells is synthesized at a

high rate and possibly on a continuous basis (11). It is conceivable that M DNA in mammalian cells serves as a template for a vital cellular protein that is required throughout the cell's life cycle. The circular dimer could be advantageous in this case by providing twice as much mRNA per RNA polymerase initiation site. The high level of large transcription products after a short radioactive pulse in the HeLa system suggest that an extensive portion of the M DNA genome is expressed (11). It is known that in rat liver M DNA, one strand of the duplex is copied exclusively (12).

It is also possible that the circular dimer is providing a purely quantitative advantage in the malignant systems studied to date. This argument could be strengthened by demonstrating that there is more M DNA per cell in malignant tissue than in the appropriate control cell. Such a conclusion has been reached in certain induced tumor systems in rodents (13). It is not known whether this increase in M DNA is due to an increased number of monomeric molecules or to the existence of complex forms.

This question can be related specifically to the leukemic cell. Quantitative differences in mitochondria-associated enzymes between normal and leukemic cells have been reported (14, 15, 16). Differences in mitochondrial morphology and number have also been ascribed to the malignant leukocyte (17). The problems with much of these data are: (a) most differences in enzymatic activity are not consistent among different laboratories; (b) the results are not uniform for different patients with the same disease; and (c) the appropriate controls are

not employed. It is hard to imagine that one should not find differences between a mixed population of leukemic cells and mature cells from normal donors, as have been reported. Controls present an acute problem in myelogenous leukemia, as it is impossible at the present time to effect a clean separation of the various cell types in the myeloid series. Since the protein products from M DNA in mammalian cells are unknown, it is not possible to assign any reported difference in protein levels or occurrence to M DNA anomalies.

This work has provided one interesting clue to the role of the circular dimer. It seems well established that cytotoxic drug treatment of a patient afflicted with chronic myelogenous leukemia reduces the frequency of the circular dimer. We do not know whether the M DNA is connected with the etiological aspect of leukemia, or is merely the result of some other cellular aberration. In either event, the reduced frequency could be due to one or both of the following two factors: the leukemic cell is "cured" of circular dimers by some effect of therapy; the leukemic cell is destroyed by therapy and normal leukocytes proliferate. The most direct approach to answer this question would be to study the mode of action of the drug employed. In the case of chronic myelogenous leukemia, Myleran is the drug most often used. The exact role of Myleran in altering cell physiology is unknown; but it is clear, that as a bifunctional alkylating agent it could alter the DNA structure. Similarly, most of the drugs found to be effective in both chronic and acute myelogenous leukemia interfere with DNA function or biosynthesis. These facts do not answer the above question. Instead,

our attention must be directed to the cell population as a function of therapy.

Chronic myelogenous leukemia is a disease of the marrow elements which usually results in an altered peripheral blood cell distribution. Since all M DNA studies reported here utilized peripheral leukocytes, we cannot compare these results with kinetic studies that have utilized bone marrow specimens. Data on the peripheral leukocytes (18, 19, 20) indicate that: (a) Leukemic myeloid cells have a longer life span than normal leukocytes. (b) Generation times for leukemic myeloid cells are longer than for normal leukocytes. (c) Mitotic activity of immature myeloid cells in the peripheral system is inhibited. (d) A dual population of cells may be present, with one population having diploid character, and the other population displaying the Ph′ chromosome in the case of chronic myelogenous leukemia and aneuploidy in the case of acute myelogenous leukemia. (e) After chemotherapy the patient's peripheral myeloid cells may retain some type of chromosomal anomaly or a normal karyotype may prevail. By definition, the cell differential returns to normal values after successful treatment. The M DNA distributions before chemotherapy suggest that higher frequencies of circular dimers will be found in more immature cell populations. In contrast, the circular dimer frequency after long-term treatment in chronic myelogenous leukemia, and after short- and long-term treatment in acute myelogenous leukemia, is low, regardless of cell differential. This suggests that the cell population is "cured" of circular dimers upon treatment. It would be interesting to

correlate M DNA frequencies after treatment with chromosomal studies on the total cell population to eliminate the possibility that the frequency drop was merely a dilution effect.

The following areas are worthy of future investigation. (a) Analysis of the M DNA distribution when a patient escapes treatment and returns to the fully leukemic state should be made. (b) M DNA studies on bone marrow samples should be compared with marrow chromosomal analysis and peripheral blood data. (c) The study of M DNA distributions in lymphogenous leukemia should be completed. The first publication in this section reports the existence of circular dimers in a patient with chronic lymphogenous leukemia. This work is being expanded, since lymphocytes can be cultured more effectively than myeloid cells and are an extensively researched tissue. (d) The circular dimer form should be established in a tissue culture system. This would provide a continuing source of cells that could be subjected to experimental parameters not available in the in vivo leukocyte system. This is being actively pursued at the present time with the aim of answering some of the questions opened by the original observations of this new M DNA form. Investigators in this laboratory have screened a variety of existent cell lines and animal tumors for the circular dimer form. Most results have been negative, but the circular dimer was recently observed in a virus-transformed mouse cell (21) and in an established cell line from a mouse melanoma (22). It is hoped that this will expedite research in this particular area.

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D. Physical Properties of Circular Dimer Mitochondrial DNA.

This section is the preprint of

Homology and Structural Relationships
Between the Dimeric and Monomeric Circular Forms
of Mitochondrial DNA from Human Leukemic Leukocytes

by David A. Clayton, Ronald W. Davis, and Jerome Vinograd.

This paper has been accepted for publication by the
Journal of Molecular Biology.

1. Introduction

One species of complex mitochondrial DNA, the circular dimer, occurs in human leukemic leukocytes (Clayton & Vinograd, 1967, 1969) and in human tumors (C. A. Smith, private communication), but does not occur in a variety of normal tissues (Clayton, Smith, Jordan, Teplitz & Vinograd, 1968). As the name implies, the circular dimer was considered to be a structure containing two mitochondrial genomes. This provisional assignment was based on the observations that the contour length of the dimer was twice the length of the monomer and that the base compositions of the monomer and dimer were similar. The latter was inferred from the observation that a mixture of unequal amounts of the two species formed a symmetrical band in a buoyant cesium chloride density gradient. In this communication we report the results of experiments designed to answer the questions: Does the dimer consist essentially of two mitochondrial DNA (M DNA) genomes linked in tandem and arranged in the form of a closed circular duplex? If so, does the dimer contain deletions, insertions, or regions of nonhomology?

In these studies we compare monomers and circular dimers that occur in mitochondrial DNA preparations from peripheral leukocytes of a patient with chronic myelogenous leukemia. The mitochondrial DNA from this patient, M. C., contained approximately 30% circular dimers, 60% monomers, and 10% catenated oligomers. We find that the buoyant densities of the separated monomers and circular dimers are identical within experimental error, a result

that indicates that the base compositions of the two duplexes are the same within ± 1.0%. The buoyant densities in alkaline cesium chloride of the corresponding heavy and light complementary strands were also the same within the experimental error. This result indicates that the base compositions of the corresponding separate strands in the two forms are likely to be very similar.

The degree of homology between monomer and dimer M DNA was examined by both buoyant density and electron microscope procedures. A high molecular weight hybrid was formed upon annealing approximately equal amounts of light monomer and heavy dimer strands. The buoyant density of the hybrid is indistinguishable from that of the native duplex, indicating that a degree of homology greater than 90% exists between the circular dimer and the monomer forms.

A mixture of singly nicked monomers and singly nicked dimers was successively denatured and reannealed in formamide solutions. The products were then examined in the electron microscope by the procedures developed by Davis & Davidson (1968), Davis, Simon & Davidson (1969), and Westmoreland, Szybalski & Ris (1969) to detect single-stranded loops and bushes. The electron micrographs showed that continuous regions of heterology, or deletions or insertions, greater than approximately 100 base pairs, were absent. A new kind of circular DNA structure resembling a "figure 8" was formed in these experiments. We call this DNA species which contains one dimer strand and two monomer strands a fused dimer.

A circular dimer can, in principle, be formed from two

monomeric genomes in two different ways. One method leads to a head-to-tail tandem arrangement of monomer units (Fig. 1(a)). The other leads to a head-to-head arrangement (Fig. 1(b)). We have used electron microscope procedures to distinguish between these models.

2. Materials and Methods

(a) Isolation and purification of M DNA

Mitochondrial DNA was isolated from purified mitochondria of peripheral leukocytes obtained from a donor with untreated chronic myelogenous leukemia by methods reported previously (Clayton & Vinograd, 1969). The portions of the cesium chloride-ethidium bromide (CsCl-EB) gradients subtended by the upper and lower bands were collected at weekly intervals and pooled (fraction A). An alkaline buoyant analysis of fraction A showed that there was less than 5% nuclear DNA present in the pooled unfractionated M DNA sample. The pooled samples were again centrifuged to equilibrium and collected into an upper band (fraction B) and a middle and lower band (fraction C).

(b) Separation of circular dimer and monomer M DNA

Circular dimers were separated from monomers by preparative sedimentation velocity in CsCl-EB (Watson, Bauer & Vinograd, 1969). Fraction B, containing about 3 μg of nicked M DNA, was

Figure 1. Representation of the two modes of formation of the circular dimer from two monomers. (a) Head-to-tail tandem arrangement of monomer units. (b) Head-to-head arrangement of monomer units.

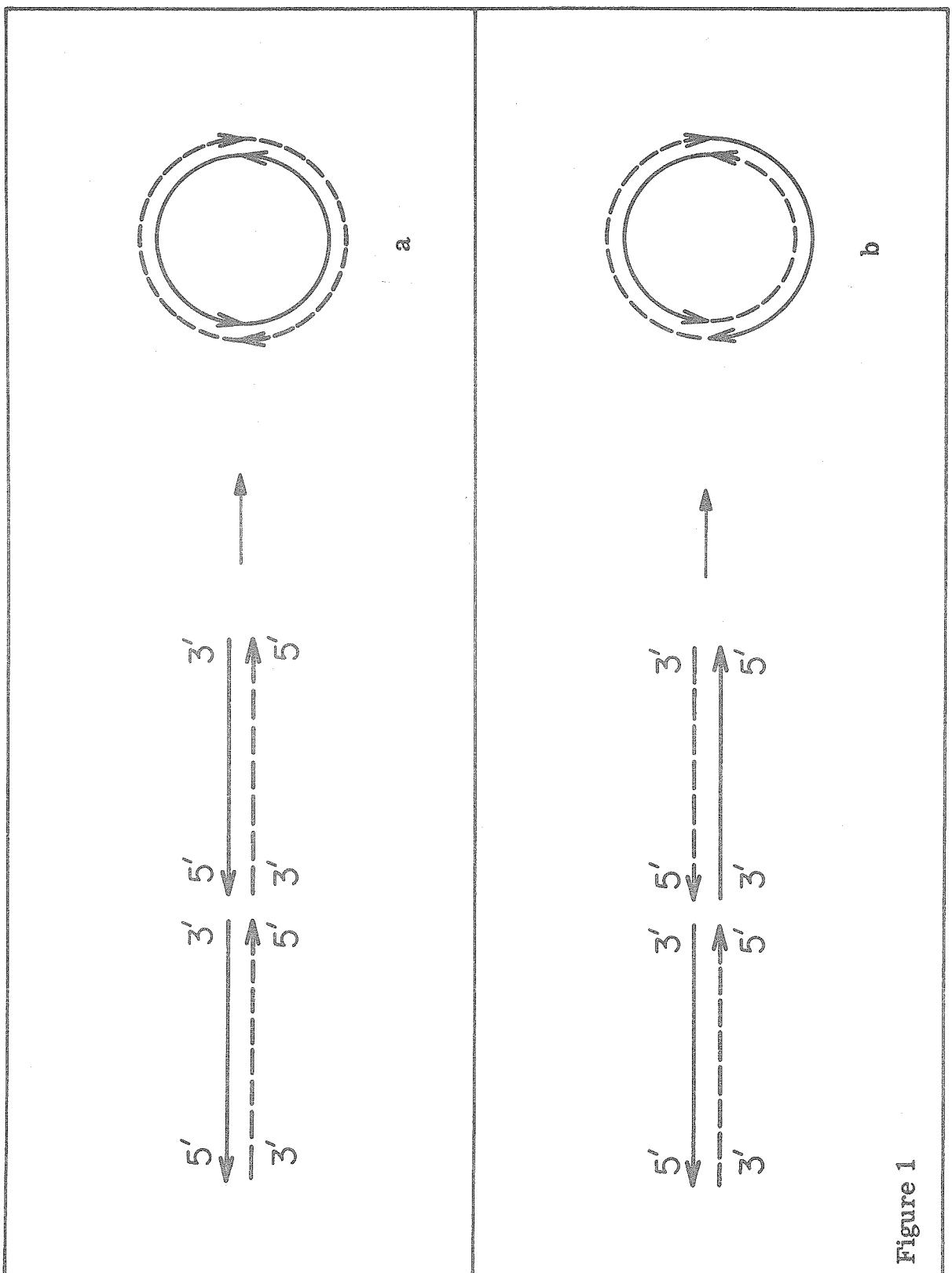

Figure 1

diluted to 5 ml. with 0.01 M-Tris, pH 7.5, and 0.001 M-EDTA, and centrifuged for 12 hr at 40 Krpm in an SW50.1 rotor. The M DNA pellet was allowed to resuspend in 0.4 ml. of supernatant and placed on 4.5 ml. of CsCl, ρ = 1.40 g/ml., 0.01 Tris, pH 7.5, 150 μg/ml. EB, and centrifuged for $3\frac{1}{4}$ hr at 45 Krpm in an SW50.1 rotor. The 33 S nicked circular dimers and 26 S nicked monomers were detected by fluorescence and were separated by approximately 4 mm in the centrifuge tube (Fig. 2). The two M DNA species were isolated by drop collection and the EB removed by passage through a small column of Dowex-50 cation exchange resin. The small amount of heterogeneous nuclear DNA was removed in this preparative velocity experiment (Fig. 2), as is shown in the buoyant analyses of fractions B_1 and B_2 (Fig. 3).

Purified closed circular dimer DNA was obtained from fraction C in a similar sedimentation velocity experiment. The closed dimer (fraction C_1) was freed of dye and concentrated as described above.

Examination of the purified nicked circular dimers (fraction B_2) in the electron microscope revealed 85% circular dimers, 12% monomers, and 3% ambiguous and catenated dimers. A similar analysis of the purified nicked monomer sample (fraction B_1) gave 97% monomers and 3% circular dimers. These samples were used for the determination of the neutral and alkaline buoyant densities and for the hybridization experiments analyzed by centrifugation as described below.

Figure 2. The separation of nicked circular dimer (lower band) and nicked monomer (upper band) M DNA after velocity sedimentation in a cesium chloride-ethidium bromide solution. Centrifugation conditions are described in Materials and Methods, section (b). The tube was photographed in ultraviolet light.

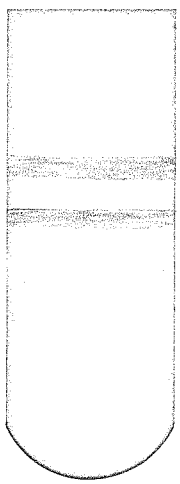

Figure 2

(c) Analytical centrifugation

Buoyant density experiments were performed in Beckman model E ultracentrifuges equipped with photoelectric scanners at 44,000 or 44,770 rev./min at 25°C for 24 hr. Neutral and alkaline buoyant densities were calculated from the distances between the M DNA peaks and a crab dAT marker. In these calculations the buoyant density of the marker was taken to be 1.669_6 g/ml. at neutral pH (Bauer & Vinograd, 1968) and 1.728 g/ml. at high pH. The buoyant density gradient (Vinograd & Hearst, 1962) was used in the calculations.

The band sedimentation velocity experiment was performed in 2.85 M-CsCl, pH 12.5, 20°C, 35,600 rpm and recorded with the photoelectric scanning system in a Beckman model E ultracentrifuge.

(d) Hybridization observed in the electron microscope

The nicked circular M DNA mixture used for the electron microscope hybridization studies was prepared by illuminating a cellulose nitrate SW50 tube containing a single band of freshly prepared closed circular M DNA with a 100W incandescent light bulb at 8 inches for 36 hr. The tube contained 300 μg/ml. EB and 4.5 M-CsCl, 0.01 M-Tris, pH 7.5, that had been centrifuged at 43 Krpm for 24 hr. The closed DNA had been isolated as a lower band and had been rebanded in a second tube. Approximately 50% of the closed circular M DNA was photochemically nicked as estimated by the ratio of upper to lower band material upon recentrifugation to equilibrium in

CsCl-EB. The DNA from the upper band material (fraction D_1) was freed of EB and pelleted as described above. The foregoing procedures were designed to prepare an M DNA sample in which all molecules were nicked at least once, and seldom twice, as discussed in the results.

Approximately 0.05 μg of M DNA (fraction D_1) in 10 μl. was denatured by dialysis against 95% formamide and 0.01 M-EDTA at pH 8.3 for 1 hr at 24°C, and was reannealed by dialysis for $2\frac{1}{2}$ hr against 50% formamide, 0.20 M-Tris, pH 8.0, 0.02 M-EDTA. The reaction was then quenched by dialysis for 2 hr against 0.1 M-NaCl and 0.01 M-EDTA, pH 7.3. The dialysis mixture was divided into two samples. One sample was adjusted to contain 0.1% cytochrome C and 0.5 M-NH_4Ac at pH 7.0 in a final volume of 50 μl. This sample was then spread onto 0.25 M-NH_4Ac at pH 7.0. (This procedure will be referred to as the aqueous technique.) The second sample of renatured DNA was adjusted to contain 0.1% cytochrome C, 0.1 M-NH_4Ac, 0.01 M-Tris at pH 8.0, and 40% formamide. The latter sample was then spread onto 0.01 M-Tris at pH 8.0 and 10% formamide. (This procedure will be referred to as the formamide technique.) The films were picked up on Parlodion-coated specimen grids and were either stained with uranyl acetate (Davis & Davidson, 1968) or shadowed with platinum-palladium (Pt-Pd).

Nicked circular PM2 viral DNA, prepared by the method of Espejo & Canelo (1968), was a gift from R. Watson. The sample obtained as an upper band from a CsCl-EB density gradient was

lightly nicked and contained on the average about three single-strand scissions per molecule. This was shown in an analytical sedimentation velocity experiment in alkaline CsCl, pH 12.5. Approximately 20% of the DNA was in the form of single-strand circles and approximately 50% as single-strand linears. The rest of the material sedimented as smaller molecular weight linear species. Denaturation, reannealing, and specimen preparation was performed as described above.

Electron microscopy was routinely employed as a check on the form of the M DNA samples. Specimen grids of native duplex M DNA were prepared as described previously (Clayton & Vinograd, 1967). A Philips EM 300 electron microscope equipped with an auxiliary Plumbicon TV system was used.

(e) Reagents, enzymes, and marker DNA

Optical grade CsCl from the Harshaw Chemical Company, Cleveland, Ohio, and ethidium bromide from Boots Pure Drug Co., Ltd., Nottingham, England, were used without further purification. All other chemicals were reagent grade. DNase I and RNase A used in the mitochondria isolation were purchased from the Sigma Chemical Company, St. Louis, Missouri. Cytochrome C (Lot 45461) was purchased from Calbiochem, Los Angeles, California. Crab dAT isolated by the Cs_2SO_4-$HgCl_2$ method (Davidson et al., 1965) from Cancer antennarius sperm was a gift from R. Hyman.

(f) Measurement of pH

A Beckman Research model pH meter with an E. H. Sargent combination small-probe glass electrode was used. The pH meter was standardized at 24° C with Beckman saturated $Ca(OH)_2$ buffer at pH 12.63 before the pH determinations of alkaline CsCl solutions were performed. The buffer was re-read after a determination. The drift did not exceed ± 0.05 pH unit.

3. Results

(a) Neutral buoyant densities of circular dimer and monomer M DNA

It is well known that there is usually a correspondence between the buoyant density of DNA and its base composition (Schildkraut, Marmur & Doty, 1962). We have previously reported a buoyant density for leukocyte M DNA of 1.700 g/ml. and a corresponding calculated base composition of 46 mole percent guanine-cytosine. This analysis was performed on a mixture of M DNA forms. Figure 3 presents scans of absorbance profiles of analytical cells containing the separated circular dimers and monomers. A marker DNA, crab dAT, was present in each experiment. The calculated buoyant densities of the monomer and the circular dimer species, 1.6996 ± 0.0005 g/ml., are in good agreement with the value reported earlier for the mixture. The overall base compositions of the circular dimer and monomer are, therefore, identical within the experimental error

Figure 3. Photoelectric scans of (a) purified monomeric and (b) purified circular dimeric leukemic leukocyte M DNA with a crab dAT marker in neutral buoyant cesium chloride, 25°C, 44, 770 and 44, 000 rpm, respectively. The field is directed to the right. The light band contains dAT and the dense band M DNA.

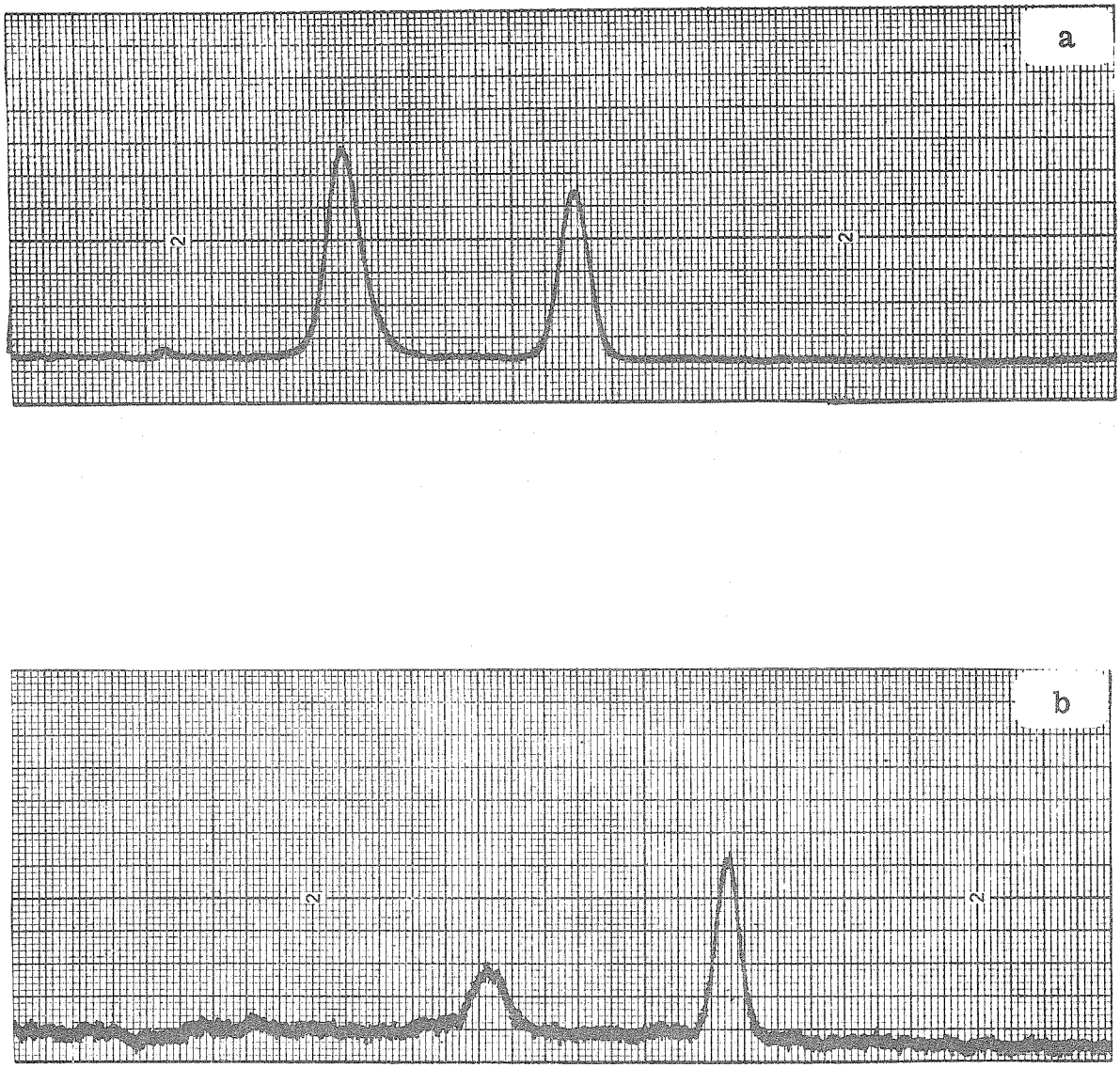

Figure 3

limits of ± 1.0 mole percent guanine-cytosine (GC). A difference of 0.001 g/ml. in buoyant density corresponds to a difference of 1 mole percent GC (Schildkraut, Marmur & Doty, 1962).

(b) Alkaline buoyant densities of the complementary strands in circular dimer and monomer M DNA

Centrifugation of nicked circular or linear DNA in buoyant alkaline (pH 12.5) CsCl usually gives a unimodal distribution of DNA at a higher density than the native duplex, due to the titration of guanine and thymine residues and subsequent loss of the duplex structure (Vinograd, Morris, Davidson & Dove, 1963). Human M DNA in such experiments forms a bimodal distribution. Each band contains one of the two M DNA complementary strands (Corneo, Zardi & Polli, 1968). The difference in buoyant density of the two strands is thought to be principally the result of a difference in the guanine plus thymine content of each strand. Scans of the buoyant profiles of purified circular dimers (fraction B_2) and purified monomers (fraction B_1) are presented in Figure 4. The buoyant densities of the light strand and heavy strand of the circular dimer were calculated from results of an experiment in which crab dAT was added as a density marker (Fig. 4(a)). The light and heavy strands are 1.738 and 1.779 g/ml., respectively. The separation between the complements is the same, 0.041 g/ml., in the dimer samples with and without the marker (Figs. 4(a) and (b)). The separation between complementary strands in the preparation of monomers is also 0.041 g/ml. (Fig. 4(c)). In Figure 4(d) the marker obscured the small amount of light monomer

Figure 4. Photoelectric scans of leukemic leukocyte M DNA in buoyant alkaline cesium chloride, pH 12.5, 0.05 M-K_3PO_4, 25°C. The field is directed to the right. (a) The least dense band is crab dAT. The band at intermediate density is the light strand of purified circular dimer M DNA (fraction B_2). The dense band is the heavy strand of purified circular dimer M DNA. (b) The light strand and heavy strands of purified circular dimer M DNA (fraction B_2). (c) The light and heavy strands of purified monomers (fraction B_1). (d) A mixture of purified monomers (fraction B_1) and crab dAT. The M DNA light strand is observed as a shoulder on the dense side of the marker. The heavy strand forms a broad band near the center of the pattern (↓). All of the experiments were at 44,770 rpm, except (c), which was at 44,000 rpm.

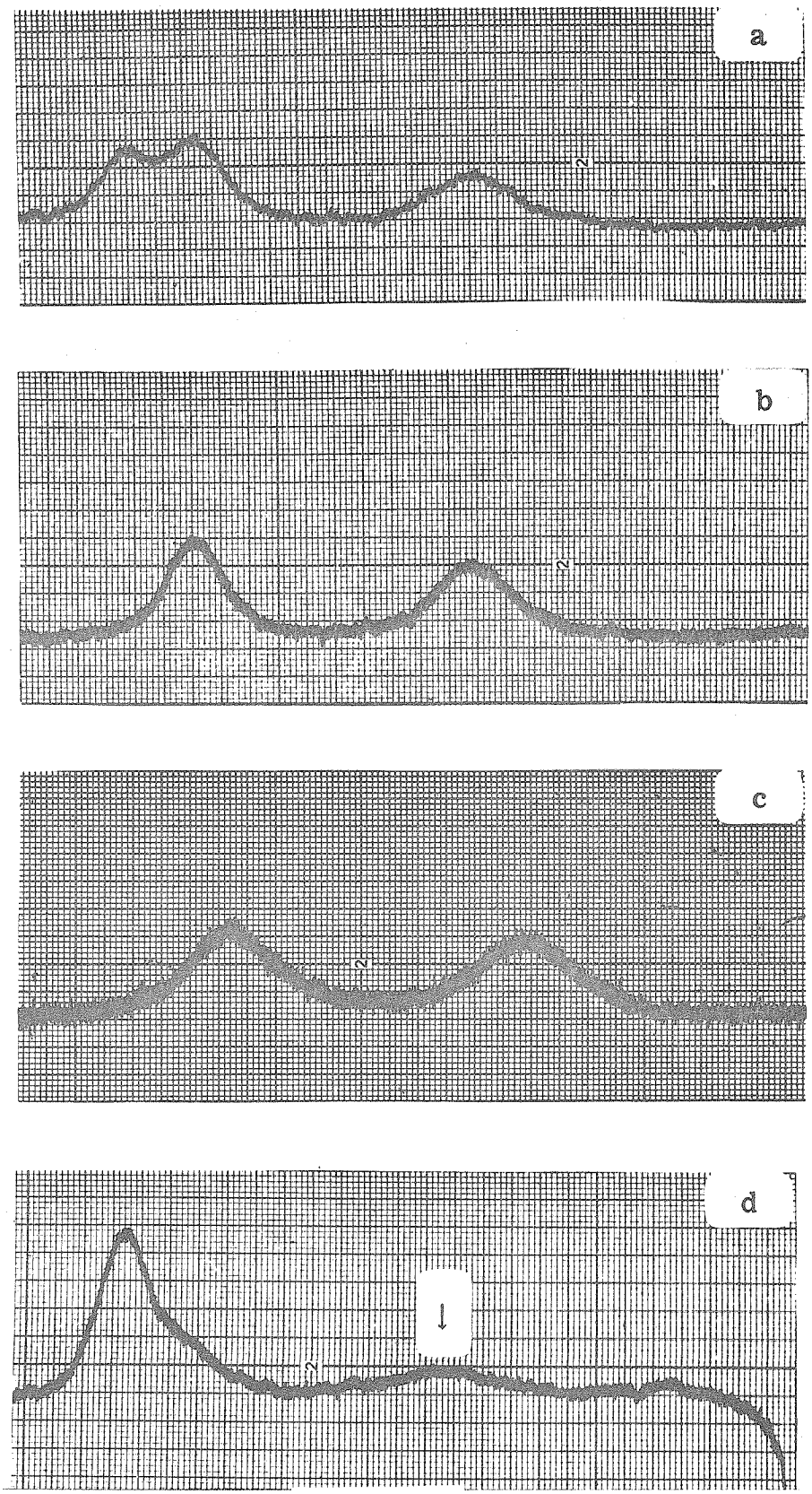

Figure 4

strand present. The maximum in the center of the diagram corresponds to 1.779 g/ml. for the heavy monomer strand. The buoyant density of the light monomer strand, 1.738 g/ml., was calculated from the separation between strands observed in Figure 4(c) and from the value for the heavy strand in Figure 4(d). We conclude that the base compositions of the corresponding circular dimer and monomer complementary strands are likely to be very similar, if not identical.

It might be argued that the presence of the light and heavy bands (Figs. 4(a) and (b)) in the buoyant pattern obtained with dimer DNA at high pH and the absence of bands midway between them rules out the head-to-head structure (Fig. 1(b)) for the dimer. Each of the dimer strands in this model, if intact, should have a buoyant density close to the mean of the heavy and light monomer strands. Unfortunately, the mitochondrial DNA strands cannot be assumed to be full-length in these experiments because of the well known sensitivity of M DNA to alkali. The molecular weight, calculated from the band width at the six-tenths height, is about one-third that expected for full-length strands. Such fragmented dimer strands, if derived from the head-to-head structure, should form skewed bands at the positions of heavy and light monomer strands and elevate the base line between the bands. The bands, however, are fairly symmetrical and there is little material between. It can also be argued that material with intermediate buoyant density would be entirely absent if the two bonds joining the monomeric genomes were

were specially alkali sensitive, a possibility which has not been ruled out. The electron microscope studies (Materials and Methods, section (d)), performed with intact strands, show that the model in Figure 1(b) cannot be correct. The centrifuge results cited above are consistent with this conclusion.

(c) The reannealing of light monomeric and heavy dimeric M DNA complements

A direct analysis of the ability of circular dimer single strands to hybridize with monomer single strands can be obtained by examining the buoyant density patterns of artificial mixtures of heavy dimer and light monomer strands that have been subjected to reannealing conditions. The heavy strand of purified circular dimers and the light strand of purified monomers were isolated by alkaline buoyant density centrifugation in the preparative ultracentrifuge.

Approximately 0.5 μg of nicked circular dimer (fraction B_2) and 0.5 μg of nicked monomer (fraction B_1) M DNA were placed in separate alkaline buoyant CsCl solutions, 1.760 g/ml., 0.05 M-K_3PO_4, pH 12.5, in Polyallomer tubes. In addition, approximately 0.5 μg of previously isolated light strands of [^{3}H]HeLa M DNA was mixed with the circular dimer sample and approximately 0.05 μg of heavy strands of [^{3}H]HeLa M DNA was mixed with the monomer sample. The specific activity of the HeLa DNA strands was approximately 10^5 cpm/μg. The corresponding buoyant densities of

complementary strands of HeLa M DNA and leukemic leukocyte M DNA in alkali are known to be similar (R. L. Hallberg, private communication). The alkaline gradients were centrifuged for 65 hours at 20°C and at 31.5 Krpm in an SW50.1 rotor. Forty-μl. fractions were collected, and 5-μl. aliquots were counted to locate the position of the [^{3}H]HeLa M DNA strands (Fig. 5). The fractions containing the separate strands of the circular dimer and monomer leukocyte M DNA were appropriately pooled (Fig. 5), and either cross-hybridized or "self-annealed" by dialysis against 50% formamide, 0.20 M-Tris, pH 7.5, 0.01 EDTA for ten days. The samples were dialyzed against 0.50 M-NaCl, 0.01 Tris, pH 7.5, and 0.005 EDTA to remove the formamide and then pelleted for twelve hours at 40,000 rev./min in an SW50.1 rotor as described earlier. The pellet was suspended in concentrated CsCl solution, 0.01 M-Tris HCl, pH 7.5, and examined at buoyant equilibrium in the analytical ultracentrifuge.

The resultant hybrid molecules formed a single sharp band with a buoyant density of 1.700 g/ml. (Fig. 6(a)). This value is the same as that obtained for both the monomeric and dimeric native duplex M DNA's. We conclude that a high molecular weight duplex species has formed. This species contains heavy strands from the circular dimer and light strands from the monomer. No banded material was observed at positions for neutralized single strands of light monomer, 1.707 g/ml. (Fig. 6(b)), or heavy dimer.

The self-annealed light strands of the circular dimer were examined as a check for contamination and for any evidence of self-annealing (Fig. 6(b)). The material formed a band with a buoyant

Figure 5. [^{3}H]HeLa light and heavy M DNA complements were used to detect the position of light and heavy M DNA complements in leukocyte M DNA, as described in the text. Figure 5(a) is a radioactive pattern of HeLa M DNA complements obtained by R. L. Hallberg in this laboratory using the conditions employed in (b) and (c). The peaks have a separation of 13 fractions. Since the monomer sample, (b), has a band maximum for the [^{3}H]HeLa heavy strand at fraction 20, we expect the band maximum for the light leukocyte monomer strand to be located at fraction 33. Similarly for the circular dimer sample, (c), the band maximum for the [^{3}H]HeLa light strand was found at fraction 32. The band maximum for the heavy dimer strand is expected to be at fraction 19. Therefore, fractions 30 through 40 in (b) were pooled and used as the monomer light-strand sample and fractions 12 through 22 in (c) were pooled and used as the dimer heavy-strand sample. The light dimer strand (fractions 24 through 40 in (c)) was isolated and used as a control.

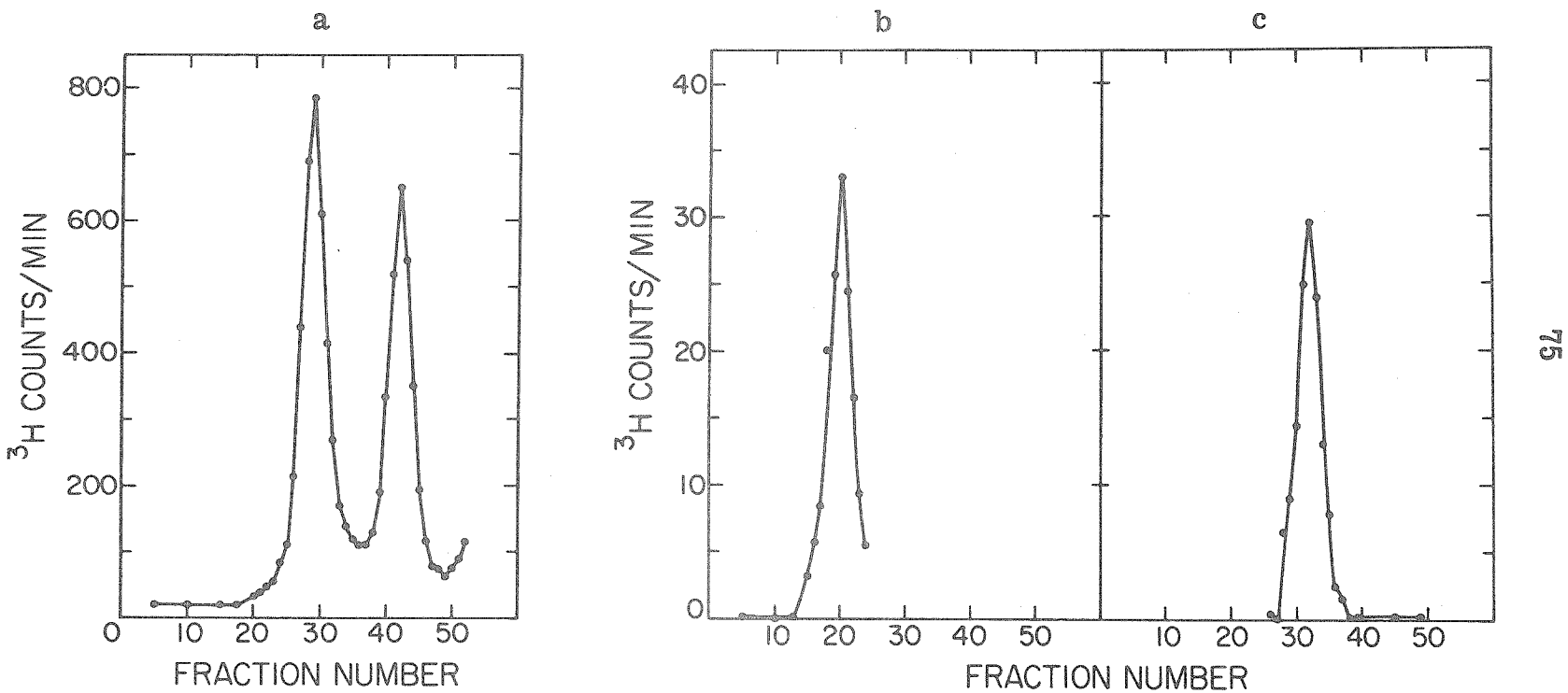
a
b
c
800
600
400
200
0
40
30
20
10
0
10 20 30 40 50
^{3}H COUNTS/MIN
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Figure 5

Figure 6. Photoelectric scans of annealed M DNA in neutral buoyant cesium chloride at 44, 770 rpm, 25°C. The field is directed to the right. The light band in each case is crab dAT added as a marker. (a) Annealed mixture of approximately equal amounts of heavy dimer strand and light monomer strand plus crab dAT. (b) Self-annealed light dimer strand plus crab dAT. (c) Annealed mixture of denatured monomer and dimer M DNA plus crab dAT. The mixture contained 88% dimer and 12% monomer.

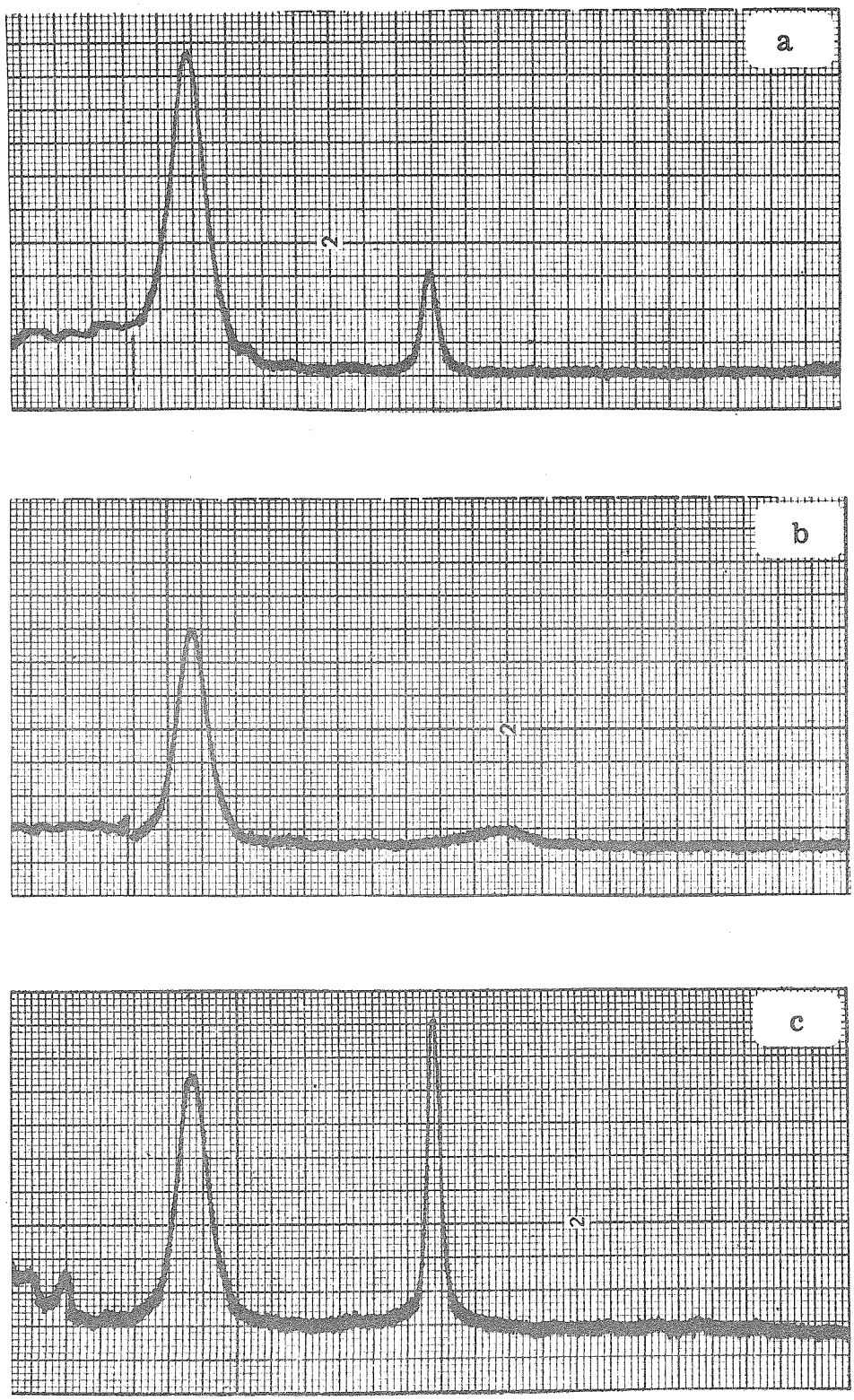


Figure 6

density of 1.707 g/ml. The light strand of HeLa M DNA forms a band at neutral pH with the same buoyant density (R. L. Hallberg, private communication). No contaminating heavy single-stranded DNA or duplex DNA was detected. The sample of 88% dimers and 12% monomers from the alkaline buoyant density experiment in Figure 4(b) was neutralized, annealed, and centrifuged in a neutral buoyant CsCl gradient. The hypersharp band with a buoyant density of 1.700 g/ml. (Fig. 6(c)) indicates that a mixture of annealed dimer and monomer strands regains a duplex structure analogous to the hybrid molecules in Figure 6(a).

In view of the similarity of the neutral and alkaline buoyant behavior between leukocyte and HeLa M DNA, we take 1.707 g/ml. as the buoyant density of the neutralized light strand (Fig. 6(b)) and 1.726 g/ml. as the buoyant density of the heavy strand (R. L. Hallberg, private communication). The mean, 1.717 g/ml., is 0.017 g/ml. higher than the value for the native duplex. The hybrid structure with the same buoyant density must, therefore, within experimental error, consist of pure duplex. Regions of nonhomology or imperfect annealing in the concatenate should have raised the buoyant density of the hybrid. We estimate the uncertainty in $\Delta\Theta$ in these experiments to be about ± 0.001 g/ml. Based on this uncertainty and assuming linearity between the fractional duplex content and the fractional buoyant increment (Rownd, Lanyi & Doty, 1961), we estimate that at least 90% of the mass of the buoyant species is in a duplex structure. We conclude, therefore, that any heterologous regions, if present,

cannot exceed 10% of the total in the monomeric and dimeric M DNA forms. A summary of the sedimentation properties of circular monomeric and dimeric forms is given in Table 1.

(d) Electron microscopy of reannealed M DNA

The apparent identity of base compositions of the circular dimer and monomer forms of leukemic leukocyte M DNA and the existence of at least 90% homology between the two forms suggests that the circular dimer is essentially a double-sized copy of the monomer genome. To test this hypothesis further, we have employed the electron microscope technique for the detection of heterologous regions in reannealed DNA.

Heteroduplex molecules containing one strand from one M DNA molecule and the complementary strand from another were prepared by renaturing a mixture of denatured (fully strand-dissociated) monomers and dimers. Prior to denaturation at least one single-strand scission was introduced into each DNA molecule. Since a second scission in a strand of the duplex results in fragmented single strands which interfere with the analysis, this study was carried out with a mixture of lightly nicked molecules (fraction D_1). The composition of this fraction is given in Table 2. All of the duplex forms contained at least one scission, as indicated in Materials and Methods, section (d). We have calculated with the aid of the Poisson relation that at least 88% of the monomeric and

TABLE 1

Sedimentation properties of the circular monomeric and dimeric forms of human leukemic leukocyte M DNA

	Monomer	Dimer
$\underline{s}^0_{20,w}$, closed, Svedbergs	—	51.6 ± 0.7*
$\underline{s}^0_{20,w}$, nicked, "	25.6 ± 0.4†	33.1 ± 0.5‡
$\underline{s}^0_{20,obs,alk.}$, closed, "	80**	112**
Θ, g/ml., duplex, neutral	1.700	1.700
Θ, " , light strand, alk.	1.738	1.738
Θ, " , heavy strand, "	1.779	1.779
Θ, " , light strand, neutral	—	1.707

* Clayton & Vinograd (1967). †Previously unpublished data. ‡Hudson, Clayton & Vinograd (1968). The sedimentation solvent was 2.85 M-CsCl at 20°C. The sedimentation coefficients have been fully corrected to standard conditions and are expressed as values for the sodium form.

** Clayton & Vinograd (1967). These sedimentation coefficients are the observed uncorrected values in 2.85 M-CsCl, 0.05 M-K_3PO_4, pH 12.5, 20°C.

TABLE 2

Electron microscope frequency analysis of M DNA forms before denaturation and after annealing

Form	Before denaturation (%)*	After annealing (%)
Circular monomers	71	75
Circular dimers	24 ± 3	4 ± 2
Catenated dimers	3.5 ± 1	9.5 ± 2†
Tailed molecules	0	3 ± 1
Linear duplexes	1.5 ± 1	1 ± 0.9
Ambiguous molecules	0	8.5 ± 2
Total no. of molecules classified	800	800

* The error values indicate the interval which contains the true mean at a level of confidence of 95%.

† Most of these molecules are considered to be fused dimers, as described in the text.

80% of the circular dimeric strands were present during the re-annealing experiment as full-length linear or circular single strands. The remainder must have been present as linear fragments of varying size.

In order to avoid introducing extra single-strand scissions, which occur at high pH or at elevated temperature, the M DNA was denatured and renatured with formamide. A micro-procedure, using only 0.05 μg of DNA, was employed. The renatured DNA sample was mounted on a specimen grid for electron microscopy by the formamide technique (Davis, Simon & Davidson, 1969). The formamide was incorporated into the mounting solution to melt out the random base interactions of single-stranded DNA. The heterologous regions are more readily detected with this mounting procedure than with the aqueous technique. Several kinds of duplex DNA's, but little single-stranded material, were observed. Plate I shows a typical field as viewed in the electron microscope. All duplex molecules were examined for heterologous regions (Figs. 7(a) and (b)). Several hundred molecules were studied, but no heterologous regions were seen in any of the duplex forms. We conclude that the sequence similarity between the M DNA's is so great that no detectable heterologous regions occur. It has been our experience that deletions, additions, or substitutions as small as 50 to 100 base pairs can be detected by this procedure.

In a separate experiment to test if the procedure used here does indeed denature the DNA and allow for strand dissociation, the

Plate I. Typical field of renatured M DNA as viewed in the electron microscope. Three circular duplex molecules of monomer length, a circular duplex molecule of two monomer lengths, a single-stranded circular monomer molecule (upper-right corner), and a linear duplex molecule of slightly less than monomer length and with single-stranded ends (lower-right corner) are shown. The DNA was mounted on specimen grids by the formamide technique and shadowed with Pt-Pd.

Plate I

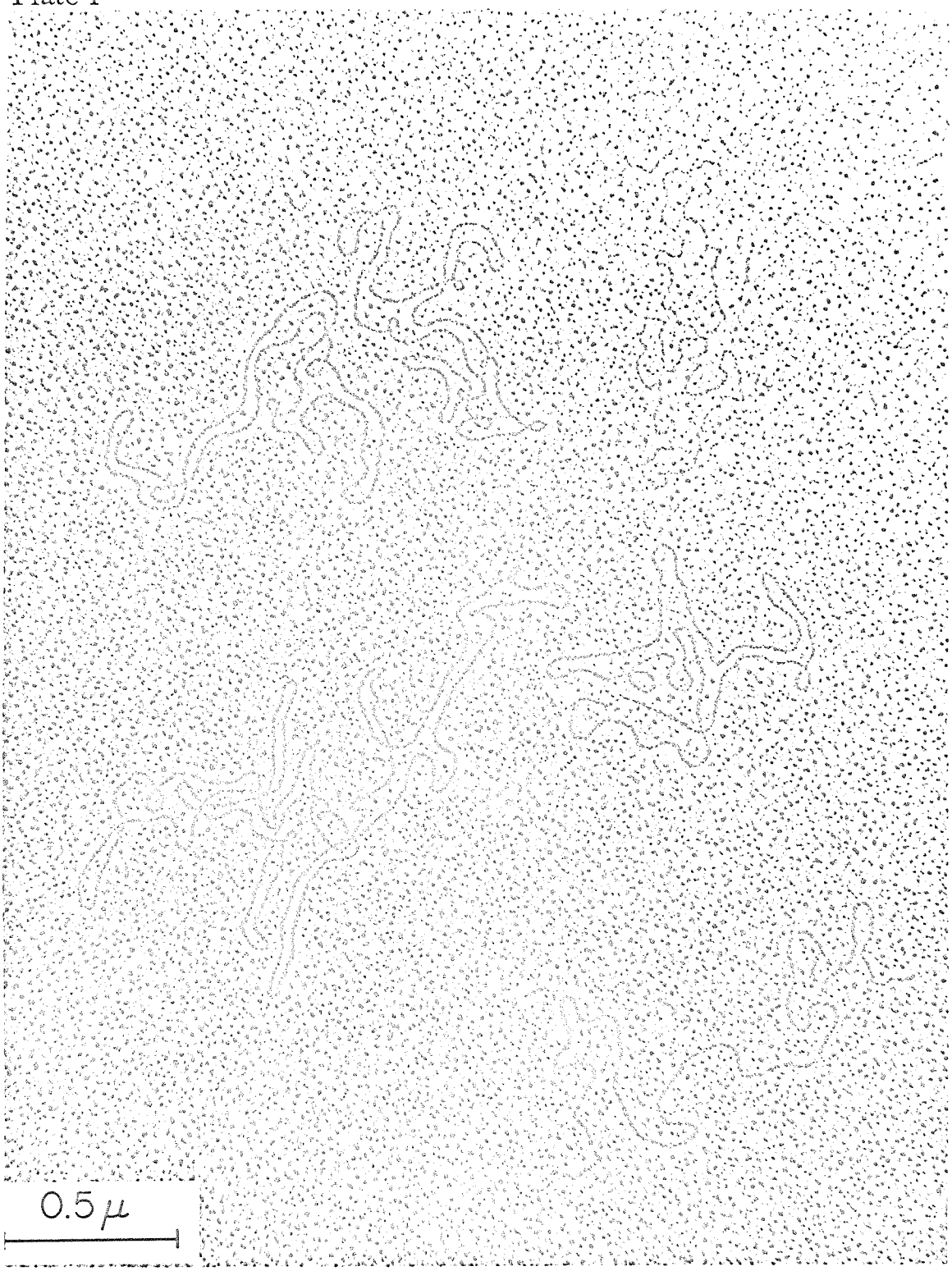


Figure 7. Representations of three ways of forming a nonduplex region. (a) A region of nonhomology. (b) An insertion or deletion. (c) A gap region.

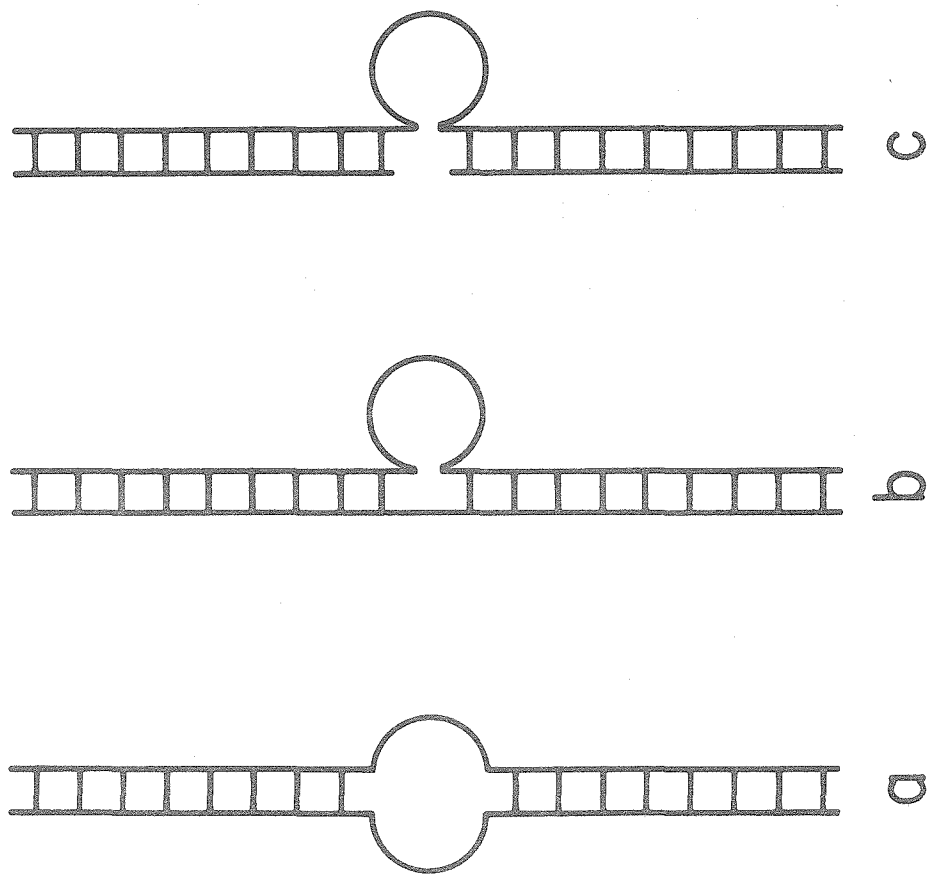


Figure 7

reannealing step was omitted after denaturation. The resulting sample contained only 6% of the DNA molecules in the form of circular duplex monomers. These were presumably renatured molecules that formed during the quenching step. The remainder were identified from their appearance as single strands. We conclude, therefore, that the complementary strands in the mixture experiment had separated from each other upon denaturation and had reformed duplexes on reannealing.

In order to visualize the topology of the duplex forms more readily, the reannealed mixture of DNA's was also mounted on specimen grids for electron microscopy by the aqueous technique. The formamide technique often causes acute tangling of the DNA and makes topological identification difficult. The duplex molecules were scored as monomers, dimers, etc. Plate II shows the three major classes of duplex molecules found in this study. It should be recalled that single strands of DNA, when mounted by the aqueous techniques, collapse into what appears to be a "bush" due to random base interactions.

If no hybridization between monomer and dimer strands had occurred, we should have found monomer and dimer circular duplexes and catenanes in approximately the same proportion as in the original sample. We should also have found some linear duplexes formed from originally circular duplexes in which both strands were nicked. Most of the latter kind of molecules should have cyclized in these experiments. We did observe, however,

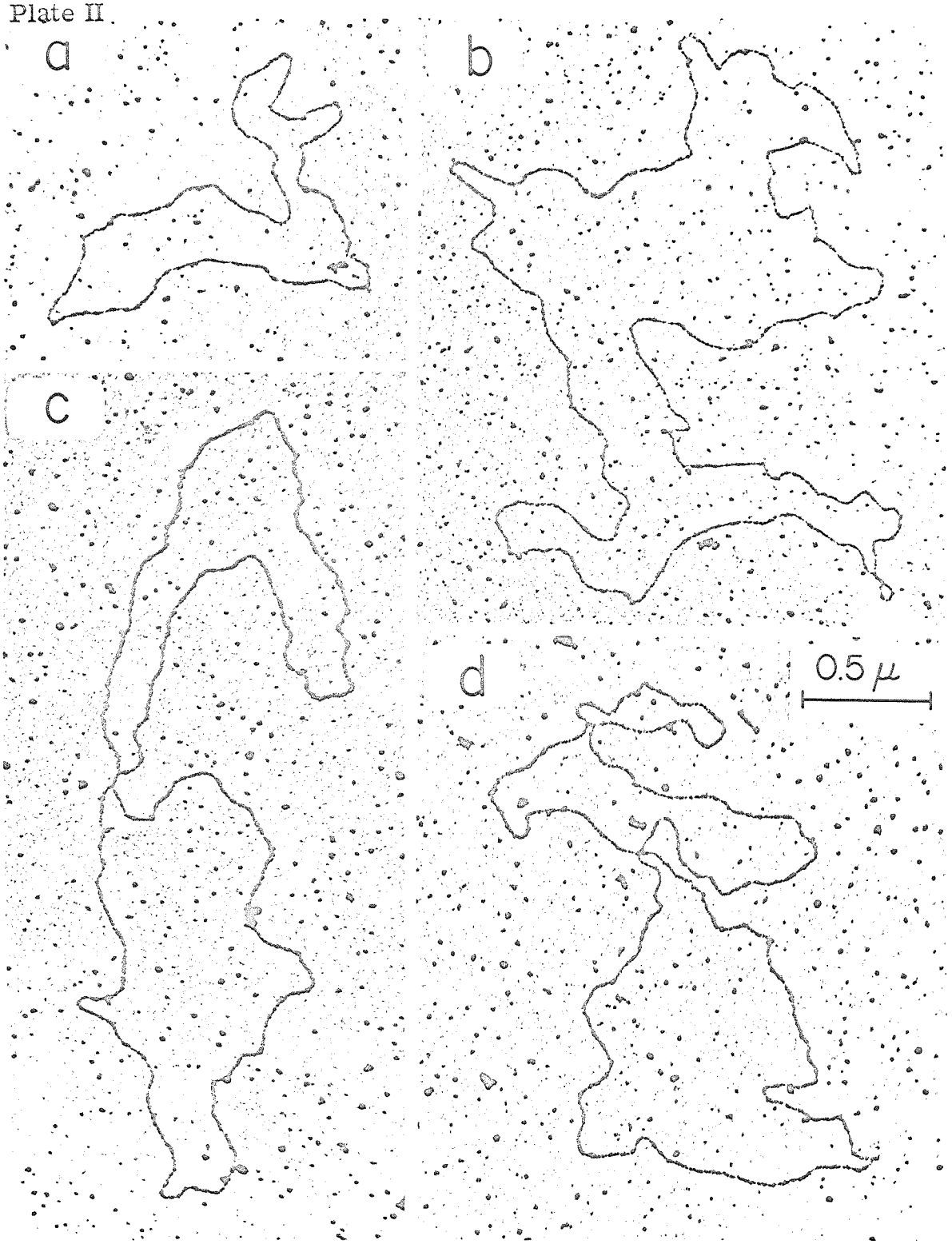
Plate II. Major classes of renatured M DNA. (The DNA was mounted on specimen grids by the aqueous technique and stained with uranyl acetate.)

(a) Renatured circular monomer.

(b) Renatured circular dimer.

(c) and (d) Renatured "figure 8" molecules. Such molecules are considered to be fused dimers, as explained in the text.

Plate II



that the frequency of the circular dimer (Plate II(b)) decreased from 24 to 4%, and the frequency of "figure 8" molecules (Plates II(c) and (d)) increased from 3 to 9.5% (Table 2). The frequency of monomers (Plate II(a)) remained essentially the same: 71 and 75%. Circular monomer duplexes with one or two double- or single-stranded tails were also observed at a frequency of 3% (Plate IV).

The absence of 5-μ linear duplexes is regarded as strong evidence for the head-to-tail structure of the dimer (Fig. 1). The head-to-head dimer should have preferentially reannealed by a monomolecular mechanism to form uniform linear duplexes of 5-μ contour lengths. The two hairpin regions would be too small to appear as bushes. No 5-μ linear duplexes were observed. Also, no 5-μ linear duplex molecules were present in the sample of denatured DNA that was slowly quenched. The head-to-head dimer should have annealed under these conditions.

We consider next the various pathways for the formation of heteroduplex molecules from full-length single strands. Figure 8 presents schematically some possible modes of formation of heteroduplexes from the four main types of full-length single strands present in the mixture during annealing. These may pair in three ways. Reannealing between circular complements is regarded as unlikely and is not considered. Heteroduplex formation between a linear monomer and a circular dimer is shown in Figure 8(a_2). When a second linear monomer anneals with (a_2), an intermediate structure, (a_3), is formed. Regions 1 and 2 of the circularly permuted linear

Figure 8. Representation of various modes of formation of heteroduplexes containing full-length monomer strands and one full-length dimer strand. These duplexes are called fused dimers and are shown in the fourth column. The paired strands in the first column anneal to form the structures containing two single strands in the second column. These nucleate with a third single strand as shown in the third column and anneal to form fused dimers. The shaded areas represent hydrogen-bonded duplex regions. The unshaded annuli represent nonduplex regions. The nucleation sites are indicated by transverse lines. The carats (v) represent single-strand scissions. A detailed description of the winding that occurs in the formation of the fused dimer is given in the text.

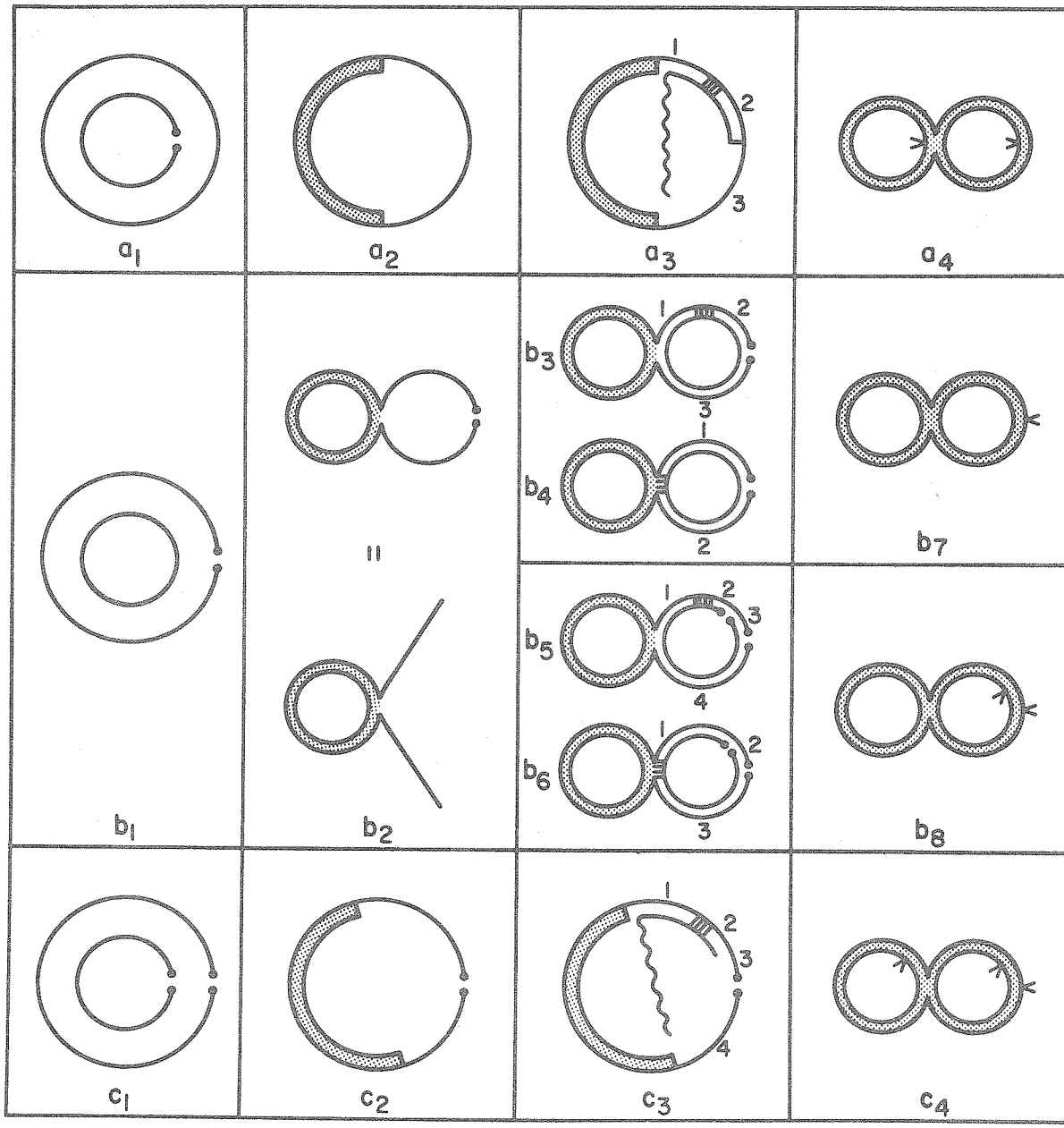


Figure 8

strand wind around the complementary regions in the circular dimer strand to form a hydrogen-bonded duplex, and leave an unannealed tail complementary to region 3. This tail winds around the dimer strand after a second nucleation anywhere in region 3. There are no topological restrictions in this process because of the presence of swivels at both ends of this region. The duplex, (a_4), contains two single-strand scissions at the positions indicated by the carats (v). Such "figure 8" molecules have the appearance of catenanes in electron micrographs. We shall refer to these molecules as fused dimers.

The intermediate (Fig. 8(b_2), made up of a linear dimer and circular monomer strand could appear with varying tail lengths, depending upon the site of nucleation. We illustrate the formation of fused dimer molecules from an intermediate with equal-size single-stranded tails. Four illustrations of the modes of formation of fused dimers are given in Figures 8(b_3) through (b_6).

Figure 8(b_3) illustrates the nucleation of a monomer circle with a site on a single-stranded tail. Duplex formation in region 2 can occur by winding of this chain-end around the circular strand. Duplex formation in region 1 can occur by rotation of the partially duplex circular monomer around the linear strand in region 1. The linear tail in region 3 can then reanneal to complete the formation of the fused dimer, (b_7), with one single-strand scission. Figure 8(b_4) illustrates nucleation immediately adjacent to the junction region. Winding of each of the tails then results in the formation of the fused dimer.

Figures 8(b_5) and (b_6) illustrate intermediates that arise when similarly permuted linear strands nucleate with single-stranded regions in (b_2). Winding in (b_5) occurs in the regions 1, 2, and 4, and leaves cohesive ends in region 3 unreacted. A cyclization reaction, such as described by Thomas & MacHattie (1964) for the circularly permuted strands of T2 DNA and by Hershey & Burgi (1965) for the cohesive ends of lambda DNA, then occurs and leads to a fused dimer, (b_8). Fused dimers can also be formed as in Figure 8(c) from a linear dimer and two linear monomer strands.

The results of careful inspection and measurement of twenty "figure 8" molecules are presented in Table 3; two examples are shown in Plates II(c) and (d). Seventeen of these molecules are two-ring systems in which the individual lengths of the monomers are within one standard deviation of the mean length. Neither bushes nor tails were observed in this group. Such molecules must have been formed as described above from heterologous strands, unless they were derived from the small number of catenanes (3%) originally present. These catenanes would have formed pairs of interlocked single-stranded rings upon denaturation and could have reannealed with complementary linear monomer strands. If catenanes alone were responsible for the formation of "figure 8" forms, we should have expected to find them at approximately the original frequency. Instead, such molecules accounted for 10% of the duplex forms. Most of the "figure 8" molecules, therefore, have been formed by reannealing of monomer strands with dimer strands. This observation gives

TABLE 3

Normalized contour lengths of annealed human leukemic leukocyte M DNA*

Monomer	Monomer circles in fused dimers	
1.08	0.96, 1.01	1.04, 1.01
1.01	1.01, 1.01	0.99, 0.87‡
0.99	1.00, 0.96	1.03, 0.99
0.98	0.98, 0.98	0.96, 0.96
1.02	0.98, 1.00	0.91, 0.91
1.00	0.99, 0.98	0.99, 0.83‡
1.00	1.07, 1.08	0.99, 0.99
1.01	1.10, 1.10	0.98, 0.65‡
1.03	1.01, 1.03	0.99, 1.01
0.98†	1.09, 1.07	1.05, 1.05
0.94†		
1.00†		
0.99†		
0.98†		
1.00 ± 0.031 S.D.	1.01 ± 0.056 S.D.	

* The contour lengths were normalized with the mean value of the contour length of the simple monomers.

† Monomer with single-stranded tail.

‡ Circle with bush region. These lengths were not included in the determination of the mean length.

support to the finding that the monomeric and dimeric forms hybridize.

Three of the twenty "figure 8" molecules appeared to contain a single bush in one circular duplex. Such bushes correspond to gap regions which result from the incorporation of short linear dimer or monomer strands into the hybrid (Fig. 7(c)). The contour lengths of the three duplex circles containing bushes were significantly shortened to 2.9, 3.7, and 3.9 μ.

The "figure 8" molecules shown in Plates II(b) and (c) are either renatured catenanes or fused dimers. Since fused dimers are heteroduplexes between a dimer and two monomer strands, it is important to specifically examine these molecules for heterologous regions. Although it is difficult to positively identify a "figure 8" molecule when mounted in the presence of formamide, all possible candidates were carefully studied. Plate III shows a renatured monomer, a renatured dimer, and two "figure 8" molecules. Again, no heterologous regions were found.

So far, we have considered only those renatured molecules which were formed from intact single strands. As previously stated, these experiments were carried out with a mixture of lightly nicked molecules. Approximately 12% of the monomeric strands and 20% of the dimeric strands contained two single-strand scissions and were, therefore, present as fragmented single strands. These strands will, of course, also renature with other whole and fragmented strands and form incomplete duplexes with gaps as shown in Figure 7(c) and Plates IV(d) and (e). The incomplete duplexes, unlike the complete

Plate III. Search for heterologous regions. (The DNA was mounted on specimen grids by the formamide technique and shadowed with Pt-Pd.)

(a) Renatured circular monomer and circular dimer. No heterologous regions can be seen.

(b) and (c) Renatured "figure 8" molecules considered to be fused dimers, as explained in the text. No heterologous regions can be seen.

Plate III

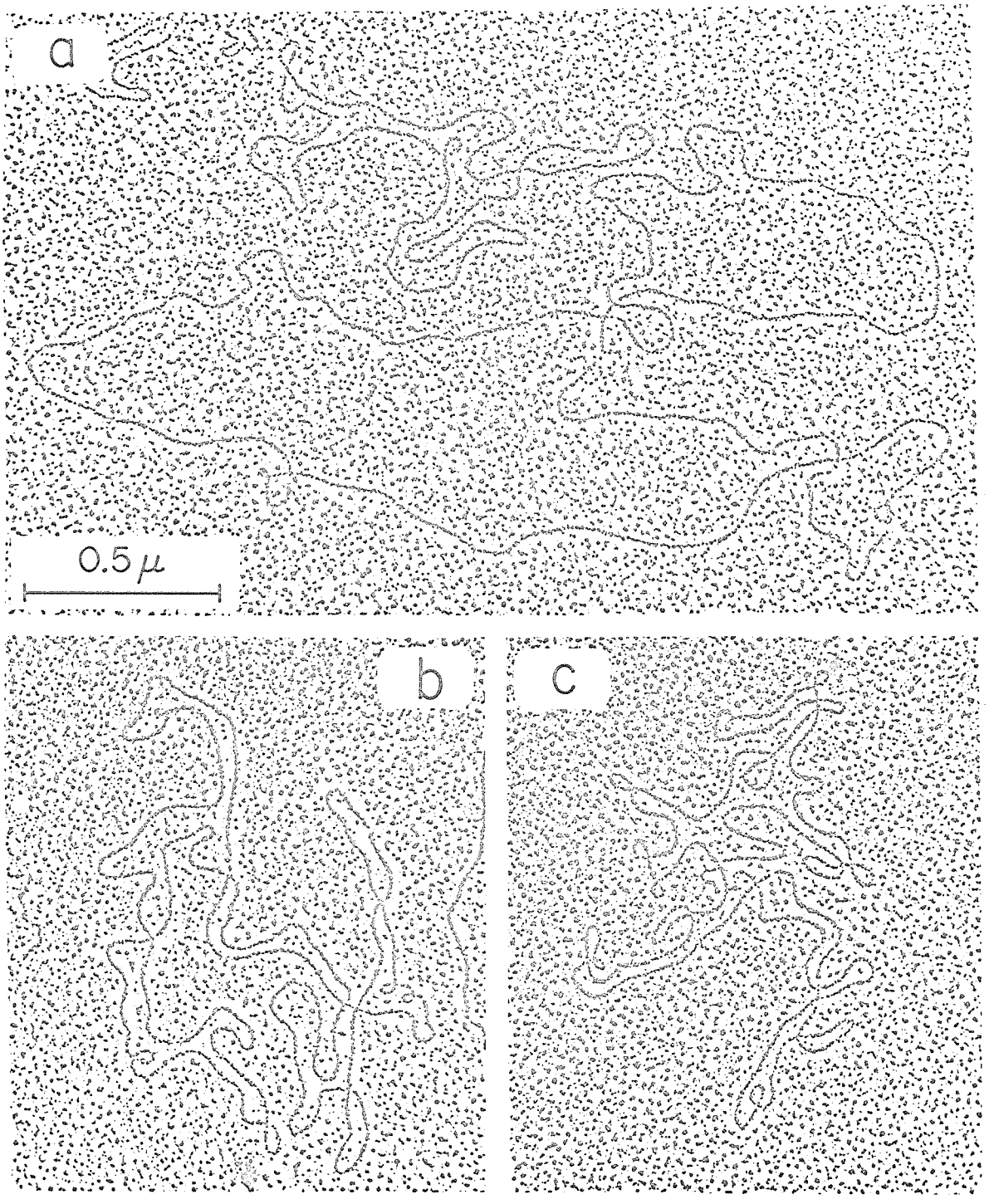


Plate IV. Incomplete duplex molecules.

(a) and (b) Renatured circular duplex monomer molecules with a single-stranded tail. These molecules presumably correspond to the form, (b_2), in Figure 8, and, therefore, are intermediates in the formation of a fused dimer. The molecules were mounted on specimen grids by the formamide technique and shadowed with Pt-Pd.

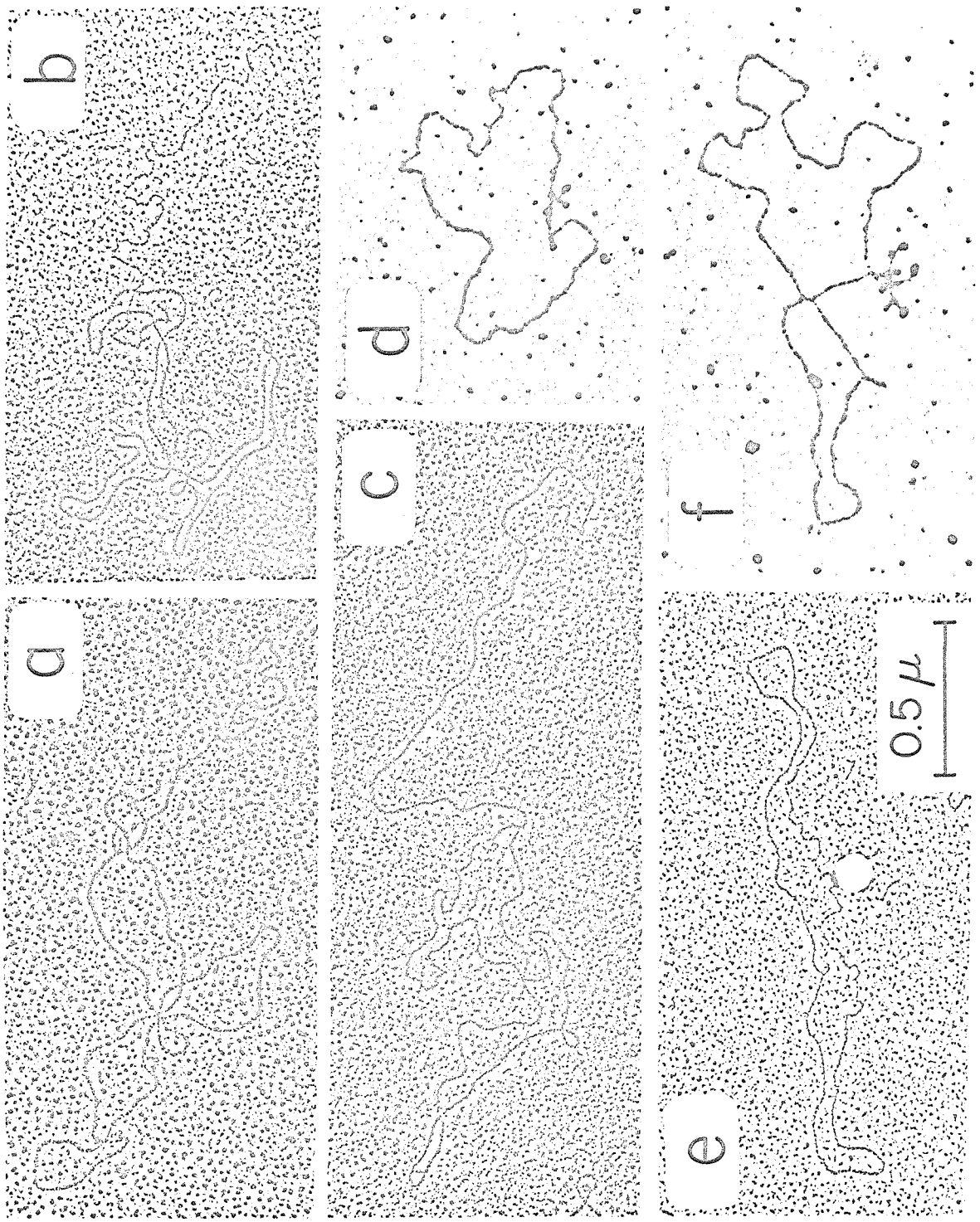
(c) Renatured circular (partial) duplex monomer molecule with a duplex tail. The right end of the duplex tail appears to be single-stranded. The left end of the duplex tail appears to be attached to the circular monomer as a duplex; however, at the joint, the upper part of the circle appears to be single-stranded, while the lower part appears to be double-stranded. This molecule may be the result of renaturing a linear dimer with two monomers, one or more of which was a fragmented molecule. The DNA was mounted as in (a) and (b).

(d) Renatured molecule showing a single-stranded gap (Fig. 7(c)). This molecule is the result of renaturing a whole monomer strand with a short strand. The DNA was mounted by the aqueous technique and stained with uranyl acetate.

(e) Same as in (d), except mounted as in (a).

(f) Presumably the same type of intermediate that is shown in (a) and (b), but mounted as in (d).

Plate IV



duplexes, can continue to undergo renaturation with the formation of branched molecules and large aggregates. The presence of such molecules considerably complicates the electron microscopic analysis. It is essential in these experiments, therefore, to utilize very lightly nicked DNA (one single-strand scission per duplex). As expected, incomplete duplex and aggregated molecules were observed in the electron microscope (Plates IV(d) and (e)). Since they were present in low relative frequency, they could be unambiguously identified, and did not interfere with the analysis of the renatured molecules derived from full-length single strands.

Another interesting class of incomplete duplexes are the intermediates in the formation of the heteroduplex from one dimer and one monomer strand. There were very few of these intermediates present, since the renaturation was carried almost to completion. A few examples that were found are shown in Plates IV(a), (b), and (f). These intermediates are composed of one complete duplex monomer circle with a single-stranded DNA tail and, presumably, correspond to the structure in Figure 8(b_2). Also, a few monomer circular molecules with duplex tails were observed (Plate IV(c)).

The "figure 8" molecules were not formed when a sample of a nicked, but otherwise homogeneous, circular duplex DNA from PM2 virus (Espejo & Canelo, 1968) was reannealed. The nicked viral DNA (prepared as described in Materials and Methods, section (e)) was analyzed in alkaline CsCl by the band velocity procedure in the analytical ultracentrifuge. The DNA contained 20% circular strands and

50% homogeneously sedimenting linear strands. The remaining 30% was present as heterogeneous slowly moving linear strands. The nicked viral DNA with a molecular weight of about 6 million daltons was denatured and reannealed by the procedures used in the study of the monomer-dimer mitochondrial DNA system. The concentration during hybridization was 5 μg/ml. A second experiment was performed at 25 μg/ml. The first renatured sample contained 56% duplex circles, 15% PM2-size linear molecules, 16% large aggregates, 9% circles with tails, and 3% single-stranded molecules. The more concentrated renatured product consisted largely of aggregates. The remainder was a mixture of PM2-sized circular and linear duplexes and circles with tails. "Figure 8" molecules were not observed in the experiments with PM2 DNA. This result supports the validity of our classification of such molecules as monomer-dimer hybrids.

4. Discussion

This research was undertaken with the object of determining the structural relationship and homology between the circular monomers and circular dimers that occur in mixture in mitochondrial DNA samples prepared from leukocytes from patients with myelogenous leukemia. The study was carried out with M DNA from blood samples taken periodically from a single patient who had not been treated with cytotoxic drugs. The problems of homology and structure were

examined with two experimental approaches: (1) a centrifugal examination of the composition of the purified monomer and dimer species; (2) an examination of hybrids between monomers and dimers by electron microscope and centrifugal methods. Dawid & Wolstenholme (1968) employed a simpler version of the buoyant density procedure to detect qualitatively a sequence homology between Xenopus laevis and chick M DNA's. They also examined the renaturation products of purified X. laevis M DNA by electron microscope and centrifugal methods. They observed that the renatured products contained duplex circles with the contour length of native X. laevis M DNA, shortened duplex circles with single-strand regions, linear molecules of varying sizes, and aggregates.

(a) Base composition

We have deduced from the buoyant behavior of separated monomers and dimers that these duplex species have the same base compositions. The equivalence of the buoyant densities in the analytical ultracentrifuge follows from the equivalence of the difference in buoyant density between each species and a marker DNA. The measurement error in each experiment corresponds to an error of approximately $\pm 0.5\%$ in the guanine-cytosine (GC) content of the DNA. The limit of detection for a difference is, therefore, $\pm 1\%$ in GC content.

The buoyant densities of the light and heavy strands in alkaline CsCl containing a marker DNA were also the same within the

accuracy of our measurement. In this case, we cannot clearly assess the quantitative aspects of the degree of similarity of base composition of the corresponding complements. The buoyant density difference (0.041 g/ml.) between light and heavy complements is due in part to a thymine bias between the strands. The dense strand (R. L. Hallberg, private communication) contains approximately 40% more thymidylate residues than does the light strand. Since all four types of residues contribute to the buoyant density of a titrated strand with unknown weighting factors, the experimental results—identical alkaline buoyant densities of light and heavy strands—could have been obtained by combinations of several differences in base composition that somehow compensate. On the other hand, the simpler interpretation strongly supported by the hybridization experiments is that the base compositions of the corresponding separate strands are, indeed, very similar.

(b) Homology

The results of experiments in which mixtures of light monomer and heavy dimer strands were reannealed and the buoyant density of the hybrid examined in the analytical ultracentrifuge clearly demonstrate that within the limits of detection these strands reanneal to form perfect hybrid duplexes. If the duplex had contained 10% single-stranded DNA, we should have observed a detectable shift of 0.001 g/ml. toward higher density. The results of these experiments are especially significant for the interpretation of structure of the various

molecular forms of hybridized DNA observed in the electron microscope.

The isolation of the separate complements from alkaline buoyant gradients centrifuged in the preparative ultracentrifuge induces a few scissions into each strand and provides materials suitable for the formation of the highly concatenated hybrid duplexes discussed above. The presence of such concatenated structures, which may be branched, interferes with the detectability of heterologous regions in the electron microscope. The electron microscope hybridization studies were, therefore, performed with mixtures of monomer and dimer molecules that had been carefully nicked and fractionated. Upon denaturation, this material then formed circular single strands and largely full-length linear strands. These mixtures were then reannealed and formed circular monomers, circular dimers, and fused dimers. Only the fused dimers are necessarily hybrid duplexes. These forms were separately inspected for the presence of heterologous regions and measured for contour length. A significant number of such molecules had the correct circular contour length and contained no heterologous regions. The absence of heterologous regions in these selected hybrid molecules reveals that deletions, additions, or insertions do not occur over lengths of DNA larger than 50 to 100 nucleotides. Smaller stretches, which do not give detectable heterologous regions, could, of course, occur.

If we now consider both the electron microscope and centrifuge

analysis of the hybrids together, we conclude that the total heterology cannot exceed 10%; and, if any heterology at all is present, it must be dispersed in short noncontiguous sections of less than 50 to 100 nucleotides.

(c) The head-to-tail structure of the circular dimer

The strongest evidence for the head-to-tail arrangement of monomer genome in the circular dimer was obtained in the electron microscope experiment with the slowly quenched denatured DNA sample. If the dimer contained a head-to-head arrangement, the linear dimer strands should have, by this treatment, annealed to 5-μ linear duplexes with hairpins at both ends. Since no linear duplex molecules were observed in this experiment in which a small number of circular monomer duplexes formed, we conclude that the monomer sequences are arranged in the form of a head-to-tail circular concatenate. Five-micron linear duplexes were also not observed in the extensive study of the completely reannealed DNA.

(d) The homogeneity of base sequence in monomeric mitochondrial DNA

Mitochondrial DNA in animals, from the sea urchin to man, occurs in the form of circular duplex molecules approximately 5 μ in contour length and in the form of multiples of this unit length. The unit length contains about 16,000 nucleotide pairs. This DNA, if homogeneous and if engaged entirely in the formation of mRNA

for protein synthesis, can specify no more than 5,300 amino acid residues, a number that is inadequate to account for the synthesis of a complete mitochondrion. Most cells, however, contain a large number of M DNA molecules (e.g., 250 to 500 in a HeLa cell) and each could conceivably be different, a possibility that is unlikely in view of the well established rapid renaturability of M DNA. The standard method at the present time for determining the amount of genetic information in a unit mass of DNA is based on a correlation between the second-order rate constant for annealing of denatured DNA under standard conditions and the complexity of the DNA (Britten & Kohne, 1965; Wetmur & Davidson, 1968). Borst, Ruttenberg & Kroon (1967) performed such measurements and reported that the apparent genome size of chick liver M DNA was approximately eight-tenths the size of the molecule. This result indicates that chick liver M DNA is essentially homogeneous.

The results described in this study, while focused on the monomer-dimer hybrids, also yield significant information regarding the homogeneity of the leukocyte M DNA monomer and dimer species individually. The complete reannealing of the denatured M DNA mixture, as observed in the buoyant density experiment (Fig. 7(c)), and the failure to observe heterologous regions in the renatured monomers or the renatured dimers (Plates I through IV), clearly demonstrates that the monomer and dimer species are not in themselves mixtures of partially homologous DNA's. Either these DNA's are essentially homogeneous or they consist of classes of DNA's that

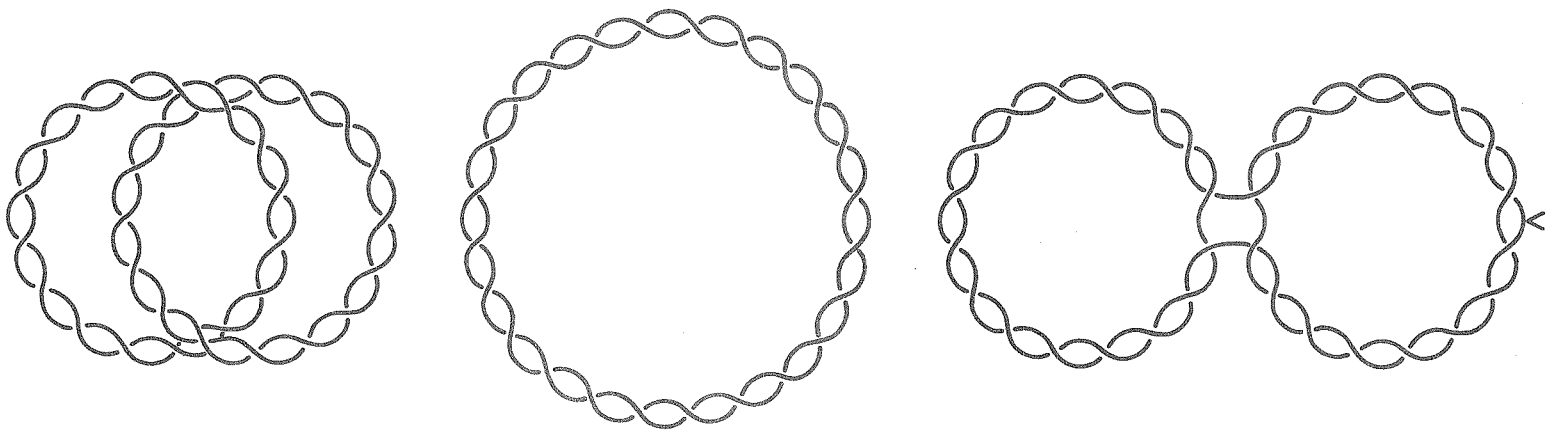
are so slightly homologous that they do not reanneal with each other. The latter alternative is held to be less likely in view of the further requirements that the DNA's in the putative classes also must have the same single-strand base composition and molecular weight. Both the above results and the kinetic result of Borst, Ruttenberg & Kroon (1967) suggest that M DNA should be regarded as homogeneous with an information content that corresponds to its molecular weight. The presence of point mutations or small deletions or insertions that could not have been detected are, of course, not ruled out.

(e) Three structural forms of dimeric mitochondrial DNA

Figure 9 presents three different structural forms of dimeric M DNA diagrammatically. The topologically bonded catenated dimer (Fig. 9(a)) and the circular dimer (Fig. 9(b)) occur naturally. The fused dimer prepared in small amounts in this work contains a four-stranded junction region represented in Figure 9(c) with five unpaired bases in each strand. The actual number of unpaired bases in the junction regions is not known at present. An inspection of two juxtaposed space-filling duplex models suggested that the junction could be formed with less than five unpaired bases per strand. The junction region in the fused dimer formally corresponds to a fourfold or X-type branch from which four duplexes emanate. It may be contrasted with the threefold or Y-type branch point postulated as a formal intermediate

Figure 9. Representation of three structural isomers of dimeric mitochondrial DNA. The fused dimer is shown as a duplex containing a linear dimer strand and two circular monomer strands. The carat (v) represents a single-strand scission.

STRUCTURAL ISOMERS OF DIMERIC
MITOCHONDRIAL DNA



CATENATED
DIMER

CIRCULAR
DIMER

FUSED
DIMER

Figure 9

at the origin and, also, at the growing point of DNA partially replicated by a semiconservative mechanism as discussed by Cairns (1963). The completion of replication of a circular duplex by the Cairns mechanism formally involves a fusion of the origin and the growing point and the formation of a fourfold junction region.

Bloomfield (1966) and Fukatsu & Kurata (1966) have calculated the effect of permanently associating short regions in a circular duplex in such a way as to form molecules which contain equal-sized duplex loops. These calculations were performed to assess the hydrodynamic effects of superhelical turns in a closed circular duplex. They calculated that a molecule containing two loops should sediment about 15 percent faster than the corresponding parent molecule, a result which can be checked experimentally when larger quantities of fused dimers are prepared and purified.

(f) "Normal" and "abnormal" mitochondrial DNA

As stated in the introduction, we have referred to the circular dimer as an "abnormal" form, because it occurred in leukemic leukocytes and did not occur in the appropriate controls. It must be emphasized that the present study compares the information content of the circular dimer to the circular monomers in the same patient. A similar study of the homology relationships between circular dimers from a leukemic patient and circular monomers from a nonleukemic (i. e., "normal") person remains to be performed.

Summary

Mitochondrial DNA occurs in multiple circular forms in human leukemic leukocytes. As part of an inquiry into the structural relationships between the forms, the 5- and 10-μ circular duplexes were separated and characterized by sedimentation procedures. The buoyant densities in neutral cesium chloride were indistinguishable, indicating that overall base compositions of the two species are the same. The buoyant densities of the corresponding heavy and light strands in the two species were also indistinguishable. Together, the results suggest that the dimer consists of two monomer genomes.

Reannealing of equal amounts of fragmented light monomer and heavy dimer complementary strands isolated from alkaline buoyant cesium chloride gradients leads to a high molecular weight duplex with a buoyant density indistinguishable from the parent duplexes. It is concluded from this result that the degree of homology between monomer and dimer is at least 90%.

Denaturation of a mixture of lightly nicked monomer and dimer duplexes, followed by reannealing in formamide solutions, leads to mixtures of circular monomer and dimer duplexes and heteroduplexes which contain one dimer strand and two monomer strands. These heteroduplexes occur in the form of a "figure 8" molecule, a new DNA structure which we have called the fused dimer. Neither heterologous regions, nor insertions, nor deletions were found in a careful examination of the electron micrographs.

It is concluded from this part of the work that heterologous regions, insertions, or deletions exceeding 50 to 100 nucleotides in length do not occur in the heteroduplexes. At this level of insight, the dimer is a circular concatamer of two monomer genomes. In addition, the results show that leukemic leukocyte mitochondrial DNA monomers (as well as dimers) are substantially homogeneous in base sequence.

The electron microscope studies also show that monomer genomes in the dimer are connected in a head-to-tail structure, rather than in a head-to-head structure.

ACKNOWLEDGEMENTS

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Part II

USE OF ALKALI METAL SALTS OF TRICHLOROACETIC ACID AS BUOYANT DENATURING SOLVENTS FOR DNA

INTRODUCTION

Buoyant density centrifugation has been used extensively in the characterization of nucleic acids. This general method suffers from the lack of a suitable denaturing solvent for DNA and RNA. Alkaline buoyant cesium chloride gradients cannot be used for RNA. If DNA has depurinated, it will also hydrolyze at these sites (1). It would be desirable to have a nonhydrolytic strand-separating solvent for the analysis of RNA and DNA structure. The recent discoveries that some DNA's consist of complementary strands that resolve into two bands in buoyant alkaline cesium chloride (2, 3, 4) has given new impetus to this problem. Isolation of intact complementary strands would allow definitive hybridization experiments to be performed. The purpose of this work was to find a suitable reagent for a buoyant, neutral analysis of the denatured form of DNA at room temperature.

It is known that certain salts (such as, the alkali metal salts of Cl_3CCOO^-, SCN^-, ClO_4^-, and I^-) are powerful denaturants of DNA. Hamaguchi and Geiduschek have shown that the order of effectiveness of these salts in denaturing DNA did not correlate with effects on the activity coefficients of nonpolar compounds. They concluded that the effects of these salts on the activity coefficients of purine and pyrimidine bases in DNA could not account for their denaturing ability. The phenomenon was attributed to the salt's effectiveness as a "hydrophobic bond" breaker and subsequent effect on the structure of water (5). Robinson and Grant (6) later pointed out that the purine and pyrimidine bases in DNA are polar as evidenced by their dipole moment and,

therefore, the activity coefficient change should be re-evaluated. They determined the activity coefficients of thymine, adenine, cytosine, deoxyadenosine, and adenosine from solubility measurements at 25°C. The results and conclusions were as follows. (a) The large differences in activity coefficients observed with different salts are mainly a function of the anion. (b) The order of effectiveness of a salt on all the bases is nearly the same. (c) There is a good correlation between the effects of concentrated salt solutions on the activity coefficients of the bases and their effects on the denaturation of DNA. (d) The activity coefficient change stabilizes the denatured form relative to the native duplex due to increased exposure to solvent in the denatured form. (e) The mechanism of the activity coefficient change is unknown. It is suggested, but not proved, that the anions may decrease the activity coefficient of the bases by a direct interaction with the polar groups in the bases (6).

This part of the thesis will describe the results obtained in a preliminary investigation of the properties of the alkali metal salts of trichloroacetic acid used as a buoyant system for DNA. It is shown that these salts are indeed active denaturing agents for calf thymus DNA at neutral pH and room temperature. Both native and denatured calf thymus DNA were banded in buoyant gradients in the analytical ultracentrifuge. It is also demonstrated that rubidium trichloroacetate does not introduce single-strand scissions in closed circular polyoma viral DNA.

MATERIALS AND METHODS

Reagents

Reagent grade trichloroacetic acid (lot #37006, 37360) was obtained from J. T. Baker Chemical Co. Reagent grade sodium and potassium hydroxides were purchased from Mallinckrodt Chemical Co. Reagent grade lithium hydroxide (lot #782841) was from Fischer Chemical Co. Optical grade cesium hydroxide was either from Trona Chemical Co. (lot #10678) or Harshaw Chemical Company. Rubidium carbonate was purchased from Gallard-Schlesinger (optical grade, lot #B4077).

DNA

Calf thymus DNA, type I, lot #125B-0950, was purchased from Sigma Chemical Company. Polyoma viral DNA was a gift from R. J. Radloff.

Preparation of salts

Each alkali metal salt of trichloroacetic acid was prepared in the following general manner. The appropriate base was titrated to neutrality with trichloroacetic acid in a minimum of volume. The titration was considered complete when a diluted sample of the salt registered neutral pH on a Beckman Research pH meter with E. H. Sargent combination electrode. The solution was then purged for one hour with nitrogen gas and the pH readjusted as necessary. This neutral solution was then successively filtered through "Norite" until clear. This removed an often present yellow color of unknown origin. The solution

was then passed through a 1.2 μ Millipore filter and evaporated in a rotary flask evaporator until a slurry was formed. This slurry was transferred to a drying dish and dried in a vacuum dessicator over P_2O_5. The salt was considered to be free of water when a constant weight was obtained for a representative sample. The salt was then stored under vacuum as a powder. Solutions used in melting and buoyand experiments were prepared immediately prior to use by dissolving the dry salt in glass-distilled water.

Analytical centrifugation

Analytical buoyant density experiments were performed in Beckman model E ultracentrifuges equipped with photoelectric scanners. The alkali metal salts of trichloroacetic acid of the molarity necessary for denaturation at 25°C have an optical density of approximately 0.5 at 280 mμ. It is necessary to have a monochromatic light source at 280 mμ, since the absorbance curve rises rapidly at lower wavelengths. All analytical experiments were, therefore, carried out at 280 mμ. The rotor speed was 44,000 or 44,770 rpm, and the temperature varied from 15° to 30°C depending on the estimated T_m. Titanium centerpieces were employed exclusively for reasons described under Results.

The analytical band sedimentation velocity experiment was performed in CsCl, pH 12.5, $\rho = 1.46$, at 35,600 rpm, 20°C, as described by Vinograd et al. (7).

Determination of T_m

The melting profile of calf thymus DNA in various trichloroacetate

solutions was recorded with a Gilford model 2000 Multiple Sample Absorbance Recorder.

Density determinations

Densities were determined by weighing a 300 μl micropipette containing a known molarity of salt solution. The micropipette had been calibrated with glass-distilled water. All weighings were performed on a Mettler microbalance.

RESULTS

In the course of this study it became apparent that the trichloroacetate salt system presented some experimental difficulties. These salts react with polyallomer, aluminum, Kel-F, and Epon, as evidenced by a significant increase in optical density of a trichloroacetate solution after contact with the above materials. Cellulose nitrate tubes are inert. All analytical centrifugation was performed with titanium centerpieces.

The optical density of a 4 molar solution of trichloroacetate is approximately 0.5 at 280 mμ and greater than 3 at 260 mμ. It is, therefore, necessary to analyze DNA at 280 mμ, or at one-half sensitivity. The relative viscosity of a buoyant solution of trichloroacetate is between four and six. This crude determination was obtained by comparing the flow time for the salt and glass-distilled water from a volumetric pipette. The resultant increased time needed to reach equilibrium in a buoyant system is an undesirable feature of the system. Approximately 72 hours were required to obtain a near equilibrium situation in the analytical ultracentrifuge.

RbTCA is potentially the most useful salt for studies of DNA. Unfortunately, Rb has a naturally occurring isotope (β^- emitter) with a half-life of 6×10^{10} years. This isotope accounts for 28 percent of the Rb atoms. A trial run showed that a 4.5 M solution of RbTCA contributed approximately 150 cpm in the ^{3}[H] window under standard conditions in a Packard scintillation counter. This background must be considered if labeled DNA is being analyzed.

The results of several melting experiments with calf thymus DNA in varying concentrations of trichloroacetate are given in Figure 1. It is seen that the trichloroacetate anion is a powerful denaturant. There is an apparent stabilizing effect of cesium on the native form of DNA as evidenced by the increased molarity of CsTCA needed to drop the T_m to 20°C. NaTCA and RbTCA are reasonably close in denaturing power. The small number of data points involved precludes a firm statement on the possibility of a real difference between these two salts. The width of the transition, $\sigma_{\frac{1}{2}}$, was slightly greater in CsTCA (11°) than in NaTCA and RbTCA (8 to 9°). Since the integrity of the calf thymus DNA was not monitored from one experiment to another, it is impossible to ascribe any significance to this difference. It should be noted that this DNA was not purified beyond the original sample.

Density measurements were performed on a series of trichloroacetate salts (Figure 2) to provide an estimate of the T_m in the appropriate density range for DNA. The data were assumed to be linear and to have an intercept at the origin. All of these salts at 35°C formed saturated solutions at a concentration of approximately 6 molar. If we assume that denatured DNA will have a buoyant density between $\rho = 1.60$ and $\rho = 1.75$ in this medium, it is immediately apparent that Li, Na, and KTCA are not suitable as buoyant solvents. CsTCA encompasses this density range at a molarity of 3.5 to 4. This molarity does not provide a low enough T_m to be useful in the analytical ultracentrifuge. The properties of RbTCA bridge these two extremes. The density of 4.25 to 5.25 M-RbTCA is in the range expected for denatured

Figure 1. T_m of calf thymus DNA versus molarity of three alkali metal salts of trichloroacetic acid. In each case, 20 μg of DNA was placed in the desired molarity of trichloroacetate at 10°C. The samples were degassed and placed in 0.5 ml cuvettes and absorbance measured as a function of temperature as described in Methods. It should be noted that decomposition of trichloroacetate occurred at approximately 55°C as evidenced by the evolution of chloroform.

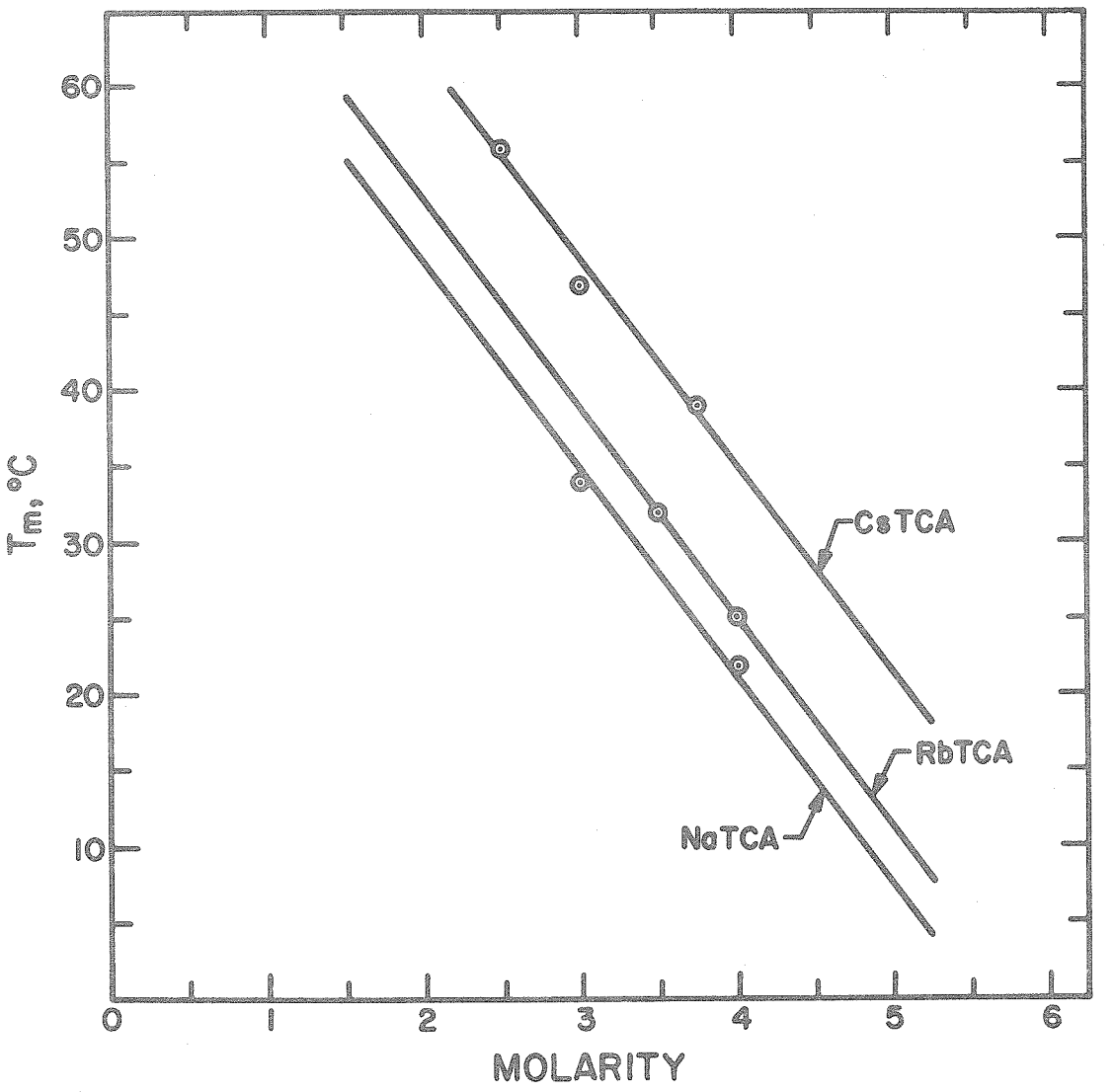

Figure 1

Figure 2. Molarity versus density for the alkali metal salts of trichloroacetic acid. Density measurements were performed as described in Methods. The density of LiTCA was determined at a concentration of 4 M only.

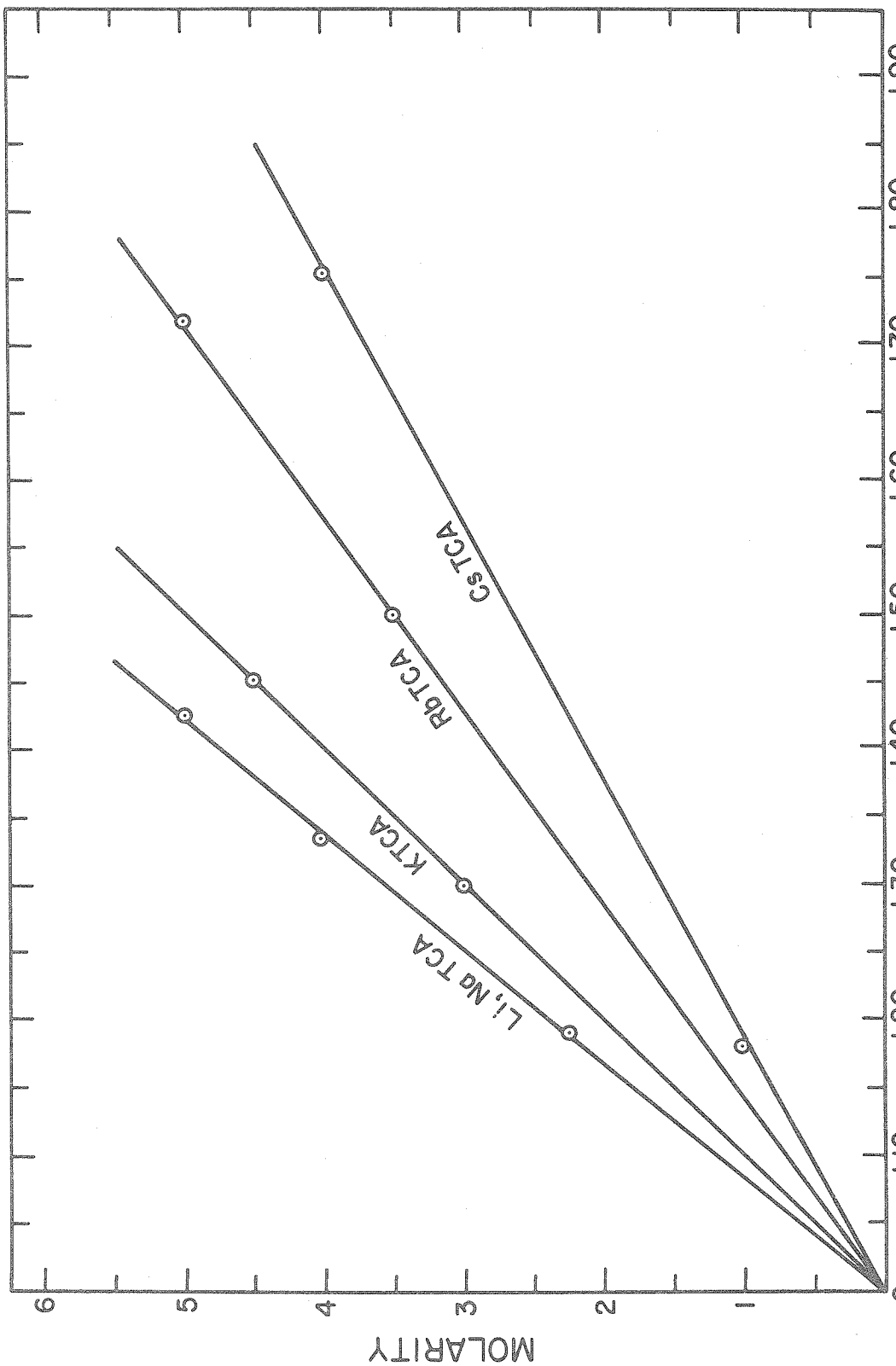
MOLARITY
DENSITY
0
1
2
3
4
5
6
1.10
1.20
1.30
1.40
1.50
1.60
1.70
1.80
1.90
CsTCA
RbTCA
KTCA
Li, Na TCA

Figure 2

DNA. It should, therefore, be possible to make adjustments in temperature and molarity to achieve either the native or denatured condition.

The possibility that RbTCA would cause single-strand scissions in DNA was tested with the following experiment. Covalently closed polyoma viral DNA was incubated at 25°C for one week in 4.5 M-RbTCA. At the end of this time period the DNA was sedimented on alkaline cesium chloride in the analytical ultracentrifuge (Figure 3). All the DNA sedimented as covalently closed polyoma DNA (8). A control sample incubated in buffer gave a similar result. It is concluded that RbTCA does not introduce nicks in polyoma DNA in a time period sufficient to perform a buoyant analysis.

Calf thymus DNA was banded in RbTCA of different molarities. The native form was buoyant in a solution of 3.5 M-RbTCA. The denatured form was buoyant in 4.5 M-RbTCA. Since the gradient in this system was not determined, it is not possible to calculate buoyant densities of this DNA. Rough estimates indicate that native calf thymus has a density of approximately 1.50, and denatured calf thymus a density of 1.65. These estimates are based on the assumption that the DNA has a buoyant density near the input density of the solution if it bands near the isoconcentration point.

Figure 3. Analytical sedimentation velocity of closed circular polyoma DNA after seven days' incubation in 4.5 M-RbTCA. The run was performed in alkaline CsCl, $\rho = 1.46$, pH 12.5, at 35,600 rpm and 20°C. The field is directed to the right. One band is seen moving down the cell at two-minute intervals. Absorbing material trailing the DNA is trichloroacetate, evidenced by its failure to sediment at higher speeds at longer times. The DNA had an uncorrected sedimentation coefficient of approximately 40 S, confirming its assignment as the covalently closed species.

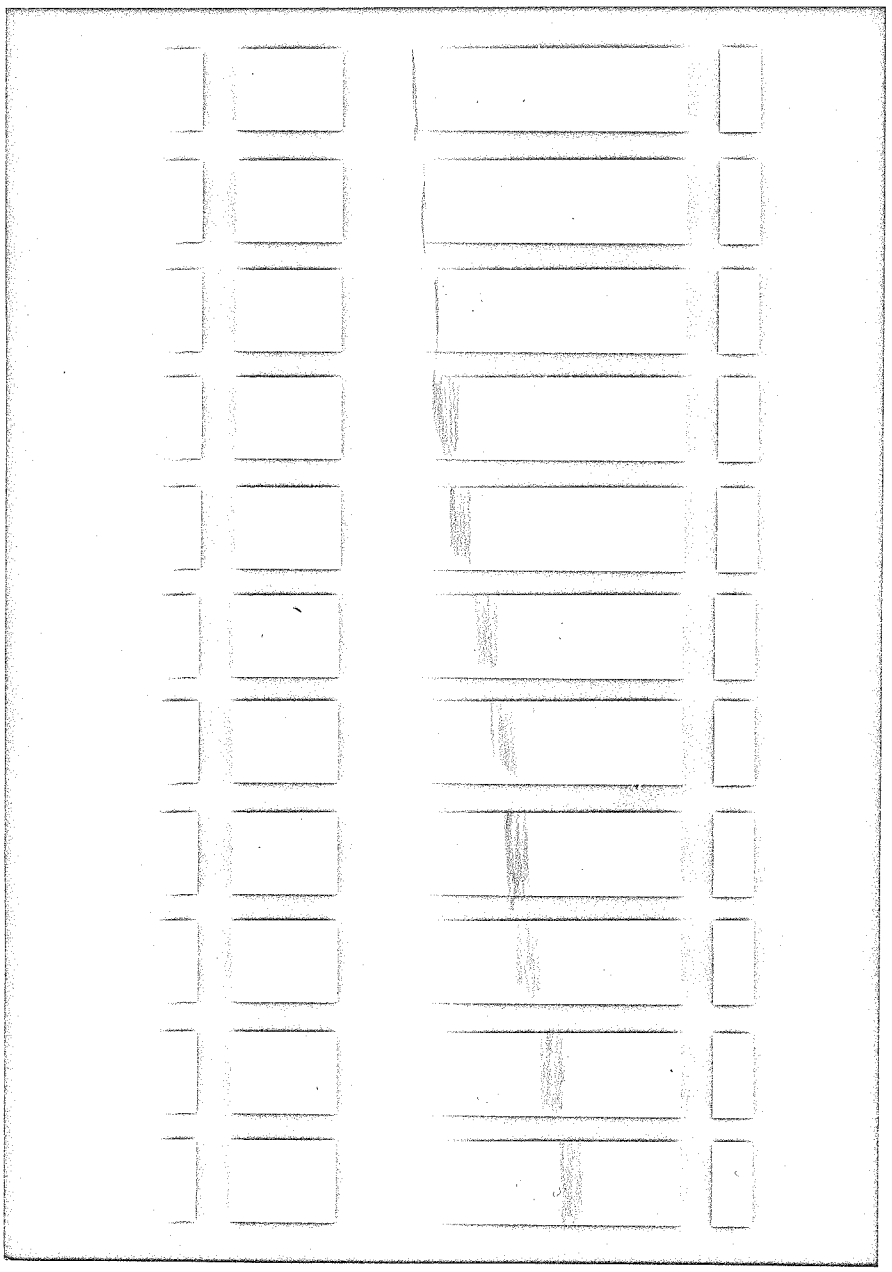

Figure 3

DISCUSSION

These preliminary results show that RbTCA is a potentially useful buoyant solvent for DNA. It is likely that most DNA's can be banded in both the native and denatured states in this medium without introducing single-strand scissions. This opens the possibility of preparatively isolating intact complements of mitochondrial or phage DNA for further experimentation. A study of the dependence of the buoyant density in RbTCA on base composition is necessary to validate this reasoning. It is also concluded that Li, Na, and KTCA are potentially useful sedimentation velocity denaturing solvents for DNA. Further work on this topic could include: (a) properties of various RNA species in trichloroacetate salts; (b) the effective gradient in a buoyant trichloroacetate system; (c) the buoyant density of a DNA as a function of base composition and structure (i.e., single-strands versus covalently closed circles); (d) a determination of refractive index as a function of density for the appropriate salt; and (e) determination of viscosity of the appropriate salt as a function of molarity.

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