STUDIES OF CIRCULAR AND CATENATED CIRCULAR DNA

Thesis by

Bruce Hudson

In partial fulfillment of the requirements for

the degree of

Master of Science

California Institute of Technology

Pasadena, California

1968

(Submitted September 25, 1968)

ACKNOWLEDGMENT

I am deeply indebted to Dr. Jerome Vinograd for his generous expenditure of time, patience and experience during my undergraduate and graduate study at Caltech.

TABLE OF CONTENTS

Catenated Circular DNA Molecules in HeLa Cell Mitochondria

by

BRUCE HUDSON
JEROME VINOGRAD

Norman W. Church Laboratory for Chemical Biology,
California Institute of Technology

Closed circular mitochondrial DNA molecules which are catenated, or connected like the links in a chain, have been identified in extracts of HeLa cells.

MITOCHONDRIAL DNA from a variety of organisms has been shown to occur in the form of closed circular duplex molecules with a uniform length of about 5μ and a molecular weight of 10 million daltons[1–3]. Closed circular duplexes display a variety of special properties which arise from the inability of the two polynucleotide strands to unwind. One of these properties, the restricted uptake of the intercalating dye ethidium bromide (EB)[4], forms the basis of a convenient method for isolating and detecting closed circular DNA molecules[5]. When this method was first applied in a study of extracts of HeLa cell mitochondria, a paucidisperse system of closed circular molecules was seen in the electron microscope[5]. Ten micron dimers accounted for 10 per cent of the molecules; higher oligomers were present in smaller amounts. The structure of these oligomers, however, was not determined, and it was possible that they consisted of monomers that were joined to each other after extraction of the DNA from the mitochondria. We now report experiments which show that mitochondrial DNA dimers and higher oligomers are stable, isolable DNA molecules that consist of independent, double stranded, closed circles that are topologically interlocked or catenated like the links in a chain. Because of the nature and stability of this topo-

logical bond, these oligomers must exist in the HeLa cell. A plausible mechanism for the formation of the catenated molecules is the physical recombination or crossing over of circular mitochondrial DNA molecules.

When ethidium bromide binds to DNA, the strands in the Watson–Crick duplex partially unwind. The unwinding results in a rotation of the molecule about the duplex axis if the DNA is linear or if it is circular and has a single-strand scission or nick. We will refer to both of these types of molecules as open molecules and to closed circular molecules as closed. A closed DNA with no site for rotation resists the uptake of dye in high concentrations and thus binds a smaller amount of dye than an open DNA. The binding of ethidium bromide, which has a low density, lowers the buoyant density of DNA in caesium chloride density gradients. The differential binding causes the closed DNA to band at a higher density than the open DNA. A mixture of closed and open DNAs forms two well separated bands after centrifugation of a mixture of ethidium bromide, caesium chloride, DNA and water. A compound molecule consisting of one closed and one nicked circular DNA would be expected to form a band approximately midway between the closed and open bands.

A fluorescence photograph of an ultracentrifuge tube containing mitochondrial DNA from HeLa cells and nuclear DNA from a subcellular fraction enriched with mitochondria is shown in Fig. 1*a*. The DNA-dye bands stand out over the background of dye because of the enhanced efficiency of fluorescence when the dye binds to DNA[6]. The positions of the upper and lower bands correspond to the previously reported[5] positions for open (upper) and closed (lower) DNAs in the conditions used. The middle band, as shown in the microdensitometer record of this photograph (Fig. 1*b*), is about equally distant from the centres of the upper and lower bands. When examined with the electron microscope, the bottom band was seen to contain about 10 per cent dimers and 90 per cent monomers, and the middle band about 60 per cent dimers and 40 per cent monomers. Higher multiples were not included in this count. The dimers contained cross-over points at or near the middle of the molecule. Representative electron micrographs of DNA molecules from the middle band are shown in Fig. 2. Dimers were observed on sparsely populated grids. Some dimers were made up of one extended molecule joined to one twisted molecule as shown (Fig. 2*c*).

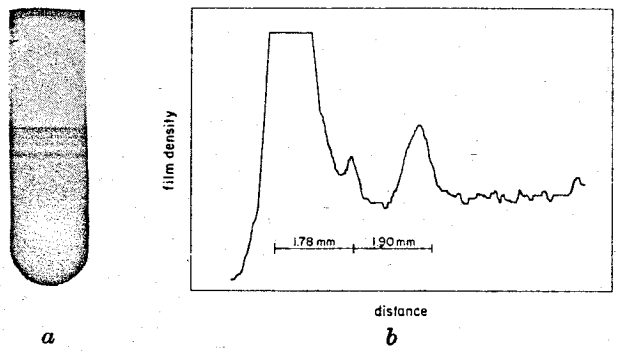


Fig. 1. A caesium chloride–ethidium bromide density gradient showing the resolved species of HeLa DNA. *a*, Preparative tube photographed in ultraviolet light through an ultraviolet filter with a Polaroid camera as described[5] but with type 46 film. The darkest band contains the open DNA. The next two bands in descending order contain the open–closed dimers and the fully closed species. The lowest band is a carbohydrate as judged by the density, turbidity and low dye binding. *b*, Microdensitometer tracing of this photograph showing the band positions. The left-hand side of the tracing corresponds to the upper part of tube. HeLa *S*3 cells were grown in suspension culture in Eagle's medium containing 10 per cent calf serum, washed in TD buffer (0·005 M *tris*, 0·15 M NaCl, 0·005 M KCl, 0·001 M NaH_2PO_4), suspended in 0·01 M *tris*, 0·01 M KCl, 0·005 M EDTA (*p*H 7·4) and homogenized at 0°–4° C in a glass homogenizer fitted tightly with a 'Teflon' pestle. The homogenate was sedimented at 3 K r.p.m. in an *SS*34 Sorvall rotor for 5 min to remove nuclei and remaining cells. The supernatant was centrifuged, usually twice more, until no further pellet was observed. The supernatant was centrifuged at 10 K r.p.m. for 20 min, and the pellet was suspended in 0·6 per cent sodium dodecyl sulphate–0·01 M EDTA. After 2 h at room temperature enough 7 M CsCl was added to make the solution 1 M in CsCl; it was cooled to 0° C, and centrifuged at 10 K r.p.m. for 30 min to remove most of the caesium dodecyl sulphate. The supernatant was adjusted to 1·55 g/ml. with solid CsCl, and ethidium bromide was added to 200 μg/ml. A sample (3–5 ml.) was centrifuged in a Spinco *SW* 50 rotor at 43 K r.p.m. at 20° C for 36 h. The bottom and middle bands from four preparations, 3 ml. of packed cells each, were pooled, stored at −70° C for 1–3 weeks and rebanded to obtain the tube shown. A significant fraction of top band material was included in the pooling. Typical yields of bottom bands were about 200–1,000 mitochondrial DNA molecules per HeLa cell. The middle band contains about 2×10^{10} mitochondrial molecule equivalents.

We conclude from these observations that the middle band contained joined molecules in which one molecule is a closed circular duplex and the other a nicked circular duplex. The presence of monomers in the middle band is accounted for by the overlap inherent in the breadth of the bands (Fig. 1*b*) and the size of the fraction taken for electron microscopy. When middle and lower bands from several experiments were pooled and rebanded after some nicking had occurred, the fractional amount of material in the middle band increased substantially while the material in the lower band decreased. Repetition of this procedure resulted in a loss of middle band material. These experiments allow us to dismiss the possibility that the middle band consists of dimers banded at the intermediate position because of a different content of guanine and cytosine.

Measurements of the DNA molecules in electron micrographs made from the middle band (Fig. 3) show that the dimers consist of two molecules both the length of the unjoined monomers. The approximate 5μ length of these molecules from subcellular fractions enriched with mitochondria indicates their mitochondrial origin. The hypotheses that dimers consist of pairs of unequal length or of two populations containing long pairs and short pairs were tested with the χ^2 test and were found not to be statistically significant. The dimers, therefore, consist of two monomeric mitochondrial molecules joined together so that one monomer may be nicked while the other remains closed. The dimers found in the lower band contain joined monomers which are both closed.

The joint between the two molecules is stable in 4·5 molar caesium chloride and survives the hydrodynamic and surface forces which develop when specimens are being prepared. We now consider two possible kinds of joints between the monomeric constituents. The first kind involves ordinary chemical bonds, either covalent or secondary, between the two constituents, and possibly including joining agents such as proteins. The alternative is that the circular monomers are joined by a topological bond formed by interlocking the two circular monomers. The electron micrographs of dimers from the middle band (Fig. 4) show the interlocked nature of the joint. The molecules were successively shadowed while the specimen was rotated and then while stationary. This procedure reveals the three dimensional details of the two overlaps that are involved in the topological bond. We conclude from photographs of this type that the dimeric mitochondrial molecules are topologically linked circular monomers.

Many rotary shadowed dimeric molecules were examined and they gave the following further evidence that dimers are not joined by ordinary chemical bonds. (*a*) The two intersections between the monomers appeared, under the electron microscope, to be overlapping fibres when the focus was varied about the setting for best focus. In most cases the relations among the levels of the fibres at the two intersections showed that the monomers were interlocked. (*b*) Photographs of fourteen molecules like those in Fig. 2 were carefully examined by us and by six other people in the laboratory; everyone agreed that there were seven molecules which were catenanes. The rest of the molecules were ambiguously identified (the two possible orientations at each intersection were chosen about equally often). In no case was there agreement that a test dimer represented overlapped molecules. The agreement in the assignment at each crossover was based on

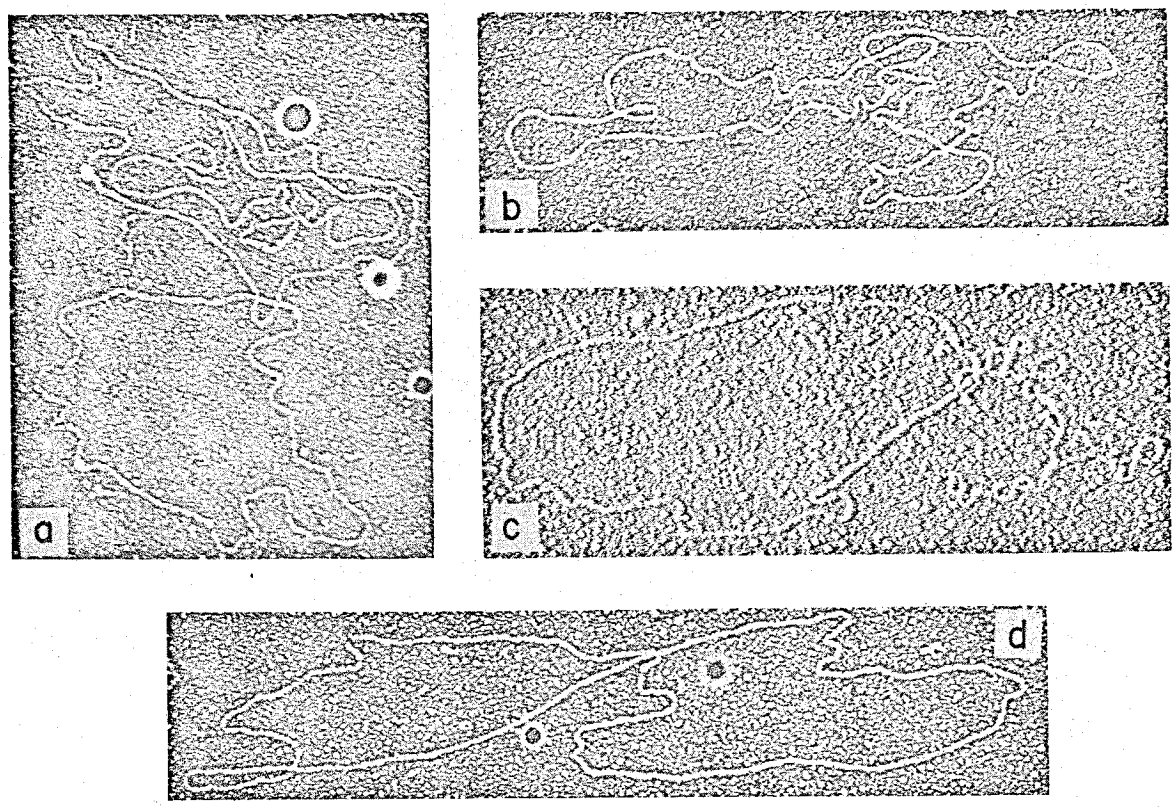


Fig. 2. Representative dimeric molecules from the middle band shown in Fig. 1. The density gradient shown in Fig. 1 was fractionated into approximately 50 μl. fractions. In these conditions the tenth fraction below the centre of the open band contains the peak fraction of the closed band[6]. In this experiment the resultant fractions were diluted with 20 μl. of 10 mg/ml. of cytochrome *c* and 80 μl. of 0·01 M EDTA, *p*H 7, and specimen grids were prepared by the procedure of Kleinschmidt and Zahn[7] with 'Parlodion' covered 200 mesh grids. The hypophase was 0·15 M ammonium acetate, *p*H 7·4. The DNA-cytochrome films were aged for 10–30 min. The grids were shadowed while rotating with platinum–palladium (except *c* which was also shadowed from a single direction) and examined in a Philips *EM* 200 electron microscope. The molecules shown were found in the sixth fraction from the open band. The fourth fraction showed a predominance of the molecules shown in Fig. 5. The eleventh and twelfth fractions contained mostly 5μ monomers with only 10 per cent dimers. (× *c.* 20,600.)

the continuity or lack of continuity of a light halo (absence of grains) along the edges of the fibres. This halo may be a result of the gathering of the cytochrome from the bulk of the film on to the DNA. (*c*) Both overlaps otherwise had the same appearance, and the fibres at the joint were not thickened as might be expected if they were held by large protein joiners. (*d*) Except when the molecules appeared to be pulled from each other and contained only one overlap, there were never sharp corners at the critical overlaps as would be expected if the fibres were joined by mechanically restrictive chemical bonds. From the foregoing we have concluded that at least most of the dimers contained in the middle band (Fig. 1) are catenanes. Our use of the term catenane to describe these compounds follows the usage of Wasserman[8]. The term concatenate has been used to describe oligomers of DNA of unknown structure[9].

Higher oligomers, $n > 2$, were also reported previously[5] and have been found in this study in the lower band and at a higher frequency in the middle band. Trimers, tetramers, pentamers and a septamer have been found, and some examples are shown in Fig. 5. The interlocking nature of these molecules was confirmed by examining the photographs and in some cases by focusing the electron microscope.

We have considered the possibility that accidental overlapping of molecules could result in apparent catenation. Such accidental overlaps occur only infrequently for circular molecules of this length. We have examined photographs of polyoma circular DNA (1·5μ), and λ phage circular DNA (15μ) as well as mitochondrial DNA, and have found that even at high surface concentrations the DNA molecules seem to avoid one another (Fig. 2*a*) rather than overlap. A plausible explanation for this effect is that the complex of DNA and cytochrome is able to move at the surface of the hypophase. Because the DNA-cytochrome films are allowed to age for from

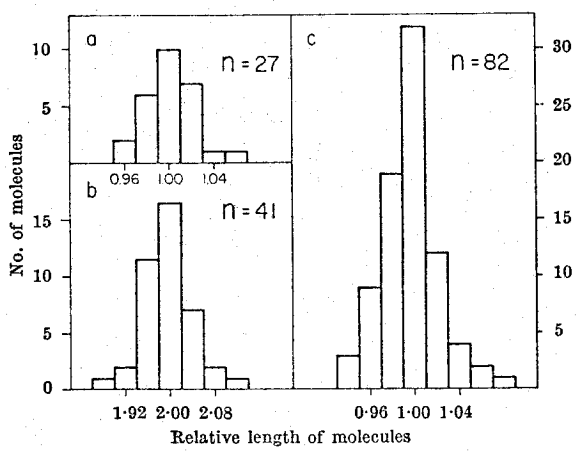


Fig. 3. Length measurements of mitochondrial DNA molecules from the middle band shown in Fig. 1*a*. Single length (monomeric) molecules. The approximately 5μ lengths were normalized by the mean length for the population. The standard deviation was 2·1 per cent. *b*, Double length (dimeric) molecules. The lengths were normalized by the factor used in *a*. The normalized mean length was 1·994 with a standard deviation 2·2 per cent about 2·000. *c*, Monomeric units contained in dimers. The lengths were again normalized by the mean length of monomers in *a*. The mean length was 0·997 with a standard deviation of 2·5 per cent about 1·000. The normalization procedure reduces the effect of drift in the magnification factor and stretching of the 'Parlodion' specimen supports. This latter factor is an important cause of scatter. In effect we are using the approximately 5·3μ monomers as internal relative standards in photographs of one grid hole. All these data were obtained with one specimen grid. Results from another grid were quantitatively similar. Length measurements were made on low gloss prints with a map measure.

10 to 30 min, the complexes are able to separate from each other and expose to the surface the most possible protein side groups. Careful examination of the dimers which had been shadowed from one direction showed that only very few apparent dimers were accidental overlaps. There is a phenomenon known as flower pattern formation which causes the aggregation of DNA into large complex structures. Flower patterns were observed infrequently on one or two of the grids and were easily distinguishable from the higher oligomers by their very compact nature.

These catenated molecules represent a new class of naturally occurring compounds. Molecules with topological bonds[10] were first identified by Wasserman[8], who formed catenated dimers of thirty carbon rings. Wang[11] prepared non-covalent catenanes by cyclizing viral λ DNA in solutions containing high concentrations of hydrogen bonded phage 186 circular molecules. Topologically bonded DNA molecules have been known for some time. Monomeric mitochondrial DNA and all other closed circular duplex DNAs contain single stranded DNA molecules which are topologically bonded to their respective complements. The pairs of single rings in closed duplex molecules are interlocked by one topological bond which is characterized by a topological winding number, α, of about 450 in polyoma[12,13] and SV 40[4] DNA and about 1,500 in closed circular mitochondrial DNA. The quantity α represents the number of times one strand winds about the other when the molecule is constrained to lie in a plane. The corresponding quantity, A, for interlocked duplexes is unity in all the catenanes described here.

In the discussion which follows the circular duplex will be considered as a single circular system (the individual polynucleotide strands will not be considered). Catenated

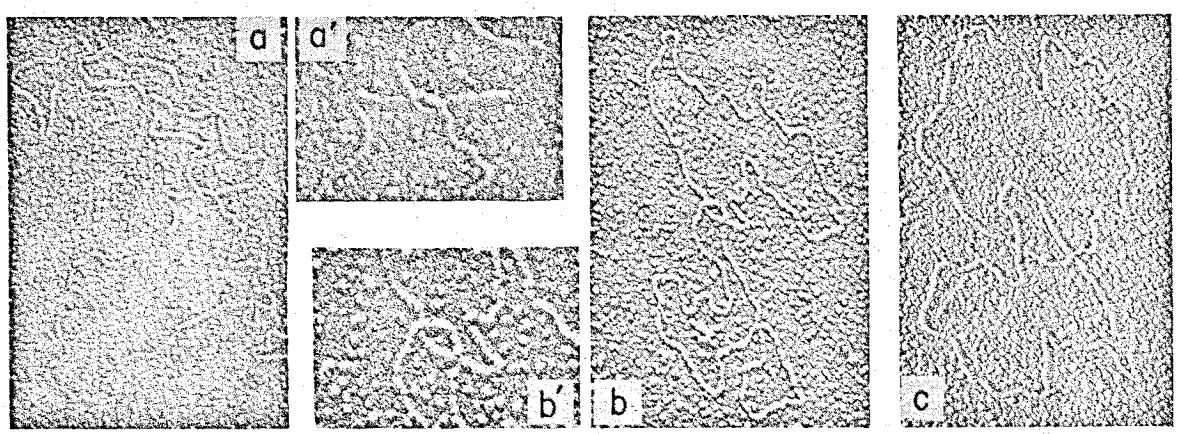


Fig. 4. The interlocking nature of catenated dimers. The specimen grid prepared as described in Fig. 2 was subsequently shadowed from one direction to reveal the three dimensional nature of the intersections. (× *c.* 18,775 except *a'* and *b'* which are × *c.* 34,600.)

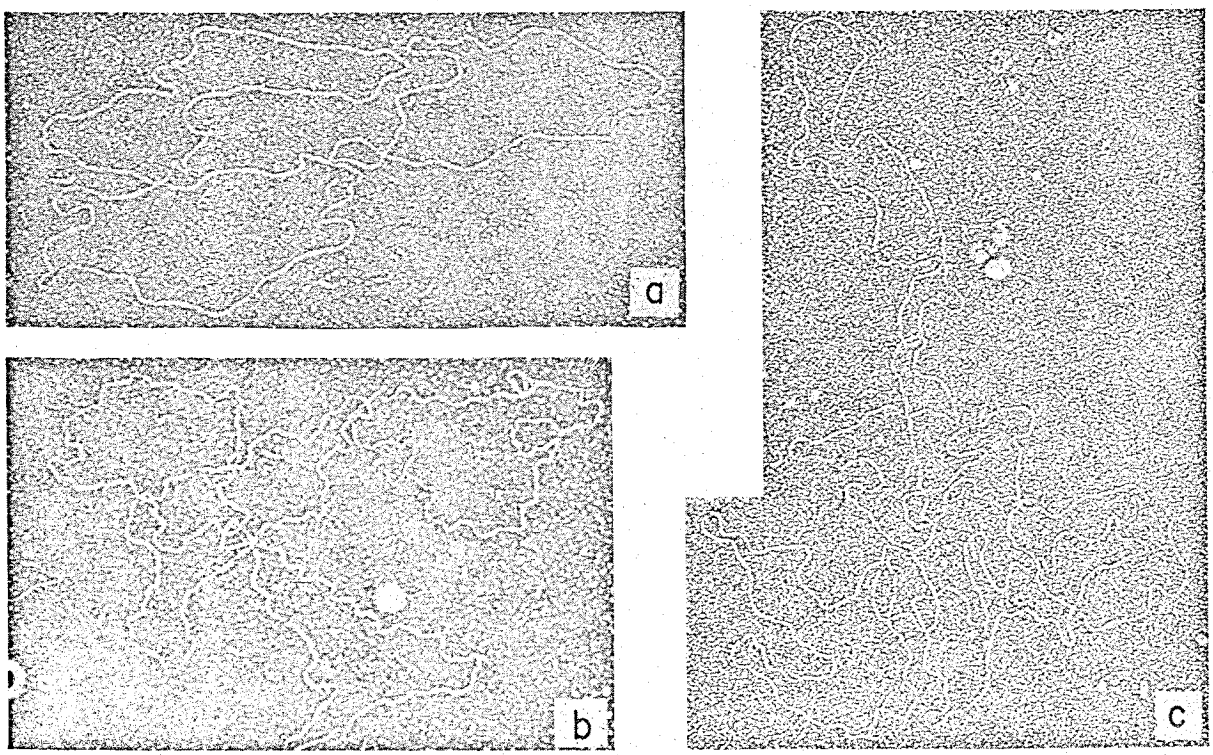


Fig. 5. Higher oligomers of mitochondrial DNA. The specimens were prepared as described in Fig. 2. *a*, A trimer b_2c. *b*, A tetramer b_2c_2. *c*, A pentamer b_2c_2e. (× *c.* 20,600.)

trimers differ from dimers in that at least one ring is joined to two others. In a linear trimer two of the molecules have a topological bonding number (TBN) of one, and the third a bonding number of two. All three molecules in the cyclic trimer have a TBN of 2. Isomerism based on the TBN results in a large number of species in the case of higher oligomers. Figure 6 presents the isomers for $n \leq 4$ together with a notation system based on the TBN. The criterion for determining the TBN is the number of circles that must be cleaved in order to free an intact circle. In this system a stands for TBN = 0, b for TBN = 1, and so on. Subscripts indicate the number of monomers of a given topological bonding number in the oligomer. The pentamer shown in Fig. 5*c* is characterized by the formula b_2c_2e. This system does not specify the topological winding number in the bonds, nor does it distinguish structural isomerism based on the three dimensional arrangement of the topological bond. Figure 7*b* shows the optical isomers for the cyclic trimer, and

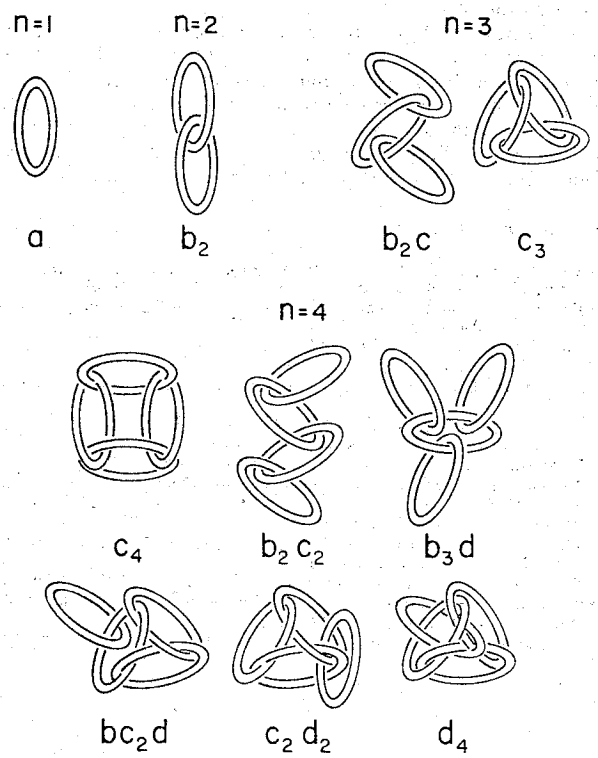


Fig. 6. Topological bonding number (TBN) isomers. The simple connexion of two circles by a single winding ($A = 1$) is regarded as the only type of bond. The formula representations are based on a for TBN = 0, b for TBN = 1, and so on.

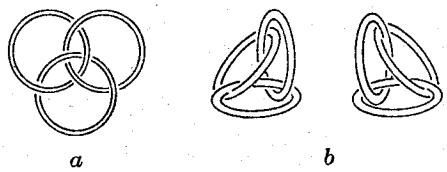


Fig. 7. Examples of topological isomers other than topological bonding number isomers[10]. *a*, A Borromean ring, a trimeric structure with topological bonds based on a linkage type other than simple catenation. *b*, The pair of enantiomers for one of the c_3 species. There is another pair of enantiomers for a second c_3 species. The latter pair differ from the one shown in the order of the overlaps of the crossings.

Fig. 7*a* represents a trimer with topological bonds based on a higher type of topological linkage than simple catenation. There is a further type of isomerism, based on the polarity of the strands in the Watson–Crick duplex, which gives rise to two polarity isomers for the catenated dimer and to many further isomers for the higher oligomers.

We now consider the origin of the oligomers in terms of known processes in which the backbone bonds of DNA are formed or re-arranged. We postulate that these molecules arise either in the course of replication or recombination. The mode of replication of closed circular molecules is not yet understood adequately to permit predictions of the possible structures that might be formed as replication intermediates or as a result of errors of replication. The breakage and reunion model for genetic recombination, on the other hand, enables us to formulate a plausible scheme for the formation of the catenated oligomers described. It also enables us to correlate the results presented here with those obtained with mitochondrial DNA from human leucocytes as described in the following article. The model also suggests the existence of structures that have not as yet been observed. The catenated molecules, according to the recombination model, result from a double recombination between two circular mitochondrial DNA molecules. This process could occur as two consecutive single recombination events or as a concerted process in which both recombination events occur within the same pairing region (Fig. 8). Higher oligomers would be formed by double recombinations between a monomer and catenated dimers. Doubly interlocked dimers ($A = 2$) should result from four recombination events. The recombination model for the origin of catenated circles predicts that circular dimers without crossing points should also occur. Such species have been observed at a very low frequency in the bottom (closed) band. It is possible that the low frequency is in part a consequence of the loss of cyclic dimers from the closed band because of statistical nicking. Also, twisted circular dimers are difficult to distinguish from catenated dimers. These circular oligomers of mitochondrial DNA

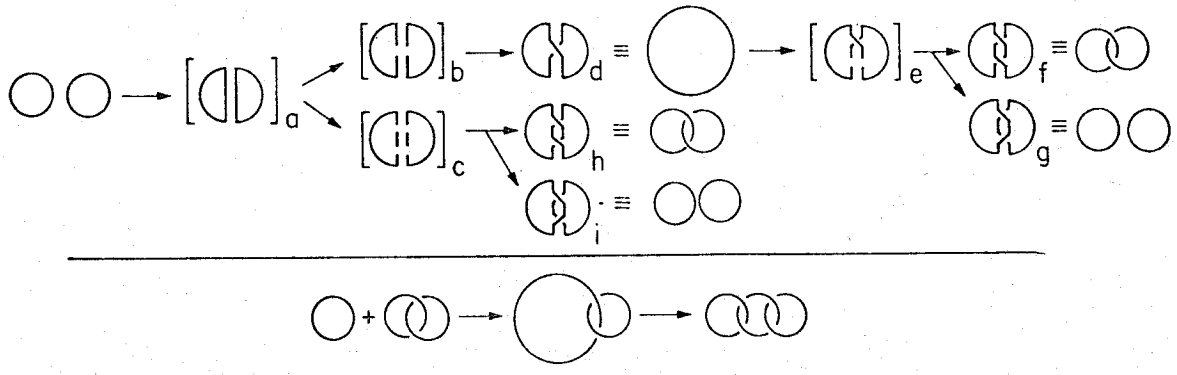


Fig. 8. The circular mitochondrial molecules first pair (*a*) and are then "broken" either once (*b*) or twice (*c*) as shown. If broken once, reunion results in an open dimer (*d*) which can pair again (*e*) and recombine. Half of the products of the second recombination will be catenanes (*f*) while the other half will be separate circles. If broken twice (*c*) half of the recombinations will result in separate circles (*i*) while half will be catenanes (*h*). Double recombination events between dimeric catenanes and monomers result in trimeric catenanes and this can be continued to higher species.

are more frequent in other systems and are discussed in more detail in the following article. Species involving 10μ circular dimers catenated to 5μ monomers should also be seen but have not yet been observed in mitochondrial DNA of HeLa cells. Double interlocked dimers ($A=2$) have not as yet been conclusively identified.

The recombination model places the various types of mitochondrial DNA species in a sequence running from circular monomers to circular dimers, to catenated dimers, to higher oligomers. In particular, it predicts that catenated dimers will occur before higher oligomers and that, as catenated dimers increase, circular dimers will decrease. The population of mitochondrial DNA is viewed as an equilibrium population, and the various possible distributions of mitochondrial species as different positions in a multiple equilibrium. The equilibrium position for HeLa cells seems to be well to the right of catenated dimers. Other equilibrium positions are known for human leukaemic leucocytes (see following article).

The sequence of events depicted in Fig. 8 depends somewhat on the particular mechanism of the recombination process. An alternative mechanism which has been proposed[9] does not involve the initial formation of a four-stranded pairing region between the duplex molecules. Instead the molecules are specifically broken, matched with another specifically broken molecule and reunited. Reciprocal recombinants are not formed in the same recombination event but are statistically equally likely. The ends of the molecule are free to move in solution. The most reasonable proposal for the specific recognition system for the matching of the broken molecules is that the initial breakage gives rise to single stranded cohesive ends on the two fragments. This model of the recombination event differs from the homologous pairing model (Fig. 8) in that catenated dimers can arise from monomers without the intermediate formation of a cyclic dimer. If linear molecules with cohesive ends are formed in low concentration and then recyclize in the presence of closed circles some catenanes will form. According to this model catenanes are only a by-product of recombination; the two members of a dimer have not undergone a recombination event with each other. In conditions of high concentrations of linear molecules with cohesive ends, cyclic dimers would form. Another mechanism for catenane and cyclic dimer formation involves the opening and reclosure of mitochondrial DNA molecules at one specific point in the molecule. This process would be similar to the closure and reopening of λ phage DNA and is presumably not related to recombination.

Genetic recombination between cytoplasmic genomes in *Chlamydomonas* has been demonstrated in the experiments of Sager[14]. Possibly the observed fusion of mitochondria in the cytoplasm[15] is a manifestation of a recombination event. Interaction between the mitochondria of different strains of maize has been reported to result in a new type of mitochondria in the hybrid strain[16]. On the other hand, because the number of DNA molecules in specific mitochondria is not known, it is possible that the recombination process described here could occur between molecules in each mitochondrion. Our results show that in some mitochondria, possibly those which are structurally or functionally abnormal, there are at least two to seven mitochondrial-length molecules. The monomeric units of the oligomers described here are all the same length and have approximately the same base composition and are therefore, probably identical genomes. Polymerization of mitochondrial DNA molecules may result in gene duplication which would otherwise not occur.

We thank S. Hudson for printing electron micrographs and measuring the molecules; L. Wenzel, C. Phillips and J. Eden for their assistance in the preparation of HeLa cells; P. Koen for assistance with the electron microscope; and R. Kent for assistance in the preparation of the manuscript. This work was supported in part by grants from the US Public Health Service.

Received October 23, 1967.

[1] Van Bruggen, E. F. J., Borst, P., Ruttenberg, G. J. C. M., Gruber, M., and Kroon, A. M., *Biochim. Biophys. Acta*, **119**, 437 (1966).
[2] Kroon, A. M., Borst, P., Van Bruggen, E. F. J., and Ruttenberg, G. J. C. M., *Proc. US Nat. Acad. Sci.*, **56**, 1836 (1966).
[3] Pikó, L., Tyler, A., and Vinograd, J., *Biol. Bull.*, **132**, 68 (1967).
[4] Bauer, W., and Vinograd, J., *J. Mol. Biol.* (in the press).
[5] Radloff, R., Bauer, W., and Vinograd, J., *Proc. US Nat. Acad. Sci.*, **57**, 1514 (1967).
[6] LePecq, J., and Paoletti, C., *Anal. Biochem.*, **17**, 100 (1966).
[7] Kleinschmidt, A. K., and Zahn, R. K., *Z. Naturforsch.*, **14b**, 770 (1959).
[8] Wasserman, E., *J. Amer. Chem. Soc.*, **82**, 4433 (1960).
[9] Thomas, C. A., *Prog. Nucleic Acid Res.*, **5**, 315 (1966).
[10] Frisch, H. L., and Wasserman, E. J., *J. Amer. Chem. Soc.*, **83**, 3789 (1961).
[11] Wang, J., *Biopolymers* (in the press).
[12] Vinograd, J., Lebowitz, J., Radloff, R., Watson, R., and Laipis, P., *Proc. US Nat. Acad. Sci.*, **53**, 1104 (1965).
[13] Vinograd, J., Lebowitz, J., and Watson, R., *J. Mol. Biol.* (in the press).
[14] Sager, R., and Ramanis, Z., *Proc. US Nat. Acad. Sci.*, **53**, 1053 (1965).
[15] Robertson, J. D., *Sci. Amer.*, **206**, 64 (1962).
[16] Sarkissian, I., and McDaniel, R., *Proc. US Nat. Acad. Sci.*, **57**, 1262 (1967).

The Sedimentation Velocity Properties of Complex Mitochondrial DNA

Bruce Hudson and Jerome Vinograd

The interlocked duplex DNA rings in a catenane appear to sediment as independent units when the rings are relaxed and as connected units when the rings are in a superhelical conformation.

Mitochondrial DNA from animal cells exists primarily in the closed circular form with a molecular weight of 10-11 million daltons. Complex forms of mitochondrial DNA from HeLa cells [1] and human leukemic leucocytes [2] have also been described. These complex forms are of two types. First, there are circular dimers which are circular molecules with twice the molecular weight and contour length of the monomers. Second, there are catenanes, consisting of two or more interlocked monomeric circles. Circular DNA molecules can have either a closed (intact) or open (nicked) circular topological structure and either a superhelical or relaxed conformation. Catenated dimers can have three topological structures and three corresponding conformations. We will refer to the three structures as doubly-open, doubly-closed, and singly-closed structures. The three structures have been identified on the basis of their differing interaction with ethidium chloride in a cesium chloride gradient.[1,3] This paper reports the results of an investigation of the conformational states of catenated DNA molecules by the sedimentation velocity method.

In order to compare our results with sedimentation results for other DNA species, we have correlated the sedimentation coefficients of closed circular superhelical DNAs of various molecular weights. Similar correlations are made for open circular DNAs. These comparisons strongly suggest that the conformation of the doubly-closed catenated dimer is so compact that the two rings are effectively mechanically connected. This also seems to be the case for triply-

closed catenated trimer and the singly-closed catenated dimer. On the other hand, the catenated dimer in which both of the submolecules are in the relaxed conformation has a sedimentation coefficient smaller than that of the relaxed monomer. The latter result indicates that the conformation of the doubly-open catenane is sufficiently relaxed that the two circles are effectively unconnected and sediment independently.

Band Centrifugation of HeLa Cell Mitochondrial DNA and the Identification of Components

The sedimentation velocity pattern for HeLa cell mitochondrial DNA is shown in Fig. 1. The numbers near the bands are the sedimentation coefficients of each sedimenting component to the nearest integer. The closed circular DNA used in this experiment was prepared as described below just prior to the sedimentation experiment. It should, therefore, consist primarily of closed circular species. An analysis with the electron microscope indicated that the sample contained, in addition to the monomer, about 11% by number catenated dimers and about 1% catenated trimers, or about 18% and 2% respectively on a mass basis. With this background information, and the assumption that nicking of the DNA molecules that had occurred was random, the above velocity pattern can be uniquely analysed with the aid of the Poisson relation. There is also the additional information that the closed and open circular monomeric species have sedimentation coefficients of 38-39 S and 27 S respectively. In the pattern in Fig. 1 the 37 S species is considered to be the superhelical monomer. It is the major species, as expected. The

Fig. 1

D

difference between the measured value, 37 S, and the reported values,[4] 38-39 S, obtained by other workers is statistically significant. It probably arises from a difference between the CsCl band velocity procedure and the NaCl boundary velocity procedure which will be discussed below. If this is the case, all of our values are expected to be slightly lower than those of other workers by about 4%. The 26 S species is identified as the relaxed circular monomer because of its sedimentation coefficient. We can estimate from the relative areas under the 26 S and 37 S bands that about 15% of the monomers have suffered single strand scissions. The catenated dimer should therefore be present for the most part in the form of the doubly-closed species. This identifies the 51 S material as the doubly-closed catenane. The area under this band is about 15% of the total, in agreement with the fraction of catenanes found by electron microscopy after allowing for the nicking that had occurred. We tentatively assign the 62 S peak to a fully superhelical trimeric catenane, the 40 S peak to a catenated dimer consisting of one superhelical and one relaxed circular submolecule, and the 23 S peak to a fully relaxed catenated dimer.

Some of the bands in this pattern are very small. Our confidence in their reality is based on the fact that these small bands are seen in most of the scans in a given experiment, they move from scan to scan and give a linear plot of log r versus t. Also, they each appeared in at least two different velocity experiments with the same sedimentation coefficient. Further evidence for the reality of the 23 S band will be presented below. The smaller bands in Fig. 1 represent less than 0.01 microgram of DNA or 2×10^8 molecules for the trimers.

A sedimentation velocity analysis of the same sample of HeLa mitochondrial DNA after storage for two weeks is shown in Fig. 2. The 37 S closed circular superhelical monomer is now a minor component while the 26 S relaxed circular monomer is the major component. Most of the monomer has been nicked. The catenated dimer should therefore be largely in the form of the doubly-open species. The 23 S component, now increased in relative amount, is identified as the doubly-open catenane, in agreement with the previous assignment. The relative areas under the curves agree with the expected composition based on electron microscopy.

Fig. 2

The sedimentation coefficient, 23 S, is also the value predicted for linear molecules of the mitochondrial molecular weight. Five μ linear molecules were not seen in the original preparation in the electron microscope. The possibility that single strand scissions gave rise to linear molecules prior to centrifugation must still be considered. Such processes seem very unlikely because of the presence of the 23 S component in the sample analysed in Fig. 1 which is predominately in the closed circular form. Previous calculations[5] have shown that linearization of 10% of a 10 million dalton circular duplex requires on the average about 150 nicks per molecule, and that closed duplexes are absent from samples which have been linearized by random nicking.[6,7] Similarly, the presence of some closed 37 S material in the sedimentation pattern shown in Fig. 2 shows that linear molecules

cannot be being generated by introduction of single strand scissions. Also, the agreement between the observed and expected amounts of 23 S material expected from electron microscopy and from the conversion of the 53 S component argues that it consists of the catenanes. The sharpness of the 23 S band and the absence of any trailing material other than the EDTA also argues strongly against any enzymatic or random hydrolytic linearization.

In order to confirm our assignment of the fully relaxed catenated structure to the 23 S band, a sedimentation velocity experiment was performed in the preparative ultracentrifuge with fully nicked HeLa mitochondrial DNA. The fractions in the region which should contain 20-30 S material were examined in the electron microscope. The results are shown in Table 1. These results show that there is an enrichment of catenanes in a region centripetal to fractions that contain monomers and are depleted in catenanes. Very little, $<5\%$, linear DNA was found on any of the grids and it was mostly small, $<2\ \mu$, and heterogeneous.

Table 1

The other form of complex mitochondrial DNA is the circular dimer. A particular patient suffering from chronic granulocytic leukemia has mitochondrial DNA which consists primarily of circular dimers. The sedimentation velocity pattern of a sample of closed circular mitochondrial DNA from this patient has been previously presented.[2] The sedimentation velocity pattern of open circular mitochondrial DNA from the same patient has been analyzed and the sedimentation coefficient of the relaxed circular form, 33 S, determined.[8]

The Determination of Standard Sedimentation Coefficients from Band Centrifugation Experiments in CsCl Solution

The standard sedimentation coefficients, $s^0_{20,w}$, for Na DNA presented here have been obtained from measured sedimentation coefficients with the correction procedure of Bruner and Vinograd.[9] This correction proceeds in two steps. A "plot" of $s\eta_{rel}$ versus ρ (in a computer program) is extrapolated linearly to $\rho = 0.998$ g/ml from the observed result $s^0_{20}\ \eta_{rel}$ in a CsCl solution with a density $\rho \approx 1.35$ g/ml and the buoyant density corrected to 20°. This linear extrapolation is based on the experimentally observed linearity of such a plot for T7 phage DNA. The extrapolated value is converted to the sodium salt value by multplication by an experimental factor determined in the experiments with T7 DNA. Our $s^0_{20,w}$ values are slightly lower than the values observed by other workers for mitochondrial DNAs. Thus, for form II determined in 1 M NaCl there is fairly general agreement[4] on a value of 27.4 ± 0.5 S, but our value is 26.1 ± 0.2 S. Dawid[10] has measured the sedimentation coefficients of the mitochondrial DNA from Xenopus laevis and Rana pipiens in both NaCl and CsCl solutions and has recalculated the cesium to sodium DNA correction factor. His value differs from the previous value by 3%. If we use his value for the correction factor, we calculate a sedimentation coefficient of 26.9 S for form II, in good agreement with published results by others. This is also the case for form I. It has been found recently in this laboratory[25] that the plot of $s\eta_{rel}$ versus ρ for SV 40 DNA is not quite linear for concentrated CsCl solutions but curves slightly

downward. Some downward curvature can, with hindsight, be seen in the data of Bruner and Vinograd. This curvature probably accounts for the different apparent values of the cesium to sodium DNA conversion factor and for the slight deviations between our sedimentation coefficients and those determined in NaCl.

Correlations between Molecular Weight and Sedimentation Coefficients

Several correlations between the molecular weight of a DNA molecule and its sedimentation coefficient have been presented for linear, open circular and closed circular, superhelical DNAs. Crothers and Zimm[11] have investigated the dependence of the sedimentation coefficient of linear DNA as a function of molecular weight determined by light scattering methods for the lower molecular weights and more approximate methods for the higher molecular weights. Gray, Bloomfield and Hearst[12] have presented a semi-empirical justification of the equation of Crothers and Zimm. They have also extended this semi-empirical treatment to the case of relaxed circular DNA.

Several DNAs occur or have been prepared in both linear and open circular forms and the sedimentation coefficients have been measured for both forms. The molecular weights of these DNAs can be calculated from the correlation of s versus M for linear DNAs of Crothers and Zimm. The resulting molecular weights and sedimentation coefficients for circular DNAs can be used to test the semi-empirical treatment of Gray, Bloomfield and Hearst. This test is shown in Fig. 3, a graph of the sedimentation coefficient against the 0.445th power of the molecular

Fig. 3

weight. The ordinate scale here is linear rather than logarithmic as is customary in such correlations. The ordinate scale therefore, reveals small deviations in the values of the sedimentation coefficient. Approximate values of the molecular weights, based on electron microscopy, of two species of mitochondrial DNA are also shown. The Gray, Bloomfield and Hearst equation

$$S = 2.7 + 0.01759\ M^{0.445} \qquad (1)$$

seems to account for the observed data as well as any equation of such a simple form can. Equation (1) can be used for the determination of molecular weight of a circular DNA when the sedimentation coefficient of the relaxed circular form is known. Such molecular weights can then be used to establish a correlation between molecular weight and the sedimentation coefficient of closed circular, superhelical DNA. This consecutive procedure has the advantage that it is based on a single method for the calculation of molecular weights. The correlation of molecular weight with sedimentation coefficient for closed circular forms is shown in Fig. 4. The filled symbols are the new results obtained in this work.

Fig. 4

The sedimentation coefficient of closed circular DNA should depend on its superhelix density. The correlation of s with M shown in Fig. 4 assumes that there are no significant fluctuations in the superhelix density of the various DNA forms or that these fluctuations are not large enough to affect the sedimentation velocity. Bauer and Vinograd[24] have shown that the sedimentation coefficient of SV 40

DNA is very insensitive to superhelix density in the region near the native superhelix density. It has recently been shown in this laboratory that the superhelix density of HeLa cell mitochondrial DNA is approximately 50% lower than that of SV 40 DNA and several other mitochondrial DNAs.[25] HeLa mitochondrial DNA, nevertheless, has very nearly, if not exactly, the same sedimentation coefficient as that of other mitochondrial DNAs. Variations of approximately 50% in the superhelix density of these closed circular DNAs can be tolerated without any influence on the observed dependence of s on M.

Connectedness and Unconnectedness in Catenanes

Several conclusions can be drawn from these correlations of s with M and the data in Table 2. The sedimentation coefficient of the closed circular dimer is that expected for a dimer of the mitochondrial length. The doubly closed catenated dimer has the same sedimentation coefficient as the closed circular dimer. This is also the case for the triply-closed catenated trimer, which has the sedimentation coefficient expected for the closed circular trimer, Fig. 4. The above results imply that these catenated molecules behave as if they were connected structures with molecular weights and conformations similar to those of the appropriate circular oligomer. The singly-closed catenated dimer also behaves as though it was a connected structure. The sedimentation coefficient of this species is between those of the doubly-closed circular dimer and the doubly-open circular dimer, as would be expected for a dimer with an intermediate frictional coefficient.

The sedimentation coefficient of the doubly open catenated dimer is less than the sedimentation coefficient of an open monomer. It is possible that the two interlocked circular DNA sub-molecules have, as a unit, a very large frictional coefficient. If we make the reasonable assumption that there is no difference between the partial specific volumes of interlocked and non-interlocked circles, we can calculate the ratio of the frictional coefficient of the doubly open catenane to that of an open monomer. In this calculation the molecular weight of the catenane is set equal to twice the value of the monomer circle. Similarly, we can calculate the frictional coefficient of an open circular dimer. These results for the ratio of the frictional coefficients of the open molecules are 1.00 for the 26 S circular monomer, 1.56 for the 33 S circular dimer and 2.26 for the 23 S catenated dimer. In the absence of hydrodynamic interaction, the localization of two identical objects in a region increases the frictional coefficient for the region as a whole by two. Hydrodynamic interactions will generally lower the frictional coefficient by shielding.[26] The localization of the segments of two circular monomers into the conformation of an open circular dimer increases the frictional coefficient by only 1.56, considerably less than 2.00. In order to account for the observed sedimentation coefficient of the doubly-open catenated dimers, we must postulate that the frictional coefficient increases by a factor which is larger than two. The only way this could happen is that an extensive rearrangement of the random coil structure of the circular monomers occurs as they are conceptually combined to form a catenane.

The conformation of DNA with the highest frictional coefficient is that of a rigid linear molecule. A minimum sedimentation coefficient, 17 S, may be calculated from the Perrin equation[27] for a prolate ellipsoid with a semi-minor axis of 13 Å and a molecular weight 20×10^6. A rigid rod with about 3/4 of the length of such an ellipsoid would have the observed sedimentation coefficient of 23 S. Such a model is more extended than any conformation that can be proposed for a catenane which can be maximally extended to 50% of its contour length. The most probable conformation for a catenated molecule is two more or less interpenetrating spherical distributions of segments. This would certainly not have the high frictional coefficient calculated for a twenty million molecular weight 23 S species.

An alternative possibility is that these relaxed catenated molecules do not behave as a twenty million molecular weight unit but instead sediment as partially independent ten million molecular weight units. This possibility is plausible for several reasons. First, there is conceptually little difference between catenated molecules and a concentrated solution of linear molecules, on an instantaneous basis. A solution of 10 million molecular weight DNA molecules at 50 μg/ml has an average distance between molecular centers of about 4×10^{-5} cm. Each molecule has a radius of gyration of about 3×10^{-5} cm. Thus, the segmental distributions of the catenated submolecules inter-

the segmental distributions of the catenated submolecules interpenetrate significantly. Second, there is the argument presented above that no 20 million molecular weight molecule could have the necessary frictional coefficient. Third, there is the suggestive fact that the sedimentation coefficient of the catenanes is close to that of the monomer. If the two submolecules have independent sedimentation properties, one would expect this result.

The sedimentation coefficient of the relaxed circular catenane is smaller than that of the relaxed monomer. The most likely explanation for this is that the interpenetration of the two segmental distributions causes a perturbation of each of the distributions in the direction of expansion.

The Mechanism of Connectedness

The differing properties of catenated superhelical molecules and catenated relaxed circular molecules can be rationalized by considering a microscopic description of the sedimentation process. The motion of a polymer in a viscous solution subject to a gravitational field consists of a large number of correlated, biased walks. Each polymer segment undergoes a random walk process modified by two forces - the bias of the gravitational field and the motion of the other polymer segments. It is the correlation of the motions of the polymer segments which determines the connectedness of the molecule. If the

correlation is strong the molecule will behave as if it were a particle with the mass of the sum of all the masses of the segments. If the correlation of the motion of the segments, or groups of segments, is weak, the separate groups will behave independently. Individual molecules in concentrated solutions normally show weak correlations. The important point is that the distance scale in which correlating interactions act is of the order of the mean free path of the walk of a polymer segment in solution. This distance is very small.

Superhelical circular molecules have a more compact conformation than relaxed circular molecules. The differing behavior of catenated superhelical molecules and catenated relaxed circular molecules can be explained by the reasonable assumption that the segment density in the region of overlap is relatively high in the superhelical catenane but relatively low in the relaxed catenane. This increase in segment density increases the correlation between the two sub molecules sufficiently that the catenated superhelical molecule behaves as though it were connected.

This description of the origin of connectedness raises several questions which are subject to experimental test. If a catenated molecule in which both sub molecules are supercoiled is titrated with the intercalating drug ethidium bromide, the conformation can be varied continuously from a fully superhelical to a fully relaxed state. Is there a possibility of partial connectedness for the intermediate conformation? If so, what does the concept of molecular weight mean under these circumstances? If the diffusion coefficient of the doubly-open mitochondrial

catenanes were measured, would the application of the Svedberg equation lead to a molecular weight of 10 million? Large molecules, e.g., λ phage DNA, can be catenated to smaller circular DNA molecules, e.g., SV 40 viral DNA by the method of Wang and Schwartz.[28] Would the larger molecule "drag" the smaller in a sedimentation velocity experiment? When one of the circles is larger than another, and if "drag" occurs, the frictional coefficient for the velocity experiment would depend on the rotor speed even at infinite dilution.

The proof of structure of catenated mitochondrial DNA molecules was argued from two kinds of evidence in a previous publication.[1] The presence of a middle band in an ethidium bromide-cesium chloride gradient consisted primarily of dimers as seen in the electron microscope. Other studies of the mechanism of this interaction on the buoyant densities of closed and open DNAs allowed us to conclude that the dimeric structure in the middle band must have one of the structures shown in Fig. 5. The choice of structure (a) was based on electron microscopic examination of specially prepared specimen grids. The results presented in this paper exclude structures (b) and (c) because these structures would have higher sedimentation coefficients than the monomers. The catenated structure can now be demonstrated on the basis of evidence which is primarily physical as opposed to electron microscopy. In this respect, the present results are strongly complementary to those of Rhoades and Thomas.[15] These authors have detected and examined several intra-

cellular forms of P22 phage DNA. They found a fast form, 80 S, which, upon suffering a single strand scission resulted in a 60 S form and finally upon more hydrolysis, a 40 S form. The P22 monomer has sedimentation coefficients of 60 S and 40 S. They concluded from this two phase velocity decay pattern of the 80 S form that it was an interlocked form. The overall pattern of sedimentation coefficients agrees very well with our pattern. Their use of the isolated 80 S component removes all remaining ambiguities as to the assignment of bands with components and thus supports our assignment of sedimentation coefficients. Our previous structural results and the present sedimentation velocity results strongly support their assignment of the interlocked structure to the 80 S P22 intracellular form.

The catenated structures represent a new class of high polymers. The unusual structure of these molecules leads one to expect unusual physical properties. This has proven to be the case for transport properties.

Culture of HeLa Cells and the Isolation of Mitochondrial DNA. HeLa S3 cells were grown in suspension culture in 1 liter spinner bottles or in the three 15 liter jars of a New Brunswick fermentor, Model FS-314, in Eagle's medium containing 10% calf serum. The cells were grown in the fermentor from 1 x 10^5 cells/ml to about 5 x 10^5 cells/ml in 5 days. One to 3 x 10^{10} cells were harvested from the fermentor cultures with a laboratory model Sharples continuous flow centrifuge. The packed cells occupied 60 to 70 ml. This volume of material was processed in 6 portions. The cells were washed, homogenized and subjected to differential centrifugation as previously described except that the homogenizing medium also contained 0.25 M sucrose. The crude mitochondrial pellet from a "high speed spin", 10 Krpm, 10 minutes, Sorvall SS34 rotor, was suspended in 10 ml 0.25 M sucrose, TKM buffer (0.05 M Tris, 0.025 M KCl, 0.0025 M $MgCl_2$, pH 6.7) and 0.1 ml of 1 mg/ml bovine pancreatic DNAase I, 1 mg/ml bovine pancreatic RNAase (Sigma Chemical Co.) in 0.01 M acetate buffer, pH 5.0, 0.002 $MgCl_2$, 0.004 M NaCl was added. The mixture was incubated for 30-40 minutes at room temperature. One ml of 0.1 M EDTA was added and the mixture cooled to 0° C. Another high speed spin yielded a pellet which was washed once in 0.25 M sucrose, 0.01 M Tris, 0.01 M KCl, 0.005 M EDTA, pH 7.4, and then suspended in 5 ml of a solution of Renografin 76[29,30] (Squibb) density 1.25 g/ml, 0.25 M sucrose, 0.1 M Tris, pH 7.4, and 0.005 M EDTA. This was overlayered with 15 ml volumes of similar Renografin solutions with densities of 1.20 and 1.10 g/ml and centrifuged in the SS 34 rotor at 16 Krpm for 2-3 hours. The

middle turbid layer at the interface between the 1.20 layer and the 1.10 layer was removed with a pipet and diluted with 0.25 M sucrose, 0.01 M Tris, 0.01 M KCl, 0.005 M EDTA, pelleted and washed 3 to 8 times in the latter medium. Three ml of 1% sodium dodecyl sulfate, 0.01 M Tris, 0.005 M EDTA was added to the pellet, and the suspension was incubated for two hours at room temperature or overnight at 4° C. This suspension was then processed as previously described in a CsCl-ethidium chloride density gradient.

Some preparations contain as much as 90% of the DNA in the closed circular band in the CsCl-ethidium chloride gradient. In one such preparation the upper band was examined in the electron microscope. The material consisted primarily of 5 μ circles (80%) and the remainder was linear, predominantly less then 5 μ. Heating the sample to 60° for 5 minutes or standing at room temperature for 24 hours in 0.2 M CsCl, 0.1% cytochrome c yielded electron micrographs containing only short linear molecules. It was, therefore, concluded that the upper band contained primarily mitochondrial DNA which had been fairly extensively attacked by DNAse during the nuclease treatment of the mitochondria. Thus, these preparations before CsCl-ethidium chloride centrifugation were at least 90% pure mitochondrial DNA. It may therefore be stated that mitochondrial DNA is at least 90% closed circular in the cell. Only the bottom band, which is 100% mitochondrial DNA was used for the experiments in this communication.

Analytical Band Sedimentation Velocity Experiments

Band centrifugation was carried out according to the method of Vinograd, et al.[31] Thirty mm double sector type I centerpieces and sample volumes of 30-50 μ l were used. The sedimentation solvent was 2.85 M CsCl, .01 M Tris, pH 7.4. Sedimentation was performed at 35,600 rpm at 20° and the results recorded with an automatic photoelectric scanner. The data were analysed with a digital computer.

We wish to thank L. Wenzel and J. Edens for their assistance in the preparation of the HeLa cells, J. Ceasar for assistance in the preparation of the manuscript, and D. A. Clayton for permission to quote his unpublished results. This work was supported in part by grants from the United States Public Health Service and a fellowship to BH from the National Science Foundation.

This is contribution number 3744 from the Division of Chemistry, California Institute of Technology, Pasadena, California.

[1] Hudson, B. and Vinograd, J., Nature, 216, 647 (1967)

[2] Clayton, D. A. and Vinograd, J., Nature, 216, 652 (1967)

[3] Radloff, R., Bauer, W., and Vinograd, J., Proc. Nat. Acad. Sci., 57, 1514 (1967)

[4] Borst, P. and Kroon, A. N., Int. Rev. of Cytology, in press (1968)

[5] Vinograd, J., Lebowitz, J., Radloff, R., Watson, R., and Laipis, P., Proc. Nat. Acad. Sci., 53, 1104 (1965)

[6] Crawford, L. V., J. Mol. Biol., 13, 362 (1965)

[7] Ruttenberg, G. J. C. M., Smit, E. M., Borst, P., van Bruggen, E. F. J., Biochim. Biophys. Acta, 157, 429 (1968)

[8] Clayton, D. A. (unpublished results)

[9] Bruner, R. and Vinograd, J., Biochim. Biophys. Acta, 108, 18 (1965)

[10] Dawid, I. B. and Wolstenholme, D. R., J. Mol. Biol., 28, 233 (1967)

[11] Crothers, D. M. and Zimm, B. H., J. Mol. Biol., 12, 525 (1965)

[12] Gray, H. B., Jr., Bloomfield, V. A., and Hearst, J. E., J. Chem. Phys., 46, 1493 (1967)

[13] Burton, A. and Sinsheimer, R. L., J. Mol. Biol., 14, 327 (1965)

[14] Wang, J. C. and Davidson, N., J. Mol. Biol., 15, 111 (1966)

[15] Rhoades, M. and Thomas, C. A., Jr., J. Mol. Biol., in press

[16] Hershey, A. D., Burgi, E., and Ingraham, L., Proc. Nat. Acad. Sci., 49, 748 (1963)

[17] Wang, private communication

[18] Bazaral, M. and Helinski, D., Biochemistry, in press

[19] Ray, D. S., Preuss, A., and Hofschneider, P. H., J. Mol. Biol., 21, 485 (1966)

[20] Crawford, L. V., J. Mol. Biol., 13, 362 (1965)

[21] Rush, M. G., Kleinschmidt, A. K., Hellmann, W., and Warner, R. C., Proc. Nat. Acad. Sci., 58, 1676 (1967)

[22]Kiger, J. A., Young, E. T., II, and Sinsheimer, R. L., J. Mol. Biol., 33, 395 (1968)

[23]Young, E. T. and Sinsheimer, R. L., J. Mol. Biol., 30, 165 (1967)

[24] Bauer, W. and Vinograd, J., J. Mol. Biol., 33, 141 (1968)

[25] Gray, H. B., Jr. (unpublished results)

[26] Kirkwood, J. G. and Riseman, J., J. Chem. Phys., 16, 565 (1948)

[27] Tanford, C., in Physical Chemistry of Macromolecules, 327 (Wiley, New York, 1961)

[28] Wang, J. C. and Schwartz, H., Biopolymers, 5, 953 (1967)

[29] Schatz, G., Haselbrunner, E., and Tuppy, J., Biochem. Biophys. Res. Comm., 15, 127 (1964)

[30] Shapiro, L., Grossman, L. I., Marmur, J., J. Mol. Biol., 33, 907 (1968)

[31] Vinograd, J., Bruner, R., Kent, R., and Weigle, J., Proc. Nat. Acad. Sci., 49, 902 (1963)

TABLE 1. VERIFICATION OF THE ASSIGNMENT OF THE RELAXED CATENATED DIMER WITH THE 23 S COMPONENT.

Fraction number	Molecules counted	Percentage catenated dimers
38, 40 *	-	-
42	100	3 ± 3
44	400	2 ± 1
46	200	4 ± 3
47	100	11 ± 6
50	100	19 ± 8
52	300	41 ± 6
54	100	51 ± 10
56, 60 *	-	-

* DNA was not seen on specimen grids prepared with these fractions

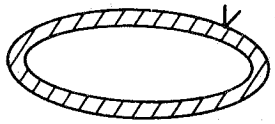
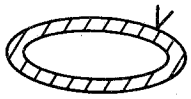
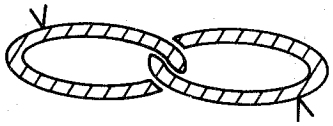
A sample of closed circular HeLa mitochondrial DNA was banded in a CsCl-ethidium bromide gradient. The material was primarily in the lower (closed) band. Upon standing at room temperature for 24 hours and rebanding, 90% of the material was in the upper (open) band. The contents of the tube were pooled, diluted, and pelleted at 41 Krpm in an SW 41 rotor for 8 hours, 20°. The pellet and bottom few drops were mixed in a total volume of 0.25 ml and layered on a 4.5 ml linear CsCl gradient (density 1.3 to 1.4 g/ml) in an SW 50 centrifuge tube. The tube was centrifuged at 45 Krpm in an SW 50 rotor for 3 hours, at 20°.

Fractions (.04 ml) were collected and numbered from the bottom of the tube. Previous calibration of this centrifugation procedure indicated that 21 S SV 40 DNA moved to approximately fraction 60. In this experiment mitochondrial DNA was found in the region corresponding to 22-28 S. The fractions listed above were processed for electron microscopy as previously described. Only unambiguous monomers and unambiguous dimers were counted in the scoring. The indicated errors are the 95% confidence limits calculated from

$$E \pm = 1.96\left(\frac{x}{n}\left(1 - \frac{x}{n}\right)\right)^{1/2}$$

where x is the number of catenanes and n is the total number of molecules scored.

TABLE 2. SEDIMENTATION COEFFICIENTS OF RELAXED AND SUPERHELICAL HUMAN MITOCHONDRIAL DNA FORMS.

	S		S
	62.3 ± 1.4 (5)		37.1 ± 0.3 (8)
	51.3 ± 0.5 (7)		33.4 ± 1.0 (2)
	51.6 ± 0.7 (4)		26.1 ± 0.1 (10)
	39.7 ± 1.3 (2)		23.0 ± 0.4 (4)

The solid figures represent closed superhelical molecules and submolecules. The cross-hatched figures with the V-mark represent open relaxed molecules and submolecules. The standard sedimentation coefficients, $S^{0}_{20,w}$ are for NaDNA and given in Svedbergs. The errors are the standard deviation of the results obtained in the number of experiments shown in parentheses.

LEGENDS

Fig. 1 Band sedimentation velocity patterns of HeLa cell mitochondrial DNA containing largely closed circular molecules. The sedimentation is from left to right. The absorbance at the top of the cell is due to EDTA in the sample. The scans were made approximately at the indicated times after attaining the nominal rotor speed. The numbers near the bands are the standard sedimentation coefficients of each component to the nearest integer and are expressed in Svedbergs for NaDNA.

Fig. 2 Band sedimentation velocity patterns of HeLa cells mitochondrial DNA containing largely open circular molecules. The sedimentation is from left to right. The scans were made approximately at the indicated times after attaining nominal rotor speed. The numbers near the bands are the standard sedimentation coefficients of each component to the nearest integer and are expressed in Svedbergs for NaDNA.

Fig. 3 The sedimentation coefficients of relaxed circular DNA as a function of molecular weight. The line is calculated according to eq. (1). The standard sedimentation coefficients are for NaDNA at infinite dilution. When measured in NaCl the experimental values have been corrected by the Svedberg procedure; when measured in 2.8-3.0 M CsCl the values have been corrected by the method of Bruner and Vinograd.[9] The DNAs are 1, Polyoma (Gray and Vinograd, unpublished); 2, RF-ϕx[13]; 3, Monomeric mitochondrial DNA from HeLa cells, this work; 4, Circular dimers from human leukemic leukocytes[8]; 5, Viral lambda b_2b_5c;[14] 6, Intracellular P22 monomers;[15]

7, lambda c.[23] The molecular weights were calculated from the sedimentation coefficients of the linear species[11] for DNAs 1, 5,[16] 6 and 7. The result obtained by light scattering was used for 2. Electron microscope measurements of contour length were used for 3 and 4. The error bars represent our estimate of the uncertainty in the latter two values.

Fig. 4 The sedimentation coefficients of closed circular DNA as a function of molecular weight. The sedimentation coefficients were corrected as described in the legend to Fig. 2. The DNAs are 1, An intracellular DNA from E. coli 15 (Wang, private communication); 2, RF ϕx[13]; 3, 4, SV 40 and polyoma respectively (Gray and Vinograd, unpublished); 5, 9, 11, Col factor E_1, monomer, circular dimer and circular trimer;[18] 6, RF-M13;[19] 7, Human papilloma;[20] 8, RF-ϕx circular dimer;[21] 10, HeLa mitochondrial monomer, this work; 12, Human leukemic leukocyte mitochondrial circular dimer;[2] 13, HeLa mitochondrial catenated dimer, this work; 14, Intracellular lambda, b_2b_5c;[22] 15, Intracellular P22 monomer;[15] 16, Intracellular lambda c;[23] 17, HeLa mitochondrial catenated trimer, this work. In all cases the molecular weight was calculated from the sedimentation coefficient of the relaxed circular form with eq. (1), except 13 and 17 in which M was set equal to a multiple of the monomer molecular weight. The exponent for the abcissa was obtained from the slope of a double logarithmic plot of the data. The intercept and coefficient were obtained by a least squares fit of the data shown above. The final equation for the superhelical forms is $S = 7.44 + 2.43 \times 10^{-3} M^{0.58}$.

The correlation in Fig. 4 differs from that in Clayton and Vinograd[2] because of a change in the value of a data point (14 above) from a preliminary value and the addition of new data points.

Fig. 5 The lines represent the individual strands of DNA.

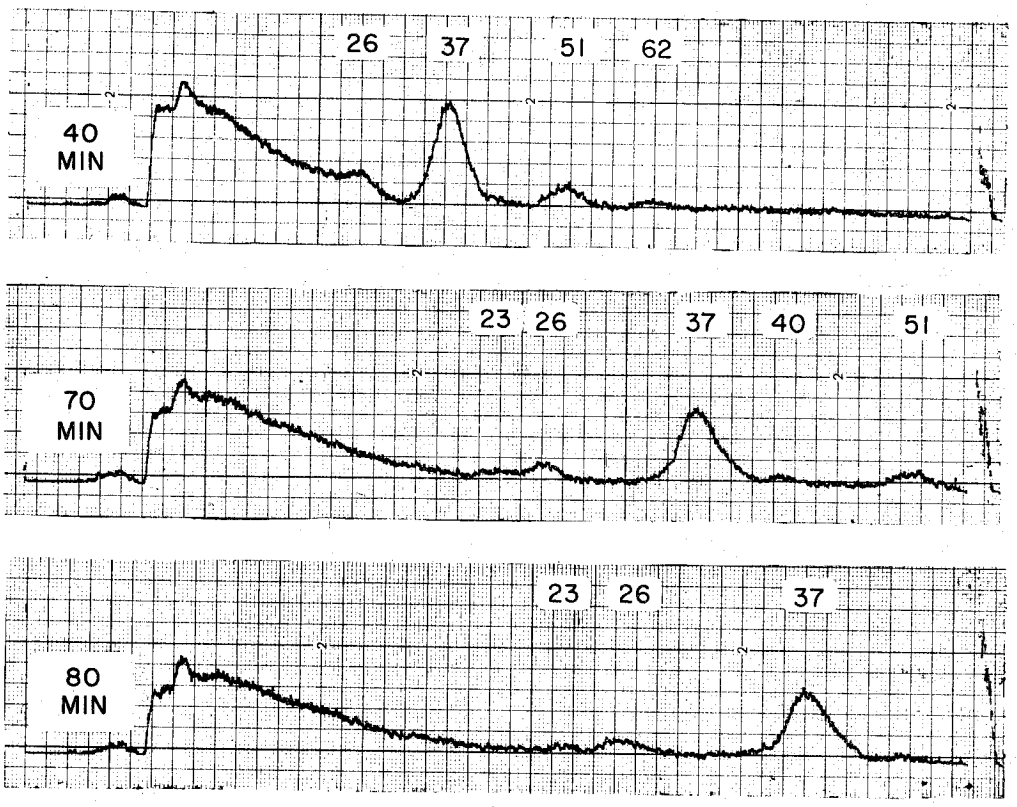


FIG. 1

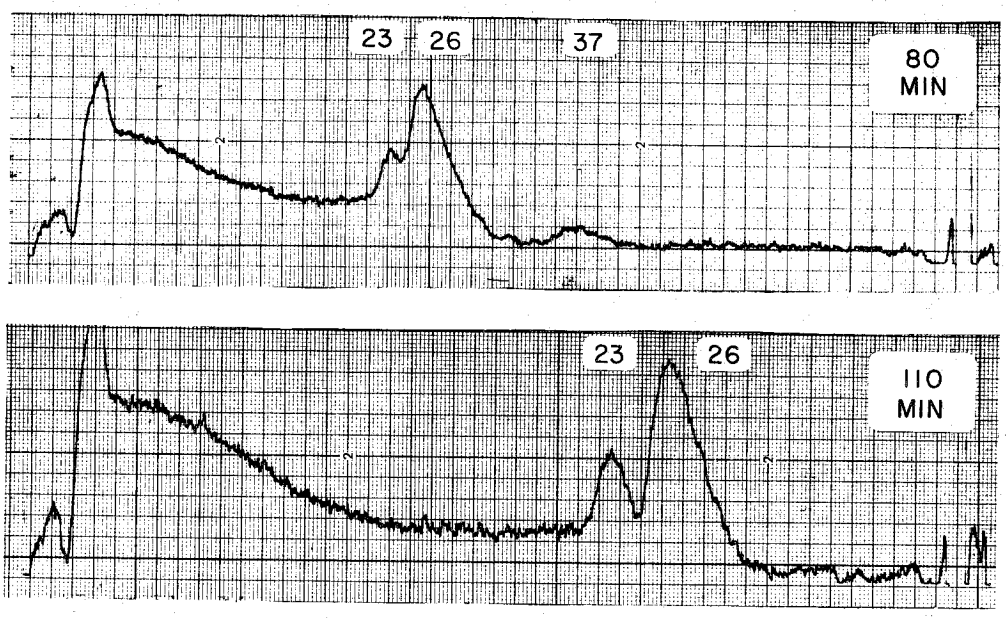


FIG. 2

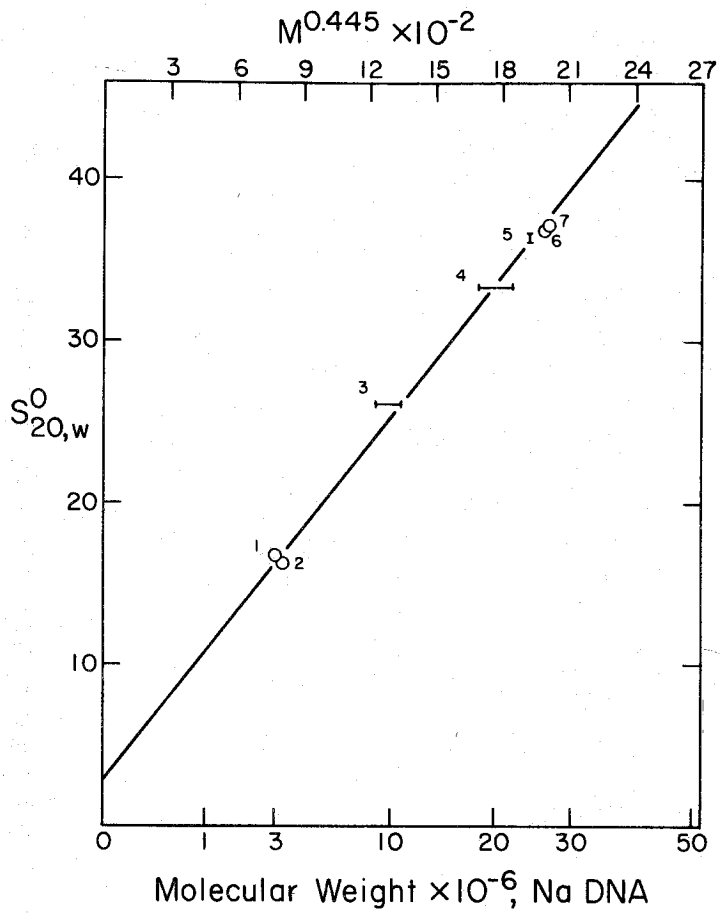

M^0.445 ×10^-2
3
6
9
12
15
18
21
24
27
40
30
20
10
S°20,w
1
2
3
4
5
6
7
0
1
3
10
20
30
50
Molecular Weight ×10^-6, Na DNA

FIG. 3

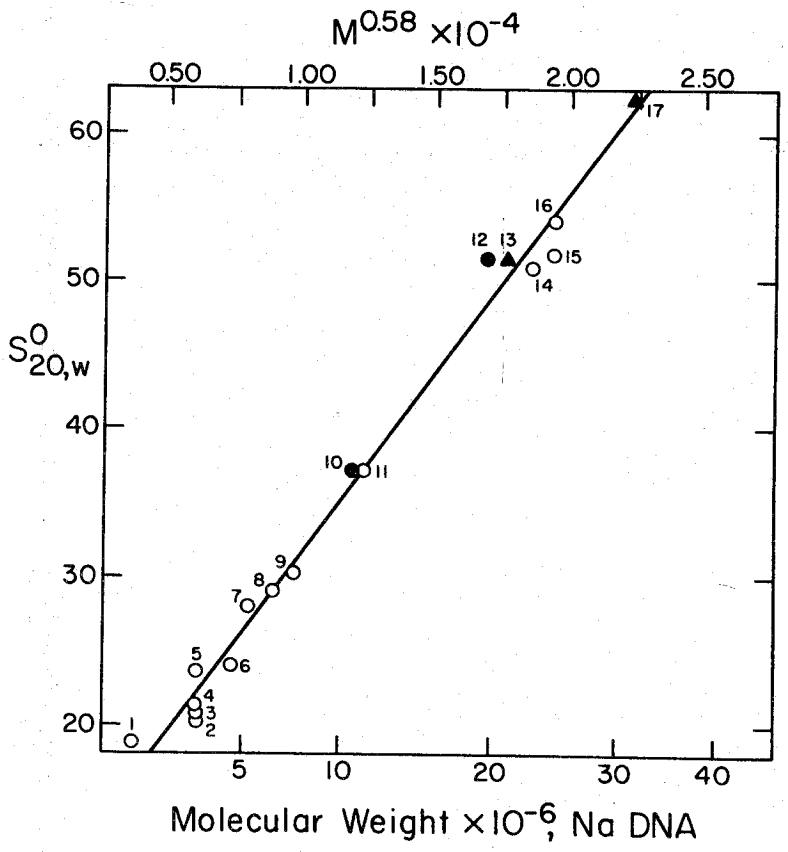

M^0.58 ×10^-4
0.50
1.00
1.50
2.00
2.50
60
50
40
30
20
S°20,w
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
5
10
20
30
40
Molecular Weight ×10^-6; Na DNA

FIG. 4

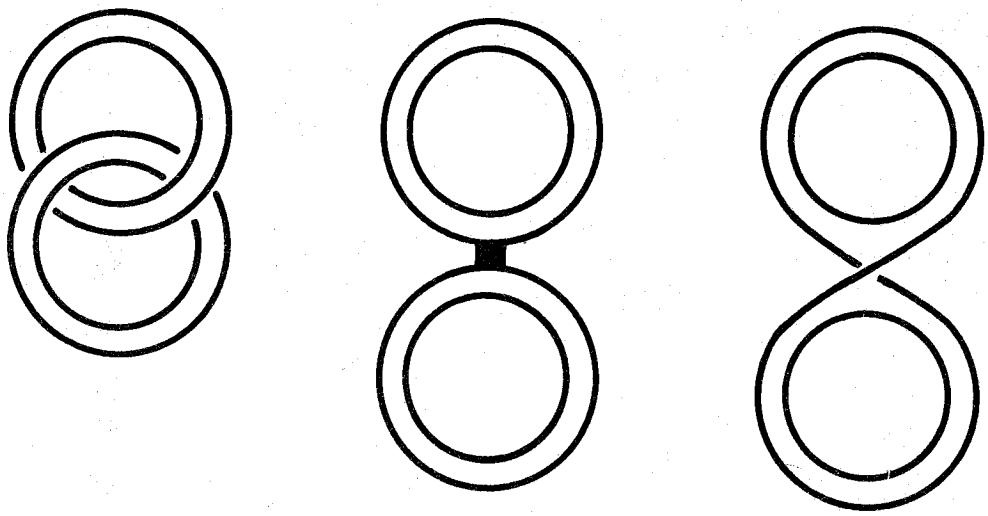
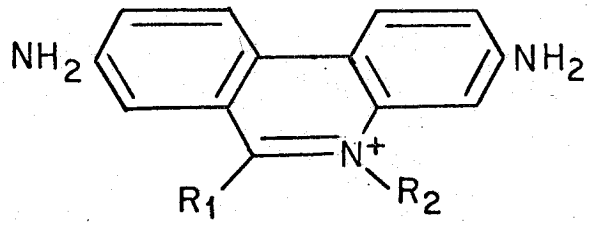


FIG. 5

The Use of an Ethidium Analogue in the Dye-Buoyant Density Procedure for the Isolation of Closed Circular DNA: The Variation of the Superhelix Density of Mitochondrial DNA.

Bruce Hudson, William B. Upholt, Joseph Devinny, and Jerome Vinograd

The dye-buoyant density method[1] has proven to be a reliable and efficient procedure for the detection and isolation of closed circular DNA. This communication reports the results of a study of variations in the method designed to increase the resolution of closed circular species from open species while at the same time preserving other useful features of the method, particularly reproducibility and sensitivity for the detection of DNA by inspection or photography of fluorescent bands. The method previously described employed ethidium bromide, EB, (3,8-diamino-6-phenyl-5-ethyl phenanthridinium bromide, I, with $R_1=C_6H_5$ and $R_2=C_2H_5$) as the "dye" component. We have found that the analogue of ethidium with $R_2=C_3H_6N(C_2H_5)_2$, which we refer to as propidium chloride, PC, enhances the resolution between closed and open DNA by a factor of approximately 1.8 relative to ethidium bromide.



I

The increased resolution appears to be independent of the molecular weight and only slightly dependent on the superhelix density of the DNA. The superhelix density, σ_0, is defined as the number of superhelical turns per ten base pairs under the assumption that the angle of the duplex is the same in the closed and open forms.[2]

The magnitude of the separation between the closed and open (nicked or linear) DNA should be sensitive to the superhelix density of the DNA.[3] This has been found to be the case. We have concluded from the results obtained in this study that there are differences in superhelix densities between mitochondrial DNAs from various sources. The superhelix densities of mitochondrial DNAs from HeLa cells and sea urchin eggs appear to be about two-thirds as large as those from rat and rabbit liver.

Materials and Methods. — Analogues of ethidium bromide: The analogues of 3,8-diaminophenanthridine used in this study were kindly provided by Dr. T. I. Watkins of Boots Pure Drug Co., Ltd. We have successfully repeated the straightforward synthesis of 3,8-diamino-5-diethylaminopropyl-6-phenylphenanthridinium iodide[4] from 3,8-dinitro-6-phenylphenanthridine.

Photography and measurement: After centrifugation, the dye-CsCl gradients were illuminated with ultraviolet light as described previously[1] and photographed through a Wratten 16 filter with either a Polaroid camera and type 46 L film, ca. 10 sec. at f 11, 5 3/4", or with a single lens reflex camera and Ektachrome film, ca. 1 sec. at f 1.4, 6". The resulting transparencies were measured in a Nikon 6 projection comparator.[5] A separation on the film could be reproducibly measured to $\pm$ 0.005 cm. The magnification factor for the fixed photographic arrangement was determined by photography of a rule and by measurement of the centrifuge tube width.

Fluorescence measurements: Fluorescence intensities were measured on a Farrand fluorimeter. The uncorrected values for different dyes were compared.

Preparation of DNAs: Viral SV 40[2] and the mitochondrial DNAs[6,7] were prepared as described elsewhere. Crab dAT from Cancer antennarius sperm was a gift from R. Hyman. M. lysodeikticus was a gift from T. W. Thompson.

Polynucleotide ligase:[8,9,10] The enzyme was prepared from E. coli K 12 strain 1100 by a modification of the method of Gefter, Becker and Hurwitz,[10] assayed by the adenylate binding method of Hurwitz[11] and Little et al.[12] using the units of Hurwitz.[11]

Closure of SV 40 DNA by ligase: Purified SV 40 I DNA (54 μg/ml) was converted to SV 40 II DNA with DNAase I (Bovine Pancreas, Sigma Chemical Company) at a concentration of 1.63×10^{-5} μg/ml in 0.015 M NaCl, 0.012 M $MgCl_2$, 0.01 M tris, 0.007% BSA, 0.001 M EDTA pH 8 for 30 minutes at 30°. SV 40 II DNA was purified by the dye-CsCl buoyant method. SV 40 II DNA was closed with polynucleotide ligase in essentially the medium described by Olivera and Lehman[8] for 20 minutes at 30° C. SV 40 L1 DNA was prepared with SV 40 II DNA at 24 μg/ml using 2 units/ml of ligase. SV 40 L2 DNA was prepared with SV 40 II DNA at 26 μg/ml and 5 units/ml of ligase with 6.8 μg/ml of ethidium bromide added.[14]

SV 40 L1 DNA was purified by the dye-CsCl buoyant method and SV 40 L2 DNA by sedimentation through a sucrose gradient.

Results and Discussion. — The buoyant density of a DNA species at equilibrium in a cesium chloride gradient containing a gradient of a reacting solute is approximately equal to the mass of the buoyant complex divided by its volume.[2]

$$\theta = \frac{1 + \Gamma' + \nu'}{\bar{v}_3 + \Gamma' \bar{v}_1 + \nu' \bar{v}_4} \tag{1}$$

In Eq. (1) Γ' is the preferential hydration of the CsDNA in grams water per gram CsDNA, ν' is the mass of dye bound per gram CsDNA and the $\bar{v}$'s are the partial specific volume of water (1), CsDNA (3), and the dye (4). To an approximation of about 2% for the case that the partial specific volume of the dye is about equal to that of water, $\Delta\theta$, the difference in buoyant density between closed (I) and open DNA (II), is given by

$$\Delta\theta = \frac{\Delta\Gamma' (\bar{v}_3 - \bar{v}_1) + \Delta\nu' (\bar{v}_3 - \bar{v}_4)}{(\bar{v}_3 + \bar{\Gamma}' \bar{v}_1 + \bar{\nu}' \bar{v}_4)^2} \tag{2}$$

where $\Delta\Gamma' = \Gamma'_{I} - \Gamma'_{II}$, and $\bar{\Gamma}' = (\Gamma'_{I} + \Gamma'_{II})/2$, $\bar{\nu}' = (\nu'_{I} + \nu'_{II})/2$ and $\Delta\nu' = \nu'_{I} - \nu'_{II}$. The quantity $\Delta\nu'$ is not zero at high levels of an intercalating dye because of the restricted binding[2] of the dye to closed circular DNA. It is this effect which gives rise to the buoyant density difference between the closed and open forms of a circular DNA. A measure of the resolving power of the system for open and closed DNA at high dye concentrations is given by $\Delta\theta$ to a good approximation.

Analogues of ethidium: A variety of analogues of ethidium were tested for an increase in $\Delta\theta$. Table 1 presents the results obtained with SV 40 DNA. The individual separations have been normalized with the value for ethidium chloride (EC) measured in the same experiment in order to cancel out small variations due to time, temperature, speed, and radial distance. The ratio $\Delta r_{XC}/\Delta r_{EC}$ is designated χ.

Table 1

The variations in Δr presented in Table 1 appear to be due to differences in $\Delta\nu$, the difference in the amount of dye bound to the closed and open forms on a molar basis. This conclusion was reached by making reasonable estimates for Γ', $\Delta\Gamma'$, $\bar{v}_4$ and the density gradient for each derivative and comparing the resulting calculated $\Delta\theta$, Eq. (2), with the $\Delta\theta$ calculated from the known quantities for ethidium bromide.[2] The variations in Δr could not be accounted for at constant $\Delta\nu$ within the assumptions of the derivation of Eq. (1). *

The magnitude of $\Delta\nu$ at saturating concentrations of intercalating dye depends on the magnitude of the restriction forces which arise in closed circular DNA because of the positive free energy of superhelix formation.[2] The value of $\Delta\nu$ thus appears to increase progressively for the analogues listed in Table 1 in rough correlation with the bulkiness of the substituents on the phenanthridinium ring. The simplest explanation for the increase in $\Delta\nu$ is an increase in the unwinding angle associated with intercalation. For example, an increased unwinding angle for propidium chloride over ethidium chloride would cause an increased restriction for form I and an increased separation.

Choice of experimental conditions: The properties of propidium chloride, PC, ($R_1 = \phi$, R_2 = diethylaminopropyl) relative to EC were studied in more detail. The separation between closed and open SV 40 DNA in an ethidium chloride–cesium chloride density gradient is approximately constant at high dye levels. The separation becomes smaller, however, if the free dye concentration in the region of the bands falls below about 75 μg/ml. This can occur in preparative ultracentrifuges because of the large redistribution of the dye in the gradient. We have found that the originally described conditions,[1] ρ = 1.55 g/ml and 100 μg/ml EB, are barely adequate to maintain the needed free dye concentration at the bands. Raising the initial EB concentration from 200 to 500 μg/ml has no observable effect on the separation of SV 40 DNA components. In the case of PC, it was found that the separation increased upon raising the concentration from 100 to 300 μg/ml, but then remained constant to 500 μg/ml. At these high dye levels, however, the sensitivity for detection by fluoresence is somewhat reduced because of the fluorescence of free dye.

The optimum choice of an initial dye concentration also depends on the initial density. Low dye concentrations can be used if the density is sufficiently high that the bands form near the top of the cell where the dye is concentrated. A final choice of 300-500 μg/ml of dye either EB or PC, and density of 1.58 g/ml was decided upon. Centrifugation for 24 hours at 43 Krpm in a 3.0 ml volume in an SW 50 rotor is sufficient for preparative purposes although 48 hours was routinely used in this study. The details of the centrifugation are unimportant and a wide variety of rotors (including angle rotors) have been used in this laboratory. A tube containing a reference DNA in the open and closed form was always included in the same rotor for purposes of quantitative comparison.

The effect of molecular weight and superhelix density on the separation enhancement of PC over EC: The increased separation of SV 40 DNAs in PC as compared with EC is shown in Fig. 1. The same result, Table 2, was obtained in a similar experiment with HeLa cell mitochondrial DNA (M DNA), which has a molecular weight approximately three times higher than SV 40 DNA. The ratio of separations with PC over EC, χ, is 1.8 in each case. This is the expected result, since all of the properties which determine the buoyant density of a DNA-dye complex are intensive and there should be no effect of molecular weight.

Fig. 1

Table 2

We have prepared two artificial forms of closed SV 40 DNA to test the effect of the superhelix density on χ. A preparation of SV 40 I DNA was treated with pancreatic DNAase at very low levels to produce some nicked SV 40 II with 3'-hydroxyl groups at the hydrolysed bond. Species II was then purified and closed with the enzyme polynucleotide ligase.[8-10] The first product, SV 40 L1 DNA, was obtained by enzymatic closure in moderately low salt. The second product, SV 40 L2 DNA, was obtained similarly except for the presence of ethidium bromide during the action of the ligase.

SV 40 L1 DNA has a low superhelix density. Viral λ DNA closed under the incubation conditions used to prepare SV 40 L1 DNA has been calculated to have about 15% of the superhelix density of SV 40 I.[13] SV 40 L1 DNA should have a similarly low superhelix density. SV 40 L2 DNA, on the other hand, has a high superhelix density because it was closed in an underwound condition. The extent of this underwinding,

and the final superhelix density, depends on the amount of dye bound to the DNA at the instant of closure.[14] Rough calculations demonstrate that the superhelix density of SV 40 L2 DNA should be substantially higher than that of SV 40 I DNA.

The separations of these forms of SV 40 DNA have been determined in EC and PC in order to see if there was any effect of the initial superhelix density on χ. Table 2. There seems to be no large effect, although a trend may be indicated.

The side chain nitrogen in PC is probably protonated at pH 7. Comparison with other tertiary amines separated from a positive nitrogen by 3 carbons indicates a pK of about 9. We have, therefore, investigated the effect of pH on the separation. No difference was found in three tubes at pH of 6, 7, and 10. This result is significant only in regard to the use of the method and does not necessarily reveal the nature of the species which actually binds to DNA.

Separation of open DNAs with different base composition: The buoyant density difference between DNAs which have different buoyant densities in dye-free CsCl and similar dye binding constants is expected to be smaller in these four component systems because of the effects of dye binding and hydration. Eq. (1). Figure 2 shows a tube containing crab "dAT" DNA and M. lysodeikticus DNA in a propidium chloride-CsCl density gradient. The density gradient in these tubes is about 0.10 gm/cm^4. The observed separation is about one half as large as that corresponding to the 0.055 g/ml buoyant density difference observed in the absence of dye. Reduced separations have been obtained for a variety of DNAs in ethidium chloride-CsCl density gradients.

Fig. 2

Fluorescence: The fluorescence enhancement[15] of EC and PC were compared at 365 mμ and found to be the same. This implies that the detection sensitivity of PC and EC should also be equal. However, in practice it seems that PC is somewhat less sensitive than EC for detecting small amounts of DNA. The reason seems to be that the binding constant is lower for PC and that higher free dye concentrations are needed to saturate the DNA. This raises the background level.

Removal of dye: Several methods are available for removal of EC and PC. Removal is greatly facilitated by high salt because of the reduced binding affinity of DNA for dye. Dialysis against low ionic strength medium is inefficient. The dye is easily removed from the DNA in the 4.5 M CsCl by passage through a Dowex 50 column,[1] or by extraction with isopropanol[16] or isoamyl alcohol followed by ether extraction. The extraction methods have the advantage that they are quick and can be used on very small samples with little dilution.

Variation of the buoyant separation with superhelix density: Consider the three forms of SV 40 DNA which have been made as described above. SV 40 L1 has a low superhelix density, SV 40 I has an intermediate superhelix density and SV 40 L2 has a high superhelix density. The binding of an intercalative dye to these molecules is shown schematically in Fig. 3, in which the horizontal coordinate represents the superhelix density. The figures at the left represent the molecules before addition of dye. A certain amount of dye, ν_c, binds to the molecule and converts it to the open conformation.

Fig. 3

This amount depends on the initial superhelix density since $\nu_c = (\pi/10\phi)\sigma_0$,[2,17] where ϕ is the unwinding angle of the base pairs upon binding one molecule of the intercalating dye. The relaxed closed circular molecules bind further dye, ν_s , and are eventually "saturated" in that the free energy of the dye binding is effectively counterbalanced by the free energy of superhelix formation.[2] If ν_s is a constant with respect to σ_0 , the amount of dye bound at saturation, $\nu = \nu_c + \nu_s$, should depend on σ_0 and closed circular molecules with high (negative) superhelix densities would exhibit small separations in a dye-buoyant density system. A high initial superhelix density results in increased binding at saturation and a decreased buoyant density. There is, however, a limit for the sum, $\nu_c + \nu_s$, which can not exceed $\nu_{II,\,max}$, the maximum number of binding sites, expressed in moles dye per phosphate, on the nicked circular form. As the limit is approached for molecules with high superhelix density, the assumption that ν_s is independent of σ_0 may no longer be valid.

The effect of variations of the superhelix density is demonstrated by the results obtained with closed SV 40 DNAs. Figure 4 presents the experiment in which the three closed forms and the open form were banded in dye-cesium chloride gradients containing either EC or PC. The experiments have also been conducted with each of the DNAs separately to be sure of the assignment of the bands. The result is that SV 40 L2 DNA, the high superhelix density DNA, shows a very small buoyant separation from the open form. The native DNA, SV 40 I DNA, shows an intermediate separation and SV 40 L1 DNA, the low superhelix density DNA, shows a large buoyant separation. The trend of these data is in the direction expected, Δr varies inversely with σ_0 . The sensitivity of the separation to a given change in the superhelix density is about 1.8 times as large for PC as for EC.

Fig. 4

Table 3

The buoyant separation between SV 40 I and II in an EC-CsCl gradient was measured 6 times under standard conditions, Table 3, and found to be 0.297 ± .004 cm (S.E.). The separation is thus very reproducible. In three experiments with HeLa M DNA in EC it was found that the separation, 0.340 ± .005 cm, was slightly, but significantly, larger than the SV 40 separation. This was also the case in PC where there was no doubt about the larger separation for HeLa cell M DNA, 0.608 cm, compared with 0.545 cm for SV 40 DNA,

Fig. 5 Fig. 5. An experiment with a mixture of SV 40 I, SV 40 II, HeLa M I and HeLa M II DNAs in EC showed a slight splitting of the bottom, but not the top, bands. The obvious interpretation of these results is that HeLa cell M DNA has a lower superhelix density than SV 40 I DNA.

The separation between closed and open DNAs in a dye-CsCl density gradient should also depend on the buoyant density of the DNA in the absence of dye, and hence on the G + C content of the DNA. This effect has been estimated to result in about a 1/2% increase in the closed to open separation of DNA per 1% increase in G + C content [3] for the case in which the binding constant is independent of G - C and $\bar{v}_4 \approx 1$.

We have measured the buoyant separations of rat and rabbit liver M DNA in EC and have found them to be about the same or slightly less than SV 40 DNA, Table 3. The separation for chicken liver M DNA is

Fig. 6 smaller than that for HeLa M DNA, Fig. 6. The separation measured for sea urchin (L. pictus) M DNA is considerably larger than for rat and rabbit liver M DNAs, but less than for HeLa M DNA, Table 3. This implies that the superhelix densities of HeLa cell and sea urchin

M DNA are lower than that of the liver M DNAs. The results are in agreement with those of Smit and Rutt enberg[18] in that there appears to be little difference in superhelix density between SV 40 DNA and rat liver DNA. Furthermore, these results confirm the expectation that if two DNAs have equal superhelix densities, they will have an equal separation in these systems.

The superhelix density of the in vitro closed SV 40 L1 DNA should be about the same as that measured for in vitro closed λ DNA made under the same conditions. The latter value is 15% of the value of viral SV 40 DNA. The superhelix density of HeLa cell M DNA can be estimated to be 61% or 69% of that of SV 40 viral DNA if a linear interpolation is made with the data for EC or PC respectively. A similar estimate with the data for PC of 82% can be made for sea urchin egg DNA. These numbers indicate the magnitude of the variations in superhelix densities and should not be considered as accurate determinations. The linearity of superhelix density with separation has not yet been established, nor has the effect of base composition been examined. It has been shown in this laboratory that the minimum in an EB-sedimentation velocity titration of HeLa cell M DNA occurs at a much lower free dye concentration than in a titration with SV 40 I DNA,[19] confirming the above interpretation.

All closed circular DNA molecules isolated to date contain superhelical turns. Chicken[20] and rat[18] liver mitochondrial DNAs, papilloma viral DNAs,[21, 22] SV 40 viral DNA,[2] and polyoma DNA[5, 17] have

superhelix densities which are approximately equal, although there are small but definite differences between SV 40 and polyoma DNAs.[19] The superhelix densities of HeLa cell and sea urchin egg mitochondrial DNAs are considerably lower. The biological problem of the origin of superhelical turns[23] has not been solved. Wang[13,24] has shown that about 15% of the superhelix density of SV 40 DNA can be accounted for by changes in the average angle between base pairs due to changes in ionic strength, and that temperature has a small effect on superhelix density. Since the internal physical chemical environment of cells is unknown, the relative contribution of the former effect cannot be assessed. Any proposed mechanism for the origin of superhelical turns must include the potential for a variability.

Summary: — The separation between open and closed circular DNA in buoyant CsCl gradients containing intercalating dyes depends on the superhelix density of the closed form. These separations are about 1.8 times larger with propidium chloride than with ethidium chloride. The superhelix densities of mitochondrial DNA from HeLa cells and L. pictus eggs appear to be about two-thirds that of mitochondrial DNA from rat and rabbit liver.

It is a pleasure to thank T. I. Watkins and Boots Pure Drug Co., Ltd. for providing the ethidium analogues and the 3,8-dinitro-6-phenyl phenanthridine, and L. Pikó for supplying the L. pictus M DNA. We thank L. Wenzel and J. Edens for assistance in the culture of HeLa cells and J. Ceasar for assistance in the preparation of the manuscript. We also wish to thank Professor J. Hurwitz for generously providing advice and facilities to one of the authors, WBU, who prepared the polynucleotide ligase while a guest at Albert Einstein Medical College, Bronx, New York.

This is contribution number 3743 from the Division of Chemistry, California Institute of Technology, Pasadena, California.

REFERENCES

[1]Radloff, R., Bauer, W., and J. Vinograd, these PROCEEDINGS, 57, 1514 (1967).

[2]Bauer, W., and J. Vinograd, J. Mol. Biol., 33, 141 (1968).

[3]Bauer, W., Ph.D. thesis, page 241, California Institute of Technology, 1968.

[4]Watkins, T. I., J. Chem. Soc., 3059 (1952).

[5]Vinograd, J., Lebowitz, J., and R. Watson, J. Mol. Biol., 33, 173 (1968).

[6]Hudson, B., and J. Vinograd, manuscript in preparation.

[7]Piko, L., Blair, D. G., Tyler, A., and J. Vinograd, these PROCEEDINGS, 59, 838 (1968).

[8]Olivera, B. M., and I. R. Lehman, these PROCEEDINGS, 57, 1426 (1967).

[9]Zimmerman, S. B., Little, J. W., Oshinsky, C. K., and M. Gellert, these PROCEEDINGS, 57, 1841 (1967).

[10]Gefter, M. L., Becker, A., and J. Hurwitz, these PROCEEDINGS, 58, 240 (1967).

[11]Hurwitz, J., private communication.

[12]Little, J. W., Zimmerman, S. B., Oshinsky, C. K., and M. Gellert, these PROCEEDINGS, 58, 2004 (1967).

[13]This calculation is based on the value of the binding constant of ethidium bromide and the minimum in a sedimentation velocity-EB titration of λ phage DNA closed under conditions similar to those used to make SV 40 L1 (Wang, private communication), and the dye concentration at the minimum in a sedimentation velocity titration for SV 40 DNA performed under similar conditions in this laboratory (Gray, H. B., Jr., private communication).

[14]This method for the preparation of closed circular DNAs with variable superhelix densities has been independently recognized and used by Wang (private communication).

[15]Le Pecq, J.-B., and C. Paoletti, J. Mol. Biol., 27, 87 (1967).

[16]Cozzarelli, N. R., Kelly, R. B., and A. Kornberg, these PROCEEDINGS, 60, 992 (1968).

[17]Crawford, L. V., and M. J. Waring, J. Mol. Biol., 25, 23 (1967).

[18]Smit, E. M., and G. J. C. M. Ruttenberg, in Borst, P., and A. M. Kroon, Int. Rev. Cytol., in press.

[19]Gray, H. B., Jr., private communication.

[20]Ruttenberg, G. J. C. M., Smit, E. M., Borst, P., and E. F. J. van Bruggen, Biochim. Biophys. Acta, 157, 429 (1968).

[21]Crawford, L. V., and M. J. Waring, J. Gen. Virol., 1, 387 (1967).

[22]Bujard, H., J. Mol. Biol., 33, 503 (1968).

[23]Vinograd, J., and J. Lebowitz, J. Gen. Phys., 49, 103 (1966).

[24]Wang, J. C., Baumgarten, D., and B. M. Olivera, these PROCEEDINGS, 58, 1852 (1967).

---

* Footnote to Page 43

The values of the quantities in Eq. (2) were estimated as follows. The partial specific volume was estimated from the known value for ethidium bromide[2] by assuming that the effective volume of the side chains could be taken as the reciprocal of the density of the parent amine or hydrocarbon. The buoyant densities of the DNA-dye bands were estimated from the known

values for ethidium bromide, the density gradient and the positions of the bands in the tubes. These buoyant densities were then used to calculate the value of Γ' for each band using the same procedure as has been used for the ethidium bromide system.[2] Unfortunately, the values of Γ' depend quite critically on the estimated values of the buoyant densities of the DNA-dye complexes. The degree of solvation of the upper band in the propidium chloride gradient, $\Gamma'_{II,\ PC}$, is particularly difficult to estimate because it lies outside the range of accurately interpolated experimental data.[2] The conclusion that the increased separation of PC over EC is due to an increased $\Delta\nu$ is, therefore, only tentative. It is possible that the effect is due to the increased mass of the propidium molecule coupled with an increased differential solvation, $\Delta\Gamma'$.

TABLE 1. Effects of substituents in the diaminophenanthridinium ring system, I, on the buoyant separation between closed and open viral SV 40 DNA in dye-CsCl density gradients.

R_1	R_2	$\chi = (\Delta r_{XC}/\Delta r_{EC})$
C_2H_5	C_2H_5	0.70
ϕ	CH_3	0.92
ϕ	C_2H_5	1.00
ϕ	C_3H_7	1.01
$pNH_2\phi$	C_2H_5	1.00
ϕ	$C_3H_6N(C_2H_3)_2$	1.80

The dye concentrations were 300-500 μg/ml, and the initial density was 1.55-1.58 g/ml. The samples were centrifuged in an SW 50 rotor at 43 Krpm for 48 hours at 20°.

TABLE 2. The buoyant separation, Δr, between the closed and open forms of four DNAs in cesium chloride containing ethidium chloride, EC, and propidium chloride, PC.

DNA	Rotor	Δr_{PC} cm	Δr_{EC} cm	$\chi = \frac{\Delta r_{PC}}{\Delta r_{EC}}$	$\bar{\chi}$
SV 40 L1	SW 50.1†	0.822	0.429	1.91	1.91
	SW 50.1*	0.895	0.470	1.90	
SV 40 I	SW 50	0.532	0.300	1.77	1.80
	SW 41	0.570	0.310	1.84	
	SW 50.1†	0.619	0.345	1.79	
	SW 50.1*	0.669	0.370	1.81	
SV 40 L2	SW 50.1†	0.347	0.201	1.72	1.72
	SW 50.1*	0.389	0.228	1.71	
HeLa M DNA	SW 50	0.608	0.345	1.76	1.76

The dye concentration for all experiments was 330 μg/ml and the initial density was 1.58 gm/cm^3. Three ml volumes were used in all experiments except in the SW 41 rotor where 6 ml were used. All runs were at 20° C for 48 hours except for the SW 41 which was run for 72 hours. The nominal rotor speed for the three rotors used was 43 Krpm for the SW 50, 35 Krpm for the SW 41, and 40 Krpm for the SW 50.1. The measured speed in the experiment marked with an asterisk* was 39 Krpm as compared with 40.5 Krpm in the experiment marked with a dagger.† This difference accounts for the variation of Δr in the two experiments.

TABLE 3. The buoyant separation between the open and closed forms of six DNAs relative to the comparable separation for SV 40 viral DNA measured in each experiment.

		$\Omega = \Delta r / \Delta r_{SV\ 40\ I}$	
	Material	Ethidium chloride	Propidium chloride
1.	SV 40 L1 DNA	1.26 ± .01 (2)	1.33 ± .01 (2)
2.	HeLa M DNA	1.12 ± .005 (2)	1.12 (1)
3.	L. pictus M DNA		1.07 (1)
4.	SV 40 I	1.00	1.00
5.	Rabbit liver M DNA	0.97 (1)	
6.	Rat liver M DNA	0.96 (1)	
7.	SV 40 L2 DNA	0.60 ± .02 (2)	0.57 ± .01 (2)

Typical values of $\Delta r_{SV\ 40}$ are given in Table 1. The average value of $\Delta r_{SV\ 40}$ was 0.297 ± .004 (S.E.) in six experiments in an SW 50 rotor at 43 Krpm. The number of determinations of each ratio is given in the parentheses. For details of composition and centrifugation see text.

LEGENDS

Fig. 1 Fluorescent bands of open (upper) and closed (lower) SV 40 DNA in a buoyant CsCl density gradient containing (a) ethidium chloride and (b) propidium chloride. The initial densities were 1.58 g/ml. The tubes were centrifuged in the same rotor under standard conditions, Table 2.

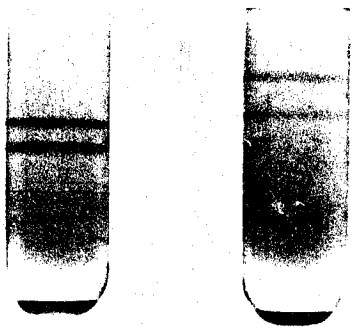
Fig. 2 Effect of base composition on the buoyant separation of open DNA in propidium chloride. Upper band, crab "dAT" DNA; lower band, M. lysodeikticus DNA. Centrifugation was performed under standard conditions, Table 2.

Fig. 3 Diagrammatic representation of the binding of an intercalating dye to three closed circular DNAs with differing initial superhelix densities. The dye free molecule is shown at the left, the relaxed molecule in the center and a highly twisted molecule at the right.

Fig. 4 The effect of superhelix density on the buoyant density in dye-CsCl gradients. Fluorescent bands of SV 40 DNA in buoyant CsCl containing (a) ethidium chloride and (b) propidium chloride. In order of increasing densities the bands are SV 40 II DNA, SV 40 L2 DNA, SV 40 I DNA and SV 40 L1 DNA.

Fig. 5 A comparison of the buoyant separations of HeLa cell M DNA (a) and SV 40 I DNA (b) in propidium chloride-CsCl density gradients.

Fig. 6 A comparison of the buoyant separations of (a) HeLa cell M DNA and (b) chicken liver M DNA in ethidium chloride-CsCl density gradients.



a b

FIG. 1

FIG. 2

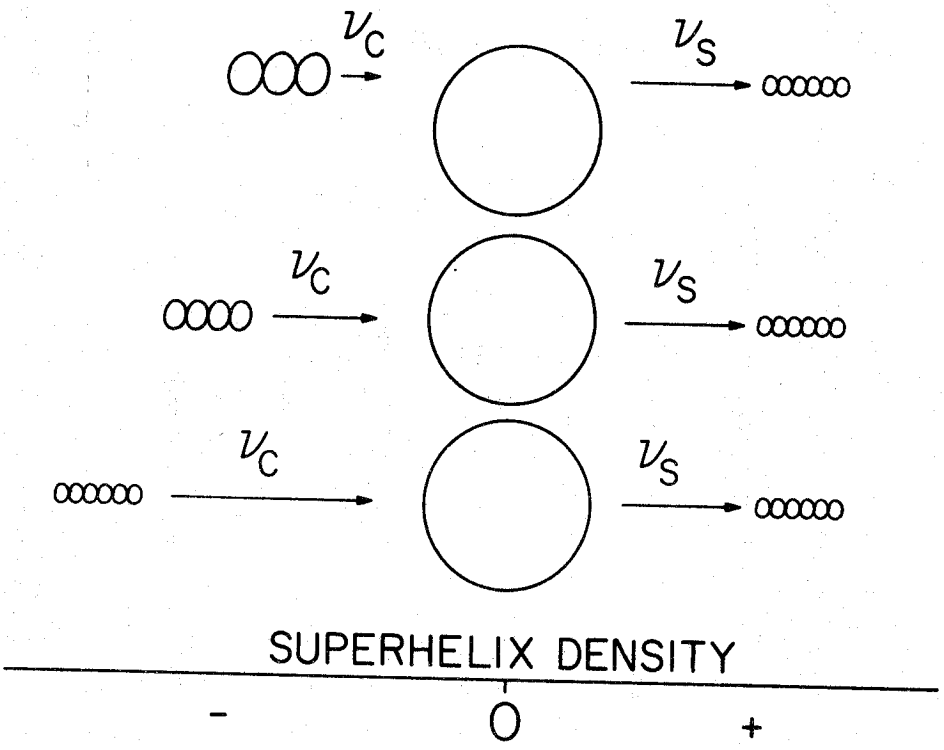
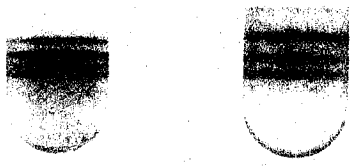
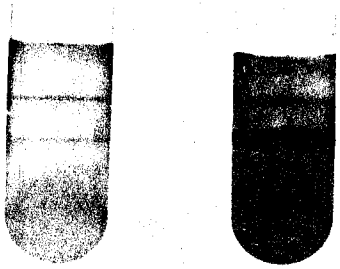


FIG. 3



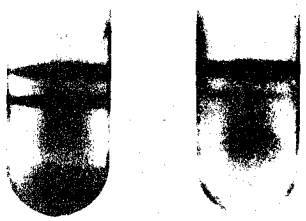
a b

FIG. 4



a b

FIG. 5



a b

FIG. 6

The Binding of Ethidium Bromide to DNA

Several authors have investigated the binding of ethidium bromide to DNA.[1,2,3] The data obtained by spectroscopic, spectrofluorimetric and buoyant density methods have been plotted according to Scatchard. For a single type of non-interacting site the fractional occupation of possible sites, ν, the free dye concentration, c, and the intrinsic binding constant, k, are related by

$$\frac{\nu}{c} = k(\nu_{max} - \nu) \qquad (1)$$

The Scatchard plot of ν/c versus ν should, therefore, be linear with slope -k and intercepts $k\nu_{max}$ and ν_{max}. Similar data have been obtained for the aminoacridines[4] and actinomycin.[5] The general result is that the plots of ν/c versus ν are not linear but are more or less convex when viewed from the origin.

Non-linear Scatchard plots can be interpreted in two ways: there is more than one type of non-interacting binding site, each type with a different k or there are interactions between the identical binding sites. It has been proposed[1,2,3] that DNA contains both primary (intercalative) and secondary binding sites for ethidium bromide. This discussion outlines the argument for the alternative proposal that there are interactions between initially identical sites.*

The chief motivation for a reexamination of the two site binding proposal was the fact that extrapolation of the initial linear portion of the Scatchard plot for ethidium bromide to obtain $\nu_{1,max}$ yields a value of 0.40 on a base pair scale.[1,2,3] This means that 40% of the

---

* This possibility was first considered by Waring.[1]

base pairs of DNA are sites for strong binding. This proportion seems to be independent of the base composition of the DNA. The author found this result uninterpretable at the microscopic level.

Repulsive interaction between bound molecules will result in a atchard plot with downward curvature. A comparison of two binding models will illustrate this curvature and other features of an interacting site Sc atchard plot. For model I we chose half of the polymer repeat units to be binding sites which do not interact. For model II the binding of each molecule excludes the binding to a neighboring site but all repeat units are initially potential sites. This is the simplest case of repulsive interaction between bound molecules. Assume that the intrinsic binding constants are the same for both models. Model I will result in a linear calculated Sc atchard plot running from $k\nu_{max} = 0.5\,k$ to $\nu_{max} = 0.5$. For model II we must consider the limiting cases of high and low c. As $c \to \infty$, $\nu \to 0.50$ because every other repeat unit will be occupied. As $c \to 0$, $\nu \to 0$ and $\nu/c \to k$ because, at $c = 0$, the effective fractional number of available sites is 1. For independent sites, S, $[S] = [S]_0 (1 - \nu)$. Substitution into the Mass action equation results in (1) (except that ν is defined here on a site basis). For model II, at low c, $[S] = [S]_0 (1 - 3\nu)$ because the binding of one molecule reduces the number of sites by 3 instead of 1. This results in $\frac{\nu}{c} \sim k(1 - 3\nu)$, $c \to 0$. The initial slope of the calculated Scatchard plot for model II is -3k. This situation is shown in Fig. 1 from which it can be seen that the plot must be convex from the origin.

The complete shape of the Scatchard plot for model II can be calculated from statistical mechanics. The problem of interacting elements in one dimension has been solved by several authors for various rather general cases.[6] The result for model II is

$$\nu = \frac{\sqrt{1 + 4kc} - 1}{2\sqrt{1 + 4kc}} \tag{2}$$

The important feature of this equation is that most of the curvature in a plot of ν/c versus ν occurs as the line approaches the ν axis. The Scatchard plot thus has an initial nearly linear portion with a fairly sharp curve at the end. A fit of equation (2) to the data obtained by Bauer for ethidium bromide is shown in Figure 2. Model II appears to fit all of the data more closely than an independent site model.

The nearest neighbor exclusion model for the binding of ethidium bromide to DNA has several advantages over the independent site model proposed in the literature. The most important feature of this interacting site model is that all of the base pairs in the DNA molecule are potential binding sites. This explains the reported independence of the binding constant of the base composition of the DNA. It also removes the awkward result $\nu_{max} = 0.40$. The interacting site model is also simpler because it requires only one binding mechanism instead of two. The source of the interaction between bound molecules can be explained in several ways, the simplest of which is coulombic repulsion of adjacent ethidium molecules.*

---

* This is very likely to be the correct explanation. A rough calculation shows that the interaction energy is of the right magnitude. This predicts longer range and less absolute interactions than used in model II and a Schatchard plot which flattens out near the ν axis and continues beyond $\nu = 0.5($

Any interacting site model, providing the interactions decrease rapidly with increasing distance between bound molecules, predicts that, at high ν values, the bound molecules will be more or less equally spaced along the polymer. They will be exactly equally spaced for the limiting case of nearest neighbor exclusion. An analog of ethidium bromide bearing a heavy atom should result in a well defined x-ray fiber pattern for the DNA dye complex.

REFERENCES

1. Waring, M. J., J. Mol. Biol., 13, 269 (1965)
2. Le Pecq, J. and Paoletti, C., J. Mol. Biol., 27, 87 (1967)
3. Bauer, W. R., Thesis, California Institute of Technology (1968)
4. Drummond, D. S., Simpson-Gildmeister, V. F. W., and Peacocke, A. R., Biopolymers, 3, 135 (1965)
5. Muller, W. and Crothers, D. M., J. Mol. Biol., 35, 251 (1968)
6. Hill, T., Statistical Mechanics, (McGraw-Hill, New York, 1956)
   Steiner, R. F., J. Chem. Phys., 22, 1458 (1954)
   Scatchard, G., Ann. N.Y. Acad. Sci., 51, 660 (1949)
   Lifson, S., J. Chem. Phys., 40, 3705 (1964)
   Steiner, R. F. and Beers, R. F., Polynucleotides (Elsevier, New York, 1961)
7. Crothers, D. M., Biopolymers, 6, 575

LEGENDS

Fig. 1 Schematic Scatchard plots for two dye binding models discussed in the text.

Fig. 2 A Scatchard plot for the binding of ethidium bromide to DNA taken from Bauer.[3] The dashed straight line is the best least squares fit of the data except for the four solid points which were excluded. The solid curved line is a fit of equation 2 ($k = 7 \times 10^3$). Note that the scales in this figure are in units of nucleotides instead of nucleotide pairs. These numbers on both axes should be multiplied by 2 for comparison with the rest of this discussion.

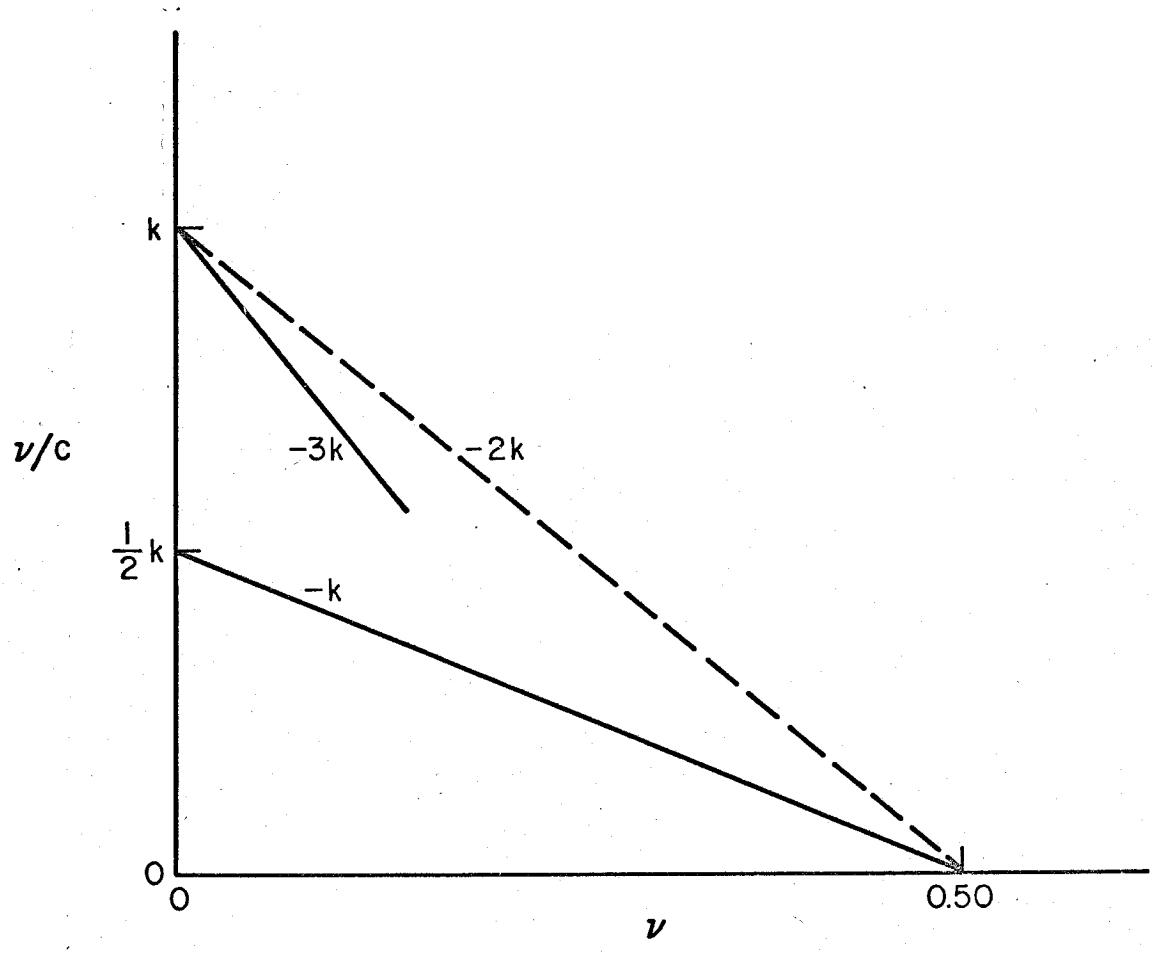


Figure 1

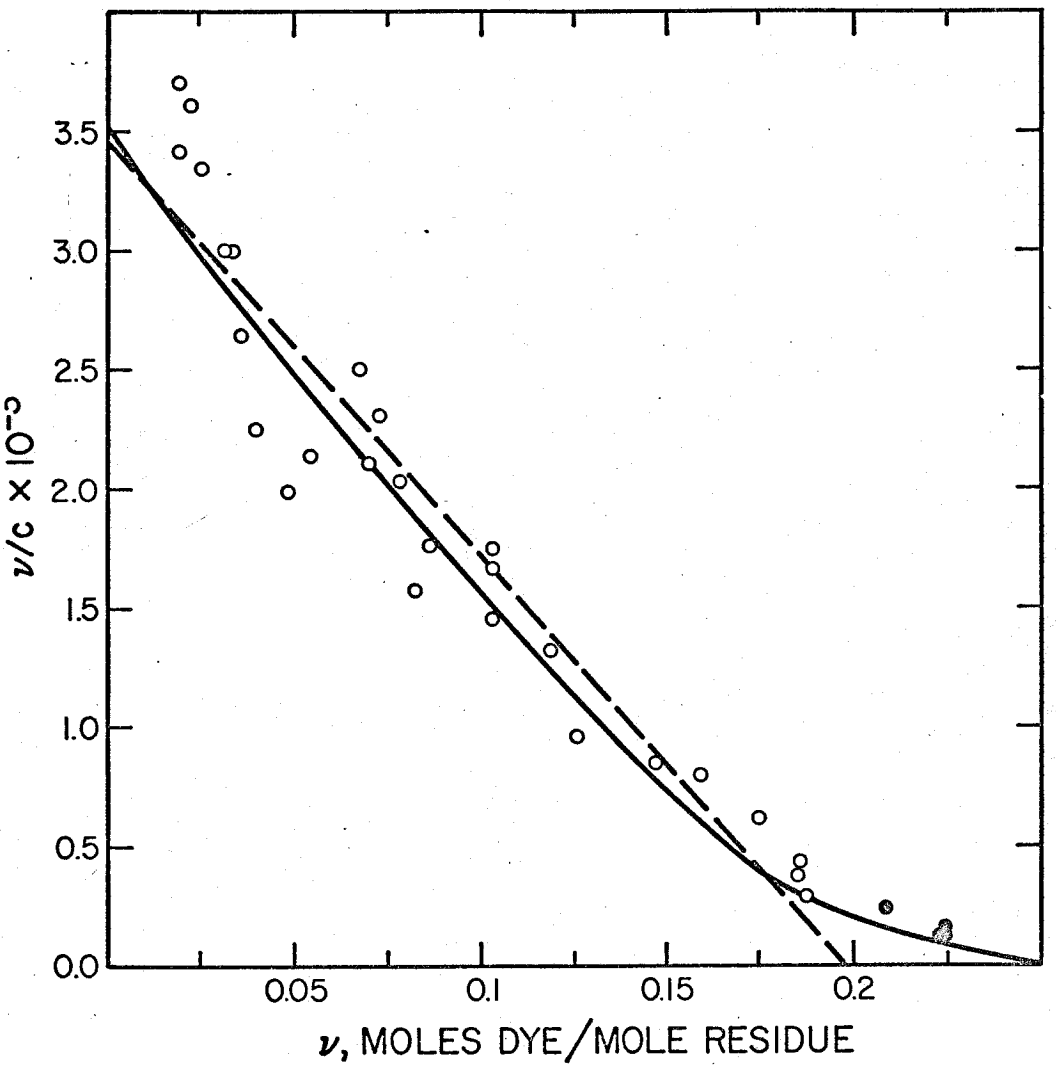

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ν/c × 10⁻⁵
0.05
0.1
0.15
0.2
ν, MOLES DYE/MOLE RESIDUE

Figure 2