Flexural Rigidity of Double-stranded DNA as Measured by Electron Microscopy

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ABSTRACT: The flexural rigidity ε of double-stranded DNA has been calculated to be $\varepsilon = 1.2 \times 10^{-19}$ dyne cm^{-2} from a study of the results of electron-microscopic measurements. A re-examination of the theoretical background of the analysis of the electron micrographs of DNA taken by the protein-monolayer technique is done on the basis of a stiff-chain model. An appropriate interpretation of the results is given in terms of two parameters: one is the flexural rigidity of the DNA molecule and the other is the "excluded area" which allows for the interaction between the DNA molecules and a surface-denatured protein monolayer. Employing the "diffusion method" introduced by Lang, *et al.*, the contour length and the end-to-end distance were measured from micrographs of DNA molecules. The materials studied were DNA of the λ-phage on a cytochrome-C monolayer. For this case, the "excluded area" β_{II} has a numerical value of 0.016 μ^2.

KEY WORDS   DNA / Stiff Chain / Electron Micrograph / Flexual Rigidity / Statistical Length / Persistence Length /

A considerable amount of information about the conformation of linear macromolecules in solution can be obtained by means of physicochemical measurements of such quantities as viscosity, sedimentation constant, light scattering, and flow dichroism. To analyze the results of these measurements, it is necessary to use an appropriate model so as to link certain attributes of the individual molecules with the macroscopic quantities measured as ensemble averages.[1] Recent advances in electron microscopy enables us to make direct observations of the individual shapes of certain macromolecules. These new techniques enable direct measurements of molecular quantities to be made.[2] For double-stranded DNA, such quantities as the contour length of a molecular chain and the molecular weight have been determined, using the reasonable assumption that DNA has the B-form helical structure in both fibres at high humidity[3]

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and in solution.[4]

Difficulties arise in any attempt to measure a quantity relating to the three-dimensional molecular conformation from the two-dimensional molecular image of DNA obtained by means of electron microscopy. One of the most important quantities to obtain is a quantitative estimate of the changes in molecular conformation produced by a reduction of entropy (degree of freedom in the conformational space) or by disturbances raised by the flow and surface-tension of water during the preparation of a sample.

Lang, *et al.*,[5,6] who developed the "diffusion method" for preparing DNA samples as a revision of the protein-monolayer technique for DNA sampling, minimized the disturbances by means of a diffusion-absorption process of DNA on to a plane of protein layer. Although this device has successfully removed one difficulty mentioned above, the effect of the reduction of entropy still remained to be considered by means of an appropriate theoretical framework. In regard to this problem they assumed that

(1)   the molecules in solution exist as random coils of equal contour length, and

(2) the end-to-end distance of the average coil is parallel-projected on to the plane.

Their data were expressed by the relationship derived from a theory of Katchalsky and Lifson,[7]

$$(\bar{h}^2)^{1/2} = \frac{bL}{A}(1 + \sqrt{1 + A^3/b^2L}) \quad L > 10^{-5}\,\mathrm{cm}$$

where $\bar{h}^2$ is the mean square end-to-end distance in solution, A is the Kuhn statistical element (the length of a unit segment), L is the contour length of a molecule and b is a quantity which allows for the electrostatic repulsion between the parts of an unbranched polyelectrolyte. According to Lang, *et al.*, by using the assumptions given above, the mean square end-to-end distance in the two-dimensional plane, $\bar{a}^2$ was expressed as

$$(\bar{a}^2)^{1/2} = (2 \cdot \bar{h}^2/3)^{1/2}$$

The values of the parameters thus obtained were[6]

$$A = 1.85 \times 10^{-5}\,\mathrm{cm}$$

and

$$b = 1.68 \times 10^{-6}\,\mathrm{cm}$$

The parameter A, which is the Kuhn statistical length, is about three times larger than the value of $717\,\text{Å}$, obtained by Hearst and Stockmeyer[8] from a theoretical analysis of the sedimentation constant within the same range of ionic strength (at $I = 0.2$) on the assumption that long-range interaction was negligible. Since the electrostatic repulsion increases the end-to-end distance, the mean square end-to-end distance given by the above equation is more than three times as large as that suggested by Hearst and Stockmeyer.

Recently, Hays, Magar, and Zimm[9a] re-evaluated the persistence length (just half of the Kuhn statistical length) from light scattering measurements and from hydrodynamic data. The former method has given a value of persistence length of $900 \pm 200\,\text{Å}$ and the latter $600 \pm 100\,\text{Å}$. On the other hand, Eisenberg[9b] has also pointed out that the persistence length appears to be 1500—$1900\,\text{Å}$ which is three to four times higher than those ever accepted.

The present authors have re-examined the diffusion method, especially from its theoretical

analytical side, and have obtained information about the stiffness or flexural rigidity of double-stranded DNA molecules. The present treatment possesses a certain validity in describing the conformations from the two-dimensional electron microscopic picture for various cases of flexible molecules.

THEORY

This section presents an attempt to improve the existing theoretical methods devised for the interpretation of the two-dimensional molecular images of double-stranded DNA molecules observed by electron microscopy.

Gaussian Chain and Stiff Chain Models

Let the chain consist of segments of an effective number n and of statistical length b. Here the segment does not mean the nucleotide unit. Then the total length of the chain is

$$L = nb \tag{1}$$

and the end-to-end vector $\mathbf{R}$ of the chain is

$$\mathbf{R} = \sum_i \mathbf{u}_i b \tag{2}$$

where $\mathbf{u}_i$ is the unit vector which is parallel to the longitudinal axis of the i-th segment.

The distribution function of the end-to-end vector is

$$W(\mathbf{R})\,\mathrm{d}\mathbf{R} = \int \cdots \int e^{-U/kT}\,\mathrm{d}(\boldsymbol{\omega}) \tag{3}$$

where U is the potential energy of the chain and the integration is carried over all conformational space $\boldsymbol{\omega}$ in fixing the end-to-end vector at $\mathbf{R}$. The potential energy is written as

$$U = \sum_i u_i + \sum_{i<j} u_{ij} \tag{4}$$

where u_i is the internal potential energy of the i-th segment, and u_{ij} denotes the interaction between i-th and j-th segments.

If n tends to infinity and if only short-range interaction is taken into consideration, the distribution function $W(\mathbf{R})$ is approximated by means of the central limit theorem as a form of Gaussian function.[10] Thus we can write two- and three-dimensional spreads of the vector $\mathbf{R}$ using constants n and b as follows:

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$$W(\boldsymbol{R}_{\rm II}) \propto \exp\left(-\boldsymbol{R}_{\rm II}^2/n_{\rm II}b_{\rm II}^2\right) \tag{5a}$$

$$W(\boldsymbol{R}_{\rm III}) \propto \exp\left(-3\boldsymbol{R}_{\rm III}^2/2n_{\rm III}b_{\rm III}^2\right) \tag{5b}$$

where suffices II and III indicate two- and three-dimensional cases, respectively.

While the Gaussian-chain model is obtained by replacing the potential energies by their averages, the stiff-chain model is obtained when each of them is taken to be the elastic energy.[11~13] By neglecting the tensile and compressive energies of the chain, the potential energy is written as

$$U = \frac{1}{2}\varepsilon \sum_{i=1}^{n} K^2 b \tag{6}$$

where ε and K are the flexural rigidity and the curvature vector of the i-th segment, respectively, and the former is assumed to be constant for all of the segments. If n tends to infinity while L remains constant (at the limit of infinitesimal segment-length) the summation can be replaced by an integration. The energy is thus written as

$$U = \frac{1}{2}\varepsilon \int_0^L K^2 \, \mathrm{d}s \tag{7}$$

where s is the contour co-ordinate fixed along the chain.

The relationships between the position vector r, the tangential vector u, and the curvature vector K are,

$$\boldsymbol{u} = \partial\boldsymbol{r}/\partial s \quad \text{and} \quad \boldsymbol{K} = \partial\boldsymbol{u}/\partial s = \partial^2\boldsymbol{r}/\partial s^2 \tag{8}$$

The partition function is thus rewritten as

$$Q = \int \exp\left\{-\frac{\varepsilon}{2kT}\int_0^L \left(\frac{\partial\boldsymbol{u}}{\partial s}\right)^2 \mathrm{d}s\right\} \mathrm{d}(\boldsymbol{\omega}) \,. \tag{9}$$

By means of the calculus of variation, Saito, et al.,[12] derived the partial differential equation,

$$\frac{\partial Q}{\partial s} = D\varDelta_u Q \tag{10}$$

where $D = kT/2\varepsilon$, $\varDelta_u$ is the Laplacian operator in u-space, and we have the restriction

$$\boldsymbol{u}^2 = 1 \tag{11}$$

The Green's function of eq 10 in the three-dimensional case is,

$$G(s, \theta, \varphi \,|\, 0, 0, 0)$$
$$= \sum_n \sum_m \mathrm{e}^{-n(n+1)Ds} Y_{nm}(\theta, \varphi) Y_{nm}(0, 0) \tag{12}$$

Using eq 12, the mean square end-to-end distance is given by

$$\langle \boldsymbol{R}_{\rm III}^2 \rangle = (\mathrm{e}^{-2DL} - 1 + 2DL)/2D^2 \tag{13a}$$

and the mean square curvature[14] is calculated to be

$$\langle \boldsymbol{K}^2 \rangle = 4D^2 \tag{14a}$$

which is substituted for the integrand of eq 7. The mean potential energy is thus expressed as

$$\langle U_{\rm III} \rangle = kTDL \tag{15a}$$

By employing a similar procedure, the above mentioned quantities can be derived by means of the two-dimensional Green's function,

$$G_{\rm II}(s, \theta \,|\, 0, 0) = \frac{1}{\pi}\left(\frac{1}{2} + \sum_{n=1}^{\infty} \mathrm{e}^{-n^2 Ds} \cos n\theta\right) \tag{12a}$$

and expressed as

$$\langle \boldsymbol{R}_{\rm II}^2 \rangle = \frac{2}{D^2}(\mathrm{e}^{-DL} - 1 + DL) \tag{13b}$$

$$\langle \boldsymbol{K}_{\rm II}^2 \rangle = D^2 \tag{14b}$$

$$\langle U_{\rm II} \rangle = \frac{kT}{2}\frac{DL}{2} \tag{15b}$$

Eq 4, 15a, and 15b all indicate that the mean potential energy $\langle U \rangle$ is proportional to the total length of the chain, L, at a large n. By equating the coefficients of these equations we obtain

$$\frac{\langle u_{\rm II} \rangle}{b_{\rm II}} = \frac{kT}{2}\frac{D}{2} \tag{16a}$$

$$\frac{\langle u_{\rm III} \rangle}{b_{\rm III}} = kTD \tag{16b}$$

Equating the mean potential energy of a segment $\langle u \rangle$ with the elastic potential energy, we obtain the following relationships in the two- and three-dimensional cases by using the equipartition of energy theorem

$$\langle u_{\rm II} \rangle = \frac{kT}{2} \tag{17a}$$

$$\langle u_{\rm III} \rangle = kT \tag{17b}$$

$$b_{\rm II} = \frac{2}{D} \tag{18a}$$

$$b_{\rm III} = \frac{1}{D} \tag{18b}$$

These relationships are also confirmed by the correspondence of the mean square end-to-end distances of the two models. If the number of segments n and the non-dimensional length DL are larger, eq 5a, 5b and 13a, 13b indicate that

$$\langle R_{II}^2 \rangle = n_{II} \cdot b_{II}^2 = b_{II}L \simeq \frac{2}{D}L \qquad (19a)$$

$$\langle R_{III}^2 \rangle = n_{III} \cdot b_{III}^2 = b_{III}L \simeq \frac{1}{D}L \qquad (19b)$$

Thus we obtain the important relationships between the segment length and the numbers of the segments in the two- and three-dimensional cases,

$$b_{II} = 2b_{III} \simeq \frac{2}{D} \qquad (20)$$

$$n_{II} = \frac{n_{III}}{2} \simeq \frac{DL}{2} \qquad (21)$$

Excluded Area Effect

In the preceding discussion we have used only the first summation on the right-hand side of eq 4, which expresses the potential energies of short-range interactions of among the adjacent parts located within the same segment. Let us consider now the effect of the long-range interactions between the remote parts of the chain (expressed in terms of a contour co-ordinate s upon the mean square end-to-end distance).

Using the Gaussian approximation, eq 4 is written as

$$U = n\langle u \rangle + \sum_{i>j} u_{ij} \qquad (22)$$

If the potential u_{ij} is approximated as

$$u_{ij} = \beta kT\delta(|r_{ij}|) \qquad (23)$$

the increment of the mean end-to-end distance is estimated, as shown by Fixman,[15] who derived the equation for the three-dimensional case, to be

$$\left(\frac{\langle R_{III}^2 \rangle}{\langle R_{III}^2 \rangle_0} \right)^{\frac{3}{2}} = 1 + 2\beta_{III}n_{III}^{\frac{1}{2}} \left(\frac{3}{2\pi b_{III}^2} \right)^{\frac{3}{2}} \qquad (24)$$

where β_{III} is the excluded volume and

$$\langle R_{III}^2 \rangle_0 = n_{III}b_{III}^2 \qquad (25)$$

Following his derivation, we obtain a similar expression for the two-dimensional case, namely,

$$\left(\frac{\langle R_{II}^2 \rangle}{\langle R_{II}^2 \rangle_0} \right)^2 = 1 + \beta_{II}n_{II}\frac{1}{\pi b_{II}^2} \qquad (24a)$$

$$\langle R_{II}^2 \rangle_0 = n_{II}b_{II}^2 \qquad (25a)$$

where β_{II} is interpreted as the "excluded area" and the relationships expressed by eq 20 and 21 should be noted thereby. If we translate eq 24a into the stiff-chain model, we obtain the approximation for $DL \ll 1$, that

$$\langle R_{II}^2 \rangle = \frac{2L}{D}(1 + KDL)^{1/2} \qquad (26)$$

where K is a non-dimensional parameter expressing the excluded area effect and given by

$$K = \frac{1}{8\pi}D^2\beta_{II} \qquad (27)$$

Eq 24a or 26 shows that the mean square end-to-end distance in the two-dimensional case is proportional to the 3/2's power of the chain length L in the limit as n tends to infinity: while in the three-dimensional case, it is proportional to the 4/3's power.

MATERIALS AND METHODS

DNA

DNA was extracted with phenol from the λ c1 phage. The preparation of the phage and the extraction of DNA were performed as described previously.[16] DNA samples which are cut mildly by passing the solution through a pipet were also prepared, since data on DNA molecules having a shorter length than that of the whole molecule were also needed for the analysis. The DNA was dialysed against 0.2 M-ammonium acetate and stored at −20°C. To exclude the effect of the cohesive ends of the λ-DNA, the solution was heated for five minutes at 70°C and cooled quickly just before use.

Electron Microscopy

Samples for electron microscopy were prepared by the diffusion method introduced by Lang, et al.,[6] with only minor changes. Barriers and troughs (10 × 10 × 1 cm) were coated with paraffin. Cytochrome-c was purchased from Wako Jun-Yaku Co. Ltd. Tokyo, Japan. The samples measured in the present analysis were obtained from a layer of cytochrome-c which sat for 30 minutes on a solution having a DNA

concentration of 2×10^{-7} g/ml in 0.2 M-ammonium acetate (pH 7.05) at 23°C.

The electron microscope used was a JEOL Model JEM 7 A, by which a series of 24 micrographs can be easily taken. All micrographs were taken at an instrumental magnification of 4000; they were projected and traced at a final magnification of 33300. Length and distances were determined from the tracing using a map measure (Sakurai Co. Ltd., Tokyo).

RESULTS AND DISCUSSION

Errors Involved in Determining the Lengths and the End-To-End Distances of DNA

The magnification was determined by the use of polystyrene latex spheres of $0.557 \pm 0.0108 \mu$ diameter (Dow Chemical Co. Inc., Midland, Michigan). The measurement of the lengths and the end-to-end distances rested on three sets of 24 micrographs. Each set contained four fields of spheres. The molecular images were traced under such conditions that the diameters of four spheres, which were randomly selected, fell in the range of 18.0–19.0 mm.

The estimated experimental errors were 1.2% (from the standard deviation of the diameters of the spheres), 2.7% (in determining the final magnification), 2% (in determining the lengths and distances), and 2% (in tracing). The total error, which is given by the geometrical mean of the above mentioned errors, was less than 5%.

Diffusion–Adsorption Process of DNA Molecules

As shown in Table I and Figure 1, the mean square end-to-end distance was found to be proportional to the 3/2's power of the chain length of the range of the present experiment. The distributions of end-to-end distances obtained for various values of chain length are shown in Figures 2 a—g.

In these measurements, we tried to find a flexible point and/or an inherent distribution of the different degrees of flexibility along the DNA strand, if any, because the DNA used is known to have a non-uniform but unique distribution of GC-content. However, we could not find such a characteristic within the precision of our experiment.

Table I.

Contour length $L \pm S_L(\mu)$[a]	N (no. of molecules)	Mean square end-to-end distance $\langle R_{II}^2 \rangle \pm S_R(\mu^2)$
0.25 ± 0.04	98	0.0358 ± 0.0017
0.50 ± 0.05	56	0.0853 ± 0.0071
1.00 ± 0.10	111	0.243 ± 0.015
2.00 ± 0.20	112	0.588 ± 0.054
4.00 ± 0.40	88	1.66 ± 0.14
8.00 ± 0.80	71	5.92 ± 0.48
16.0 ± 1.6	47	12.7 ± 1.6

[a] S_L and S_R are the standard deviation of contour length and mean square end-to-end distance, respectively.
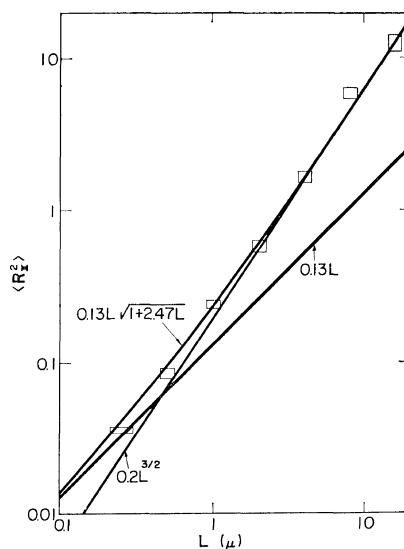

Figure 1. Mean square end-to-end distances of DNA molecules.

These results are numerically the same as those obtained by Lang, et al.[5] But in the present study the relationship, $\langle R_{II}^2 \rangle \propto L^{3/2}$, is obtained with more certainty, since the standard deviations of both mean values are much smaller. The equation of Lang, et al., given in the introductory section, seems less probable since it presents a relationship, $\langle R^2 \rangle \propto L^2$, for the case of $L \gg b$.

Another difficulty with the previous theoretical treatments is the interpretation of the process of absorption of DNA on to a protein layer. If it is caused by the interaction of positively
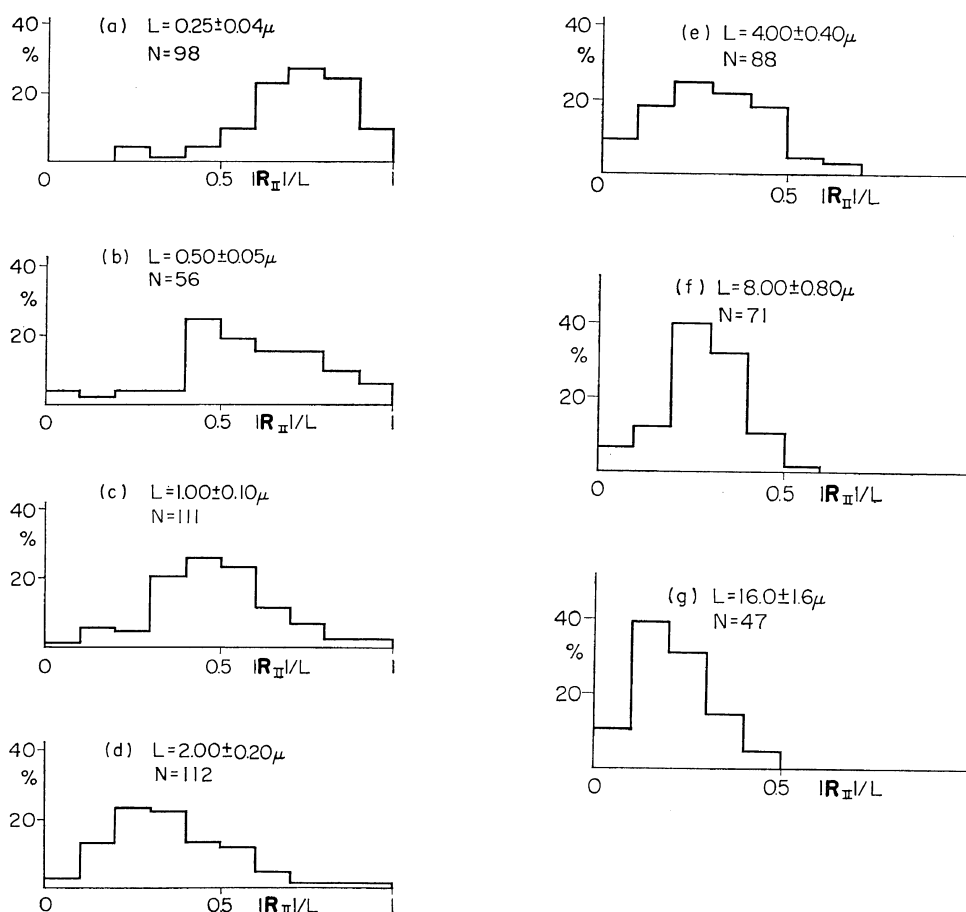
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Figure 2. Distribution of end-to-end distances. N, number of DNA molecules measured.

charged protein and negatively charged DNA, as assumed previously, the second assumption quoted in the introduction seems improbable, because it assumes a parallel projection of the end-to-end distance. A DNA molecule approaches through the adjacent region, which has higher concentrations of anions, and then fixes on to the positively charged layer in order to neutralize the charges on both sides to compensate for the reduction of entropy. As expressed in eq 20 and 21, if a linear molecule is fixed on a plane as a parallel projection, the free energy will increase. Since Cytochrome-c film allows a measurement of its surface pressure by conventional methods of surface chemistry,[17] it seems unreasonable to assume that the film has enough rigidity to resist the two-dimensional Brownian motion of the DNA molecules, which, otherwise, takes place in order to maintain (or restore) thermal equilibrium.

If the Brownian motion in a plane is assumed to be a dominant process in determining the conformation of a DNA molecule in the electronmicrograph, then the observed relationship, $\langle R_{II}^2 \rangle \propto L^{3/2}$, is appropriately interpreted as an "excluded area effect", which in turn suggests the above mentioned interaction between the protein film and the DNA.

Estimation of Parameters in Terms of a Stiff-Chain and Excluded-Area Scheme

The relationship between the apparent non-dimensional chain length $D'L$ and the counter length L is given by eq 13b and plotted in Figure 3. As shown in Figure 4, $D'L$ (obtained by the relation given in the Figure 3 and by using $\langle R_{II}^2 \rangle$ observed) is approximately propor-
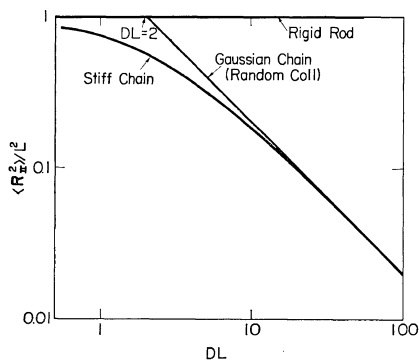
Figure 3. Relation between "Apparent non-dimensional chain length" $D'L$ $(= 2L^2/R_{II}^2)$ and DL.

tional to the square root of L. This suggests that the long-range interaction is a repulsive one. In Figure 4, the value L corresponding to $D'L = 2$ in ordinate where energy per one degree of freedom becomes $kT/2$ (see eq 16a), gives a real value of D (as $D = D'$ at $L = 2/D'$), because at that length, the interactions between the remote parts in the DNA chain apparently vanish in the present model. Thus we obtain $D = 15.4\,\mu^{-1}$ or $1/D = 0.065\,\mu$.

Using this value for D, the parameter K in eq 26 is found to have the numerical value 0.154 for the best fit of the theoretical equation with the empirical result

$$\langle R_{II}^2 \rangle = 0.13\sqrt{1 + 2.47L}$$

The excluded area thus obtained is, $\beta_{II} = 0.016\mu^2$, which is about 10 times larger than that of a segment of the B-form structure given by a parallel-projection on to a plane parallel to the

main axis. This suggests that cytochrome-c is a "good solvent" for DNA, and supports the assumption that DNA and cytochrome-c interact in order to compensate for the reduction of entropy.

While K is a highly solvent-dependent parameter, D is thought to be structure-dependent and not highly affected by the solvent provided the chain has the B-form structure. In fact, the value, $1/D = 650\,\text{Å}$, shows a good agreement with λ $(=1/D) = 717\,\text{Å}$ given by Hearst and Stockmeyer[8] when the excluded volume was assumed to be zero (*i.e.*, at theta temperature). Thus, in solution, the excluded-volume effect seems to be negligibly small.

By substituting in eq 10 the values, $D = 15.4\,\mu^{-1}$, $T = 296°\text{K}$, and $k = 1.38 \times 10^{-16}\,\text{erg/deg}$, the flexural rigidity of DNA is calculated to be $\varepsilon = 1.2 \times 10^{-19}\,\text{dyn cm}^{-2}$, which possesses a certain validity in describing the conformation of DNA solution. Cohen and Eisenberg[18] first estimated the ε to be $2.5 \times 10^{-19}\,\text{dyn cm}^{-2}$ which is almost twice that obtained by us. Also the value of the statistical lengths given above is much shorter than those from light scattering and hydrodynamic data. These discrepancies might come from the reduction of electrostatic charges on the DNA strand at the protein surface, or it might show a difference of flexural rigidity between that in solution and on the protein membrane.

It is also of interest to determine the folding energy of DNA into a phage particle or phage head from the viewpoint of flexural rigidity.[19] The mechanical energy required to fold a DNA
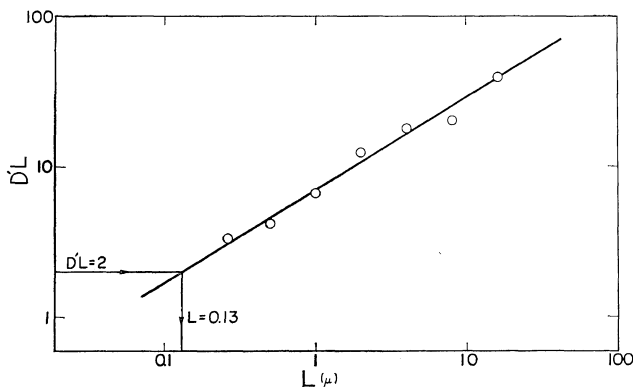

Figure 4. Experimentally obtained relation between $D'L$ and L.

molecule of a length L into a curvature K is shown in eq 7. By using the flexural rigidity of T_2-phage DNA as calculated above, the energy of rounding up into an 800 Å diameter solenoid (which is the geometry of the head of T_2-phage) is calculated to be 2 cal/mol-nucleotide pair. On the other hand, according to Kilkson and Maestee,[20] the DNA in a phage head has a super helical form of 80 Å diameter. For this case, if we use 40 Å as the smallest radius of curvature, this gives 200 cal as the flexural energy of folding per mol of nucleotide pairs. This agrees well with other measurement.[19] In either of the cases mentioned above it can be said that, as far as flexural rigidity is concerned, the folding energy of DNA into a phage particle or head can be compensated by the interaction (electrostatic is seemed to be major source) between DNA and coat proteins, because the interaction between nucleotide units and basic protein is of the order of kT ($=600$ cal at room temperature).

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