Sucrose Gradient Analysis: Computer Simulation and Measurement of the Parameters Involved in the Sedimentation of DNA Molecules

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The Montecarlo method is used to computer simulate a random distribution of molecular lengths generated by inducing T4 DNA fragmentation through the decay of ^{32}P atoms introduced in the molecule.

Taking into account the experimental conditions we find that the value of α for alkali sucrose gradients is 0.46 ± 0.02 and does not depend on the running time. Our findings also prove that the computer simulation can be utilized to analyze sedimentation profiles of DNA molecules fragmented *in vivo*.

Introduction

Much work has been performed to establish some empirical relation between the sedimentation coefficient (S) through sucrose linear gradients and the molecular weight (M) of DNA [1–8]. The proposed relationship [1] $S = K M^{\alpha}$ or the equivalent one $S_1/S_2 = (M_1/M_2)^{\alpha}$ allows the calibration of S against molecular weight, provided α is known and a reference DNA is available.

On the basis of accurate measurement and on some hypothesis, α has been determined [7] to be 0.35 for native DNA, 0.55 for neutral denaturated DNA and 0.40 for alkaline DNA. It was also shown [2] that the distance sedimented (D) is proportional to the sedimentation coefficient, so that

$$\frac{D_1}{D_2} = \left(\frac{M_1}{M_2}\right)^{\alpha}. \qquad (1)$$

On the other hand the sucrose gradient sedimentation technique is a powerful method to determine the mass or the length of intact and fragmented molecules, provided a suitable calibration is available. Indeed the parameters involved in relationship (1) depend on the experimental conditions, on the mass range to be considered and particularly on the form of the analysed molecules. The latter problem becomes particularly relevant if denatured DNA has to be analysed because single-strand DNA molecules undergo dramatic transitions between extended and compact forms as a function of pH and ionic strength [7, 9].

In this paper we present a simple procedure to calibrate the method. This procedure allows determination of α, with the use of only one marker molecule and overcomes the difficulties introduced by the molecular form factor.

The procedure is divided in two parts. A random distribution of molecular lengths is generated by inducing T4 DNA fragmentation through the decay of ^{32}P atoms introduced in the molecule [3, 10].

The second part of the procedure consists in computer simulation of the same process by using the Montecarlo method taking into account our experimental conditions. The sedimented distance distribution, as function of α, is obtained by using the relationship (1). Finally, the α is obtained by fitting the calculated to the experimental distribution.

On the other side the computer simulation method, calibrated with respect to a known distribution under our experimental conditions, will be used to analyse, with appropriate hypothesis, sedimentation profiles of T4 DNA molecules fragmented *in vivo* [11].

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Sample Preparation and Random Endonucleolytic Cleavage of T4 DNA Molecules

Because of the biological problem we are interested in [11, 12], we had to explore the distribution of random nicks in the interval between 1 and 15.

It was convenient to prepare at least two T4 DNA stocks with different values of the average number of ^{32}P isotopes per single-strand molecule (N_0) because of the ^{32}P isotope half life and of the times involved in preparing and analysing the samples.

T4 radioactive lysates were prepared as follows: *E. coli* B^E cells were grown at 37 °C in TMM low phosphate medium [13] containing two different amounts of ^{32}P labeled orthophosphate (6.75 mCi/ml and 1.67 mCi/ml). Therefore assuming that T4 native double strand DNA contains 3.3×10^5 bases [14], we obtain $N_0 = 12$ and $N_0 = 25$ respectively.

Bacteria were then infected with $T4^+$ phages at a multiplicity of infection of 1/100 and, following a multicycle growth, lysis of the culture was attained within 4 hours. T4 lysates were subsequently purified and phage DNA was extracted following standard procedures [15].

In order to measure the N_0 values, an aliquot of each preparation was phenol extracted and the specific activities of the DNA were measured (Table I A). N_0 were also independently calculated from the slope of the line obtained by plotting the number of surviving phages at various ^{32}P decay times (Table I B): we assumed that 10% of the ^{32}P decays produce double strand breaks, which are lethal events [16].

Table I. Determination of the average number N_0 of ^{32}P isotopes per T4 DNA single strand molecules. The first column shows the N_0 values for the two DNA preparations as calculated through the ^{32}P specific activities of the medium used and the number of basis per single strand T4 DNA. Columns A and B show the N_0 values as measured through: A) specific activity at 260 mμ of the extracted DNA, B) slope of the surviving phages curves. The errors represent the dispersion of three independent experiments.

N_0 expected	N_0 measured	
	A	B
12	10±2	15±5
25	28±4	30±5

After phage lysis, DNA molecules were alkali denatured to prevent double strand breaks, neutralized and stored at 4 °C. Since the ^{32}P decay breaks the corresponding phosphodiester bond (nick) and the ^{32}P atoms are randomly distributed along the molecule, samples of the same DNA preparations at various times give us sets of molecular fragments of different length randomly distributed. The average number of nicks at the time t is given by the relationship

$$m(t) = N_0(1 - e^{-t/\tau}). \qquad (2)$$

and the number of nicks per molecule follows the Poisson distribution around $m(t)$.

Sucrose Gradient and Sedimentation Profiles

5 – 20% (w/v) linear sucrose gradients (0.2 M KOH, 0.1% sarkosyl, 0.015 M EDTA, 1 M NaCl) were performed in polyallomer tubes with total volume of 4.9 ml.

Samples of 0.1 ml of lysis buffer (0.1 M EDTA, 0.1 M Tris-HCl pH 7.5, 0.01 M NaCl) containing 10^{-2} μg of denatured DNA were deposited on the gradient with a peristaltic pump to avoid DNA shearing.

After centrifugation, gradients were automatically fractioned by collecting seven drops per fraction from the bottom of the tube, corresponding to a volume of 0.17 ml. In this way and under our centrifugation conditions (SW 50.1 rotor, 15 °C, 33.000 rpm, 180 min), the distribution of intact T4 DNA molecules is well represented by a gaussian-like profile with a half-height width of three fractions. Such a peak can be displaced along the gradient by varying the centrifugation time without substantial shape change. 50 μg of denatured calf thymus DNA was added as carrier to each collected fraction. Samples were then precipitated in the cold with 5% Cl_3COOH, filtered onto fiber glass/C discs, washed with 70% alcohol, dried and the radioactivity was measured in a liquid scintillation counter. Recovery of counts was always over 90%.

Fig. 1 shows the sedimentation profiles for various decay times [*i.e.* for various values of the average nick number (m_e)]. It is evident that the length distribution moves toward the top of the gradient and becomes narrower as m_e increases. It should also be mentioned that an appreciable amount of radioactivity appears at the top of gradients. Since we agree with the considerations of reference [3] to neglect this, the profiles shown at the top of the experimental gradients were obtained

through graphic interpolation with appropriate assumptions.

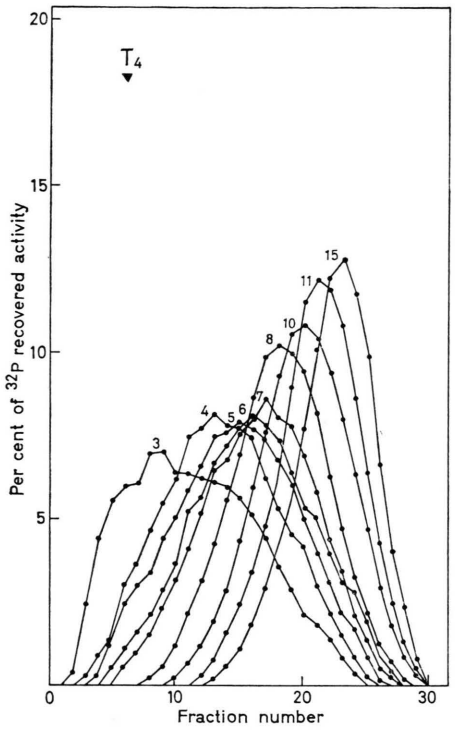

Fig. 1. Sedimented distance distribution S of single strand DNA fragments resulting from an average number m_e of nicks randomly generated in T4 native DNA. Sedimentation increases from right to left. The arrow indicates the position of intact single strand T4 DNA molecule. The numbers correspond to the average numbers (m_e) of nicks as calculated from the data reported in Table I. The experiment was done in duplicate.

Computer Simulation

The process previously described, can be computer simulated by the Montecarlo method.

The molecular population is divided in K classes containing $n(k)$ molecules. $n(k)$ follows the Poisson distribution with a given m as expected value. Each molecule of l_0 length, belonging to the class $n(k)$- is subdivided into $(k+1)$ fragments of l length by randomly positioning k nicks.

Successively for each fragment l the corresponding sedimented distance D is calculated by the relationship $D/D_0 = (l/l_0)^\alpha$ where D_0 is the position reached by the unnicked molecule of l_0 length. Each length is further spread out following a gaussian distribution through the appropriate number of fractions, as experimentally determined.

In such a way we obtained a set of sedimentation distributions, as a function either of m or α, which allows us to determine α and calibrate the method. In Fig. 2 the distributions for the m values indicated and for $\alpha = 0.46$ are shown.

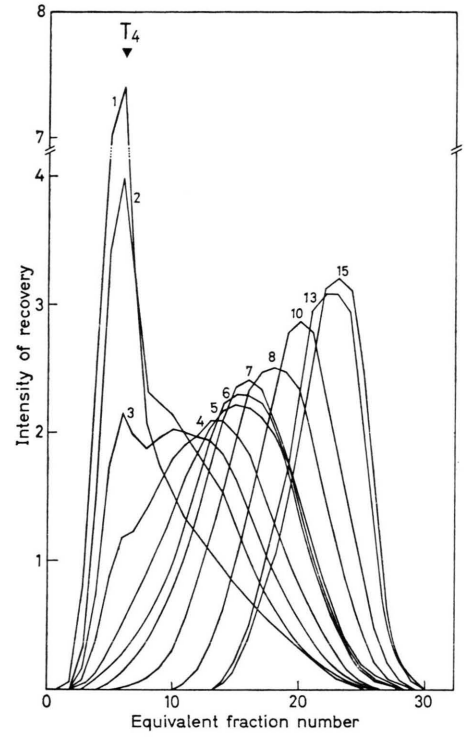

Fig. 2. Computer simulated distributions of the sedimented distances of molecular fragments resulting from an average number m of breaks randomly generated. The numbers correspond to expected values (m) in the Poisson distribution. The sedimentation distances are calculated by the relationship (1) with $\alpha = 0.46$.

Comparison between Simulated and Experimental Distributions

The α parameter is determined by minimizing the sum of the squared differences Ψ^2 between the experimental and corresponding simulated profiles for each fraction. In Fig. 3 the behaviour of this sum, calculated for the distribution with $m_e = 4$ and $N_0 = 12$ is shown. The minimum is attained for $\alpha = 0.46$ and from Fig. 3 we assume .02 as error.

With this α value we globally compared the whole set of the experimental distributions, corresponding to the two sets of m_e values (3, 4, 5, 6, 7, and 8, 10, 11, 15) as calculated from the ^{32}P decay law

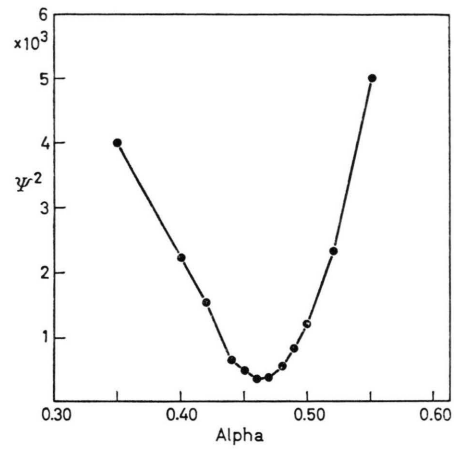

Fig. 3. Sum of the squares difference (Ψ^2) between computer simulated and experimental profiles as a function of the α parameter. Ψ^2 is calculated for $m_e=4$. The minimum is attained for $\alpha=0.46$ and ± 0.02 can be assumed as error.

($N_0=12$ and $N_0=30$), with the simulated distributions for various m values. The results of this comparison are reported in Table II where m_c indicates the m value which minimizes the sum of the squared differences Ψ^2. The N_0 values can be determined directly from the m_c values, by using the relationship (2). The mean values so obtained (12 ± 2, 31 ± 2) are in agreement with the data reported in Table I.

In order to analyse the α dependence from the time of centrifugation, DNA samples were also sedimented for 270 and 360 min. We found that α is constant in this running time interval.

Conclusions

Many authors have analysed the validity range of the relationship (1) and various determinations of the α parameter were done by using standard molecules of known mass. The method we present in this paper utilizes, as reference masses to determine α, fragments of randomly distributed lengths of the same molecule in which we are interested in analysing the unknown distribution of *in vivo* fragmentation [11, 12]: the molecular form and the experimental conditions are then automatically taken into account. This procedure does not require more than a single molecular weight marker to determine the molecular weight scale.

Our findings can be summarized as follows:

a. α is almost constant when the centrifugation time varies between 180 and 360 min. This result gives us the possibility to displace the distribution according to the experimental needs.

b. The α value for alkaline denatured DNA under our experimental conditions, in the range 1 to 1/15 of the T4 single strand DNA mass (M.W.: $56\cdot 10^6$ daltons) is 0.46 ± 0.02 to be compared

Table II. Comparison between the experimental and simulated alkali sucrose sedimentation distributions of randomly broken DNA. m_e and m values are those reported respectively in Figs 1 and 2. The errors in the m_e column reflect the indetermination in the measurement of N_0 as reported in Table I, column A.

m_e	Ψ^2	m	m_e
	4900	2	
	1452	2.5	
3.0 ± 0.5	2928	3	2.5
	7943	3.5	
	13135	4	
	8479	3	
	3519	3.5	
4.0 ± 0.5	377	4	4
	2322	4.5	
	3469	5	
	12699	4	
	7219	4.5	
5.0 ± 0.5	3684	5	5
	9423	5.5	
	24139	6	
	31394	5	
	15612	5.5	
$6\ \pm 1$	9175	6	6
	10308	6.5	
	11197	7	
	1267	6	
	919	6.5	
$7\ \pm 1$	399	7	7
	1502	7.5	
	3301	8	
	6367	7	
	5915	7.5	
$8\ \pm 1$	1526	8	8.5
	942	8.5	
	1760	9	
	9370	8	
	6560	8.5	
$10\ \pm 1$	1544	9	10
	790	10	
	3543	11	
	3943	10	
	2402	11	
$11\ \pm 2$	1453	11.5	11.5
	3642	12	
	6420	13	
	84230	12	
	45030	13	
$15\ \pm 3$	11973	14	15
	1492	15	
	9422	16	

with $\alpha = 0.40$ as reported in reference [7], $\alpha = 0.38$ in reference [8] and $\alpha = 0.35$ in reference [3]. This further emphasizes that α depends on the characteristics of the molecules and of the sucrose gradients used. Therefore α must be measured every time this technique is used to determine molecular lengths or masses.

c. Due to the exponential form of the relationship (1) the resolving power increases as a function of the sedimented distance. Under our condition we obtained $\Delta l/l = 15\%$ at $l = l_0$ and $\Delta l/l = 60\%$ at $l = l_0/15$.

These findings show that the whole procedure is adequate. In such a way the method of comparing computer simulated and experimental distribution can be used to analyse, with appropriate hypothesis, sedimentation profiles of T4 DNA molecules fragmented *in vivo*.

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