Analytical Ultracentrifugation of T4r Bacteriophage DNA in Preformed Sucrose Density Gradients*

JOEL ROSENBLOOM† AND VERNE N. SCHUMAKER

From the Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia

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The sedimentation coefficients of high molecular weight deoxyribonucleic acid (DNA) isolated from bacteriophage and bacteria are apparently dependent upon the rotor speed of the ultracentrifuge. Explanation of this singular finding is made difficult by the possibility that convective disturbances may exist in these extremely dilute solutions. A density gradient engine which we have used to establish sucrose gradients in the analytical ultracentrifuge has been built. Using preformed gradients to prevent convective disturbances, we have studied DNA isolated from T4r bacteriophage over a wide range of concentrations and a wide range of rotor speeds. We find a distinct difference between the results of experiments made in this fashion and control runs made in the absence of a superimposed gradient. In the presence of a gradient there is a rapid loss of a fraction of the original starting material. The extent of this initial loss, which clearly is not sedimentation of individual DNA molecules, increases with increasing rotor speed and increasing starting concentration. The remainder of the DNA left behind in solution behaves normally. It moves with a sedimentation coefficient that is independent of rotor speed but strongly dependent upon concentration. A phase change which is dependent upon rotor speed has been hypothesized to explain this initial rapid loss of material. Small aggregates, formed by molecular entanglement, would sediment more rapidly than the monomeric DNA molecules, collide with the latter, and grow in size. This aggregation is considered to be reversible with a rate of disentanglement proportional to the surface area of the aggregates. As the aggregates grow, the monomer concentration declines, and thus the probability of collision, and hence the rate of growth, is reduced. When the rate of growth and the rate of disentanglement become equal, there will be equilibration of mass between the monomeric molecules and the aggregated phase. According to this hypothesis, the collision rate of the aggregates with the monomers is a function of rotor speed. Therefore the solubility coefficient of the aggregated phase will also be a function of rotor speed.

The sedimentation coefficients of high molecular weight deoxyribonucleic acids isolated from bacteriophage and bacteria are apparently dependent upon the rotor speed of the ultracentrifuge, the observed coefficient increasing with speed. This observation was first reported by Hearst and Vinograd (1961) who also found the speed effect to be strongly concentration dependent for T4r DNA,[1] disappearing at a concentration of about 6 μg/ml; the increase in sedimentation coefficient was accompanied by excessive boundary spreading and loss of the plateau region. Burgi and Hershey (1961) have found that the sedimentation coefficient of T2 DNA drops rapidly at rotor speeds below 30,000 rpm, but that above that speed the sedimentation coefficient is independent of concentration; they also note that the sedimentation coefficient is generally lower and less reproducible with Epon or Kel-F centerpieces (Beckman) as opposed to aluminum ones. Eigner, *et al.* (1962) found a similar speed dependence with T6 DNA studied at the single concentration of 20 μg/ml; these authors also reported a small speed dependence with high molecular weight DNA isolated from *E. coli*.

No satisfactory explanation of these singular findings has been proposed. Interpretation of the results is made difficult by the possibility that convective disturbances exist in these extremely dilute solutions. Even though great precautions have been taken to properly align the cell and to minimize thermal gradients, convection might occur since there is no stabilizing force in the plateau region and only a small density change in the boundary region.

A density gradient engine which we have used to establish sucrose gradients in analytical ultracentrifuge cells is described in this paper. Using preformed gradients to prevent convective disturbances, we have studied DNA isolated from T4r bacteriophage over a wide range of concentrations and a wide range of rotor speeds. We find a distinct difference between the results of experiments made in this fashion and control runs made in the absence of a superimposed gradient. In the presence of the gradient, it is possible to observe the sudden loss from the solution of a portion of the DNA. The amount of DNA lost from solution depends upon the initial concentration and upon the speed of the ultracentrifuge. As will be discussed, we believe this loss is due to a phase transition with a fraction of the DNA behaving like a precipitate. The remainder of the DNA left behind in solution behaves normally; it moves with a sedimentation coefficient that is independent of rotor speed, but strongly dependent upon concentration.

METHODS

Gradient Centrifugation.—Gradients were formed by a gradient engine constructed from readily available commercial materials (Fig. 1). Calibration of the gradients while the centrifuge is in operation may be achieved by the use of either the schlieren or ultraviolet optical systems (Fig. 2). We have formed gradients ranging in steepness from 1.70 to 3.3% and from 17.5 to 28.8%. All have been stable and the only difficulty has been an excessive schlieren effect with the steeper gradients and consequent loss of light from the optical path. Use of a 1° negative wedge window circumvents this difficulty.

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† Post-doctoral Fellow of the National Institutes of Health, Bethesda, Maryland.

[1] Abbreviation used in this work: DNA, deoxyribonucleic acid.

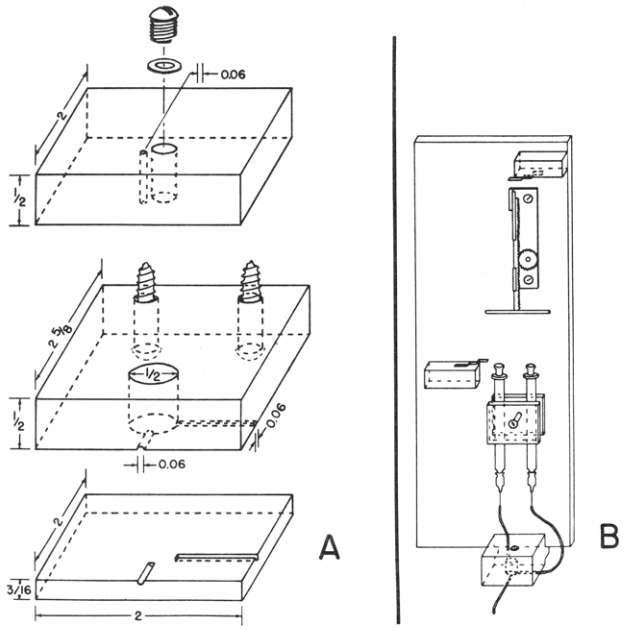

FIG. 1.—A 1/3-rpm synchronous reversible motor is mounted behind a 6 1/2 × 16–in. board and geared to a moving T bar. Positioned on the board are two tuberculin syringes which are emptied by the downward movement of the T bar. These syringes are fitted with appropriately sized needles or their plastic equivalents. Polyethylene catheters lead from the needles to the mixing chamber which is also rigidly affixed to the board. The chamber, Fig. 1A, constructed of plexiglass, is made in the following way. A 1/2–in. diameter well is first drilled through a 1/2–in. thick block of plexiglass, on top and bottom of which is then glued two other pieces of plexiglass. Holes to accept the polyethylene catheters are drilled such that one leads into the bottom of the well, and another into the top. A third hole for the outflow catheter is drilled leading out of the bottom of the well. A large-bore threaded hole in the top permits easy cleaning of the chamber and is sealed with a stainless steel screw and rubber washer. The polyethylene catheters are affixed to the mixing chamber with epoxy glue. Stirring is achieved by a Teflon-covered magnetic stirring bar. The entire system is air-tight. In our device the T bar moves with a linear speed of 0.81 in./minute (0.35 cc/minute), and the catheters easily accept a No. 20 needle (0.9 mm diameter). We have found that the stirring bar need revolve only slowly to attain adequate mixing. In forming the gradients the more concentrated solution is placed in the mixing chamber. The diluent is placed in the syringe whose catheter leads into the bottom of the well. The second syringe is filled with an equal volume of air. This arrangement produces a linear gradient, while omission of the air syringe produces a logarithmic gradient. We routinely use an initial volume of 0.5 cc in the mixing chamber although the apparatus may be operated with smaller volumes. It is only necessary to enlarge the entrance hole of conventional cells to accept a No. 19 needle (No. 55 drill) to adapt them to this type of gradient work. The valve type of synthetic-boundary cell (Pickels, *et al.*, 1952) may be used directly. In the formation of gradients the outflow catheter is placed inside the cell in contact with one of the radial walls so that the liquid runs down the side. The rotor is then rotated 90° and the filled cell is carefully set in place. Some mixing occurs when the rotor is rotated for placement in the centrifuge.

The gradients measured in the cell always show a diminution in steepness from the theoretical values along with some flattening at the meniscus and near the bottom of the cell. While they are linear only in the mid-portion of the cell, a significant gradient remains throughout.

In sedimentation velocity runs the concentration

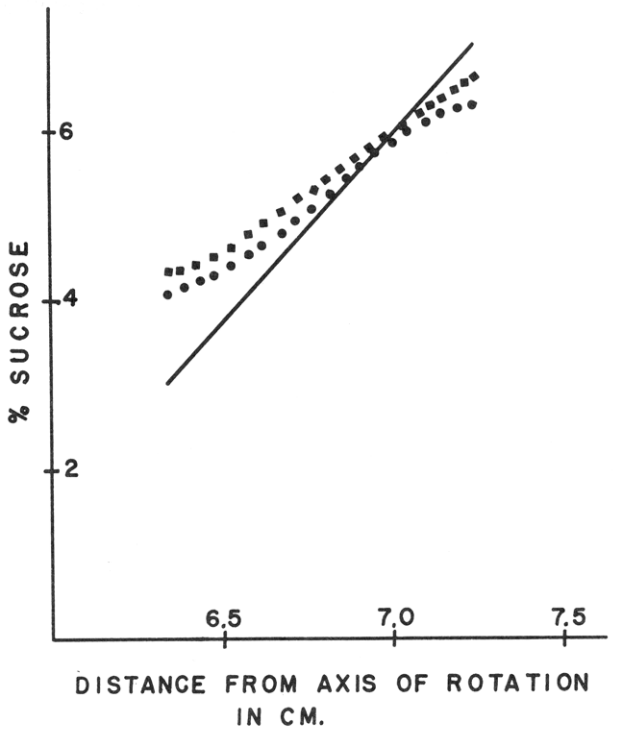

FIG. 2.—Typical sucrose gradient. Squares indicate calibration of gradient by use of adenosine marker in the concentrated sucrose solution in the mixing chamber and use of the ultraviolet optical system. Dots indicate calibration by use of the schlieren optical system following the procedure of Schachman (1957). The solid line is the gradient that would have been observed in the absence of mixing in the ultracentrifuge cell.

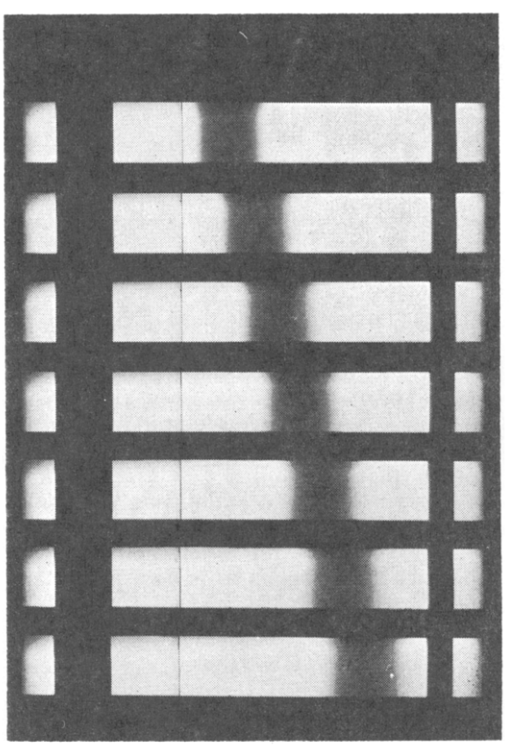

FIG. 3.—An illustration of zone centrifugation in the analytical ultracentrifuge using crystalline catalase $OD^{1\ cm}_{410m\mu} = 0.925$ in the cup and a sucrose gradient in the lower portion of a synthetic-boundary cell. The conventional bromine-chlorine filter has been replaced by a 410-mμ interference filter in the ultraviolet optical system.

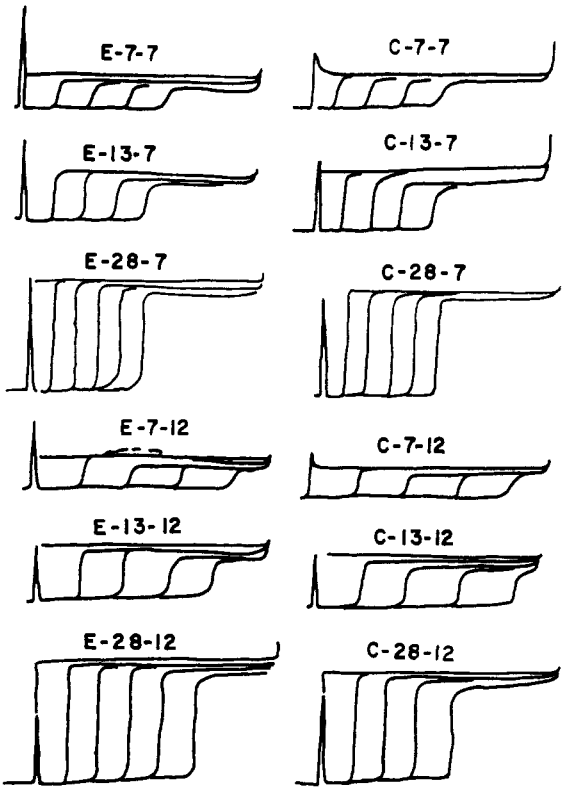

FIG. 4.—Composite densitometer tracings for runs at 7717 and 12,590 rpm, code-labeled as described in text. Sedimentation is from left to right. Not all tracings from any given run are shown, selection was made for clear presentation.

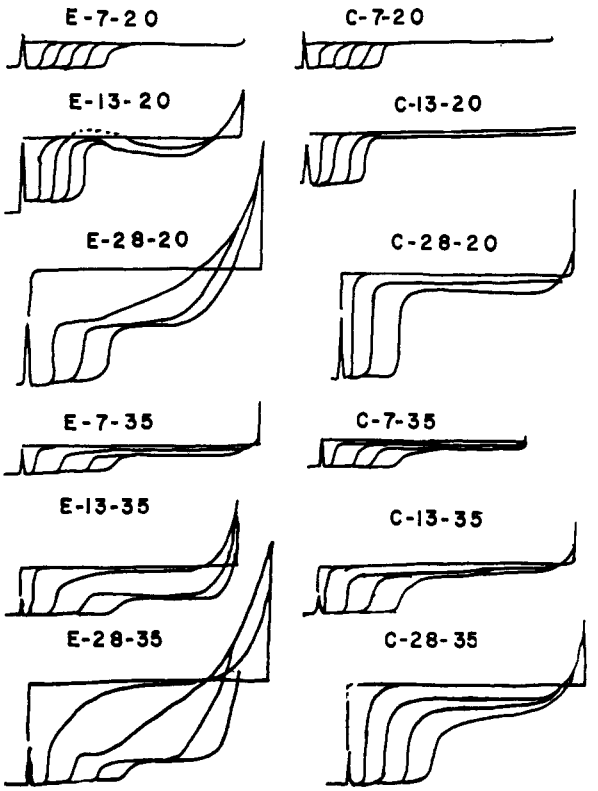

FIG. 5.—Composite densitometer tracings for runs at 20,410 and 35,600 rpm.

of the macromolecules is made uniform throughout the cell simply by having equal concentrations in both the concentrated and diluent gradient solutions.

One may also perform zone centrifugation experiments simply by placing the solution of the macromolecules in the cup of a synthetic-boundary cell and a density gradient in the bottom compartment (Fig. 3).

Isolation of the DNA.—T4r phage were grown and harvested as described by Cohen and Arbogast (1950). The crude phage preparation was treated with 1 μg/ml deoxyribonuclease for 30 minutes at room temperature in 0.85 g/100 ml NaCl, 0.005 M Tris, *p*H 7.6, and 0.01 M $MgSO_4$; the phage was then purified by differential centrifugation. The DNA was isolated by the method of Mandell and Hershey (1960) except that a ground-glass stoppered vial was used and contact with metal by the DNA was thus eliminated throughout. The DNA was dialyzed in boiled washed tubing against 0.05 M Tris, *p*H 7.6, and 0.2 M NaCl. The DNA was then stored at 0°. All dilutions were performed by pouring and weighing. An extinction coefficient of 18.1 cm^2/mg sodium nucleate was used to convert optical density measurements to concentrations (Rubenstein *et al.*, 1961).

RESULTS

The first group of experiments consisted of a total of twenty-four runs; twelve of these runs were made using a sucrose gradient of 4.1 to 6.3%, and twelve control runs were made in a uniform 5% sucrose solution. In the latter group of runs the analytical cells were filled by the gradient device with the stirrer turning and a 5% sucrose solution in both the mixing chamber and diluent syringe. Contact with metal was prevented by using polyethylene connections in the gradient engine and a Kel-F centerpiece in the analytical cell. The dimensions of the gradient engine indicate a wide margin of safety in regard to shear degradation of the DNA (Burgi and Hershey, 1962; Hershey, *et al.*, 1962; Levinthal and Davison, 1961). We have observed no alteration in sedimentation coefficient upon passage through the device.

Each set of twelve runs consisted of three groups (three different concentrations) of four runs (four different speeds). Each run has been given a code designation as follows. The letter E indicates a gradient, while the letter C indicates a uniform 5% sucrose solution. The first number designates the initial concentration of the DNA in μg/ml, and the second number the speed of revolution of the ultracentrifuge in thousands of rpm. Thus E-7-7 indicates a 4.1–6.3% sucrose gradient, an initial concentration of 7.2 μg/ml, and a speed of 7717 rpm. Figures 4 and 5 are composite tracings of the densitometer recordings of all twenty-four runs.

At 7717 rpm and 12,590 rpm, the composite tracings with and without the gradient are comparable at all concentrations, and the radial dilution law is obeyed reasonably well. At both speeds the measured sedimentation coefficients are nearly the same in the presence and the absence of the gradient, and a marked dependence of sedimentation coefficient upon concentration is noted.

At the higher rotor speeds, there is a distinct change in the sedimentation behavior of the DNA in the presence of the gradient. At 28.1 μg/ml and at 20,410 rpm (E-28-20) there is an initial sudden loss of approximately half of the original material. The remaining DNA sediments normally, with a well-defined plateau region. At 35,600 rpm these differences are accentuated. At an initial concentration of 28.1 μg/ml (E-28-35) there is a very rapid loss of 75% of the original

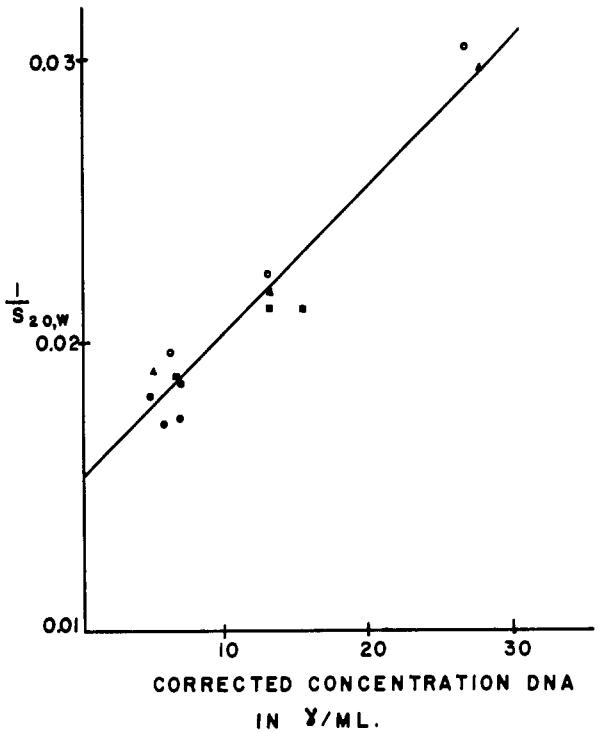

FIG. 6.—Plot of the inverse of $s_{20,w}$ irrespective of speed as a function of concentration found midway during the run. Open circles, 7717 rpm; triangles, 12,590 rpm; squares, 20,410 rpm; dots, 35,600 rpm. The straight line is the least squares line for this data and extrapolates to $s_{20,w}$ of 66.6. All runs were performed in preformed sucrose gradients.

material, again the reappearance of a plateau region, and relatively normal sedimentation of the remaining DNA, although marked boundary spreading was seen at the conclusion of the run. Excessive boundary spreading of the remaining DNA was noted in all runs at 35,600.

The corresponding control runs made in the absence of a gradient do not exhibit the sudden loss of material which is seen in the presence of the gradient. However, in these control runs the concentration in the plateau region decreases much more rapidly than predicted by the radial dilution law.

At lower concentrations the same effects can be observed, although they are less pronounced. At 35,600 rpm and at 13.3 and 7.2 μg/ml a smaller fraction of the total DNA is lost; it is interesting to note that approximately the same amount of DNA is left behind in solution (E-13-35 and E-7-35). At 20,410 rpm in the presence of the gradient the sudden loss of material can be observed at an initial concentration of 13.3 μg/ml, but not at 7.2 μg/ml. A peculiar concentration inversion appears to occur in the former run (E-13-20). We occasionally see such inversions, and we believe that at intermediate speeds and concentrations the initial loss of material may extend only part way up the cell, leaving the concentration relatively unchanged at the meniscus.

In these runs made at higher speeds in the presence of a gradient (E-28-35, E-13-35, E-28-20, E-13-20) there also appears a considerable "pile-up" of DNA at the cell bottom during the first minutes of the run. This "pile-up" decreases with time as though the material were slowly compressed into a pellet at the bottom of the cell.

In Figure 6 are plotted the reciprocals of the sedimentation coefficients observed in the experimental runs as a function of the actual concentration of DNA left in solution. It is seen from Figure 6 that the sedimentation coefficient is essentially independent of rotor speed and strongly dependent upon concentration as long as $1/s$ is plotted as a function of the actual concentration of DNA found midway in the run in the presence of the gradient.

Sedimentation coefficients were measured by choosing points on the boundaries where the concentration was approximately half that of the plateau region found at each time considered. In runs in which the bulk of the material is rapidly lost, the reported sedimentation coefficient is that of the material which remained behind and appeared to sediment normally. Conventional ln x vs. time plots gave reasonably good straight lines in all cases, and sedimentation coefficients were corrected to $s_{20,w}$. In the gradient runs the average sucrose concentration through which the boundary had moved was used in making the correction.

Sedimentation coefficient values obtained for the control runs agreed in general with those reported by Hearst and Vinograd and by Hershey. At low speeds a marked dependence of sedimentation coefficient on concentration is observed; at high speeds little or no concentration dependence is seen; at intermediate speeds a less pronounced concentration dependence is found.

In an effort to influence the magnitude of the initial loss of DNA at the higher rotor speeds, additional runs have been made in the presence of 1.6 and 2.8 M NaCl, 1% formaldehyde, and 0.006 M citrate, *p*H 7.6. The 2.8 M NaCl seems to decrease slightly the percentage of DNA which is rapidly lost; the other reagents were ineffective. Table I summarizes these results. (Included in this table are runs made upon two other preparations of T4r DNA and a preparation of T6 DNA.)

This unusual behavior is manifested only by very high molecular weight DNA. Paired control and

TABLE I

A SUMMARY OF THE DATA OBTAINED IN ADDITIONAL RUNS MADE IN THE PRESENCE OF PREFORMED SUCROSE GRADIENTS

Experiment	DNA Initial (μg/ml)	DNA Remaining (μg/ml)	DNA Remaining (%)	ω(rpm)	$s_{20,w}$	Special Feature
1	18.7	8.6	46.0	25,980	53.6	Preparation 1 T4r DNA
2	16.6	11.7	70.5	20,410	45.4	Preparation 2 T4r DNA
3	19.1	8.1	42.4	25,980	55.9	T6 DNA
4	28.1	6.3	22.4	35,600	55.0	T4r DNA (control for experiments 5–8)
5	28.1	7.6	27.0	35,600	50.7	1.62 M NaCl
6	28.1	6.5	23.1	35,600	57.4	0.006 M Citrate, 0.2 M NaCl, *p*H 7.6
7	28.1	6.4	22.7	35,600	61.0	1% Formaldehyde, 0.2 M NaCl
8	28.1	10.4	37.0	35,600	50.7	2.78 M NaCl

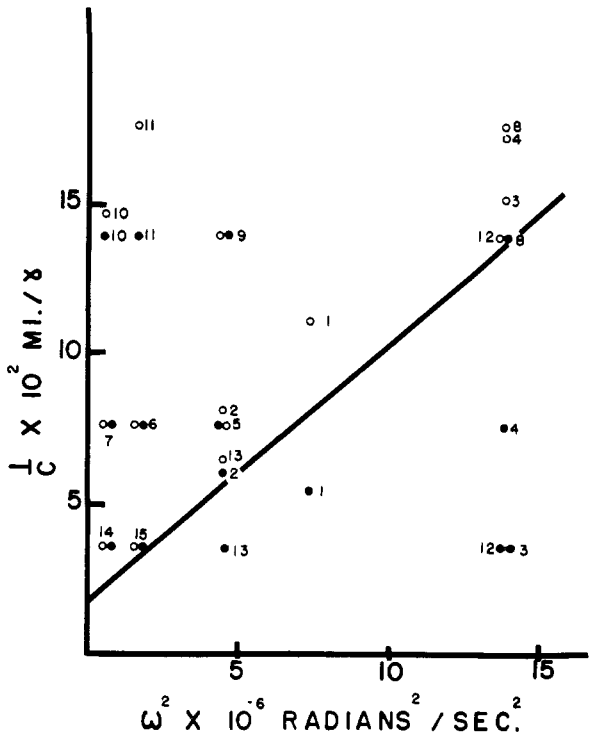

FIG. 7.—Plot of the reciprocal of the initial concentrations (solid circles), and the reciprocal of the final concentrations after loss of material (open circles) as a function of the square of the rotor speed. All runs were in sucrose gradients. The numbers identify values taken from the same run.

experimental runs using thymus DNA, sedimentation coefficient 25 S, were identical.

DISCUSSION

From our results it appears that: (1) In the absence of a sucrose gradient convective disturbances markedly distort the results observed at the higher rotor speeds. (2) In the presence of the gradient a portion of the DNA is rapidly swept to the bottom of the cell at the higher rotor speeds. (3) The DNA which remains in solution after the initial sudden loss sediments in a relatively normal fashion with a sedimentation coefficient that is independent of rotor speed.

Let us first consider the initial sudden loss of the DNA. This might be attributed to: (1) a slow reversible aggregation of the DNA for which the equilibrium position depends solely upon concentration, and is independent of rotor speed, but for which the rate of attainment of equilibrium is slow compared to the duration of a high speed ultracentrifuge run; (2) a change in shape of the individual DNA molecules caused by the high centrifugal field; (3) a phase transition which occurs in the presence of a high centrifugal field.

Each of the above three possibilities will now be considered. We may eliminate hypothesis one since the weight average sedimentation coefficient measured in the plateau region for such a system is independent of rotor speed (Schumaker, 1955). Our data shows this is not the case. For example, in run E-28-35, where the plateau region is briefly maintained, the weight average sedimentation coefficient measured initially is 76.6. This is much larger than the values of 34.1 and 33.0 found in the corresponding runs made at 12,410 and 7717 rpm (E-28-12, E-28-7).

The second possibility, that a change in the shape of the individual DNA molecules is responsible for the sudden loss of material, is not consistent with the observation that a constant amount of DNA remains behind and sediments normally at a given rotor speed. Since this amount is a variable fraction of the original DNA it is unlikely that there are two distinct classes of molecules present: those which change shape in the centrifugal field, and those which maintain their shape.

The third possibility seems to offer the best explanation for our results. Indeed, the occurrence of a phase transition is indicated by the observation that at a given rotor speed a constant concentration of DNA remains behind in the dispersed phase independent of the initial DNA concentration. Such a phase transition could be caused by (a) attractive forces linking the individual molecules into a framework, (b) repulsive (electrical) forces mutually orienting the individual molecules and possibly causing changes in configuration, and (c) steric interactions.

The forces which would be involved in (a) and (b) must be weak, for the effect vanishes at low concentrations. Therefore, they would most likely involve hydrogen bonds, electrostatic interactions, or polyvalent cation chelates. We have attempted to minimize such interactions with high salt, formaldehyde, and citrate. The results tabulated in Table I indicate that these reagents caused small or negligible effects, and therefore it seems unlikely that such linkages are involved in a phase transition. We are left then with the hypothesis that the sudden loss of material at high speeds is due to a phase change caused by steric interactions among the molecules.

In an attempt to portray this phase change, we have plotted the results from our experimental runs in Figure 7. The axis of ordinates in this figure is the reciprocal of the concentration, the axis of abscissas is the square of the rotor speed. The initial concentration of DNA at the beginning of the run is indicated in this figure by the closed circles; the final concentration which remains after the bulk of the aggregated material has been removed from solution is indicated by the open circles. From inspection it may be seen that none of the open circles lies in the lower right-hand half of the diagram. We have therefore drawn a line separating Figure 7 into two regions. The upper portion represents concentrations sufficiently low that all the DNA remains in solution. The lower half represents concentrations which are sufficiently high that the DNA separates into two phases. The aggregated phase is then swept from solution by the centrifugal field leaving behind a soluble phase whose concentration is represented by the corresponding open circle. From Figure 7 it can be seen that the concentration above which two phases separate is a function of the rotor speed.

We suggest the following mechanism to explain the observed dependence of the phase transition upon the rotor speed. The formation of small aggregates, dimers, trimers, etc. is due to an equilibrium reaction involving random collisions and molecular entanglement. These small aggregates sediment appreciably faster than the monomers from which they are derived; as they move through the solution they will collide with other molecules, some of which will stick to the surface of the aggregates and cause them to grow in size. This in turn will increase the sedimentation velocity. As the aggregates grow in size the concentration of monomer units falls, reducing the probability of collision. These processes continue until there is equilibration of mass between the monomeric DNA molecules and the aggregates. In this formula-

tion the collision rate of the aggregates with the monomers will be increased as the centrifugal field is increased. The equilibrium position is thus a function of rotor speed.

We may express the foregoing in mathematical terms for the equilibrium reaction:

$$A(M) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A$$

where (M) represents the concentration of monomer units in solution and A represents the surface area of the aggregated phase. We assume the rate of formation of monomers to be proportional to the surface area of the aggregated phase and thus write:

$$\text{rate of formation} = k_2 A$$

We assume the rate of disappearance of monomer to be proportional to the concentration of monomer and the surface area of the aggregated phase and write:

$$\text{rate of disappearance} = k_1(M)A$$

At equilibrium these are equal and

$$(M)k_1A = k_2A$$
$$(M) = k_2/k_1 \qquad (1)$$

The concentration of monomer is therefore independent of the quantity of aggregate present. The right-hand side of equation (1) is the solubility coefficient of the monomer. If (M) is less than k_2/k_1 an aggregated phase will not be formed. If (M) is greater than k_2/k_1 the excess monomer will aggregate leaving a concentration of soluble monomer equal to k_2/k_1. It is now necessary to include in the expression for the solubility coefficient a term which depends upon the rotor speed. As discussed above, the rate of collision between the aggregate and monomer increases with the sedimentation velocity of the aggregate. Therefore we may write:

$$k_1 = k_1^0 + s_A\omega^2 rb \qquad (2)$$

where s_A is the sedimentation coefficient of the large aggregates, ω^2 is the square of the rotor speed, r is the distance from the axis of rotation, and b is a measure of the "sticking" of the monomer to the surface of the aggregate once collision has taken place. Substituting equation (2) into (1) and taking the reciprocal we get:

$$\frac{1}{(M)} = \frac{k_1^0}{k_2} + \frac{s_A\omega^2 rb}{k_2} \qquad (3)$$

This equation indicates that the reciprocal of the solubility coefficient is directly proportional to ω^2.

In Figure 7, there are not sufficient experimental points to define with precision the phase transition line, nor can we say with any assurance that the line is straight. Indeed, the sedimentation coefficient of the aggregate, s_A, will increase slowly with aggregate size, and larger aggregates will probably be produced at higher rotor speeds. Therefore it would be expected that the line would bend upward at the higher rotor speeds. The line drawn in Figure 7 was deliberately placed below all the open circles in order to dramatize the phase transition.

If the hypothesis which we have advanced is correct, then there will be some concentration at which the DNA will separate into an aggregated phase and a soluble phase in the absence of a superimposed centrifugal field. From a careful inspection of our data we suspect that this phase transition will occur between the limits of 50 and 100 μg/ml for T4r DNA. This phenomenon would complicate the isolation and purification of extremely high molecular weight DNA, which may exist in bacteria and cell nuclei of higher organisms.

Measurement of Sedimentation Coefficients of High Molecular Weight DNA.—Since the observed rapid loss of material increases with increasing rotor speed and DNA concentration, we recommend that low rotor speeds and low DNA concentrations be used. Since there is also a marked dependence of sedimentation upon concentration, we recommend that extrapolation be made to infinite dilution.

Acknowledgment

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