ZONE CENTRIFUGATION IN A CESIUM CHLORIDE DENSITY GRADIENT CAUSED BY TEMPERATURE CHANGE

VERNE N. SCHUMAKER *and* JUDITH WAGNILD

From the Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia. Dr. Schumaker's present address is, the Department of Chemistry, Division of Biochemistry, University of California at Los Angeles.

ABSTRACT In this communication is described a new technique for the determination of sedimentation coefficients of macromolecules banded in equilibrium density gradients. Initially, the macromolecules are banded in the analytical ultracentrifuge at a low temperature of about 5°C. After equilibrium has been obtained, the temperature is increased to 25°C. The equilibrium band will now sediment to a new equilibrium position in the ultracentrifuge cell: (*a*) By following the position of the migrating band as a function of time, sedimentation coefficients may be determined. (*b*) If several species having different sedimentation coefficients are present in the original band, then during the course of the migration the band may split into several new bands which eventually reunite at the final equilibrium position. (*c*) If different chemical species of macromolecules such as nucleic acids and carbohydrates are present, in general they will exhibit different temperature density relationships, and can move different distances and directions in response to temperature change.

INTRODUCTION

During the past decade, the technique of zone sedimentation velocity has been widely used for the separation and characterization of macromolecules of biological interest. The preparatory centrifuge and swinging bucket rotor have been employed in most of these studies. The analytical ultracentrifuge, on the other hand, has not been widely used in zone sedimentation velocity experiments in spite of the great advantages afforded by the optical system. In part, this delay has been caused by the difficulty of establishing a preformed density gradient in the analytical cell. Recently, however, several different methods of establishing density gradients have been developed [Vinograd, Bruner, Kent, and Weigle (1963), Rosenbloom and Schumaker (1963), Baldwin and Shooter (1963), Meselson and Nazarian (1963)].

In this communication we wish to describe another method of performing zone

centrifugation in analytical cells. Macromolecules are banded in the usual manner in concentrated CsCl or other salts but at a low temperature, usually 5°C. At equilibrium, a well characterized density gradient is present throughout the cell. If the molecular weight is very great, the band of macromolecules will be narrow and the position of its center can be located with great precision. The temperature is now raised from 5° to 25°C. If the temperature coefficients of expansion for the solution and for the macromolecules are not identical, then at the higher temperature the band will no longer be in the equilibrium position. Therefore, it must sediment or float to a new equilibrium position. Observation of the movement of of the peak yields the following data: (*a*) Sedimentation coefficients may be determined by measuring the velocity of the peak during the migration. Using this technique, we have obtained a value of $S^\circ_{20,w}$ for DNA isolated from T_4 bacteriophage of 60.5S. (*b*) If two macromolecular components having appreciably different sedimentation coefficients are present in the original band, there will occur a transient separation of the original band into two bands during the course of the migration to the new equilibrium position. In this manner, we have been able to resolve a prepared mixture of "whole" and "blended" molecules of DNA isolated from bacteriophage T_4. (*c*) In experiments in which a concentrated CsCl solution was added directly to a lysate of *Escherichia coli,* a second, hypersharp band appeared which did not absorb appreciably in the ultraviolet region, but which was well resolved with the schlieren optical system. We suspect that this material is a bacterial carbohydrate. This second band moves toward the meniscus when the temperature is raised, while the DNA band moves in the opposite direction toward the cell bottom. It is possible that this technique will be of use in the identification of different chemical species of high molecular weight by observation of the differences in their coefficients of expansion in response to temperature change as reflected by the direction and magnitude of the shifts in equilibrium position.

THEORY

Sedimentation Velocity in Concentrated Salt Solutions. In this communication, the symbols employed are identical with those of Hearst and Vinograd (1961 *a* and *b*), and Hearst, Ifft, and Vinograd (1961) in their elegant studies on the effects of hydration and pressure on macromolecules banded in a density gradient. The sedimentation coefficient of the macromolecules, *s,* may be written as:

$$s = \frac{V}{\omega^2 r} = \frac{M_s(1 - \bar{v}_s\rho)}{Nf} = \frac{M_s\bar{v}_s(\rho_s - \rho)}{Nf'\eta} \tag{1}$$

where V is the velocity of the macromolecules; M_s, $\bar{v}_s$, and ρ_s are the molecular weight, partial specific volume, and density of the solvated macromolecules, respectively, and f' is the frictional coefficient of the macromolecules divided by the solution

viscosity, so the $f'\eta = f$. The term $(\rho_s - \rho)$, which appears on the right-hand side of equation (1), represents the difference between the solvated density of the macromolecules and the solution. This term is a function of pressure, water activity, the compositional density of the solution, and the density of the macromolecules. Under standard conditions, the sedimentation coefficient of the macromolecules may be written:

$$s_{20,w} = \frac{M(1 - \bar{v}\rho_w)}{Nf''\eta_w} \tag{2}$$

where M and $\bar{v}$ are the molecular weight and partial specific volume of the uncharged, anhydrous macromolecule and the subscript, w, refers to water. The term, f'', is the frictional coefficient of the macromolecule in a dilute salt solution. Dividing equation (1) by equation (2) gives:

$$\frac{V}{K} = \frac{f''}{f'} s_{20,w}(\rho_s - \rho) \tag{3a}$$

where

$$K = \left\{\frac{M_s}{M} \frac{\bar{v}_s}{(1 - \bar{v}\rho_w)} \frac{\eta_w}{\eta} \omega^2 r\right\} \tag{3b}$$

Equation (3*a*) is now in a form suitable for the reduction of the experimental data. The velocity of the migrating band can be measured from consecutive photographs. If the various terms of which K is composed are known, then a plot of V/K as a function of $\rho_s - \rho$ may be constructed. The slope of this plot at any point will be equal to $(f''/f')s_{20,w}$. Should the shape factor of the macromolecules remain unchanged in going from dilute to concentrated salt solutions, then the sedimentation coefficient, $s_{20,w}$, can be determined.

Determination of the Bouyant Density of DNA as a Function of Temperature. At equilibrium, the density at a distance, r, from the center of rotation was determined from the following equation:

$$\rho_T = \bar{\rho}_T + \frac{\omega^2}{2\beta_T}(r^2 - \bar{r}^2) \tag{4}$$

where ρ_T is the density of the solution at the temperature, T, at the geometrical center of the ultracentrifuge cell. It has been shown that the solution at the geometrical center of the cell has a density almost identical with the density of the solution originally placed in the centrifuge (Ifft, Voet, and Vinograd, 1961). The variation with temperature of the mean density, $\bar{\rho}_T$, was determined by interpolation from the tabulated values in the international critical tables. The value of β_{298K} was obtained from the data of Ifft, Voet, and Vinograd, (1961), for a CsCl solution with a density of 1.70 at 25°. To calculate β_T at other temperatures, equation (4) may be differentiated to give the compositional density gradient:

$$(\partial\rho/\partial r)_{\text{comp.}} = \frac{\omega^2 r}{\beta_T} \tag{5}$$

From thermodynamic considerations, the compositional density gradient may also be written for a uni-univalent electrolyte:

$$(\partial\rho/\partial r)_{\text{comp.}} = (\partial\rho/\partial c)_{T,P}\left(\frac{dc}{dr}\right) = (\partial\rho/\partial c)_{T,P}\frac{M(1 - \bar{v}\rho)\omega^2 r}{2RT\left(1 + \frac{d\ln\gamma}{d\ln c}\right)_{T,P}} \tag{6}$$

where M, $\bar{v}$, and c now refer to the electrolyte (CsCl).

The compositional density gradient may be eliminated between equations (5) and (6), and the resulting expression solved for β_T. Dividing this equation by the corresponding equation for β_{298K} and ignoring the temperature dependence of the term $d\ln\gamma/d\ln c$, yields:

$$\beta_T = \beta_{298}\frac{T(1 - \bar{v}\rho)_{298}(\partial\rho/\partial c)_{298}}{298(1 - \bar{v}\rho)_T(\partial\rho/\partial c)_T} \tag{7}$$

Values of β_T calculated according to equation (7) are given in the second column of Table I.

TABLE I

THE VALUES OF β AS A FUNCTION OF TEMPERATURE FOR A CsCl Solution of Density of 1.70.

Temperature	Values of $\beta \times 10^{-9}$	
	By equation (7)	Observed equation (8)
degrees		
0	1.061	—
9	1.110	1.100
19	1.161	1.155
29	1.210	1.218
39	1.264	1.279

There is a second independent method for the determination of β_T. Values of β_T may be calculated directly from the schlieren patterns. Dividing equation (5) by the corresponding equation for β_{298K} yields:

$$\frac{\omega^2}{2\beta_T} = \frac{\omega^2}{2\beta_{298}}\frac{(\Delta\rho/\Delta c)_T(\Delta c/\Delta n)_T(dn/dr)_T}{(\Delta\rho/\Delta c)_{298}(\Delta c/\Delta n)_{298}(dn/dr)_{298}}\left(\frac{\bar{r}_{298}}{\bar{r}_T}\right) \tag{8}$$

where the $d\rho/dr$ have been expanded to give $(\Delta\rho/\Delta c)(\Delta c/\Delta n)(dn/dr)$. The dn/dr were calculated by the following procedure. The heights of the schlieren baselines were measured at the geometrical centers of the cell in the CsCl runs; from these heights were subtracted the heights of the schlieren baselines measured at calculated positions of equal pressure at equivalent temperatures in a water run. The $(\Delta c/\Delta n)$ were calculated from measurements made at the various temperatures using an Abbé refractometer upon 53.35 and 58.52 weight per cent CsCl solutions. We found a small drop of 2 per cent, barely significant at the level of accuracy of our refrac-

tometer, in the refractive index increment in going from 10° to 40°C. The values of $(\Delta\rho/\Delta c)$ were calculated from the density concentration data of the international critical tables using the 55 and 60 weight per cent solutions. The pressure dependence of the latter two terms remains uncorrected; the measurements were made at 1 atm. Only the ratio of such terms appears in equation (8); therefore, the effect of measuring at 1 atm. rather than inside the cell should be negligible. In Table I the values of β_T obtained experimentally using equation (8) are given in the third column. These experimentally determined values of β_T were used to calculate the compositional density of the solution at the location of the DNA band at the different temperatures as shown in Fig. 1.

A pressure density gradient is superimposed upon the compositional density gradient, and, as is pointed out by Hearst, Ifft, and Vinograd, it is sufficiently accurate to add a pressure correction to the compositional density gradient. This pressure correction is appreciable since the DNA peak moves as much as 3 mm in the cell in the experiments reported in Fig. 1. The following expression was used to evaluate

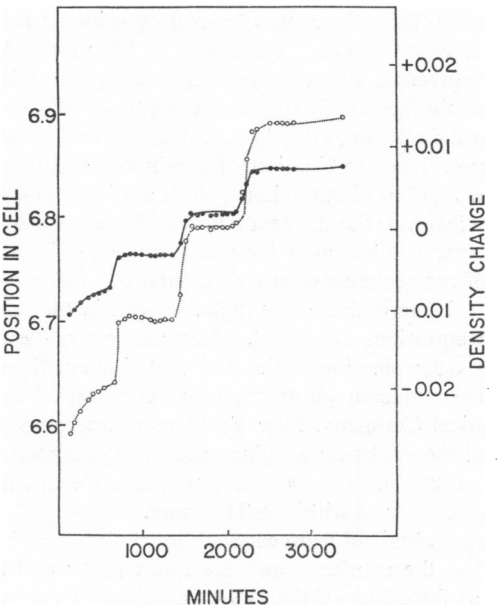

FIGURE 1 In this figure are plotted as a function of time the positions within the ultracentrifuge cell (○) and the changes in the density (●) of DNA isolated from *E. coli* 15T- and banded in a CsCl solution having an initial density of 1.7056, by centrifuging at 44,770 RPM. The temperatures recorded were 0 to 360 minutes, 9°C; 360 to 616 minutes, a drift from 9 to 11°C; 616 to 720 minutes, 9 to 19°C; 720 to 1376 minutes, 19°C; 1376 to 1504 minutes, 19 to 29°C; 1504 to 2056 minutes, 29°C; 2056 to 2288 minutes, 39°C, and the temperature readings remained at 39°C until the conclusion of the experiment. The densities were calculated as described in the text and are reported above as the changes in density from the value found at 25°C.

the density of the hydrated macromolecules $\rho_{T,s}$ at a temperature, T, corrected to the pressure at the geometrical center of the cell:

$$\rho_{T,s} = \bar{\rho}_T + (1/\beta_T + (\kappa - \kappa_s)\rho_{298^\circ,s}^2)\frac{\omega^2(r^2 - \bar{r}^2)}{2} \quad (9)$$

For T_4 DNA, Hearst, Ifft, and Vinograd (1961), find the difference in the compressibilities of the solution and the DNA, $(\kappa - \kappa_s)$, to be 17.7 10^{-6} atm^{-1}.

MATERIALS AND METHODS

Determination of Sedimentation Coefficients. A Spinco-Beckman model E analytical ultracentrifuge equipped with both ultraviolet and schlieren optical systems was used in these studies. A 12 mm "regular" cell with a 4° single sector filled Epon centerpiece was fitted with sapphire windows to minimize distortion. To avoid shear degradation of the bacteriophage DNA, the ultracentrifuge cells were filled in the following manner; after tightening the cell, the "B ring" was unscrewed and the upper window holder and window were removed. The DNA solution was then poured into the cell with the aid of a small stirring rod. The window and holder and B ring were replaced and the cell assembly tightened. In a typical experiment, the DNA band was formed by spinning a 0.1 to 1 μg/ml solution of DNA in 56.45 wt per cent CsCl for 24 hours at 44,770 RPM and at 5° in the analytical ultracentrifuge. The temperature was then increased from 5° to 25°C by turning off the refrigeration, turning on the heater, (a standard component of the rotor indicator control system supplied by Spinco-Beckman, Palo Alto, California) and removing the vacuum pump and diffusion pump fuses. The combination of the heater and the air friction caused the temperature to increase 20°C in about 1 hour. Both schlieren and ultraviolet photographs were taken at 16 minute intervals for the next 8 hours. A final photograph was taken 8 hours later. Only those photographs taken after the temperature had increased to 25°C were used in the determinations of the sedimentation coefficients. For the very high molecular weight DNA isolated from $T_{4,+}$ bacteriophage, the diphasic peak in the schlieren pattern is visible even at the lowest concentrations employed. Measurements made on the schlieren photographs were used in the determination of the sedimentation coefficients for the whole molecules of $T_{4,+}$ DNA. The schlieren photographs were measured on a Bausch and Lomb (Bausch and Lomb Optical Company, New York) two dimensional microcomparator; the ultraviolet absorption photographs on a Spinco analytrol equipped with the microanalyzer attachment. Microcomparator measurements are reproducible to within $\pm$0.010 mm in the cell and analytrol measurements to within $\pm$0.015 mm.

The following data were obtained from each schlieren photograph: the radial coordinates of the left reference edge, the meniscus, and the DNA peak; and the vertical coordinates of the schlieren image at the center of the DNA peak, and at the geometrical center of the cell, and the vertical coordinate of the schlieren image in the center of the reference edge. From the heights of the schlieren baselines at the peak positions were subtracted the heights of the schlieren baselines at calculated positions of equal pressure in a water run. The resulting height, Z_t, is proportional to the compositional density gradient. The definitions, derivations, and a discussion of the compositional and the effective density gradients is given by Hearst and Vinograd (1961), and Hearst, Ifft, and Vinograd (1961). The equations have been derived by these authors assuming that equilibrium has been obtained. From the experiments reported in this communication, the CsCl gradient is not at equilibrium but slowly changes toward the final equilibrium distribution while the DNA peak migrates

down the cell. Careful inspection of the schlieren photographs indicates that to an excellent degree of approximation the compositional density gradient varies linearly with distance over the short distances between the peak position, r_p, and the position of the geometrical center of the cell, $\bar{r}$, during the interval prior to establishment of equilibrium:

$$(d\rho/dr)_r = (d\rho/dr)_{\bar{r}} + \left\{(d\rho/dr)_{r_p} - (d\rho/dr)_{\bar{r}}\right\} \frac{r - \bar{r}}{r_r - \bar{r}} \tag{10a}$$

or

$$Z_r = Z_{\bar{r}} + (Z_{r_p} - Z_{\bar{r}}) \frac{r - \bar{r}}{r_p - \bar{r}} \tag{10b}$$

The density of the solution at the peak position, r_p, can be written as the integral:

$$\rho = \bar{\rho} + \int_{\bar{r}}^{r_p} (d\rho/dr)_r \, dr \tag{11a}$$

Upon substitution of equation (10*b*) into equation (11*a*) and integrating:

$$\rho = \bar{\rho} + \frac{k}{2} (r_p - \bar{r})(Z_{\bar{r},t} + Z_{r_p,t}) \tag{11b}$$

where $\bar{\rho}$ is the compositional density at the geometrical center of the cell. This value is assumed to be a constant after the temperature has been increased to 25°C, and equal to the density of the solution initially placed into the cell. Therefore, the density-distance plot is assumed to pivot around the geometrical center of the cell. We have tested this assumption for our temperature change studies by point-by-point integration of the mass throughout the cell, and have found it to be correct with experimental error. $Z_{\bar{r},t}$ is the value of Z at the geometrical center of the cell at time, t. $Z_{r_p,t}$ is the value of Z measured at the peak position, r_p, at time, t. The k is the constant of proportionality which converts refractive index units to density gradient units in the equation $d\rho/dr = kZ$. This constant may be evaluated numerically from the data taken from the last photograph:

$$k = \frac{w^2 \bar{r}}{\beta Z_{\bar{r},\infty}},$$

where $Z_{\bar{r},\infty}$ is the value of Z at the geometrical center of the cell in the last photograph taken at least 18 hours after the conclusion of the temperature transition when the equilibrium distribution of CsCl has been effectively obtained. Equation (11*b*) now may be used to determine the compositional density of the solution, ρ, at the position of the migrating peak at any time, t.

As a first approximation to the value for the density of the solvated macromolecules, ρ_s, it is convenient to use $\rho_{s,\infty}$, which is a value equal to the density of the solution at the final position of the peak many hours after the conclusion of the migration when the entire system is again at equilibrium. Then the term $(\rho_{s,\infty} - \rho)$ could be used in equation (3*a*) to determine the sedimentation coefficient of the macromolecules. However, for DNA in aqueous CsCl, the use of $\rho_{s,\infty}$ instead of ρ_s gives approximate values for $(\rho_s - \rho)$ which are too large by about 20 per cent; changes in water activity and in pressure which occur while the migrating macromolecules move through the solution reduce the effective buoyant density of the DNA. [The variation in water activity causes a decrease in the solvation of the DNA; this is reflected as a 24 per cent decrease in the term $(\rho_{s,\infty} - \rho)$. Due to differences in compressibility between the solvated macromolecules and the surrounding solution, variation in pressure causes an increase in the term $(\rho_{s,\infty} - \rho)$ of about 5 per cent.]

To make the water activity correction, it is necessary to multiply the term $(\rho_{s,\infty} - \rho)$ by the term $(1 - \alpha)$. The symbol α is defined by Hearst and Vinograd (1961*b*) as $(\partial\rho_{s,\infty}/\partial a)_p(\partial a/\partial\rho)$, where a is the activity of water in the CsCl solution. To make the pressure correction, it is necessary to add a term which to a good degree of approximation may be written $(\kappa - \kappa_s)\rho^2_{s,\infty}\omega^2(r^2_{p,\infty} - r^2_{p,t})/2$. The symbol $\kappa - \kappa_s$ represents the difference between the compressibilities of the solution and the solvated macromolecules. If the symbol ψ is now defined as $\psi \equiv (\kappa - \kappa_s)/(1 - \alpha)$, it is possible to write an equation for $(\rho_s - \rho)$ which takes into account both hydration and pressure terms:

$$(\rho_s - \rho) = \left[(\rho_{s,\infty} - \rho) + \frac{\psi\rho_{s,\infty}{}^2\omega^2(r_{p,\infty}{}^2 - r_{p,t}{}^2)}{2}\right](1 - \alpha) \qquad (13)$$

The values of ψ and α for DNA isolated from T_4 bacteriophage have been evaluated by Hearst, Ifft, and Vinograd, (1961): $\psi = 23.0\ 10^{-13}$ (dynes/cm^2)$^{-1}$, and $(1 - \alpha) = 0.76$. For the determination of sedimentation coefficients reported in this communication, the values of $\rho_s - \rho$ has been computed by equation (13). The value of $\rho_{s,\infty}$ has been taken as the value of the compositional density at the final equilibrium position of the macromolecules obtained approximately 18 hours after the conclusion of the migration. Values of ρ used in the right-hand side of equation (13) have been computed by the use of equation (11*b*).

Determination of sedimentation coefficients by use of equation (3*a*) requires evaluation of K, given by equation (3*b*). Values used for the various terms used are: $(1 - \bar{v}\rho_w) = 0.451$; $\bar{v}_s = 0.5889$. The relative viscosities of CsCl solutions were measured at $\rho = 1.64$, $\rho = 1.70$, and $\rho = 1.74$. The three values obtained lie upon a smooth curve the equation of which is $\eta\text{CsCl}/\eta_{w,T} = 1.64396 - 1.4433\rho + (2/3)\rho^2$. The temperature correction for the viscosity of water must also be applied so that the viscosity term in equation (3*b*) becomes: $(\eta/\eta_w) = (\eta\text{CsCl}/\eta_{w,T})(\eta_{w,T}/\eta_{w,20°})$, where the relative viscosity is assumed to be independent of the temperature. The ratio of the molecular weights of the cesium nucleate to the sodium nucleate is 1.307. This ratio must be multiplied by a solvation term $(1 + \Gamma')$, where $\Gamma' = 0.28$ *gm water/gm DNA*, (Hearst and Vinograd, 1961), to obtain $M_s/M = 1.673$.

Preparation of an E. Coli Lysate. *E. coli* $T^-A^-U^-$ were grown in 20 ml of a minimal salts medium supplemented with 1 mg/ml glucose, 2 gm/ml thymine, 20 gm/ml arginine, and 10 gm/ml uracil to $2 \times 10^{+8}$ bacteria/ml (turbidity = 60 per cent). The cells were chilled, centrifuged, and washed twice with 0.1M Tris, pH8, and once with 17 per cent sucrose. The cells were centrifuged and the pellet resuspended with the aid of a vortex mixer in 0.08 ml of 17 per cent sucrose. Then 0.1 ml of a 2 gm/ml lysozyme solution were added, and 2 minutes later 0.1 ml of a 20 mg/ml solution of versene were added. The mixture was incubated for 5 minutes at 37°C. Then, 0.025 ml of a 5 per cent sodium dodecyl sulfate solution in 45 per cent ethanol were added and the incubation continued 12 minutes. The lysate contains DNA and a presumed polysaccharide at 25 to 50 times the concentration suitable for the ultracentrifuge run and must be diluted accordingly.

Preparation of $T_{4r}+$ DNA. The $T_{4r}+$ bacteriophage were grown and harvested as described by Cohen and Arbogast (1950) and the DNA isolated by the method of Mandell and Hershey (1960) using distilled phenol and peroxide-free ether.

RESULTS

Density-Temperature Relation for DNA. In Fig. 1 are shown the results of raising the temperature of the solution after obtaining equilibrium in a DNA-CsCl density gradient experiment. As the temperature is raised, the DNA band sinks deeper into the solution. A combination of effects determines the direction and

magnitude of the movement of the banded macromolecules. For example, as the temperature is raised from 9° to 29°C: (*a*) the solution expands, and the density at the geometrical center of the cell, ρ_T, drops from 1.7174 to 1.7026. This density change is in the right direction to cause the DNA band to move toward the bottom of the cell. (*b*) The compositional density gradient becomes a little less steep at the higher temperature; 0.134 to 0.124 (gm/cm^3)/cm. This change in density gradient may tend to cause the band to move either up or down the cell, or not at all, depending upon the location of the band. Since the compositional density plot "pivots" about the density at the geometrical center of the cell, a reduction in the density gradient would cause a band initially located above the center to move toward the meniscus, and a band initially located below the center to move toward the cell bottom. (*c*) For DNA in aqueous CsCl, the pressure correction term always adds to the compositional density gradient; the increase in the density gradient is about +5 per cent. (We have assumed that this pressure correction term is temperature independent.) Therefore, a band moving deeper into the solution will move only 95 per cent as far as it would be calculated to move in the absence of the pressure gradient.

All of these factors are taken into account in equation (9). The filled circles in Fig. 1 represent a plot of the change in this hydrated density of the DNA macromolecules, $\rho_{T,S}$, as a function of temperature. Since solution expansion, change in compositional density gradient, and pressure have been corrected for in calculating $\rho_{T,S}$, this plot should represent the change in the density of the hydrodynamic unit as a function of temperature. It is interesting to note that the hydrodynamic unit becomes more dense as the temperature is raised.

Direction of Migration of Different Macromolecular Components. Lysozyme-treated *E. coli* are lysed with sodium dodecyl sulfate. The resulting lysate, which also contains the products of the lysozyme digestion, is diluted and then mixed with a concentrated CsCl solution to give a final density of 1.70. When studied in the analytical ultracentrifuge, two bands of macromolecules appear (Fig. 2). The more dense of the two bands strongly absorbs ultraviolet light, and represents the DNA component. As usual, during the temperature transition the DNA component moves down the cell toward the cell bottom. The second band has a hydrated density of 1.667. We shall call this second band "X" component. The material in the second band absorbs ultraviolet light weakly or not at all. From a comparison of the sizes of the schlieren diphasic peaks for the DNA component and for the "X" component, it may be estimated that there is roughly 5 times as much material in the "X" band as in the DNA peak. The photodensitometer tracing of the ultraviolet absorption pattern indicates that the DNA band absorbs over 10 times as much ultraviolet radiation as does the "X" band. Moreover, some of the apparent light absorption by the "X" component will be due to a scattering of light by the macromolecules. Therefore, the specific extinction coefficient of "X" component is at least 50 times less than it is for nucleic acid.

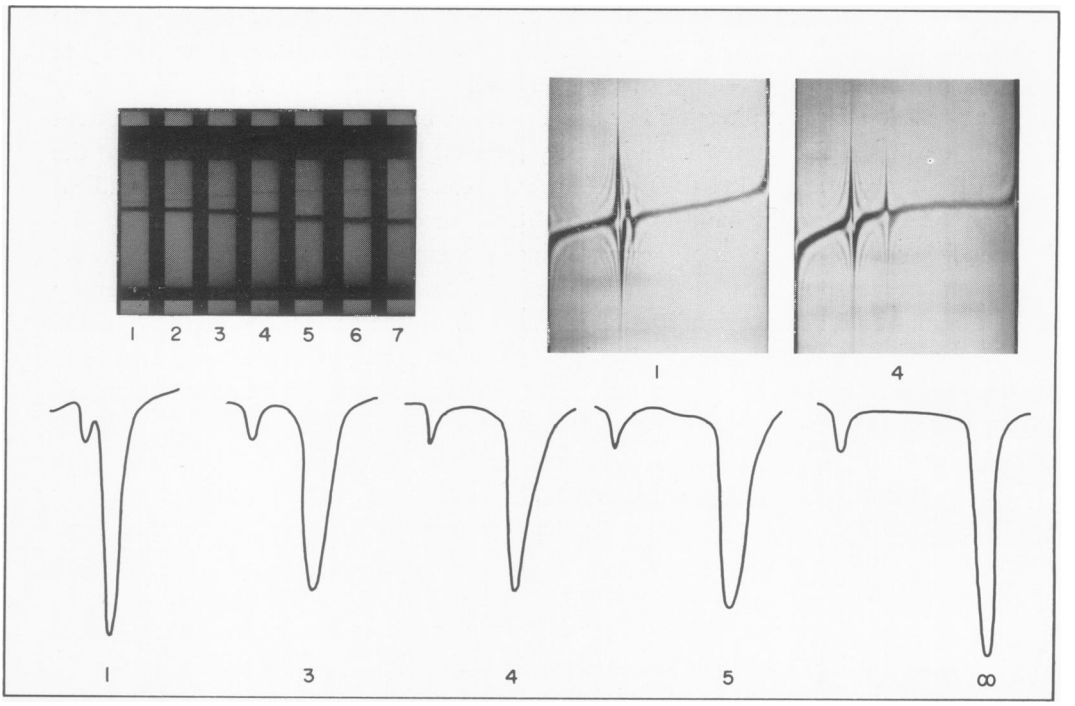

FIGURE 2 Two bands appear in a CsCl density gradient run of lysozyme-treated *E. coli* lysed with sodium dodecyl sulfate. Consecutively numbered photographs were taken at 64 minute intervals. Ultraviolet absorption and schlieren photographs show normal behavior of the DNA component. The second component appears to be roughly five times as concentrated as the DNA (estimated from the schlieren pattern) and only weakly UV absorbing. It also shows a positive temperature coefficient of expansion. For the ultraviolet absorption photograph, the cell bottom is toward the bottom of the page, for the schlieren photograph, it is to the right. See text for interpretation.

Because the "X" component has a low specific extinction coefficient, it cannot represent RNA or DNA. The high density of "X" component is evidence that it represents neither a pure lipid nor a pure protein. Bacterial carbohydrates, on the other hand, do have densities in this range, and do not absorb appreciably in the ultraviolet. Therefore, we believe this material represents a bacterial carbohydrate.

Upon changing the temperature from 5° to 25°C, the "X" component moves nearer the meniscus. This is opposite to the direction moved by the DNA component. The temperature coefficient of expansion for the hydrated "X" component is positive in sign, while that for hydrated DNA is negative. The sedimentation coefficient which may be calculated from the velocity of transition for "X" component is very high; we estimate it to be over 200 S. Either the material is of very high molecular weight or else it represents an aggregated product of smaller particles.

Concentration Dependence. The shapes of the migrating peaks are markedly altered at high concentration by the dependence of the sedimentation coefficient upon concentration. In Fig. 3 is shown a comparison of peak shapes at a fivefold difference in concentration. Peak shape is clearly changed in run 1957. At the edges of the migrating zone, the macromolecules move more rapidly because of their lower concentrations; therefore, the leading edge is pulled away from the body of

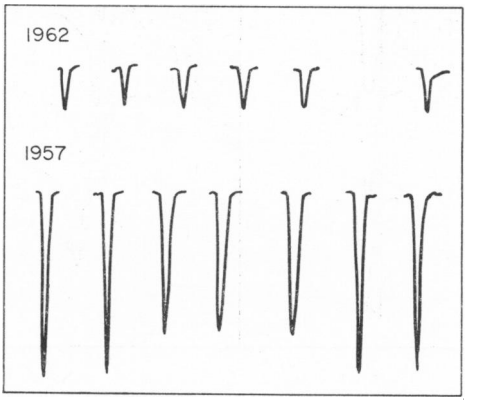

FIGURE 3 Peak shapes are compared in two temperature change experiments using T_4 ϕ DNA at starting concentrations of 1γ/ml (1957) and 0.2γ/ml (1962). The densitometer tracings are of photographs taken at 64 minute intervals, and run from left to right. The low concentration tracings show no marked change in shape; at 5 times that concentration the shapes are clearly changed and peak heights lowered during band migration. See text for explanation.

the peak while the trailing edge catches up with the peak. As a result, the rear of the peak rises abruptly, while the front is skewed in a forward direction. In addition, there appears to be a sharp diminution in peak height.

Multicomponent Systems. If two macromolecular components are present in the original band, and if these two components have much different sedimentation coefficients, then the original band will split into two bands during the period of migration to the new equilibrium position. In Fig. 4 are shown several examples of this effect; (4*a*) *E. coli* DNA isolated by the method of Marmur, (1961), was precipitated with streptomycin, resolubilized, and dialyzed. After this treatment, a second component is usually seen which moves much more rapidly than the main body of the DNA. (This second peak may represent an aggregate caused by interaction between residual streptomycin and the DNA.) (4*b*) Whole molecules isolated from bacteriophage T_{4r+} were mixed with an equal portion of bacteriophage DNA sonicated for 5 minutes at full power in a raytheon sonicator R-223. It is interesting to note that the sonicated fragments did not band precisely in the same place, but that the center of the band of sonicated DNA is shifted slightly toward higher densities.

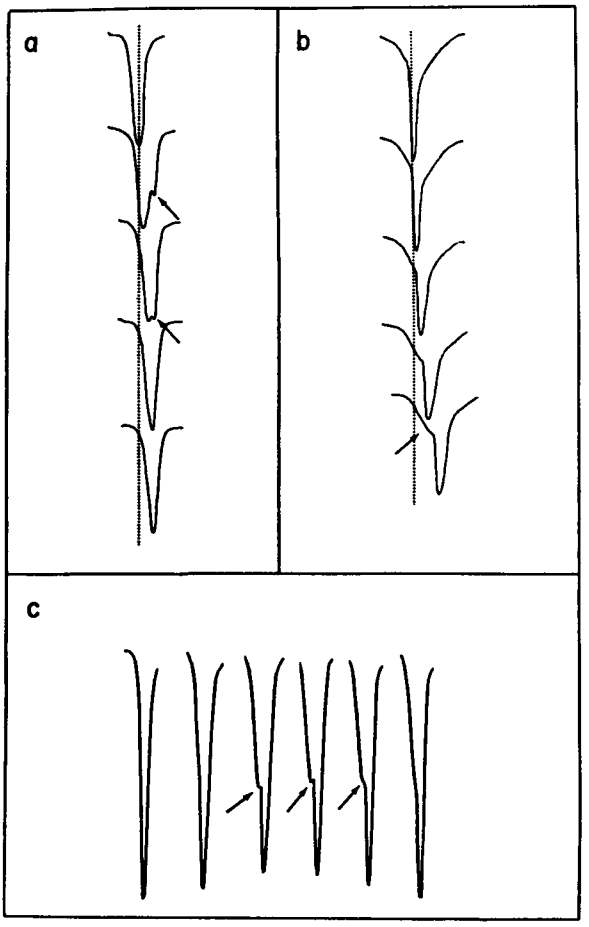

FIGURE 4 Analytrol tracings of consecutive ultraviolet photographs of the migrating DNA bands. (*4a*). DNA isolated from *E. coli* 15T^-. After equilibrium was reached at 19°C at 1:00 P.M., the rotor was heated at 29°C. The photodensitometer tracings shown above were made from ultraviolet photographs taken at (from top to bottom): 2:04 P.M., 23.1°C; 4:12 P.M., 29.0°C; 6:20 P.M., 29.0°C; 9:08 P.M., 29.0°C; and 12:20 A.M., 29.0°C. (*4b*). DNA isolated from bacteriophage T_4r+. Half of this DNA was sonicated and then mixed with an equal quantity of unsonicated DNA. Pictures were taken at (from top to bottom): 7:36 P.M., 9.2°C; 8:40 P.M., 16.6°C; 9:44 P.M., 22.3°C; 10:48 P.M., 28.3°C; and 12:24 A.M., 27.9°C. (*4c*). DNA isolated from bacteriophage T_{4r+}. Half of this DNA was blended at top speed in a virtis homogenizer, and then mixed with an equal quanity of unblended bacteriophage DNA. After obtaining equilibrium at 13°C at 12:07 P.M., the rotor was heated to 33°C. Pictures were taken at (from left to right): 1:11 P.M., 18.9°C; 3:19 P.M., 30.1°C; 5:27 P.M., 33.3°C; 6:13 P.M., 33.3°C; 7:35 P.M., 33.3°C; and 9:30 P.M., 31.8°C. Arrows are drawn to indicate the presence of the double peak formed from the bacterial DNA, the pronounced shoulder of sonicated DNA and the separate band of blended DNA; all of which appear during the migration caused by the temperature change.

This may be an indication that some denaturation has occurred. After banding at 9°C, the temperature was raised to 29°C, and the migration of the peak followed as a function of time. The whole molecules sediment more rapidly than the sonicated DNA, and a distinct shoulder is produced at the trailing edge of the whole molecule peak. (4*c*) Whole molecules of bacteriophage T_4 + DNA were mixed with an equal portion of bacteriophage DNA mixed at top speed in a vertis high speed blender. The sedimentation coefficient of the blended molecules is 18 S. During the temperature change, the original, single band temporarily splits, indicating the presence of at least two distinct macromolecular components.

Determination of Sedimentation Coefficients. As discussed above, sedimentation coefficients may be obtained from these temperature change experiments by measuring the velocity of migration of the peak as it moves to a new equilibrium position. For this purpose, it is convenient to plot the peak position, r_p, as a function of time. Such a plot is shown in Fig. 5 for a sample of shear degraded bacteriophage T_4 + DNA. A smooth curve may now be drawn through the points. Better values of r may now be determined from the curve at equal time intervals, Δt, and the average velocity at the center of each interval can be computed as $\Delta r/\Delta t = V$. The velocity, V, is now divided by K, where K is given by equation (3*b*). The bouyant density of the solvated macromolecules, $(\rho_s - \rho)$, is evaluated from equation (13). The values of V/K are now plotted as a function of $\rho_s - \rho$. In Fig. 6, such a plot is shown for the r *vs.* t data of Fig. 5. To demonstrate the maximum errors which might be obtained, the data of Fig. 5 were not smoothed; instead, "raw" values of $\Delta r/\Delta t$ were computed directly from the experimental points depicted in Fig. 5. The slope of the least squares line obtained from the V/K *vs.* $(\rho_s - \rho)$ plot will now give $(f''/f')s_{20,w}$. If the macromolecules do not change shape in the concentrated salt solution, $(f''/f') = 1$, then from the slope is obtained the sedimentation coefficient. The data plotted in Fig. 5 are taken from run No. 2033 in which the bacteriophage DNA sample had been previously blended for 1 minute at the lowest speed in the 5 ml glass microflask of a virtis homogenizer (The Virtis Company, Gardiner, New York). In a conventional boundary centrifuge run, a value of 38.8 S was obtained for this sample of DNA. In run 2039, a sample of the same DNA preparation was blended for 5 minutes at top speed in the homogenizer and a value of $s_{20,w}$ of 20.0 S was obtained by the temperature change method as compared with a value of 17.7 S by conventional boundary centrifugation. An estimation of the errors which are involved in computing $s_{20,w}$ from the experimental data is not readily made. On Fig. 6 are drawn lines between which the confidence limits on slope are ±90 per cent. When these lines are mapped on the r *vs.* t plot (Fig. 5) it becomes apparent that the data are much better than the impression given by the V/K *vs.* $(\rho_s - \rho)$ plot. We believe that this is due to the fact that when plotting consecutive values of Δr, each error appears twice, but in opposite directions. Taking the least squares line through the points nearly cancels these consecutive errors. In an attempt to estimate the accuracy and precision of this method of determining sedimentation coefficients, seven runs

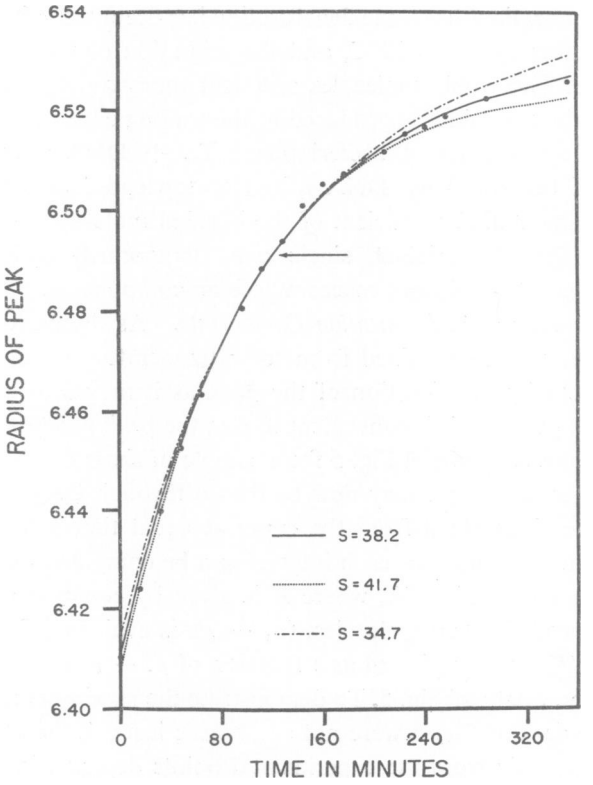

FIGURE 5 The distance of the peak from the center of rotation is plotted as a function of time, t_0, being the time at which 25°C was attained. Schlieren photographs were taken at 16 minute intervals thereafter and r, measured from these plates, is represented by the dots. In order to generate the theoretical curves it was necessary to place one point on the graph arbitrarily as a starting point. We chose the midpoint marked by an arrow. Each subsequent point was then determined from the equation $r_2 = r_1 \pm Vt$; ($r_3 = r_2 \pm Vt$ etc.) where V was calculated from $V/K = s\ (\rho_s - \rho)$ for $S = 38.2 \pm 3.5$.

were made with well characterized preparations of whole molecules of DNA. In a series of extensive studies made upon these preparations by conventional boundary ultracentrifugation the best value of $s^\circ_{20,w}$ was found to be 61.3 S. In Fig. 7 are shown the values of $1/s$ *vs.* C_{ave} obtained by the temperature change technique. The concentrations employed in Fig. 7 are values of the average concentration for the Gaussian band, and are computed from the maximum concentration at the band center divided by 1.414: $C_{ave} = C_{max}/\sqrt{2}$. The solid line drawn through the data of Fig. 7 extrapolates to a value of $s^\circ_{20,w} = 60.5$ S. The standard deviation of the individual values of $s_{20,w}$ from the best line is ± 3.7 per cent which is a reasonable value for the precision of the temperature change method.

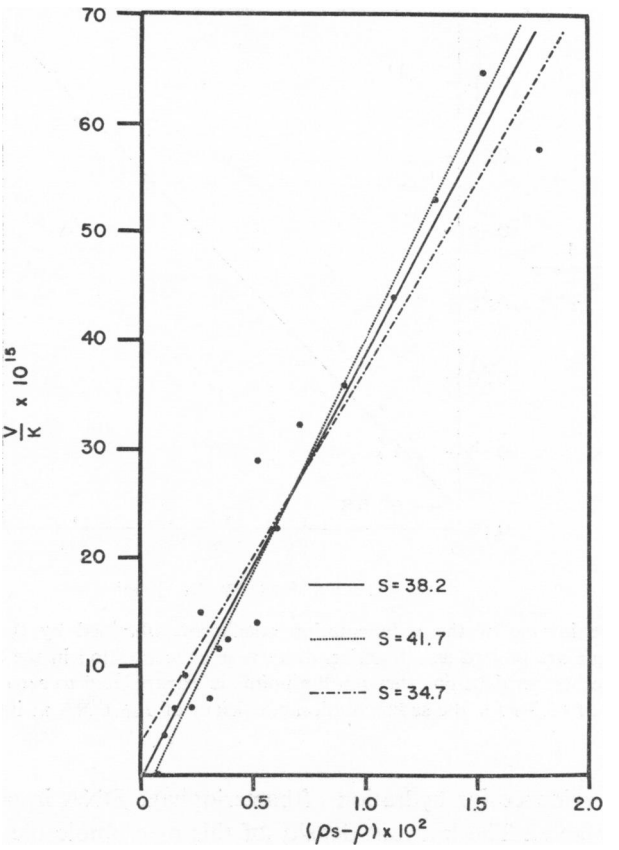

FIGURE 6 Experimentally determined points of V/K *versus* $(\rho_s - \rho)$ have been plotted and the best straight line through them determined by the least mean squares method. The lines representing the extremes of the 90 per cent confidence limits are also shown.

DISCUSSION

The Sedimentation Coefficient. In order to determine the sedimentation coefficient, a number of corrections and assumptions have been made which are not regularly applied to sedimentation data obtained in the conventional manner. A correction for pressure is made which reduces the apparent S by about 5 per cent; a correction for the hydration of the macromolecules, $M(1 + \Gamma')$, is made causing a reduction in the apparent S of 28 per cent; and a correction for the change in the hydration with water activity must be applied causing a $100/(1 - \alpha) = 32$ per cent increase in apparent sedimentation coefficient. If these corrections are all neglected, the apparent value of $s^0_{20,w}$ will be only about 2 per cent too high. Therefore, it is important either to make all these corrections or not to make any corrections—in either case, the final value is just about the same.

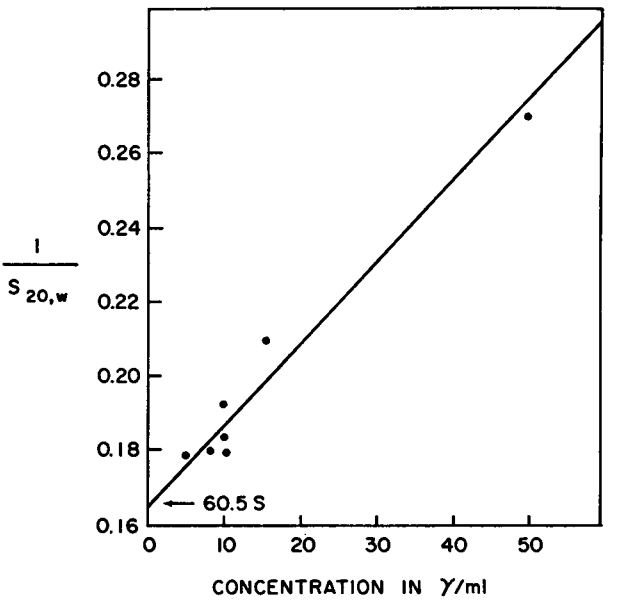

FIGURE 7 The inverse of the sedimentation coefficients obtained by the temperature change technique are plotted as a function of average concentration in the band ($C_{ave} = C_{max}/\sqrt{2}$). The best straight line through the points is extrapolated to zero concentration giving a value of 60.5 *s* for the sedimentation coefficient of $T_4\phi$ DNA at infinite dilution.

However, the evidence for hydration of bacteriophage DNA in concentrated salt solutions is very good. The bouyant density of this macromolecule in concentrated aqueous CsCl is about 1.70, whereas the reciprocal of the partial specific volume for Cs-DNA is 2.09 (Hearst, 1962). If in equation (1), the bouyant molecular weight, $M_s(1 - \bar{v}_s\rho)$, is calculated using the reciprocal of 1.70 for $\bar{v}_s$, then to be consistant, the hydration term $(1 + \Gamma')$ must be included in the expression: $M_s(1 - \bar{v}_s\rho) = M(1 + \Gamma')(1 - \rho/1.70)$.

The evidence in favor of making the pressure correction for the density of the solvated macromolecule is also quite good. Hearst, Ifft, and Vinograd have performed simple and elegant experiments showing that the pressure correction, while it is quite small, is readily and accurately determined. Justification for making the correction for the change in the water activity and hence the degree of hydration (*i.e.*, the α term) rests largely upon the work of Hearst and Vinograd, who have examined the bouyant density of DNA in a variety of cesium salts. These authors find that the bouyant density, and hence the degree of solvation, is a sensitive function of water activity. Recently this work has been confirmed by Hearst using an isopiestic technique (private communication). Therefore, the correction has been applied to our sedimentation data; it is large and effectively cancels the other two terms. Since the final value for the sedimentation coefficient of T_{4r} + DNA agrees closely with the values

obtained with conventional techniques, and since the hydration correction, $1 + \Gamma'$, and the pressure correction undoubtedly must be applied, we believe that our experiments represent a confirmation of the necessity of making the correction involving α. If the α correction were not applied, the values of the sedimentation coefficient would be 32 per cent too small, and a final value of $s^0_{20,w}$ would be 46 S instead of 60.5 S. This is more than 5 times the probable experimental error.

The frictional coefficient ratio, f''/f', has been assumed to be equal to 1 in these experiments. Here again, the primary justification for this assumption is that the value of $s_{20,w}$ which is obtained agrees with that measured by conventional techniques.

For high molecular weight DNA, the sedimentation coefficient has been found to be a sensitive function of the speed of the ultracentrifuge (Rosenbloom and Schumaker, 1963). This phenomenon has been shown to be due to a speed-dependent aggregation. According to a hypothesis put forth to explain this phenomenon, small aggregates—dimers, trimers, etc., may be formed by random collision. In a centrifugal field, these small aggregates have a sedimentation velocity much greater than their velocities due to thermal agitation. The rate of collision of these aggregates with monomers is markedly increased, and, like rolling snow balls, the aggregates grow in size. If this hypothesis is correct, then the aggregation phenomenon depends upon sedimentation *velocity* rather than the speed of angular rotation of the centrifuge rotor. By conventional techniques, we have recently shown this to be the case. As the density of the solution is increased, the sedimentation velocity of the molecules decreases, and, in like manner, the rotor speed dependent aggregation decreases. Therefore, in the experiments reported in this communication where the sedimenting boundaries are traveling at 1 per cent or less of the normal sedimentation velocities in the absence of a density increasing solute, no rotor speed-dependent aggregation should be observed. As may be seen from the data of Fig. 7, the sedimentation characteristics of high molecular weight bacteriophage are well behaved and "normal." No evidence for molecular entanglement can be seen, although rotor speeds and concentrations were employed far in excess of those necessary to cause the effect in dilute salt solutions.

The Density of DNA. The apparent increase in the density of DNA with the increase in temperature is probably a reflection of dehydration of these macromolecules with advancing temperature. Normally, substances expand when heated. For example, the partial specific volume of proteins increases by 5 to 10×10^{-2} per cent for each degree increase (Cox and Schumaker, 1961). Assuming that the thermal expansion of DNA is of the same magnitude, then it is possible to calculate from the observed density changes of the hydrated unit that approximately 20 per cent of the original water of hydration has been lost in going from 9° to 39°C. A better estimation of the hydration change must await precise measurement of the change in the partial specific volume of DNA in concentrated aqueous CsCl as a function of temperature. It seems clear, however, that water of hydration is lost as the temperature is raised. This result is in marked contrast to the results of studies on the protein,

RNase, for which it is found that the degree of preferential hydration increases by about 10 per cent as the temperature is raised from 5° to 40°C. It may well turn out to be the case that biological polymers of different types preferentially bind water in various amounts and with various degrees of tenacity. Temperature change experiments as reported in this communication might then serve as a means of identification for these polymers as well as providing a measure of their homogeneity and sedimentation coefficients.

Comments of the First Referee. The authors feel that two comments made by the referee will be of value to those using the technique described in this paper.

(*a*) "I consider it poor procedure to stop the mechanical pump without allowing air into the chamber. In my experience this has sometimes led to serious contamination of the chamber by diffusion pump oil. Furthermore, a slightly leaky pump will allow forepump oil to back up into the vacuum system."

(*b*) "A correction has been applied by subtraction of the values of the gradient of a water-filled cell for the same pressure and temperature. This correction neglects differences between the compressibilities of the CsCl solutions and those of water (and, in case the windows form a liquid prism out of the cell, the refractive index differences between water and salt solutions). A better correction can often be applied by subtraction of the gradient in the same (or similar run without solute) experiment at very early times after reaching speed, but before redistribution affects the central region of the cell."

Received for publication, March 1, 1965.

BIBLIOGRAPHY

BALDWIN, A. L., and SHOOTER, E. M., 1963, *in* Ultracentrifugal Analysis in Theory and Experiment, (J. W. Williams, editor), New York, Academic Press Inc., 143.

COHEN, S. S., and ARBOGAST, R., 1950, *J. Exp. Med.*, **91**, 607.

COX, D., and SCHUMAKER, V. N., 1961, *J. Am. Chem. Soc.*, **83**, 2433.

HEARST, J., 1962, *J. Mol. Biol.*, **4**, 415.

HEARST, J., IFFT, J., and VINOGRAD, J., 1961, *Proc. Nat. Acad. Sc.*, **47**, 1015.

HEARST, J., and VINOGRAD, J., 1961*a*, *Proc. Nat. Acad. Sc.*, **47**, 999.

HEARST, J., and VINOGRAD, J., 1961*b*, *Proc. Nat. Acad. Sc.*, **47**, 1005.

IFFT, J. B., VOET, D. H., and VINOGRAD, J., 1961, *J. Physic. Chem.*, **65**, 1138.

MANDELL, J. D., and HERSHEY, A. D., 1960, *Anal. Biochem.*, **1**, 66.

MARMUR, J., 1961, *J. Mol. Biol.*, **3**, 208.

MESELSON, M., and NAZARIAN, G. M., 1963, *in* Ultracentrifugal Analysis in Theory and Experiment, (J. W. Williams, editor), New York, Academic Press Inc., 131.

ROSENBLOOM, J., and SCHUMAKER, V. N., 1963, *Biochemistry*, **2**, 1206.

VINOGRAD, J., BRUNER, R., KENT, R., and WEIGLE, J., 1963, *Proc. Nat. Acad. Sc.*, **49**, 902.