at pH 12 or higher, nucleic acids are completely denatured (Mathieson and Matty, 1957), it seems unlikely that the denaturing effect of Me_2SO is playing a role in the enhanced RNA degradation.

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Binding Properties of Dye-Tagged Polylysine Complexed to Deoxyribonucleic Acid*

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ABSTRACT: Emission properties of dansyl-labeled polylysine and dansyl-labeled polylysine complexed to DNA have been studied. Perrin plots of the fluorescence of the complex show three regions. The first, which occurs at high viscosity and low temperatures, is the same as a portion of the Perrin plot of the free dansyl-polylysine. The second region is of constant polarization, in which the anisotropy is appreciably above that of the free dansyl-polylysine. The third is a region concave upward, characteristic of a thermally activated rotation about one or more single bonds. In the dissociation of the complex by most salts, the fraction of polylysine bound is shown to be the same function of the anisotropy though salts of widely differing dissociating power are used. In general the turbidity is not proportional to the amount of complex present. It depends on the type of salt in the solution and on the salt concentration. It decreases with increasing excess DNA. The scattering unit, however, is well defined. Excess DNA solubilizes the complex and lowers the turbidity.

The interaction of polylysine with DNA has been studied extensively by a variety of techniques and the following properties are known. (1) At low salt concentrations, polylysine–DNA complex formation is irreversible, in the sense that DNA added, after the complex is formed, will not exchange with the DNA in the complex in times of the order of 1 day (Tsuboi *et al.*, 1966). At 1 M NaCl exchange is rapid (Leng and Felsenfeld, 1966; Shapiro *et al.*, 1969). (2) The complex has a definite stoichiometry of one lysine to one DNA phosphate (Olins *et al.*, 1967). (3) Polylysine stabilizes the DNA against melting (Raukas, 1965; Ohba, 1966; Tsuboi *et al.*, 1966; Olins *et al.*, 1967, 1968). (4) The binding is cooperative (Raukas, 1965; Tsuboi *et al.*, 1966; Leng and Felsenfeld, 1966; Olins *et al.*, 1967; Shapiro *et al.*, 1969). (5) In 1 M salt, the interaction is selective for (A-T)-rich DNAs (Leng and Felsenfeld, 1966; Shapiro *et al.*, 1969). (6.) The optical rotatory properties of solutions of complex are markedly different than those of DNA solutions (Cohen and Kidson, 1968; Inoue and Ando, 1968, 1970; Shapiro *et al.*, 1969).

Our laboratory has recently published a study of the interaction of DNA and polylysine, using the technique of polar-

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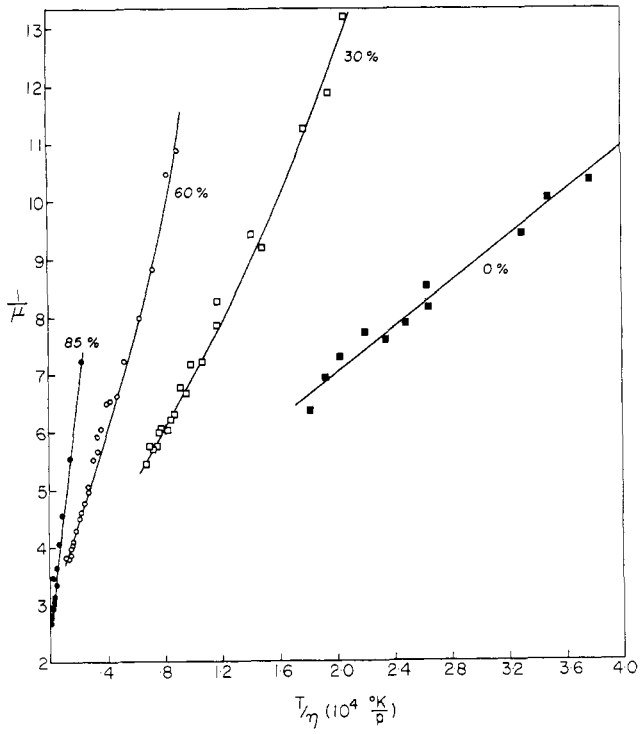

FIGURE 1: Perrin plot of dansyl-labeled polylysine. Dansyl concentration is 8.2×10^{-6} M and the lysine to dye ratio is 21.7. All solutions are 0.5 M NaCl. The per cent glycerol is as indicated.

ization of fluorescence (Evett and Isenberg, 1969). We showed that under simple but reasonable assumptions, the amount of polylysine bound to DNA may be determined from the fluorescence properties of a dye conjugated to a small fraction of the lysines. This technique permits the fraction of polylysine bound to DNA to be determined in a relatively rapid fashion. This makes it possible to follow changes in binding as solution parameters are altered. We presented salt dissociation curves using a variety of cations and anions, and demonstrated that dissociation is sensitive to the type of ion used. Evidence was given that the presence of labels on a small fraction of the ϵ-amino groups did not alter the binding to a measurable extent.

This paper will extend our work. We will present Perrin plots for both complexed and free dansyl-polylysine.[1] These will show that the polarization, as a function of temperature, differs from the polarization as a function of inverse viscosity. The origins of the depolarization phenomena will be discussed in terms of internal degrees of freedom of the complex.

We will also report that the fraction of polylysine bound to DNA is, for many salt titrations, a single function of the anisotropy of emission, independent of the markedly different dissociating properties of these salts. Consequently it is possible to directly calibrate dissociation in terms of an intensive parameter of the emission, which may be measured almost instantaneously.

Polylysine–DNA complexes are slightly turbid. The turbidity changes as salt is added. If an initial turbidity is measured at a moderately low salt concentration, in our case 0.05 M NaCl in 0.1 M cacodylate buffer (pH 5.5) then we have shown (Evett and Isenberg, 1969) that the fractional decline of this turbidity very nearly follows the dissociation of the polylysine from the DNA. In this sense, the turbidity is almost a measure of the amount of complex present. However, as will be emphasized in the present paper, the turbidity does not measure the concentration of complex in general. Rather turbidity is also a function of the concentration and of the type of salt in the solution and of the amount of excess DNA present. In some instances turbidity is not even monotonic with the fraction of polylysine bound.

Materials and Methods

Reagents. Poly-L-lysine was synthesized by the *N*-carboxyamino acid anhydride method (Becker and Stahmann, 1952; Katchalsky and Sela, 1958). The average DP was 140. Poly-L-lysine was also purchased from Yeda Research and Development Co. Analysis of this polylysine by chromatography on a CM-cellulose column, using an exponential sodium chloride gradient (Stewart and Stahmann, 1962), indicated a single range of molecular weights centered around a DP of 275.

Salmon sperm DNA was purchased from Calbiochem, and calf thymus DNA from Worthington Biochemical Corp. DNA was further purified by standard ethanol and 2-propanol precipitation procedures.

Glycerol was spectroquality grade purchased from Matheson, Coleman & Bell. Dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) was purchased from Aldrich Chemical Co. Dansyl chloride was conjugated to polylysine by a standard procedure previously described by (Evett and Isenberg, 1969).

Dansyl-polylysine was complexed to DNA by a modification (Evett and Isenberg, 1969) of the salt gradient dialysis procedure of Huang *et al.* (1964). A final stage in this method involves the centrifugation of the sample to separate "precipitated" from "soluble" complex. We have consistently found that, with our procedure, the concentration of complex in the supernate was 10^{-4} M in lysine. It may be noted that 10^{-4} M is the order of magnitude of the solubility of the "soluble" complex reported by Olins *et al.* (1967). In the work on turbidity as a function of excess DNA, reported below, no centrifugation step was used in the preparation of samples except as described in the text.

Salts were added as powder and corrections for water of hydration were made, when necessary, by standard quantitative analyses of weighed amounts of material.

Anisotropy and intensity of fluorescence were measured using an instrument built in our laboratory and previously described (Evett and Isenberg, 1969). All measurements were made at 25.0 ± 0.2° unless otherwise noted.

Turbidity was measured in a Cary Model 14 spectrophotometer at 500 nm, using a 0–0.1-absorbance slide-wire.

Results

For depolarization due to Brownian rotatory diffusion of a rigid molecule, with no internal degrees of freedom, the anisotropy[2] is a function of T/η (Weber, 1952a,b, 1953). Figure 1

[1] Abbreviations used are: dansyl, 1-dimethylamino-naphthalene-5-sulfonyl; DP, degree of polymerization.

[2] As discussed by Jablonski (1960), the anisotropy, which may be defined by eq 1 is for all purposes a better measure of the angular

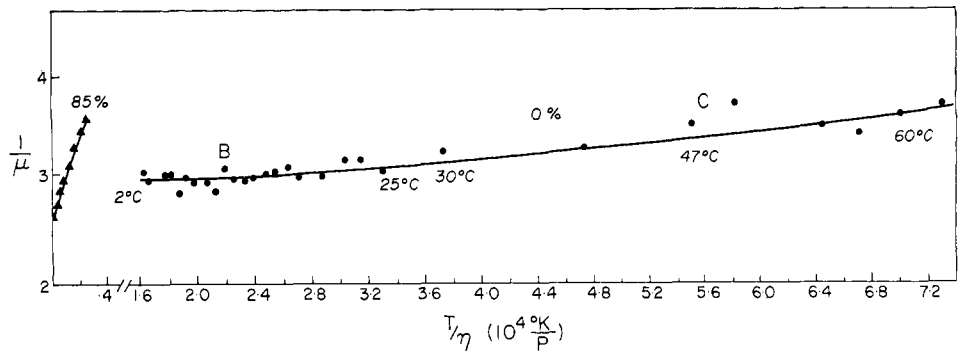

FIGURE 2: Perrin plot of the dansyl-polylysine–DNA complex in 0 (●) and 85% (▲) glycerol. DNA-(P)/lysine is 5.71, [DNA((P)] = 9.5×10^{-4} M, and both samples are 0.3 M in NaCl.

shows a Perrin plot for dansyl-labeled polylysine. This clearly shows that the inverse anisotropy is not a function of T/η. Figure 2 shows two Perrin plots for the complex, one in buffer without glycerol, and one in 85% glycerol.[3] For the complex, also, the inverse anisotropy is not a function of T/η. Measurements of emissions from samples with no glycerol yielded a curve having a relatively constant value of anisotropy from about 2° to about 20°. At higher temperatures there is a slow rise with temperature. It should be noted that in the relatively flat, low-temperature portion of the curve, the anisotropy is appreciably lower than that of a completely rigid chromophore. This degree of freedom may be quenched by raising the viscosity of the medium, which we have done by adding glycerol. Figure 3 shows Perrin plots for the complex and for the dye-tagged polylysine both in 85% glycerol. Three regions of the plot for the complex may be noted: a high-temperature region with a curvature that is concave upward, an intermediate region of relatively constant anisotropy, and a low-temperature region that approaches a complete freezing-in of all rotatory degrees of freedom. It may be seen that this last region is the same for the dansyl-polylysine–DNA complex as it is for dansyl-polylysine by itself. These regions will be analyzed in the Discussion section of this paper.

In discussing salt induced dissociation, our notation will be the same as developed previously (Evett and Isenberg, 1969; Ellerton and Isenberg, 1969).

Let μ be the anisotropy of the light emitted by the sample where

$$\frac{1}{\mu} = \frac{1}{p} - \frac{1}{3} \tag{1}$$

Let μ_1 and μ_2 be the anisotropy of the emission from bound and free dansyl-polylysine. In all cases, we will use the same subscripts, 1 and 2, for bound and free polylysine, respectively.

Let f be the fraction of dansyl-polylysine bound to DNA. Let ϵ_i and q_i be the molar extinction coefficient and quantum yield for state i.

It has been shown (Evett and Isenberg, 1969) that

$$f = \frac{I(c)}{I(0)} \frac{\epsilon_1(0)q_1(0)}{\epsilon_1(c)q_1(c)} \frac{\mu - \mu_2}{\mu_1 - \mu_2} \tag{2}$$

c is the concentration of salt added to a state that we label with the symbol zero. It should be emphasized that this zero state is an initial one, and is not necessarily one devoid of salt. It is simply any state for which all of the polylysine is bound to DNA.

In analogy to a similar equation derived elsewhere (Ellerton and Isenberg, 1969) it is easy to show that

$$\frac{\epsilon_2 q_2}{\epsilon_1(0)q_1(0)} = \frac{1}{1 - f}\left[\frac{I}{I(0)} - \frac{\epsilon_1 q_1}{\epsilon_1(0)q_1(0)} f\right] \tag{3}$$

We now assume that the emission of a molecule of bound

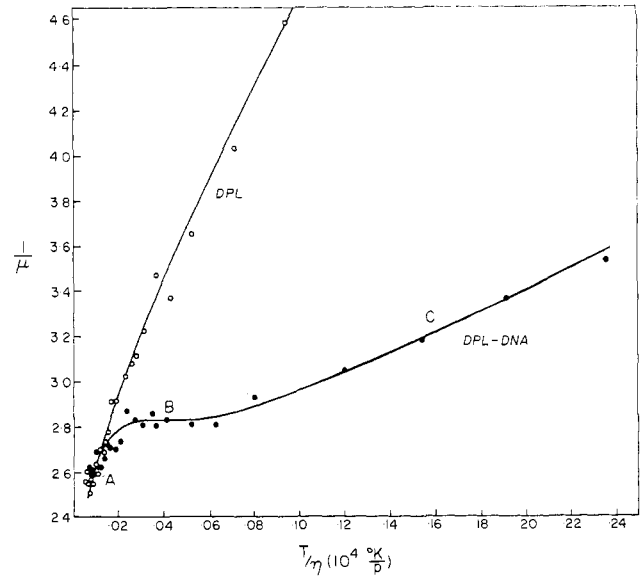

FIGURE 3: Perrin plot of dansyl-polylysine (DPL) and the dansyl-polylysine–DNA complex, both in 85% glycerol.

anisotropy of the emitted light than the commonly employed polarization. The reader may note that the definition used by some authors is simply related to ours by a factor of two-thirds. The customary Perrin plot may be labeled as inverse anisotropy *vs.* T/η. $\mu = 3/2(I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$, where $I_\parallel$ and $I_\perp$ are the emissions with polarizations parallel and perpendicular to the polarization of the exciting beam.

[3] Solvent changes, such as the addition of glycerol will, in general, result in more than simple viscosity alterations. The bearing of such changes on the interpretation of our data will be examined in a later portion of this paper.

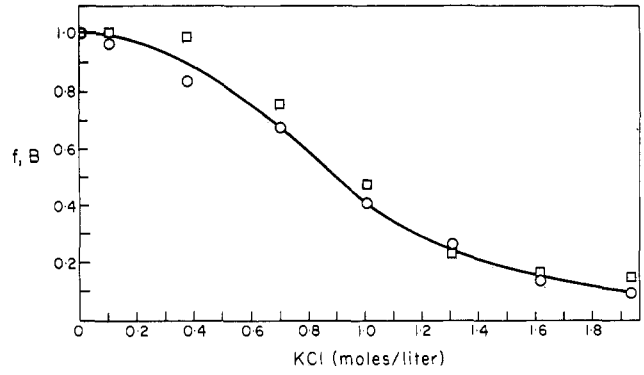

FIGURE 4: KCl dissociation of the dansyl-polylysine–DNA complex. Initial solvent was 0.05 M NaCl–0.1 M cacodylate buffer (pH 5.5). Fraction of dansyl-polylysine bound (○) and, B, the fractional decrease in turbidity (□) are shown.

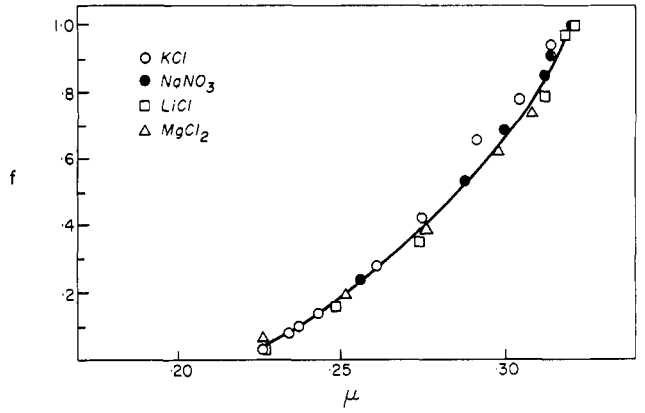

FIGURE 6: Fraction of dansyl-polylysine bound as a function of anisotropy: dissociation by KCl (○), LiCl (□), $NaNO_3$ (●), and $MgCl_2$ (△).

dansyl-polylysine is independent of the salt concentration (Evett and Isenberg, 1969). Then

$$\frac{\epsilon_1(c)q_1(c)}{\epsilon_1(0)q_1(0)} = 1 \tag{4}$$

We then have

$$= \frac{I(c)}{I(0)} \frac{\mu - \mu_2}{\mu_1 - \mu_2} \tag{5}$$

and

$$\frac{\epsilon_2(c)q_2(c)}{\epsilon_1(0)q_1(0)} = \frac{1}{1-f}\left[\frac{I}{I(0)} - f\right] \tag{6}$$

Equation 5 permits the determination of the fraction of dansyl-polylysine bound to DNA from experimental data. Equation 6 permits the determination of the salt dependence $\epsilon_2 q_2$, which measures the emission of that part of the dansyl-polylysine which is dissociated.

Figure 4 shows f for the KCl dissociation of a sample having an initial state of 0.05 M NaCl and 0.1 M cacodylate buffer (pH 5.5). Figure 4 also shows the fractional change in turbidity, denoted by *B*. It may be seen that the two curves are closely similar, as reported previously for a large number of monovalent salt titrations (Evett and Isenberg, 1969).

Figure 5 shows a plot of the intensity of emission per dissociated molecule. $\epsilon_2 q_2$ is very nearly constant. This constancy has the following consequence. It has been shown (Evett and Isenberg, 1969) that, if one *assumed* that $\epsilon_2 q_2$ were strictly constant, one could derive the equation

$$f = \frac{1}{1 + \frac{I(0)}{I(\infty)} \frac{\mu_1 - \mu}{\mu - \mu_2}} \tag{7}$$

If the end parameters, $I(\infty)$ and μ_2, are the same for any given set of samples, eq 7 implies that, for this set, f is a function of μ only.

We have found that, for a particular complex, the end parameters are the same when a variety of salts are used to dissociate the complex. For these salts, f should be a universal function of μ. This is found to be the case and Figure 6 shows such a plot for four different salts, including the divalent salt $MgCl_2$. It should be noted that the actual dissociation curves for these salts are different as shown in Figure 7.

We have previously shown (Evett and Isenberg, 1969) that small additions of Mg^{2+} result in a marked increase in the

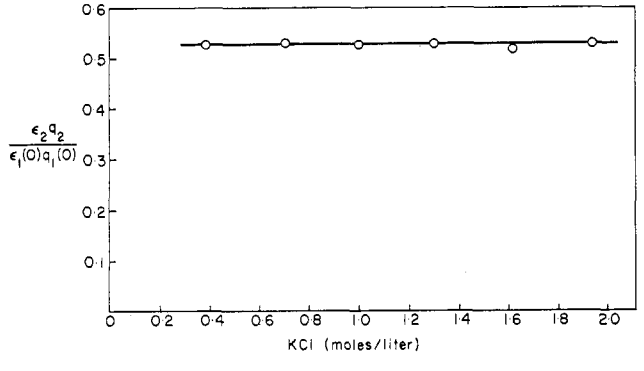

FIGURE 5: The intensity of emission per free dansyl-polylysine molecule.

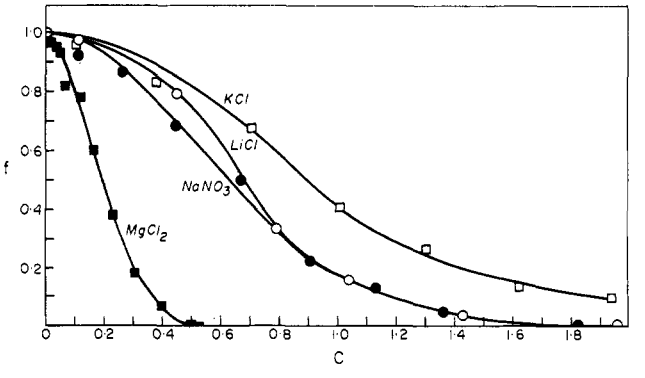

FIGURE 7: Salt dissociation of the dansyl-polylysine–DNA complex by $MgCl_2$ (■), $NaNO_3$ (●), LiCl (○), and KCl (□).

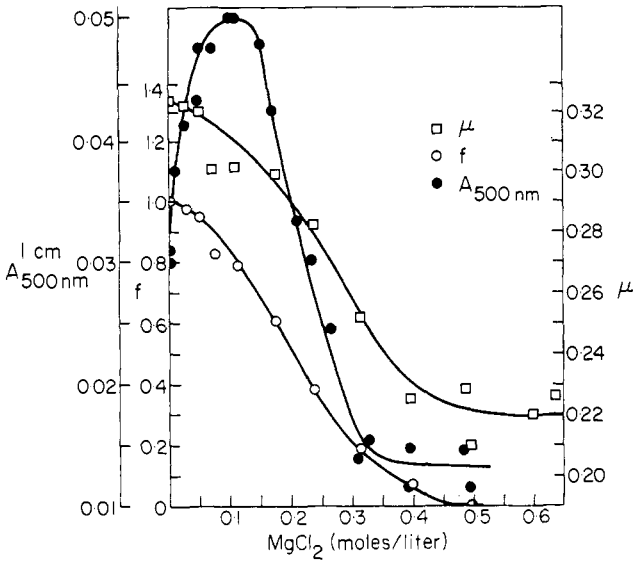

FIGURE 8: $MgCl_2$ dissociation of the dansyl-polylysine–DNA complex: anisotropy (□), fraction of dansyl-polylysine bound (○), and turbidity (●).

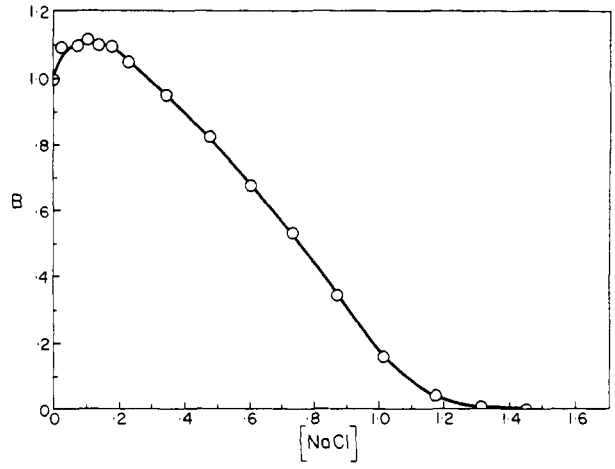

FIGURE 9: Fractional change in turbidity, B, as a function of NaCl concentration. Initial solution conditions were 0.01 M cacodylate buffer (pH 5.5), no NaCl.

turbidity of polylysine–DNA samples. The increase occurs before there is any appreciable dissociation of the complex (Figure 8). Increases in turbidity also occur when monovalent salts are added, but are of considerably smaller magnitude (Figure 9). Upon raising the concentration of NaCl to 0.03, the turbidity increases by about 10%. This is followed by a relatively flat region and then by a turbidity decrease which nearly parallels the dissociation (Evett and Isenberg, 1969).

Olins *et al.* (1967) and Leng and Felsenfeld (1966) studied the solubility characteristics of polylysine–DNA complexes as a function of the lysine to phosphate ratio, holding the DNA constant. Their work yielded valuable data on the stoichiometry, reversibility, and cooperativity of complex formation. We report here data on the turbidity of complexes as a function of phosphate to lysine ratios but, in our case, holding the lysine concentration constant. At low salt, since all of the lysine is bound, the amount of complex is determined by the lysine concentration. Our data report, therefore, on the variation of turbidity as a function of excess DNA.

A set of samples, 10^{-4} M in lysine, having DNA concentrations varying from 10^{-4} M phosphate to 30×10^{-4} M phosphate, was salt annealed to a final concentration of 0.05 M NaCl and 0.01 M cacodylate buffer (pH 6.2).

The sample with 1:1 stoichiometry yielded only a granular precipitate after gradient dialysis. The precipitate contained practically all of the complex. For example, the supernatant of this sample showed no detectible turbidity at 500 nm. The insolubility of a 1:1 complex at low salt was reported previously by Olins *et al.* (1967). The turbidity of all of the other samples was measured. The samples were then centrifuged at 390*g* for 15 min, and the turbidity of the supernatant fractions was again measured. Figure 10 summarizes the data. Excess DNA clearly lowers the turbidity of a sample. Also, it may be noted that centrifugation resulted in little or no decrease in turbidity, except for the two points at lowest phosphate to lysine ratios. Consistent with this was the observation that no visible pellet was obtained, except for these latter samples.

The decrease in turbidity and the solubilization of the complex by excess DNA probably reflect the same phenomenon.

Inoue and Ando (1970) have reported that clupeine–DNA complexes are solubilized by excess DNA and Olins (1970) reported a similar finding for lysine-rich histone–DNA complexes. The phenomenon that is described is therefore of a rather general nature.

Discussion

The Perrin plots (Figures 1 and 2) show that the inverse anisotropy is not a function of T/η, but separately depends on the two parameters. When the anisotropy is a function of T/η, one may conclude that the depolarization of fluorescence results from overall rigid body rotatory diffusion (Weber, 1952a,-b, 1953). When the anisotropy is not a function of T/η, analysis of the data becomes less straightforward. As emphasized by Stryer (1968), a variation of solvent composition may result

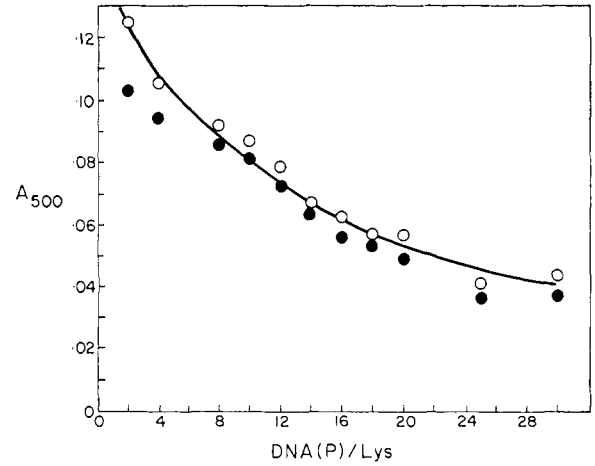

FIGURE 10: Turbidity as a function of the DNA(P)/Lys ratio of the solution. All samples are 10^{-4} M in lysine. Turbidity was measured before and after centrifugation for 15 min at 390*g* (○ and ●, respectively).

in more than a simple viscosity change. Indeed, a demonstration, as we report, that the viscosity is not a function of T/η implies alterations other than viscosity changes.

We believe that, at present, a detailed interpretation in terms of any specific model is premature. However, it seems reasonable to rationalize the data as consistent with depolarizations due to thermally activated rotations about saturated bonds. The depolarization due to such rotations has been discussed, and analyzed, in a number of papers, in recent years (Gottlieb and Wahl, 1963; Wahl and Weber, 1967; Rawitch *et al.*, 1969).

Our results for the free dansyl-polylysine are similar to those found by Gill and coworkers (Gill and Omenn, 1965, 1967; Omenn and Gill, 1966; Gill *et al.*, 1967).

The depolarization of the complexed dansyl-polylysine is sharply reduced from that of free polypeptide, except at low temperatures and high glycerol concentrations.

Three regions of the plots may be distinguished: a region concave downward at low temperatures, a flat portion, and a concave upward section at higher temperatures. These are labeled A, B, and C in Figures 2 and 3.

The conformation of the complex may alter in going from a solvent without glycerol to one containing 85% glycerol. Despite such possible changes, however, it still appears reasonable to assume that, qualitatively, regions B and C correspond to one another in both solvents.

Region A is, within experimental error, the same for the dansyl-polylysine that is either complexed or free. We therefore interpret this region as one directly involving one or more degrees of freedom in the side chain containing the dye. As the temperature is raised the depolarization increases. In region B, the depolarization resulting from this degree of freedom evidently saturates, and a flat section of the curve results.

Downward curvatures in Perrin plots, at low values of T/η, have been found by Knopp and Weber (1969) in their studies of macroglobulins tagged with pyrene butyric acid. Similar observations were also reported by Wahl and Weber (1967) using dansyl-labeled globulin. In these studies the depolarization due to bond rotation was superimposed on a depolarization resulting from rigid body rotatory diffusion, a phenomenon analyzed theoretically by Gottlieb and Wahl (1963).

Due to the high molecular weight of the DNA complexes, depolarization due to rigid body rotations are not to be expected (Ellerton and Isenberg, 1969). The curvature of region A may therefore be understood by a model involving depolarizations due to bond rotations only.

Region C may be tentatively interpreted as a new, thermally activated, degree of rotational freedom. There is little that can be said about this degree of freedom at the present time. It may represent a new local movement of the side chain containing the dye. However, it is possible that this movement is related to a recently reported thermally activated motion observed in proflavine–DNA complexes (Ellerton and Isenberg, 1969) and the relaxation reported for ethidium bromide–DNA complexes (Wahl *et al.*, 1970). A significant depolarization exists in the emission from these complexes, even at low dye to phosphate ratios, where practically all of the dye intercalates. This depolarization may be due to local motion of the DNA helix. We may speculate that both the depolarization reported for the dye complexes, and that reported here, may be due to the same motion of a portion of the DNA. However, we have no evidence to support such a speculation.

In our earlier paper (Evett and Isenberg, 1969), and in this one, the fraction of polylysine bound to DNA was determined using a two-state model. The model states that any polylysine molecule is either completely bound or completely free. The concentration of any other state is assumed to be negligibly small compared to these

The two-state model does not necessarily imply, however, that the dissociated state is the same, independent of the means of dissociation. We find, however, that for a variety of salts, this is true. Figure 6 clearly shows this. The curve is the same even for two such dissimilar salts, as $MgCl_2$ and KCl, which dissociate the complex quite differently (Figure 7). Figure 6 has useful implications for future studies using polarization of fluorescence. It implies that a dissociation may be calibrated directly in terms of the anisotropy of emission. This, in turn, means that changes in the dissociation, at any point in the dissociation curve, may be determined directly without the necessity of obtaining the entire curve.

Shapiro *et al.* (1969) have reported extensive investigations of the aggregation, and phase separation, of stoichiometric preparations of polylysine–DNA at 1 M NaCl. They report the formation of particles which, on the basis of a spherical model, have radii of 1700 Å. The particles show little size dispersion. They have a sedimentation coefficient in the range of 5000–10,000 S. These particles are responsible for the turbidity of the sample. Shapiro *et al.* (1969) report that the particles contain all, or almost all, of the complex.

The turbidity of the samples studied by us is a function both of the type and amount of salt present, and of the concentration of excess DNA. There is no simple relationship between the amount of complex present and the turbidity. Excess DNA will result in a turbidity decrease. Small concentrations of Mg^{2+} will yield appreciably more turbid samples. If, however, we severely define the conditions of measurement, namely, hold the total DNA concentration constant, and take as an initial state one having a small amount of a monovalent salt, then we find that the turbidity very nearly measures the degree of complex present. Salt dissociation curves presented in a prior paper (Evett and Isenberg, 1969) and in this one, taken under the above conditions, show that the turbidity and the amount of complexed polylysine closely parallel one another. The parallelism implies that, throughout an entire salt dissociation, the complexes that remain undissociated at any given salt concentrations, still form well defined structures with little heterogeneity.

The ability to alter solution parameters over wide ranges and to correlate any measurement, such as turbidity, with the changes in amount of complex present, obviously depends on being able to measure the concentration of complex quickly and accurately. Depolarization of fluorescence techniques appear to offer distinct advantages in doing this.

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On the Basis of Specific Fragmentation of Ribonucleic Acid by Nucleases*

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ABSTRACT: The limited exposure of rabbit reticulocyte RNA to T_1 ribonuclease produces a series of stable intermediates of degradation, which can be characterized by electrophoresis in polyacrylamide gels. Explanations of this effect in terms of secondary or tertiary structures are ruled out by fragmentation patterns under conditions leading to the elimination of base-pairing. In particular, the RNA in the presence of 3% formaldehyde is much more rapidly degraded, but a sharp pattern of zones is formed which is very similar to that produced by the native material. No differences are observed when the digestion is performed at 63°, when single-strand stacking is to a great extent eliminated, or after chemical methylation of guanine residues, or after introduction of excess amounts of polycytidylic acid under conditions leading to binding. This last, as well as the absence of significant amounts of guanylic acid in the digests, renders any explanation of specific fragmentation in terms of long runs of guanine residues improbable.

It is suggested that the degree of lability of any bond is determined by subsites on the enzyme, which recognize a series of adjoining residues; such situations have been reported for other hydrolytic enzymes. This interpretation is supported by experiments with the closely related nuclease, N_1, which has the same primary specificity and other properties as T_1. Limited digestion with N_1 nuclease leads to a well-defined pattern of zones, which is however unrelated to that produced by T_1. Limited digestion of a specific tRNA by these two nucleases also reveals some differences. This indicates that, as expected, the systems of subsites on the two enzymes are not identical.

The ability of nucleases under the right circumstances to break down RNA into large fragments by cleavage at labile regions has been known for a considerable time (Huppert and Pelmont, 1962; McPhie *et al.*, 1966; Gould, 1966a,b; Delihas and Bertman, 1966, and much subsequent work). The use of polyacrylamide gel electrophoresis to provide high resolution according to molecular weight of RNA species over a wide size range revealed that the fragments were indeed highly stable intermediates, each essentially monodisperse (McPhie *et al.*, 1966; Gould, 1966a, 1967). This type of behavior has

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