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Title: STUDIES ON THE INTERACTION OF POLYLYSINE WITH DEOXYRIBONUCLEIC ACID

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The interaction of polylysine with DNA may be divided into two separate phenomena: the primary interaction involved in formation of a well-defined complex, and secondary interactions leading to aggregation of these complexes. Most studies on the interaction of polylysine with DNA have used techniques which measure properties of the aggregated phase resulting from complex formation, and the properties of the interaction have been inferred from these measurements.

When polylysine complexes with DNA its rotational freedom is decreased and consequently the fluorescence depolarization of a chromophore attached to the polylysine also decreases. The calculation of the fraction of polylysine bound from fluorescence depolarization measurements were found to be independent of the degree of aggregation of the complex, and independent of factors affecting

aggregation. This demonstrates that it is, therefore, possible to measure the primary interaction between polylysine and DNA directly by fluorescence depolarization. In contrast, sedimentation and turbidity measurements more closely relate to the state of aggregation of the complex rather than the formation of the complex itself.

The interaction has been reported in the literature to be irreversible at low ionic strength, i.e. 0.0 M to about 0.4-0.8 M NaCl, and reversible at high ionic strength, 1.0 M NaCl. This study shows that the fraction bound, of polylysine, at 1.0 M NaCl is a function of the path taken to reach 1.0 M NaCl. It is also shown that, when the DNA concentration in the sample is raised and complexes formed, the binding decreases even though, on an equilibrium model for the interaction, more binding sites would become available. Both of these findings demonstrate that the interaction between polylysine and DNA is irreversible.

pH studies indicate pK shifts of the chromophore occur as the complex is dissociated by NaCl. This is interpreted as charge effects of the DNA phosphates. Neutralization of the phosphates by salt ions changes the pK of the chromophore.

The turbidity is found to be sensitive to many experimental parameters and is not a quantitative measure of the complex.

It is also shown that rhodamine B conjugates, as well as dansyl conjugates, are useful for studies of dye-labelled polylysine-DNA interactions.

Studies on the Interaction of Polylysine with Deoxyribonucleic Acid

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INTRODUCTION

Since the suggestion, in 1950, by Stedman and Stedman (74) that the histones act as gene repressors, many laboratories have investigated the relationship between the basic proteins of the nucleus and genetic expression of DNA. The demonstrations that histones inhibit the template efficiency of DNA for DNA-dependent RNA synthesis (2, 4, 5, 32, 48) and DNA-dependent DNA synthesis (29, 86) support this suggestion.

If the function of the histones is repression of the expression of genetic information, then the large number of bits of genetic expression suppressed in the differentiated cells of a higher organism and different species implies there should be great heterogeneity in the histones. The sequencing of histone IV from calf thymus and pea seedling (20, 52) show the proteins to be nearly identical, however. Of the 102 residues of the protein, only two replacements are found, and these are conservative replacements (arginine for lysine and isoleucine for valine) requiring only a single base change in the genetic code. This homology, in spite of wide species difference, suggests a very strongly correlated structure-function relationship for histone IV. In contrast cytochrome c from wheat and bovine have

about one-third of their residues different and yet are functionally the same.

Limited heterogeneity of all the histones is reported for calf thymus by Panyim and Chalkley (58), who find only five major electrophoretic fractions, and only 12 subfractions when the major ones are further resolved. Stellwagen and Cole (75), however, suggest the lysine-rich histone may be rather heterogeneous and report they exhibit phenotypic specificity.

Though histones have not been found in procaryotic cells, protein-nucleic acid complexes have recently been isolated from bacterial cells (14, 77, 78) and the complexed DNA found to have reduced template activity in comparison to deproteinized DNA (62). Most DNA-associated proteins of bacterial systems have not yet been characterized well enough to know how specifically they bind to DNA.

Huang and Bonner (31) report the isolation of histone-bound RNA from pea seedlings and suggest it is the high specificity of RNA combined with the general inhibitory gene activity of histones that controls gene expression. Other workers also report isolation of RNA in the DNA-protein complexes in bacterial systems (77, 78) and higher organisms (23). Commerford and Delihas, however, report they are consistently unable to find RNA-histone in mammalian tissues and suggest that found by Bonner and coworkers have a more

specialized function in pea seedling (16). The reconciliation of these conflicting reports has still to be made.

RNA is not the only type of macromolecule capable of highly specific interaction with DNA. The recent isolation of the lac (24, 65) and λ phage (60) repressors and the demonstration that they are proteins and bind to specific sites on the DNA (8, 25, 59, 61, 66) provides specific examples of highly selective interaction of a protein with DNA for the control of genetic expression of DNA.

Protein and Polypeptide Interactions with DNA

As mentioned previously, the histones inhibit the template activity of DNA. Template activity of DNA in chromatin is only between 10 and 15% of that of the same DNA after deproteinization (5, 6). The selective removal of histones by increasing ionic strength (48, 54, 85) results in a step-wise increase in template activity (6, 48). Related to this is the observation by Littau, et al. (47) that the removal of histones, especially the lysine-rich fraction, results in an increase in extended or non-condensed chromatin (47), and the demonstration that mRNA synthesis occurs primarily on the extended portions of the chromatin (46).

Liau, Hnilica, and Hurlbert (45) report that addition or removal of histones cause respective decreases or increases in the amount of RNA synthesized by nucleoli. The addition or removal of

histones also effects the composition of the RNA produced. In low-histone nuceoli the RNA produced is similar in composition to the nucleolar DNA and richer in A-T content than ribosomal RNA, which is the product of high-histone nucleoli. This result is suggestive of selectivity, by the histones, for A-T rich DNA.

Such a specificity toward A-T rich DNA is also suggested by the studies of Crampton, Lipshitz, and Chargaff (17, 18) on nucleohistone and reconstituted nucleohistone, where reconstitution was achieved by salt dissociation of the nucleohistone, and subsequent lowering of the ionic strength to reform the histone-DNA complex. With both preparations they report an increase in the A-T/G-C ratio of the DNA extracted by increasing ionic strength. Skalka, et al. (70) report that the efficiency of inhibition by histones, polylysine and protamine increase as the A-T content of the DNA primer increases.

The recent isolation and sequencing of the glycine-arginine rich (GAR) histone (20, 52) makes it possible to attempt to analyze various aspects of the interaction of this histone with DNA. The sequence of this histone reveals that the basic amino acids are clustered toward the amino terminus of the molecule and that the other end contains most of the hydrophobic side chains. Studies by Moskowitz, et al. (51) using the basic amino acid clusters, obtained by proteolytic digestion of the molecule, show they are not as

effective as the whole histone molecule in inhibiting the DNA-primed RNA synthesis. At levels where the whole GAR histone produced 100% inhibition, one cluster caused only 25% inhibition and another was even slightly stimulatory. The authors interpret these results as evidence that ionic binding alone does not account for the inhibition, and that the whole histone molecule is required because of the presence of other factors in the interaction.

Conformational changes in polypeptide or DNA may occur upon complexing. Clewell and Helinski (14) find removal or digestion of the protein associated with DNA in E. coli results in the conversion of the DNA from a supercoiled circular form to an open circular DNA. They do not suggest a model for the observation but note that deproteinized DNA cannot compete for the protein in the complex. The binding is therefore irreversible. More will be said later in this paper concerning the possibility of irreversible structure formation through protein-DNA interactions.

According to Zubay and Doty (87) the maximum linear dimension of DNA is about twice that of deoxyribonucleoprotein. They consider the basic unit of deoxyribonucleoprotein (DNP) of calf thymus to be a DNA molecule, molecular weight 8 million, and a protein complement of approximately the same weight. Sedimentation studies on calf thymus DNP show a fairly symmetrical distribution around 50 S for 70% of the preparation, with the remaining 30%

appearing as a diffuse leading edge of the sedimentation boundary. These results are interpreted by the same model by Zubay and Doty, and the large-sedimentation-coefficient material is attributed to aggregation by crosslinking of the basic DNP particles.

These findings were challenged by Itzhaki and Rowe (36) who presented electron micrographs suggesting that DNP in solution is an ordered aggregate, which if sufficiently disrupted, appears to break down to the units proposed by Zubay and Doty. Itzhaki and Rowe propose that chromation is composed of discrete DNP particles and the units of Zubay and Doty are the result of disruption of weak ordering forces forming the DNP particles. Itzhaki and Rowe consider the disruption to be denaturation of the DNP particles.

Tuan and Bonner (80) report that the removal of histones from DNP results in a 10% increase in the molar extinction coefficient of the DNA, which they interpret as indicating a change in the orientation of the bases of the DNA when it is complexed with the histones. The largest decrease found in their studies occurred with the removal of the more arginine-rich histones. This result is consistent with flow dichroism studies (53) by Ohba that presumably show the presence of arginine-rich histones in DNP cause tilting of DNA bases.

Bradbury and coworkers (9, 10) report that infrared absorption studies are consistent with the DNA in deoxyribonucleoprotamine and deoxyribonucleohistone being in the B form.

Olins (55) finds no change in the circular dichroism of DNA when it is complexed with the fl histone and concludes the DNA is in the B configuration when so complexed. It should be pointed out that the effect of removal of histones from deoxyribonuclehistone on the conformation of the DNA need not be the same as the addition of histones to form complexes.

The studies of the conformation of histones have not provided substantial evidence relating to histone conformations in the complex. As pointed out by Bradbury, et al. (11), it is not possible to interpret the ORD measurements in terms of a given amount of a given conformation. In their studies they have combined ORD, NMR and infrared spectroscopy measurements and report that all but the lysine-rich histones are capable of forming alpha-helical conformation in some segments of the molecule. The lysine-rich histone is characterized by its ability to form extended chain conformations, possibly due to its high proline content, which would disrupt alpha-helical configurations.

The above interpretation is consistent with the observations of Barr and Butler (2) and Johns and Butler (38) that the lysine-rich fraction is the most efficient, by far, of the histone fractions in precipitating DNA from solution. They interpret this ability to precipitate DNA so efficiently as resulting from an extended configuration of the lysine-rich histone enabling it to crosslink a large number of

DNA molecules. It may be mentioned again, however, that the mode of interaction between histone fractions when they form complexes with DNA are not necessarily the same as that when the histones are involved in native DNP particles.

Titration studies (82) of nucleohistone and deoxyribonucleohistone report the 80% of the lysine and arginine residues of DNP do not titrate. All the carboxyl, imidazole and tyrosyl groups do titrate, indicating they lie on the surface of the complex and are freely accessible to the solvent. The pK' for the carboxyl and tyrosyl groups of the histone are about one unit higher in the complex than found for free histone, and this is interpreted to suggest these groups are in the negatively charged environment of the phosphates of the DNA. In the free histone the pK' values for the lysine and arginine groups were lower than normally found for these groups, indicating they are grouped together, rather than being randomly distributed in the molecule, in agreement with the sequence analyses that have recently shown this to be the case for the arginine-rich histone (20, 52).

The solubility of DNP and DNA-histone complexes are very dependent on the ionic strength of the solution. As the ionic strength is increased from 0 to about 0.15 M NaCl the solubility decreases to a few hundredths of that at 0 ionic strength (3, 53, 87), thereafter gradually increasing up to 0.5 M where the lysine-rich histones begin

dissociating, and the solution at the same time begins to appear clearer.

Olins (55) studied the solubility of reconstituted f1-DNA complexes. The complexes were formed by the method of salt-gradient dialysis, originally developed by Huang, Bonner and Murray (32) to prepare soluble complexes of reconstituted DNP. In salt-gradient dialysis samples are mixed at high ionic strength where no interaction occurs and then the solutions are lowered in ionic strength by dialysis against decreasing salt concentration until the ionic strength is low enough for the complex to be completely formed.

Olins found that, if the peptide cation/DNA anion ratio (+/-) was greater than 0.3, gradient dialysis to 0.1 M NaCl resulted in the formation of an insoluble complex. If dialysis was carried down to 0.001 M sodium cacodylate buffer, no NaCl, complexes having (+/-) ratios all the way up to unity were soluble. The soluble complexes prepared at 0.001 M cacodylate buffer could be raised to 0.1 M NaCl with no loss of solubility. This suggests that an irreversible step in the formation of the soluble complex occurs between 0.1 and 0 M NaCl.

Johns and Forrester also studied the solubility of DNA complexes with the lysine-rich histone (39) and other histone fractions (40). DNA was considered as soluble if it remained in the supernatant after the solution was centrifuged at 2500g for 1 hour. Direct mixing

in 0.14 M NaCl causes precipitation of all the DNA when the peptide anion/DNA(P) ratio is 0.7 or greater. At a (+/-) ratio of 0.7, in glass distilled water, only 30% of the DNA is precipitated. In distilled water a (+/-) ratio of about 1.2 is the most effective ratio, precipitating 60% of the DNA. Ratios greater than this resulted in increased solubility. If mixing occurs between 0.4 and 0.5 M NaCl the solubility abrubtly changes from 0 to essentially 100%, corresponding with the observation by Ohlenbusch, et al. (54) that the lysine-rich histones dissociate and can be selectively removed from DNP between these NaCl concentrations. The decrease in solubility for a sample prepared at 0 ionic strength and subsequently raised to 0.15 M NaCl was not reversible when the solution was dialyzed back to 0 ionic strength unless EDTA was also present.

Johns and Forrester (39) find the same qualitative behavior for the other histones, though the lysine-rich histones were found to be the most efficient fraction in precipitating DNA, as has been observed in many laboratories (38, 40, 47, 53). In distilled water a ratio of histone is reached that maximally precipitates the DNA and the addition of histone beyond this ratio redissolves the complex. Less DNA is precipitated by mixing with histone at 0 ionic strength and then raising the ionic strength to a given value than is precipitated by direct mixing at that ionic strength, suggesting again, that some structural arrangement of the complex occurs irreversibly.

Irreversibility will be analyzed further in the Discussion section of this thesis.

The effect of solubilization of the complex of the lysine-rich histone and DNA by addition of excess histone beyond that required for maximal precipitation of the DNA has also been observed by Sluyser and Snellen-Jurgens (71). Boublik, Sponar and Sormova suggest that lysine-rich histones may be effective cross-linkers of DNA (7). They report, based on light scattering, viscosity and sedimentation data, that lysine-rich histone-DNA complexes show a substantial change in DNA shape, greater compactness in the complex, accompanied by little change in molecular weight. The circular dichroism data of Olins (55) indicated no change in the base orientation of lysine-rich histone-DNA complexes. The change in the shape of the DNA may therefore be due to formation of higher structures not affecting base orientation, e.g., supercoiling of the DNA. Crosslinking may be a reasonable mechanism of supercoiling.

A crosslinking model for the interaction of the lysine-rich histones with DNA is consistent with the observation by Littau et al. (47) that the disappearance and reappearance of the condensed chromatin structure correlates with the removal or addition, respectively, of the lysine-rich histones. They interpreted the condensation of chromatin by lysine-rich histones to be due to crosslinking by the histone.

Histone-DNA interaction is complex since histones contain hydrophobic and acidic as well as basic amino acids. In an attempt to separate and simplify aspects of the interactions a number of investigators have turned to the study of basic polypeptides and DNA.

The binding of polylysine to DNA has been studied by Evett (21) and Evett and Isenberg (22) by fluorescence depolarization of dye-labelled polylysine. Their studies demonstrate the applicability of such measurements to investigations of binding of dye-labelled polypeptides with DNA under a wide range of experimentally alterable conditions. Analysis of the data by assuming a two state model (see Materials and Methods) allows one to determine the fraction of dye-labelled polylysine bound as a function of the experimental conditions. Salt dissociation curves were presented for a variety of salts. The relative effectiveness of ions in dissociating the complex were found to follow the Hofmeister series, which has been shown to be the order of effectiveness of salts in denaturing a wide variety of polymers (81).

Salt addition resulted in a change in the turbidity that correlated well with the fraction of polylysine bound for the monovalent salts used. The divalent metal salts however, were characterized by high initial increases in turbidity, and often resulted in the formation of insoluble complexes that could not be studied.

Evett and Isenberg (22) point out that changes in rotatory

diffusion, which results in the depolarization of fluorescence of the dye, are more directly related to the physical process of complex formation than are other changes in the system, such as intensity changes, or turbidity of the sample. This comment suggests that polarization measurements are fundamentally related to the processes of complexing and dissociation of the dye-labelled polylysine.

Evett and Isenberg (22) reported dissociation of the polylysine-DNA complex by NaCl began when the NaCl concentration was about 0.3 M and was complete at about 1.2-1.4 M. Miller and Inbar (50) report similar results on the basis of ion exchange measurements of precipitated DNA-polylysine interaction products. Up to about 0.4 M NaCl no polylysine-DNA ionic bonds were disrupted and by 1.2-1.4 M NaCl all the bonds had been disrupted.

Equilibrium dialysis studies by Latt and Sober (42) report the binding is largely electrostatic based on the observation that Na^+ strongly inhibits the binding of polylysine to poly(I+C) and poly(A+U), the Na^+ being able to compete strongly for the phosphate binding sites.

The binding of lysine, arginine, tetralysine and the ions Na^+, Li^+, Cs^+ and K^+ are found to bind equally tightly to DNAs of any base composition in equilibrium dialysis measurements by Shapiro, Stannard and Felsenfeld (69). Tetramethylammonium ion (TMA^+) however is found to bind more tightly to A-T rich DNA. They suggest this specificity arises due to weak hydrophobic forces between TMA^+

and A-T rich DNA. It is also noted that tetrabutylammonium ion, which is too large to fit into the major groove of DNA, does not exhibit any base composition selectivity in its binding to DNA.

Solubility properties of the complex of basic polypeptides with nucleic acids are similar to those of the histone-DNA complexes. Sober and coworkers (72) find direct mixing of polylysine with RNA in low ionic strength results in formation of a precipitate having 1:1 stoichiometry between lysyl residues and nucleotides when equivalent amounts of polylysine are added. Soluble complexes result when the lysine/nucleotide ratio is less than 0.5.

Evett (21) found the turbidity of polylysine-DNA complexes prepared by gradient dialysis to 0.0 M salt showed a slight increase when the monovalent salt concentration was raised to about 0.05 M but the turbidity then decreased as salt concentration further increased, in the same fashion as the fraction of polylysine bound decreased. He suggested the slight initial increase may reflect a change in the state of aggregation or solubility of the complex.

Tsuboi, Matsuo and Ts'o (79) find the complex less soluble at 0.03 than at 5.6×10^{-4} M NaCl when the polylysine is complexed by direct mixing with DNA at these ionic strengths. Matsuo et al (49), Cohen and Kidson (15) and Olins, Olins and von Hippel (56) all report complexes formed by direct mixing have lower turbidity and higher solubility at low ionic strength than those prepared by

gradient dialysis. Others (Raukas (63) and Inoue and Ando (34)) however, report it was necessary to use sonicated DNA in order to produce soluble complexes by direct mixing at low ionic strength. This apparent contradiction may be reconciled by the work of Tsuboi, Matsuo and Ts'o (79). They found that the rate of addition of polylysine to the DNA solution in direct mixing preparations affected the stoichiometry of the complex. If polylysine is added quickly, two species of DNA result: one essentially completely complexed with polylysine and another that is essentially free. Slow addition, with very complete mixing resulted in a distribution of DNA from essentially completely complexed to free DNA. This suggests that the binding is irreversible at low ionic strength and the polylysine binds with the first DNA it comes in contact with. The inability to produce soluble complexes reported above may be due then, to mixing the solutions so rapidly and with a high enough concentration of components that the polylysine is irreversibly bound to several DNA molecules simultaneously, causing a large crosslinked network that is insoluble.

Inoue and Ando (34) find that in the presence of free DNA molecules, 1:1 complexes of DNA with clupeine are dispersed in solution and precipitated only by high speed centrifugation. However, in the absence of free DNA, the complexes precipitate without centrifugal force, even at 0.1 times the concentration of that in the

solution having free DNA. Thus free DNA appears to have the effect of solubilizing the complexed DNA whenboth species are present on the same solution, even though the complex is irreversibly formed.

Spitnik, Lipshitz and Chargaff (73) found that when DNA was extracted from polylysine-DNA complexes by increasing the ionic strength, the first DNA extracted was richer in G-C than subsequent fractions, suggesting the interaction of polylysine was preferentially greater with A-T rich DNA. A-T specificity in the interaction with histones was also suggested by the earlier demonstration by Brown and Watson (12) that G-C rich DNA was extracted first by increasing salt gradient elution of DNA from a histone-kieselguhr column. Similar specificity by polylysine for A-T rich DNA was found by Ayad and Blamire (1) and Helleiner (28) using salt gradient elution of DNA from polylysine-kieselguhr columns.

Felsenfeld and co-workers (44, 68) report that, under appropriate conditions, polylysine exhibits an almost perfect selectivity for A-T rich DNA, as judged by preferential precipitation. Cohen and Kidson (15) find the magnitude of spectral changes in the ORD of DNA when complexed with polylysine increases as A-T content increases. Olins, Olins and von Hippel (56) and Raukas (63) have found polylysine preferentially stabilizes A-T rich regions of DNA against thermal denaturation. It may be noted, however, that neither of these latter observations need be interpreted in terms of selective

interaction of polylysine for A-T rich DNA. It may only mean the interaction with A-T pairs is different than it is with G-C pairs, and is not necessarily selective.

The interaction of polylysine with DNA has been demonstrated to be irreversible at low ionic strength. Tsuboi, Matsuo and Ts'o (79) found that when polylysine was added to DNA at low ionic strength and then DNA labelled with ^{32}P subsequently added and the mixture put aside for 19 hours at 5°C or 30 minutes at 25°C, the labelled DNA was found to migrate the same as free DNA upon electrophoretic analysis, i. e. it could not compete for the polylysine after the complex had formed. Polylysine-DNA complexes prepared by salt-gradient dialysis show a biphasic thermal denaturation profile when the polylysine has more than about eight residues and there is less than a stoichiometric amount of polylysine (34, 56, 57, 63, 79). Von Hippel and co-workers have taken this existence of two DNA species as evidence of irreversible binding of the polylysine to the DNA (56). If the polylysine were reversibly bound, all of the DNA should be affected the same way by the polylysine with regard to melting, and only one melting transition would be observed.

Spitnik, Lipshitz and Chargaff (73) observed that direct mixing at low ionic strength produced soluble complexes if the concentration of components was less than 10^{-4} M, and insoluble complexes if they were greater than 10^{-4} M, but that it was possible to raise the

concentration of the soluble complex (after it was formed) to greater than 10^{-4} M with no loss in solubility. This also suggests the interaction leading to the formation of the complex is irreversible at low ionic strength. (The complex here refers to the system resulting from the interaction of polylysine with DNA.)

A further indication of irreversibility of the interaction of the complex at low ionic strength is given by Olins et al. (57). Complexes of polylysine having an average degree of polymerization ($\overline{DP}$) greater than about 20 gave much broader melting curves for the complexed DNA when prepared by direct mixing at low ionic strength than when prepared by gradient dialysis. They say that samples prepared by gradient dialysis, though in an irreversible state at the time measurements are taken, reflect the binding specificities characteristic of the interaction at high salt, reversible-equilibrium conditions, and that the characteristics of the high salt equilibrium have been "frozen in" when the salt concentration is lowered. Direct mixing at low ionic strength never allows the system to be in the state of reversible, equilibrium interaction and therefore one does not expect the samples prepared in this manner to behave like those prepared by gradient dialysis, if the interaction at low salt is irreversible, in agreement with their observations.

Felsenfeld and co-workers (44, 68) find that the addition of polylysine to DNA at 1.0 M NaCl results in the formation of an

aggregated phase which is pelletable by 25,000g in 20 to 30 minutes. DNA added after such complexes are formed is found to exchange with the DNA in the complex, although the exchange is not complete, and is less complete as the percentage of DNA complexed in the original solution approaches 100. They interpret this exchangeability to mean that the interaction leading to complex formation and the interaction leading to formation of the aggregated phase are reversible. It is not necessary to consider complex formation reversible because of the exchangeability of DNA, however. Leng and Felsenfeld (44) say that the selectivity by polylysine for interaction with A-T rich DNA also indicates the interaction must be reversible. If it were not reversible polylysine would bind with the first DNA molecule it encountered and no selective mechanism could operate. Exchangeability and reversibility will be considered in greater detail in the discussion.

The stoichiometry of the interaction is of interest as it may suggest the mechanism of the interaction. Felsenfeld and co-workers (44, 68) report the concentration and chain length of the polylysine, the concentration and type of salt and the pH of the solution are all parameters affecting the conditions under which stoichiometric precipitation of DNA takes place. For example, the NaCl concentration at which stoichiometric precipitation occurs for polylysine having $\overline{DP}$ of 7 is 0.85 M compared to 1.0 M for polylysine of $\overline{DP}$ = 100.

At salt concentrations below that giving 1:1 stoichiometry, the ratio of lysine to DNA is less than 1:1 and at higher salt concentrations the ratio of lysine to DNA in the precipitate is greater than 1:1. High pH (10.8) requires high excesses of polylysine ($\overline{DP}$ = 100) and low ionic strength (10^{-2} M NaCl) to precipitate DNA by centrifugation. This is presumably due to decreases in the electrostatic interaction because of the decrease in charges on the lysine residues. Thus stoichiometry at high ionic strength, or other solvent conditions where the reaction is "reversible," is rather ill-defined. 1:1 stoichiometry for the interaction appears to be a property of the experiment and not a fundamental characteristic of the interaction in these cases.

The evidence that the complex is stoichiometric at low ionic strengths, where the binding is irreversible, is more firmly established. When complexes are formed either by gradient dialysis or direct mixing, and have less than a stoichiometric amount of polypeptide, a biphasic thermal denaturation of the DNA is observed. The higher melting-temperature transition has been shown to be due to the melting of DNA complexed with polypeptide (56). The percentage of the DNA undergoing the second thermal transition is equal to the stoichiometry of the solution (34, 56, 57, 63, 79).

Von Hippel and co-workers (56, 57) find for their solutions (prepared by salt-gradient dialysis), that it is possible to

subfractionate the solution into two DNA species by high speed centrifugation, one being "naked" DNA having no associated polypeptide and the other, DNA having characteristics of the complex, which they call "purified complex." The peptide cation/DNA anion ratios of the "purified complex" for poly-L-ornithine, poly-L-lysine, poly-L-arginine and poly-L-homoargine were 0.9-1.2, 1.0-1.1, 1.0-1.3, and 1.3-1.7, respectively. Except for poly-L-homoarginine, the results suggest the complex is stoichiometric within experimental error.

When soluble complexes of polylysine-RNA prepared by direct mixing of polylysine with RNA in a lysine/nucleotide ratio of 0.5 were digested with RNase, a precipitate formed which was found to contain equivalent portions of lysyl residues and nucleotides (Sober et al. (72)). The length of the "protected" RNA fragment was equal in length to the length of the polylysine, which in addition to indicating the stoichiometry of the complex, also rules out crosslinking as the cause of precipitation in this system. Firstly, the complexes were soluble before digestion with RNase and the polylysine is irreversibly bound, therefore, it could not redistribute to form insoluable complexes; and secondly, if precipitation resulted from random crosslinking the average chain length of a protected RNA segment would be expected to be the ratio of total to uncomplexed phosphates, and therefore be insensitive to polylysine chain length.

The ability to separate the solution into DNA essentially all free and DNA essentially fully complexed in the above report by von Hippel and co-workers suggests the interaction leading to formation of the complex is cooperative. Similarly, Inoue and Ando (34) find, for polyarginine and clupeine complexes, when arginine:phosphorous input ratios are less than unity for samples prepared by gradient dialysis, the DNA molecules can be fractionated into an essentially free species and those fully complexed. In contrast, samples prepared by direct mixing (Tsuboi, Matsuo and Ts'o (79)) indicate the polylysine does not bind cooperatively, but is more randomly distributed on the DNA. This is probably due to the fact that in direct mixing preparations the polylysine and DNA never are in solvent conditions where "reversible" binding can occur and the random binding reflects the "first encounter" distribution of the polylysine with the DNA in solution.

Felsenfeld and co-workers (44) say the binding must be cooperative in order to account for the selectivity for A-T rich DNA and also the stoichiometry of the interaction. That is, if polylysine preferentially interacts with A-T rich DNA the reaction must be followed by the cooperative binding of polylysine to the rest of the A-T rich DNA molecule rather than to A-T rich regions of an A-T poorer DNA if the A-T rich DNA is to be preferentially precipitated as a 1:1 stoichiometric complex.

The later report by Felsenfeld and co-workers (68), however, that tetralysine exhibits A-T selectivity provides an example where the conditions of the above argument must be weakened. The stoichiometry of selectivity by tetralysine is 6, or greater, to 1. It has also been demonstrated by von Hippel and co-workers (57) that tetralysine does not bind cooperatively, being more like salts, spermine and diamines which do not bind cooperatively, in its interaction with DNA. Sober et al. (72) also finds polylysines shorter than heptalysine do not exhibit cooperative binding behaviour.

Various techniques have been used to study the conformational changes that occur in DNA and polypeptides when they form complexes. Hydrogen-exchange studies by Lees and von Hippel (43) show that about one-fourth of the interchain DNA hydrogens are shifted into an "instantaneously" exchanging class on interaction with poly-L-lysine, though the exchange rate of the remaining hydrogens is essentially unaffected. They interpret this as evidence of a conformational change of the DNA as a result of complex formation. The peptide hydrogens exchange appreciably more slowly in the native DNA-polylysine complex, suggesting that these hydrogens are partially shielded from the aqueous environment in this system.

Inoue and Ando (33, 35) find slight changes in the ORD spectra of DNA when complexed with clupeine or $(\text{arginine})_{20}$ by direct mixing. Complexes with polylysine and polyornithine (both $\overline{DP} = 20$)

however show very large changes. Shapiro, Leng and Felsenfeld (68) report changes in the ORD spectra of DNA when complexed with polylysine for samples prepared by gradient dialysis from 1.5 M down to 0.85-1.0 M NaCl. The ORD changes observed were qualitatively but not quantitatively reproducible, suggesting again the system they are studying is not reversible. In agreement with this interpretation is their observation that samples prepared by direct mixing have similar changes in ORD but are of considerably smaller magnitude. Inoue and Ando (35) report that the ORD spectra they measured were obtainable with good reproducibility under constant conditions of sample preparation. It seems unlikely that the changes observed are artifacts due to scattering because of turbid samples. The least turbid sample, polylysine-DNA, yielded the greatest change.

ORD measurements on polylysine-DNA complexes, prepared by salt-gradient dialysis down to 0.3 M NaCl, also indicate changes in the ORD spectra of the DNA (Cohen and Kidson (15)). The spectral change occurs in proportion to the relative amounts of DNA free and complexed and the changes also are dependent on the A-T content of the DNA, the red shift of the negative peak being clearly related to the A-T content of the DNA. (They also report reproducible results.) These authors attribute the spectral changes caused by binding of polylysine to either a change in the pitch of the helix or a tilting of the bases. Inoue and Ando (35) however, note that too little

information correlating the conformational changes of DNA with the ORD spectra is available to interpret the results in terms of any specific model.

The terms "complex," "aggregate," and "precipitate" are used in describing the product of DNA-polylysine interaction. Some comment on the use of these terms might be appropriate. Most workers use the term complex to refer to the association of a polylysine molecule or molecules with a DNA molecule to form a well-defined structure. In many cases, however, the same term is then applied to the aggregation of these structures and the complex is often taken to mean the large aggregated particles, of which the polylysine-DNA structures are the components. In this thesis, insofar as it appears possible to do, the various entities will be differentiated and referred to by different terms.

Felsenfeld and co-workers (68) report the sedimentation coefficient of the separate aggregated phase to be between 5,000 and 10,000 S for samples prepared by gradient dialysis from 1.5 M to 0.85 M NaCl. The report the particle (aggregated phase) is highly hydrated, 96% water by weight. Matsuo et al. (49) find the polylysine-DNA "complex" prepared by direct mixing at low ionic strength to be soluble in the usual gravitational field but insoluble at 5,000g. Electron microscopy of the "complex" show it to be an aggregate of variable size. Polylysine-RNA complexes gave similar results

except the size of the complex was much more uniform as judged by electron microscopy.

Several studies have been made on the interaction of polylysine with polyacids other than nucleic acids and on the nature of the polylysine itself in various solvents leading to conformational changes. Davidson and Fasman (19) have extended the polylysine polynucleotide interaction studies to the polylysine-single-stranded polyadenylic acid complex. Stoichiometric complexes are formed and ORD measurements show the complex to have highly ordered structure. Formation of the stoichiometric complex is insensitive to ionic strength over the range 0 to 0.2 M NaF. Moreover, complex formation is insensitive to the degree of charge of the polylysine over the range 100% to 40%, demonstrating the interaction leading to complex formation is not exclusively electrostatic.

Davidson and Fasman (19) also report that, as 1:1 stoichiometry of the complex is approached, aggregation occurs with time, and this aggregation causes further changes in the ORD spectra beyond the changes observed after initial complex formation. Similar results were indicated by Cohen and Kidson (15) who reported centrifugation at 12,000g removed the "insoluble," aggregated complex and caused a shift in the negative peak from that observed for the remaining "soluble" complex. Davidson and Fasman suggest this may represent some structural character of the aggregated phase.

Felsenfeld and co-workers (68) have also suggested the ORD spectra they observed could be due to long-range order present in the aggregated phase, rather than necessarily indicating a change in DNA conformation.

The interaction of polylysine with a number of polyacids results in aggregation and precipitation of the complex when charge equivalence is approached (Gratzer and McPhie (27)). The ability of dioxane, which lowered the dielectric constant of the solution from 81 to 56, to clear the solution suggests the aggregation is due to hydrophobic interactions rather than crosslinking, as lowering the dielectric constant should enhance electrostatic site binding and consequently not disrupt existing crosslinking. The binding of polyacids that have little intrinsic secondary conformation, e.g. poly(acrylic acid) and poly(phosphoric acid), form complexes with polylysine in which the solutions show a cotton effect with a trough at 233 nm, interpreted as alpha helix formation. No such trough is found when the polyacid is poly(uridylic acid), RNA, native or denatured DNA.

In summarizing the literature several general features emerge:

1. Complexes are generally turbid and relatively insoluble at fairly low ionic strength (73). Direct mixing produces more soluble, less turbid complexes than salt-gradient dialysis (57). Direct mixing with unsonicated DNA sometimes produces fibrous precipitates (34) whereas soluble complexes are formed when the DNA is sonicated

prior to mixing (63). The relationship between complexes formed with sonicated and unsonicated DNA is unknown. The complex is generally insoluble when charge equivalence between polycation and polyanion is reached (27, 56, 77).

2. The complex is reported to be stoichiometric, reversible and cooperative at 1.0 M NaCl (44). At low ionic strength the interaction is irreversible and stoichiometric (19, 56). Samples prepared by gradient dialysis also indicate the interaction is cooperative, at least at some point before the binding becomes irreversible.

3. The binding is a function, not only of the ionic strength of the solution, but also is dependent on which salt is used. The demonstration that the effect of the type of salt used on the dissociation follows the Hofmeister series indicates that more than electrostatic interactions are involved in the binding of polylysine to DNA (21, 22).

4. Changes in ORD spectra of DNA when complexed with polylysine suggest conformational changes might occur when polylysine binds (15, 35), but these changes may also be indicative of the formation of long-range order in the aggregated phase resulting from formation of complexes (19, 68). Possible changes in the DNA conformation are also suggested by the shifting of one-fourth of the DNA interchain hydrogens to the instantaneously exchanging class when the complex is formed (43). Marked stabilization of the double stranded structure of DNA against thermal denaturation results from

formation of a complex with polylysine (56, 63).

5. The interaction of polylysine is preferential for A-T rich DNA (1, 28, 44, 73) and A-T rich regions of DNA are preferentially stabilized against thermal denaturation (56).

Several areas of contradiction are noted in the literature surveyed. For example, Olins, Olins and von Hippel (57) find the interaction is not cooperative if the polylysine has a chain length less than eight residues, and certainly is not cooperative for tetralysine. Yet Shapiro, Leng and Felsenfeld (68) find tetralysine will preferentially precipitate A-T rich DNA, and they say selectivity indicates that the interaction is cooperative. It may be seen that these investigators use different methods to measure the result of the interaction. Felsenfeld and co-workers' measure of the cooperativity is defined operationally by sedimentation of an aggregated phase resulting from the interaction of polylysine with DNA at 1.0 M NaCl. All the properties of the aggregated phase are apparently assumed to reflect properties of the interaction of polylysine with DNA.

The studies of von Hippel and co-workers were made under irreversible conditions and binding is operationally defined here as the ability to stabilize the DNA against thermal denaturation. The properties of the irreversible state are assumed to reflect the properties of the interaction under reversible, equilibrium conditions.

In all the studies made there is no mention made of the

possibility of the existence of more than one type of complex, although a wide variety of solvent conditions and preparational procedures are employed in the studies. It has not been demonstrated that the aggregated phase studied under varying solvent conditions is the same aggregated phase or that the properties of the aggregated phase can be used to infer the nature of the interaction of polylysine with DNA at the polymer-polymer level.

MATERIAL AND METHODS

Analysis of Fluorescence Polarization and Turbidity Data

The analysis of NaCl dissociation of the complex as measured by fluorescence depolarization has been described by Evett and Isenberg (22). Analysis is based on the addition law for polarization (Weber (83)) which states that, for a multicomponent solution,

$$\mu = \sum_i \mu_i \phi_i$$

where μ_i* is the anisotropy of the light emitted by the i^{th} species, μ is the measured anisotrophy and ϕ_i is the fraction of light emitted by the i^{th} species. A two-state model is used which assumes there are essentially only two species of dansyl polylysine: that bound and having a characteristic anisotropy, μ_1; and that which is free, also having a characteristic anisotropy, μ_2. On the basis of this model it was shown that the fraction bound, f(c), is given by

$$f(c) = \frac{I(c)}{I(0)} \frac{\epsilon_1(0)q_1(0)}{\epsilon_1(c)q_1(c)} \phi_1$$

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* As presented by Jablonski (26), the anisotropy is a better measure of the angular anisotropy of the emitted light than the commonly employed polarization. The anisotropy is defined as $\mu = \frac{3}{2} \frac{E - B}{E + 2B}$ where E and B are the intensities of emitted light with polarizations parallel and perpendicular to the polarization of the exiciting beam, respectively.

where I(c) is the intensity of emitted light at a given salt concentration, c; I(0) is the intensity of emitted light before the addition of salt, when all polylysine is assumed to be bound; $\epsilon_1(0)\, q_1(0)$ and $\epsilon_1(c)\, q_1(c)$ are the molar extinction coefficients and quantum yields, respectively, at the initial and subsequent salt concentrations. It is assumed that

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

i.e., the bound dansyl polylysine does not have any large direct interaction with the salt causing variation in these properties of the dye. It may then be shown that f(c) is related to measurable parameters by the following relationship:

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

where μ is the measured anisotropy and the other parameters are as defined previously. The studies by Evett and Isenberg (22) demonstrated the applicability of this method of studying the interaction of polylysine and DNA and also showed, that under the conditions their measurements were made, the assumptions made in the derivation were consistent with the data.

The fractional decrement of turbidity, B(c) may be defined (22) as:

$$B(c) = \frac{A(c) - A(\infty)}{A(0) - A(\infty)} ,$$

where A(c), A(0) and A(∞) are the turbidities of the solution at a given salt concentration, c, the initial turbidity and the final turbidity, respectively. The study demonstrated a very close agreement between B(c) and f(c) under appropriate conditions.

Preparation of Samples

Polylysine (molecular weight = 62,000, degree of polymerization = 300) was purchased from Miles-Yeda Ltd. (code 8120 B, Lot No. LY104). Analysis of this polylysine by Spencer L. Baird, Jr., using an exponential sodium chloride gradient elution from a carboxymethyl cellulose column (76), indicate a single range of molecular weights centered around a degree of polymerization of 275.

Dansyl polylysine (DPL) was prepared by one of two methods. The first is a modification of a standard method (22, 84) for conjugation of dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) to polylysine. The following is a typical preparation: 21.2mg of polylysine was dissolved in 10 ml water and the pH of the solution adjusted to 9.2 with 2N NaOH. Double distilled deionized water was used in all studies.

3.1 mg of dansyl chloride (Aldrich Chemical Co.) was dissolved in 25.0 ml of Baker "GC-Spectrophotometric Quality" acetone. 10.5 ml of the dansyl chloride-acetone solution was dripped from a separatory funnel at a rate of approximately 0.5 ml/min. into the

polylysine solution, with continuous stirring. The solutions were placed in the dark during the addition of the dye and the mixture was allowed to continue stirring an additional 2 hours after the addition of the dansyl chloride-acetone solution had been completed. The acetone was removed by flash evaporation and the DPL solution then passed through a Sephadex G-10 column to remove any unreacted dye. The lysine/dye ratio was calculated from lysine concentration determinations made on a Beckman amino acid analyzer and dansyl concentration determined from $0.D._{330}^{1\ cm}$ measurements on a Cary Model 14 Spectrophotometer.

The second method uses celite as an inert carrier of dansyl chloride (67), eliminating the use of acetone. Celite (Johns-Mansville Celite 503) was first washed by soaking 15 hours in 0.1 M EDTA, rinsed 10 times in glass-distilled water and then dried in vacuo over P_2O_5. Dansyl chloride was then absorbed on celite. Absorption of the dye was accomplished by suspending the celite in a methanol solution of dansyl chloride and then removing the methanol by flash evaporation. Dye was absorbed on the celite in a weight ratio of 3.82 mg dye/gm. celite . Labeling of polylysine then followed the same procedure used with the acetone method except the appropriate amount of celite to give the required concentration of dansyl chloride was substituted for the dansyl chloride-acetone solution. The mixture was stirred fast enough to insure uniform

suspension of the celite for about 9 hours. The solution was then centrifuged to precipitate the celite. The supernatant was subsequently treated in the same manner as in the previous method after the step removing the acetone by flash evaporation.

Rhodamine B-polylysine (RBPL) was prepared in essentially the same manner as dansyl-polylysine.

Rhodamine B sulfonyl chloride was prepared as outlined by Chadwick, McEntegart and Nairn (13). One gm of Lissamine Rhodamine B200 (K & K Laboratories) was ground together with 2 gm PCl_5 with a mortar and pestil for 5 minutes under the hood. Ten ml of Baker Spectroquality grade acetone was added and the solution stirred an additional 5 minutes. The solution was thenfiltered and diluted to 250 ml with acetone. 0.525 mls of this solution was then added to 10 ml acetone and the resulting solution dripped into a stirring sample of 10^{-2} M lysine (pH 9.2) as outlined in the procedure for the preparation of DPL. After the acetone was removed by flash evaporation, the material was passed through a Sephadex G-10 column and the dye to lysine ratio determined in the same manner as for DPL.

The method of preparation and degree of labeling of the dye labelled polylysines used in these studies are indicated in Table I.

Samples of DPL-DNA and RBPL-DNA complexes were prepared

Table I. Preparation and Labeling of Polylysine

Preparation	Method Used	Lysine Residues Per Dye Molecule
DPL(I)	Celite	34.4
DPL(II)	Celite	34.2
DPL(II)	Acetone	20.5
DPL(IV)	Acetone	50.2
RBPL	Acetone	40.0

by salt-gradient dialysis (22, 32, 56). In general the procedure was as follows: DNA and dye-labelled polylysine to give the desired final concentrations were measured out in separate beakers. Salt and buffer were added to bring the solutions to the appropriate volume, at 2.6 M NaCl. The polylysine solution was then slowly added to the DNA solution, while stirred continuously. The mixture was then put in dialysis bags and dialyzed against solutions of decreasing salt concentration. The details of each salt-gradient dialysis are given in Tables II-VI. Dialysis bags (Union Carbide, "18/100") were prepared by boiling for at least one hour in the following series of solutions: once in glass distilled water, twice in 0.05 M EDTA, twice more in glass distilled water. They were stored in glass distilled water and given a final rinse with buffer immediately before use.

Direct-mixed samples were prepared in the following manner: To 3.0 ml of 10^{-3} M DNA solution, at the desired NaCl concentration, 3.0 ml of 2×10^{-4} M DPL solution, at the same NaCl concentration

was added dropwise from a syringe at a rate of about 0.3 ml/min. The DNA solution was rapidly stirred on a cyclo mixer during the addition of the DPL.

Salt dissociations were made by adding dry salt (reagent grade, J. T. Baker Chemical Co.) to the sample in either a beaker or a fluorescence cuvette. The solution was stirred 5 min to dissolve the salt. The concentration of salt in the solution, after any given addition of salt, was calculated from the weight of salt added, the volume of the solution and the previous salt concentration. The volume of solution was obtained from the net weight of the solution and the density of the solution, corrected for density changes due to the addition of salt. All measurements were corrected for dilution due to the addition of salt. The salt was dried by heating at 90°C in a vacuum oven for 24 hours.

Turbidity measurements were made at 500 nm with a Cary Model 14 spectrophotometer using a 0 to 0.1 absorbance slide wire.

Anisotropy and intensity measurements were made using an instrument built in this laboratory and previously described (22). Several modifications have been made on the instrument for these studies. A 200 watt high pressure mercury lamp (Ushio Electric Inc., type 200 D) was used for part of this work in place of the xenon lamp that was initially used. Measurements of I_V and I_H (intensity of the vertically and horizontally polarized components of the emission)

were made directly and the anisotropy and intensity of emission calculated from these. Both signals were routed, alternately, through one lock-in amplifier (Princeton Applied Research model HR-8, with modifications by P.A.R. to decrease noise and increase stability). Measurements were made at 365.2 nm to take advantage of the high intensity of the Hg line emitted at this wavelength by the lamp. Artifacts due to stray light were minimized or eliminated by use of a double monochromator in the exciting beam and long-wave pass interference filters (Optics Technology Inc.) having a cutoff at 501 nm placed in the emission beam.

Sedimentation velocity measurements were made on a Beckman Model E analytical ultracentrifuge equipped with the Beckman photoelectric scanning system and multiplex. An AN-J type rotor was used, making it possible to take data on three samples simultaneously.

Sedimentation of "insoluble" complex to separate it from "soluble" complex (Olins et al. (56)) was done on a Sorvall Superspeed RC2-B centrifuge using a type SS-34 rotor.

In the following tables the details of preparation of the complexes used in this study are given. The complexes have been assigned a letter designation for convenience of reference. In cases where a simple name or abbreviation is more descriptive of the complex, such a name has been also assigned to aid in orienting the reader.

Table II. Details of Preparation of Complex A

Before Dialysis	
Lysine to dansyl ratio (DPL(II))	34.2
Calf thymus DNA(P) molarity	5×10^{-4}
DPL molarity in lysine residues	1×10^{-4}
DNA(P)/lys ratio	5
Dialysis	
NaCl Molarity sequence of dialysate changes (0.01 M cacodylate buffer, pH 6.50): 1.2 (5 hr) → .9 (8.5 hr) → .72 (8 hr) → .50 (22 hr) → .3 (24 hr) → .05 (24 hr) → .05 (10 hr). Total time 4^{+} days.	
Characterization	
The sample had low turbidity and no visible precipitated material and was not centrifuged.	
$A^{1\ cm}_{550\ nm}$	.0548
Calf thymus DNA(P) molarity	5×10^{-4}
DPL molarity	1×10^{-4}
DNA(P)/lysine	5
This sample will be named Complex A.	

Table III. Details of Preparation of Complex B

Before Dialysis	
Dansyl polylysine	DPL I
Calf thymus DNA(P) molarity	5×10^{-4} M
DPL molarity in lysine residues	1×10^{-4} M
DNA(P)/lys. ratio	5:1
Dialysis	
NaCl molarity sequence of dialysate changes (cacodylate buffer: .01 M, pH 6.0).	
1.2 (5 hr) → 0.8 (10.5 hr) → 0.4 (9 hr) → 0.3 (6 hr) → 0.1	
11 hr) → .05 (6 hr) → 0.05 (67 hr). Total dialysis time: 4^{+} days.	
Centrifugation and Characterization	
Solution centrifuges 90 min. at 30,000 g (5°C)	
Supernatant	
DNA(P) molarity	2.27×10^{-4}
Lys molarity	$\sim 10^{-5}$
DNA(P)/lysine	22.7
Precipitate (resuspended by gentle stirring in .05 M NaCl, .01 M cacodylate buffer).	
DNA(P) molarity	2.73×10^{-4}
Lysine molarity	9×10^{-5}
DNA(P)/lysine	3.08

These samples will be named Complex B (resus), B(sn) and B(orig) to designate the resuspended pellet, the supernatant and the original salt-gradient dialyzed complex before centrifugation.

Table IV. Details of Preparation of the RBPL-DNA Complex

Before Dialysis	
Lysine to dye ratio (RBPL)	40.0
RBPL molarity in lysine residues	1×10^{-4}
Calf thymus DNA(P) molarity	5×10^{-4}
DNA(P)/Lysine ratio	5

Dialysis

NaCl molarity sequence of dialysate changes (0.01 M cacodylate buffer, pH 6.25).

1.2 (16 hr) → 1.0 (40 hr) → .8 (28 hr) → .7 (24 hr) → .6 (28 hr) → .5 (27 hr) → .4 (18 hr) → .2 (28 hr) → .1 (5 1/2 hr) → .05 (84 hr). Total time: 11 1/2 days.

Characterization

The solution was slightly turbid, but had no visible precipitated material. It was not centrifuged.

Table V. Details of Preparation of Series of Complexes having Different DNA(P)/Lysine Ratios

Before Dialysis	
Lysine to dansyl ratio (DPL(III))	20.5
DPL molarity in lysine residues	1×10^{-4}
Calf thymus DNA(μ) molarity	$1, 2, 4, \ldots, 18, 20, 25, 30 \times 10^{-4}$
DNA(P)/Lys. ratio	1, 2, 4, ... 18, 20, 25, 30

Dialysis

NaCl molarity sequence of dialysate changes (0.01 M cacodylate buffer, pH 6.00).

1.2 (10 hr) → 1.1 (10.5 hr) → 1.0 (12.5 hr) →.85 (10 hr) → .7 (15 hr) →.55 (7 hr) →.4 (17 hr) → .2 (47 hr) → .1 (31 hr) → .05 (18 hr) → .05 (24) → .05 (48) → .05 (24 hr). Total dialysis time: 11 1/2 days.

The samples were not centrifuged. This series will be designated D/L(1), D/L(2),..., etc., where the member in parentheses indicates to the ratio of DNA(P) to lysine residues.

Table VI. Details of Preparation of a Series of Complexes Salt-Gradient Dialyzed to Different Final NaCl Concentrations

Before Dialysis	
Lysine to dansyl ratio (DPL (IV))	50.2
DPL molarity in lysine residues	1×10^{-4}
Calf thymus DNA(P) molarity	5×10^{-4}
DNA(P)/Lys. ratio	5

Dialysis

15 ml aliquots of the DPL-DNA mixture were put in dialysis bags and dialysis begun in 2.6 M NaCl, .01 M cacodylate buffer. After 10 hrs one bag was removed (designated as Complex E(2.6)) and the NaCl concentration lowered to 2.4 M. After 7 hours one bag was removed and dialyzed against 1 liter of 2.4 M NaCl, .01 M cacodylate buffer, and the other dialysate lowered to 2.2 M. This process was continued, lowering the NaCl concentration by 0.2 M increments until 0.2 M was reached, and then samples at .1, .05 and 0 M NaCl were prepared. Each sample was dialyzed against the NaCl concentration ±.05 M of its designation 7 hrs, and then an additional 7 hrs against 1 liter of that NaCl concentration ±.001 M.

The samples will be called Complexes, S.A.(c) where c is the final NaCl concentration to which the complex was dialyzed.

EXPERIMENTAL RESULTS

The affect of pH on the anisotropy for DPL(I) is shown in Figure 1. The helix→coil transition is clearly seen in the change in anisotropy as a function of pH. The midpoint of the transition occurs at pH 9.9, in very good agreement with the transition midpoint (pH 10.0) reported by Rifkind (64). These results are in excellent agreement with those reported by Evett (21) using dansyl polylysine having a $\overline{DP}$ = 106.

The anisotropy, intensity and fraction of DPL bound, f(c), for Complex A for a series of NaCl dissociations are shown in Figures 2, 3, and 4, respectively. The data for all three parameters is remarkably reproducible for the three salt dissociations. A comparison of the NaCl concentration at which f(c) = 0.5 with the values obtained by Evett (21) for his Complex E are in good agreement (0.90 compared with 0.86-0.88) and excellent agreement is found between the curves over the entire range of salt concentrations.

Complex B (resus) was prepared for a pH titration of a series of NaCl dissociations. In the preparation it was observed that when gradient dialysis reached 0.8 M NaCl the samples began to appear turbid, and the turbidity increased as the concentration was lowered to 0.3, although there was never a visible precipitate formed, nor any observable settling of material to the bottom of the dialysis bag.

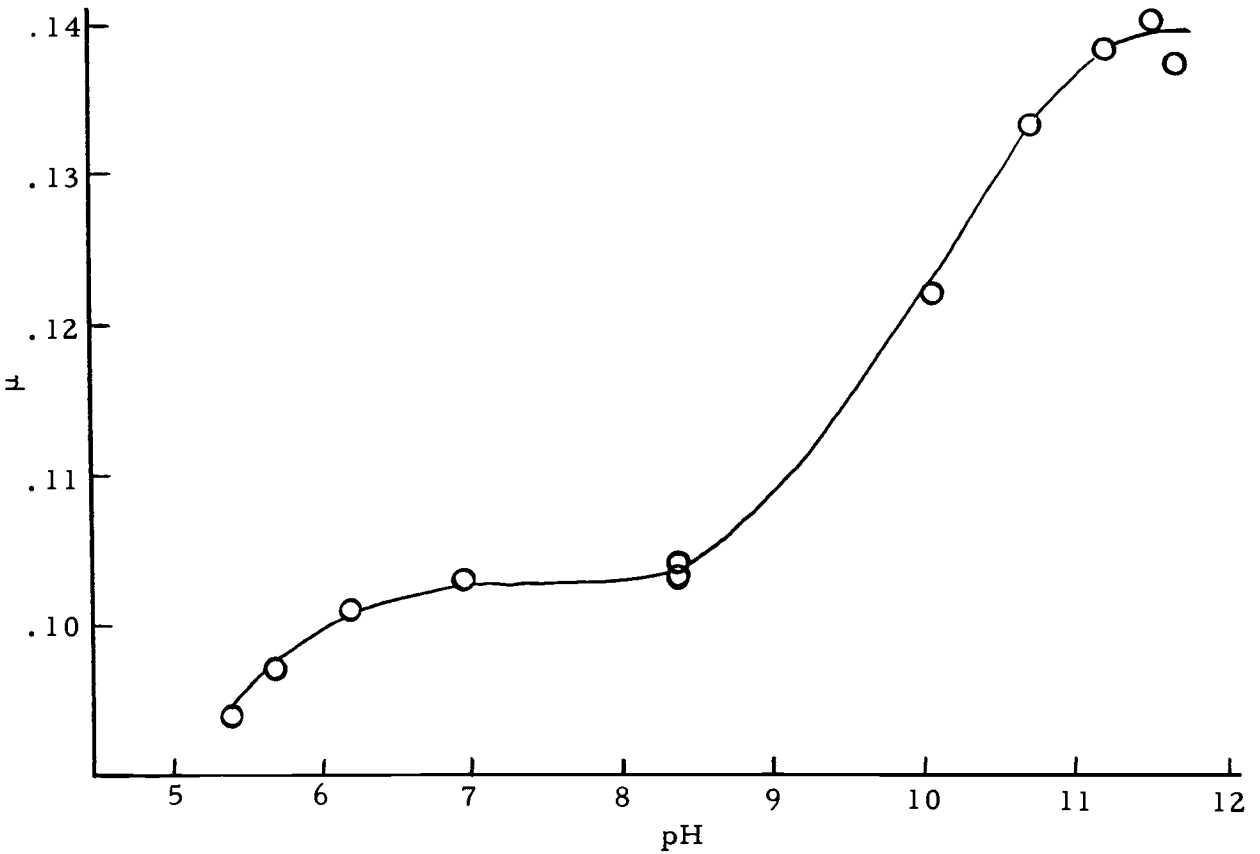

Figure 1. Effect of pH on anisotropy of DPL(I). Lysine concentration = 1.24×10^{-4}M. Exciting wavelength = 335 nm.

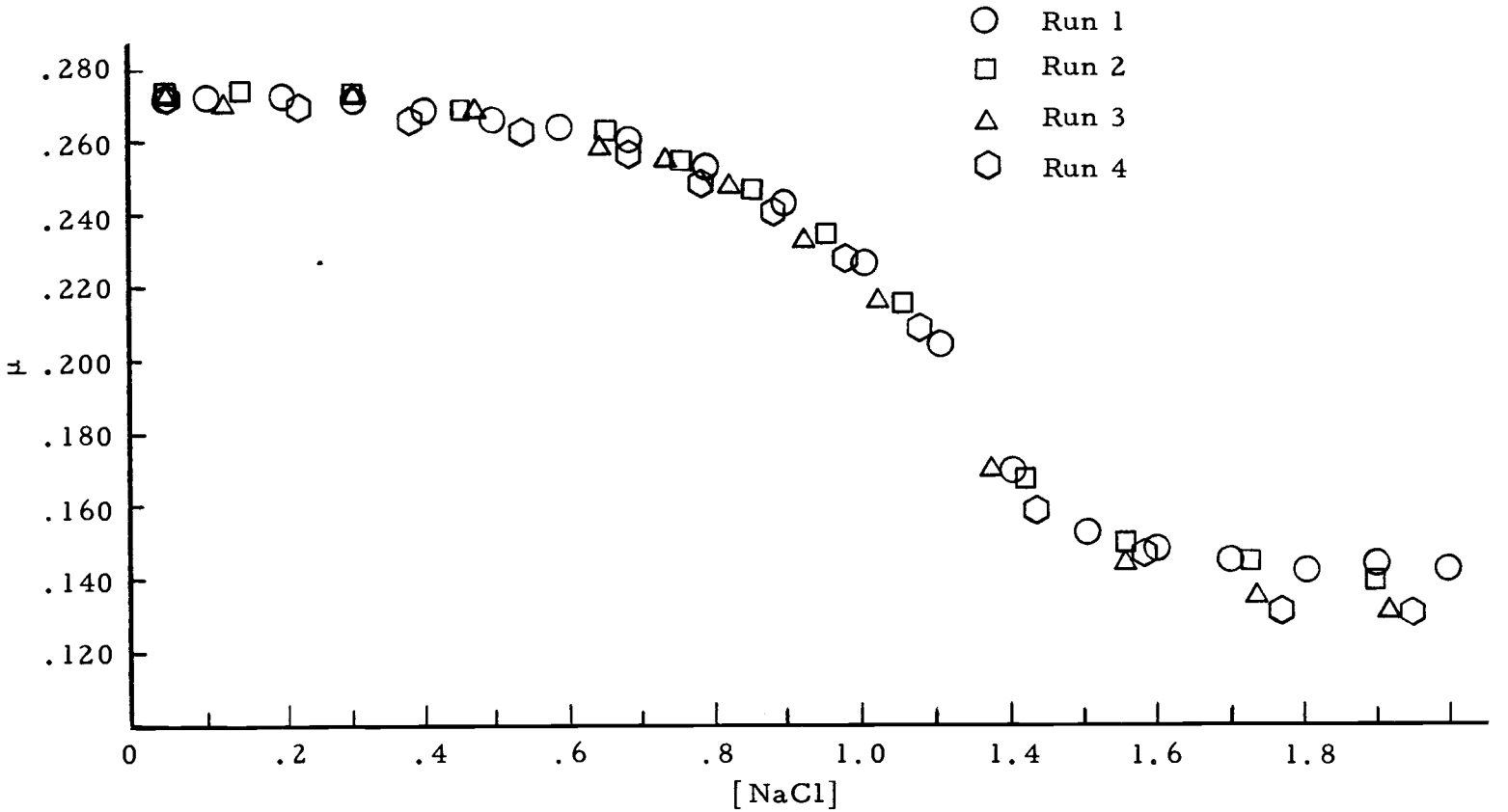

Figure 2. NaCl dissociation of Complex A: Anisotropy.

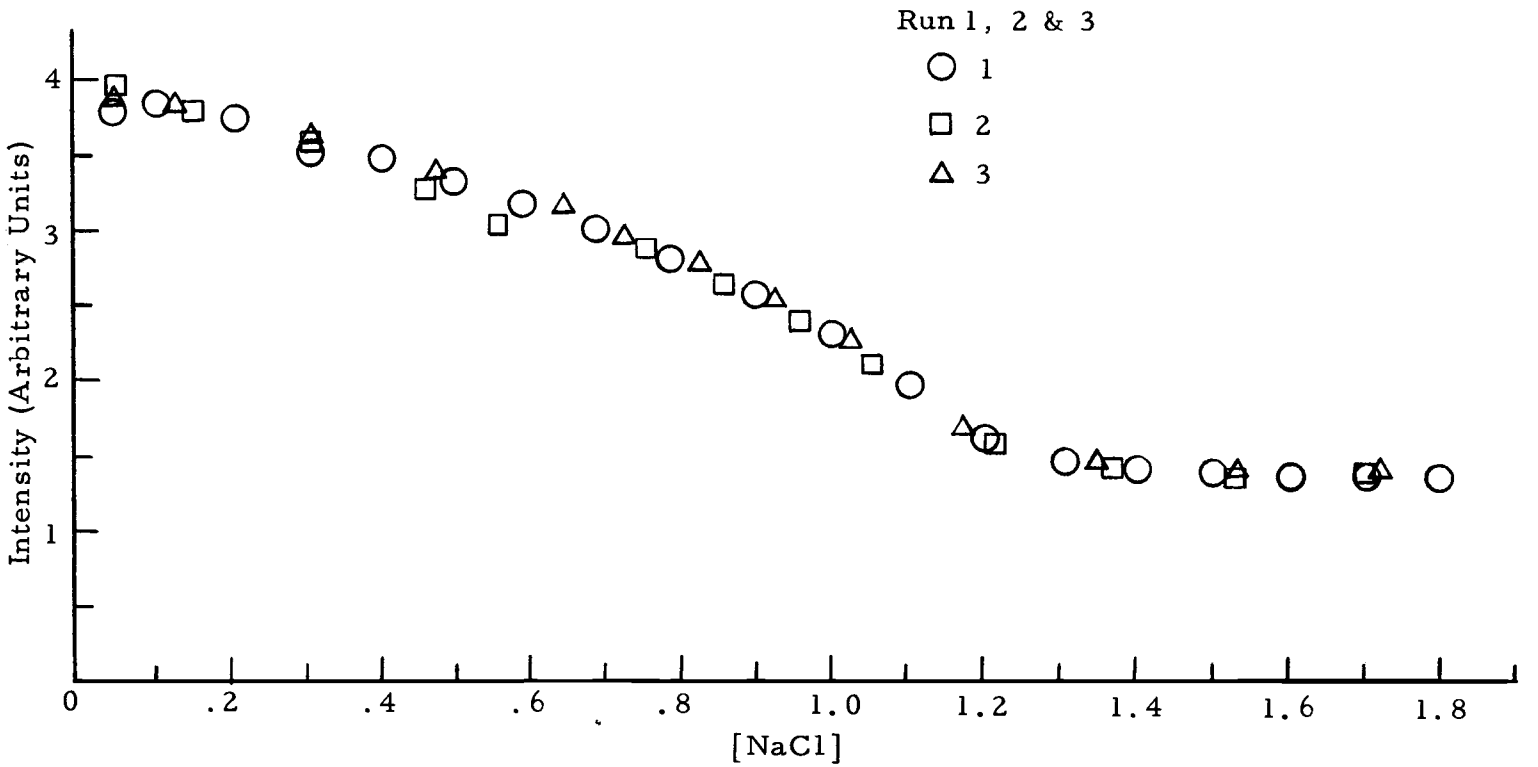

Figure 3. NaCl dissociation of Complex A: Intensity.

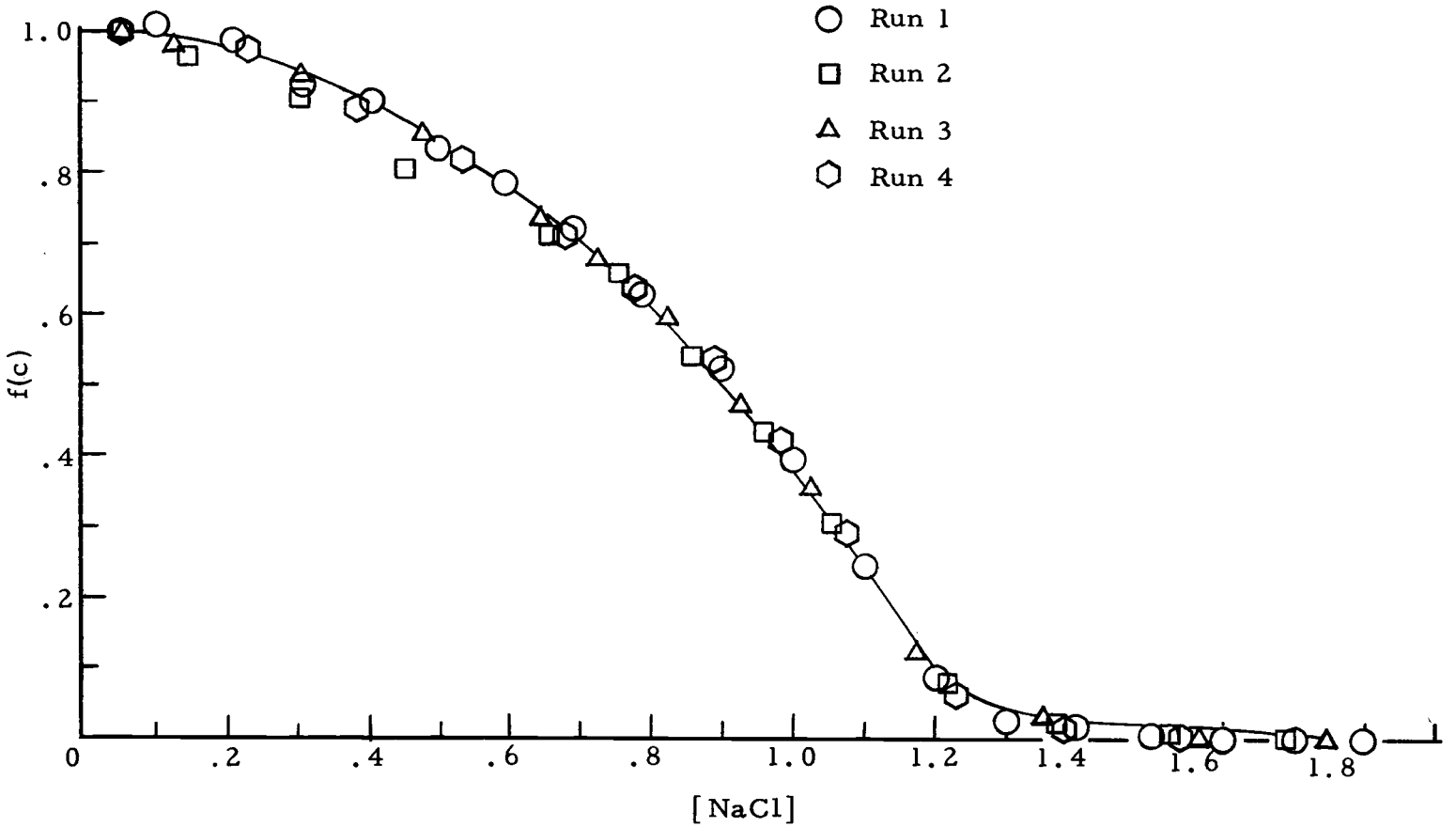

Figure 4. NaCl dissociation of Complex A: Fraction bound.

Centrifugation cleared the turbidity and resulted in precipitation of 90% of the DPL and 55% of the DNA, as indicated in Table III.

Measurement of the intensity and anisotropy of emission of complexes B (resus), B(sn), and B(orig) gave the results shown in Table VII.

Table VII. Data on B(resus), B(sn), and B(orig)

Sample	Anisotropy	Intensity (Relative Units)
B (orig)	0.272	11.57
B (resus)	0.279	10.36
B (sn)	0.247	1.19

The data presented in Table VII demonstrates that more than one complex exists using the following analysis: If two different complexes exist, then the anisotropies should obey the Weber addition law. The sum of the products of the percentage of the whole complex each fraction comprises and its anisotropy should equal the anisotropy of the solution before the complexes were separated. The following calculation shows that this is indeed the case:

$$\mu = \phi_1 \mu_1 + \phi_2 \mu_2$$

$$= \left(\frac{10.36}{11.57}\right)(.279) + \left(\frac{1.19}{11.57}\right)(.247)$$

$$= .274$$

Experimentally, $\mu = 0.272$ was found for the original solution.

Figure 5 shows a comparison of the NaCl dissociation of Complex B (resus) at pH 6.50 with the dissociation of Complex A. As judged from f(c), the two complexes appear to be identical within experimental accuracy. Thus, on this basis, there does not appear to be a difference between the whole (nonfractionated) solution of complex (Complex A), the precipitable complex (Complex B (resus)), and the supernatant fraction of the complex (Evett's (21) Complex E.) We conclude that although the solubilities of the aggregated phase of the complexes are different, no difference is observed in the interaction of the polylysine and DNA as judged by the dissociation of the complex.

Figures 6 through 12 show the effect of pH on the anisotropy and intensity of emission of Complex B (resus) when dissociated by NaCl. The wavelength of the exciting light was 360 nm in these studies. It is seen that the intensity at low pH rises with salt at low salt concentrations whereas anisotropy values do not change until about 0.4 M NaCl is reached. At neutral pH there is no rise, but there may be a small rise at higher pH values.

The initial flat portion of the anisotropy curves mean that the rotational relaxation time and the excited state lifetime of DPL are not changing, providing evidence that the complex has not begun to

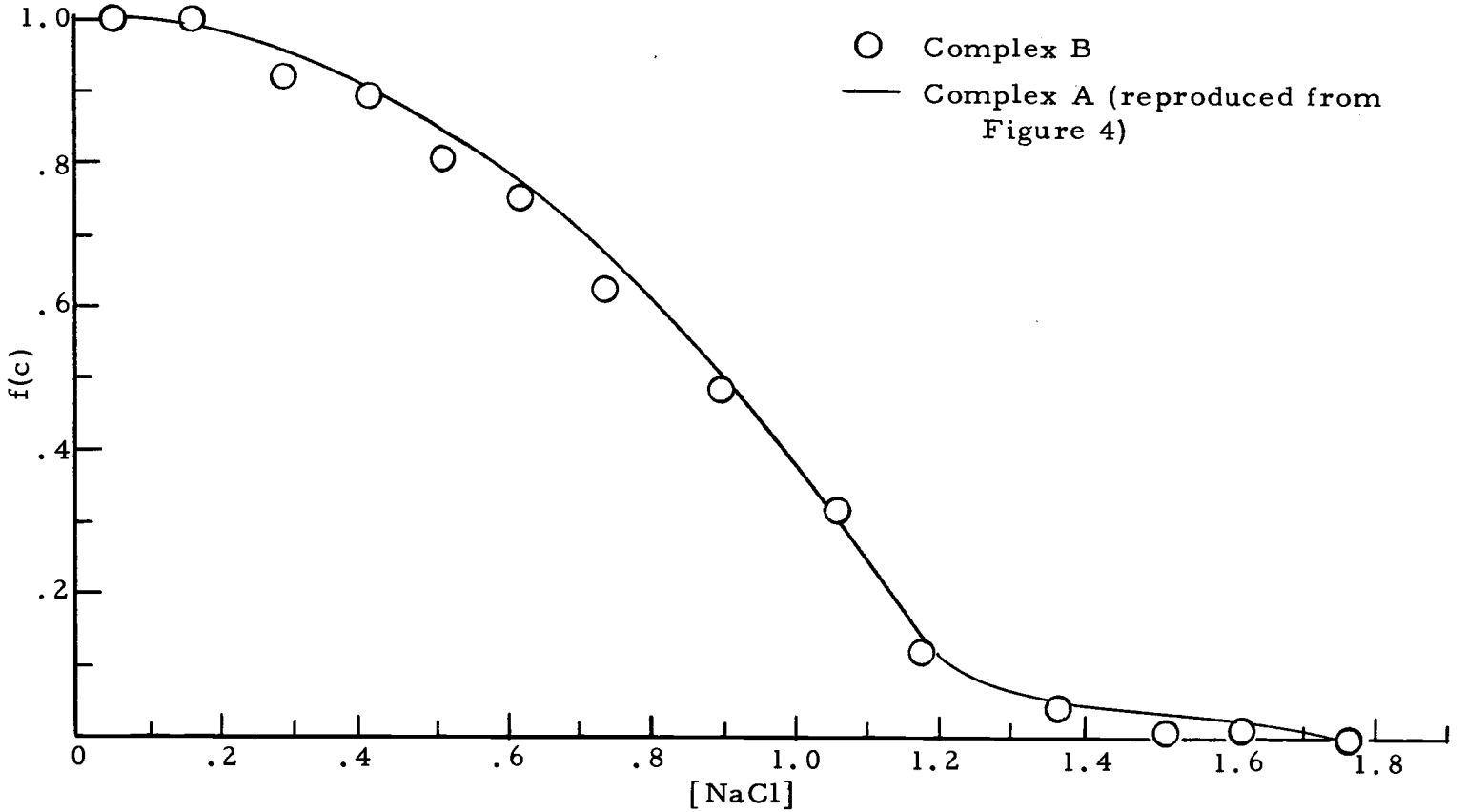

Figure 5. Fraction bound as a function of NaCl concentration for Complexes A and B (resus).

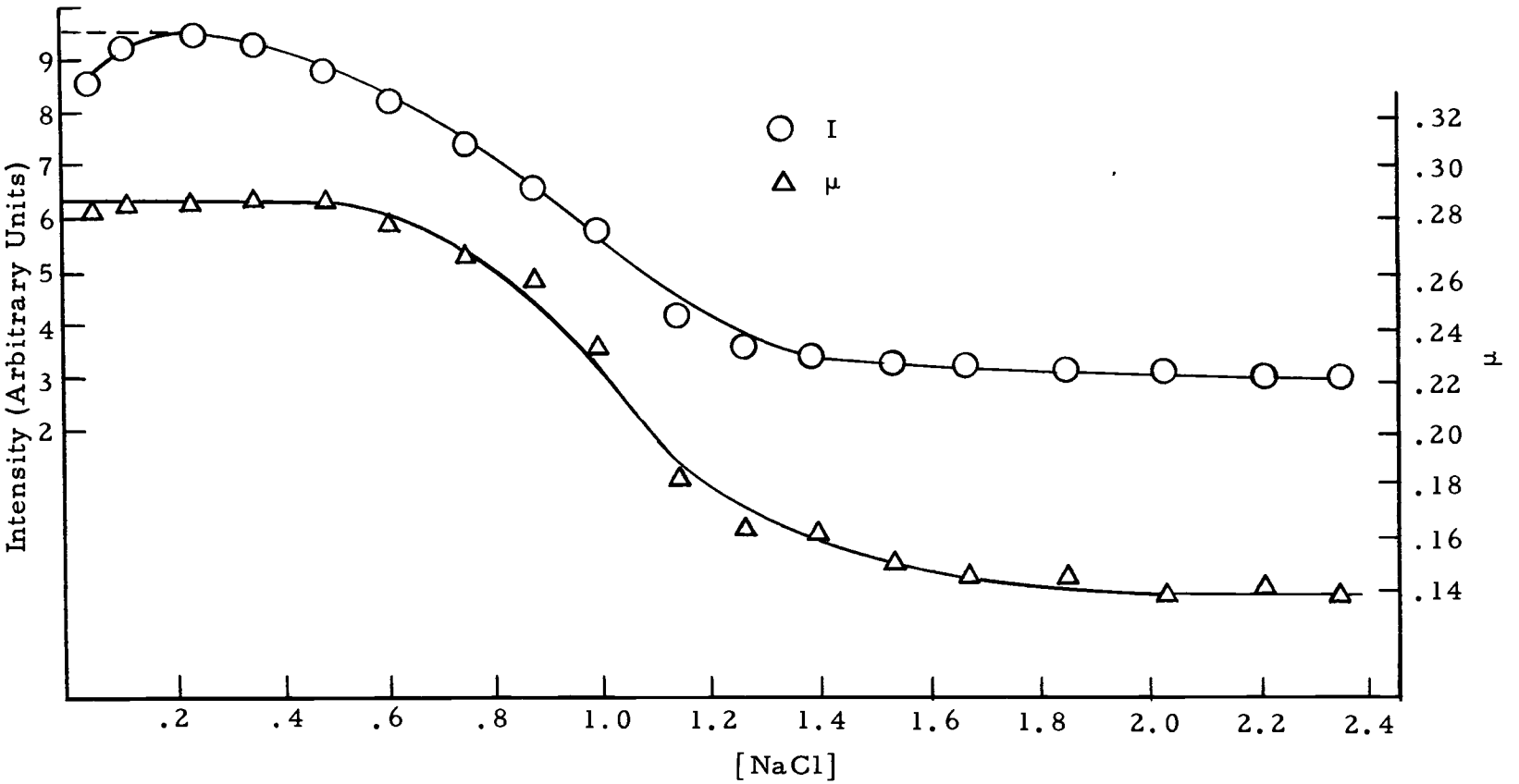

Figure 6. NaCl dissociation of Complex B (resus) at pH 5.0: Anisotropy and intensity.

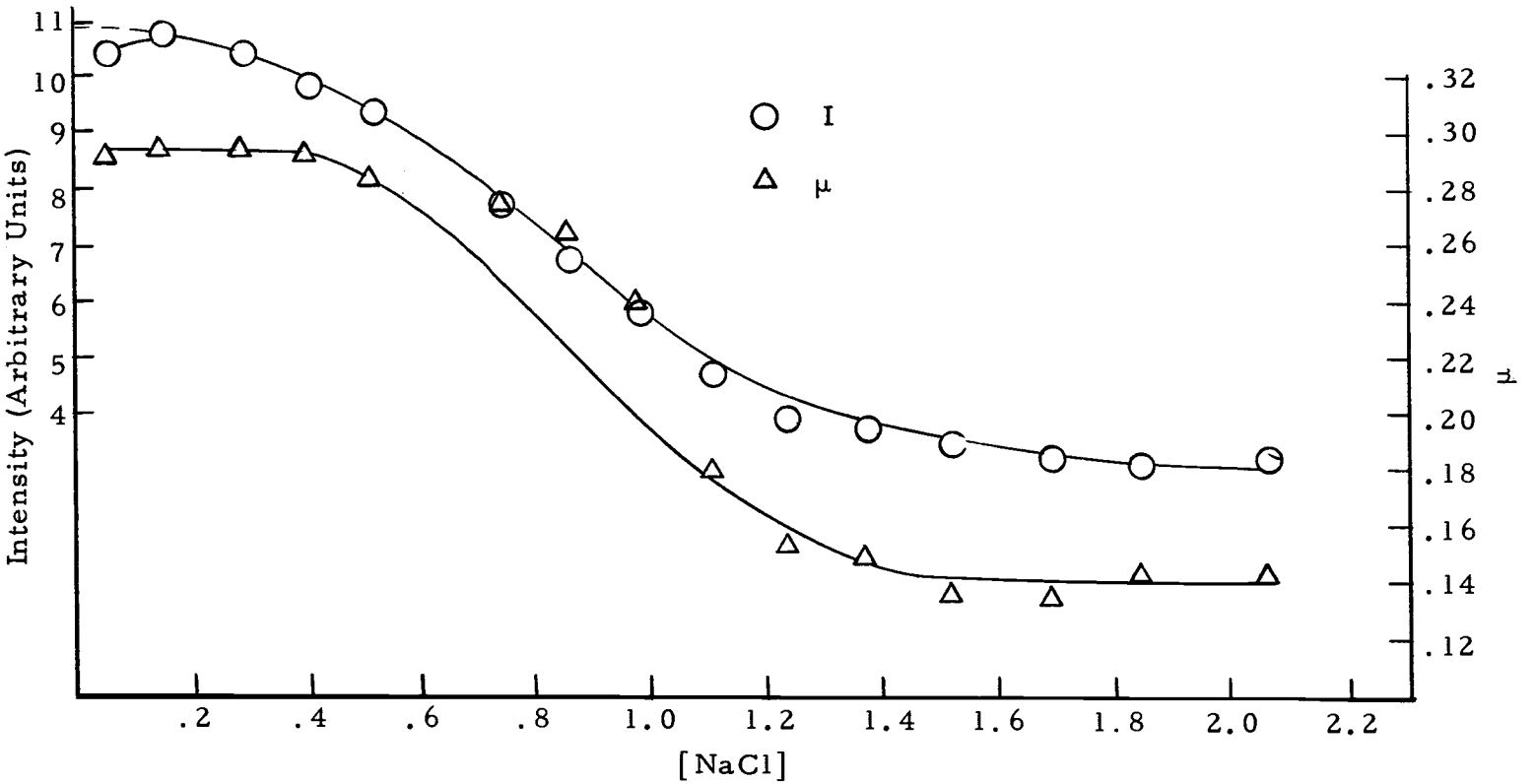

Figure 7. NaCl dissociation of Complex B (resus) at pH 5.5: Anisotropy and intensity.

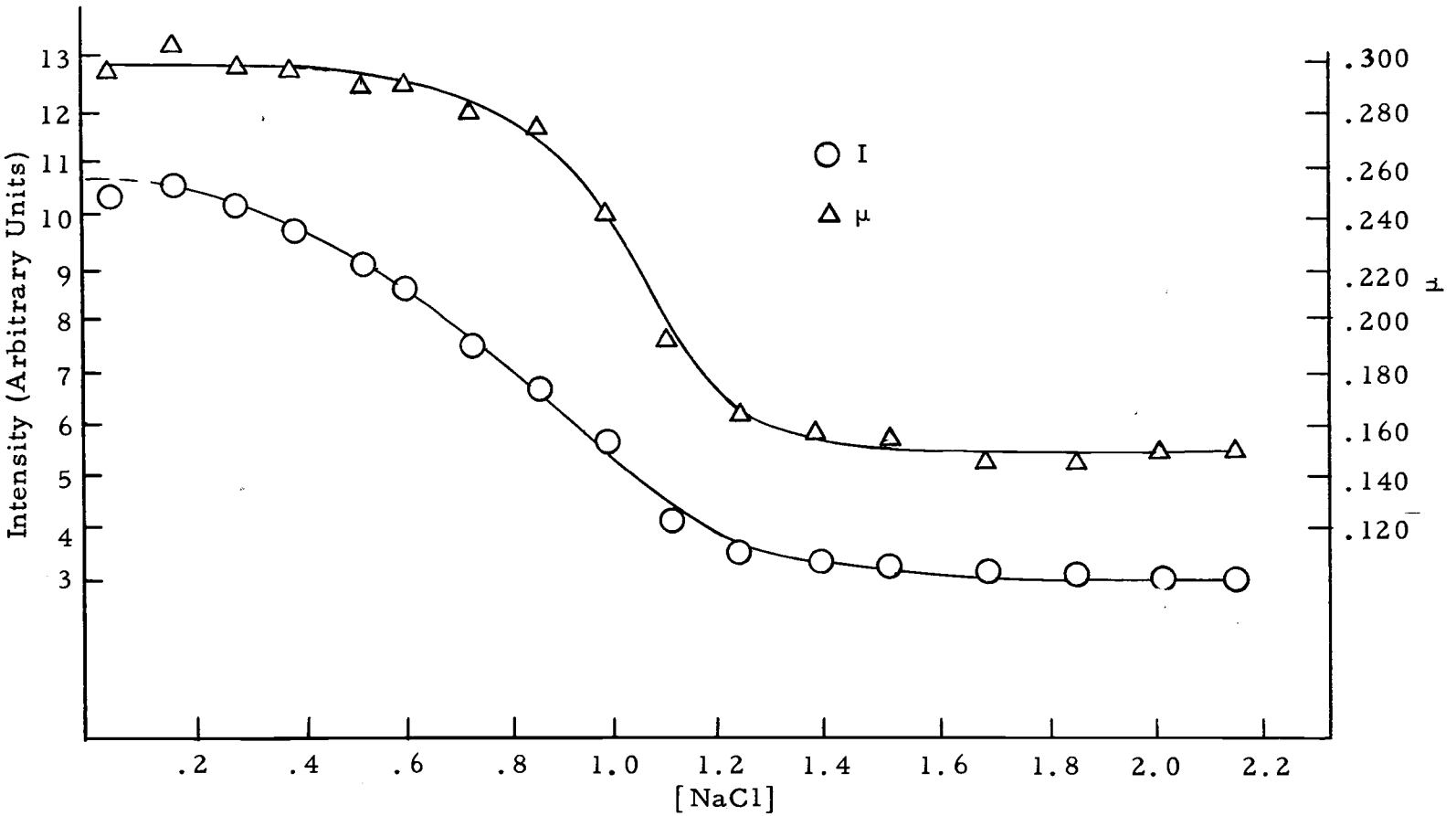

Figure 8. NaCl dissociation of Complex B (resus) at pH 6.0: Anisotropy and intensity.

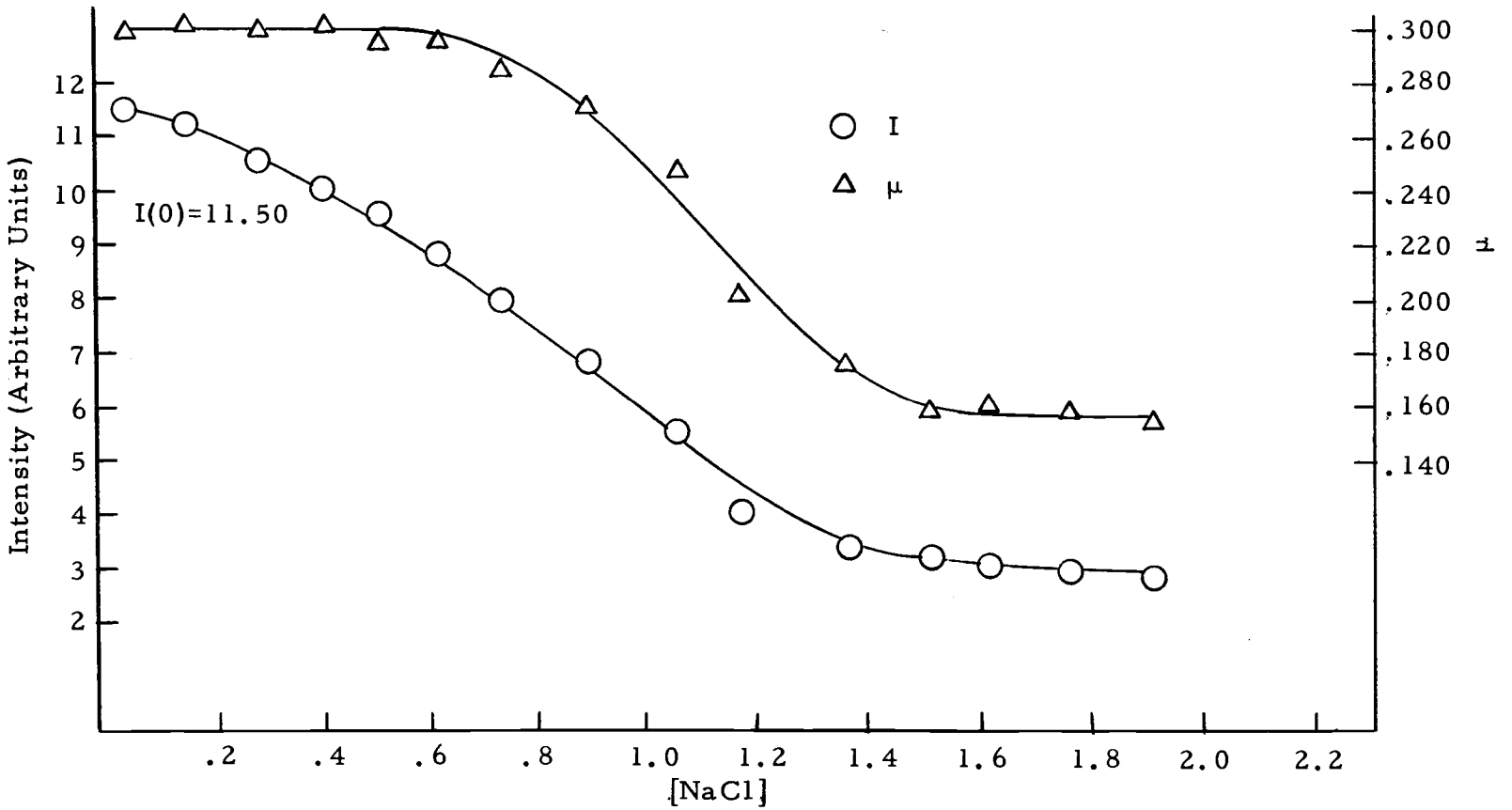

Figure 9. NaCl dissociation of Complex B (resus) at pH 6.5: Anisotropy and intensity.

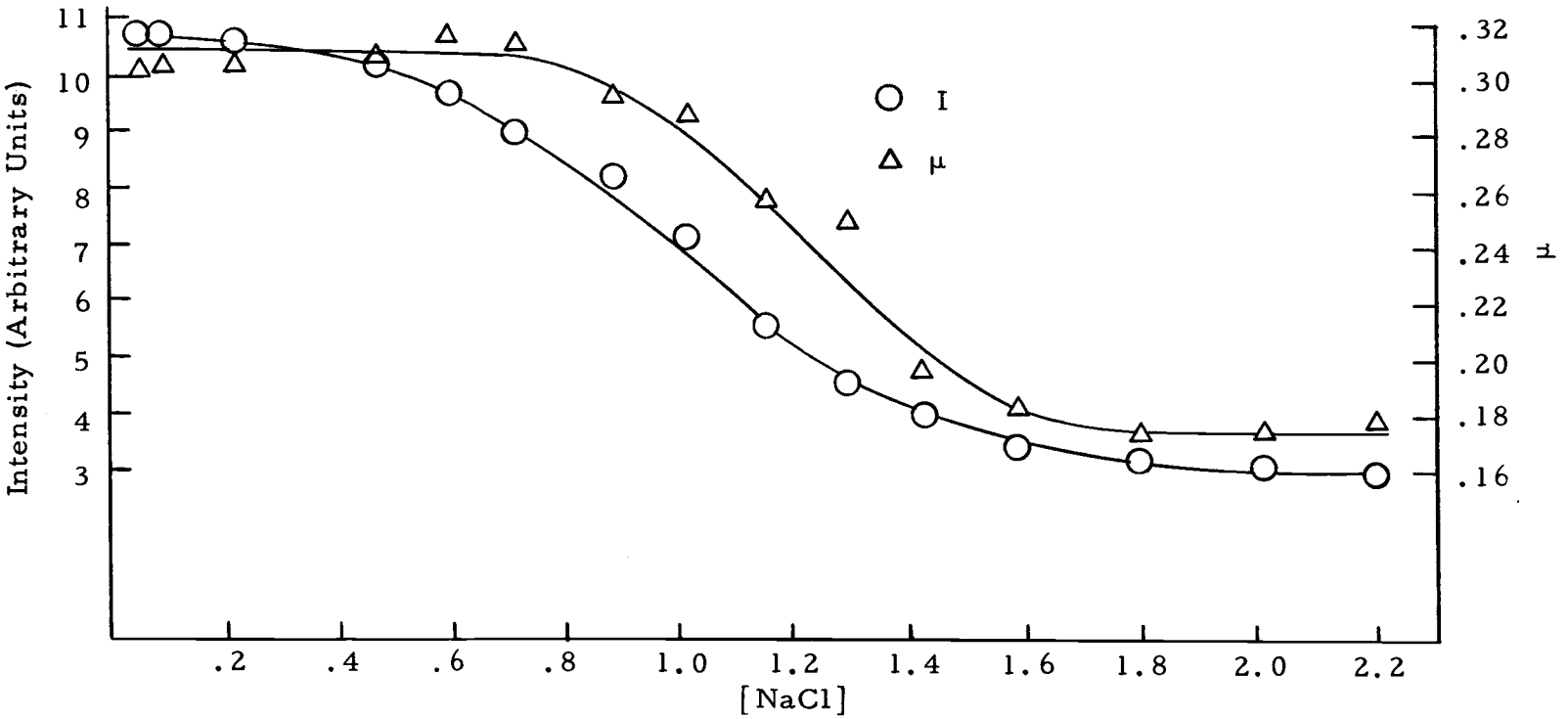

Figure 10. NaCl dissociation of Complex B (resus) at pH 7.0: Anisotropy and intensity.

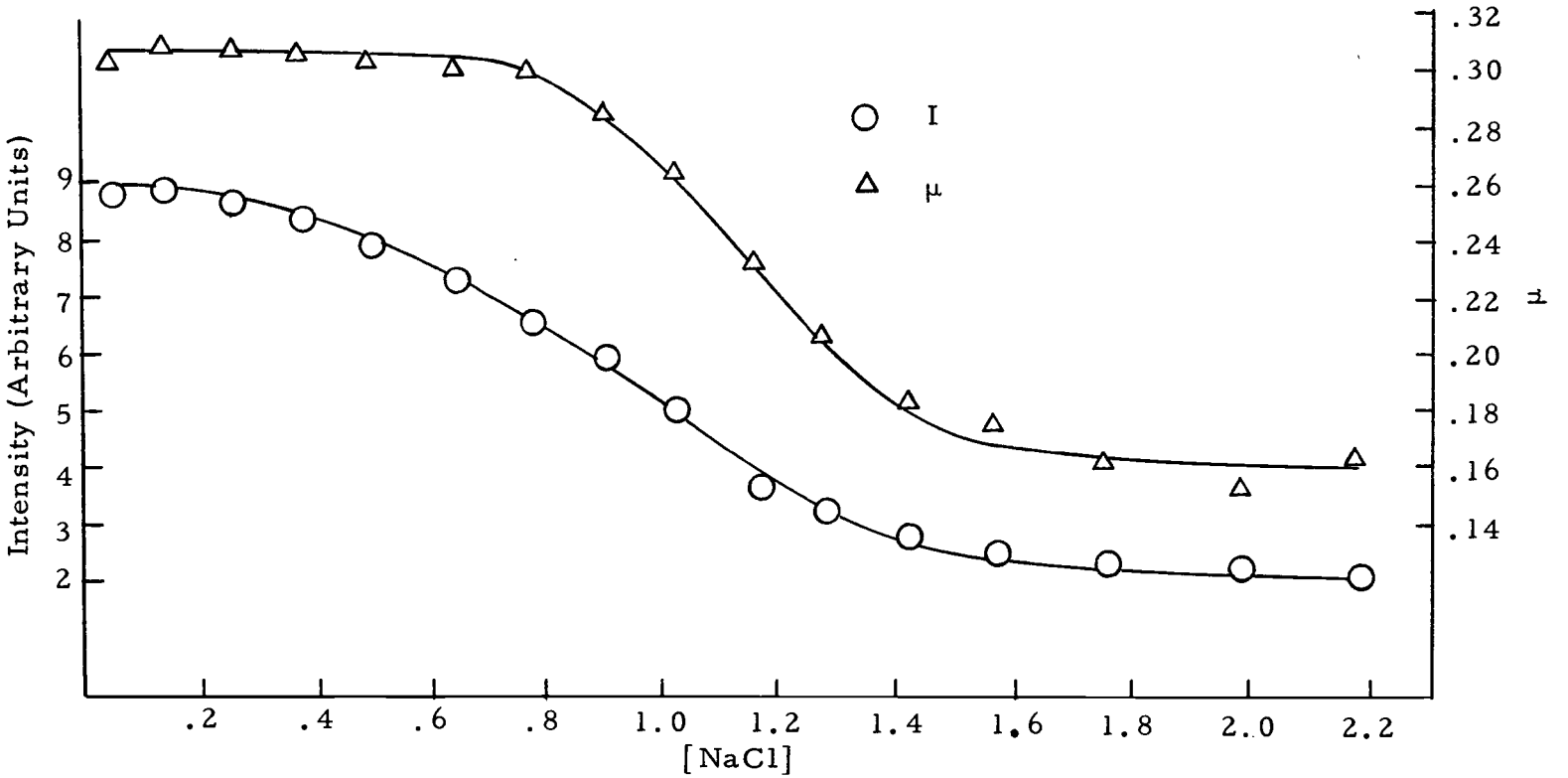

Figure 11. NaCl dissociation of Complex B (resus) at pH 7.5: Anisotropy and intensity.

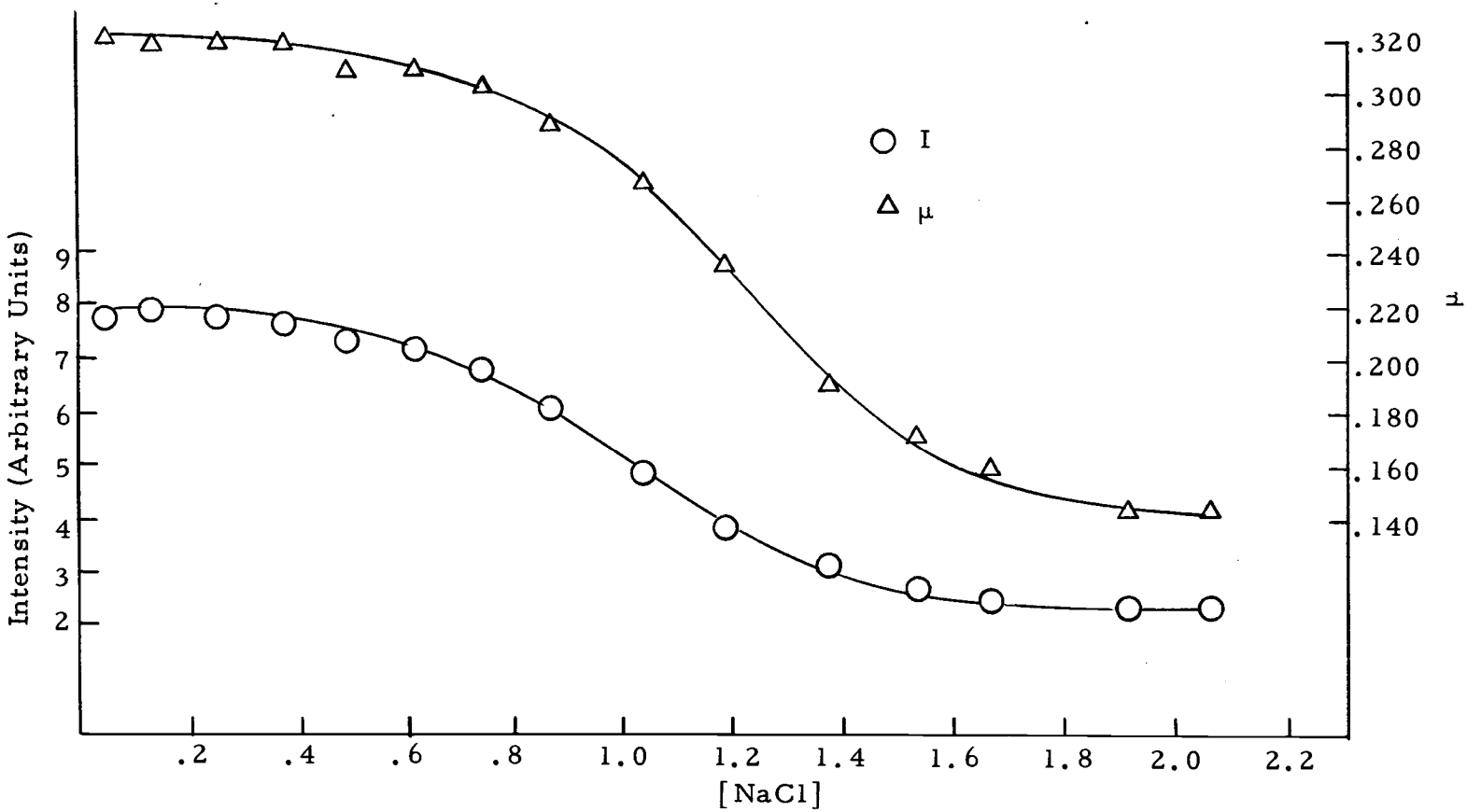

Figure 12. NaCl dissociation of Complex B (resus) at pH 8.0: Anisotropy and intensity.

dissociate in this range of NaCl concentrations. The constancy of the excited state lifetime further means the quantum yield of the dye is not changing. As the intensity of the emission is proportional to the product of the quantum yield and the molar extinction coefficient, the changes in intensity reflect changes that are occurring in the molar extinction coefficient of the dye as the salt concentration changes at acid pH. Similar effects on the molar extinction coefficient have been reported by Gill, McLaughlin and Omenn (26) on studies of dansyl polylysine. They found under conditions where the anisotropy of emission did not change, that changes in intensity correlated with changes in the ultraviolet absorption by the dye.

The above provides a method of measuring the extinction coefficient when it is not easily measurable by ultraviolet absorption. The dansyl concentration in these samples is only about 2.6×10^{-6} M and the optical density of the solution is only about 0.01. The samples are turbid and the apparent optical density due to light scattering is several times greater (.04-.06), making it difficult to detect even a 50% change in the extinction coefficient of the dye. Such measurements can be made with high sensitivity, as demonstrated above, using fluorescence intensity.

Examination of Figures 6 through 12 reveals that the largest intensity perturbation occurs with the low pH solutions. Klotz and Fiess (41) find the pK_a of dansyl-glycine to be 3.99 in water and in

protein conjugates it varies by as much as 2 units, indicating it is sensitive to environment. The larger affect on intensity at pH 5.0 and 5.5 than at higher pH's in this study is interpreted to mean the dye is in a region of negative charge, namely DNA phosphates. The intensity change reflects a change in the number of dansyl molecules protonated as the NaCl concentration increases and "shields" the dye from the negative charge of the DNA. The demonstration that this interpretation is correct will await further studies on the pH effects at lower pH values. This interpretation also predicts that studies on other DNA-protein systems will show similar effects on intensity if the chromophore used has a pK_a sufficiently close to the pH of the solution in which measurements are being taken.

It was pointed out, in the section describing methods of analysis of fluorescence polarization data, that an assumption made in deriving the equation by which f(c) is calculated was that the molar extinction coefficient of the bound dye was constant and not a function of the salt concentration. This assumption has been violated in the measurements on Complex B (resus) and it must be determined if this causes appreciable errors in the analysis.

The reason the changes in extinction coefficient seen here were not observed by Evett and Isenberg (20) is that the latter workers did not use solutions at sufficiently low pH and further, the measurements were made at the wavelength of maximum absorbance, 345 nm,

rather than on the shoulder at 360 nm (Figure 13). Slight shifts in the excitation spectra will not affect the molar extinction coefficient at 345 nm nearly as much as that at 360 because of the difference in the steepness of the curve at these two points.

In calculating f, when necessary, an initial intensity, I(0), was determined by extrapolation of the monotonic portion of the I vs c curve (Figures 6 through 12). f(c) values were not sensitive to this choice.

In Figure 14 the fractional decrement of turbidity, B(c), is shown for the pH series of salt dissociations. At pH values greater than 6.00 the curves are not monotonic functions of NaCl concentration and B(c) obviously does not represent the fraction of polylysine bound.

It was noticed during this series of salt dissociations that the turbidity decreased when the pH was raised. Figure 15 shows the effect of pH on turbidity. The decrease is found to not be reversible when the sample was back-titrated. This effect is not understood. It may be related to the observation by Hofstee (30) that proteins form insoluble complexes with DNA when the pH is considerably below their isoelectric points, but that complexes formed in solutions slightly alkaline with respect to the isoelectric point remained soluble when the pH was subsequently lowered. This observation is similar to the effect seen in Figure 15.

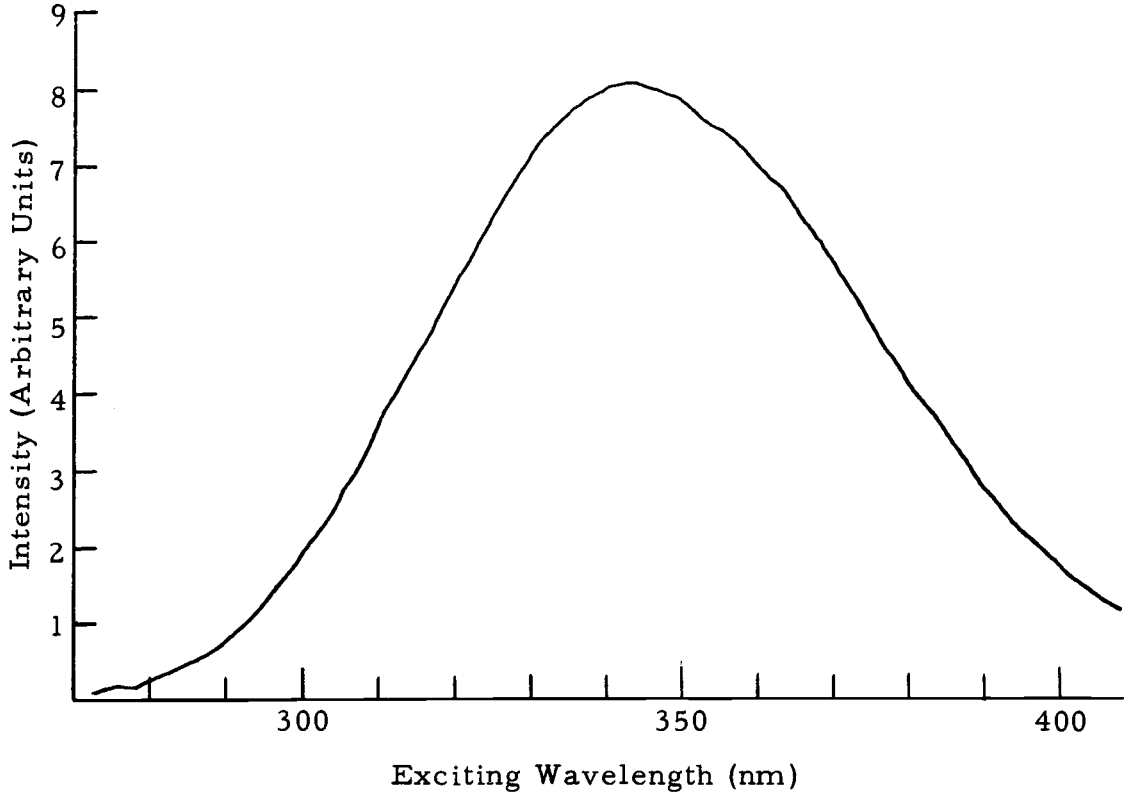

Figure 13. Excitation spectra of Complex A.

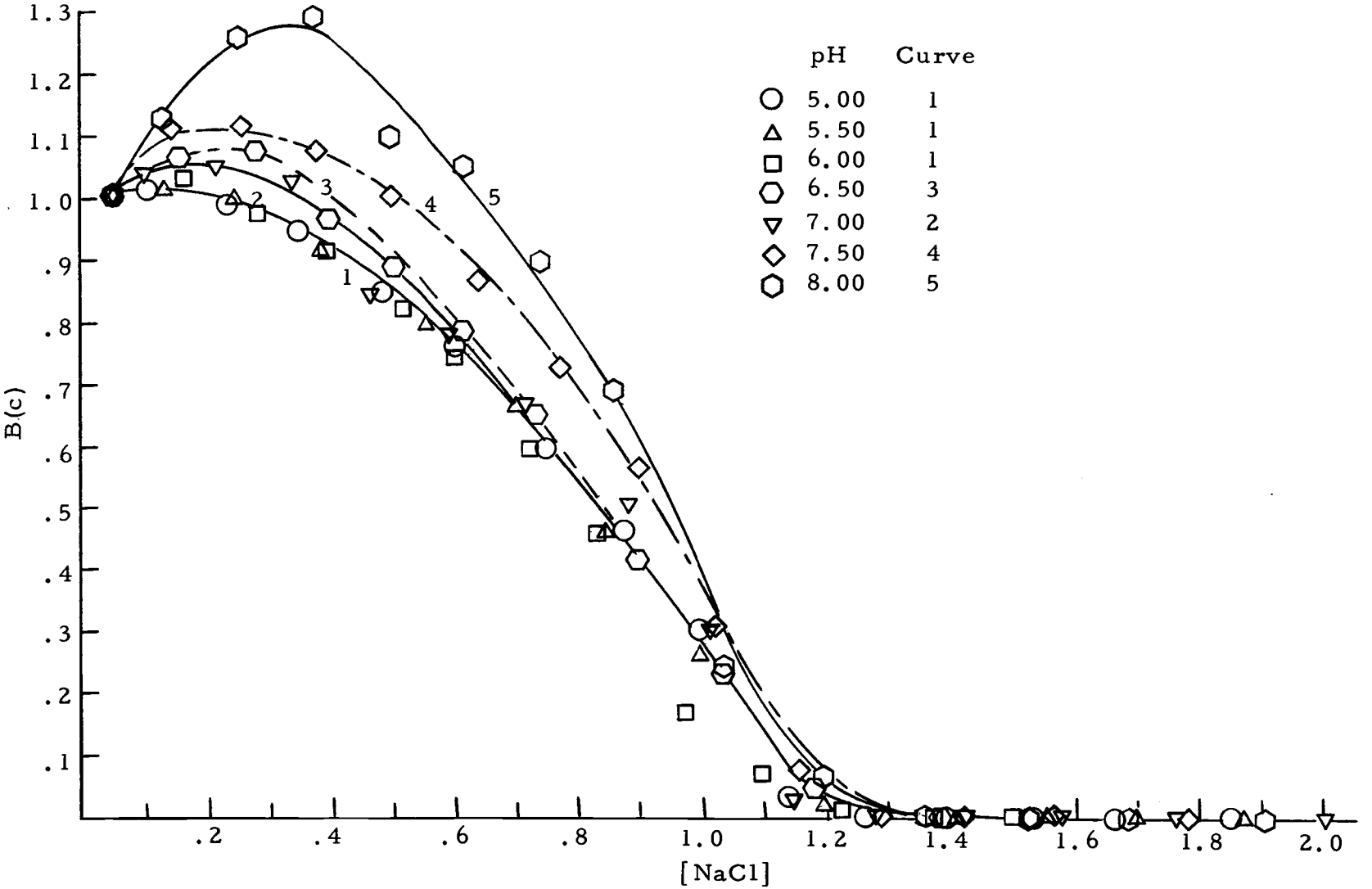

Figure 14. Effect of pH on NaCl dissociation of Complex B (resus) as measured by fractional decrement of turbidity.

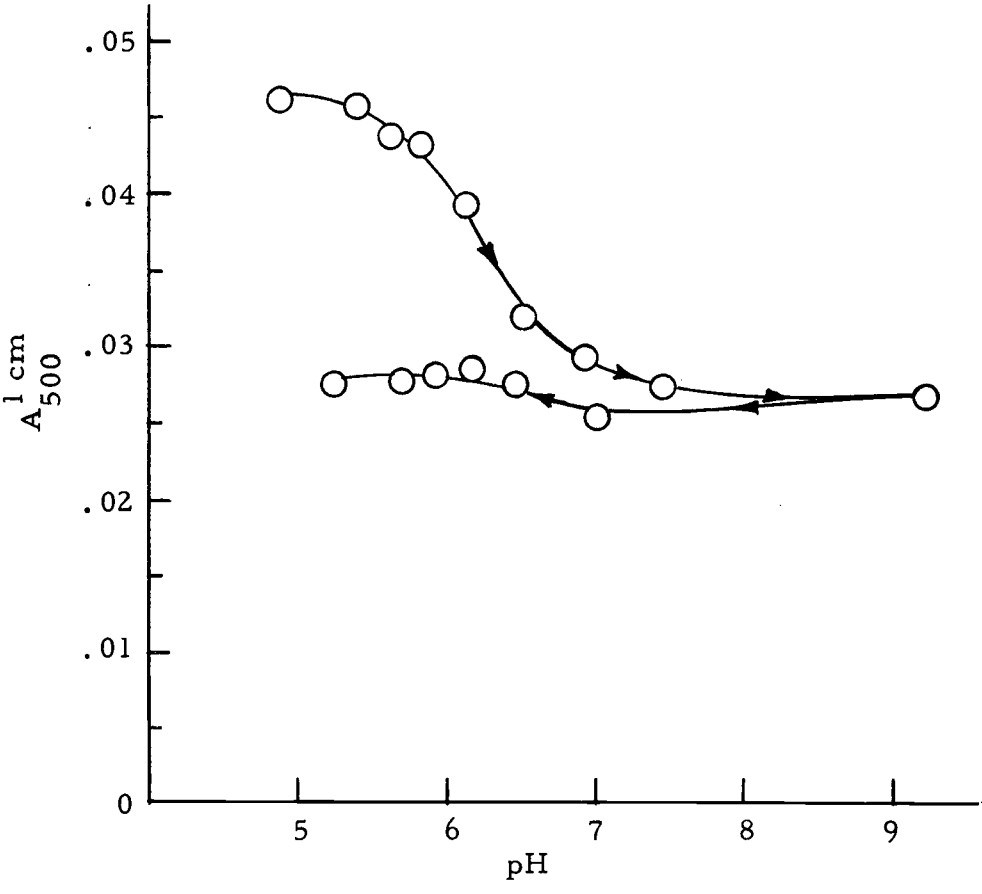

Figure 15. pH affect on turbidity of Complex B.

The change in turbidity as a function of pH also indicates turbidity is not a measure of the amount of complex present.

The effect of NaCl concentration on the anisotropy and intensity of DPL(IV) (10^{-4} M in lysine) obtained by changing the NaCl concentration over the same range used for salt dissociation studies is shown in Figure 16. The wavelength of the exciting light was 365.2 nm. The intensity is presented as the ratio of intensity at a given NaCl concentration to that at 0 M NaCl. Both the anisotropy and the intensity are seen to be functions of the ionic strength of the solution. Similar data for RBPL is shown in Figure 17. The NaCl dissociation of RBPL-DNA (details of preparation in Table VII) is also presented in Figure 17.

For RBPL-DNA the anisotropy of RBPL is identical to that of RBPL in the absence of DNA at NaCl concentrations greater than about 1.4 M. The free fraction of RBPL may do so over the entire salt range. In such a case it is no longer strictly correct to characterize the free state by a constant anisotropy, μ_2, but rather, it is necessary to use $\mu_2(c)$ for the final state at a given salt concentration c, where $\mu_2(c)$ is obtained from the curve of anisotropy of RBPL versus NaCl concentration in the absence of DNA. The calculation of f(c) was made in two different ways. In the first procedure μ_2 was assumed constant, equal to 0.218, approximately at the minimum of μ vs c. In the second procedure $\mu = \mu(c)$ for RBPL in

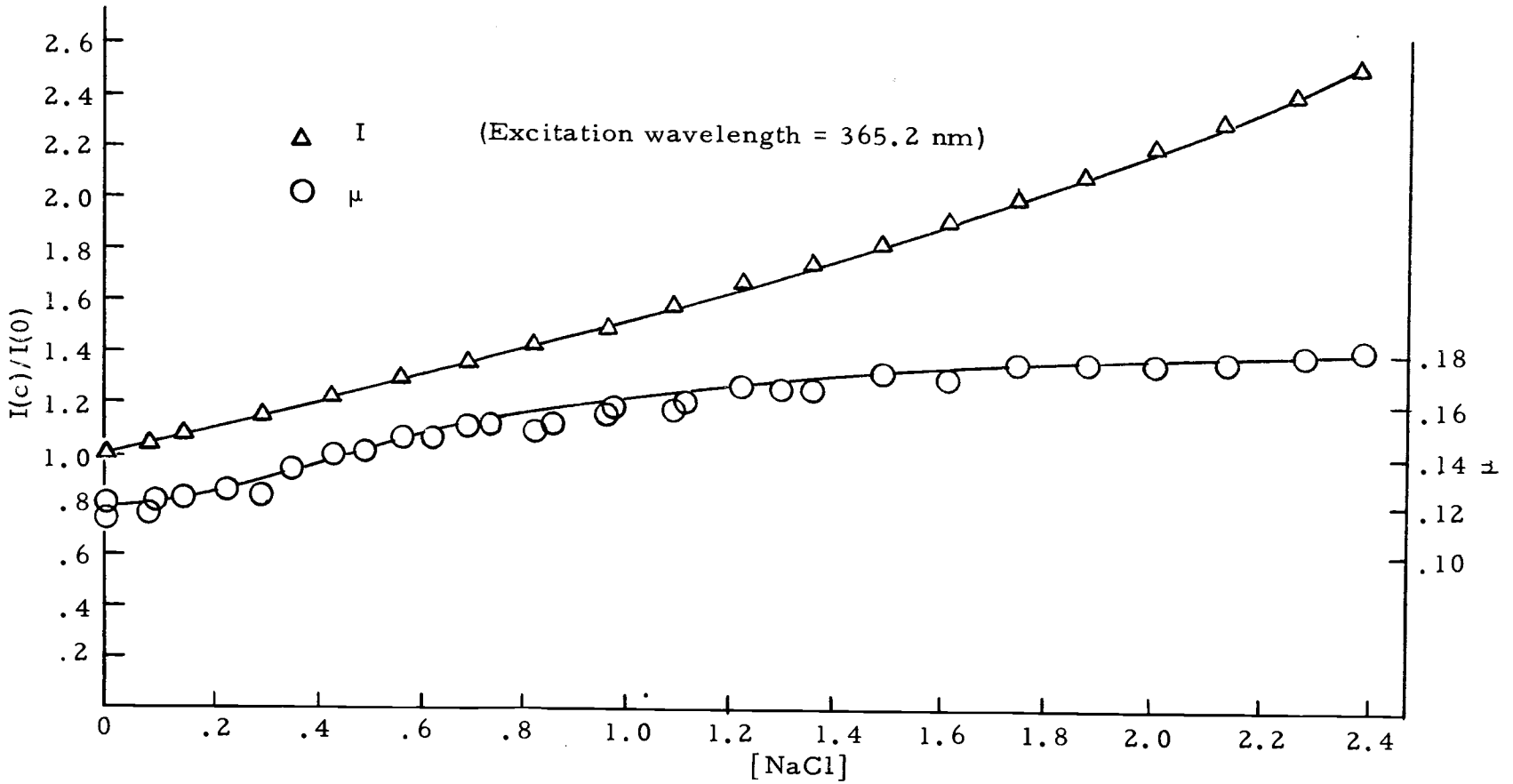

Figure 16. Effect of NaCl concentration on anisotropy and intensity of DPL(IV) in the absence of DNA.

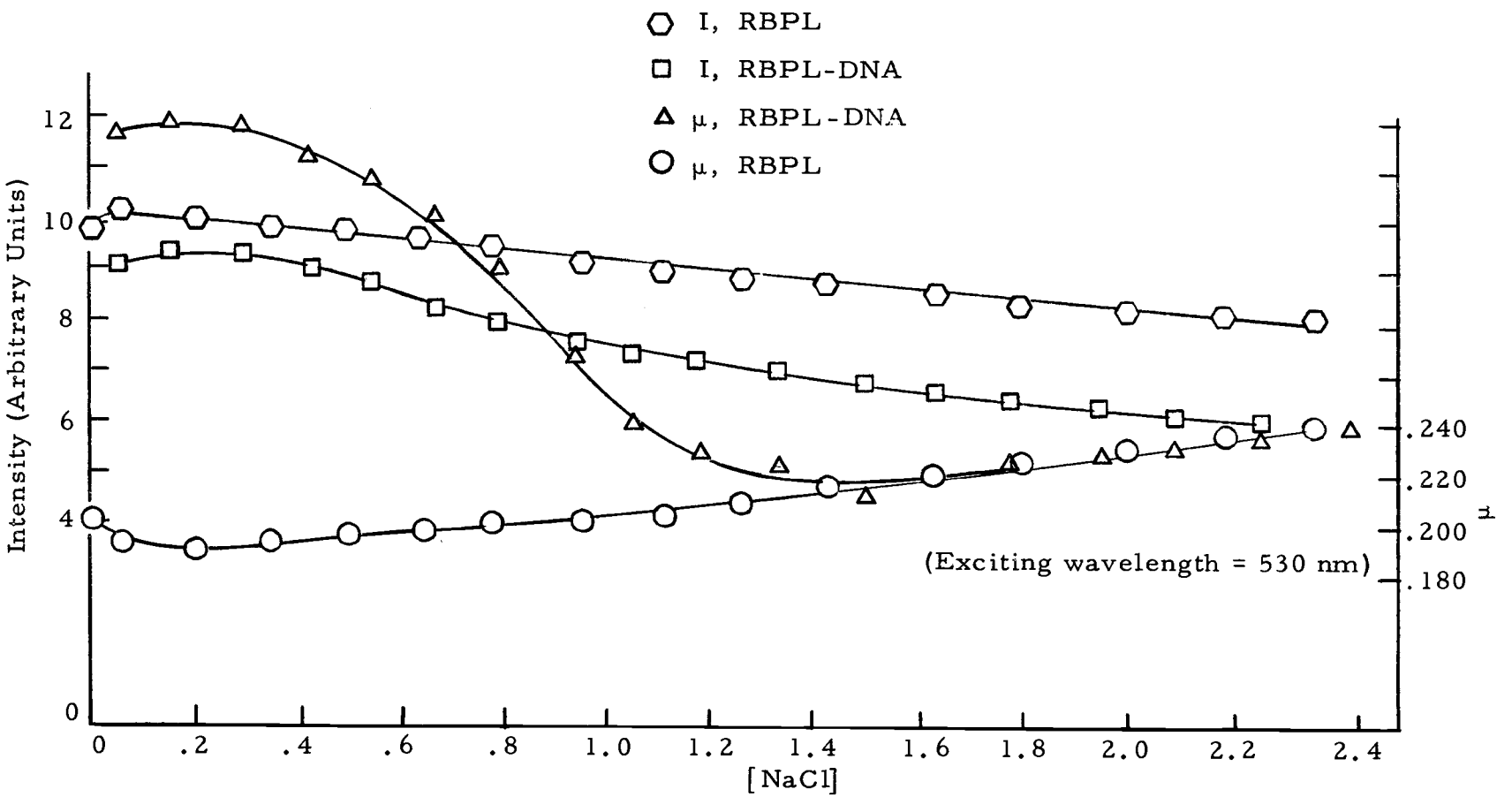

Figure 17. Effect of NaCl concentration on anisotropy and intensity of RBPL and RBPL-DNA.

the absence of DNA was assumed. The results, shown in Figure 18, indicate a lack of sensitivity to this assumption, since both values agree to 5% or better. The data also indicates that there is essentially no interaction between RBPL and DNA above 1.4 M NaCl for RBPL-DNA.

It may also be noted that the dissociation curve for the RBPL-DNA system is very close to, perhaps within experimental error the same as, the dissociation shown by the DPL-DNA system. (The dissociation curve of Complex A is reproduced in Figure 18 as the broken line.)

A series of samples were prepared as indicated in Table V to investigate the effect of different ratios of DNA to polylysine. During the course of the dialysis a granular precipitate was observed to form in D/L(1). The other samples were also turbid, though no visible precipitate formed. Turbidity measurements on the samples before and after centrifugation for 15 minutes at 390g are shown in Figure 19. Centrifugation resulted in the removal of some material from solution for samples D/L(2) and D/L(4). (A visible precipitate was observed in the bottom of the tubes in these two cases also.) The slight decrease in turbidity for the other samples is the same and is probably due to a slight clarification of the solution through removal of dust or small air bubbles. The decrease in turbidity with increasing DNA concentration suggests either the complex is not

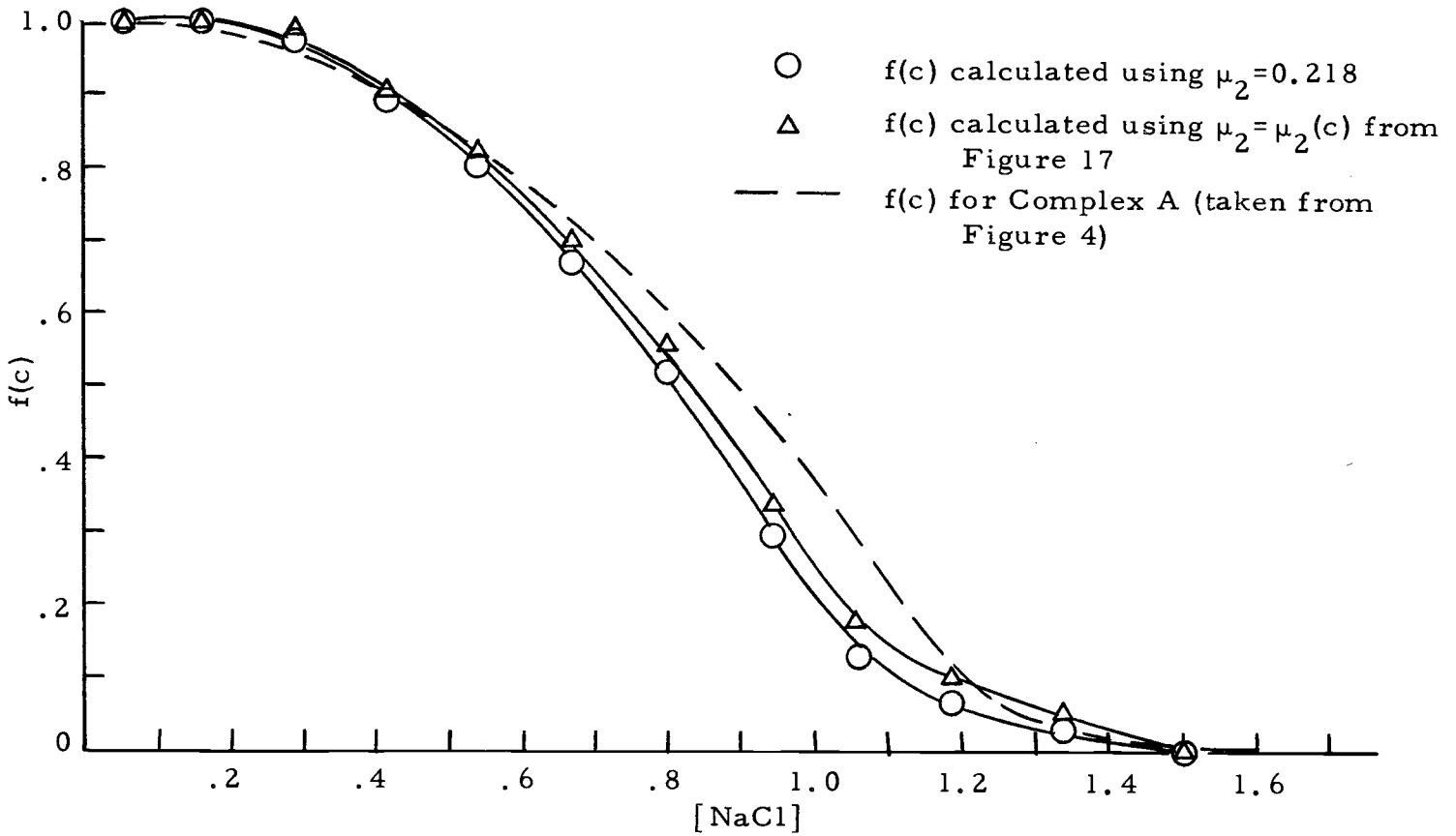

Figure 18. Effect of values of μ_2 on calculation of fraction bound.

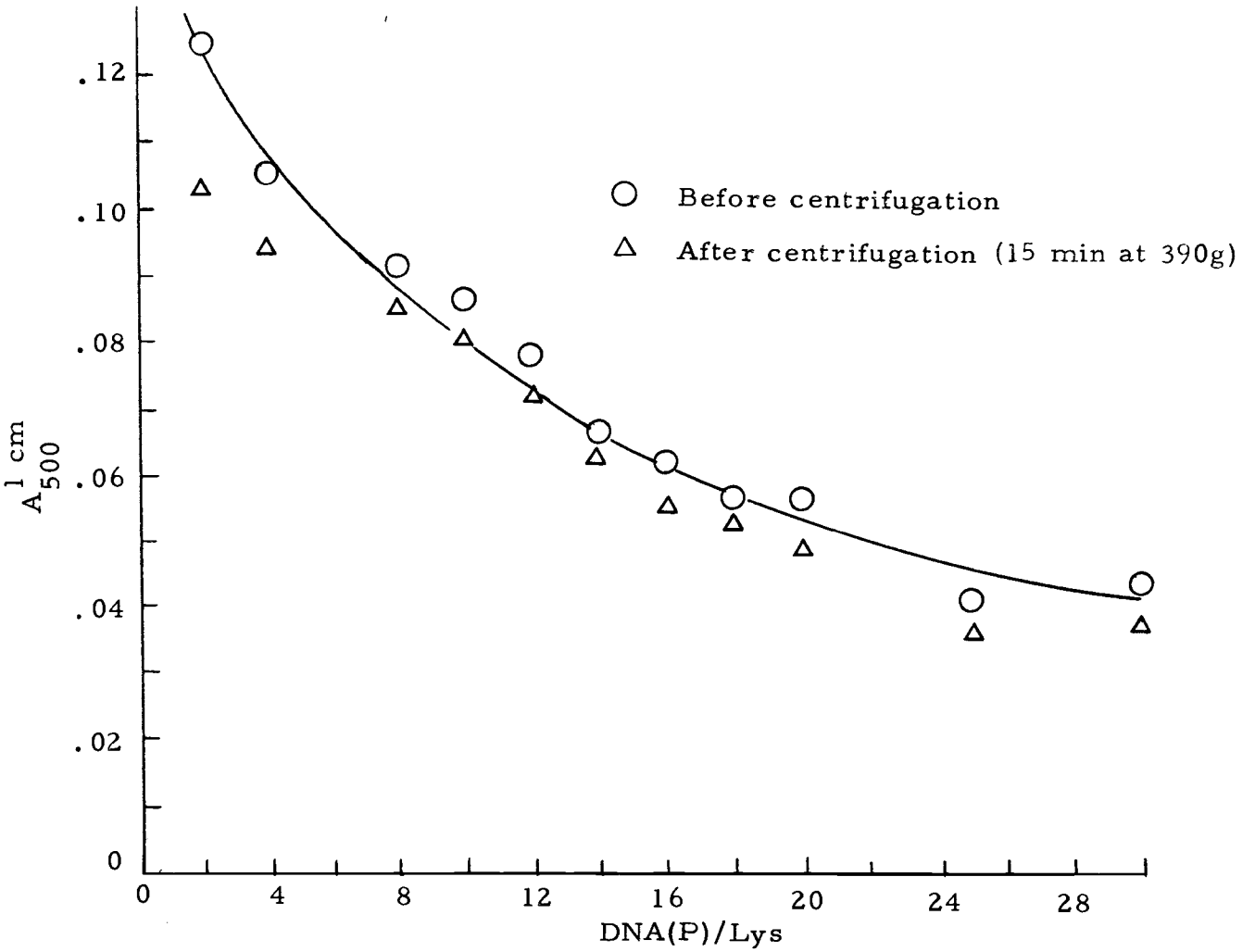

Figure 19. Effect of the DNA(P)/Lysine ratio on the solubility of the complex.

stoichiometric or that free DNA interacts with a stoichiometric complex in such a way as to solubilize it. This has been observed by Inoue and Ando (34) for DNA-clupeine complexes.

A typical dissociation is shown in Figure 20. A slight decrease in μ_1 was observed as the DNA(P)/lysine ratio increased (Figure 21).

Analysis of the NaCl dissociation of these complexed indicated the dissociation occurs later (i.e. at higher NaCl concentration) with each sample up to D/L(8) - D/L(10) and then dissociation began occuring earlier as the DNA(P)/lysine ratio further increased. The results are summarized in Figure 22. The percent of polylysine bound at 0.5, 0.75 and 1.0 M NaCl is plotted as a function of the DNA(P)/lysine ratio of the samples. The data indicates that the binding of polylysine increases as the number of binding sites increases, up to an eight- to ten-fold excess of binding sites, and then the binding begins to decrease as the concentration of DNA (binding sites) further increases.

This result is not consistent with a model of reversible binding under conditions where equilibrium has been attained. If measurements were made under equilibrium conditions, and the interaction is reversible, the fraction of polylysine bound should increase as the DNA concentration increases. To check the possibility that a steady state for the complex had not been attained during one of the salt dissociations, a sample at intermediate salt was prepared, measured

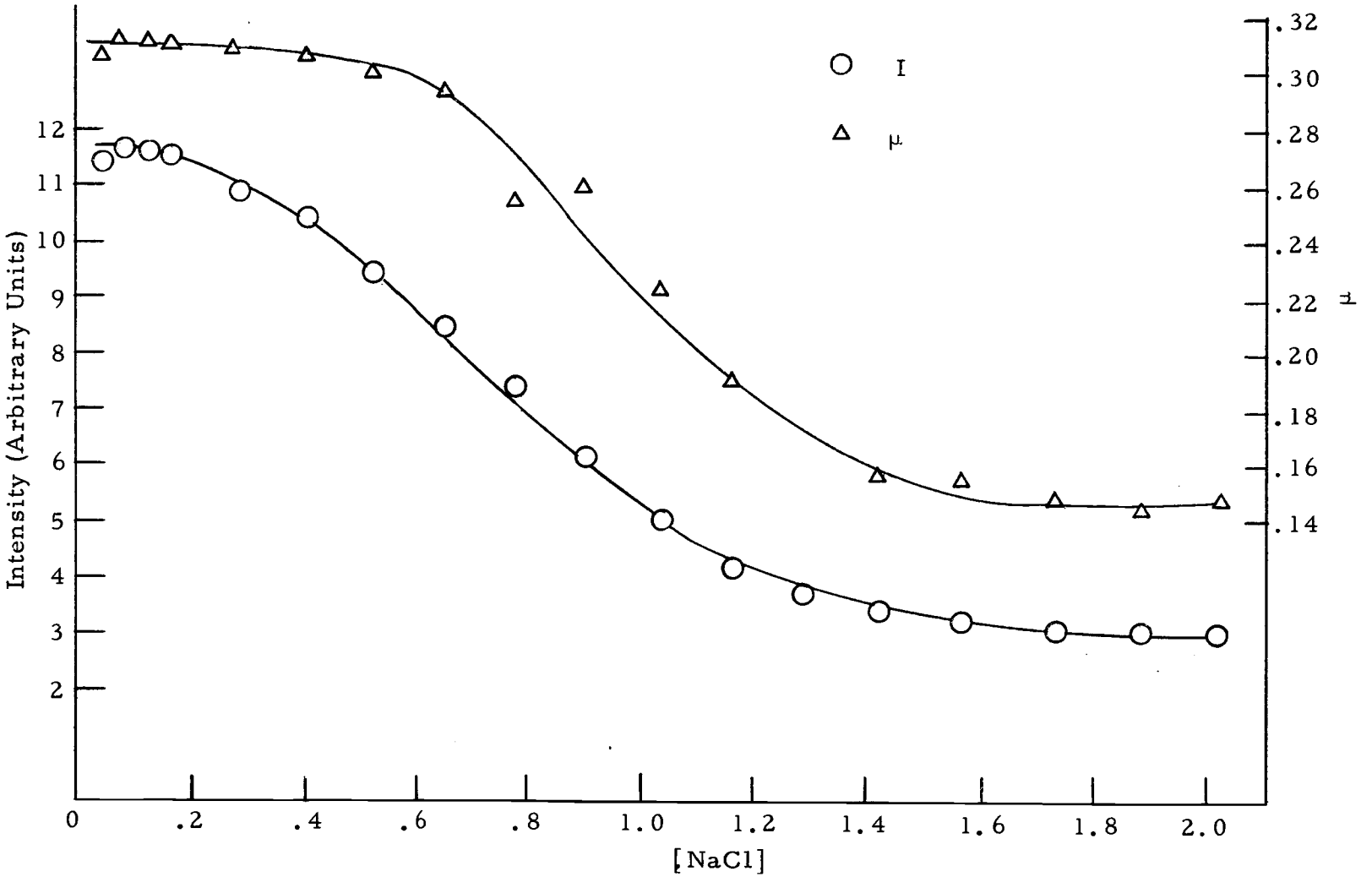

Figure 20. NaCl dissociation of D/L(8): Anisotropy and intensity.

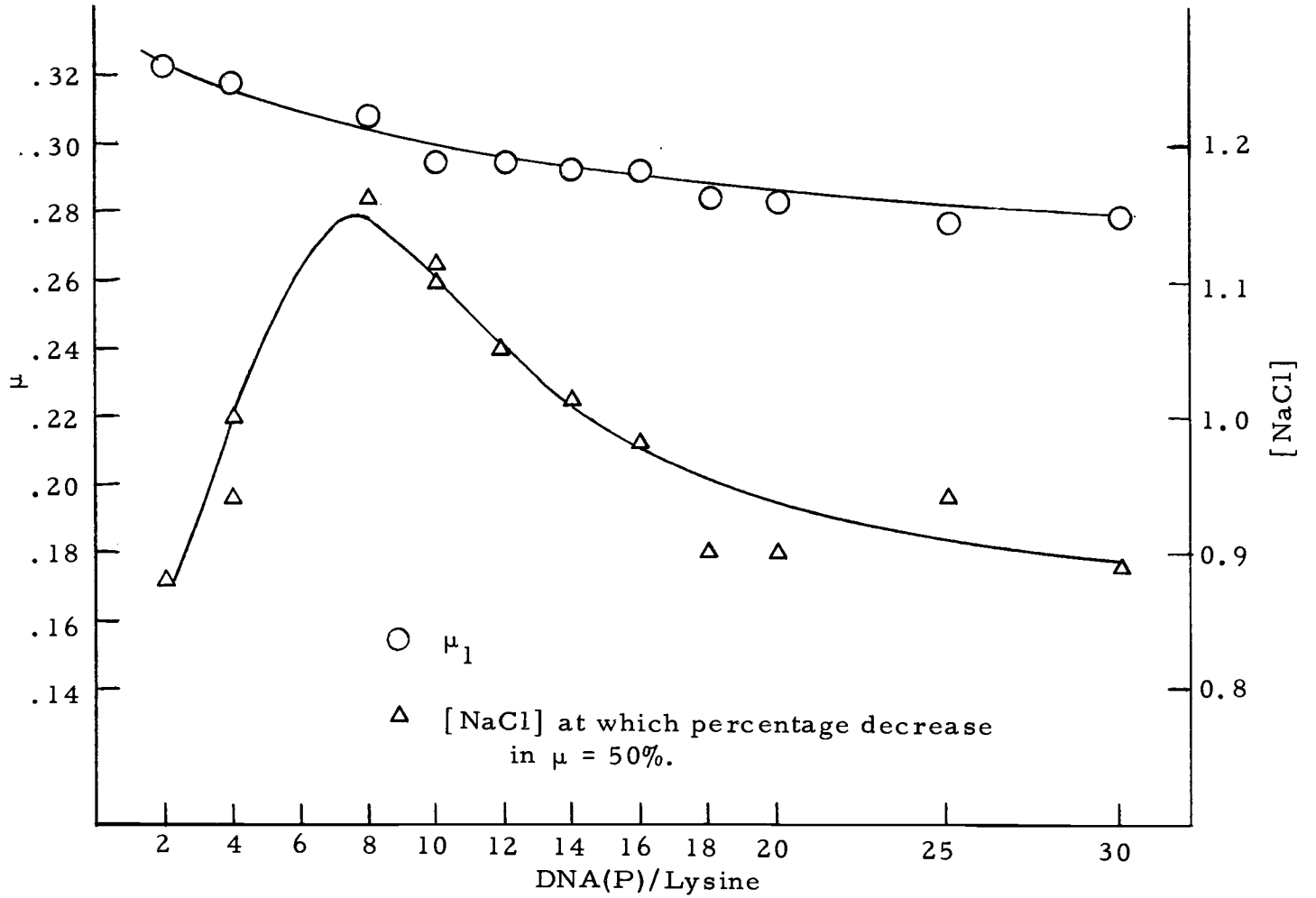

Figure 21. μ_1 of D/L series of complexes and [NaCl] at which percentage decrease in μ = 50% for NaCl dissociation of D/L(2)-D/L(30).

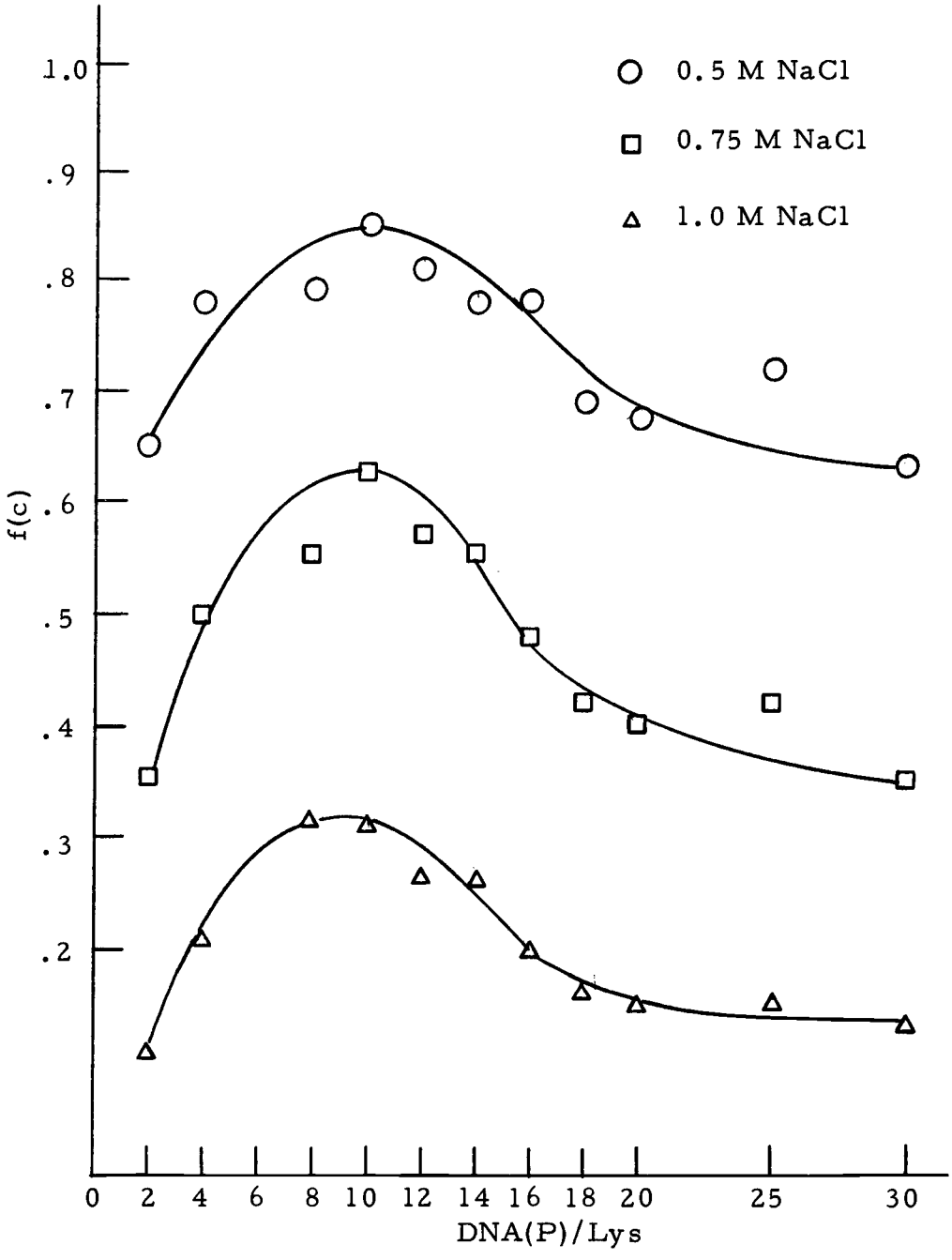

Figure 22. Fraction of polylysine bound at 0.50 M, 0.75 M and 1.0 M NaCl for NaCl dissociation of the D/L series of complexes.

immediately after preparation and then twice more at hourly intervals. The measurements were identical in all three cases for both the intensity and anisotropy, indicating the system being measured had reached a stationary value.

The remaining possibilities for explaining the data are either that the model used in analysis of the data is incorrect, or the binding is not reversible. The following discussion will show, however, that the data indicates that above DNA/lysine = 8 the binding of lysine is actually less at higher DNA concentrations and this result is independent of any particular model for the interaction. Figure 23 shows percentage change in μ vs salt concentration for the D/L complexes. Above D/L = 8, percentage in μ vs c is closer to the ordinate the higher D/L is. Within a wide range of possible models, this result indicates qualitatively easier salt dissociation for greater DNA concentration. Figure 21 shows the salt concentration at which the μ change is 50% complete vs D/L. The most reasonable conclusion, therefore, is that complex formation and dissociation is not reversible.

Additional evidence on questions of reversibility is provided by salt dissociations of samples prepared in different ways. Figure 24 shows dissociations for three differently prepared samples--salt annealed to 0.05 M NaCl (Complex A), salt annealed to 0.00 M NaCl (Complex S.A.(0)), and directly mixed at 0.00 M NaCl (Complex D.M. (0)). In addition points are shown for the series of S.A. and

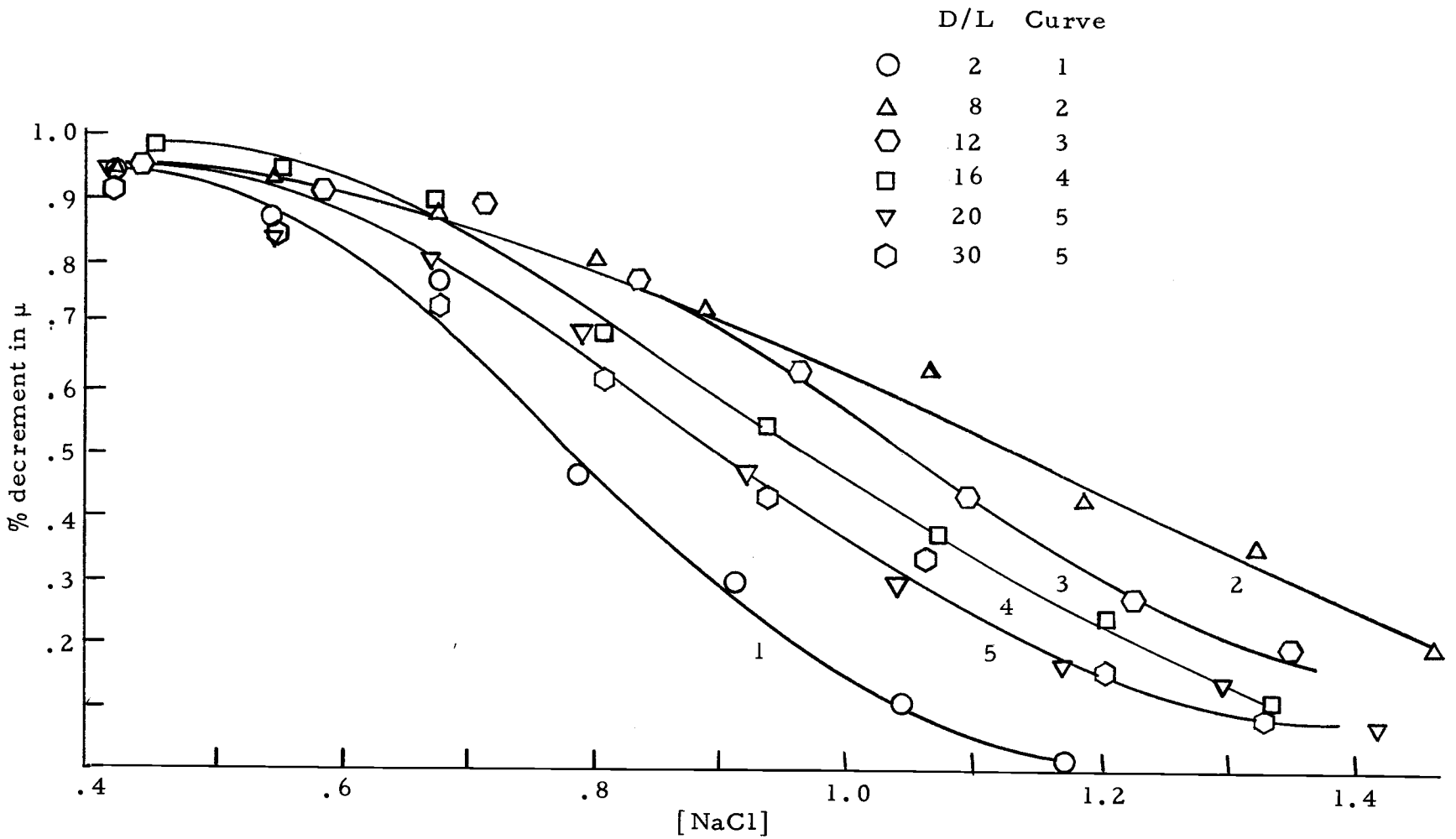

Figure 23. Percentage change in μ as a function of NaCl concentration for the NaCl dissociation of D/L(2), D/L(8), D/L(12), D/L(16), D/L(20) and D/L(30).

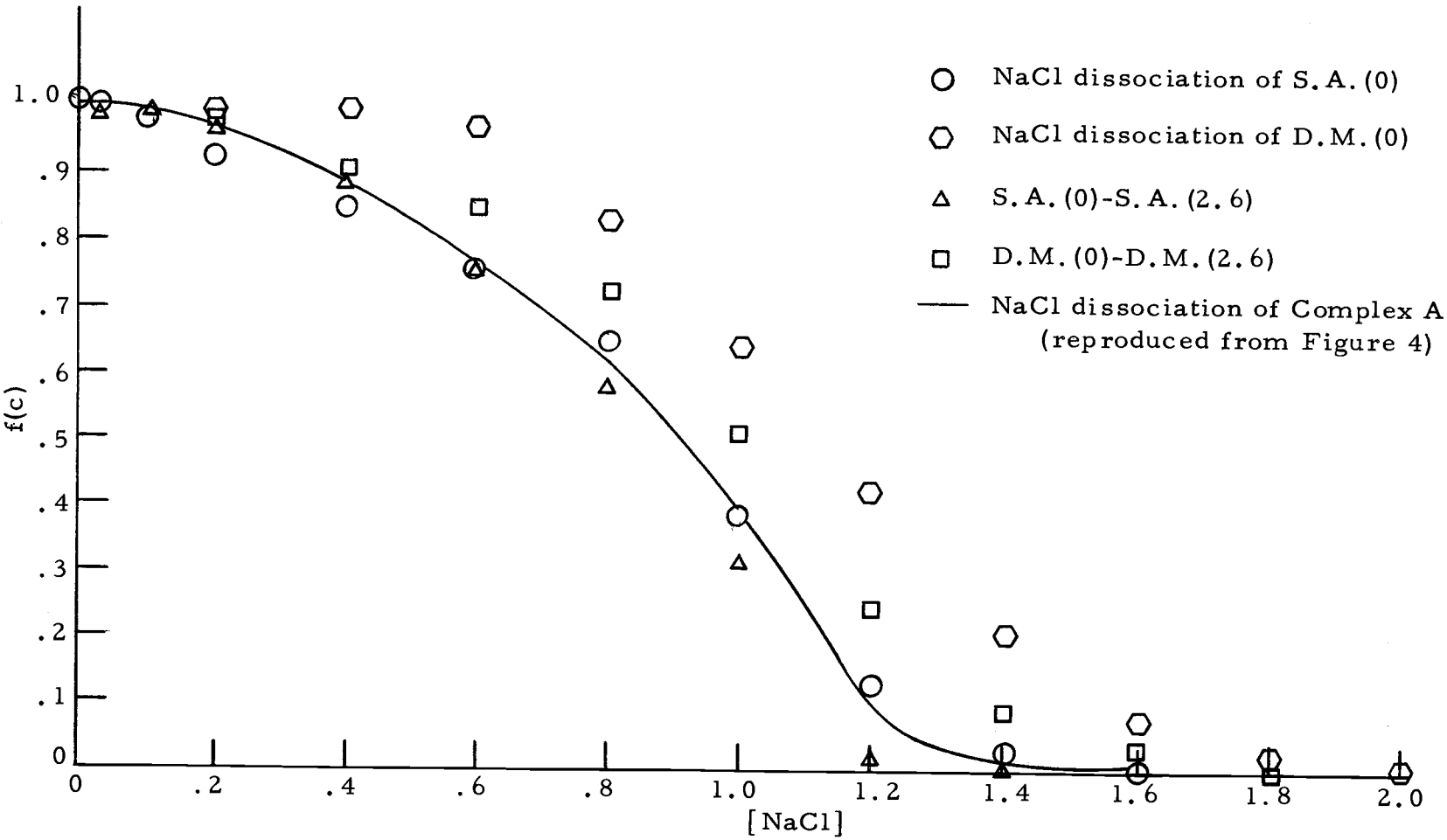

Figure 24. Comparison of fraction of polylysine bound for NaCl dissociation of complexes prepared in different ways.

and D.M. complexes, where f(c) is calculated by using each complex in the series as if it were a point in a salt dissociation. Several features are noteworthy: (1) There is no detectable difference for samples dialyzed to 0.05 M and 0.00 M salt. (2) Samples prepared by direct mixing dissociate at the highest salt concentrations. (3) One does not obtain the same f(c) values using the S.A. and D.M. series of complexes. One again concludes that the interactions are irreversible.

It should be noted, however, that the D.M. complex series and the S.A. series must be considered as preliminary results only. The anisotropies found at high salt were lower for both series of complexes that what is obtained after salt dissociation of annealed or directly mixed samples at zero salt (Figures 25 and 26). A noteworthy feature is that, above 1.4 M NaCl both series of complexes yielded anisotropy values equal to that of DPL in the absence of DNA. This might seem to imply that samples prepared by gradient dialysis or direct mixing, at low salt, are not really dissociated completely when "salt dissociated" to 2.6 M NaCl. No evidence is at hand to support such an assertion but, it should be noted, that contrary evidence does not exist either, and the point must await future investigations.

The various types of samples show distinguishable turbidity characteristics (Figure 27) which differ from one type of preparation

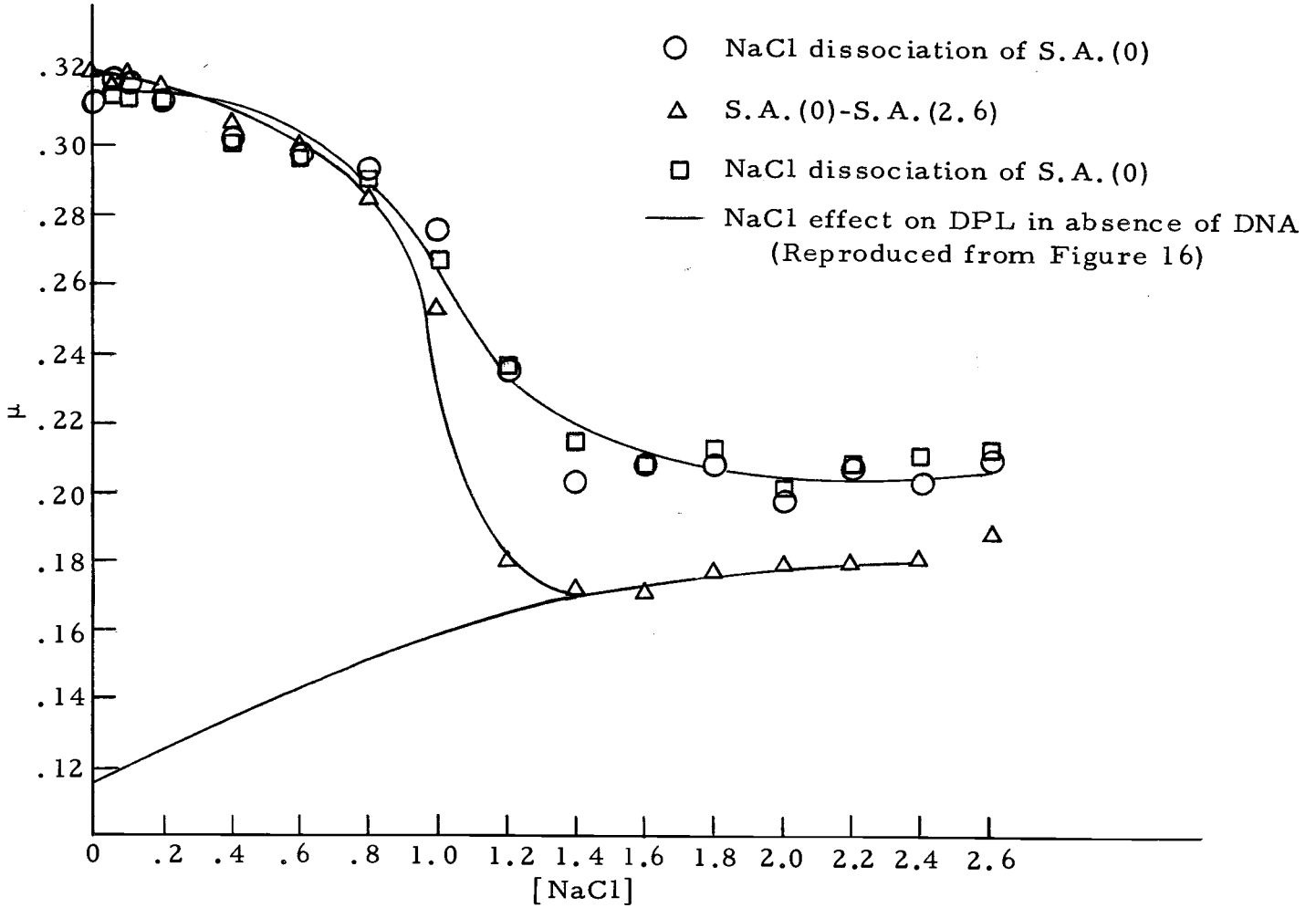

Figure 25. Anisotropy of S.A.(0)-S.A.(2.6) and NaCl dissociation of S.A.(0).

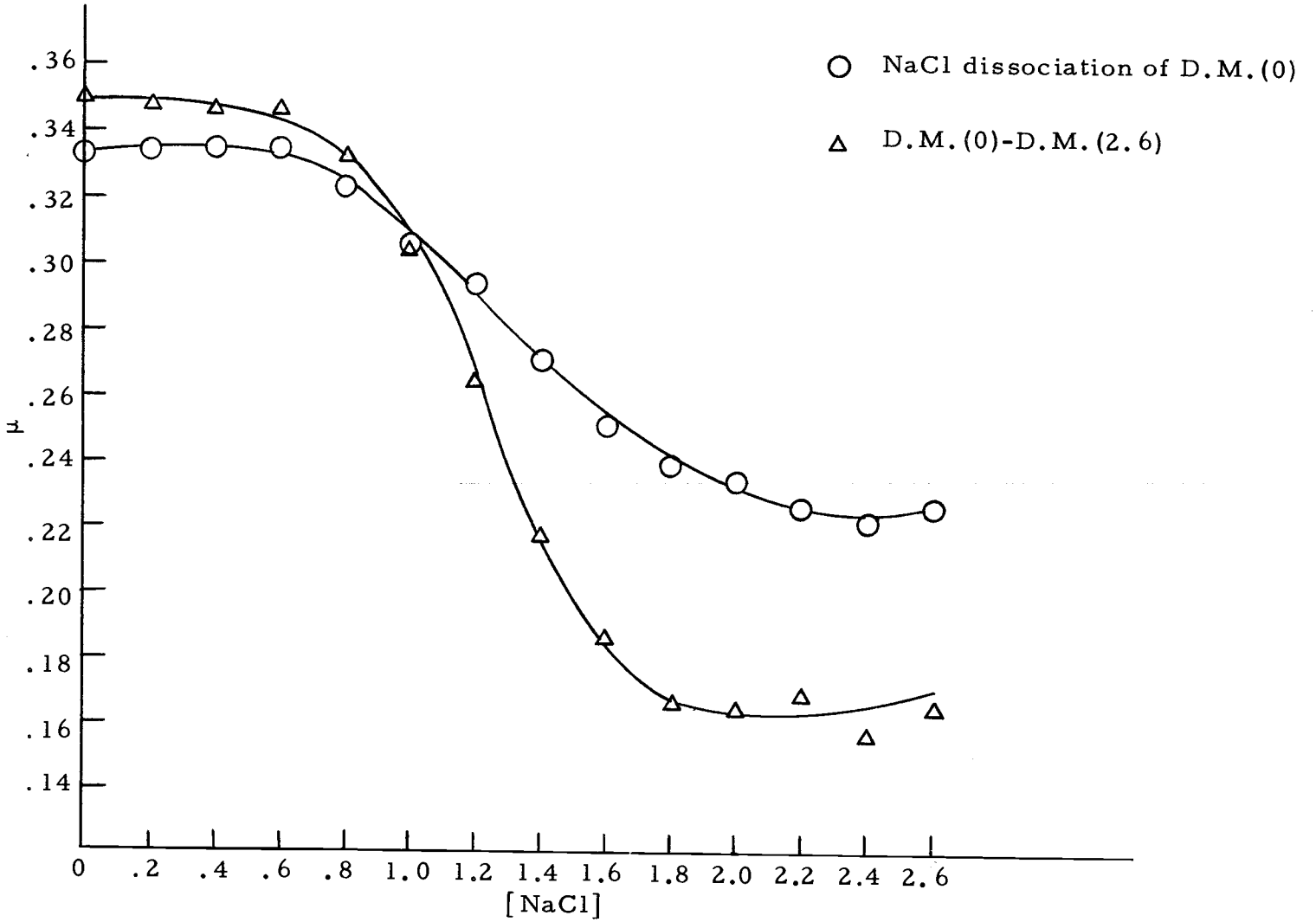

Figure 26. Anisotropy of D.M.(0)-D.M.(2.6) and NaCl dissociation of D.M.(0).

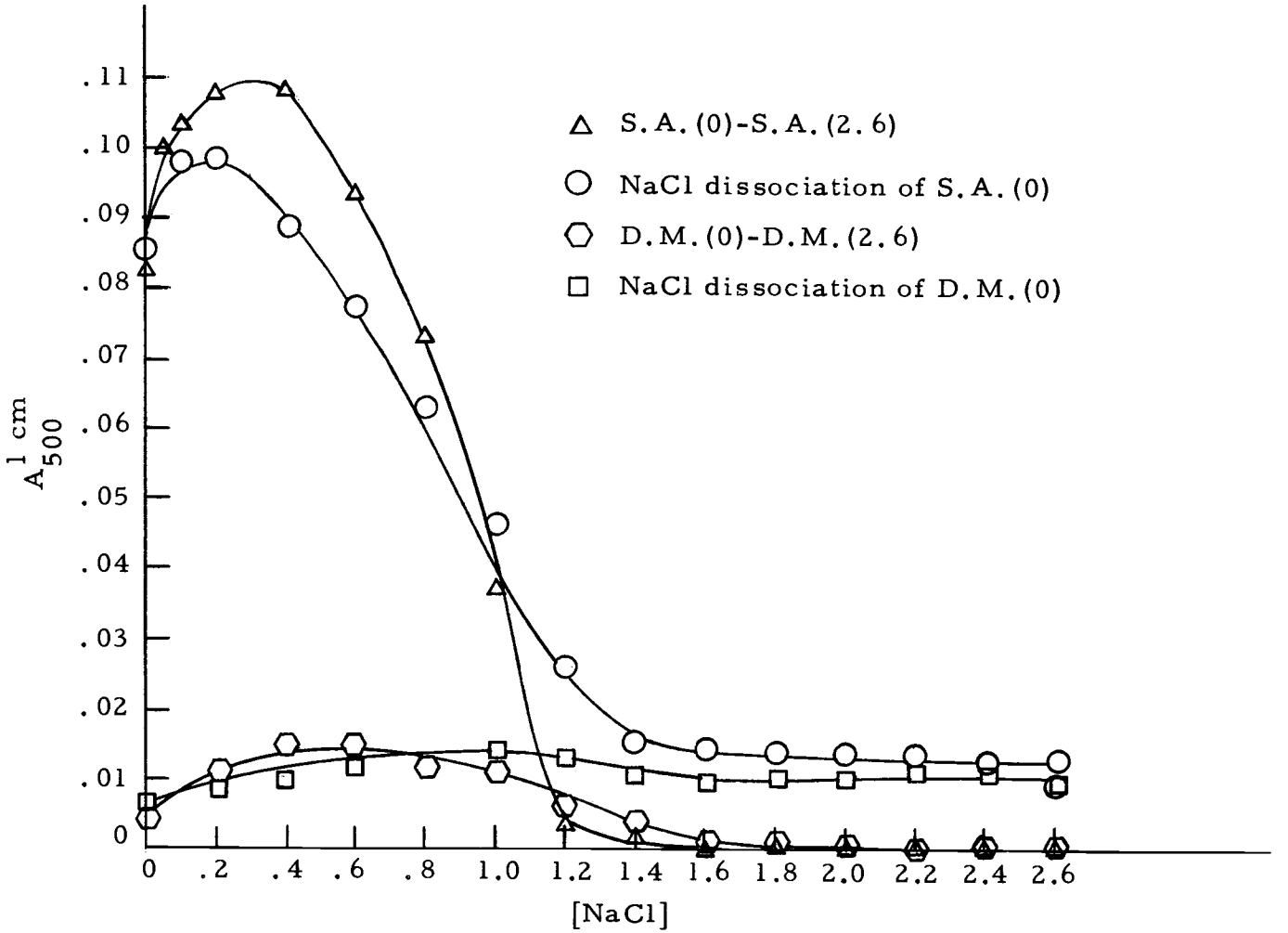

Figure 27. Turbidity of S.A.(0)-S.A.(2.6) and D.M.(0)-D.M.(2.6) and of NaCl dissociations of S.A.(0) and D.M.(0).

to another, consistent with the above suggestion.

Preliminary measurements with the analytical ultracentrifuge indicate that the method of preparation affects the size and size distribution of the aggregated phase. Samples prepared by salt gradient dialysis have a large distribution of sedimentation coefficients, for DNA(P)/lysine ratios greater than two. The S values range from about 1000 to 20,000-30,000. The DNA not complexed with the fast moving "boundary" has an S value just slightly higher than S for DNA alone. As the NaCl concentration increased up to 0.4 M, the S values were observed to increase in a parallel fashion with turbidity and then began decreasing as the NaCl concentration was further increased. At 1.0 M NaCl the stoichiometry of the complex was about unity and the distribution of S values was not quite as spread, now ranging from about 900 to 1600. The S.A. series of complexes and a NaCl dissociation cf S.A.(0) gave similar results.

In contrast to salt-gradient annealed complexes, those prepared by direct mixing had close to 1:1 stoichiometry at all salt concentrations for the fast sedimenting component and the S values for this component were lower and not as dispersed as those for the annealed samples. At 1.0 M NaCl the direct mixed samples were like those prepared by gradient dialysis at 10 M.

A crude calculation of the sedimentation coefficient of the material that is pelleted by the centrifugation at 30,000g for 90

minutes shows it to be a minimum of about 800 S. The sedimentation coefficient of the calf thymus DNA used in these studies was determined to be 14 S. The precipitate therefore represents a highly aggregated system, calculation of the DNA(P)/lysine ratio of the resuspended precipitate, i.e. B(resus), indicates a stoichiometry of 3.1:1. The salt-gradient dialysis procedure used in this study does not give the 1:1 stoichiometry von Hippel and co-workers (56) report for their preparations by salt-gradient dialysis and subsequent clarification by centrifugation at 1700 g for 15 minutes. The difference may be due to the difference in dialysis times and gradient steepness. My salt gradients are much more gradual than those of von Hippel and co-workers. They begin dialyzing against 0.4 M NaCl and reduced to 0.3 in 4 hrs. At 0.4 M NaCl I find the complex is still ~ 100% formed. Their procedure may be more like direct mixing than mine, and result in 1:1 stoichiometry.

DISCUSSION

As pointed out in the literature survey, a number of paradoxes exist in the results of studies of the interaction of polylysine with DNA. For example, at 1.0 M NaCl, added DNA will exchange with DNA in the complex and hence the interaction leading to formation of the complex and the aggregated phase are considered reversible (44). Yet reproducible results are not obtained from ORD measurements (68). A-T specificity exhibited by the interaction was used as a basis for the conclusion that the interaction is reversible and cooperative, but A-T specificity was also exhibited (68) by tetralysine that was shown to not bind cooperatively (57).

The measurements made in these studies were by techniques that measure properties of the aggregated phase, e.g. light scattering, sedimentation, and perhaps even ORD. The aggregated phase is not the primary result of the interaction of polylysine with DNA, and therefore its properties need not necessarily reflect properties of the initial interaction, i.e., the formation of the polylysine-DNA complex.

Fluorescence polarization studies have made it possible to study parameters of the system that are more closely a function of the primary interaction resulting in complex formation. It also appears that this technique is relatively insensitive to secondary

interactions, occuring subsequent to complex formation, that lead to aggregation of the complex. This was shown in the demonstration that the fraction bound measured on the supernatant, precipitated and whole fractions of complex were identical. It is also shown in the analysis of Mg^{++} dissociations in which there is an initial large aggregation upon the addition of salt, an aggregation not reflected in μ values (22). The use of fluorescence polarization makes it possible to examine the important thermodynamic point: Is the interaction reversible?

The result of studies by fluorescence polarization indicate that the formation and dissociation of the complex is not reversible. It is indicated, however, that the complex is a well-defined unit. Though turbidity and sedimentation indicated several different sizes or states of aggregation, the salt dissociation of the different aggregated complexes gave identical results.

It was shown in the presentation of results that the conclusion of irreversibility of the interaction leading to complex formation is independent of a choice of models for the interaction. As has been indicated several times in this thesis, there has been a hesitancy in the literature to describe the interaction as irreversible, even though some results have suggested this might be the case. This is perhaps because an irreversible system is also often ill-defined. This is not necessarily the case, and, as has been demonstrated in

this study, the complex is well defined.

The properties of the aggregated phase, that have been measured by other techniques, are not inconsistent with an irreversible interaction.

The interpretation that the binding is reversible is based on the demonstration that DNA is exchangeable into and out of the aggregated phase resulting from the interaction of polylysine with DNA at 1.0 M NaCl. This exchangeability was considered the necessary and sufficient proof that complex and aggregated phase formation are reversible.

Exchangeability does not mean reversibility however; it is merely consistent with reversibility. The following example may serve to illustrate the point. The exchangeability of a component of a piece of apparatus means it is possible to replace that component by a similar component without completely disassembling the entire apparatus. Thus, the apparatus may be considered irreversibly assembled, yet the component is readily exchangeable. In the same sense it is possible for DNA to exchange into and out of the complex and the aggregated phase without the formation of complex or aggregated phase being reversible.

Much of the difficulty in interpreting studies on the properties of the complex arises because a very restrictive model has been implicitly assumed. This model is that when polylysine interacts

with DNA it forms a complex that consists of a DNA molecule which has a series of polylysine molecules associated with it in a very specific manner, e.g. aligned next to it or wound around it. The binding results from matching up of the charged phosphate and amino groups plus, perhaps, some hydrophobic interactions in the groove of the DNA double strand. Exchangeability, in terms of this model, does require the interaction to be reversible. Selectivity, for the interaction described by this model, indicates the interaction "has" to be reversible and cooperative. Stoichiometry is expected in terms of this model when selectivity for A-T rich DNA is manifested. Yet, A-T specificity was reported for tetralysine, for which interaction was neither stoichiometric nor the binding cooperative. The more pertinent observation about the selectivity by tetralysine, was that selectivity was not manifested until the polylysine-DNA system formed the aggregated phase, and the A-T rich DNA-polylysine complex was pelletable. This feature is held in common in all the systems where A-T selectivity was observed.

A less restrictive model is to consider only that the complex is a well-defined structure resulting from the interaction of polylysine with DNA. As illustrated above, the formation of the complex does not have to be reversible to allow for exchangeability of DNA in this system.

An explanation for A-T selectivity can be offered in terms of

the less restrictive model that is consistent with irreversibility, and does not require stoichiometry or cooperativity. If an irreversible complex or aggregated phase occurs when polylysine is mixed with two species of DNA having different base compositions, equal amounts of each DNA may occur in the aggregated phase. It requires only a slightly stronger interaction of polylysine with A-T rich regions of DNA compared to G-C rich regions to result in the inclusion of the A-T rich DNA in the aggregated phase, because in such a case A-T rich DNA will not exchange out of the aggregated phase as readily as G-C rich DNA. The only feature of the interaction required in this model is that the formation of the aggregated phase be irreversible, for if it were not, and the aggregated phase could reversibly form and dissociate, there would be no mechanism for enriching the aggregated phase with the A-T rich species of DNA. New aggregated phases would be continually forming and those partially enriched in A-T rich DNA would be dissociating.

The above explanation is testable in the following way. Determination of the G-C content of the aggregated phase as a function of time after the DNA and polylysine are mixed will indicate whether or not an A-T enrichment of the aggregated phase occurs with time, as would be predicted by the above model.

This model was given only to indicate that reversibility, cooperativity and stoichiometry need not necessarily be required to

provide specificity, the point being that when selectivity is described in terms of a very restrictive model, one must begin making conclusions about the interaction on the basis of the model rather than evidence.

We propose that it is more reasonable, at the present time, to choose a very general model, with details to appear later as more data accumulate.

The hesitancy to describe the interaction as irreversible may be due to the connotations that term carries, for it is implied by irreversibility that the system is not well-defined or amenable to quantitative analysis, and suggests the interaction only goes in one direction. It may be pointed out, however, that in many biochemical pathways irreversible reactions are found at the key point in the pathway for control of the overall pathway. The structure of biological systems, such as mitochondria, must also be considered to be irreversibly formed. In both cases the system involved is certainly well-defined. And it seems very reasonable that the interactions involved in control of the genetic information of the cell may have irreversible characteristics.

A number of separate, but significant, comments may be made about the work reported here, particularly in relation to possible future studies. As pointed out in the presentation of results, analysis of the data by the two state model of Evett and Isenberg (22)

is insensitive to minor violations of the assumptions of constancy of ϵ_1 and μ_2. The insensitivity to these small perturbations does not mean, however, that the method is insensitive to changes in the dissociation of the complex, as was demonstrated by the wide variety of dissociation curves obtained by Evett (10) when using different salts. The observed insensitivity may mean these perturbations are related to secondary interactions of the complex. It is technically significant in that the method is insensitive to small accidental perturbations.

The ability to detect small changes in the molar extinction coefficient indirectly by intensity measurements has been demonstrated. This technique makes it possible to measure small extinction coefficients in the presence of high turbidity, an exceedingly difficult procedure by direct measurements. Use of this technique in this study provided evidence that a change occurs in the molar extinction coefficient of dansyl in the DPL-DNA complex at pH 5.0 and 5.5 when the salt concentration is increased from 0.05 M to about 0.2 M NaCl. This was interpreted as suggesting that the dye is in close proximity to the negative charge of the DNA phosphates. If this result is accepted then the possibility is raised for studying small pK shifts by fluorescence methods.

The results indicate that the Rhodamine B conjugate is also useful for fluorescence polarization studies of the polylysine-DNA

interaction. The excitation maximum of RBPL is about 550 nm, compared to 350 nm for DPL, and it is therefore possible to measure both of these dyes in the same solution independently. Such a technique could have application in competition or exchange studies for example.

This study shows that turbidity is not a quantitative measure of the complex, nor is it related to the primary interaction between polylysine and DNA, except under very restricted conditions. The polylysine concentration of all preparations used in this study was 10^{-4} M and thus the concentration of complex is comparable in all preparations. The turbidity, however, was shown to be dependent on the pH of the solution (Figure 15), the salt concentration (Figures 14 and 24), the DNA concentration (Figure 19), and the method of sample preparation (Figure 24).

The behavior of turbidity, both in this study and in the literature, closely correlates with the solubility of the complex. In general, turbidity is high when solubility is low and vice versa. The turbidity changes under conditions where no dissociation occurs, demonstrating it does not measure the amount of complex present. It more closely reflects the secondary interactions leading to aggregation. Because of this it may be possible to study the secondary interactions by using turbidity and fluorescence polarization measurements together, and at least partially separate the primary and secondary interactions.

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