STUDIES ON THE INTERACTION
OF DESOXYRIBONUCLEIC ACID
WITH ACRIFLAVINE

DISSERTATION

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I. INTRODUCTION

An ever increasing proportion of the researches on polyelectrolytes appearing in the literature in recent years have been more or less quantitative studies of the interaction of polyelectrolytes of many types with substances ranging from simple inorganic ions to other polyelectrolytes. The immediate object of the present investigation was to study the interaction of a highly polymerized preparation of the naturally occurring polyelectrolyte desoxyribonucleic acid (DNA) with a simple, well-defined, organic cation, employing techniques suitable for the system chosen. It was desired to obtain quantitative information as to the degree and strength of the binding of the cation by DNA and the thermodynamic constants of the reaction at several values of the experimental variables, ionic strength, pH, and temperature, preferably as a result of two or more independent experimental and theoretical approaches. After some searching, the fluorescent dye acriflavine, 3,6-diamino-10-methyl-acridinium chloride, was selected as the cationic interactant, and its complexing with DNA isolated from calf thymus was studied as a function of component concentrations and environmental conditions both by means of the distribution of the unbound dye between the aqueous phase and an immiscible organic solvent and by means of the quenching

of the fluorescence of the dye on its interaction with DNA.

It is believed that a body of data on the interaction of desoxyribonucleic acids from various sources with a large variety of molecules would supplement physico-chemical measurements on DNA alone and might yield valuable information concerning as yet unknown details of DNA structure and concerning its macromolecular configuration in solution and, particularly, in the nuclei of living cells. Further, from such accumulated data, taken under diverse experimental conditions, a great deal might be learned concerning the mode of interaction of DNA with proteins and other biological substances, the nature of the forces involved, the environmental conditions favoring interaction, and, ultimately, the role of DNA in cell metabolism and in mitosis and meiosis.

II. BACKGROUND

A. Interaction Studies

By far the largest number of polyelectrolyte interaction studies are concerned with the interactions of proteins, and a great many of these have been thoroughly reviewed and evaluated by Klotz (1), by Goldstein (2), who dealt particularly with pharmacological interactions, and by Putnam, in articles on interactions with detergents (3) and on protein denaturation (4). Besides answering the specific questions at which they were directed, these studies, taken as a whole, have made significant contributions to research on the interactions of polyelectrolytes in general. First, quite a number of experimental methods have been developed for the detection of interaction with a macromolecule and its quantitative evaluation in various types of complexing systems. Second, theoretical treatments of binding by polyelectrolytes have appeared and

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- (1) I. M. Klotz, in The Proteins, Vol. I, Part B, eds. H. Neurath and K. Bailey, Academic Press, New York, p. 727 (1953).
 - (2) A. Goldstein, J. Pharmacol. Exptl. Therap. 95, 102 (1949).
 - (3) F. W. Putnam, Advances in Protein Chem. 4, 79 (1948).
 - (4) F. W. Putnam, in The Proteins, Vol. I, Part B, eds. H. Neurath and K. Bailey, Academic Press, New York, p. 807 (1953).

have been expanded and modified so that increasingly complex types of interactions have been accounted for satisfactorily.

Some of the concepts gleaned from the work on proteins, especially on relatively pure and well-characterized proteins, are of general applicability. While most of the interactions studied are basically electrostatic in nature, affinity is enhanced when the structure of the interactant molecule offers opportunities for secondary binding: Indeed, secondary forces alone are sometimes sufficient to permit the binding of neutral molecules by proteins (5). Whenever the interaction of a polyelectrolyte is under consideration, careful attention must be given to the factor of competition of other substances present in solution for the binding sites involved. The buffer ions, impurities, even the solvent itself may compete with the interactant under study to an extent dependent upon the relative affinities of the substances for the binding sites on the polyelectrolyte and the relative concentrations present. Studies of competitive binding or of the binding of one substance by means of its displacement of another may be very instructive, except that, as Karush has pointed out (6), such studies are greatly complicated by the fact that two

(5) I. M. Klotz and J. Ayers, *Disc. Far. Soc.* 13, 189 (1953).

(6) F. Karush, *J. Am. Chem. Soc.* 72, 2714 (1950).

interactants may have widely different affinities for particular sites on the polyelectrolyte. Although a great many opportunities remain unexploited, a good beginning has been made toward uncovering detailed structural features of proteins by studying their complexing properties. Data is being amassed on the relative affinities of proteins for organic molecules differing in size, nature of reactive group, substituent position, and so forth (7, 8, 9). Hypotheses have been advanced to account for these differences in reactivity and, also, for the differences in the binding capacity of different proteins for the same substance in the reversible, non-specific sort of interaction which is emphasized in the reviews mentioned. Among these hypotheses we may note two which attempt to explain the remarkable affinity of serum albumins for a large number of unrelated anions: that of Klotz and his coworkers (10) suggesting that the low content of serum albumins in hydroxyl groups precludes internal hydrogen bonding with the amino and carboxyl groups needed for electrostatic binding; and that of Karush (11) proposing

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- (7) J. D. Teresi and J. M. Luck, J. Biol. Chem. 174, 653 (1948).
- (8) I. M. Klotz, J. Am. Chem. Soc. 68, 2299 (1946).
- (9) I. M. Klotz, R. K. Burkhard, and J. M. Urquhart, J. Phys. Chem. 56, 77 (1952).
- (10) I. M. Klotz, Cold Spring Harbor Symp. Quant. Biol. 14, 97 (1950).
- (11) F. Karush, J. Am. Chem. Soc. 72, 2705 (1950).

an unusual configurational adaptability for serum albumins that would permit them to accommodate interactant molecules of many sizes and shapes. In contrast, extensive study in the field of the extremely specific immunological reactions (12, 13) has yielded, in accordance with the general "lock-and-key" concept, quite detailed ideas about specific and, presumably, very rigid configurations on the surfaces of individual proteins complementary to the structures of the particular haptens with which they interact (14). Similar structural information should arise from studies of the intermediate enzyme-substrate complexes, which likewise involve specific interactions. It is evident that the factors mentioned here, steric factors, degree of macromolecular flexibility, and requirements for special configurations in the neighborhood of binding sites will be as important in interpreting any polyelectrolyte interaction as they have been found to be in studying binding to proteins.

Research on the interactions of natural polyelectrolytes other than proteins and of synthetic polyelectrolytes is still in the beginning stages. A review of some of the recent polyelectrolyte interaction studies is

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- (12) K. Landsteiner, The Specificity of Serological Reactions, Harvard University Press, Cambridge, Mass. (1945).
- (13) W. C. Boyd, in The Proteins, Vol. II, Part B, eds. H. Neurath and K. Bailey, Academic Press, New York, p. (1954).
- (14) L. Pauling, Endeavor 7, 43 (1943).

included in the report from this laboratory on a light scattering investigation of the interaction between egg albumin and a synthetic cationic polyelectrolyte (15), and the relatively meager literature on binding by nucleic acids will be examined at the end of this section.

The equilibrium methods which have been developed for the study of reversible, non-specific associations giving rise to stable complexes can be divided into two groups (1, 2). One group depends upon alteration of the properties of a macromolecule: pH changes in the case of proteins and other polyampholytes, changes in spectra, refractive index, light scattering, or optical rotation, changes in osmotic pressure, viscosity, surface tension, sedimentation or electrophoretic properties, etc. The other group takes advantage of changes in the properties of a small interacting molecule on interaction: increase in solubility of a slightly soluble substance, decrease in electrical mobility of an ion, decrease in diffusion current in polarographic reduction, or changes in diffusion properties, spectrum, or biological activity. In certain cases, it is possible to measure the concentration of unbound low molecular weight interactant, either by means of a suitable electromotive cell or by separating and analyzing a polymer-free portion of the solution. The separation is sometimes

(15) I. J. Heilweil, Doctoral Dissertation, The Ohio State University (1954).

effected by ultrafiltration or ultracentrifugation, but, more usually, quantitative information is obtained by equilibrium dialysis or partition analysis.

In the equilibrium dialysis technique (16), the macromolecule is confined on one side of a membrane impermeable to it but fully permeable to the small interactant molecules. When equilibrium has been attained, the unbound low molecular weight interactant is distributed across the membrane in accordance with the Donnan equation, so that the total amount of unbound substance may be calculated if the polymer-free solution is analyzed and the volumes on both sides of the membrane are known. The amount bound is then easily obtained from the total amount of interactant present in the system. In most studies involving ionic interactants, the Donnan effect is made negligible by the addition of simple electrolytes, but it must be kept in mind that the results obtained for the thermodynamic constants of the reaction in such experiments are, because of competition for binding sites by the added ions, a function both of the ionic strength of the medium and of the nature of the specific ions added. Quite often, the membrane takes up considerable amounts of the dialyzing component, so that control experiments must be run to determine a

(16) I. M. Klotz, F. M. Walker, and R. B. Pivan, J. Am. Chem. Soc. 68, 1486 (1946).

correction for this adsorption. If the membrane uptake does not vary too greatly from membrane to membrane, the accuracy of the equilibrium dialysis technique depends only upon the accuracy of the method of analyzing for the interactant.

Partition analysis, recently adapted for the study of the interactions of macromolecules by Karush (17), involves the distribution of one of the interactants between the aqueous phase and an immiscible liquid in which the second interacting species, usually a polyelectrolyte, is insoluble. The concentration of the distributed species is determined in the organic phase, and the concentration of unbound substance in the aqueous phase is calculated by means of the separately determined partition coefficient. Inherent in the method is the assumption that the partition coefficient for the free interactant is not changed by the presence of the polyelectrolyte in the aqueous phase. Furthermore, if the organic phase is at all soluble in water, it may, in some cases, compete for binding sites on the polyelectrolyte or alter the binding properties of the polyelectrolyte in some way. Provided that a suitable organic phase can be found, the method offers several advantages. There are no restrictions upon the sizes of the molecules of the two interacting species. Membrane difficulties are avoided altogether, and, since the Donnan effect is absent,

(17) F. Karush, J. Am. Chem. Soc. 73, 1246 (1951).

determinations may be made at zero or at very low ionic strength. Finally, the time required^o for equilibration is much shorter than in the equilibrium dialysis method, a factor which could be of considerable importance in working with molecules that are unstable. The details of the application of these methods in the present work will be discussed in the section on experimental procedures (IV).

B. Structure and Properties of DNA

Desoxyribonucleic acids, along with associated proteins, are the major constituents of chromosomal material, the material believed responsible for the transmission of hereditary characteristics. They have been isolated for study from many tissues by several methods, some of which have recently been examined by Frick (18). Since the physico-chemical properties of DNA have been found to be very sensitive to factors in their preparation and storage, such as pH, ionic strength, temperature, enzyme inhibition, stirring technique, and many others, the latest preparative procedures have been devised with the aim of producing DNA samples free of contamination, especially by protein and ribonucleic acid, and in a state of polymerization like that existing in vivo, as far as this state can be deduced.

(18) G. Frick, Biochim. Biophys. Acta 13, 374 (1954).

With the exception of a few details, the chemical structure of DNA has been established by conventional techniques of organic and biochemistry, and the results are presented by Davidson (19) and in a monograph on nucleic acids (20), both of which contain, as well, references to the vast earlier literature on DNA. A later discussion of some features of chemical structure may be found in a paper by Butler (21). Briefly, it may be said that the DNA chain has a backbone of alternate 2-desoxy-D-ribose and phosphate groups joined through phosphate diester linkages. Each sugar group has attached one of the five purine or pyrimidine bases which have been detected in DNA from many sources, adenine, guanine, thymine, cytosine, and 5-methyl-cytosine. The phosphate ester of the base-sugar glycoside is called a nucleotide, and several thousand nucleotides comprise a DNA molecule. Contrary to earlier opinion, the order of nucleotides is probably irregular, and the exact sequence is still the subject of much speculation. The careful paper chromatographic analyses of Chargaff and his colleagues (22) have revealed certain remarkable regularities

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- (19) J. N. Davidson, The Biochemistry of the Nucleic Acids, Methuen and Co., Ltd., London (1950).
(20) Symp. Soc. Exp. Biol. 1 (1947).
(21) G. Butler, Can. J. Med. Sci. 31, 222 (1953).
(22) E. Chargaff, C. F. Crampton, and R. Lipshitz, Nature 172, 289 (1953).

in the molar contents of the various purines and pyrimidines in DNA: while animal species differ slightly in the amounts of the several bases present in their nuclei, the various tissues from any individuals of the same species have a constant average composition; in every tissue studied, it has been found that the purine content equals the pyrimidine content, the cytosine plus adenine content equals the thymine plus guanine content, and the adenine to thymine and guanine to cytosine ratios are both unity.

In contrast, the macrostructure of DNA is being investigated by the many methods currently being developed for the study of the physico-chemical nature of polyelectrolytes and is still far from being completely elucidated, though considerable progress has been made of late (23, 24, 25). Investigations are made on the newer, less degraded preparations of DNA, or, more exactly in neutral solutions, of the sodium salt of DNA, in an effort to clarify such features as the amount of chain branching, the degree and manner of internal hydrogen-bonding, a concept introduced by Gulland and his group in their series of physico-chemical studies (26), the degree and type of electrostatic

(23) Trans. Far. Soc. 50, 290 (1954).

(24) Proc. Nat. Acad. Sci. 40, 747 (1954).

(25) K. G. Stern, Bull. Soc. chim. biol. 34, 648 (1952).

(26) J. M. Gulland, et al., J. Chem. Soc. 1947, 1129 ff.; 1949, 1406 ff.

interaction between neighboring chains, the molecular weight, shape, and degree of chain flexibility, as functions of environmental conditions.

Although the results and interpretations of different workers sometimes seem to conflict, certain general trends of opinion in the recent research may be noted. Most viscosity studies (27) take into account the extraordinarily high structural viscosity of DNA and the consequent necessity for extrapolating to zero shear. Butler and his associates (28), working at very low DNA concentrations at several ionic strengths, present evidence that electrostatic interactions between DNA molecules exist at very low ionic strength. Moreover, sedimentation (29, 30) and diffusion (31) studies, also, indicate the existence of intermolecular interactions of some sort down to low DNA concentrations. Because of the high asymmetry of the DNA molecule, recent light scattering work (32, 33) has

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- (27) M. J. Callanan and W. R. Carroll, *J. Poly. Sci.* 11, 53 (1953).
 - (28) J. A. V. Butler, B. E. Conway, and D. W. F. James, *Trans. Far. Soc.* 50, 612 (1954).
 - (29) K. G. Stern and S. M. Atlas, *J. Biol. Chem.* 203, 795 (1953).
 - (30) V. L. Koenig and J. D. Perrings, *J. Coll. Sci.* 8, 452 (1953).
 - (31) J. A. V. Butler and D. W. F. James, *Nature* 167, 844 (1951).
 - (32) J. W. Rowen, *Biochim. Biophys. Acta* 10, 391 (1953).
 - (33) M. E. Reichmann, S. A. Rice, C. A. Thomas, and P. Doty, *J. Am. Chem. Soc.* 76, 3047 (1954).

emphasized angular scattering measurements, and the general consensus of opinion appears to be that the molecular weight of highly polymerized samples of DNA are in the range of several millions, that the shape is that of a slightly flexible rod or a rather stiff chain even in solutions of a relatively high ionic strength, and that there is a high intermolecular attraction down to the lowest concentrations measured. Electron microscopical examinations (34, 35) seem to confirm that the DNA macromolecule may exist as a long, thin, rather inflexible fiber, but little is as yet known of the effect on the apparent structure of the method of specimen preparation for electron microscopy. A number of X-ray analyses of DNA samples prepared in various ways have yielded several very promising ideas about the structure of the DNA fiber and of concentrated DNA solutions (36, 37, 38, 39). In the most recent interpretations, there seems to be

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- (34) R. C. Williams, *Biochim. Biophys. Acta* 9, 237 (1952).
(35) H. Kahler and B. J. Lloyd, *Biochim. Biophys. Acta* 10, 355 (1953).
(36) W. T. Astbury, *Symp. Soc. Exp. Biol.* 1, 66 (1947).
(37) D. P. Riley and G. Oster, *Biochim. Biophys. Acta* 7, 526 (1951).
(38) L. Pauling and R. B. Corey, *Proc. Nat. Acad. Sci.* 39, 84 (1953).
(39) R. E. Franklin and R. G. Gosling, *Acta Cryst.* 6, 673, 678 (1953).

agreement that the molecular structure is helical in nature, and there is considerable evidence (37, 39) that the macromolecules aggregate, probably through their phosphate groups, to form more or less ordered systems depending upon the water content. Crick and Watson (40) have proposed an exceedingly attractive hypothetical macrostructure for DNA which, while it will no doubt require a good bit of modification and elaboration, appears to be in accord with the general features of the X-ray evidence, the surprising analytical results of Chargaff and his collaborators (22), and a large part of the reliable physico-chemical data, including recent observations on infrared absorption (41). In addition, it suggests the outlines of a molecular mechanism, long sought, for the role of a relatively simple substance like DNA in the transmission of a complex system of hereditary factors. The Watson and Crick proposal envisions two polynucleotide chains wound helically about a common fiber axis and joined by hydrogen bonding between pairs of nitrogen bases, one from each chain, whose atoms lie in the same plane approximately perpendicular to the fiber axis. As a consequence of steric considerations, adenine may be paired only with thymine and guanine only with cytosine or

(40) F. H. C. Crick and J. D. Watson, Proc. Roy. Soc. A 223, 80 (1954).

(41) G. Frick and A. Rosenberg, Biochim. Biophys. Acta 13, 455 (1954).

5-methyl-cytosine; thus, the sequence of nucleotides is determined for one chain by the sequence in the other, but any sequence is permissible on the first chain. It is noteworthy that the sugar phosphate backbone forms the outside of the helix in the proposed structure, so that the phosphate groups occur on the periphery of the helix and are therefore easily accessible for interchain interactions and binding of cations.

C. DNA Interaction Studies in the Literature

The expectation has already been expressed that accumulated data on the interactions of DNA should ultimately yield information concerning features of macrostructure. This section is intended as a brief summary of some of the interaction studies already in the literature. As in the preceding section, emphasis is given to investigations of the last few years, which, in turn, will serve as a guide to older researches.

Quantitative studies on the long-recognized association of inorganic cations with the negatively charged phosphate groups of the DNA backbone are just beginning to appear (1954) and will undoubtedly contribute significantly in the elucidation of the role of salts in intermolecular interactions, enzymatic degradation, competitive interactions, and other phenomena. The extent of binding of

alkali ions per DNA phosphate group, roughly 0.5 in neutral solutions, does not appear to depend upon the state of aggregation of DNA (42, 43). The binding of a series of divalent cations has been studied conductimetrically and by means of the effect of ionic association in lowering the absorption maximum of DNA at 260 mμ (44, 45). Cavalieri (45) believes that magnesium ions interact both at the DNA phosphate groups and at the nucleotide amino groups. Using viscosity, light scattering, electrophoresis, and equilibrium dialysis techniques, Katz (46) investigated the rather unusual reaction of DNA with mercuric ions, which is accompanied by aggregation without precipitation in mercuric chloride solutions. The compositions of several of the insoluble rare earth complexes of DNA have been determined (47). The experiments and commentary of Neuberg and Roberts (48) are

-
- (42) J. Shack, R. J. Jenkins, and J. M. Thompsett, J. Biol. Chem. 198, 85 (1952).
- (43) A. Veis, J. Phys. Chem. 57, 189 (1953).
- (44) J. Shack, R. J. Jenkins, and J. M. Thompsett, J. Biol. Chem. 203, 373 (1953).
- (45) L. F. Cavalieri, J. Am. Chem. Soc. 74, 1242 (1952).
- (46) S. Katz, J. Am. Chem. Soc. 74, 2238 (1952).
- (47) K. G. Stern and M. A. Steinberg, Biochim. Biophys. Acta 11, 553 (1953).
- (48) C. Neuberg and I. S. Roberts, Arch. Biochem. 20, 185 (1949).

a good indication of the very numerous reactions of DNA with cations and the almost unlimited possibilities for studying them. It appears that Neuberg and Roberts did not consider competitive effects in their discussion of the solubility of water-insoluble DNA complexes in solutions containing relatively high concentrations of alkali ions.

Since the interactions of DNA with proteins are generally believed to be primarily electrostatic in nature, perhaps it would not be out of place to mention several such studies at this point. Attempts are often made to discover whether native nucleoproteins and reconstituted nucleoprotein complexes prepared from protein and DNA salts have the same properties at the same concentrations of simple salts: Alexander (49) studied the constitution and behavior of DNA-protamine complexes, and the reassociation of DNA and histone has recently received further attention (50). From their observation that the measured X-ray scattering curves of nucleoprotein samples agreed, essentially, with the curves obtained by summation of experimental scattering curves for the individual nucleates and proteinates, Riley and Arndt (51) concluded that the nucleoproteins they

(49) P. Alexander, *Biochim. Biophys. Acta* 10, 595 (1953).

(50) G. F. Crampton, R. Lipshitz, and E. Chargaff, *J. Biol. Chem.* 206, 499 (1954).

(51) D. P. Riley and U. W. Arndt, *Nature* 172, 294 (1953).

studied were addition complexes, linked electrostatically, for the most part, without major structural change in the components. The interaction between calf thymus DNA and bovine serum albumin below pH 5.5 has been studied electrophoretically by Goldwasser and Putnam (52) and by Geiduschek and Doty (53), who made use of recent theoretical derivations concerning the application of light scattering to multi-component systems. The indications are that the reaction is in good part electrostatic and that the nucleic acid undergoes little or no change in shape upon association with this protein. Steiner (54) studied the interactions between DNA and the proteins serum albumin and lysozyme by means of fluorescence polarization.

In the last decade or two, reports have begun to appear rather frequently in the literature of researches especially designed for the investigation of the interactions in solution between DNA and dyes, drugs, and other small organic ions or molecules. Few general conclusions should as yet be drawn from these studies, not only because relatively few results are available and because the experimental conditions have not always been comparable, but also

(52) E. Goldwasser and F. W. Putnam, J. Phys. Coll. Chem. 54, 79 (1950).

(53) E. P. Geiduschek and P. Doty, Biochim. Biophys. Acta 9, 609 (1952).

(54) R. F. Steiner, Arch. Biochem. Biophys. 46, 291 (1953).

because the interactions are often found to depend markedly upon the state of aggregation of the DNA, and the DNA preparations used in the different studies have varied widely. Michaelis (55, 56) considered the interaction in solution of various nucleic acids with cationic dyes on the basis of the changes in the absorption spectra of the dyes and inferred that dye cations are bound at the DNA phosphate groups singly or in pairs but probably not as higher aggregates. From the difference in absorption properties of free "methyl green", ethyl hexamethyl para-rosaniline, and of methyl green in combination with DNA, Kurnick and Foster (57) were able to study the stoichiometry of DNA-methyl green complexing. They emphasize that methyl green does not seem to combine with depolymerized DNA (58). Cavalieri and his collaborators were probably the first workers to apply the equilibrium dialysis and partition analysis techniques to the study of reversible interactions of DNA. They studied the interaction of a DNA sample prepared according to the method of Hammarsten with the dyes rosaniline hydrochloride, closely akin to methyl green, and trimethyl-

(55) L. Michaelis, Cold Spring Harbor Symp. Quant. Biol. 12, 131 (1947).

(56) L. Michaelis, J. Phys. Coll. Chem. 54, 1 (1950).

(57) N. B. Kurnick and M. Foster, J. Gen. Physiol. 34, 147 (1950).

(58) N. B. Kurnick, J. Am. Chem. Soc. 76, 417 (1954).

(p-(p-hydroxy-benzeneazo)-phenyl)-ammonium chloride (59, 60) under different conditions of pH, ionic strength, and temperature, and they interpreted the results in terms of two groups of binding sites on DNA, presumably primary and secondary phosphate groups, with differing binding affinities. In several experiments (60, 45), these workers attempted to determine whether magnesium ions, bovine serum albumin, desoxyribonuclease, and others associate to any significant extent with DNA by measuring the degree of displacement of the dyes from DNA by these substances. As we have noted before, however, such results must be interpreted with a great deal of caution.

The equilibrium dialysis studies of Allenby and Collier (61) on the binding of uncharged thiazines to yeast nucleic acid and to nucleoprotein, while they do not directly concern us here inasmuch as DNA was not investigated, are of interest because they indicate that binding may occur at sites other than the phosphate groups, sometimes by mechanisms other than Coulombic attraction.

Several investigations have been reported of the strong interactions between DNA and quinoline and acridine

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- (59) L. F. Cavalieri and A. Angelos, J. Am. Chem. Soc. 72, 4686 (1950).
(60) L. F. Cavalieri, A. Angelos, and M. E. Balis, J. Am. Chem. Soc. 73, 4902 (1951).
(61) G. M. Allenby and H. B. Collier, Arch. Biochem. Biophys. 38, 147 (1952).

derivatives. Interactions of this kind may, at least in part, account for the observed effectiveness of acridines in inhibiting the growth of microorganisms (62), viruses, and even mouse tumors (63). In a comprehensive paper, Albert, et al. (62), analyzed the characteristics of molecular structure in acridine compounds which appear to enhance their antibacterial activity, and these observations should be of value in selecting specific compounds for quantitative interaction studies involving well-characterized nucleic acids. According to quantitative spectrophotometric and equilibrium dialysis results (64, 65), doubly-charged cationic derivatives of 4-aminoquinoline and 9-aminoacridine, with the positive charges on the ring nitrogen and the diethylamino nitrogen of the rather complicated aliphatic side chains, associate reversibly and to a considerable extent with highly polymerized DNA and nucleoprotein, the affinity being less for yeast nucleic acid. The acridine compound is bound more strongly than the quinoline derivative, and the side chains of these antimalarials are not

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- (62) A. Albert, S. D. Rubbo, R. J. Goldacre, M. E. Davey, and J. D. Stone, *Brit. J. Expt. Path.* 26, 160 (1945).
- (63) M. R. Lewis and P. P. Goland, *Am. J. Med. Sc.* 215, 282 (1948).
- (64) F. S. Parker and J. L. Irvin, *J. Biol. Chem.* 199, 897 (1952).
- (65) J. L. Irvin and E. M. Irvin, *J. Biol. Chem.* 206, 39 (1954).

necessary to the interactions. Some time ago, Oster and Grimmson (66) noted spectral shifts implying association of acriflavine and similar compounds with nucleic acids. Oster (67) examined the quenching of the fluorescence of acriflavine and of several other acridines by highly polymerized and commercial preparations of calf thymus DNA and attributed the strong quenching effect, greatest for the most highly polymerized sample, to interaction involving both electrostatic and secondary bonding. Acriflavine has been chosen as the cationic interactant in the quantitative work to be presented here, and fluorescence quenching data is used to derive the constants for the binding by a high molecular weight preparation of DNA according to a hypothesis somewhat different from that of Oster. The results are corroborated by independent partition analysis experiments.

(66) G. Oster and H. Grimmson, Arch. Biochem. 24, 119 (1949).

(67) G. Oster, Trans. Far. Soc. 47, 660 (1951).

III. THEORETICAL CONSIDERATIONS IN POLYELECTROLYTE INTERACTION STUDIES

Quite a few attempts have been made to account theoretically for binding by polyelectrolytes in terms of theories of solutions, theories of adsorption, molecular models, etc. Among these we may note the application by Schellman (68) of the Bjerrum ion association theory to the binding of spherically symmetrical ions by proteins, with the assumption that the ion pair is shielded by the protein from the polar solvent so that the dielectric constant in the region of the binding site is reduced. Hill (69) has treated the binding in solution of ions or molecules by proteins, or, indeed, by any molecule capable of binding one or more molecules of another kind, on the basis of a recent quantum mechanical general theory of solutions involving all the forces operating in solution between molecules of any kind. From somewhat different considerations, Steiner (70) has derived equations which permit the calculation of the adsorption isotherm for binding by a one-

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- (68) J. A. Schellman, J. Phys. Chem. 57, 472 (1953).
(69) T. L. Hill, in Ion Transport Across Membranes, ed. H. T. Clarke, Academic Press, New York, p. 189 (1954).
(70) R. F. Steiner, J. Chem. Phys. 22, 1458 (1954).

dimensional array of sites, which could well be a nucleic acid or any other polyelectrolyte molecule, from measurements of the concentration of free adsorbate and estimates of the energies of interaction for first and second nearest neighbor pairs. Theoretical treatments such as these will no doubt be of great value in attempts to deduce a molecular mechanism for interaction in a given system for which experimental data is available and to describe the forces involved. In this section, the discussion will be confined to the bases for the calculations of constants of binding which have been carried out in connection with the experiments to be described.

A. Multiple Equilibria and the Law of Mass Action (71a, 71b)

The equilibria involved in the reversible interaction of one molecule (or ion) of species A with a number of molecules (or ions) of species B may be described by the equations

(71a) J. Bjerrum, Metal Ammine Formation in Aqueous Solution, P. Haase and Son, Copenhagen (1941).

(71b) J. C. Sullivan and J. C. Hindman, J. Am. Chem. Soc. 74, 6091 (1952).

[1] Cont'd.

$$\begin{aligned}
 AB_{n-1} + B &\rightleftharpoons AB_n, & k_n &= [AB_n]/[AB_{n-1}] c \\
 & & &= [AB_n]/(k_1 k_2 \dots k_{n-1}) [A] c^n
 \end{aligned}$$

where n = maximum number of molecules of B Bound to a single molecule of A; i.e., maximum number of sites on A available to B under the conditions of the experiment

k_i = equilibrium constant for the association
 $AB_{i-1} + B \rightleftharpoons AB_i$

c = molar concentration of unbound B

$[AB_i]$ = molar concentration of the i^{th} complex, AB_i .

Implicit in these equations is the assumption that the sites on A act independently; that is, that the intrinsic affinity of any site for a molecule or ion of B is not affected by the fact that other sites on A have reacted. As is generally the case in Mass Law treatments of multiple equilibria, the thermodynamic activity coefficients have been assumed to be constant at constant ionic strength.

If we define, as a measure of the extent of interaction of B with A, the average number of B molecules combined with one A molecule, r ,

$$r = \frac{\text{number of moles of bound B}}{\text{total number of moles of A}}, \quad [2]$$

then we obtain directly from the Mass Law equations

$$r = \frac{k_1 c + 2k_1 k_2 c^2 + \dots + n(k_1 k_2 \dots k_n) c^n}{1 + k_1 c + k_1 k_2 c^2 + \dots + (k_1 k_2 \dots k_n) c^n} \quad [3]$$

If c and r are determined experimentally, by the equilibrium dialysis or partition analysis methods for example, the successive association constants k_i may be calculated in a number of ways, several of which will be described below.

Mention may be made here of the development by Steiner (72), from similar Mass Law considerations, of equations for the application of several of the ordinary physico-chemical techniques, such as light scattering, electrophoresis, etc., to associating systems in which the macromolecule is polydisperse.

B. The Method of Steiner for the Determination of k_i

Without any assumption as to the value of n or as to the relationship between the successive association constants, k_i , the values of these constants may be obtained quite easily from a series of values of r measured at several values of c . In the method devised by Steiner (73), the denominator in the definition of r in equation [3] is set equal to λ :

$$\lambda = 1 + k_1 c + k_1 k_2 c^2 + \dots + (k_1 k_2 \dots k_n) c^n \quad [4]$$

(72) R. F. Steiner, Naval Medical Research Institute Report Project NM 000 018.06.27, 11, 799 (1953).

(73) R. F. Steiner, Arch. Biochem. Biophys. 47, 56 (1953).

Then r becomes

$$r = \frac{c}{\lambda} \frac{d\lambda}{dc}$$

so that

$$\ln \lambda = \int_0^c \frac{r}{c} dc \quad [5]$$

Values of λ at different values of c may be obtained by graphical integration of a plot of r/c versus c . The initial slope of a graph of these values of λ plotted against c is k_1 , the equilibrium constant for the binding of the first molecule of B,

$$k_1 = \left(\frac{d\lambda}{dc} \right)_{c=0} \quad [6]$$

k_1 , k_2 , etc., may be obtained by successive differentiation, in accordance with the definition of λ , but, of course, the degree of accuracy decreases with each step. Much the same method was employed by Fronaeus (73a, 71b) in studying complex ions.

C. The Method of Scatchard for the Determination of k_i

In a paper by Edsall, et al. (74), on the tetra-coordinated imidazole complexes of zinc and copper, a method developed by Scatchard is presented which, if n is

(73a) S. Fronaeus, Acta Chem. Scand. 4, 72 (1950)

(74) J. T. Edsall, G. Felsenfeld, D. S. Goodman, and F. R. N. Gurd, J. Am. Chem. Soc. 76, 3054 (1954).

known, allows the rapid determination of the first and last association constants and the estimation of intermediate association constants from data on r and c . The method is generally applicable to any system where n sites are available for binding, without regard to the relative values of the association constants.

The quantity Q is defined:

$$\begin{aligned}
 Q &= \frac{r}{(n-r) c} \\
 &= \frac{k_1 + 2k_1 k_2 c + \dots + i (k_1 \dots k_i) c^{i-1} + \dots + n (k_1 \dots k_n) c^{n-1}}{n + (n-1) k_1 c + \dots + (n-1) (k_1 \dots k_{n-1}) c^{n-1}} - [7] \\
 &\quad - \frac{n (k_1 \dots k_n) c^{n-1}}{(k_1 \dots k_{n-1}) c^{n-1}}
 \end{aligned}$$

From this it follows directly that

$$\lim_{c \rightarrow 0} Q = k_1 / n \quad [8]$$

and

$$\lim_{\substack{c \rightarrow \infty, \\ r \rightarrow n}} Q = n k_n \quad [9]$$

so that, if the data are plotted as $\log Q$ against r , the intercepts at $r = 0$ and at $r = n$ yield k_1 and k_n , respectively. Intermediate association constants are estimated from the limiting slopes of the curve.

The curve of $\log Q$ versus r may have any shape, depending upon the relationships among the values of the association constants. When these are all equal, except

for statistical factors which will be discussed in the succeeding section, equation [7] becomes $Q = k_1/n$ for all values of c , and the $\log Q$ plot is a horizontal straight line. In the case where the successive constants, after correction in accordance with statistical considerations, bear a constant ratio to one another, the curve is not quite linear and is symmetrical about $n/2$. Regardless of the shape of the curve, however, the method is suitable whenever extrapolations can be made with sufficient accuracy.

The value of n , required for the calculation of Q , may, under favorable circumstances, be estimated from a graph of r against $\log c$ (71a): Such a curve is asymptotic to $r = 0$ at low values of c and to $r = n$ at high values of c , and should be symmetrical about $n/2$. In many cases, however, measurements cannot be made at free concentrations high enough to determine the shape of the curve accurately. Another way to estimate n will be given below, but this method, too, is inaccurate when data at high c are lacking. It is fortunate that the value of k_1 obtained from the $\log Q$ plot is not very sensitive to the value chosen for n .

D. The Cases of One and Two Intrinsic Association Constants

Klotz (75), Scatchard (76), and others have

(75) I. M. Klotz, Arch. Biochem. 9, 109 (1946).

(76) G. Scatchard, Ann. N. Y. Acad. Sci. 51, 660 (1949).

treated the special case in which the intrinsic binding affinity of each site on A for a molecule or ion of B is the same, and the sites are independent. Under these circumstances, the equilibrium constants k_i are related to the intrinsic association constant, K, the equilibrium constant for the interaction of one molecule of B with any one particular site available on A, through a statistical factor (71a):

$$k_i = \frac{n - i + 1}{i} K \quad [10]$$

Thus, when the intrinsic affinities of all sites are equal, the likelihood of interaction decreases as more and more molecules of B are complexed, only because the number of sites left available on the molecule of A is decreased, and the number of possible dissociations is increased. Now, on replacing the constants k_i in equation [3] for r by their equivalents in terms of K, we obtain for r

$$r = \frac{n Kc}{1 + Kc} \quad [11]$$

which may be rearranged to give

$$\frac{r}{c} = nK - Kr \quad [12]$$

Equation [12] indicates that a plot of r/c against r should be linear, with slope $-K$ and intercepts nK , or k_1 (equation [10]), on the r/c axis, and n on the r axis. It is noteworthy that equation [11] has the form of the Langmuir adsorption isotherm.

If, for a given system, r/c is not a linear function of r , then either the binding sites on A do not act independently, or they differ sufficiently to have different intrinsic affinities for the molecule or ion B, or both of these factors operate. It cannot be emphasized enough that data must be obtained over as wide a range of r as possible, in order that deviations from linearity will show up significantly. In the case of an ionic interactant B, the affinity of A for a B ion may be altered due to the electrostatic effect of the ions of B already bound. This so-called electrostatic interaction, just one of the possible deviations from statistical binding at sites of equal intrinsic affinity, is often corrected for by the application of a method developed by Scatchard (76).

When the binding sites on A are independent but have different intrinsic affinities for B, equation [3] for r in terms of the equilibrium constants k_i is valid, as we have indicated. r may be given, also, in terms of the intrinsic association constants, K_i

$$r = \sum_{i=1}^n \frac{K_i c}{1 + K_i c}, \quad [13]$$

equation [13] reducing to equation [11] when all the K_i 's are equal. Examination of equations [3], [7], and [13] reveals that the intercepts of a plot of r/c versus r on the y and x axes yield, respectively, k_1 and n . In most

cases, however, the extrapolations, especially the one necessary to obtain n , are uncertain because data cannot be obtained over a sufficiently wide range of r , and the curvature is often considerable at the ends of the curve. More reliable values of k_1 are frequently obtained by the methods of calculation of Steiner and Scatchard already discussed.

Scatchard, et al. (77), and Karush (11) have been able to fit binding data on the assumption that the sites could be divided into groups, each with a single intrinsic association constant. Equation [13] becomes, for this case

$$\frac{r}{c} = \frac{n_1 K_1}{1 + K_1 c} + \frac{n_2 K_2}{1 + K_2 c}, \quad [14]$$

and we have the relations

$$\lim_{\frac{r}{c} \rightarrow 0} r = n = n_1 + n_2 \quad [15]$$

and

$$\lim_{r \rightarrow 0} \frac{r}{c} = n_1 K_1 + n_2 K_2 \quad [16]$$

Using two sets of experimental values for r and c , preferably taken near the ends of the range of r , in equation [14], and the best values for the intercepts $(r)_{r/c=0}$ and $(r/c)_{r=0}$

(77) G. Scatchard, J. H. Scheinberg, and S. H. Armstrong, Jr., J. Am. Chem. Soc. 72, 540 (1950).

that can be obtained by a visual fit of the data plotted as r/c versus r , we may solve equations [14], [15], and [16] for the four constants of binding and then calculate a theoretical r/c versus r curve. This curve may indicate a better choice of intercepts for a second calculation, and the procedure may have to be repeated several times in order to fit the theoretical curve to the data as closely as possible. Thus, if the two-constant hypothesis accounts for given binding data, the intercepts, and hence the values of k_1 and n , as well as the intrinsic binding constants and n_1 and n_2 , are determined so as to be in agreement with all the data available.

E. Proposed Relations Between Intrinsic Association Constants

It has been proposed by Karush and Sonenberg (78, 11) that the intrinsic association constants, for some instances of interaction where these differ for the independent binding sites, may be related by a theoretical equation based upon the assumption of a Gaussian distribution of the free energies of binding. This equation involves the two parameters K_0 , the average binding constant, and σ , a measure of the spread of the values of K , the equation reducing to equation [11] for the one-constant case when

(78) F. Karush and M. Sonenberg, J. Am. Chem. Soc. 71, 1369 (1949).

$\delta = 0$, and it contains an integral which must be evaluated numerically in order to determine a theoretical binding curve using the initially determined values of K_0 and δ . Karush and Sonenberg (78) were able to describe the data for the binding of alkyl sulfates by bovine serum albumin on the basis of this hypothesis. They pointed out that, since binding curves calculated even for high values of δ do not deviate far from linearity in certain ranges, data limited to such ranges might erroneously be interpreted in terms of a single intrinsic association constant.

Sips (79, 80) has derived binding isotherms for systems involving independent sites with differing intrinsic constants by considering several other distributions of the free energies of binding, one of which is very close to a Gaussian distribution. He has obtained expressions in which the integrals have been solved analytically and which have the form of Freundlich adsorption isotherms:

$$\frac{r}{n} = \frac{kc^a}{1 + kc^a} \quad (0 < a \leq 1) \quad [17]$$

$$\frac{r}{n} = \left(\frac{c}{c + k} \right)^a$$

where a and k are constants dependent upon temperature. It is evident that both these equations reduce to the Langmuir adsorption isotherm when $a = 1$. For some systems, according

(79) R. Sips, J. Chem. Phys. 16, 490 (1948).

(80) R. Sips, J. Chem. Phys. 18, 1024 (1950).

to Sips, the second equation may contain a sum of terms like that on the right, each with a different value for the constant a .

F. Fluorescence Quenching Upon Interaction

The mechanism of diffusion controlled Fluorescence quenching has received a great deal of attention in recent years, but relatively little seems to have been written concerning the relation between the extent of interaction, in solution, of a fluorescent compound with another substance and the attendant alteration in intensity of fluorescence. On the other hand, protein binding studies have been carried out (81) utilizing changes in the polarization of fluorescence upon interaction, the phenomenon of fluorescence polarization having been considered from both theoretical and experimental viewpoints by Weber (82) and appearing to offer many opportunities for further work. In the present investigation, a number of determinations of fluorescence intensity were made on the DNA-acriflavine system under a variety of experimental conditions, and we shall deal here with several rather simple relationships which have been of use in attempts to interpret these measurements in terms of interaction constants.

(81) D. J. R. Laurence, *Biochem. J.* 51, 168 (1952).

(82) G. Weber, *Biochem. J.* 51, 145, 155 (1952).

If the quantum yield of fluorescence of a fluorescent molecule in solution differs markedly from the quantum yield of the same molecule when it is bound to some complexing agent, and if the quantum yield per molecule is the same for all bound molecules and remains constant under the same conditions of pH, ionic strength, temperature, etc., independent of the relative concentrations of the two interactants, it would be possible to determine the concentration of free and of bound fluorescent substance in any solution containing the interactants from a measurement of the intensity of the fluorescence of the solution. Under these circumstances, r/c versus c curves could be prepared from fluorescence intensity data alone, in accordance with the following development.

Equation [18] is often written to describe the quenching of a fluorescent substance; the particular form used here is taken from a study by Heintz (83) of the dependence of the quenching of the fluorescence of a dye by fractions of a certain synthetic polymer upon the molecular weights of the fractions.

$$\frac{F_0}{F} = \frac{c_0 \phi}{c \phi + c_b \phi^1} = \frac{c_0}{c + p c_b} \quad [18]$$

(83) E. Heintz, J. Phys. Radium 15, 219 (1954).

Here, F_o = fluorescence intensity of the fluorescent compound at concentration c_o when no quencher is present

F = measured fluorescence intensity of the solution containing fluorescent compound at total concentration c_o and quencher

c_o = initial or total molar concentration of fluorescent compound.

c = equilibrium molar concentration of free fluorescent compound

c_b = equilibrium molar concentration of bound fluorescent compound

ϕ = quantum yield of fluorescence per mole of free fluorescent compound

ϕ^1 = quantum yield of fluorescence per mole of bound fluorescent compound

p = ϕ^1/ϕ = ratio of quantum yields of bound and free fluorescent compound, assumed the same for all bound molecules.

Remembering that $c_o = c + c_b$, it follows that

$$c = \frac{c_o \left\{ 1 - \frac{F_o}{F} p \right\}}{\frac{F_o}{F} (1 - p)} \quad [19]$$

When c is negligible compared with c_b , equation [18] becomes

$$\left(\frac{F_o}{F} \right)_{c=0} = \frac{1}{p}, \quad [20]$$

so that p may be determined by measuring F in the presence of large amounts of quencher. Thus, provided p is constant with concentration, then the value of c , and consequently of r , may be determined for a solution of a given c_o and $[A]_o$,

where $[A]_0$ is the initial or total concentration of quencher, by measuring the fluorescence intensities of three solutions: the solution under consideration, a solution of the same c_0 but containing no quencher, and a solution at c_0 containing a large excess of quencher.

Oster (68⁷) used his data on the quenching of the fluorescence of acriflavine by DNA to determine an equilibrium constant for the reaction

where A is DNA, the quencher, and B is the fluorescent dye, acriflavine. If this were the reaction proceeding, c_b would be equal to $[AB]$, the equilibrium concentration of complex. An equilibrium constant K could then be written

$$K = \frac{[AB]}{[A] c} \quad [22]$$

and equation [18], written in the form

$$\frac{F_0}{F} - 1 = \frac{(1-p)[AB]}{c + p[AB]}, \quad [23]$$

would become, at exceedingly low concentrations of quencher when $[AB]$ is insignificant in comparison with c ,

$$\left\{ \frac{F_0}{F} - 1 \right\}_{[AB] \rightarrow 0} = (1-p) K [A] \quad [24]$$

Measurement of the fluorescence intensities of a series of solutions of constant dye concentration c_0 and quencher concentrations varying from zero to a concentration high enough

to permit calculation of p according to equation [20] would then give a curve of $(F_0/F - 1)$ versus $[A]_0$ whose initial slope would yield K . The assumption is made that $[A]$ is approximately equal to $[A]_0$ at relatively low dye concentrations.

In the light of the discussion on the multiple equilibria involved in the binding of small molecules or ions by a macromolecule, it would seem unlikely that equation [21] is adequate to describe the equilibrium situation for the DNA-acriflavine system, except at exceedingly low concentrations of dye. The first complex, AB , would be expected to exist alone in equilibrium with free dye and free DNA, with no AB_2 , AB_3 , ..., AB_n present, only in solutions very dilute with respect to dye. It is easy to see, then how quenching data may be used to obtain k_1 , the equilibrium constant for the formation of the first complex. Quenching curves, that is, graphs of $(F_0/F - 1)$ against $[A]_0$, are obtained at several dye concentrations c_0 , as low as is feasible. The initial slopes divided by $(1-p)$ are then plotted against c_0 , the extrapolated value at $c_0 = 0$ being taken as k_1 . At infinitely low dye concentration, too, the assumption that $[A] = [A]_0$ becomes valid.

IV. EXPERIMENTAL PROCEDURES

A. Preparation and Properties of DNA

The sample of calf thymus DNA, or, more accurately, the sodium salt of DNA, used in the studies to be described was prepared according to the procedure carefully detailed by Schwander and Signer (84). Several additional hints were taken from light scattering studies from Doty's laboratory (85, 86). It might be helpful to anyone undertaking the preparation to have a brief outline of the procedure which includes some of the details of application that seem worth mentioning.

The thymus glands were removed from the calves not more than twenty minutes after decapitation, immediately cut into small pieces which were mixed with a large quantity of crushed dry ice, and transported to the laboratory in a tightly-covered Dewar. 450 g. of the frozen tissue was run through an aluminum meat grinder, the remainder being stored in the deep-freeze at -40° C. for two weeks and used at that time in the preparation of a second batch. The extraction of nucleoprotein from the tissue with 1 M NaCl, the three

(84) H. Schwander and R. Signer, *Helv. Chim. Acta* 33, 1521 (1950).

(85) P. Doty and B. H. Bunce, *J. Am. Chem. Soc.* 74, 5029 (1952).

(86) B. H. Bunce, Doctoral Dissertation, Harvard University (1951).

precipitations of the nucleoprotein by dilution to 0.14 M NaCl, corresponding to the ionic strength of minimum solubility of nucleoprotein, and the dissociation, precipitation, and filtering off of the protein portion in saturated NaCl solution, were carried out essentially as described by Schwander and Signer. Since these steps require almost a month during which time it is essential to keep enzymatic degradation to a minimum, great care was taken to insure that the temperature of the preparation never rose above 0° C. All the work was carried out in a cold room regulated at 4° C. The mincing of the still-frozen ground tissue was carried out in a pre-cooled Waring blender, each portion of tissue being mixed with partially frozen 1 M NaCl solution and run for no longer than half a minute (35), so that the blending did not heat the solutions sufficiently to melt all the ice. All solutions used through the final filtration to remove protein were 0.01 M in sodium citrate, the desoxyribonuclease inhibitor recommended by Schwander and Signer, and all solutions and liquids were pre-cooled to below 0° C. before use. At every stage, the glass vessels containing the preparation were kept in ice baths and stirred efficiently to maintain temperature equilibrium, and frozen cubes of saturated NaCl solution were kept in the 8" Buchner funnels during the vacuum filtrations to remove protein. For the extensive stirring of large volumes of solutions required throughout the procedure, a Plexiglas

paddle with two vanes about 5" x 1" was constructed and used in connection with a variable-speed motor powerful enough to keep the viscous liquids well stirred. The speed of stirring was never greater than 100 revolutions per minute.

Alcohol precipitation of the nucleate from the filtered saturated NaCl solution was facilitated (85) by diluting the nucleate solution 1:1 with water before pouring one volume into 1 1/2 volumes of grain alcohol through a narrow-stemmed funnel. By this method, fine nucleate fibers were formed, and wound about the wooden dowel stirrer rod so that they could be separated from the supernatant very easily. Difficulty was experienced in carrying out the next precipitation of the nucleate redissolved in distilled water by adding one volume of the water solution to two of 95% alcohol as recommended by Schwander and Signer; analysis of the supernatant of a trial portion treated in this way revealed that over 90% of the nucleate had remained in solution. Almost complete precipitation was achieved, however, by pouring one volume of the water solution into four of alcohol saturated with NaCl.

The nucleate fibers, after the preliminary drying with absolute alcohol and anhydrous ether, were dried and stored somewhat differently than described by the other authors. In order to avoid contamination by desiccants, the preparations were dried in a vacuum desiccator at 4° C.

for about a week, using no drying agent. It was felt that the preparations would be best preserved if kept as dry as possible, and so they were stored in covered glass containers at 4° C. in a desiccator over P_2O_5 . The P_2O_5 was kept in a dish tightly covered with lens paper and was left to settle several hours before each change, so that the dust would not contaminate the nucleate.

The product was in the form of very white, tangled fibrous masses, which were conveniently torn apart for sampling by means of glass needles. In the first preparation, the yield from 450 g. of thymus was 4.0 g., while, in the second, the same amount of starting material yielded 8.6 g. of dry nucleate, probably because the precipitates were better separated from the supernatants the second time when the nature of the substances and the procedure were familiar. Schwander and Signer (84) estimated the protein content of their preparations to be well below 1%, and they found the viscosities at several intermediate rates of shear to be higher than those for any of the other preparations studied. Light scattering studies (85, 33, 87) indicate, too, that DNA samples prepared according to the Schwander and Signer procedure are somewhat less degraded than samples prepared by earlier methods. The molecular weight of the first

(87) M. E. Reichmann, R. Varin, and P. Doty, J. Am. Chem. Soc. 74, 3203 (1952).

sample whose preparation is described above was determined by I. J. Heilweil of this laboratory from angular light scattering measurements made in 0.2 M NaCl. Using his measured refractive index increment of 0.160, he obtained a molecular weight of 10×10^6 .

The Beckman Model DU spectrophotometer with water-cooled lamp housing was used in making absorption measurements in the visible and ultraviolet. The extinction coefficients for DNA in doubly distilled water at $259 \text{ m}\mu$, the wavelength of maximum absorption in the ultraviolet, were found to be 248 and 238 (100 ml./g. cm.) at a slit width of 1 mm. for the first and second preparations, respectively. All extinction coefficients are given here in terms of grams of solute per 100 ml. of solution. The average deviation of three separate determinations of each value was well under 1%. Although these values are somewhat higher than earlier ones given in the literature for DNA, recent determinations (33) on similarly prepared DNA samples have tended to be in agreement with the values reported here. It was determined that Beer's Law is obeyed at $259 \text{ m}\mu$ to concentrations at least as high as 0.005 g./100 ml. At $231 \text{ m}\mu$, the wavelength at which a minimum in the ultraviolet absorption occurs, the extinction coefficients were determined to be 92.8 and 89.4, the ratio E_{259}/E_{231} being 2.67 in both cases. According to Mirsky

and Pollister (88), a ratio as high as this indicates low protein content. The extinction coefficient in 0.2 M NaCl was found to be 204 for the first sample, the ratio of the coefficient in water to that in 0.2 M NaCl being 1.22, which may be compared with the value of 1.21 obtained by other workers (33).

The extinction coefficients were based upon dry weight of DNA, determined by two independent methods which gave results agreeing within 1%. One small sample was dried to constant weight in a vacuum oven at 100° C. for eight days. A second sample of almost two grams was dried at 4° C. in a vacuum desiccator for about a month and weighed every few days under the same conditions. A drying curve of sample weight versus the reciprocal of drying time was prepared, so that the dry weight of any sample of DNA could be determined by comparison of its weight with the weight of the reference sample measured under the same conditions of humidity.

DNA solutions used in the studies reported here were prepared from the fibrous solid in the following way: Dissolution in pre-cooled distilled water was achieved by means of magnetic stirring at moderate speeds, for eight to twelve hours in the case of dilute solutions. After the

(88) A. E. Mirsky and A. W. Pollister, J. Gen. Physiol. 30, 117 (1946).

concentration of the water solution was determined from duplicate measurements of the absorption at $259\text{ m}\mu$, appropriate volumes of this DNA solution and of stock buffer solution were diluted with distilled water in a volumetric flask to yield a solution of the desired DNA concentration, pH, and ionic strength. DNA solutions older than one week were discarded; indeed, all major experiments were set up using freshly dissolved DNA.

B. Purification and Properties of Acriflavine

Acriflavine, also called euflavine, trypanflavine, and neutral acriflavine, is meant here to refer to 3,6-diamino-10-methyl-acridinium chloride,

Commercial preparations of acriflavine contain about one third by weight of proflavine hydrochloride, the corresponding unmethylated compound. Since it was desired to study the binding of a pure compound whose degree of ionization is independent of pH, purification was undertaken according to the method given by Albert (89). Because acriflavine undergoes photooxidation rather rapidly, especially

(89) A. Albert, The Acridines, Edward Arnold & Co., London, p. 197 (1951).

in dilute solution, solutions were kept in the dark as much of the time as possible. A total of 45 g. of commercial acriflavine were dissolved in two liters of distilled water, 25 g. of a product of National Aniline and 20 g. from the Abbott Laboratories. Moist silver oxide precipitate was stirred into the solution, the mixture was allowed to stand at 4° C. in the dark for 24 hours, and the silver precipitate was removed by vacuum filtration at 4° C. The pH of the filtrate was adjusted to 7.0 with about 30 ml. of 2 N HCl, and then the solution was concentrated to about 200 ml. by vacuum dehydration and, finally, lyophilized to dryness. The dried material was recrystallized twice from boiling methyl alcohol and once from boiling water, and the water was removed by vacuum desiccation at room temperature for two days. The yield was 14.4 g.

In an attempt to determine the efficacy of the purification procedure, paper electrophoresis of the original commercial sample and of the purified material was carried out in 0.1 N HCl as described by Lederer (90). Proflavine and acriflavine should be separated in several hours, the acriflavine moving ahead of the proflavine, but separation could not be detected, either in the original or in the purified samples, although the electrophoresis was allowed to proceed for about 24 hours. The spot

(90) M. Lederer, Anal. Chim. Acta 6, 267 (1952).

corresponding to the purified sample, however, moved somewhat faster than the one for untreated dye, indicating a higher acriflavine content in the pure material.

Analysis for chlorine, carried out by Miss Mary Renoll of this laboratory, gave 13.52% chlorine in the purified preparation, which may be compared with 13.65% calculated for $C_{14}H_{14}N_3Cl$.

Absorption curves were determined for the purified sample in water and in n-hexanol, the organic phase used in the partition analysis experiments. For these measurements, and for all absorption measurements made on acriflavine solutions, a filter was mounted between the sample and the phototube compartments on the Beckman Du spectrophotometer in the filter holder provided with the attachment for the measurement of fluorescence. This filter had high transmissions in the ultraviolet and blue ranges where acriflavine absorbs but did not transmit the green wavelengths of the fluorescence spectrum. In water, maxima were observed at 263 $m\mu$ and at 452 $m\mu$, the extinction coefficients being 1960 and 1640 (100 ml./ g. cm.), respectively, the latter measured at a slit width of 0.1 mm. These values are based on dry weight of purified acriflavine, obtained by heating the powdered dye to constant weight at 105° C., and represent the average of several separate determinations which agreed within 1%. The extinction coefficients at 452 $m\mu$ in buffered solutions

differ somewhat from the value in distilled water, and these coefficients are given in the appendix with the experimental data for the partition analyses. It was found that the extinction coefficient at $452\text{ m}\mu$ does not change upon saturating an aqueous solution with n-hexanol. At $452\text{ m}\mu$, deviations from Beer's Law become significant at concentrations higher than approximately 1×10^{-3} g./100 ml. In n-hexanol, equilibrated against the various buffer solutions used at 25°C. , the absorption maximum for acriflavine occurs at $470\text{ m}\mu$ in the visible range, and the extinction coefficient was found to be 2340 (100 ml./g. cm.) independent of the saturating buffer. In dry n-hexanol, the extinction coefficient is roughly 10% higher. Beer's Law is obeyed for concentrations at least as high as 5×10^{-4} g./100 ml. in n-hexanol; it was not found necessary to measure solutions of higher concentration.

C. Equilibrium Dialysis Procedure

Equilibrium dialysis experiments were run not only to confirm the partition analysis results for the binding of acriflavine by DNA, but also to find out whether other substances interact with DNA to any appreciable extent. A number of techniques were devised to make it possible to determine the concentration of interactants after equilibration spectrophotometrically in the near ultraviolet with

negligible errors due to traces of DNA and contaminants from various sources.

A known volume of buffered DNA solution of the desired concentration, usually 3-5 ml., was confined within a dialysis sack made of Visking cellophane sausage casing, 3/4" in extended diameter. Equal lengths of casing were always measured off carefully for each experiment, so that the amount of adsorption on each sack would depend only upon the concentration of dialyzing substance and inherent variations in adsorption properties along the length of the casing as it is manufactured. Because it was found that impurities absorbing quite considerably in the ultraviolet come off the casing and contaminate the solution, and that the contamination is greater at higher ionic strengths, the lengths of casing were washed before use: several hours in running distilled water, an hour or so in each of three or four changes of buffer ten times as concentrated as that to be used in the experiment, and about an hour in each of three or four changes of the buffer at the concentration to be used. The washing in buffer was accomplished by mechanical shaking of the casings with an appropriate volume of buffer in a glass-stoppered bottle. It was found that boiling the casing to remove diffusible ultraviolet-absorbing substances often caused the membrane to pass DNA, but the washing procedure described left them impermeable to DNA and resulted in negligible ultraviolet absorption in

blank dialysis experiments containing only buffer on both sides of the membrane. Rubber gloves were worn in handling the casings, once they were washed. In filling the sacks, precautions were taken that DNA should not be present on the outer surface, and, to remove any traces of DNA that might have spilled over in knotting the casing end, as well as to remove any dialyzable components in the DNA solution, the filled sacks were shaken at 4° C. with several changes of pre-cooled buffer in a glass-stoppered bottle, usually overnight.

The sacks of DNA solution were then placed in cylindrical, glass pharmaceutical bottles of 30 or 40 ml. capacity, each containing a known volume of buffered solution of the interactant under investigation at a desired concentration. Care was taken to tuck the sacks well under the surface of the liquid so that the whole length of casing would adsorb uniformly. The bottles used had almost leak-proof snap-on caps of polyethylene, which was found not to adsorb any of the substances studied nor to give off any objectionable contaminants. Because the cellophane membrane carries an unknown amount of imbibed and adherent liquid, the total volume of solution in the system was determined by weighing the "bottled" setup, subtracting the weight of the bottle and the average weight of the bottle caps and oven-dried casing length, and correcting for the presence of buffer salts.

Controls were set up in the same manner, several bottles containing DNA solution inside the dialysis bag and buffer outside, as checks for possible leakage of DNA into the outer solution, and several containing buffer inside and interactant solution outside, these to be analyzed for equal distribution on both sides of the membrane. In cases where the interactant was adsorbed by the casing to any appreciable extent, blanks of the second kind were prepared so as to cover the entire range of free interactant concentrations, c , to be studied. The amount of interactant adsorbed was plotted versus free interactant concentration so that binding data at any c could be corrected for the loss due to membrane adsorption.

The bottles were shaken lengthwise in a horizontal plane on a mechanical shaker at a speed of about 60 cycles per minute, either in the cold room at $4 \pm 1^\circ \text{C}$. or in a constant temperature room at $25 \pm 1^\circ \text{C}$. The time required for equilibration was determined for each system and was usually about 24 hours at 4°C . Measurable degradation of DNA to dialyzable fractions was not found to occur during this period at the low temperature.

Since the ionic strength was at least 0.1 in all runs, so that the Donnan correction was negligible, spectrophotometric analysis of the outer solutions at the appropriate wavelength yielded r in accordance with the equation

$$r = \frac{c_o V_o - c V_t - C}{[NA]_o V_i} , \quad [25]$$

where

- c_o = initial, or total, molar concentration of dialyzable interactant
- c = equilibrium concentration of free interactant
- $[NA]_o$ = initial, or total, concentration of DNA
- V_o = initial volume of the solution of interactant placed outside the sack
- V_i = volume of DNA solution placed inside the sack
- V_t = total volume of solution in the system determined by weighing
- C = amount of interactant taken up by the length of dialysis casing at concentration c .

D. The Procedure for Partition Analysis

N-hexanol was selected as the organic solvent for the partition analysis study of the DNA-acriflavine system. It was discovered that different batches of Technical Grade n-hexanol, even batches obtained from the same company at the same time, often extracted different amounts of acriflavine from an aqueous solution. As Karush pointed out (17), the indications are that organic acid impurities are responsible for this: a Carbide and Carbon product that yielded a much higher partition coefficient than an Eastman Kodak sample became almost identical with the Eastman sample after it was washed with NaOH; and a much higher

partition coefficient could be obtained with the Eastman n-hexanol by adding a very small amount of lauric acid. In view of these observations, several batches of Eastman Kodak Technical Grade n-hexanol were combined to give an ample amount for all the experiments reported. A quantity sufficient for all the work planned in a given buffer solution was treated in portions in a separatory funnel as follows, the portions being thoroughly mixed before any was used: About 200 ml. of the commercial n-hexanol was washed three times with roughly equal volumes of 0.01 N NaOH, three times with distilled water, and three times with approximately 200 ml. portions of the buffer. A milliliter or two of buffer was added to the combined portions, and the solvent, usually rather cloudy at this point, was shaken up, cooled almost to 0° C., and then stored at the temperature to be used in the experiment, becoming clear as it warmed.

Potassium phosphate buffers were used exclusively in this investigation, and the concentrations of the salts necessary in a solution of desired pH and ionic strength were calculated according to the data published by Green (91). A stock solution ten times as concentrated was then prepared and diluted 1:10 as required. pH measurements were made using the Beckman Model G pH meter standardized against Beckman pH 7.00 standard buffer.

(91) A. A. Green, J. Am. Chem. Soc. 55, 2331 (1933).

Partition analysis equilibrations were carried out in the same polyethylene-capped bottles as were used in the equilibrium dialysis experiments, the caps being unaffected by the n-hexanol and giving very little trouble with respect to leakage. Five milliliters of DNA solution of twice the desired final aqueous concentration and five milliliters of a solution of acriflavine, also at twice the desired aqueous concentration, both in the same buffer, were carefully pipetted into each bottle. In almost all the experiments, the final DNA concentration was 0.005 g./100 ml., a few determinations at 0.01 g./100 ml. being made to check the effect of varying the DNA concentration. Since dilute acriflavine solutions were found to decrease in concentration with time, though slowly, solutions were made up fresh on the day a particular equilibration was to start, and their exact concentrations were determined spectrophotometrically either that day or the next. The range of acriflavine concentrations was chosen, for each set of experimental conditions, so that the lowest free dye concentration in the organic phase was measurable with an average deviation of less than three per cent by the method to be described and the highest concentration was below that which would precipitate the DNA in the aqueous layer. A stock solution of acriflavine was prepared just before use by dissolving an approximately weighed amount of the solid dye in a known volume of buffer, and this was

diluted to yield a series of acriflavine solutions at concentrations near those selected so as to give an even spread of r values. To the aqueous phase in the bottle, ten milliliters of washed n -hexanol, saturated with buffer at the temperature of the experiment, was added from a buret, slowly so that the liquid would drain.

A series of blanks containing no DNA was prepared in much the same way for the determination of the partition coefficients of acriflavine between the two phases. For each dye concentration, ten milliliters of buffer-saturated n -hexanol was added to ten milliliters of acriflavine solution in the buffer under consideration. The acriflavine concentrations were chosen so that the range of concentrations in the organic layer after equilibration would be about the same as the range in the partition analysis experiments at the same ionic strength, pH, and temperature.

The bottles, carefully capped so as to make a dry seal, were shaken for four hours at $4 \pm 1^\circ \text{C.}$ or at $25 \pm 1^\circ \text{C.}$ just as in the equilibrium dialysis runs, except that the bottle rack was covered to make it light tight.

Because it was necessary to measure dye concentrations much lower than could be determined accurately from the absorption at $452 \text{ m}\mu$ or at $470 \text{ m}\mu$, even with absorption cells five centimeters in length, which require rather large volumes of test solutions, analyses were carried out in two ways: spectrophotometrically in 1 cm. absorption

cells for the more concentrated solutions, and by the measurement of the fluorescence intensities of the very dilute ones. The fluorescence measurements were made on an Aminco high sensitivity photometer, American Instrument Company, Silver Spring, Md., designed by Oster (92). In order that volumes less than 10 ml. could be measured, a small cylindrical cell, about 1 cm. in diameter, was cemented to a square base of lucite which just fit into the square recess of the photometer. The cell was fitted with a snug glass cap to hinder evaporation and was painted black over its back surface to minimize reflection and give a small solvent correction when used with the largest incident and receiver slits available. Instead of standardizing the instrument against a standard fluorescent solution, all fluorescence measurements were made relative to a reading arbitrarily selected for one of the neutral density filters at an angle of 0° with respect to the transmitted beam: the galvanometer needle was adjusted to read this value with the sample cell removed, the neutral density filter in position, and the phototube at 0° ; and then the filled sample cell was set in place, a yellow-green exit filter was substituted for the neutral density filter, and the phototube was rotated to the 90° position for the fluorescence determination. All the fluorescence work was

(92) G. Oster, Anal. Chem. 25, 1165 (1953).

carried out at about 27° C. using incident light of wavelength $436\text{ m}\mu$.

For each buffer studied, two curves were prepared of fluorescence, in arbitrary photometer scale units, versus spectrophotometric extinction values for acriflavine solutions in buffer and in buffer-saturated n-hexanol. The series of solutions in each solvent was made up by dilution of a stock solution whose extinction had been carefully determined, the concentration range covered encompassing all the concentrations which would have to be determined from fluorescence measurements.

Partition coefficients were calculated from concentration determinations in both layers of the series of blanks. It was convenient to express the concentrations in terms of extinction values, E , either measured directly or read off from the fluorescence-extinction curves after measuring the fluorescence, and to plot the ratio of the extinction in the n-hexanol layer to that in the aqueous layer, $E_{\text{org.}}/E_{\text{aq.}}$, against the extinction in the organic phase, $E_{\text{org.}}$. Then, for any partition experiment run under the same environmental conditions, the extinction value corresponding to the equilibrium concentration of unbound acriflavine in the aqueous layer containing DNA, $E_{\text{aq.}}$, could be obtained from the curve if $E_{\text{org.}}$ were measured.

In the case of the experiments at 4° C., the layers were carefully separated at that temperature and then allowed to warm to room temperature before they were analyzed. The organic layer remained clear on warming, water being more soluble in n-hexanol at higher temperatures, but the aqueous layer clouded quickly as dissolved n-hexanol came out of solution. After standing several hours at room temperature, however, the aqueous layers of the blank experiments had cleared and could be analyzed, but troublesome clouding, requiring further waiting, occurred if the solution temperature rose even slightly, for example, as the result of handling the small cells and bottles excessively.

The basic equation for the calculation of r , the number of moles of acriflavine bound to one mole of DNA, from partition analysis data may be written as

$$r = \frac{c_o V - cV - c_{org.} V_{org.}}{[NA]_o V} \quad [26]$$

where

- c_o = initial molar concentration of acriflavine in the aqueous layer
- c = equilibrium molar concentration of unbound acriflavine in the aqueous layer
- $c_{org.}$ = equilibrium molar concentration of free acriflavine in the organic layer
- $[NA]_o$ = initial, or total, concentration of DNA in the aqueous layer
- V = volume of the aqueous phase
- $V_{org.}$ = volume of the organic phase

In terms of the quantities measured in the procedure as described, since $V = V_{\text{org.}}$, r is given by

$$r = \frac{[E_o - \frac{k_{\text{aq.}}}{k_{\text{org.}}} E_{\text{org.}} - E_{\text{aq.}}]}{k_{\text{aq.}} d \frac{M}{10} \times [\text{NA}]_o} \quad [27]$$

where E_o = extinction value corresponding to c_o ; i.e., one half the measured extinction of the original dye solution before adding the DNA solution

$E_{\text{org.}}$ = extinction of the organic layer, measured directly, or determined from the fluorescence-extinction calibration curve

$E_{\text{aq.}}$ = extinction corresponding to c , determined from $E_{\text{org.}}$ and the partition coefficient at $E_{\text{org.}}$

$k_{\text{aq.}}$, $k_{\text{org.}}$ = proper extinction coefficients

d = length of absorption cell (cm.)

M = molecular weight of acriflavine = 260

E. The Measurement of Fluorescence Quenching

The photometer, standardization procedure, incident wavelength, and yellow-green exit filter used for the measurements of fluorescence intensities in solutions containing no DNA, as described in section D. were employed, also, for the fluorescence measurements involved in the study of the quenching of the fluorescence of acriflavine by DNA. Since quenching measurements are made on systems at equilibrium, however, the instrument was transferred to the constant temperature room or to the cold room,

according as measurements of binding were desired at 25° C. or at 4° C. Care was taken that the temperature of the photometer chamber was within 1° of the desired temperature.

In the early work, the small cylindrical cell was used to measure, one at a time, a series of solutions having the same buffer and dye concentrations but varying with respect to DNA concentration from zero to a value high enough to permit estimation of p , as discussed in section III. F. It was found, however, that points necessary to determine the shape of the quenching curves, $(F_0/F - 1)$ versus $[NA]_0$, were often lacking in the first run and that dilution errors in preparing the individual solutions were considerable, so a titration procedure was substituted which was more accurate and less tedious. Fifteen milliliters of buffered acriflavine solution were pipetted into the clean, dry square cell provided with the Aminco photometer. This volume was just sufficient for the determination of F_0 . A solution at the same buffer and dye concentrations but containing DNA at a suitable concentration as well was then added from a 10 ml. buret graduated to 0.02 ml. A small glass-covered magnetic stirrer in the bottom of the cell was used in mixing the solutions well after each addition, before the measurement of F was made, and the cell was kept covered with a square of plate glass as much of the time as possible. By this technique, up to ten milliliters of

DNA solution could be added in such increments as were necessary to define the quenching curve. The dye concentrations used in the quenching experiments fell on the essentially straightline portion of the fluorescence-concentration curve.

V. Experimental Results

A. Qualitative Results with Several Cationic Compounds

In the search for a compound which would interact reversibly with DNA to form soluble complexes to a measurable extent, even at relatively high ionic strengths, several cationic substances were investigated qualitatively by means of two or three methods for the detection of interaction. The results are indicated very briefly here, for they may be considered as clues to the type of interaction which DNA undergoes.

The equilibrium dialysis technique (section IV. C.), using only three or four interactant concentrations, was applied in several cases to determine whether binding occurs at ionic strength 0.1. Much quicker, although theoretically not as valid, the following ultracentrifugation procedure was used in a few preliminary tests. Two tubes, one containing a buffer solution of DNA and the test compound, and the other containing the test compound only at the same concentration, were centrifuged at top speed in a Spinco Model L preparative ultracentrifuge for at least ten hours, a time found sufficient to centrifuge down completely DNA at a concentration of about 0.1%. The supernatants were drawn off, very carefully, and analyzed spectrophotometrically for the test compound. If no difference in concentration was detected

for the two tubes, it was concluded that binding did not take place under the conditions of the experiment, but, if the supernatant concentration for the mixture was less than that for the blank, it was considered that complexing was indicated but not proved. A number of spectrophotometric titrations were run, too, on the assumption that a change in the spectrum of either the DNA or the test compound or both would probably, but not necessarily, accompany interaction between the two. In most of these titrations, absorption measurements had to be made at wavelengths at which both DNA and the test compound absorbed, so that the experimental extinctions were compared with the sum of the predicted extinctions for the constituents, which had been shown to obey Beer's Law individually. Since inorganic cations are known to lower the absorption maximum of DNA even in very low concentrations, the spectrophotometric titrations were carried out at zero ionic strength. It was not found feasible to demonstrate interaction by means of paper electrophoresis, for the high molecular weight DNA preparation could not be made to move on the papers ordinarily used in such work.

Several alkyl pyridinium bromides were prepared, purified by recrystallization, and investigated in the expectation that these quaternary salts would combine with DNA. The ethyl and butyl derivatives, however, were

not found to interact at 0.1 ionic strength at pH 7 or at pH 5.5 in equilibrium dialysis experiments covering a wide range of concentrations. Moreover, spectrophotometric titration with ethyl pyridinium bromide in the absence of buffer revealed no spectral changes at four wavelengths in the near ultraviolet. The lauryl and cetyl derivatives, on the other hand, were bound on ultracentrifugation and on equilibrium dialysis, but the range of concentrations between the lowest that could be determined spectrophotometrically and the critical micelle concentration of the pyridinium salts in the buffer used was quite narrow. The doubly charged ethylene, tetramethylene, and decamethylene bis-quaternary pyridinium bromides, prepared and very generously donated by Dr. J. L. Hartwell (93) of the U. S. Public Health Service, all caused spectral changes in the ultraviolet on being added to DNA, and equilibrium dialysis experiments, as well, indicated interaction in the case of the ethylene salt. Benzidine di-hydrochloride, however, did not interact under the same conditions of concentration and ionic strength.

B. The DNA-Acriflavine System: Partition Analysis Results

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- (93) J. L. Hartwell and M. A. Pogorelskin, J. Am. Chem. Soc. 72, 2040 (1950).

Partition analysis experiments on the DNA-acri-
flavine system were carried out under the following
conditions of ionic strength (μ), pH, and temperature (T):

- 1) $\mu = 0.002$, pH = 6.92, T = 25°C.
- 2) $\mu = 0.1$, pH = 7.04, T = 25°C.
- 3) $\mu = 0.3$, pH = 6.88, T = 25°C.
- 4) $\mu = 0.1$, pH = 5.57, T = 25°C.
- 5) $\mu = 0.1$, pH = 7.04, T = 4°C.

Since the interaction depends very little upon pH, it is considered that the pH for series 1), 2), 3) and 5) is the same and will, for practical purposes, be called pH 7 in the succeeding. A pH near 5.5 was chosen for the run intended to indicate the effect of pH upon the interaction so that comparison might be made with the study of Cavalieri (59) on ~~ra~~saniline binding and so that extensive degradation of the DNA would be avoided (26, 94). No equilibrations were carried out in the absence of added salts, because it was desired to study the effect of varying ionic strength at a regulated and approximately constant pH. The nucleic acid solution used in the experiments at the lowest ionic strength, 0.002, was dialyzed overnight against large volumes of buffer to remove any NaCl remaining from the preparative procedure, and it was determined that the pH of the DNA solution at

(94) M. E. Reichmann, B. H. Bunce, and P. Doty, J. Polymer Sci. 10, 109 (1953).

this low buffer concentration stayed constant in the presence of acriflavine and dissolved n-hexanol.

The data used in preparing the fluorescence-extinction curves for the aqueous and organic layers for the four buffers studied is given in the appendix. All fluorescence intensities, F , are given in photometer scale units and are corrected for the reading due to the solvent. Whenever possible, measurements for particular solutions were made on the photometer ranges where the scale readings were greater than 20 and less than 80. As we have noted, $E_{aq.}$ was determined at $452\text{ m}\mu$, and $E_{org.}$ at $470\text{ m}\mu$. For the organic layer, the results fall on the same curve independent of the saturation buffer, but the curves for the aqueous layers at pH 7, $\mu=0.002$ and at pH 5.5, $\mu=0.1$ differ from that at pH 7, $\mu=0.1$ or $\mu=0.3$. The curves are not quite linear, the slopes decreasing slowly as the concentration increases.

The partition coefficient curves, $E_{org.}/E_{aq.}$ versus $E_{org.}$, for all five sets of experimental conditions are shown in Figures 1 and 2, and the corresponding data are given in the appendix. The coefficients are seen to depend markedly upon pH, μ , and T, but they vary with concentration in the same general way under constant conditions: they decrease from the value at zero $E_{org.}$, rather rapidly at first, and then much more slowly.

The values of the experimentally determined

Partition Coefficient vs. E_{ORG} at Given Values of μ , pH, and

FIGURE 1

Partition Coefficient vs. E_{ORG} at $u = 0.002$, $pH = 7.0$,
and $T = 25^\circ C$.

FIGURE 2

quantities, E_o , $F_{org.}$, and $E_{org.}$, in the partition analysis experiments are tabulated in the appendix for each set of experimental conditions, together with the corresponding values of r , calculated according to equation [27], and r/c . The value for $k_{aq.}$ is given for each buffer, and d is, in all cases, 1.01 cm. Although the DNA concentration is given in g./100 ml. for reference, the value used in the calculations of r has the units moles of phosphate groups per liter, calculated using the value 331 as the average weight of a DNA nucleotide. Thus, the necessity for assuming a value for the molecular weight of DNA is avoided, and the estimated values of n indicate the fraction of the DNA phosphate groups present in the solution which are available for binding under given conditions.

In Figures 3 through 7, the data are plotted, for the different experimental conditions, as r/c versus r . Several experiments at 0.01 g./100 ml. of DNA are specially indicated in Figure 7 and are seen to fall in with the data at 0.005 g./100 ml. of DNA. In Figure 7, too, there appear the results of some preliminary partition analyses employing an 80:20 n-hexanol:n-heptane mixture by volume as the organic phase. This solvent was abandoned because it gave low partition coefficients, but^{it} is of interest that the results are in agreement with those from the experiments with n-hexanol alone. Finally,

$\frac{r}{c}$ vs. r . Partition Analysis at $\mu = 0.002$, pH = 7.0, and $T = 25^\circ\text{C}$.

FIGURE 3

$\frac{r}{c}$ vs. r . Partition Analysis at $\mu = 0.1$, $\text{pH} = 7.0$, and $T = 25^\circ\text{C}$.

FIGURE 4

$\frac{r}{c}$ vs. r . Partition Analysis at $\mu = 0.3$, $\text{pH} = 7.0$,
and $T = 25^\circ\text{C}$.

FIGURE 5

$\frac{r}{c}$ vs. r . Partition analysis at $\mu = 0.1$, $\text{pH} = 5.5$, and $T = 25^\circ\text{C}$.

FIGURE 6

FIGURE 7

Figure 7 includes several points obtained by the equilibrium dialysis technique. Although the dialysis method, at least using cellophane casing as the membrane, is not accurate enough for the quantitative study of acriflavine because the membrane takes up exceedingly large amounts of the dye, indeed, several times the amount of free dye, the results agree quite well with the partition analysis values. Evidence that the acriflavine-DNA interaction is reversible is presented in Figure 4. In an early partition analysis run, reversal experiments were carried out by removing, as exactly as possible, five milli-liters of the organic layers of several bottles after equilibration in the usual manner. Five milli-liters of fresh buffer-saturated n-hexanol were then added, and the bottles were shaken an additional four hours to reequilibrate the system. $E_{org.}$ was determined for both equilibrations, and values of r were calculated. It is seen (Figure 4) that the results for the reversal experiments fall in with those for the first equilibrations.

The curves drawn in Figures 3 through 7 were calculated according to the method described in section III. D., equations [14], [15], and [16], for the case of two groups of sites, each with a single intrinsic association constant. Although the intercepts might be chosen within rather wide limits, the theoretical curves

fit the experimental points fairly satisfactorily for any choice of intercepts that appears reasonable, except that they are a little low in the central, curving region. The values for K_1 , K_2 , n_1 , and n_2 calculated on the basis of the two-constant assumption are given in Table I at the end of section V. The values for k_1 and n obtained from the intercepts of the theoretical curves are listed in Table I, also, the results for k_1 are in quite good agreement with those obtained by the other methods of calculation. As is usually the case in binding studies, the extrapolation which determines n is less certain, and the effect on the theoretical curve of a considerable variation in the value of n selected is relatively small, so that the values reported for n are estimates only.

An attempt was made to use the adsorption isotherms of equation [17], section III. E., derived by Sips (79, 30) for two distributions of binding energies, to construct a theoretical curve which would fit the data. It was found that both equations yield curves which come down far too rapidly at low values of r , the second equation giving worse results than the first.

The method of Scatchard (74), section III. C., was easily applied to the results for all the experimental conditions excepting those at $\mu=0.002$. Figure 8 shows the plots of $\log Q$ versus r obtained for the other four cases by using, to calculate Q , the same values of n selected

Log Q vs. r at Given Values of μ , pH, and T

FIGURE 8

in the application of the two-constant method. These plots, within experimental error, are straight lines and are extrapolated to $r = 0$ without too much uncertainty to obtain values for k_1 , listed in Table I, which agree quite well with the values obtained in other ways. In the case of the data at low ionic strength, the $\log Q$ versus r plot, not shown, appears to consist of two linear portions, neither of which extrapolates to a satisfactory value for k_1 . It is difficult to say whether experimental error, which shows up very markedly in these plots, causes the curve to deviate from linearity, in which case an average straight line might be drawn through the points, or whether there is a theoretical reason for the shape of the curve. The n^{th} intrinsic association constant, k_n , is not listed in Table I, because of the greater uncertainty in the extrapolation to $r = n$. It should be noted, in this connection, that $(Q)_{r=n}$ when divided by n yields a value for k_n less than that for k_1 ; this is a consequence of the fractional units chosen for n . In an attempt to obtain a more reliable estimate of n , graphs of r versus $\log c$ were constructed, but the data do not reach values of c high enough to determine center of symmetry of the curve (at $n/2$) with accuracy. Figure 9, for the run at pH 5.5 shows the easiest of these curves to interpret; the value of n obtained happens to be the same as is estimated by

extrapolating the r/c versus r curve to $r/c = 0$.

Finally, the values of k_1 calculated from the partition analysis data by the method of Steiner (73), section III. B., are included in Table I and are seen to fall in with the other results satisfactorily. There was no difficulty in applying the method to any of the runs, and the manner in which the procedure works out is illustrated in Figures 10 through 12 for the data at $\mu = 0.1$, $p_0 = 7$, and 4°C . In Figure 10, r/c is plotted against c for the lowest values of c , the areas measured being marked off by the vertical lines. Figure 11 shows the results for λ as a function of c for all the runs and indicates clearly how the initial slopes vary under the various experimental conditions; if the curve for $\mu = 0.002$ were plotted on the same c scale, it would, of course, have by far the highest initial slope. The so-called chord-area method for graphical differentiation, used to determine $\left(\frac{d\lambda}{dc}\right)_{c=0}$, is illustrated in Figure 12. Estimates of k_2 , k_3 , etc., might be obtained by successive differentiation, but it should be remembered that the shape of the λ versus c curve depends upon the units chosen for r , and the units chosen in the calculations presented here happen to result in negative values for the association constants k_i , where $1 < i \leq n$. The k_i 's become positive, of course, if the units are chosen so that n is greater than 1.

r vs. $\log c$ at $\mu = 0.1$, $\text{pH} = 5.5$, and $T = 25^\circ\text{C}$.

FIGURE 9

$\frac{r}{c}$ vs. c at $u = 0.1$, $\text{pH} = 7.0$, and $T = 4^\circ\text{C}$.

FIGURE 10

FIGURE 11

$\frac{\Delta\lambda}{\Delta c}$ vs. c for $\mu = 0.1$, $\text{pH} = 7.0$, and $T = 4^\circ\text{C}$.

FIGURE 12

C. The DNA-Acriflavine System: Fluorescence Quenching Results

Fluorescence quenching curves were obtained both at 25°C. and at 4°C. for pH 7 at $\mu = 0.002, 0.01, 0.05, 0.1$, and 0.3, and for pH 5.5 at $\mu = 0.1$. Runs at two or three acriflavine concentrations were made under the experimental conditions that had been studied by partition analysis; a curve at a single dye concentration was determined for each of the additional cases. All the data are tabulated in the appendix. As a further check on the reversibility of the system, solutions at several different concentrations of acriflavine and of DNA and in several different buffers were measured at 25°C., then at 4°C., where more acriflavine is bound, and then at 25°C. again. The results at 25°C. checked, and so did those at 4°C., with measurements made on solutions prepared from DNA and dye stocks which were at 4°C. before mixing.

The shapes of the quenching curves varied gradually between two extreme types, according to the extent of binding occurring under the experimental conditions being investigated. Figure 13 shows the curves obtained at three dye concentrations for $\mu = 0.1$, pH 7, and 25°C., conditions under which the amount of binding is relatively low. $(F_0/F - 1)$ increases linearly with $[NA]_0$ at first and then the slope of the curve decreases as $[NA]_0$ increases further. In Figure 14, we have examples of the types of

$\frac{F_0}{F} - 1$ vs. $[NA]_0$ at $\mu = 0.1$, $pH = 7.0$, and $T = 25^\circ C$.

FIGURE 13

$\frac{F_0}{F} - 1$ vs. $[NA]_0$ at $\mu = 0.002$, $pH = 7.0$, and $T = 25^\circ C$.

FIGURE 14

quenching curve obtained when the amount of binding is high, in this case for two dye concentrations at $\mu = 0.002$, pH 7, and 25°C . There is a curved portion of continually increasing slope at low $[\text{NA}]_0$ preceding the linear portion, and the curve goes through a maximum at higher $[\text{NA}]_0$ and then slowly levels off.

In order to obtain k_1 from the quenching data by the slope method suggested at the end of section III. F., the following procedure was adopted: The initial slopes, of curves like those in Figure 13, were easily drawn, but for curves of the type in Figure 14, not the initial slopes, but the slopes of the linear portions, measured as indicated in the figure, were used. The slopes were taken in this manner both because the results for k_1 were in much better agreement with those obtained by other methods than if the initial slopes were used, and because it was suspected that the initial curvature is due, at least partly, to the discrepancy between $[\text{NA}]$ and $[\text{NA}]_0$ when binding is considerable. The maxima in some of the quenching curves indicate immediately that p is not constant with concentration for the DNA-acriflavine system. It was decided to choose, in each case, a value of p which would hold fairly well in the concentration range corresponding to the linear portion of the quenching curve: F_0/F was plotted against $1/[\text{NA}]_0$ and the rising portion of the curve was extrapolated to $1/[\text{NA}]_0 = 0$ as indicated by

equation [20]. The values of F_0/F falling below the extrapolated curve at low values of $1/[NA]_0$ were disregarded. Since p estimated in this manner is, in every case, less than about 0.1, the value of the slope divided by $(1-p)$ at any particular dye concentration, called K_{11} , is very insensitive to the value chosen for p . The graph of K_{11} versus c_0 is slightly curved, as is seen in the insert in Figure 13, but, for the cases where quenching curves were run at only two dye concentrations, a linear extrapolation to $c_0=0$ was made. Despite this approximation and the others mentioned, the results for k_1 , included in Table I, are in agreement with the values obtained from partition analysis.

Once it was established that fairly reliable first equilibrium constants could be obtained by this relatively simple and rapid quenching-slope method, it was decided to obtain quenching curves under conditions other than those studied by partition analysis in order to learn something more about the interaction. A further approximation was made in these cases: values for K_{11} were determined at only one acriflavine concentration, and the values at $c_0 = 0$ were estimated, very roughly, by choosing a slope for K_{11} versus c_0 . The choice was based upon consideration of the slopes obtained in cases when quenching was studied at two or more dye concentrations. The values for k_1 obtained in this way, together with the thermo-

dynamic constants calculated from them, are listed in Table II.

Since, as we have seen, p is not constant with concentration for the DNA-acriflavine system, it was not found possible to apply the method derived in section III. F. for the calculation of c according to equation [19]. Figure 15 shows the results for r/c versus r obtained for the two cases of Figures 13 and 14 on the basis of the values of p chosen in the application of the quenching-slope method. The calculated points are superimposed upon the curves calculated on the two-constant assumption, and it is evident that no single value of p would make the points fall on the curve at $\mu = 0.002$. At $\mu = 0.1$, on the other hand, where the variation of p is not so great at the concentrations studied, the points follow the curve rather closely over a sizable region and indicate the order of magnitude of k_1 at least.

Figure 16 represents the results for k_1 summarized in Tables I and II. It is gratifying that the results, indicated by the open symbols, which are based on quenching data at a single dye concentration and calculated using very rough approximations, form a smooth curve with the values determined carefully by both partition analysis and fluorescence quenching. As is immediately evident, k_1 decreases markedly with increasing ionic strength at low ionic strength, the dependence becoming less at higher

$\frac{r}{c}$ vs. r from fluorescence quenching data (points)

Curves calculated for partition analysis data according to the two-constant method (Figs. 3 & 4)

FIGURE 15

FIGURE 16

TABLE I
Summary of Constants for the Interaction of DNA
with Acriflavine Obtained from Partition Analysis
and from Fluorescence Quenching Experiments

μ pH T (°C.)		0.002 7.0 25	0.10 7.0 25	0.30 7.0 25	0.10 5.5 25	0.10 7.0 25.4
$k_1 \times 10^{-5}$	Partition Analysis: Two-Constant Method	50.0	3.63	1.20	1.93	9.55
$k_1 \times 10^{-5}$	Partition Analysis: Method of Scatchard	----	3.48	0.99	1.84	9.43
$k_1 \times 10^{-5}$	Partition Analysis: Method of Steiner	49.3	3.35	1.03	1.78	9.39
$k_1 \times 10^{-5}$	Quenching - Slope Method	49.0	2.90	0.92	1.96	9.22
$k_{1av} \times 10^{-5}$		49.0	3.3	1.1	1.9	9.4
$-\Delta H_1^0$ $-\Delta H_1^0$ $-\Delta S_1^0$	(kcal./mole) (kcal./mole) (cal./°/mole)	9.1	7.5 8.1 1.9	6.8	7.2	7.6
$K_1 \times 10^{-6}$		66.8	3.89	2.82	2.53	11.7
$K_2 \times 10^{-5}$		4.76	1.31	1.83	1.75	3.35
n_1		0.07	0.09	0.03	0.06	0.08
n_2		0.43	0.20	0.18	0.20	0.21
n		0.50	0.285	0.208	0.262	0.285

TABLE II

Summary of Constants
for the Interaction of DNA with Acriflavine
Obtained from Fluorescence Quenching Experiments

μ	pH	T(°C.)	$K_1 \times 10^{-5}$	$-\Delta F_1^\circ$ kcal/mole	$-\Delta H_1^\circ$ kcal/mole	ΔS_1° cal/°/mole
0.002	7.0	25 4	$\frac{49^*}{160}$	$\frac{9.1}{9.1}$	8.9	+0.7
0.01	7.0	25 4	$\frac{36}{100}$	$\frac{8.9}{8.9}$	8.2	+2.3
0.05	7.0	25 4	$\frac{7.0}{24}$	$\frac{8.0}{8.1}$	9.5	-4.9
0.10	7.0	25 4	$\frac{3.3}{9.4}$	$\frac{7.5}{7.6}$	<u>8.1</u>	<u>-1.9</u>
0.30	7.0	25 4	$\frac{1.1}{2.8}$	$\frac{6.8}{6.9}$	8.1	-4.2
0.10	5.5	25 4	$\frac{1.9}{4.9}$	$\frac{7.2}{7.2}$	7.8	-2.1

*The underlined values appear in Table I and are average values from both fluorescence quenching and partition analysis experiments.

ionic strengths. The value of k_1 at pH 5.5 is lower than that at pH 7. The thermodynamic constants ΔF_1° , ΔH_1° , and ΔS_1° for the formation of the first DNA-acriflavine complex, calculated from the averaged values of k_1 , are listed in Table I, and values based upon the approximate quenching results for k_1 are given in Table II. It is seen that ΔH_1° is negative in all cases, and that ΔS_1° is approximately zero.

VI. Discussion

Evidence in both partition analysis and fluorescence quenching experiments indicates that the interaction between the DNA and acriflavine samples studied is reversible and that equilibrium is attained rapidly on mixing the two in solution. The distinct curvature in all the graphs of r/c versus r (Figures 3 through 7) shows that the interaction involves more than one intrinsic association constant; that is, that there are differences among the binding sites on the DNA macromolecule with respect to their affinity for the acriflavine cation. It was not considered that electrostatic interactions among the binding sites play a large role, first, because the dye cations replace inorganic cations at the sites, and, second, because the electrostatic correction was inadequate to account for the curves obtained by Cavalieri and Angelo (59) for the binding of rosaniline by DNA. Although the data in the present study, as in the case investigated by Cavalieri and Angelo (59), may be fitted satisfactorily with a theoretical curve calculated on the basis of a Mass Law equation involving two intrinsic equilibrium constants, it does not necessarily follow, as Karush (11) has pointed out, that only two types of sites exist. Rather, it may probably be inferred that the sites fall into at least two groups, each of which may be represented by an intrinsic constant, but that differences.

in affinity may exist within these groups. That the situation is indeed not quite as simple as appears on first sight is indicated by the small but distinct disparity between the calculated curves and the experimental points in the region where the slope is changing rapidly (Figures 3,4,5,7), which becomes larger if a lower value is chosen for the intercept on the r/c axis, k_1 . Thus, the values of K_1 and K_2 , n_1 and n_2 should not be interpreted too literally, but they do serve to indicate that a small number of sites with high intrinsic affinity exists and that a considerably larger number of sites have affinities a great deal lower. It is of interest, in this connection, that the heterogeneity of the sites on DNA which complex acriflavine is not described by the continuous distributions of binding energies considered by Sips (79,80).

As we have noted, the three methods, all based on the Mass Law treatment of multiple equilibria (section III. A.), which were employed for the calculation of k_1 , commonly used as a basis of comparison in cases where the one-constant assumption does not apply, give results which are in satisfactory agreement. Moreover, no noticeable trend appears in the deviations of the values of k_1 calculated by any one of the methods from the average values, and the deviations are very probably attributable to uncertainties in extrapolating the data to $c = 0$. These

uncertainties, for the experiments reported here, were a good deal smaller than the differences between the equilibrium constants k_1 for different environmental conditions, but, if greater accuracy were required, partition analyses could be carried out at somewhat lower dye concentrations than were measured in this investigation if a larger cell and bigger slits were employed for fluorescence measurements in the dilute range.

N-hexanol seems very well suited for the study of the DNA-acriflavine system by partition analysis: There is no indication that it interferes in the interaction, since equilibrium dialysis experiments confirm the partition analysis results, and since the independent fluorescence quenching method yields essentially the same results for k_1 . In addition, the variation of partition coefficient with acriflavine concentration is in the proper direction to facilitate measurement at low equilibrium concentrations of the dye. The partition coefficients might be increased by the addition of some suitable compound to the organic phase, which, however, would not affect the DNA or its interaction with acriflavine.

The modification developed here of the method used by Oster (37) to calculate an equilibrium constant for the DNA-acriflavine association from fluorescence quenching data takes into account the multiple equilibria

involved in the binding and permits the determination of values for k_1 which agree with the partition analysis results. It is indicated, therefore, that the quenching-slope procedure provides a relatively rapid and reliable method for the determination of k_1 , not only for the DNA-acriflavine complexing, but also, perhaps, for other interacting systems involving multiple equilibria where fluorescence quenching occurs to an appreciable extent and where a limiting slope at infinitely low quencher concentration can be determined.

It has been mentioned that the method suggested for the calculation of c and, hence, r from quenching data (equation [19]) cannot be applied to the DNA-acriflavine system except, with proper caution, to reveal the order of magnitude of the binding under conditions such that the binding is not too great. The principal reason for the failure of the method appears to be the variation in p with the relative concentrations of the interactants, the value of p increasing as the concentration of dye relative to the concentration of DNA decreases. This variation is not evident from the quenching curves reported by Oster (67), partly, perhaps, because of differences in the samples of DNA and acriflavine studied, and partly because Oster's work was carried out at a dye concentration an order of magnitude higher than the highest concentration used in the present study. If it may be assumed

that the quantum yield of fluorescence of the free dye remains constant in the presence of DNA, while the yield for the bound molecules depends upon the strength of binding at the sites, then the fact that p is not constant would tend to confirm that the binding sites are heterogeneous with respect to their affinities for acriflavine, and the indicated variation in p would mean that the fluorescence quenching due to interaction at the most active binding sites, those taken up first, is less than that due to binding at sites of lower intrinsic affinity. In view of the hypothesis, mentioned by Bowen (95), that fluorescence quenching is usually accompanied by strong van der Waals forces between interacting molecules, it might be suggested, tentatively, that electrostatic forces are relatively more important in the binding at the very active sites. This is in conformity with the view expressed by Cavalieri and Angelas (59) that the more active sites involve secondary phosphate groups, while the ones of lower affinity involve primary groups. At high concentrations of dye relative to the DNA concentration, there are indications of some phenomenon which causes quenching of the dye in addition to the quenching due to interaction with DNA; that is, no value of p , not even

(95) E. J. Bowen, *Quart. Rev.* 1, 1 (1947).

$p = 0$, will yield a value of r calculated according to equation [19] low enough to agree with the partition analysis results in the range of high dye concentration. Although this apparent excess quenching was not investigated further, the explanation might be suggested that aggregation of acriflavine molecules and the attendant self-quenching occurs in the presence of the aligning forces of the charged DNA macromolecule at lower dye concentrations than when DNA is absent.

The results for k_1 presented in Tables I and II and in Figure 16 show that the value for the first equilibrium constant is high under any of the conditions studied. For example, the value at $\mu = 0.1$, pH 7, and 25°C ., $k_1 = 3.3 \times 10^5$, may be compared with a k_1 of approximately 2.2×10^3 for the binding of rosaniline by DNA under almost the same experimental conditions calculated from the data of Cavalieri and Angelas (59), using the same concentration units as were employed in this report. Binding constants for other DNA interactions are summarized in the paper of Allenby and Collier (61), and these, too, are considerably lower than those found for the system studied here, but it should be remembered that comparisons must be made with caution in view of the considerable variation in the DNA preparations used in binding studies.

Since, as is the case in most binding studies of this type, the values reported for n are estimates

- only, based upon the uncertain extrapolation to $r/c = 0$, it would be of little value to make a detailed comparison with the values given by other workers. It suffices to say that the value of 0.5 for n at $\mu = 0.002$, pH 7, and 25°C . is in the neighborhood of the results obtained by Cavalieri, et al. (60) for the two dyes whose binding to DNA they studied in the absence of added salt, and by Shack, et al. (42), for the binding of sodium ions.

The effect on k_1 of decreasing the pH from 7 to 5.5 is opposite to that found by Cavalieri and Angelos (59) for the binding of rosaniline. Very probably, the decrease in binding affinity with decreasing pH in the acriflavine-DNA case is in large part due to the increased competition of hydrogen ions for the binding sites. On the other hand, since rosaniline is not a quaternary salt, the shift in equilibrium in favor of the ionized hydrochloride at the lower pH would account for the increased binding observed.

The very marked dependence of k_1 upon ionic strength indicates, of course, that the interaction between DNA and acriflavine is essentially electrostatic in nature. It is evident, however, as Oster (67) pointed out, that secondary forces play a very large part in the interaction. First, as we have noted, quenching of fluorescence upon interaction indicates strong van der Waals bonding between the quencher and the fluorescent compound (95), and the

rather large negative values found for ΔH_1° are in accord with this hypothesis. Then, we may infer from the qualitative results with several organic cations, and from the other information on DNA complexing available (section II. C.), that binding by DNA is favored for molecules which offer opportunities for secondary interactions; for example molecules with long hydrocarbon chains, as in the case of the alkyl pyridinium salts, or with several aromatic rings (66), especially if these are coplanar.

In contrast with the results of Cavalieri and Angelos (59) on rosaniline binding to DNA and with the majority of findings in quantitative studies of protein complexing that there are large positive entropy changes on interaction in these cases, the entropy change, ΔS_1° , for the formation of the first DNA-Acriflavine complex is found to be zero, within experimental error, according to the partition analysis results presented here. From his investigation of the temperature dependence of fluorescence quenching, Oster (67), too, concluded that the entropy change is zero. It appears, then, that far-reaching structural changes do not occur when acriflavine interacts with DNA, as they are not believed to occur on nucleic acid-protein interaction (51).

For this reason, and, also, because equilibrium is reached very rapidly in the DNA-acriflavine system, it would seem that a proposed structural configuration for the

DNA macromolecule should place the charged phosphate groups so that they would be readily accessible for electrostatic interaction without a great deal of configurational change. Several DNA structures suggested (section II. B.), the structure of Crick and Watson (40) included, satisfy this requirement. A structure similar to the one proposed by Crick and Watson, in addition, allows that the planar hydrocarbon portion of a molecule such as acriflavine ~~to~~ slip between successive parallel planes occupied by the purine and pyrimidine bases, the quaternary nitrogen of the molecule oriented toward the DNA phosphate group, thus permitting strong van der Waals bonding in addition to the electrostatic interaction without requiring much change in the configuration of the DNA molecule. In this connection, it might be suggested that a light scattering investigation of the DNA-acriflavine interaction would, perhaps, yield information on the molecular weight, shape, and other properties of the complex, as compared to those of unreacted DNA, which would aid in the interpretation of binding data in terms of molecular structure. It is to be hoped that binding studies involving molecules tailored so as to reveal steric factors, and others employing DNA preparations which have been modified chemically or enzymatically, will contribute in revealing details of the macromolecular configuration of DNA as it exists under various environmental conditions.

VII. Summary

1. The interaction between purified acriflavine and the high molecular weight sample of DNA prepared was found to be reversible in the concentration range where soluble complexes are formed.
2. Determinations of the amount of binding were made at several values of ionic strength, pH, and temperature over a wide range of free acriflavine concentrations by the method of partition analysis, using n-hexanol as the organic solvent. Results from several equilibrium dialysis experiments are in agreement with those obtained from partition analysis.
3. The data from partition analysis shows that the binding sites on DNA are heterogeneous with respect to their intrinsic affinities for acriflavine, and theoretical binding curves calculated on the basis that two groups of sites exist, each characterized by a single intrinsic association constant, fit the binding data satisfactorily.
4. Three, methods, all based on the Mass Law treatment of multiple equilibria, were employed to calculate k_1 , the equilibrium constant for the formation of the first DNA-acriflavine complex, from the partition analysis data. For each set of experimental conditions, the values of k_1 calculated according to the three methods agree within the limits of the uncertainty in extrapolating to zero free acriflavine concentration.

5. The maximum number of sites available to the acriflavine molecule per DNA phosphate group is estimated to be 0.5 at $\mu = 0.002$ and pH 7.

6. Measurements of the quenching of the fluorescence of acriflavine by DNA under different experimental conditions were used to calculate values for k_1 . A modification of a treatment by Oster (67) of fluorescence quenching was developed which takes into account the multiple equilibria concept for complexing by a molecule with a number of independent binding sites. Application of this quenching-slope method yields values for k_1 in agreement with the independent partition analysis results.

7. The values for k_1 obtained by the two experimental techniques under the different experimental conditions investigated are: at pH 7 and 25°C., $k_1 = 49 \times 10^5$ at $\mu = 0.002$, 3.3×10^5 at $\mu = 0.10$, and 1.1×10^5 at $\mu = 0.30$; at pH 5.5, $\mu = 0.10$, and 25°C., $k_1 = 1.9 \times 10^5$; and at 4°C., $\mu = 0.10$, and pH 7, $k_1 = 9.4 \times 10^5$.

8. k_1 decreases somewhat with a decrease in pH from 7 to 5.5, probably because of increased competition of hydrogen ions for the binding sites.

9. The decrease in k_1 with increasing ionic strength, which is very marked at low ionic strength, testifies to the essentially electrostatic nature of the DNA-acriflavine interaction.

10. The evidence, from this work and from literature sources, indicates that secondary binding forces play an important role. Molecules which interact strongly with polymerized DNA usually offer opportunities for extensive van der Waals bonding. In addition, fluorescence quenching, such as occurs on the interaction of DNA with acridines, is believed to occur when van der Waals interactions between the fluorescent and quenching molecules are strong. The relatively large negative value of ΔH_1° found for the DNA-acriflavine system, about -8 kcal./mole, is indicative of van der Waals binding.

11. ΔS_1° is zero within experimental error, indicating that the macromolecular configuration of DNA does not change significantly upon interaction with acriflavine.

12. The findings reported here are generally consistent with a macromolecular structure for DNA like that proposed by Crick and Watson (40).

APPENDIX

TABLE III

Fluorescence - Extinction Data

Organic Layer

<u>E₄₇₀</u>	<u>Forg.</u>	<u>Range</u>	<u>E₄₇₀</u>	<u>Forg.</u>	<u>Range</u>
.00368	19.3	.0001		17.6	.001
.00382	20.9	.0001	.055	24.2	.001
.00401	21.7	.0001	.0601	27.0	.001
.00602	31.4	.0001	.0763	34.4	.001
.00654	34.6	.0001	.0779	33.3	.001
.00802	41.1	.0001	.0981	43.1	.001
	19.2	.0002	.101	43.2	.001
.00981	51.7	.0001	.106	45.2	.001
	24.0	.0002			
.0105	54.7	.0001			
	25.3	.0002			
.0115	59.0	.0001			
	27.3	.0002			
.0120	59.3	.0001			
	27.5	.0002			
.0160	77.3	.0001			
	35.8	.0002			
.0180	89.0	.0001			
	41.1	.0002			
.0207	46.1	.0002			
.0371	78.8	.0002			
	17.1	.001			
.0382	82.8	.0002			
	18.1	.001			
.0401	81.3	.0002			

TABLE III (continued)

Aqueous Layer

<u>E452</u>	<u>F_{aq.}</u>	<u>Range</u>	<u>E452</u>	<u>F_{aq.}</u>	<u>Range</u>
$\mu = 0.002, \text{pH } 7$			$\mu = 0.1, \text{pH } 5.5$		
.00205	14.3	.0001	.00198	16.6	.0001
.00409	29.5	.0001	.00396	31.9	.0001
.00614	42.6	.0001	.00594	47.5	.0001
	19.7	.0002		21.8	.0002
.00818	55.4	.0001	.00792	61.0	.0001
	25.6	.0002		28.0	.0002
.0123	83.0	.0001	.0119	40.9	.0002
	38.2	.0002	.0158	53.0	.0002
.0164	52.1	.0002	.0198	63.5	.0002
.0170	55.9	.0002	.0396	27.2	.001
.0409	26.8	.001	.0594	38.0	.001
.0579	37.9	.001	.0891	52.8	.001
.0851	50.0	.001	.100	59.5	.001
.102	62.1	.001			
$\mu = 0.1, \& 0.3, \text{pH } 7$					
.00203	18.1	.0001			
.00393	36.1	.0001			
.00655	57.5	.0001			
	26.4	.0002			
.00765	65.1	.0001			
	29.9	.0002			
.00917	79.0	.0001			
	36.2	.0002			
.0121	46.8	.0002			
.0203	78.0	.0002			
	17.0	.001			
.0262	20.6	.001			
.0655	48.1	.001			
.0765	55.2	.001			
.0917	65.0	.001			
.121	80.7	.001			

TABLE IV

Partition Coefficients, $E_{org.}/E_{aq.}$							
$F_{org.}$	Range	$E_{org.}$	$F_{aq.}$	Range	$E_{aq.}$	$E_{org.}/E_{aq.}$	
$\mu = 0.002, \text{ pH } 7, \text{ T } = 25^{\circ}\text{C.}$							
1.	58.6	.0001	.0115	7.7	.0001	.0011	10.7
	27.3	.0002					
2.	50.7	.0002	.0232	16.9	.0001	.0024	9.80
3.	78.5	.0002	.0377	37.6	.0001	.00539	6.94
	17.6	.001					
4.	25.9	.001	.0574	69.8	.0001	.0103	5.69
5.	30.7	.001	.0688	40.8	.0002	.0127	5.42
6.			.0915	14.8	.001	.0222	4.12
7.			.103	20.3	.001	.0307	3.34
8.			.119	27.0	.001	.0410	2.90
9.			.144	39.0	.001	.0600	2.40
10.			.187			.108	1.73
11.			.228			.187	1.22
12.			.285			.340	.838
13.			.317			.482	.658
14.			.385			.721	.534
15.			.437		1.009		.433
16.			.494		1.16		.426
17.			.549		1.46		.376
$\mu = 0.1, \text{ pH } 7, \text{ T } = 25^{\circ}\text{C.}$							
1.	36.5	.0001	.00693	25.4	.0001	.00281	2.49
2.	71.0	.0001	.0140	51.0	.0001	.0057	2.46
	32.8	.0002		23.5	.0002		
3.	61.0	.0002	.0276	45.6	.0002	.0116	2.43
4.	24.3	.001	.0536	86.9	.0002	.0240	2.23
				18.9	.001		
5.	44.5	.001	.104	39.5	.001	.0524	1.98
			.191			.130	1.47
7.			.273			.239	1.14
8.			.347			.345	1.01
9.			.414			.477	.868
10.			.481			.664	.724
11.			.580			.938	.618
12.			.662		1.23		.538
13.			.762		1.69		.451

TABLE IV (continued)

°	F _{org.}	Range	E _{org.} $\mu = 0.3,$	F _{aq.} pH 7,	Range	E _{aq.} T = 25°C.	E _{org} /E _{aq.}
1.	61.5	.0001	.0121	55.3	.0001	.00616	1.96
	28.6	.0002		25.4	.0002		
2.	31.9	.0001	.000602	28.0	.0001	.00307	1.96
3.	22.0	.001	.0483	21.0	.001	.0271	1.78
4.	41.5	.001	.0959	41.5	.001	.0551	1.74
5.			.190			.131	1.45
6.			.271			.200	1.36
7.			.370			.322	1.15
8.			.442			.404	1.09
9.			.550			.603	.912
10.			.648			.765	.847
11.			.781			1.103	.708
12.			.890			1.33	.692
			$\mu = 0.1,$	pH 5.5		T = 25°C.	
1.	64.7	.0001	.0127	41.2	.0001	.00521	2.44
	30.0	.0002		18.9	.0002		
2.	55.6	.0002	.0256	37.2	.0002	.0107	2.39
3.	23.0	.001	.0506	15.9	.001	.0227	2.23
4.	43.3	.001	.1007	32.5	.001	.0483	2.08
5.			.202	59.5	.001	.101	2.00
6.			.298			.171	1.74
7.			.399			.261	1.53
8.			.501			.370	1.35
9.			.599			.495	1.21
10.			.741			.713	1.04
11.			.856			.924	.926
12.			1.028			1.25	.822
			$\mu = 0.1,$	pH 7,		T = 25°C.	
1.	55.2	.0001	.0107	21.7	.0001	.00237	4.51
	25.6	.0002					
2.	87.7	.0002	.0423	38.4	.0002	.00970	4.36
	19.2	.001					
3.	37.9	.001	.0884	84.7	.0002	.0222	3.98
				18.4	.001		
4.			.182	42.8	.001	.0573	3.18
5.			.3000	84.4	.001	.129	2.33
6.			.399			.225	1.77

TABLE IV (continued)

	F _{org.}	Range	E _{org.}	F _{aq.}	Range	E _{aq.}	E _{org} E _{aq.}
7.			.501			.358	1.40
8. .			.630			.566	1.11
9.			.719			.736	.977
10.			.837			1.028	.814
11.			.919			1.25	.735
12.			1.038			1.65	.629

TABLE V

Partition Analysis: $\mu=0.002$, pH 7, $T = 25^{\circ}\text{C.}$, $[\text{NA}]_0 = .005\%$

$$k_{\text{aq.}} = 1720$$

$$r = .147 [E_0 - .735 E_{\text{org.}} - E_{\text{aq.}}]$$

$$\frac{r}{c} = (4.52 \times 10^4) \frac{r}{E_{\text{aq.}}}$$

E_0	$F_{\text{org.}}$	Range	$E_{\text{org.}}$	r	$\frac{r}{c} \times 10^{-5}$
.228	28.5	.0001	.0055	.0327	31.1
.317	52.7	.0001	.0102	.0451	21.6
.400	78.1	.0001	.0157	.0565	16.4
.467	47.2	.0002	.0204	.0659	13.9
.544	65.1	.0002	.0300	.0759	9.57
.641	83.7	.0002	.0400	.0888	7.38
.715	23.8	.001	.0524	.0979	5.24
.975	27.8	.001	.0617	.135	5.32
1.30	36.9	.001	.0840	.178	3.97
1.69			.125	.228	2.28
2.37			.197	.307	1.10

TABLE VI

Partition Analysis: $\mu = 0.1$, p^H 7, $T = 25^\circ\text{C.}$, $[\text{NA}]_0 = .005\%$

$$k_{\text{aq.}} = 1810$$

$$r = .139 [E_0 - .774 E_{\text{org.}} - E_{\text{aq.}}]$$

$$r/c = (4.75 \times 10^4) \cdot r/E_{\text{aq.}}$$

E_0	$F_{\text{org.}}$	Range	$E_{\text{org.}}$	r	$r/c \times 10^{-5}$
.806	22.5	.0001	.0043	.0112	3.24
.170	52.0	.0001	.0101	.0220	2.67
.215	64.1	.0001	.0126	.0278	2.68
.260	36.7	.0002	.0158	.0335	2.54
.302	44.5	.0002	.0194	.0388	2.38
.375	59.2	.0002	.0274	.0477	2.03
.505	87.4	.0002	.0421	.0631	1.67
.675	30.1	.001	.0673	.0824	1.32
.865			.0967	.103	.996
.970			.126	.112	.757
1.20			.167	.134	.608
1.40			.225	.147	.416
1.695			.292	.168	.306
2.15			.399	.193	.202
3.025			.585	.224	.111
5.00			.958	.250	.048

TABLE VII

Partition Analysis: $\mu=0.3$, pH 7, T = 25°C., $[NA]_0 = .005\%$

$$k_{aq.} = 1810$$

$$r = .139 [E_o - .774 E_{org.} - E_{aq.}]$$

$$r/c = (4.75 \times 10^4) \cdot r/E_{aq.}$$

E_o	$F_{org.}$	Range	$E_{org.}$	r	$r/c \times 10^{-5}$
.047	26.8	.0001	.00503	.0057	1.066
.080	50.8	.0001	.00975	.0094	.897
.154	45.4	.0002	.0199	.0178	.821
.311	20.0	.001	.0439	.0352	.700
.456	33.6	.001	.0758	.0492	.538
.720			.127	.0755	.455
.945			.193	.0923	.332
.970			.194	.0955	.341
1.495			.357	.127	.199
1.515			.383	.123	.175
2.70			.728	.167	.085
3.66			.979	.177	.052
3.71			.971	.187	.055

TABLE VIII

Partition Analysis: $\mu=0.1$, p^H 5.5, $T = 25^\circ\text{C.}$, $[\text{NA}]_0 = .005\%$

$$k_{\text{aq.}} = 1640$$

$$r = .154 [E_0 - .701 E_{\text{org.}} - E_{\text{aq.}}]$$

$$r/c = (4.31 \times 10^4) \cdot r/E_{\text{aq.}}$$

E_0	$F_{\text{org.}}$	Range	$E_{\text{org.}}$	r	$r/c \times 10^{-5}$
.086	39.3	.0001	.0076	.0120	1.68
.156	73.1	.0001	.0145	.0215	1.56
.271	64.4	.0002	.0300	.0365	1.25
.377	21.7	.001	.0476	.0498	1.05
.505	32.8	.001	.0737	.0648	0.857
.760			.135	.0926	.621
1.025			.210	.118	.467
1.335			.308	.145	.349
1.765			.463	.172	.227
2.320			.672	.192	.138
3.045			.904	.215	.091

TABLE IX

Partition Analysis: $\mu=0.1$, pH 7, $T = 4^{\circ}\text{C.}$, $[\text{NA}]_0 = .005\%$
 $k_{\text{aq.}} = 1810$ $[\text{NA}]_0 = .01\%$

$$r/c = (4.75 \times 10^4) \cdot r/E_{\text{aq.}}$$

E_0	$F_{\text{org.}}$	Range	$E_{\text{org.}}$	r	$r/c \times 10^{-5}$
$[\text{NA}]_0 = .005\%$, $r = .139 [E_0 - .774 E_{\text{org.}} - E_{\text{aq.}}]$					
.085	17.2	.0001	.00320	.0114	8.02
.168	36.5	.0001	.00700	.0223	7.11
.315	38.3	.0002	.0167	.0404	5.43
.460	66.7	.0002	.0312	.0596	4.07
.630	24.6	.001	.0543	.0799	2.98
.785	35.3	.001	.0800	.0977	2.34
1.065			.145	.127	1.45
1.365			.230	.154	.891
1.615			.315	.171	.579
2.025			.456	.192	.309
2.850			.704	.222	.149
4.815			1.113	.268	.063
$[\text{NA}]_0 = .01\%$, $r = .0695 [E_0 - .774 E_{\text{org.}} - E_{\text{aq.}}]$					
1.365	29.2	.001	.0652	.0902	2.73
1.615	39.2	.001	.0900	.106	2.09
2.025			.155	.129	1.35
2.850			.325	.170	.544
4.815			.782	.231	.125

TABLE X

Fluorescence Quenching Data

$[NA]_0 \times 10^5$	F_0/F	$[NA]_0 \times 10^5$	F_0/F
$\mu = 0.002, \text{ pH } 7$			

T = 25°C.

T = 40°C.

 $c_0 = 1.17 \times 10^{-7}, p = .088$ $c_0 = 9.71 \times 10^{-7}, p = .04$

.020	1.38
.050	2.28
.099	4.31
.196	7.271
.291	8.44
.471	8.92
.686	8.79
.888	8.67
1.26	8.63
1.89	8.55
2.40	8.63
3.02	8.67

.280	3.12
.840	15.09
1.40	15.78
2.80	15.00
3.50	12.77
4.89	9.60
7.01	8.11

 $c_0 = 11.4 \times 10^{-7}, p = .042$

.185	1.66
.369	2.93
.547	4.77
.725	6.64
.900	8.17
1.25	11.44
1.58	12.81
1.91	13.19
2.22	13.13
2.69	12.89
3.57	12.12
4.65	11.27
5.89	10.54
7.98	9.71
9.70	9.14
11.2	9.01

TABLE X (continued)

$[NA]_0 \times 10^5$	F_0/F	$[NA]_0 \times 10^5$	F_0/F
$\mu = 0.01, \text{ pH } 7$			
$T = 25^\circ\text{C}.$ $C_0 = 11.4 \times 10^{-7}, p = .051$		$T = 40^\circ\text{C}.$ $c_0 = 9.71 \times 10^{-7}, p = .045$	
.185	1.47	.280	2.00
.369	2.32	.840	11.64
.547	3.48	1.75	14.06
.725	4.87	2.80	12.11
.900	6.62	3.50	10.98
1.24	9.76	4.89	9.68
1.58	11.65	9.12	8.06
1.91	12.74		
2.22	12.97		
2.54	13.18		
2.99	13.04		
3.57	12.76		
4.38	12.30		
5.89	11.61		
7.98	10.89		
9.79	10.56		
11.2	10.30		
$\mu = 0.05, \text{ pH } 7$			
$T = 25^\circ\text{C}.$ $C_0 = 11.4 \times 10^{-7}, p = .088$		$T = 40^\circ\text{C}.$ $c_0 = 11.4 \times 10^{-7}, p = .075$	
.301	1.49	.301	1.76
.903	2.80	.903	5.70
1.50	4.13	1.50	8.25
2.11	5.43	2.11	8.55
6.01	8.86	6.01	9.06
12.0	8.62	12.0	8.19
22.6	8.50	22.6	7.89

TABLE X (continued)

$[NA] \times 10^{-5}$	F_o/F	$[NA] \times 10^{-5}$	F_o/F
$\mu = 0.1, p^H \ 7$			
$T = 25^\circ C.$		$T = 40^\circ C.$	
$c_o = 1.17 \times 10^{-7}, p = .102$		$c_o = 1.17 \times 10^{-7}, p = .111$	
.369	1.86	.201	2.19
.731	2.68	.502	3.78
1.45	3.91	.643	4.46
2.17	4.79	.994	5.29
3.49	5.87	1.96	6.41
9.31	7.96	2.91	6.92
14.0	8.57	4.71	7.43
18.1	8.89	8.88	7.99
22.3	8.94	12.6	8.27
		18.9	8.30
		24.5	8.20
		30.2	8.02
$c_o = 6.20 \times 10^{-7}, p = .102$		$c_o = 11.4 \times 10^{-7}, p = .094$	
.251	1.36	.601	2.66
.495	1.80	1.28	5.54
.740	2.32	1.88	6.93
1.22	3.27	2.48	7.64
1.91	4.35	6.01	7.64
2.80	5.35		
3.64	5.91		
4.68	6.62		
7.16	7.34		
10.8	7.95		
15.1	8.27		
18.8	8.43		
$c_o = 11.3 \times 10^{-7}, p = .102$			
.366	1.49		
1.10	2.52		
1.83	3.70		
2.56	4.63		
4.02	6.09		
9.15	7.95		
36.6	8.34		
73.1	8.34		

TABLE X (continued)

$[NA]_0 \times 10^{-5}$	F_0/F	$[NA]_0 \times 10^{-5}$	F_0/F
$\mu = 0.3, pH \quad 7$			
$T = 25^\circ C.$		$T = 40^\circ C.$	
$c_0 = 1.17 \times 10^{-7}, p = .102$		$c_0 = 11.4 \times 10^{-7}, p = .123$	
.369	1.33	.601	2.11
.734	1.64	1.28	3.17
1.45	2.20	1.88	4.39
2.15	2.74	2.48	4.90
3.49	3.49	6.01	6.05
6.59	4.70	12.8	6.54
9.31	5.45	24.8	6.82
14.0	6.25	37.6	6.77
17.8	6.95		
22.4	7.22		
$c_0 = 11.4 \times 10^{-7}, p = .102$			
.502	1.25		
.994	1.57		
1.96	2.21		
2.91	2.72		
4.71	3.69		
6.86	4.53		
8.88	5.10		
12.6	5.92		
15.9	6.43		
21.6	6.95		
26.3	7.24		
30.2	7.55		
$\mu = 0.1, pH \quad 5.5$			
$T = 25^\circ C.$		$T = 40^\circ C.$	
$c_0 = 1.17 \times 10^{-7}, p = .088$		$c_0 = 11.4 \times 10^{-7}, p = .106$	
.201	1.43	.601	1.94
.502	2.04	1.28	3.98
.994	2.91	1.88	5.02
1.96	4.13	2.48	5.87
2.91	4.91	6.01	6.97
4.71	5.98	12.8	7.21
6.86	6.76	24.8	7.04
8.88	7.54		
12.6	8.20		
18.9	8.91		
24.0	9.16		
30.2	9.38		

TABLE X (continued)

$[NA]_0 \times 10^{-5}$	F_0/F	$[NA]_0 \times 10^{-5}$	F_0/F
$\mu = 0.1, \text{ pH } 5.5$			
$T = 25^\circ\text{C.}$ $c_0 = 1.17 \times 10^{-7}, p = .088$		$T = 4^\circ\text{C.}$ $c_0 = 11.4 \times 10^{-7}, p = .106$	
.601	1.54		
1.28	2.40		
1.88	3.11		
2.48	3.70		
6.01	5.76		
12.8	7.13		
30.1	7.60		
56.5	7.54		

AUTOBIOGRAPHY

I, Harriet Gerletz Heilweil, was born September 8, 1928 in Brooklyn, New York, where I received my primary education. I attended Hunter College High School, 1942-1948, and then Hunter College, from 1945 to 1948, when I was granted the degree Bachelor of Arts. In 1948, I began my graduate studies at The Ohio State University, serving as Graduate Teaching Assistant in the period from 1948 to 1950. I was granted an Abbott Laboratories Fellowship for the year 1951-1952 and received the degree Master of Science in 1951. During the year 1952-1953, I was an Ohio State University Chemistry Department Fellow. I worked as Research Assistant under a grant from the United States Public Health Service in 1950 and 1951 and, again, from 1953 to the present, during which time I completed the requirements for the degree Doctor of Philosophy.