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PROPERTIES OF HELICAL POLYCYTIDYLIC ACID

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INTERACTIONS OF PURINE WITH PROTEINS AND AMINO ACIDS

PART III

BINDING OF BASIC PROTEINS TO DNA

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E. Olabisi Akinrimisi

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CONTENTS

PREFACE

.... Et je n'ai pas pris peur de ma vision, mais m'assurant avec aisance dans le saisissement, je tiens mon oeil ouvert a la faveur immense, et dans l'adulation.

St. John Perse

("Du Maître d'Astre et de Navigation" in "Amers")

This thesis is divided into three sections. The first part deals with the secondary structure of poly C in acid solution, as revealed by several of its physical-chemical properties in solution. The interpretation of the data was based on previous knowledge of the general properties of polynucleotides in solution and on the properties of poly C monomer.

The second part of the thesis deals with the interaction of purine with the proteins. Conformation changes in the proteins are easily measurable in terms of changes in optical rotation even at the visible wavelength regions. Full use was made of this fact to study the nature of interaction of purine with the proteins. The most significant aspect of the studies is not the theoretical speculation on the mechanism of the interaction, but rather the possible practical applications of the findings. This is briefly considered in the discussion.

The mechanism of urea interaction with the proteins cannot be regarded as solved in spite of the voluminous literature on the subject. Similarly, the mechanism of purine interaction with proteins cannot be regarded as solved. The speculations presented at length on the mechanism of the interactions of purine or urea with the proteins, therefore, merely represent a simplified deduction from available data--a deduction intended to stimulate more experiments and perhaps a modified interpretation of the nature of the interactions.

The third part of the thesis presents initial findings on a very complex problem--the relationship of polyvalent polymer-polymer interactions to some of their other physical-chemical and biochemical properties. The conclusions drawn from the data are not new or peculiar to current thinking about the nature of interaction of the histones or protamines to DNA. However, certain satisfactions are derived from the fact that it has been possible to carry out reliable physical-chemical measurements on the nucleohistone and nucleoprotamine systems by means of simple standard techniques. Such data are rare in the nucleohistone or nucleoprotamine literature. No doubt, the confirmation of more complex findings on these very important systems will often require these simple physical-chemical data.

PART I

PROPERTIES OF HELICAL POLYCYTIDYLIC ACID

INTRODUCTION

General Review of the Properties of Polynucleotides

Studies of the helical structures formed by the synthetic polynucleotides have provided considerable insight into the problem of the specificity and interaction of natural nucleic acids. The homopolymers (polyadenylic acid, polyuridylic acid, polyinosinic acid, and polycytidylic acid) appear to form regular helices either by themselves or in combination with another homopolymer in the series. The formation of helices in the polynucleotides involves the formation of hydrogen bonds between like and unlike base pairs. In certain cases, such hydrogen bonding is stabilized by electrostatic interaction between the nucleotide base pairs, as well as by hydrophobic interaction amongst the relatively non-polar bases. In others, however, no such attractive electrostatic interaction is involved in the pairing of the bases. In this latter case, the stacking of the bases in an organized form and the $\rangle NH....N\langle$ and $\rangle NH....O{=}C\langle$ hydrogen bonds are responsible for the stabilization of the helix. The general properties of the polynucleotides will be discussed under the above two classifications.

(a) Polynucleotides helices not stabilized by protons:

The most typical example in this group is DNA. No attempt will be made here to give a detailed description of the structure of DNA. All physical-chemical data have, so far, supported the helical structure

proposed by Watson and Crick (53) for DNA. Of particular interest, however, is the finding that DNA is not a stiff rod, as would be predicted from the Watson and Crick model. For a thin rigid rod, the radius of gyration is approximately equal to $\sqrt{12}L$. The light scattering data obtained by Doty et al. (8); C. Sadron (37); R. Steiner (41) indicate that the observed length for calf thymus DNA is of the order of two to five times shorter than that predicted from this type of calculation. In other words, native calf thymus DNA behaves more like a coiled polynucleotide than the rigid form predicted by the Watson-Crick model. In order to explain this apparent discrepancy, it has been suggested that native DNA contains base pair imperfections (45). This allows the macromolecule a reasonable combination of flexibility and rigidity. Even without such imperfection, some flexibility attributable to extremely small distortions of bond angles or distance in each residue is to be expected (Hearst, 18). The $S^{\circ}_{20} = 0.063 \times M^{0.37}$, as well as the relationship $[\eta] = 1.45.10^{-6} \times M^{1.12}$ obtained by Doty et al. (10) for calf thymus DNA supports the more flexible model. The coefficient of Mw (1.12) in the intrinsic viscosity expression is only slightly above the limit for random coiled solvent-immobilized polymer (1.0), and the coefficient 0.37 in the sedimentation coefficient is only slightly higher than the smallest value predicted for coils (0.33 - 0.50). Geiduschek and Holtzer (17) have successfully established an agreeable correlation

between the light scattering behavior of calf thymus DNA and the proposed stiff coil model.

The above mentioned property of DNA presents some rather fundamental problems which might be understood by studying the interaction of the nucleotide homopolymers. For example, how would one explain the imperfect base-pairing in DNA, if it actually exists? Will the mistake of substituting cytosine for guanine in a DNA chain prevent the formation of hydrogen bonding only where the mistake has been made, or will it introduce an additional steric factor which will affect several other base-pairs in its vicinity? Will such steric interference result in the formation of hydrogen bond pairs which cannot be accounted for by Watson-Crick model? Questions of this type, and perhaps many more, can only be properly answered by studying simple molecular models similar to DNA.

Before turning to these simple homopolymers, certain important properties of DNA need to be mentioned. The helical structure of DNA is destroyed at high temperature at neutral pH, and by both acid and bases, to give a more coiled form. This denaturation process is accompanied by an increase in ultraviolet absorption and by a marked decrease in intrinsic viscosity $[\eta]$. This decrease in $[\eta]$ is due to a shrinkage in the over-all dimensions of the molecule, especially in the presence of a moderate concentration of salt. Such a shrinkage may arise from a decrease in molecular weight and/or from a decrease in

the electrostatic expansion of the polymer. Whether or not a decrease in molecular weight (brought about by the complete separation of strands of a helical DNA) will be observed on denaturation by heat or extreme pH seems to depend upon the conditions of the experiment.

At first, it is not easy to see why lowering the pH of a DNA solution would bring about the uncoiling of the DNA helix. One would expect the addition of protons to neutralize the negatively charged phosphate groups, and therefore decrease repulsive electrostatic interaction between the phosphate groups on the helical molecule. However, it can be seen from models of DNA that the negatively charged phosphate groups at neutral pH are a part of the backbone of the double helix, and are located rather far apart in this helical structure. Thus, the repulsive charge interaction due to the negative charges on the macroion is not enough to disrupt the helix at neutral pH and in the presence of a moderate concentration of salt. The purine and pyrimidine bases, on the other hand, are situated rather close to one another--that is, inside the double helix. As a result, the free energy involved in charging the base nitrogen atoms is much higher than that needed for charging the better solvated, more exposed phosphate ions. At neutral pH, the purine and pyrimidine nitrogen atoms are uncharged. However, in acidic solution, they become charged and create a highly repulsive electrostatic interaction between the chains. This results in the uncoiling of the DNA helix.

However, DNA can denature at neutral pH in the absence of salt, since under this condition the negatively charged phosphate groups are not partially screened by salt. In basic solutions, the repulsive charge interaction due to the negative charges is high enough to disrupt the helix either in the absence or presence of salt.

The less complex polynucleotides, whose helices are not stabilized by protons, are the poly A- poly U(Warner, 52; Warner, 51), poly A- poly I (Rich, 32), poly I- poly C (Davis and Rich, 6), and poly I complexes (Rich, 32). Like DNA, the formation of helices in these polynucleotides is accompanied by a marked decrease in ultraviolet absorption (hypochromicity). These helices, as are those of DNA, are stable at moderate salt concentrations and are destroyed by heat, extreme basic, and acidic solutions. Unlike DNA, however, the denaturation process is usually reversible. Furthermore, the multiple strands appear to separate completely under all conditions of denaturation. The latter conclusion is the result of the observation that the denaturation of synthetic polynucleotides always results in the reduction of molecular weight. Again, as in DNA, the synthetic polynucleotides do not behave as rigid helical rods. There are several random omissions in the base pair hydrogen bonds, due perhaps to the random looping of the polymer.

Of all the polynucleotides, the one that has been studied most is the poly A-poly U complex. At low ionic strength, the maximum

hypochromicity appears at the equimolar concentration of poly A and poly U at pH 7 (Felsenfeld & Rich, 12; Steiner and Beers, 43; Warner, R. C., 51). The molecular complex thus formed (poly A-poly U) shows considerably greater sedimentation coefficient than either of the two components from which it has been formed. Furthermore, the molecular complex migrates electrophoretically (pH 9.6) as a single unit (Warner, 51). Miles (26) has confirmed the existence of this complex by infrared spectroscopic studies, and the X-ray studies of Rich and Davies (34) lend support to the double helical nature of poly A-poly U.

Another property of poly A and poly U, which has not been so fully confirmed, is the formation of a three-stranded complex (poly A-2 poly U). This is supposed to form in high salt, or in the presence of Mg^{++} and Mn^{++} (Felsenfeld & Rich, 12). Kinetic studies on the formation of the three-stranded complex indicate that the reaction is second order with respect to the divalent cation. Apparently, the divalent cations reduce the electrostatic repulsion amongst the negatively charged phosphate groups in the three polynucleotide chains. Similar evidences to those above have helped to confirm the occurrence of two- or three-stranded complexes in poly I, poly I and poly A, polyribothymidylic acid and poly A (Rich, 33), poly I and poly C, poly G and poly C (Mii et al., 27).

(b) Polynucleotide helices stabilized by protons:

In this group, the formation of helices appears only in acidic solution. Poly A is the most familiar member of this group. In neutral solution, poly A shows very low flow birefringence and increased viscosity at low ionic strength (Fresco and Doty, 15). If the pH of the solution is lowered to 5.5, there is a sharp change to the ordered conformation, as indicated by a decrease in ultraviolet absorption, increase in particle weight, and a very marked negative birefringence of flow (Fresco and Doty, 15; Steiner and Beers, 11). All of the above physico-chemical properties are paralleled by the titration curve of poly A. The evidence supports the conclusion that on protonation of the adenine residue of poly A, a stiff coiled double helical aggregate is formed. The ordered structure goes through a thermal helix-coil transition similar to that of DNA, but the helix is reversibly destroyed at extreme acid pH due to the protonation of the N_7 atoms of the purine molecule, and therefore, the destruction of the hydrogen bond. The X-ray diffraction photographs of Rich et al. (35) and Fresco (13) have confirmed the helical nature of poly A in acid solution. The other member of this group, poly C, is the subject of this manuscript and details of its interaction will be delayed until later.

Some Physical Methods for Studying the Interaction of Polynucleotides

(A) Hydrodynamic methods:

The hydrodynamic methods employed here (sedimentation and viscosity) can be used to obtain information about the polydispersity, molecular weight, and molecular shape of a macromolecule in solution.

(a) Ultracentrifugation

The average sedimentation coefficient $\bar{S}$ is defined:

$$\frac{1}{\omega^2 t} \ln \left(\frac{x}{x_m} \right) \qquad (1)$$

where ω = angular velocity

t = time

x and x_m, respectively, represent the radial distances at the boundary position in the cell and at the meniscus

For polydisperse synthetic polynucleotides, measurements of $\bar{S}^o$, the average sedimentation coefficient at infinite dilution; $\bar{D}^o$, the average diffusion coefficient at infinite dilution; $\bar{V}$, the partial specific volume; and ρ, the solution density, can be used to calculate the weight average molecular weight, M_W, from the equation:

$$M_W = \frac{RT\bar{S}^o}{D^o(1-\bar{V}\rho)} \qquad (2)$$

The Archibald method (3) may also be used to obtain M_W. This method depends on measuring $\left(\frac{\partial c}{\partial x} \right)$ (the concentration gradient) and C (concentration) at the meniscus or at the bottom of the cell. The Schlieren optical system gives $\left(\frac{dc}{dx} \right)$ directly. The concentrations can be obtained by integrating the concentration gradient. From this, one can determine:

$$M_w = \frac{RT}{(1-\bar{V}\rho_o)\omega^2} \cdot \frac{(dc/dx)_m}{X_m C_m} \qquad (3)$$

where: $(\frac{dc}{dx})_m$ = concentration gradient at the meniscus. All the other terms are as defined in equation 1. In order to get the true molecular weight from equation 3, it is necessary to extrapolate the molecular weight to zero concentration. There are other ways of determining molecular weight from sedimentation data. One method is to combine $\bar{S}^o$ with the intrinsic viscosity $[\eta]$. It can be shown that

$$\bar{S}^o[\eta]^{-\frac{1}{3}} M^{-\frac{2}{3}} = \Phi^{\frac{1}{3}} P^{-1} (1-\bar{V}\rho_o)/N_o \eta_o \qquad (4)$$

where: Φ and P are universal constants

η_o is the solvent viscosity, and

N_o is Avogadro's number.

The product $\Phi^{\frac{1}{3}} P^{-1}$ is constant for all random coil polymers. This equation does not give reliable results for rod-like helices, however. Most data relating $\bar{S}^o$ and M indicate that $S^o = KM^{0.33-0.50}$ for coiled polymers, whereas the relationship $S^o = KM^{0.2}$ holds for stiff rods (Nishihara and Doty, 28).

Thus, from sedimentation coefficients, one can study the extent of polydispersity of a given macromolecule, its most probable conformation (helix or coiled), or its molecular weight or change in molecular weight in solution.

Viscosity

The reduced viscosity of a polymer solution may be defined as

$$\eta_{red} = \frac{\eta_{sp}}{c} = \frac{\eta - \eta_o}{c\,\eta_o} \qquad (5)$$

where: η is the viscosity of the solution

η_o = viscosity of the solvent

c = concentration of the polymer.

For polymers, which are usually non-newtonian, the viscosity depends both on solute concentration and on the rate of shear to which the solution is subjected. Thus, it is necessary to extrapolate the viscosity of the solution to zero concentration, and to carry out the viscosity measurements at a low shear rate. Alternatively, the viscosity can be measured over a wide range of shear and then extrapolated to zero rate of shear.

The relationship for the intrinsic viscosity is

$$[\eta] = \lim_{c \to o} \left(\frac{\eta - \eta_o}{\eta_o c} \right) \qquad (6)$$

The relationship between the molecular weight and the intrinsic viscosity can give useful information about the conformation of the polymer. For example, it can be shown that the relationship $[\eta] = KM^a$ holds for all molecules in solution, where the exponent a is an adjustable constant whose numerical value is determined by the conformation of the molecule.

Values of $\underline{a}$ range from zero for a rigid sphere to 1.7-2.0 for a rigid prolate ellipsoid, and 0.5-0.8 for a flexible polymer. For nucleic acids, $\underline{a}$ = 0.8-1.2.

The molecular weight obtained from viscosity, M_v, can sometimes be used to test for the heterogeneity of a given polymer. For $\underline{a} \geq 1.0$, $M_v \cong M_W$ (M_W is the weight average molecular weight obtained from light scattering data). However, M_W/M_n is an index of heterogeneity. Therefore, for the special case where $\underline{a} = 1.0$, $M_w/M_n = M_v/M_n$.

The intrinsic viscosity of denatured DNA is very sensitive to ionic strength. The relationship $[\eta] = A(1 + \frac{B}{\sqrt{u}})$ describes the interdependence of $[\eta]$ on u for most polyelectrolytes. Here, u = ionic strength, A = $[\eta]$ at the limit of infinite dilution, and B is an adjustable constant. The strong dependence of $[\eta]$ on u for many polyelectrolytes is probably due in part to electrostatic expansion.

Similarly, the reduced viscosity of polyelectrolytes (e.g., DNA and other polynucleotides) is dependent on polymer concentration. The magnitude of the dependence (determined in part by the excluded volume brought about by the polymer) will depend on the conformation of the polyelectrolyte, and the magnitude of its interaction with the solvent. In general, this dependence is greater for denatured DNA than for native helical DNA. Whether a given coiled macromolecule will show a high or low intrinsic viscosity depends not only on the molecular weight of the polymer, but also on the interaction of the polymer with the solvent.

This can be seen from the equation:

$$[\eta] = K \alpha^3 M^{\frac{1}{2}} ,$$

where α is the parameter for solvent-solute interaction, and it is different for "good" and "poor" solvents. This dependence of $[\eta]$ on solvent type for coiled polymers is probably partly responsible for the apparent inconsistency in the relationship between the intrinsic viscosity $[\eta]$ for the native and denatured samples of different polynucleotides. For example, the intrinsic viscosity of denatured DNA is lower than that of native DNA, as is expected, whereas the intrinsic viscosity of denatured poly A is greater than that of native poly A. This point will be further discussed in subsequent sections.

(B) Optical Rotation and Rotatory Dispersion

The specific rotation of a solute dissolved in water is defined as

$$[\alpha] = \frac{100\alpha}{dc} \qquad (7)$$

where: α = observed rotation

d = length of cell path

c = concentration in gram per 100 ml.

The molar rotation,

$$[M] = \frac{M[\alpha]}{100} \qquad (8)$$

where: M = molecular weight.

The mean residue rotation = $\frac{[M]}{n}$, where n = number of monomers

in the polymer. Optical rotatory power is a function of refractive index (n); and for solutions whose refractive index is significantly changed by the solute, the expression for specific rotation becomes

$$[\alpha] = \frac{3}{n^2 + 2} \cdot \frac{100\alpha}{dc} \tag{9}$$

The Drude equations: Application of electromagnetic theory to an optically active component of molecular weight M at wavelength λ leads to the equation

$$[\alpha]_\lambda = 1800 \left(\frac{4\pi^2}{\lambda}\right)\left(\frac{n^2 + 2}{3}\right)\frac{N}{M}\beta \tag{10}$$

Rosenfeld (38) has shown that

$$\beta \cong \frac{c}{3\pi h} \sum_i \frac{R_i}{\bar{V}_i^2 - \bar{V}^2} \tag{11}$$

where $\bar{V}_i$ = exciton frequency

$\bar{V}$ = frequency of incident light

R_i = rotatory strength

Combining equations 10 and 11, $[\alpha]$ becomes

$$\left(\frac{n^2 + 2}{3}\right)\left(\frac{9600\,\pi e N}{M h \lambda^2}\right) \sum_i \frac{R_i}{\bar{V}_i^2 - \bar{V}^2} \tag{12}$$

It has been shown that the various constants in equation 12 can be condensed into a new variable, A_i, to give:

$$[\alpha] \cong \frac{(n^2 + 2)}{3} \sum_i \frac{A_i \lambda_i^2}{\lambda^2 - \lambda_i^2} \qquad (13a)$$

With $A_i \lambda_i^2 \equiv K_i$, equation 13b gives the complex Drude equation

$$[\alpha]_\lambda = \sum_i \frac{K_i}{\lambda^2 - \lambda_i^2} \qquad (13b)$$

For the simple one-term Drude equation, the above becomes $[\alpha] = \frac{K}{\lambda^2 - \lambda_o^2}$. According to Yang and Doty (54), a plot of $[\alpha]\lambda^2$ against $[\alpha]$ gives a straight line. The slope gives λ_o^2 and the intercept gives K. For compounds showing complex dispersion (two-term Drude equation),

$$[\alpha] = \frac{K_1}{\lambda^2 - \lambda_o^2} + \frac{K_2}{(\lambda^2 - \lambda_o^2)^2} \qquad (14)$$

Most polynucleotides in the helical conformation obey a one-term Drude equation.

The application of optical rotatory dispersion to studying the conformation of nucleic acids is rather new. The empirical relationship between conformational change and optical rotatory dispersion data is not always as clear as with proteins. However, the helical asymmetry in polynucleotides is an important factor in determining the high dextrorotation of helical polynucleotides. The optical activity of native DNA is many orders of magnitude greater than that of the individual mononucleotide components (Michelson et al., 25). For the major nucleotides, the average specific rotation is close to zero. Whereas, for DNA, the molar rotation $(Mp)_D$ is usually about +42,000°. The corresponding value for the oligonucleotide thymidyl-5':3'-thymidine 5' phosphate is

+2,800 in neutral solution. Thus, the phosphoester linkage is not responsible for the high $(M\,p)_D$ for DNA. This conclusion is supported by the observation that the optical activity of DNA drops considerably when the polymer is thermally denatured ($(\alpha)_D^{25°C} = +126°$ and $(\alpha)_D^{96°C} = +28$ for calf thymus DNA) without concomitant hydrolysis. Of special interest is the observation that this decrease in optical activity takes place within a narrow temperature range with the midpoint of the transition corresponding to the midpoint of the melting profile obtained by measuring the hyperchromicity of the DNA as a function of temperature (Doty et al., 7). Thus, it can be concluded that optical rotation and u.v. absorption measure essentially the same temperature dependent conformational transition.

Ultraviolet Hypochromism

Purine and pyrimidine bases occurring in natural and synthetic polynucleotides have very strong ultraviolet absorptions which are very sensitive to conformational changes in the polymers (Loofborough, 22; Stoiner and Beers, 45). The extinction coefficient of DNA is 40 per cent lower than that expected for its mononucleotide components. This relative decrease in absorbency is referred to as hypochromicity. The molar absorbency E_p (based on one gram atom of phosphorus per liter) for native DNA ranges between $6 \cdot 10^3$ to $8 \cdot 10^3$. Conditions that denature DNA (helical to coiled form) cause an increase in this value. This is described as hyperchromic effect.

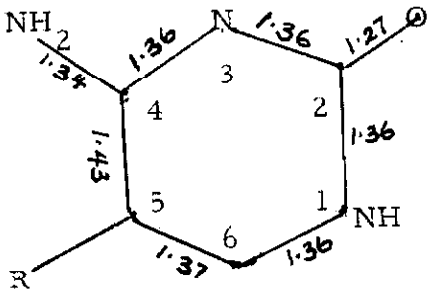
There is at present no unanimously accepted quantitative treatment of hypochromicity in nucleic acids. The theory of hypochromicity derived by Tinoco (46) and by Rhodes (36) broadly supports the most popular concept about the origin of hypochromicity, namely, that hypochromicity in the nucleic acids arises from the interaction of the bases stacked in an array perpendicular to the fiber axis. Thus, denaturation of nucleic acids would decrease such stacking, and therefore decrease the amount of hypochromicity. Insofar as there is no sound quantitative relationship between hypochromicity and structure, any variation in hypochromicity can only be regarded as an empirical index of the relative extent of conformational change. This statement will be more meaningful when the optical properties of poly C are discussed in later sections.

There are several other important physical-chemical methods for studying the interaction of macromolecules in solution, such as light scattering, flow birefringence, electrophoretic measurements, infrared spectroscopy, and several others. It is clear from the above discussion that it is usually desirable, and often necessary, to combine information from several methods of investigation in order to be able to make statements about the conformation of a given macromolecule. These statements may lead to only tentative conclusions which require the support of X-ray crystallography before they can be regarded as proved.

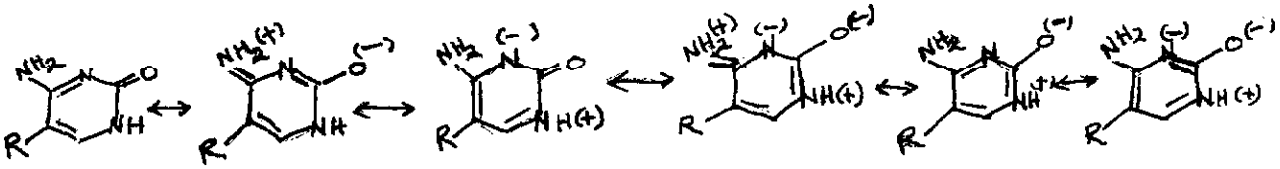
GENERAL PROPERTIES OF CYTIDYLIC ACID AND ITS POLYMER

(a) Cytidylic acid and derivatives

The pyrimidines are in general less aromatic than is benzene or pyridine. The resonance energy for pyrimidine calculated by molecular orbital methods is 26 k. cal/mole (Acheson, 1). This is about 72 per cent of the value for benzene (36 k. cal/mole). The details of the bond distances and angles in cytosine-5-acetic acid have been worked out by Marsh et al. (23), and are shown below:



From this structure, one can estimate the bond number (n) for each of the different bonds in the molecule, and therefore the relative distribution of charges around the molecule. When this is done, it becomes clear that cytosine can best be represented as a resonance hybrid to which the structure I-VI contribute the indicated amounts:



20%	20%	20%	20%	10%	10%
I	II	III	IV	V	VI

These six canonical structures of cytosine reveal and make understandable some of the most interesting properties of cytosine derivatives:

(a) The N_3 carries a great deal of negative charge in the neutral molecule. It should therefore be a potent acceptor of protons in slightly acid solution.

(b) The nitrogen of the primary amino group in cytosine is comparatively devoid of negative charge, and is therefore much less basic than the ring (N_3) nitrogen.

(c) The carbonyl oxygen is highly ionized and also constitutes a potent proton acceptor.

Cytidine monophosphate, like other mononucleotides, obeys a one-term Drude equation. The constant K in the Drude equation $(\alpha) = \frac{K}{\lambda^2 - \lambda_c^2}$ is negative. The calculated chromophore position is between 284 mμ and 326 mμ. The spectrum and rotatory dispersion of cytidine monophosphate shifts with pH across the pKa of the easily protonated ring nitrogen. Lowering the pH from 7.5 to 4.0 causes an increase in both (α) and in E max. In addition, a red shift is observed both for λ_c and for the wavelength of E max (Ts'o et al., 47).

Ts'o et al. (47) have also observed that a temperature-dependent shift in the rotatory dispersion of cytidine monophosphate occurs when the pH of the solvent is lower than the pKa of the ring nitrogen. At pH 4.85, 25°C, the spectrum of cytidine monophosphate is different from that of the same material in neutral solution. Data on rotation indicate

the same tendency. Heating of cytidine monophosphate at pH 7.5 produces no spectral shift and no temperature-dependent hyperchromic transition.

Polycytidylic Acid (poly C)

For poly C, the constant K in the Drude equation is positive. If a neutral solution of poly C is heated from 20°C to 80°C, there is a continuous drop in its specific rotation. Unlike DNA and other polynucleotides, the (α) of randomly coiled neutral poly C is very high and, in fact, higher than that of the acid form. The $(\alpha)_{589\ m\mu}^{20°C}$ of poly C at pH 7.5 is +323, whereas $(\alpha)_{589\ m\mu}^{20°C}$ at pH 4.0 is +232 (47, 2).

Temperature and pH dependent shifts in the spectrum and rotatory dispersion of poly C have been observed just as with cytosine monophosphate. However, unlike CMP or neutral poly C, acidic poly C does undergo a sharp transition on heating (47).

No simple explanation can be given for the very complex nature of the spectral behavior of CMP and of poly C in solution. It is possible that temperature, like pH, affects the relative contribution of one or more of the canonical forms to the structure of the molecule in solution. Such perturbation, if it exists, would certainly affect charge distribution around the easily protonated ring nitrogen and, as a result, its affinity for protons.

The high specific rotation of neutral poly C is equally difficult to explain. It is possible that neutral poly C does indeed possess structure

in neutral solution, but that this is not well enough ordered to yield a sharp transition on heating. There is at present no conclusive evidence to either support or contradict this hypothesis.

In any case, the fact that acidic poly C does undergo a sharp hyperchromic transition on heating is clearly indicative of a cooperative transition in an ordered structure. The fact that the neutral form of poly C does not undergo a similar transition (from measurements of (α) and E max vs temperature) implies that the neutral form does not possess the same degree of order as the acid form.

The observation by R. F. Steiner and Beers (43) of a large drop in angular dependence of scattered intensity and of a greater than 60 per cent drop in the radius of gyration of poly C and of poly A on going from the neutral form to the acid form is an anomalous one. If the neutral form of poly C is completely in random coil form and the acid form stiff and rod-like, one would expect an opposite result. However, if the so-called coiled form is relatively extended in solution, one could attribute the observed drop in the radius of gyration to a combination of two factors acting together:

(I) An increase in the diameter of the molecule without any large increase in its length accompanying formation of helix from coil.

(II) A decrease in electrostatic expansion due to the neutralization of some of the repulsive charges, as well as a repositioning of the $P\text{-}O^{(-)}$ bond at an angle that decreases electrostatic repulsion accompanying formation of helix from coil.

If one accepts the above interpretation as correct, the fact that the radius of gyration of poly C tends to decrease as a result of transition from neutral to the acid form actually supports the following hypothesis of the properties of poly C:

(A) That the neutral form of poly C is the coil form, but that the coil is almost as extended as is the helical or acid form in solution.

(B) That the acid form does have a definite secondary structure in solution.

Purpose of Experiments

Use will be made of some of the physical-chemical properties which are common to the polynucleotides in solution to study the behavior of poly C in acidic medium. Those properties which are peculiar to poly C in solution will be deliberately neglected, particularly where they neither contradict nor support the notion under examination.

The T_m of poly C in acid solutions at various pH levels, ionic strengths, and urea concentrations will be investigated by both absorbancy and specific rotation measurements. Other physical-chemical properties of poly C in acidic solution will be studied, such as optical rotatory dispersion, viscosity, and sedimentation coefficients. Particular attention will be paid to the hydrogen bonding scheme proposed by Marsh et al. (23) for cytosine-5-acetic acid in interpreting the data obtained for poly C.

EXPERIMENTAL

(A) Materials

The poly C was purchased from Miles Chemical Company, Clifton, New Jersey. The polymer was further deproteinized by the detergent-amylalcohol-chloroform method of Ts'o et al. (48). The poly C was shaken with amyl alcohol-chloroform (1:3) in the presence of 1 per cent sodium dodecyl sulfate. The deproteinization procedure was repeated three times until there was no evidence of protein precipitate in the interfacial layer. The poly C was next dialyzed against 0.2 M ammonium acetate in the cold for 40 hours and then precipitated with three volumes of cold alcohol. The precipitate was washed twice with alcohol and twice with ether, and then dried in vacuo. All other compounds were of reagent grade.

Instrumentation and Method of Analysis

Optical rotation measurements were made with a Rudolph Model 200S polarimeter equipped with an oscillating polarizer and xenon and mercury arc lamps (Ts'o et al., 48). The 20-cm polarimeter tubes utilized glass construction with water jacket and quartz window. The temperature of the polarimeter tube was read directly with a thermometer and held at any desired temperature to $\pm$ 0.1 by the flow of water through both the compartments and the tube. Measurements of specific rotations, (α), were determined from the averages of three readings of solutions of concentration of 0.4-0.6 mg/ml.

Optical density measurements were made with a Beckman DK-2 recording spectrophotometer fitted with a modified temperature control device. Quartz cells were fitted with a 20 mm inversion standard taper thermometer for direct reading of solution temperature to $\pm$ 0.1°C.

pH measurements were made with a Radiometer pH meter 22, Copenhagen, Denmark, and to an accuracy of 0.01 pH unit. The resistance of the solution was measured to an accuracy of $\pm$ 1% at 22.2°C$\pm$0.05° with a portable A.C. electrolytic conductivity bridge manufactured by Leeds and Northrop Co., New York.

Viscometer (Ubbelohde) was designed and constructed by the Cannon Instrument Co., State College, Pa. The viscometer had a water flow time of 350 seconds for each bulb, and its maximum shear gradient for water varied from 40 $sec.^{-1}$ to 210 $sec.^{-1}$. Measurements were performed at 22.6°C $\pm$ 0.05°C.

Analytical ultracentrifugation was performed in a Model E ultracentrifuge, Spinco Division, Beckman Inc., with ultraviolet absorption optics. The instrument was provided with a temperature control system. The absorption patterns, recorded on film, were traced with a Double Beam Recording Micro-densitrometer, Joyce Loebl Co., Newcastle-upon-Tyne, England. All of the sedimentation coefficients reported were corrected to 20°C, but were not corrected for the effects of solvent viscosity and density.

The molar extinction coefficient per phosphorus of poly C was determined by hydrolyzing a sample (of known absorbancy) to cytidylic acid with 0.3N potassium hydroxide for 18 hours and 24 hours at 38°C. The molar extinction coefficient obtained for the 18 hours hydrolysis was found to be the same as that obtained by 24 hours of hydrolysis. The values of E_{max} were also the same in the two cases. This indicates both that the hydrolysis is complete after 18 hours, and that no deamination of cytidylic acid takes place. The molar extinction coefficient of 2'- and 3'-CMP was taken to be 13×10^3 at 280 mμ, pH 2.0. The maximum molar extinction coefficient of poly C at 25°C and 0.1M salt determined by the above method at various pH is shown in table 1. All of the values are within 5 per cent of those previously reported by Ts'o et al. in this laboratory (47). The concentration of poly C for each experiment was determined optically by use of the experimental values for maximum molar extinction coefficient .

Table 1. Maximum Molar Extinction Coefficient of Poly C at Various pH

pH	$E_{max} \times 10^{-3}$	λ_{max} (mμ)
7.5*	6.6	267
4.85	7.2	274
4.05	7.7	274
3.65	8.0	275

* All in 0.1M salt

The recorded T_m is the temperature at the midpoint of change of the optical properties of poly C. The T_m, presumably the midpoint of the helix-coil transition, is in all cases accurate to $\pm 0.5°C$.

Results:

Optical rotation and absorption measurements of poly C in 0.1M sodium acetate, pH 4.05, at varying temperatures, yield essentially the same T_m (Fig. 1). The T_m of poly C measured by optical rotation is similarly found to be the same at other pH values (3.65, 4.85) as those determined by absorbancy. When poly C is heated at acid pH, an initial drop in optical absorbancy is always observed. This is immediately followed by a sharp rise in absorbancy (hyperchromicity). This hyperchromic spectral transition corresponds to a similar transition observable with optical rotation measurements. It may therefore be associated with a structural transition within the molecule. There is, however, no observable change in $(\alpha)589$ mμ over the temperature range in which the initial drop in absorbancy is observed. Thus, it does not appear that the initial hypochromicity is in any way related to the asymmetry of the molecule.

There are, however, several pieces of experimental evidence which suggest that the initial drop in absorbancy is due to the removal of protons at elevated temperatures from the cytidine rings of the polymer.

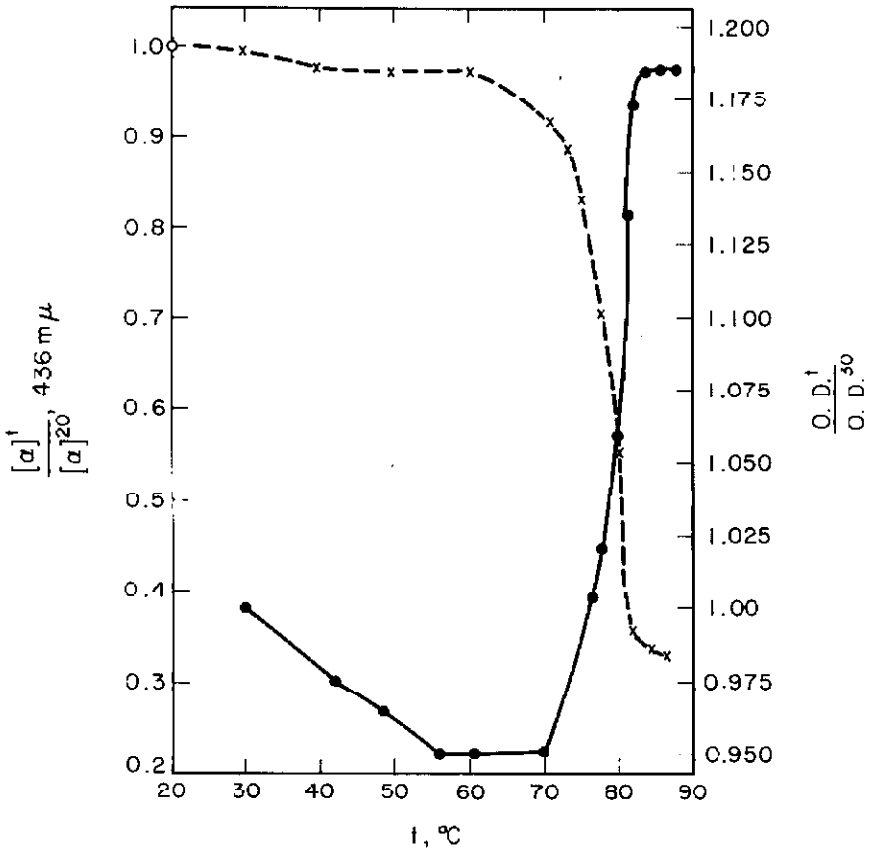


Figure 1. Profile of $\frac{[\alpha]^t}{[\alpha]^{20}}$, -X-X-, and of $\frac{O.D.^t}{O.D.^{30}}$, -●-●-, versus temperature of poly C in 0.1M Na-acetate, pH 4.08.

(I) Helmkamp et al. (19) and Ts'o et al. (48) have together shown that protons of cytidine-5-phosphate are removed at elevated temperatures. This conclusion is based on their observations, discussed above, namely:

(a) That the spectrum and rotatory dispersion of CMP shifts, in response to pH changes, across the pKa of the most easily protonated ring nitrogen;

(b) That the spectrum and rotatory dispersion spectrum of CMP shifts in response to temperature if the pH of the medium is lower than the pKa value ring of the nitrogen. The λ_c of CMP at pH 7.5 is the same at 20°C as it is at 80°C (300). On the other hand, the λ_c of CMP at pH 4.0 is 319 at 20°C and 304 at 80°C.

(II) A closer examination of the absorption spectrum of poly C clearly shows that as the temperature is slowly raised from 30° to 60°C, the minimum of its absorption spectrum increases, while the absorbancy at the maximum decreases (table 2). In table 2, a comparison of the $E_{maximum}/E_{minimum}$ ratios of poly C solutions at 25°C with the $E_{maximum}/E_{minimum}$ ratios of the same solutions at elevated temperatures is made. It is clear from these data that there is a tendency for the $E_{maximum}/E_{minimum}$ ratio of the heated poly C in acidic medium to approach that of the neutral form (pH 7.5). The $E_{maximum}/E_{minimum}$ ratio of the neutral form at elevated temperatures is essentially the same as at 25°C.

Table 2. Spectral Properties of Poly C

pH	Temp.	λ_{max}	λ_{min}	(E_{max}/E_{min}) at 25°C	(E_{max}/E_{min}) at 55-66°C
4.97 (0.1M Na Acetate)	25°	274	244	1.88	1.35
	55°	270	249		
4.80 (0.1M Na Acetate)	25°	274	244	1.95	1.39
	60°	270	249		
4.03 (0.1M Na Acetate)	25°	275	242	2.21	1.95
	66°	274	243		
7.2 (tris., 0.1M Na Cl)	25°	267	248	1.33	1.27
	66°	268	249		

(III) There is a slight but noticeable shift in the $\lambda_{minimum}$ position of poly C spectrum as the temperature is raised (table 2). The neutral form has a minimum at 248 mμ. At elevated temperatures, the minimum of the acidic form approaches 248 mμ.

However, the loss of protons from the polymer is not coincidental with the destruction of the helical asymmetry of the molecule. From the data, it would appear that there is, in acidic solvent, an initial loss of some protons from the polymer at temperatures below the T_m. Within this temperature range, the helical structure is, however, still intact. At slightly higher temperatures, the asymmetry of the polymer is destroyed within a narrow temperature range. This is observable as a

helix-coil transition both by absorption and by optical rotation measurements.

The effect of pH and ionic strength on the T_m of poly C:

The effects of changes of pH on the T_m of poly C in 0.1M sodium acetate are given in figure 2. It is clear that the stability of poly C as a function of proton concentration can be classified into two zones, i.e., the proton stabilization zone (pH 4.2 and higher) and the proton destabilization zone (pH 3.9 and lower).

(a) Proton stabilization zone

The T_m of poly C between pH 4.0 and 4.2 is constant at 82°C in 0.1M sodium acetate. Above pH 4.2, the T_m decreases with increasing pH, and at pH 4.9 is 64°C in 0.1M sodium acetate.

The effects of ionic strength (expressed as the colog of the specific conductance of the medium) on T_m are given in figure 3. At pH 4.85, in the proton stabilization zone, increase in ionic strength lowers the T_m of poly C, and therefore reduces the stability of the helix. For example, the T_m of poly C in 0.05M sodium acetate (pH 4.85) is 74°C. However, the T_m in 0.2M sodium acetate is 68°C. At pH 4.05, the T_m is relatively constant within the salt concentrations of 0.05M to 0.2M sodium acetate.

(b) Proton destabilization zone

The pH region between pH 4.0 and pH 3.6 constitutes the proton destabilization zone shown in figure 2. At pH values less than 3.6, the

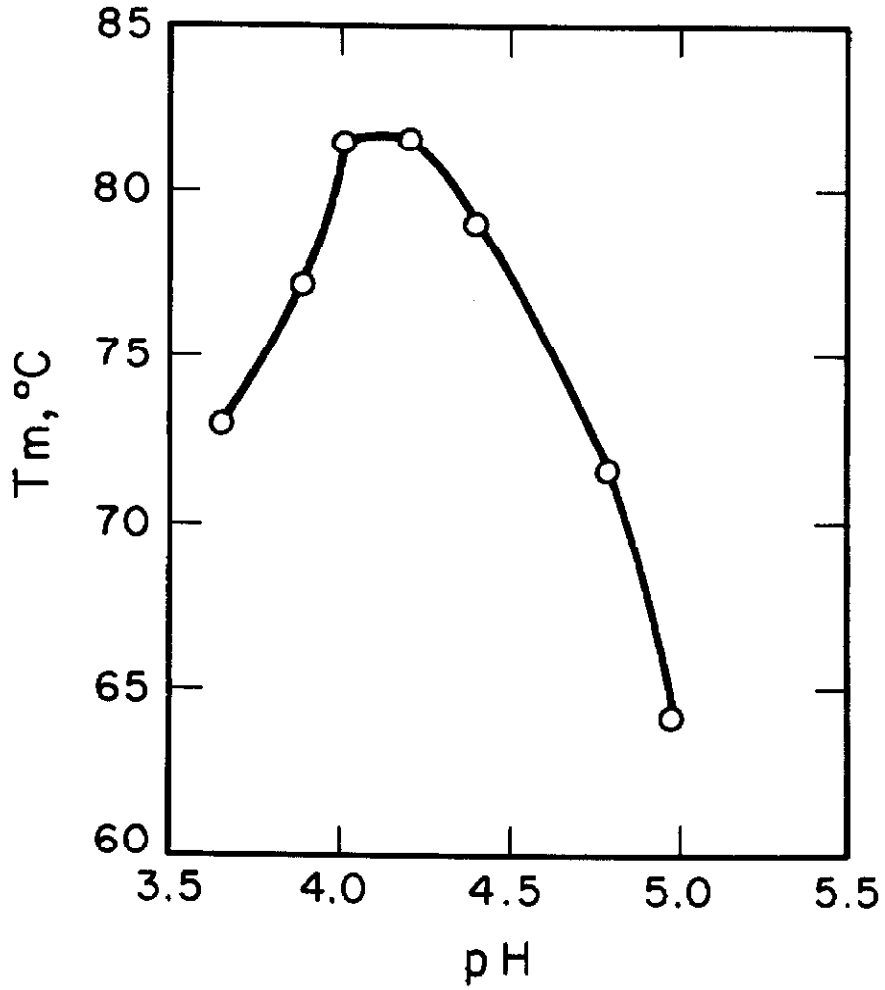


Figure 2. Variation of T_m of poly C with the pH of Na-acetate buffer (0.1M Na-acetate) in the range of pH 3.6 to pH 4.9.

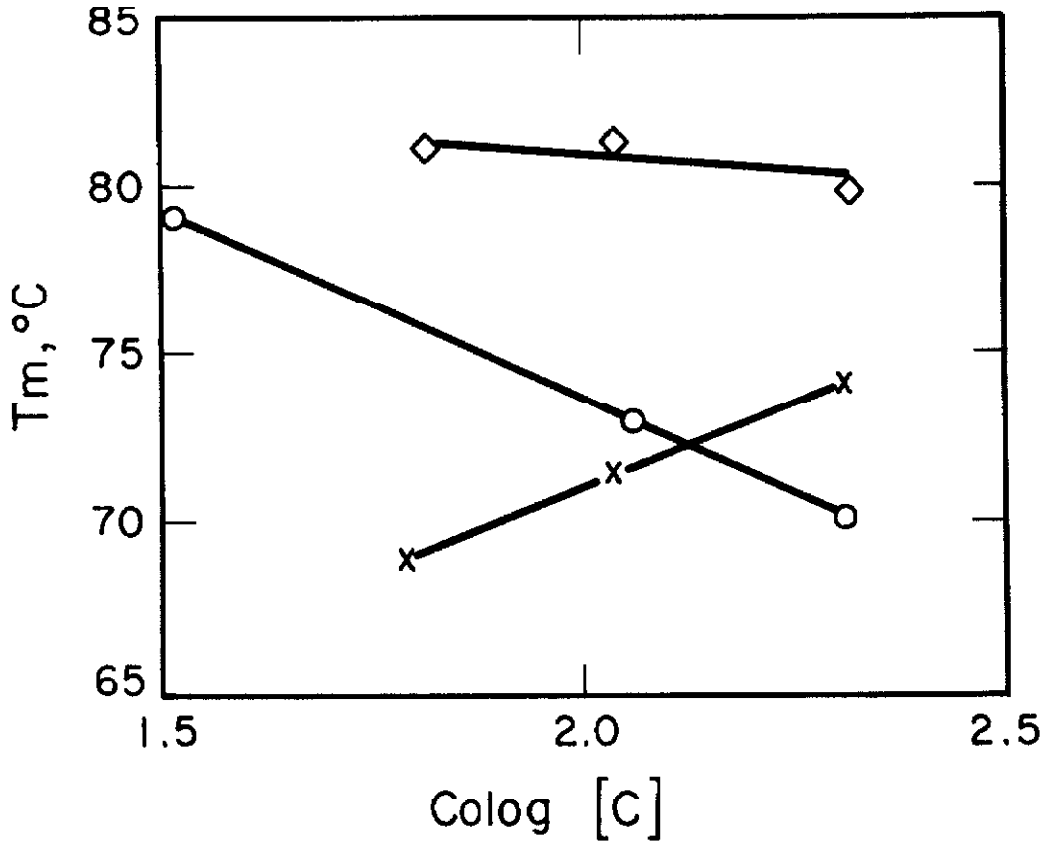


Figure 3. Dependence of T_m of poly C in acetate buffer on the concentrations of the electrolytes in solution as expressed as the colog of the specific conductance: Buffers at pH 4.85, -X-X-, and pH 4.05, -◇◇-, were prepared from the dilution of 0.2M Na-acetate to 0.05M for the variation in conductance. Buffers at pH 3.65, -O-O, consist of 0.2M NaCl and 0.1M Na-acetate at the highest conductance, 0.1M Na-acetate at the medium conductance, and 0.05M Na-acetate at the lowest conductance.

poly C precipitates from solution at elevated temperatures. As the pH of the medium is decreased from pH 4.0 to 3.6, the T_m of poly C also decreases. The T_m at pH 3.8 is 77°C, whereas the T_m at pH 3.65 is 73°C. There is no evidence of precipitation of poly C at pH 3.6 even at elevated temperatures, either by absorbency or by optical rotation measurements.

At pH 3.65, in the zone of proton destabilization, increases in ionic strength of the solution raise the T_m of poly C, and thus enhance the stability of the helix. The T_m of poly C (pH 3.65) in 0.05M sodium acetate is 70°C, whereas the T_m of the same polymer in a medium containing 0.1M sodium acetate and 0.2M sodium chloride (pH 3.65) is 79°C.

Therefore, as far as the effect of electrolytes on the T_m is concerned, poly C in the proton stabilization zone behaves like poly A (Ts'o et al., 47), while poly C in the proton destabilization zone behaves like DNA (Ts'o et al., 47). It is interesting that the transition between these two zones (i.e., the zone between the pH range 4.0 to 4.2) is not greatly affected by electrolyte concentration (figure 2).

Optical rotatory dispersion of poly C

The optical rotatory dispersion data on poly C at pH 4.9, pH 4.0, and pH 3.6 are summarized in table 3. The data agree with the values previously reported by Ts'o et al. (47) to within 5-7 per cent. The modified one-term Drude plot of the rotatory dispersion data is shown in figure 4. All of the points for the three chosen pH values lie on approximately the same one-term Drude curve.

Table 3. Optical Rotatory Dispersion Data of Poly C.

pH	$[\alpha]_{589}^{20°C}$	$[\alpha]_{436}^{20°C}$	λ_c (mμ)	K x 10^{-6}	wavelength range (mμ)
4.90[1]	225.5	584.4	303	50	320-589
4.05	198.0	531.3	306	50	320-589
3.65	184.3	485.4	305	48	320-589

[1] all in 0.1M Na acetate

If the three dispersion curves corresponding to the different pH values are separately plotted, λ_c and K of the Drude equation can be calculated respectively from the slope and intercept of the curve. λ_c at pH 4.90, pH 4.05, and pH 3.65 agree to within 1 per cent (303-306), and the parameter K for the three pH values agrees to within ± 2 per cent. It then appears that the conformation of poly C is essentially the same at the three pH values representing respectively the proton stabilization zone (pH 4.90), the transition zone (pH 4.03), and the proton destabilization zone (pH 3.6).

Hydrodynamic properties of poly C

The physical properties of poly C in neutral and acidic solutions were examined by viscosity measurements and ultracentrifugation. pH 3.75, rather than pH 3.6, was selected for representation of the proton destabilization zone in order to keep safely distant from the region in which aggregation of poly C takes place.

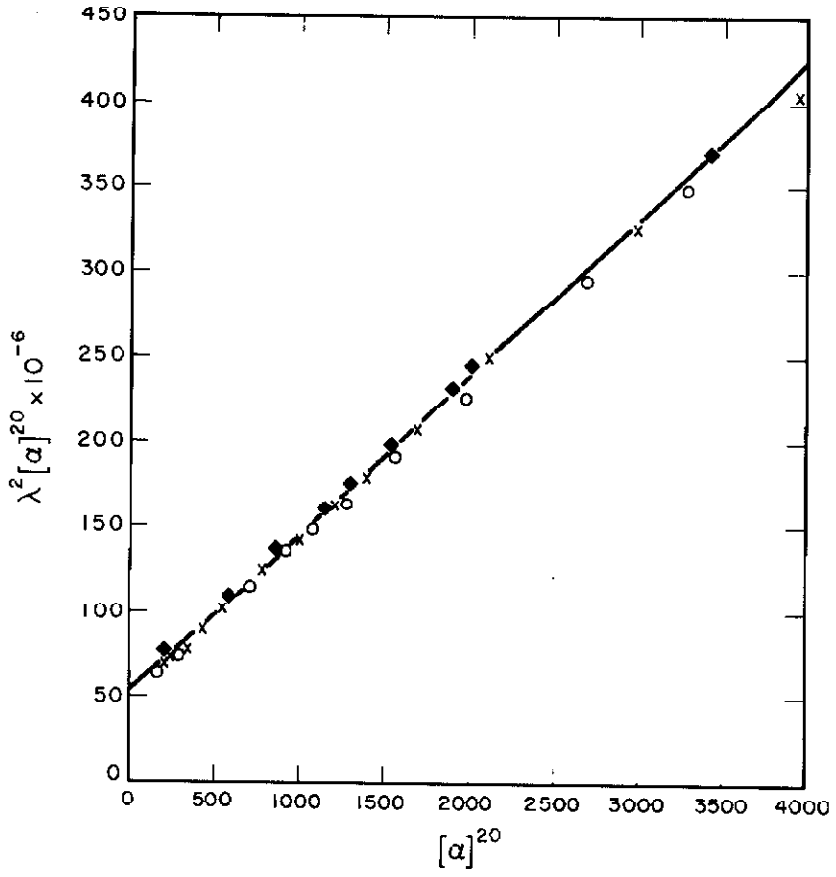


Figure 4. Modified one-term Drude plot of poly C in 0.1M Na-acetate at pH 4.90 (◆), pH 4.05 (X), and pH 3.65 (O). $[\alpha]$ was obtained at 20°C and at a symmetrical angle of 0.3.

(I) Viscosity

Viscosity measurements were made at pH 7.5, representing the neutral form, at pH 4.85, representing the acidic form in the zone of proton stabilization, and at pH 3.75, also representing the acid form in the proton destabilization zone. Dilutions were made in each case from solutions containing 0.5 to 0.6 gm/dl of poly C to about 0.15 gm/ dl of the polymer. Viscosity measurements were made at two salt concentrations (0.05 and 0.5M) at each pH and concentration of poly C. The intrinsic viscosity was obtained by extrapolating the reduced viscosity to zero concentration.

The viscosity data are plotted in figure 5 . The intrinsic viscosity of poly C at neutral pH (pH 7.5) is much higher than that of poly C at pH 4.85 and 3.75. The intrinsic viscosity of the polymer in a medium containing 0.01M tris, pH 7.5, and 0.05M sodium chloride, is 0.84 dl/gm, while the intrinsic viscosity at pH 4.85 or pH 3.75, 0.05M sodium acetate, is 0.35 dl/gm.

Furthermore, the reduced viscosity of neutral poly C is much more concentration dependent than is that of the acidic form. For example, the reduced viscosity of neutral poly C (pH 7.5, 0.05M NaCl) at a polymer concentration of 0.5 gm/dl is 1.26 dl/gm, whereas the reduced viscosity of poly C, at the same pH and ionic strength and a poly C concentration of 0.15 gm/dl, is 0.93 dl/gm. On the other hand, the reduced viscosity of poly C at pH 4.85, ionic strength = 0.05, is

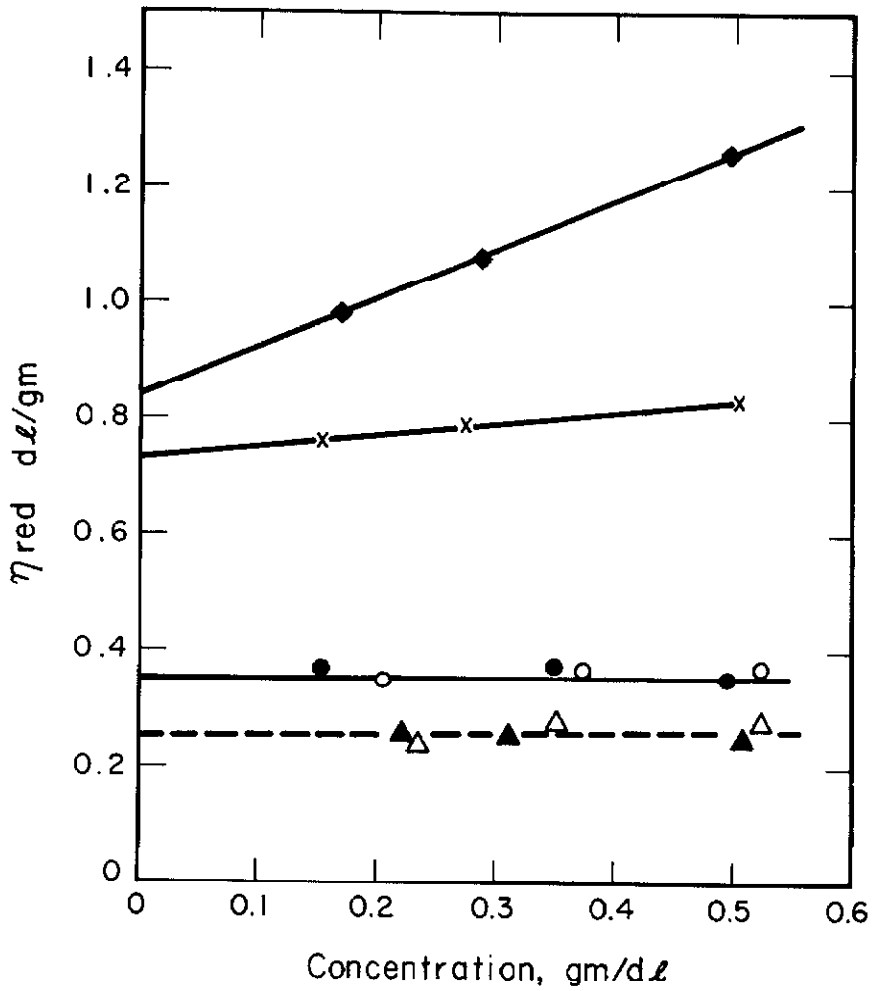


Figure 5. Reduced viscosity versus the concentration of poly C at different pH and electrolyte concentrations; pH 7.5, 0.01M tris, 0.05M NaCl -◆◆- , pH 7.5, 0.01M tris, 0.5M NaCl, -X-X-; pH 3.75, 0.05M Na-acetate (●); pH 4.85, 0.05M Na-acetate (O); pH 3.75, 0.5M Na-acetate (Δ) ; pH 4.85, 0.5M (▲).

almost the same at poly C concentrations of 0.5 gm/dl and 0.15 gm/dl. All of these observations are in agreement with the results of Steiner and Beers (43), which indicate a reduction in the radius of gyration of poly C as it changes from the neutral to the acidic form.

Changes of salt concentration over the range 0.05M to 0.5M have only a small effect on the reduced viscosity of acidic poly C. The viscosities of the polymer at pH 4.85 and at pH 3.75 are the same at the various concentrations of poly C. Similarly, the viscosity of poly C at any one ionic strength is the same at pH 4.85 as at pH 3.75. Thus, there is no difference between the viscosities of poly C in the proton stabilization zone and in the proton destabilization zone.

The viscosity of acidic poly C (pH 4.85, pH 3.75) is noticeably less in 0.5M than in 0.05M salt. The effect of ionic strength on the viscosity of neutral poly C is, however, more obvious. The reduced viscosity of poly C, 0.5 gm/dl, in a medium containing 0.01M tris (pH 7.5) and 0.05M sodium chloride, is 1.26 dl/gm. The reduced viscosity of the same concentration of poly C in a solvent containing 0.5M sodium chloride and 0.01M tris (pH 7.5) is 0.82 dl/gm. These values may be compared with those for the same concentrations of salt and for acidic poly C. The reduced viscosity of poly C (0.52 gm/dl) at pH 4.85, 0.05M salt, is 0.36 dl/gm, and the viscosity of poly C at the same pH and a salt concentration of 0.5M is 0.27 dl/gm.

The reduction in the viscosity of poly C caused by a high salt concentration (0.5M) is probably due to a reduction in the electrostatic expansion of the polymer by the high salt concentration. High salt concentration reduces the repulsive electrostatic charge on the polymer. The result is a marked reduction in reduced viscosity, especially at polymer concentrations at which polymer-polymer interaction contributes largely to the reduced viscosity. At very low concentrations of polymer, this interaction is minimized, and the effects of salt concentration on viscosity are less obvious.

The extent of repulsive charge interaction will depend on the orientation of the charges with respect to one another within the molecule. Thus, the reason why the effect of ionic strength on the reduced viscosity of acidic poly C (even at high poly C concentration) is not great may very well be attributed to the formation of some specific structural conformation in this pH region--a conformation in which electrostatic expansion is small due to the specific disposition of the charges on the molecule.

Ultracentrifugation measurements

The distribution of sedimentation coefficients of the poly C sample employed is a fairly broad one. For poly C at pH 7.5, 0.5M sodium chloride, 0.05M tris, the S_{20}, 50% is 8.4, S_{20}, 75% is 10.5, and S_{20}, 25% is 6.2. For the sedimentation studies of poly C at pH 4.85, the solutions were prepared by dilution with 1×10^{-3} sodium chloride in an acidic buffer, from two different concentrations of poly C. This was

done in order to determine the extent of formation of stable aggregates (Doty and Fresco, 9). The helix of poly C as formed at low concentrations was obtained by diluting poly C in buffer (pH 4.85) to a concentration of 0.1 mg/ml. This preparation yielded a $^{S}20$, 50% of 10.1, $^{\bar{S}}20$, 25% of 7.7, and $^{\bar{S}}20$, 75% of 13.2. The helix of poly C as formed at high concentration was obtained by diluting poly C in buffer (pH 4.85) to a concentration of 4 mg/ml, and then further diluting it to approximately 50 µg/ml for the ultracentrifugation. The $\bar{S}$ values obtained for the poly C form characteristic of high poly C concentration were not significantly different from those characteristic of low concentration, i.e., $^{\bar{S}}20$, 50% of 10.3, $^{\bar{S}}20$, 25% of 7.5, and $^{\bar{S}}20$, 75% of 12.1.

In spite of the high poly-dispersity of the poly C sample, it appears that the $\bar{S}$ values of the acidic form are slightly higher than those of the neutral form. Thus, some kind of molecular aggregate formation at pH 4.85 is indicated by the ultracentrifugation data. If such stable aggregates involving a variable number of molecules are able to exist, one would expect the sedimentation coefficient of acidic poly C from a high poly C concentration to be higher than that found for poly C from low concentration of the polymer. However, as indicated previously, the $\bar{S}$ values of the acidic form are clearly independent of the concentration of poly C in the original solution. It follows then that poly C only forms specific stable aggregates at pH 4.85--an aggregate which appears to be

a specific property of the molecule at this pH, and independent of the concentration history of the polymer. The possibility exists, however, that other less specific aggregates, which cannot be identified by ultracentrifugation, exist in the solutions.

Effect of urea on poly C

Urea is moderately effective in lowering the Tm of poly C, as shown in table 4. In contrast, it is ineffective in lowering the Tm of

Table 4. Effect of Urea on the Tm of Poly C

pH	Urea Concentration	Expected Tm without Urea C°	Tm Observed in Urea C°	Δ Tm C°	Δ Tm/M of Urea °C/M
4.85[a]	3M	71.5	63.	8.5	2.8
	5M	71.5	53.5	18.0	3.6
4.05	3M	81.3	63.	18.3	6.1
	5M	81.3	44.	37.3	7.6
3.65	3M	73.	39.5	33.5	11.2
	5M	73.	< 15[b]	> 58	> 12

a) 0.1M Na acetate buffer
b) Solution became cloudy at lower temperature. Beginning of the transition was observed.

poly A (Ts'o et al., 48). The presence of 5M urea causes a 17.5° decrease in the Tm of poly C at pH 4.85 (0.1 sodium acetate). An equivalent concentration of urea causes only a 2°C drop in the Tm of poly A at pH 4.85. The effect in both cases is due not to any change in pH caused by the presence of urea, or temperature change in the buffer.

The pH of a 0.2M sodium acetate solution buffered at pH 4.4 is essentially the same at 40° as at 20°C, independently of the 5M urea.

It is interesting to note that the effectiveness of urea increases with hydrogen ion concentration of the solution. This effect is apart from the proton effects which, as we have seen, can be divided into two contrasting pH zones. The effect of urea on poly A is independent of pH, despite the fact that the Tm of poly A in the absence of urea is strongly dependent on pH and ionic strength of the solution (Ts'o et al., 48). The drop in Tm of poly A caused by 5M urea is 2°C both at pH 4.85 and at pH 4.4, after corrections have been made for the contributions of hydrogen ion concentration to the Tm of the polymer. In contrast, the corrected decrease in Tm of poly C caused by 3M urea is 8.5° at pH 4.85, 18° at pH 4.0, and 35° at pH 3.65.

Though a direct comparison cannot be made without serious reservations, urea does appear to be far less effective in lowering the Tm of poly C than are some of the aromatic ring compounds in lowering the Tm of poly A and DNA. For example, 1M purine causes an approximately 40° to 80°C drop in the corrected Tm of poly A (Ts'o et al., 48). This is much greater than the largest value obtained for the effect of 5M urea on poly C at pH 3.65 (12-15°/molar concentration of urea). However, pyrimidine and phenol are only slightly more effective in lowering of the Tm of poly A or of DNA than is 5M urea in lowering the Tm of poly C at

pH 3.65. The decrease in Tm caused by phenol (16°/M phenol) on DNA or poly A is comparable to the decrease in the transition temperature caused by 5M urea on poly C at pH 3.65 (12-15°C/molar concentration of urea).

The effectiveness of urea (Δ Tm/M urea) increases with the concentration of urea employed, a phenomenon the opposite of that found with the aromatic ring compounds. This fact is also contradictory to the conclusion arrived at by Peller (30) on the basis of his theoretical analysis of the thermal transition of polypeptides in binary solvent systems. According to this theory, the Tm of the polymer should vary directly with the activity of the reactant, especially when K, the equilibrium constant of the reaction, is relatively temperature-independent. Schellman (39) has observed that the K of urea-polypeptide interaction is rather insensitive to temperature change. Since the activity of urea per molar concentration of urea is expected to decrease at higher concentration of urea, one would predict that the effect of urea on the Tm of polymers such as polypeptides and perhaps polynucleotides would decrease with increasing urea concentration. The analysis, however, may represent an oversimplification of the situation, since the interaction of urea with poly C (or any other polymer) will be influenced by the other components of the solvent system. Such interaction might well be a complicated function of urea concentration and of temperature.

The mechanism of urea denaturation is not at all clear. It appears, however, that urea may act both as a hydrogen bond and a hydrophobic bond breaker. The relative importance of the two effects will depend a great deal on the macromolecular structure of the polymer, its degree and nature of solvation, and the nature of the macromolecule and the solvent in which it is dissolved. Since all suggestions about the relative importance of these interdependent factors have so far been merely speculative, any attempt to explain the mechanisms of urea-poly C interaction will be equally speculative. Therefore, no such attempt will be made at this time.

DISCUSSION

(A) Helical Form of Poly C in Acidic Solution

Three lines of evidence here and in previous publications (Ts'o et al., 47; Helmkamp and Ts'o, 19) suggest that poly C in acidic solution assumes the conformation of a regular, compact, and perhaps helical, structure.

I Helix Coil Transition

The optical properties of poly C undergo an abrupt transition over a narrow range of temperature. This is indicative of a cooperative conformational change. Similar cooperative conformational changes have been observed for DNA and other polynucleotides (poly A, poly A-poly U and poly I) when they are initially in the compact helical form. In no case as yet does the coil form of any polynucleotide undergo such abrupt changes in optical properties over a small range of temperature. Since the polynucleotides are structurally very similar to one another, one would expect their bulk physical-chemical properties to be generally similar. It can be understood on a theoretical basis why the random coil form alone cannot undergo a cooperative temperature dependent transition. Warner (50) has used the theoretical treatment of Peller (30,31) and of Hill (20) to relate helix-coil transition theory of polynucleotides to common thermodynamic parameters:

Since ΔF at Tm is zero (from transition theory), $\Delta S = \left(\frac{\Delta H}{Tm}\right)$

If ΔH and ΔS are independent of temperature, $\Delta F = (Tm-T)\Delta S$

ΔF can be related to the helix coil transition parameter of Hill (20) by the equation $\Delta F = -RT \ln \frac{j_\alpha x}{j_\beta y}$.

ΔF, the free energy difference between the helix and coil form, represents the difference in free energy due to horizontal interactions between chains and that due to interaction along the length of the chain. The term $\left(\frac{j_\alpha}{j_\beta}\right)$ defines the ratio of partition functions for coil units and helical units. The term $\left(\frac{x}{y}\right)$ contains the contribution of Van der Waals interaction of stacked bases, solvent interactions, and configurational entropy change in the helix and the coil transition. The transition parameter, z^2/xy, appears in the equation

$$\Delta F_{\alpha\beta} = -RT \ln \frac{z^2}{xy} \qquad \text{(Warner, 50)}$$

It represents the probability of increase in length of an established coil sequence, as compared to the probability of forming a new one. The parameter $\left(\frac{z^2}{xy}\right)$ is usually small, and thus $\Delta F_{\alpha\beta}$ is large. It is this large $\Delta F_{\alpha\beta}$ (Steiner, 42) which causes the steepness of the transition (absorbency versus temperature) of helical polynucleotides. If one knew the fraction of coil residues Xc, values for the parameter $j_\alpha x/j_\beta y$ (= $\underline{a}$) and z^2/xy (= $\underline{b}$), one could calculate the shape of the helix-coil transition curve for polynucleotides from the equation

$$X = \tfrac{1}{2}\left[1 \pm \left(\frac{r}{r+4}\right)^{\frac{1}{2}}\right] \qquad \text{(Warner, 50)}$$

where $r = b^{-1}\left[(1-a)^2/a\right]$ (the negative sign is used for $a < 1.0$ and the positive sign for $a > 1.0$)

If one makes a reasonable arbitrary substitution for the parameter a, which by definition is proportional to the stability of the complex, one can calculate Xc as a function of temperature. If the same value of a is assumed for poly C at pH 7.5 and pH 4, but assumes b = 1 and b much less than 1.0 for the polymer at pH 7.5 and pH 4.0, respectively, curves similar in curvature to the experimental ones are obtained when Xc is plotted as a function of temperature (°K). Furthermore, the theoretical curve obtained for b = 1 is very similar to the experimental curve one obtains by measuring absorbency versus temperature for poly C at pH 4.8 in the temperature range 80-100°C. It is thus sensible to associate low values of b with more organized structure. This method has been used by Warner (50) for poly (A+U), for which case he has shown that b for helical poly (A+U) is of the order of 0.005 in 0.1μ salt over the temperature range 30-80°C.

Thus, these considerations indicate that a high value of $\Delta F_{\alpha\beta}$ is a good measure of a cooperative conformational change, as is an abrupt transition of optical properties of a polynucleotide over a narrow temperature range. Furthermore, it should be apparent from the definition of $\Delta F_{\alpha\beta}$, that whenever this kind of an abrupt temperature dependent

structural transition is observed, it must involve some kind of equilibration between coil and the helix.

II Hyperchromicity and Optical Rotation

Poly C in acidic solution has a much lower E_{max} and a higher $+(\alpha)^{20°}$ than does an equimolar amount of the corresponding monomeric form. Exposure to elevated temperatures causes an increase in E_{max} and a decrease in $+(\alpha)^{t}$. Hypochromicity can best be associated with direct interaction between the bases. This interaction may result from the stacking of the nucleotide bases in an organized structure and with a consequent marked restriction of their movement, as compared to the situation for the corresponding monomer or oligonucleotide. With an organized structure, such as helical poly C at 25°C, this effect will be much more pronounced than with a disordered form such as that obtained by heating the polymer. Similarly, one can associate the higher dextrorotatory power of acidic poly C (20°C), as compared with that of the heated form, with a greater asymmetry of the molecule at the lower temperature. This, however, is only a relative statement. The data show that the E_{max} of neutral poly C is very low, and that the $+(\alpha)^{25°}$ is very high even under these conditions. No simple explanation can be given to this apparent contradiction. As mentioned earlier, however, it is possible that there is a considerable interaction amongst the polymer molecules even at neutral pH. Such interaction need not imply helix formation. Hypochromicity, taken by itself, is not sufficient evidence

for the existence of helical conformation. For example, the dinucleoside phosphates derived from 6-dimethyl amino-purine show a 35 per cent hyperchromicity on degradation (Michelson, 24). Evidently no helical conformation can be associated with this dinucleotide. However, the behavior of hypochromicity or hyperchromicity with respect to certain well-defined variables, such as temperature, nature of solvent, or pH, can, if used wisely, serve to identify the existence of a helix. Pertinent to this argument is the observation that the $(E_{max})^{85°}/(E_{max})^{25°}$ for poly C at pH 7.2 (0.1μ salt) is 1.09, whereas the $(E_{max})^{85°}/(E_{max})^{25°}$ for the same polymer at pH 4.03 ($\mu = 0.1$) is 1.22. This observation is consistent with the finding that denatured DNA, which presumably represents the more disordered form of DNA, exhibits less hyperchromicity on heating from 25° to 100°C than does the native helical form.

III Viscosity

The lower viscosity of acidic poly C as compared with the neutral form has been discussed. The relative dependency of viscosity on polymer and electrolyte concentration as the neutral form of poly C is changed to the acidic form has also been considered. A similar pattern of behavior has been observed for helical poly A, poly A-poly U, and poly I. The observed decreased dependency of the acidic form on polymer and electrolyte concentration can only mean that the acidic form possesses a more compact structure than does the neutral form. This conclusion, combined with the other evidence and arguments presented

above, leads one to the conclusion that the structure of acidic poly C is not only regular and compact, but also helical.

(B) Proposed Scheme for the Pairing of Cytosines of Helical Poly C

The physico-chemical data presented above provide some information on the hydrogen bonding scheme which helical poly C may assume. These data indicate that the hydrogen bonding pattern of acidic poly C in solution may be similar to that of cytosine-5-acetic acid in crystal form, as determined by Marsh et al. (23). This scheme (fig. 6) consists of a pair of cytosine residues, hydrogen-bonded together across the center of the helix. The amino group of the first cytosine residue (N_7) is hydrogen-bonded to the carbonyl oxygen (O_8) of the second cytosine residue, the amino group (N_7) of the second cytosine residue is hydrogen-bonded to the carbonyl oxygen (O_8) of the first cytosine residue. In addition, a single proton is located between, and shared across the center by, the N_1 atoms of two cytosines. Thus, the helix is stabilized both by hydrogen bonding of amino to keto groups, and by charge effects due to the additional proton on the two ring nitrogens. This structure involves protonation of only one-half of the cytosines and is not possible in the unprotonated or in the fully protonated form of poly C.

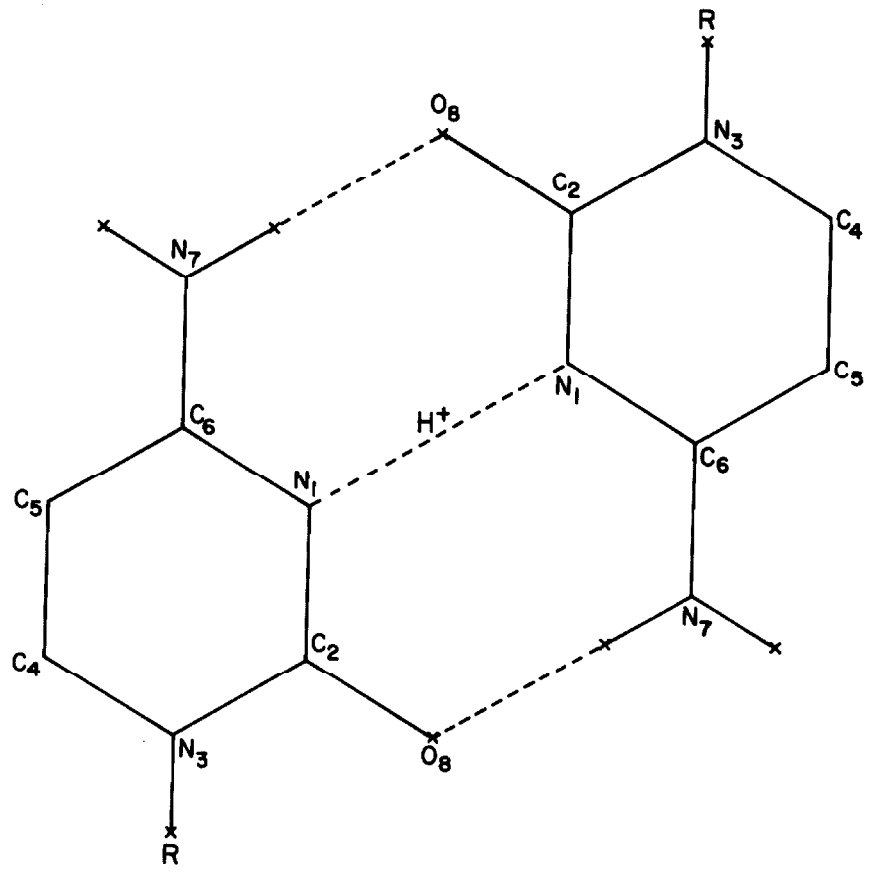


Figure 6. Diagram of the proposed scheme for the pairing of cytosines of helical poly C.

FURTHER CONSIDERATION OF THE SCHEME

The relative distribution of charge around the cytosine molecule has already been discussed. It has been clearly shown that the ring nitrogen (N_1) is surrounded by a negatively charged environment compared with the other nitrogen atoms. It thus possesses the highest pKa value. Hence, any addition of protons in the pH range 5.5 to 4.0 will go to the ring nitrogen (N_1). In support of the proposed scheme, therefore, the data show that the initial addition of protons to the N_1 nitrogen of the cytosine (pH 5.5-4.2) tends to stabilize the helix. This is to be expected if the $N_1H^{(+)}$....N hydrogen bonding is important in the formation of the helix. Furthermore, in the zone of proton stabilization, i.e., in the pH zone above the pKa of the N_1 nitrogen, addition of electrolyte weakens the helix by reducing the electrostatic attraction. This electrostatic shielding provided by high salt will tend to reduce the affinity of poly C for protons, and therefore decrease the stability of the helix in this zone of insufficient proton availability. After a certain number of protons have been added, further addition of protons will tend to destabilize the helix. In this zone of proton destabilization, addition of electrolytes strengthens the helix by reducing electrostatic repulsion of the type $(\overset{+}{N}H....\overset{+}{H}N)\text{---}\rightarrow(\overset{+}{N}H + \overset{+}{N}H)$. This reduction of affinity of poly C for protons by electrostatic shielding therefore stabilizes the

poly C helix in this zone of excess protons. The transition of poly C from the proton stabilization zone to the proton destabilization zone does not involve any gross change in structure or conformation. This is clearly shown by the similarity of the data for pH 4.85 and pH 3.75 from optical rotatory dispersion, viscosity, and ultraviolet spectroscopy measurements.

It might be asked if protonation of the amino group, especially at very low pH, can make any significant contribution to the interaction of poly C in solution. It is intuitively easy to see that protonation of the ring nitrogen (N_1) will grossly decrease the pKa of the amino nitrogen, and therefore decrease the probability of its being protonated at the pH range 4.0 and higher. At much lower pH, protonation of the amino nitrogen, as well as of the ring nitrogens, would lead to a gross destabilization of the cytosine molecule, and therefore, to a marked change in ultraviolet spectrum and dispersion constants of the molecule. Such a drastic change is not indicated by the data. It is therefore reasonable to conclude that only one proton per cytosine is added to the polymer molecule in the pH range 4.0 to 5.5, and that this proton goes almost exclusively to the N_1 nitrogen.

Langridge and Rich (21) have recently and independently arrived at the same hydrogen bonding scheme for the helical poly C from data based on X-ray fiber diagrams, titration, and infrared spectroscopy measurements. Poly C fibers prepared at pH's above 5.5 show no

indication of internal order. This conclusion is based on the findings that the molecule in this pH region (above pH 5.5) shows no sharp reflections and gives approximately zero birefringence. Below pH 5.5, however, the polymer shows high negative birefringence and sharp reflections. The diffraction pattern in this pH range indicates that the molecule is highly oriented. In contrast to DNA, the reflection of poly C does not change with humidity (0-100%). This suggests that very little water is accommodated in the poly C lattice.

The helical form thus obtained has an apparent pitch of 18.65 Å, and about 6 residues per helical turn. Adjacent asymmetric units in the polymer chain may be related by a translation of 3.11 Å along the helix axis and by a rotation of 60°. The diameter of the helical molecule is about 13.5 Å. This relatively large diameter indicates that the molecule is not single-stranded. Since adjacent nucleotide residues are related by a 60° rotation (rather than 30° rotation), it is concluded that the strands are antiparallel. Fibers drawn at very low pH (proton destabilization zone) exhibit less ordered diffraction patterns than those drawn above pH 4.0. This is compatible with the previous interpretation (Akinrimisi et al., 2) that excess protons tend to destabilize the poly C helix.

A molecular model has been constructed (Langridge and Rich, 21) in which the bases are tilted slightly from the helical axis in a propeller-like fashion to preserve the two-fold rotation axis. This model shows

that the two antiparallel chains are very close together in the helical conformation. This observation appears to explain the importance of the $N^{+}H....N$ hydrogen bonding which, probably because of its short length and high energy, may act to bring the two strands close together in the helical conformation. Thus, the data on poly C and the interpretation of the data presented above is fully supported by the X-ray diffraction patterns of Langridge and Rich. Recently, Hartman and Rich (unpublished) have reached similar conclusions on the helical nature of poly C from physico-chemical studies of the polymer in acid and neutral solutions.

SUMMARY

The T_m of polycytidylic acid in acidic solutions of various pH levels, ionic strengths, and urea concentrations has been investigated by measurements of absorbency and specific rotation versus temperature. Other physico-chemical properties of polycytidylic acid in solution were also studied, such as optical rotatory dispersion, viscosity, and sedimentation coefficients. The data support the notion that the polycytidylic acid in acidic solution assumes a helical structure with a hydrogen bonding scheme similar to that of the cytosine-5-acetic acid in crystal. This scheme consists of a pair of hydrogen bonds formed between the amino groups and the keto groups of the two cytosine residues in the helix, with an added proton in between shared by the two ring nitrogen atoms.

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PART II

INTERACTIONS OF PURINE WITH PROTEINS AND AMINO ACIDS

INTRODUCTION

General Properties of Proteins in Organic Solvents

Studies of protein structure in non-aqueous solvents and in water-organic compound mixtures are important for the following reasons:

(a) Useful information can be obtained about the nature of the interactions between the protein solute and its solvent. If the properties of the solvent are well defined and clearly understood, such information can lead to useful conclusions about the most probable structure of the same or other protein molecules in another well defined solvent.

(b) Isolation procedures for some proteins involve the use of certain organic solvents. It is important to know what kind of structural modifications take place when the proteins are dissolved in these non-aqueous solvents.

(c) Many proteins are closely associated with organic compounds in the living systems (lipid, nucleic acids, etc.). It is possible that this organic environment has a great deal to do with the conformation, and therefore the reactivity of these proteins.

The conformation of any given protein is determined, in part, by the nature of the solvent-solute interactions. The most important of these are electrostatic interaction, hydrogen bonding, and hydrophobic interaction. These three factors act cooperatively and dependently to determine the conformation of proteins in solution. The relative

contribution to the free energy of the native protein conformation of any of these factors will vary with the nature of the solvent, and the structure of the protein in question. To simplify the discussion, however, these three factors will be considered separately.

I. Electrostatic Interaction

The most important electrostatic interaction affecting the stability of proteins is the attractive-repulsive type. The electrostatic contribution to the free energy, $\bar{F}_e$, of the protein conformation is the work done in placing charges on the macromolecule. Thus, $\bar{F}_e$ of the protein is a function of Z, the charge on the macromolecule, and of D, the dielectric constant of the solvent. The Debye-Huckel equation:

$$\bar{F}_e = \frac{Z^2 \epsilon^2}{2D} \left(\frac{1}{b} - \frac{K}{1 + K_a} \right) \text{ (Cohn and Edsall, 7)}$$

gives only a rough measure of $\bar{F}_e$, and even then, for a spherical, compact, and solvent impermeable model of protein molecule. (a = distance of closest approach of charged molecule to protein; b = radius of protein molecule; ϵ = unit charge; and K = the function of ionic strength in the Debye-Huckel theory.) It is obvious that most proteins do not conform to this hypothetical model. Hill (15), Tanford (40), and a few others have derived several modifications of the Debye-Huckel theory that may be used for the analysis of the electrostatic free energy of proteins in aqueous solution. In general, the analyses are cumbersome, and often

inadequate for making precise predictions about the relationship of $\bar{F}_e$ to conformational changes in proteins.

Certain proteins, e.g. ribonuclease, do not expand even in acid solution (Buzzel and Tanford, 5). On the other hand, the conformation of some other proteins (the serum albumins) is very sensitive to changes in pH and ionic strength (Yang and Foster, 53; Tanford et al., 40). In contrast to these, ovalbumin and pepsin expand irreversibly after acquiring a considerable amount of charge (Gibbs et al., 14; Endelhoch, 10). Thus, it is not possible at this stage to make a general theoretical prediction on the relationship of $\underline{Z}$ or $\underline{D}$ to the conformational changes in proteins. When proteins are dissolved in organic solvents, especially in the absence of salts, the validity of any such predictions becomes even more dubious.

In spite of these complications, however, certain generalized statements, based on empirical observations, can still be made on the relationship of $\underline{Z}$ and $\underline{D}$ to $\bar{F}_e$ of proteins in different solvents (Singer, 39).

(a) If $\underline{D}$ is about the same for a non-aqueous solvent as in water, the quantity $(\bar{F}_e)_s/(\bar{F}_e)_{H_2O}$ will tend to increase positively in the presence of large $\underline{Z}$ (the parameters $(\bar{F}_e)_s$ and $(\bar{F}_e)_{H_2O}$, respectively, represent the electrostatic free energy in the non-aqueous solvent and in water). This increase will in most cases lead to the destabilization of the native conformation of the proteins in the non-aqueous solvent.

(b) If $\underline{Z}$ and $\underline{D}$ both make significant contributions to $\bar{F}_e$ in both solvents (aqueous and non-aqueous), the end result is usually very difficult to predict.

(c) In weakly protic or non-protic solvents with small $\underline{D}$, the quantity $(\bar{F}_e)_s$ is less than $(\bar{F}_e)_{H_2O}$, and therefore the electrostatic tendency for the proteins' conformation to be destabilized in these solvents is substantially decreased under its magnitude in water.

II. Hydrogen Bonding

It has been suggested that intrapeptide hydrogen bonds, taken by themselves, cannot make substantial contribution to the stabilization of the native conformation of proteins in aqueous solution (Kauzman, 18; Schellman, 33; Klotz, 20). The carbonyl group in the peptide amide is a relatively good proton acceptor. On the other hand, the amide-NH group is a very weak proton donor. Thus, the resulting hydrogen bonding between the -C=O and -NH group in proteins is a rather weak one, especially in aqueous solution (Schellman, 33). Furthermore, compounds with the same proton accepting capacity as the -C-NH group will compete effectively in accepting protons from the -NH group of protein amides. Thus, organic solvents like carboxylic acids and amides (formamide, urea) should be effective in disrupting the hydrogen bonds in native proteins, while solvents like the alcohols should be less effective. Water, however, accepts and donates more than one proton per molecule. It should therefore be fairly effective in disrupting the internal hydrogen bonding in proteins.

It should be obvious from the above that the destabilization of the helical conformation of any protein by hydrogen bond exchange is a very complex operation, especially in a binary solvent system. It involves a competitive hydrogen bond formation in all chemically possible permutations with the different components of the solution. The nature of the interactions depends on the chemical nature and relative concentrations of the components. This complex relationship probably explains why urea is not very effective in participating in an effective hydrogen bond exchange with amides or peptides in aqueous solution (Schellman, 33).

III. Hydrophobic Interactions

Hydrophobic interactions have been implicated as an important factor responsible for the stability of protein conformation in aqueous solution (Whitney et al., 52; Klotz et al., 21, 22; Kauzman, 18). Consider a system of type:

(Pure liquid hydrocarbon) $\rightleftarrows$ (Hydrocarbon in non-aqueous solvent mole fraction = X) (1)

The unitary free energy change of the system, $\Delta F_u = -RT \ln X_s$ (Kauzman, 18), where X_s = the mole fraction of the hydrocarbon in saturated solution. ΔF_u in this reaction can be large and positive. However, in the reaction:

(Hydrocarbon in water, mole fraction = X) $\rightleftarrows$ (Hydrocarbon in non-aqueous solvent, mole fraction = X) (2)

ΔF_u^t, the unitary free energy change in an isothermal transfer process, equals (ΔF_u) non-aq. $-(\Delta F_u)_{H_2O}$. In general, the quantity ΔF_u^t is negative. The same basic treatment may be applied to proteins in solution with protein side-chains representing the hydrocarbon system. The quantity $\Sigma \Delta F_u^t$ (taken over all non-polar residues) in proteins can attain rather large negative values in water, depending on the protein structure. However, if protein is dissolved in an organic solvent, the value $\Sigma \Delta F_u^t$ can approach zero, or becomes positive. This follows from the conclusion drawn from reaction 1.

When any hydrocarbon is dissolved in water, water tends to form an ordered structure around it (Claussen, 6; Müller et al., 26). This usually is accompanied by a positive free energy change, sometimes without a large positive change in enthalpy. Apparently, the observed change in free energy is brought about almost exclusively by the considerable loss in unitary entropy, ΔS_u, of the water. Proteins, with their exposed non-polar side-chain, will probably behave similarly in aqueous solution.

From the above, it follows that the stabilization of the native conformation of proteins should diminish in organic solvents that can form hydrophobic bonds with protein side-chain, unless there are other factors neutralizing this effect. Even though this conclusion is based on deductions from an oversimplified model, i.e., the simple hydrocarbons, some evidence will later be presented to show that the conclusion is quite applicable to proteins.

It has been pointed out that the total hydrogen bond energy in the α-helix of native proteins should be relatively weakened in aqueous solution. If, however, the hydrogen bonds are buried in a non-polar environment, they should be less affected by the aqueous atmosphere. This is probably the case in most globular proteins.

Unfortunately, the chemical nature of hydrophobic interaction is very vague, and therefore, unlike the other short-range forces previously discussed, no quantitative measurements are available for its relative contributions to the native conformation of the proteins. In order to make the discussion on hydrophobic interaction more specific, hydrophobic bonds will be defined in terms of the London dispersion forces (Waugh, 49; Kauzman, 18). Consider the interaction of the molecules A-A and B-B strictly in terms of the London dispersion forces. The two molecules can be treated as solid spheres with radii r_A and r_B, respectively. In the reaction A-A + B-B $\rightarrow$ 2 AB,

$$\Delta E = K\left[\frac{(I_A \alpha^2)}{2^7 r_A^6} + \frac{(I_B \alpha_B^2)}{2^7 \cdot r_B^6} - \left(\frac{2 I_A I_B \alpha_A \alpha_B}{(I_A + I_B)(r_A + r_B)^6} \right) \right]$$

$\underline{\alpha}$ = the polarizability and $\underline{I}$ = the ionization potential of the molecules. With $(r_A) = \beta$; $(I_A / I_B) = \underline{\delta}$, one can define a positive parameter,

$$P = (K I_A / 2^7 \cdot r_A^6)\left(\alpha_A - \frac{\sqrt{\delta}\, \alpha_B}{\beta^3}\right)^2$$

$$E = P + \left(\frac{K I_A \alpha_A \alpha_B}{2^6 \beta^3 r_A^6}\right) \left[1 - \frac{2^7 \beta^3 \sqrt{\delta}}{(1+\beta)^6(1+\delta)}\right]$$

It can be shown that the last quantity in the bracket to the right has a value which can never be greater than 1.0 for β and δ between zero and infinity (Kauzman, 18). Therefore, ΔE is always positive, Furthermore, a closer examination of the last equation in the series indicates that the dispersion forces acting between groups tend to bring identical groups closer together. The magnitude of interaction is also strongly dependent on the polarizability of the interacting molecules.

Certain properties of hydrophobic interactions are not obvious from a molecular definition such as the one above. The thermodynamics of hydrophobic interaction reveal some of its characteristic properties, and some of these properties will be summarized below (Kauzman, 18):

(a) Hydrophobic bonds are stabilized largely by entropy effects. It has been estimated (Kauzman, 18) that about 20 entropy units accompany the transfer of a non-polar aliphatic side-chain of proteins from an aqueous environment to a non-polar environment.

(b) The transfer of an aliphatic side-chain from water to a non-polar region in protein is endothermic to the extent of 100 to 200 cal/mole groups. For aromatic side-chains (phenylalanine), the transfer is more nearly athermal.

(c) The free energy change in the transfer of the non-polar side chain from water to a non-polar environment is usually exergonic.

(d) Hydrophobic bonds involving aliphatic side-chains are more stable at room temperature than at 0°C because of the endothermicity of the transfer of non-polar groups from water to non-polar environment.

(e) The transfer is expected to be accompanied by a volume expansion up to about 20 ml/mole of aliphatic side-chain and slightly less for the aromatic side-chain.

Most of the above properties are distinguishable from the hydrogen bonding and electrostatic properties. They can therefore be used as a qualitative guide for establishing the notion that hydrophobic bonds exist in protein side-chain. A few experiments will be mentioned to illustrate this approach.

The rate of protein denaturation in urea has been observed to have a large negative temperature coefficient (Jacobsen et al., 16; Simpson and Kauzman, 38). This observation is in agreement with one of the properties of hydrophobic interaction summarized above, namely, that the hydrophobic bonds involving aliphatic side-chains are more stable at 25°C than at 0°C.

Lauffer et al. (23) observed that the protein of TMV (pH 6.5, 0.1M salt) is reversibly aggregated into rods at room temperature and disaggregated at 0°C. Similar reversible disaggregation has been observed for sickle cell hemoglobin at low temperature (Murayama, 27) and for collagen at 0°C (Fessler, 13). Apparently, these aggregations involve the interactions of non-polar side-chains, leading to a large positive

unitary entropy and a small positive enthalpy change. The enthalpy change brings about the observed temperature dependence. The interaction of sodium dodecyl sulfate with bovine serum albumin causes a volume increase of 6.7 ml per mole of dye bound (Rosenberg and Klotz, 30). Such volume change is as predicted for a reaction involving hydrophobic bonds. Evidences of this kind and several others clearly indicate that proteins can form hydrophobic bonds not only between side-chains on the molecule, but also with other molecules interacting with the protein. The next problem then is to establish the relative importance of hydrophobic bonds in the maintenance of protein conformation in solution. Here also, a few experiments will help to prove the point in question.

RNAase completely unfolds in ethylene glycol containing salt (Sage and Singer, 36, 37). The hyperchromicity characteristic of the three anomalous tyrosines in RNAase is grossly diminished, and the pK_a of the phenolic-OH group is shifted from 6.9 to 6.1. All the tyrosine -OH in the molecule titrate similarly. What these phenomena imply is that in ethylene glycol, the hydrophobic regions of the molecules are all disorganized and all the tyrosine residues in the RNAase become accessible to water. However, the constant $-b_o$ (which measures helical content) for RNAase is 92° in ethylene glycol and 105° in water. Thus, RNAase has only unfolded in ethylene glycol, but has not lost most of its helical content. This, however, does not necessarily imply that the same helical regions exist in water as in ethylene glycol.

A large volume fraction of ethylene glycol in water is required to bring about conformational change in proteins, whereas only a small amount of dioxane is required for the same purpose (Weber and Tanford, 50). This is in accordance with the extent of their miscibility with organic solvents, and therefore their hydrophobic potential. But this cannot be regarded as a general rule. Formamide is very immiscible with hydrocarbon solvents, yet it is as effective as dioxane in effecting conformational changes in proteins. Here, however, the high dielectric constant of formamide will have to be taken into consideration in explaining this anomaly.

Doty and co-workers (8) found that a number of proteins are directly soluble in 2-chloroethanol. The helical content ($-b_o$) of the proteins tends to increase in this solvent over that in water, and the effect is reversible. The effect of increase in b_o is probably the result of decreased hydrogen bonding with the solvent, and also decreased electrostatic repulsive interaction due to low dielectric constant of the solvent. However, in spite of the weaker inter-peptide hydrogen bond in water over that in 2-chloroethanol, or greater electrostatic interaction due to higher dielectric constant in water, globular proteins still possess highly folded, compact helical structure in water. From this, it is only reasonable to conclude that some factor other than hydrogen bonding is involved in structural formation. It has been suggested that this additional factor is hydrophobic.

Finally, Fasman (11) carried out the titration of different non-ionic forms of polypeptides dissolved in chloroform with dichloroacetic acid (DCA) and trifluoroacetic acid (TFA). For the polypeptide poly-β-benzyl L-aspartic acid, poly-ε-carbobenzyl-oxylysine, about 7 per cent and 38 per cent of the acids, respectively, are required to disrupt the helix, whereas poly-L-leucine is still completely helical in 100 per cent TFA and DCA. Apparently, the leucine side-chain is helping to maintain the helix in intact configuration because of the strong hydrophobic interaction between the leucine side-chain.

Studies by Tanford and others (41, 43) show that the conformational change occurring in β-lactoglobulin at pH = 3.0, ionic strength = 0.02, on addition of non-aqueous solvents, goes through an intermediate structural change of type:

$$\underset{\text{I}}{(\text{native})} \rightleftarrows \underset{\text{II}}{(\text{unfolded with intact helix})} \rightleftarrows \underset{\text{III}}{(\text{refolding with higher helical content})}$$

Stage I $\rightleftarrows$ II is due to hydrophobic interaction of the solvent causing the molecule to unfold. Stage II $\rightleftarrows$ III is probably due to decreased dielectric constant of the solution resulting in decreased electrostatic repulsive interaction, higher ion pair, and hydrogen bond formation.

For convenience, therefore, one can represent the free energy contribution of the several factors involved in maintaining protein conformation in aqueous solution as follows:

F_s = free energy due to loss of entropy

F_e = free energy due to electrostatic interaction

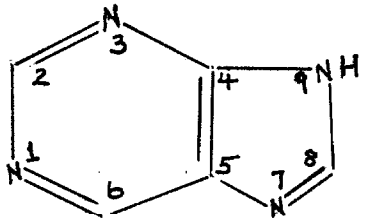
F_{HI} = free energy due to hydrophobic interaction

F_{HB} = free energy due to hydrogen bond

$F_s + F_e + F_{HI} = F_{total}$

In several proteins in organic solvents, or organic compound-water mixtures, $F_{total} - F_{HB}$ is still negative, whereas $F_{total} - F_{HI}$ is positive (i.e., destabilization of conformation). Thus, while F_{HI} actually represents the highest stabilization factor, F_{HB} probably makes the formation of a specific conformation most likely (Singer, 39).

Properties of Purine



Purine is a bicyclic aromatic compound. It is very soluble in water, but only slightly soluble in most organic solvents. The results of molecular orbital calculations around the purine molecule indicate that position 8 on the imidazole portion of the molecule has the lowest pi-electron density. This is because the pyrimidine portion of the molecule withdraws electrons from the imidazole portion. This asymmetric distribution of charges on the molecule gives rise to a molecular permanent dipole in purine.

The physical interaction of purine derivatives with several poly-

cyclic hydrocarbons has been studied by means of infrared spectroscopy (Booth et al., 1).

Small but noticeable shifts in the infrared absorption spectra of caffeins and tetramethyl uric acid can be observed when they form crystalline aromatic hydrocarbons. A similar and comparable shift is observed in purine when it is dissolved in chloroform in the presence of several of these polycyclic aromatic compounds.

It has been shown that aqueous purine will dissolve carcinogenic hydrocarbons such as 3:4, benz-pyrene, benzidines, dibenzacridines. Booth and co-workers (1) have also shown that the solubility of certain polycyclic hydrocarbons is increased in a solution of sodium-deoxyribonucleate. Weil-Malherbe (51) has prepared crystalline molecular compounds of 1,3,7,9-tetramethyl uric acid with pyrene, 3:4, benzpyrene, and coronene.

Burton and Richards (3) concluded from their studies that purine-polycyclic hydrocarbon complexes owe their stability to the lower energy of the crystal lattice of the complex as compared to those of the components, and not to any specific force of attraction between the molecules. However, infrared studies of Booth et al. (2) on these complexes seem to contradict this point of view. For example, the frequency of the IR band for caffeine at 1701 and 1658 cm^{-1} (which are due to the carbonyl group on caffeine), are reduced by about 5-10 cm^{-1} on formation of a complex. On the other hand, the bands at 1026 and 974 cm^{-1}, which are

mostly due to the C-N bonds closest to the C=O bonds are shifted to higher frequency in the same complex by about 4-7 cm^{-1}. Finally, the aromatic C-H bands in the purine derivative which has been assigned to the out-of-plane deformation mode of the C-H bond (750-850 cm^{-1}) are increased in the complex by about 8-12 cm^{-1}. Similar shifts have been observed for some other purine derivatives when they form complexes with the polycyclic aromatic hydrocarbons. The various changes in the frequencies can be related to changes in the corresponding bond force constants, arising from the deformation of the bonds, due perhaps to changes in bond distance, bond dipole, and bond dissociation energy. Such alterations in the properties of the bonds suggest that the purine derivative is involved in intermolecular physical interaction with the polycyclic compounds. In addition, the interaction is specific, and involves a force of attraction between the interacting molecules.

The aromatic compounds do in general have large polarizabilities in the plane of the molecules. Therefore, if any two of such molecules should lie close to each other, they would be attracted to each other because of their mutual polarizabilities. Any such dipole association would be further stabilized by resonance hybridization. This probably accounts for the rather high stability of the purine-polycyclic complexes. It does not appear that hydrogen bonding is of any importance in the formation of these complexes. For example, 3:4, benzpyrene interacts strongly with some purine derivatives. The structure of this polycyclic

is such that it cannot form hydrogen bonding with the purine derivatives.

The above mentioned properties of purine are of importance in understanding the observations of Ts'o et al. (44) concerning the interaction of purine with the nucleic acids. They have been able to show by means of optical rotation, that purine and several of its derivatives decrease the melting temperature of DNA and poly A. The effect of purine on these polynucleotides is of many orders of magnitude greater than that of urea. This behavior of purine has been interpreted as resulting from an extensive hydrophobic interaction between purine and the polynucleotide molecules in aqueous solution. The purine apparently disrupts the ordered water structure around the bases of the helical polynucleotides, and at the same time weakens the hydrophobic stacking of the polynucleotide bases. This results in the observed decreased stability of the polynucleotide helices.

Purine has also been observed to be extensively associated in aqueous solution. The osmotic studies of Ts'o et al. (48) suggest that the aggregation of aqueous purine proceeds not only to the dimer stage, but further to higher polymer forms. Furthermore, the various steps in the polymerization process are goverened by the same equilibrium constant. The association process may be described as shown below:

$$B_1 + B_1 \rightleftarrows B_2 \qquad K_1 = B_2 / (B_1)^2$$

$$B_2 + B_1 \rightleftarrows B_3 \qquad K_2 = B_3 / (B_1)(B_2)$$

$$B_n + B_1 \rightleftarrows B_{n+1} \qquad K_n = B_{n+1} / (B_n)(B_1)$$

$$K_1 = K_2 = K_3 = \text{----------} = K_n$$

$$B_n = K^{n-1} (B_1)^n .$$

Schweitzer et al. (35) have used proton magnetic resonance to study the interaction of purine in solution. They observed that the chemical shifts of the protons at positions 2, 6, and 8 on the purine molecule change with the concentration of purine in solution. Such concentration dependent chemical shifts have been attributed to a considerable solute-solute interaction in aqueous purine. The dilution shift is claimed to arise from a partial overlap of the purine rings. The partial ring overlap results in the cancellation of the induced field reinforcements at a ring proton when it is in the environment of another ring proton, since the two induced components are parallel in opposite directions. Only an overlap of this nature will explain the direction of the chemical shifts. The model does not support the notion that hydrogen bonding is involved in the interaction. In the absence of any quantitative measurements of the energy of the interactions, pi-pi electron non-bonding interactions and dipole-dipole interactions have been suggested to be involved in the aggregation.

Properties of Urea as Denaturant for Proteins

The denaturation of protein by urea has been extensively studied by several investigators (Kauzman, 18, 19; Schellman, 33; Klotz et al., 21, 22). In this section, the mechanism of protein denaturation by urea will be briefly discussed.

The earlier investigators attributed the denaturation of urea to the formation of hydrogen bonds with the peptide linkages (>C=O..HN<). The analysis to be presented below will show that this mechanism is unlikely in aqueous solution. Schellman (33) studied the thermodynamics of urea polymerization in solution. In a dilute solution of urea, the interaction will not proceed far beyond the dimer stage:

$$2 \text{ urea (aq.)} \rightleftarrows (\text{urea})_2 \text{ (aq.)} \qquad (1)$$

Schellman suggested that the interaction can be represented as shown below:

$$>C=O....H\text{-}O\text{-}H + H_2O....H\text{-}N< \rightleftarrows H_2O....H\text{-}O\text{-}H + >C=O....H\text{-}N< \qquad (2)$$

It is conceivable that this type of interaction can result in a linear or cyclic dimer. Each cyclic dimer has two hydrogen bonds, whereas each linear dimer has one hydrogen bond of type >C=O....HN<. Therefore, the thermodynamics of reaction 1 will be different from the thermodynamics of reaction 2. The thermodynamic values obtained per mole of urea for reaction 1 by Schellman are: $K = 4.1 \times 10^{-2}$; $\Delta F = 1890$ cal, $\Delta S_u = -5.4$ e.u., $\Delta H = -2090$ cal, and $\Delta V = 3.2$ ml.

A new set of parameters are defined for reaction 2 as follows: $\underline{h}$ is the enthalpy change (reaction 2); S_d = entropy change due to the formation of a cyclic dimer from a linear dimer; $\underline{v}$ is the volume change accompanying reaction 2, and $S_{\underline{u}}$ is the unitary entropy change in reaction 2. Since the cyclic dimer forms twice as many hydrogen bondings as the linear dimer, the relationship between ΔH and $\underline{h}$ is

$$\Delta H = (f_1 + 2f_2)\, h = (1 + f_2)\, h$$

where f_1 and f_2, respectively, represent the fraction of linear and cyclic dimers.

$$f_2/f_1 = \exp\left[-(h - T\Delta Sd)/RT\right]$$

Sd = -3 to -6 (Schellman, 33). With $\frac{f_2}{f_1}$ = 1.9 to 0.59, and ΔH = -2090 cal, $\underline{h}$ = -1300 to -1500 cal.

Similarly, if one assumes that the volume change due to the dimer is twice that due to the monomer, one can write the relationship:

$$\Delta V = (f_1 - 2f_2)\, v = (1 + f_2)\, v$$

and $v = 2.1 \pm 0.2$ ml. Now from $\Delta S_u = (S_u + f_2 S_d)$,

$$S_u = -3.3 \pm (0.1) \text{ e.u.}$$

From $\underline{h}$ and S_u, one can calculate $\underline{f_u}$, which is the free energy of hydrogen bonding in aqueous urea.

$$f_u = h - Ts_u;$$

at 25°C, $\underline{f_u} = -400 \pm 100$ cal.

This value is obviously too small to explain the effectiveness of urea as

a denaturant. In support of the above conclusions, Levy et al. (24) have recently reported that the free energy of carboxyl-carboxylate hydrogen bonding of several dicarboxylic acids is the same in water as 7M urea at 25°C.

The most currently acceptable mechanism for urea denaturation is that urea affects the intermolecular hydrophobic bonds stabilizing the conformation of proteins in solution (Kauzman, 18). Klotz et al. (21) suggest that the effect of urea is on the water structure around the non-polar side-chain of the protein molecule. Urea affects the solvent-macromolecular solute interaction which is required for the stabilization of the protein molecule in solution. Apparently, the extensive perturbation of this solvent-macromolecular solute interaction by urea results in the denaturation of the proteins (Klotz et al., 22).

The notion that urea breaks hydrophobic bonds is supported by several experimental observations. Simko (Kauzman, 18) observed that the solubility of diethyl ketone is 40 per cent greater in 8M urea than in water. Schlenk (34) reported that the solubility of n-valeric acid is markedly increased in the presence of urea at 25°C. Denatured proteins usually become more soluble in concentrated urea solution than in water (Kauzman, 18). The aggregation of certain proteins tends to decrease at lower temperatures (Murayama, 27; Fessler, 13; Lauffer et al., 23). These observations are compatible with the properties of hydrophobic interaction discussed in previous sections. Recently, Whitney and Tanford (52) have studied the effect of urea on the solubility of several amino

acids. All the amino acids with long hydrocarbon or aromatic side-chain are extensively solubilized by urea.

The above mechanism does not, however, explain all the other secondary reactions of urea with proteins. Urea has been noted to promote -SH exchange in proteins. Occasionally, this leads to an extensive aggregation of the proteins in solution (Echols et al., 9). However, a contrary reaction has often been observed--namely, that urea causes the dissociation of certain complex proteins in solution (Burk, 4). Unless the first reaction is associated with impurities in urea and the opposing reaction attributed to the breaking of hydrophobic bonds, our presently accepted mechanism of urea denaturation of proteins will have to be modified in order to be able to explain these contrasting observations.

Mention should be made of the very complex kinetics of protein denaturation by urea. Kauzman and co-workers (40) have studied the kinetics of urea denaturation of ovalbumin. The effective reaction rate can be represented as below:

$$-d[N]/dt = k[N]C^{m}$$

where: N = concentration of native protein, and $\underline{C}$ is the concentration of urea. The parameter m decreases with increasing temperature. At 0°C, m = 15, at 30°C, m = 12.5, and at 50°C, m = 8. The relationship between the intrinsic binding constant $\underline{K}$ and $\underline{m}$ is shown below:

$$m \cong \frac{nK}{1 + KC}$$

where: N = number of sites to which urea is bound.

Since $\underline{m}$, by definition, is independent of urea concentration, it follows then that a decrease in $\underline{m}$ means a decrease in K. Thus, the expected result is not observed, that the interaction of urea with protein side-chain should have a negative temperature coefficient. This suggests that the mechanism of urea denaturation might be more complex than the mere breaking of hydrophobic bonds, depending on the structure of the protein.

EXPERIMENTAL APPROACH

The approach used to study the interactions of purine with the proteins, as well as the purpose of the studies, will be discussed in this section. The relationship between optical rotation and macromolecular conformation has been considered in Part I. Proteins do, in general, have negative rotation. The specific rotation of most native proteins varies between -30° and -70° at 589 mμ. The specific rotation of most denaturated proteins is usually about -100° (± 50°) at 589 mμ. The negative increase in rotation accompanying the thermal denaturation of several proteins takes place within a narrow temperature region. The relationship of this to helix-coil transition has been fully discussed in Part I.

Optical rotation measurements in the visible region have been successfully employed in the study of the interactions of nucleic acids with various UV absorbing compounds of biological importance (Ts'o et al., 44). This technique is very sensitive to changes in conformation of biopolymers, and optical activity versus temperature profiles of biopolymers can be measured in this wavelength region without interference from chromophores. Following this approach, optical rotation has been measured as a function of temperature for DNA and poly A (Ts'o et al., 44). The data show that the helix-coil transition temperatures (T_m) of thymus DNA and helical poly A are lowered by pyrimidines, purine, nucleosides, and several of their analogs and derivatives. The

effectiveness of these compounds in lowering the T_m of nucleic acids was found to be greater than that of urea. The order of effectiveness amongst them indicates that hydrophobic stacking interactions are of importance.

Understanding of the nature of interactions between nucleic acids or nucleotides and proteins or amino acids is becoming of increasing biochemical importance. An approach of the type outlined above will be used to investigate the effect of purine on the secondary structure of proteins. The free purine base, rather than the purine nucleotide, will be used, because, in spite of the high solubility of purine, additional complications due to charge effects are minimal. Also, as shown by Ts'o et al. (44), the effects of purine on nucleic acid are the same as those of adenosine and guanine. Bovine serum albumin (BSA), lysozyme, and ribonuclease are chosen as representative proteins.

Since hydrophobic forces and stacking interactions appear to be chiefly responsible for interactions between purine and nucleic acids, one might expect the effect of purine on proteins to be principally upon the side-chains, and especially on aromatic residues. The secondary structure of proteins might be expected to be less sensitive to purine than that of nucleic acid. The latter expectation is based on what is known about the nature of interaction of purine with other molecules in solution (Ts'o et al., 44, 48; Schweitzer et al., 35). The two expectations above will be shown to be consistent with the experimental data that will be presented in later sections.

Various biochemical reactions, such as those between transfer RNA and amino acid activating enzymes, involve both nucleic acids and proteins or amino acids in close association. In such reactions, it is important to assess the role of the secondary structure of these biopolymers. Changing the environment, such as raising the temperature, usually affects both biopolymers indiscriminately and non-specifically. We therefore need to search for compounds which preferentially affect the secondary structure of nucleic acids, leaving proteins unattacked. Data presented below indicate that the secondary structure of protein is retained at purine concentration which denatures nucleic acids. Such a system for the selective denaturation of nucleic acids at room temperature may allow one to control and investigate the contribution of secondary structure of nucleic acids in such biochemical reactions.

EXPERIMENTAL

MATERIALS:

Purine was purchased from Cyclo Chemical Corp., Los Angeles, Calif. For further purification, purine was dissolved in boiling absolute ethanol. Purified charcoal was added to the amount of 3 per cent by weight of the purine. The mixture was boiled for 1-2 minutes, filtered and distilled under reduced pressure to remove the alcohol. The purine crystallized out on cooling to room temperature. The elemental analysis of the purine shows that it contains 46.6% nitrogen. It has an λ_{max} of 260 mμ and an E_{max} of 6100 at pH 1.0. The ratio of λ_{max} at 280 mμ to λ_{max} at 260 mμ is 0.36. Its melting point is 212-215°C. It is chromatographically homogeneous.

The ribonuclease (5X crystallized) and bovine serum albumin (5X crystallized) were purchased from Sigma Chemical Company, St. Louis, Missouri. Lysozyme (3X crystallized) was purchased from Worthington Biochemical Corp., New Jersey.

The amino acids and all other chemicals used were of reagent grade.

Instrumentation and Method of Analysis

Optical rotation measurements were made with a Rudolph 200S polarimeter equipped with an oscillating polarizer and xenon and mercury arc lamps (Ts'o et al., 45). The 20 cm polarimeter utilized glass construction with water jacket and quartz window. The temperature of the

polarimeter tube was read directly with a thermometer and held at any desired temperature ± 0.1°C by the flow of water through both compartments and the tube. Measurements of specific rotation were determined at protein concentrations of 8-10 mg/ml.

pH measurements were made with a radiometer pH meter 22, Copenhagen, Denmark, and to an accuracy of ± 0.1 pH units.

Protein solutions of known concentration were added to purine or urea solution (except at the highest purine concentration, in which a known weight of protein was added directly) to bring the urea or purine solution to a known molar concentration (moles per liter). The molar concentrations of purine or urea were converted to molal concentration by means of the equation:

$$m = \frac{c}{d - 0.001\,cM} \qquad \text{(Robinson and Stoke, 29)}$$

where m = molality; c = concentration in moles/liter; M = molecular weight of solute; d = density of the solution. The molar to molal conversion for purine and urea is shown in table 1. The density of different concentrations of purine and of urea were determined in water to obtain d in the above expression. Figure 1 shows the variation of d with purine and urea concentrations. The molal activity coefficients determined by Ts'o et al. (48) for purine and those determined by Scatchard et al. (32) for urea were used to calculate the activity of the purine or urea in solution.

Table 1. Values of Molarity (M) in Molality (m) for Purine and Urea

Purine		Urea	
Concentration (C) in moles per liter (Molar)	Molal concentration (m)	Concentration (C) in moles per liter (Molar)	Molal concentration (m)
0.10	0.10	0.50	0.51
0.20	0.20	1.0	1.1
0.30	0.31	1.5	1.6
0.40	0.42	2.0	2.2
0.50	0.52	2.5	2.8
0.60	0.63	3.0	3.5
0.75	0.80	4.0	4.9
1.0	1.1	5.0	6.5
1.5	1.7		
2.0	2.4		
3.0	4.0		

The extinction coefficients (E %, 1 cm) of the proteins were based upon dry weight determinations. A known weight of protein was dissolved in buffer (0.1M K-phosphate, pH 6.54), and concentration per ml determined spectrophotometrically. A known volume of the protein solution was concentrated at 80°C and then dried in vacuo at 80°C until the dry weight of the protein was constant. The weight of the buffer salt obtained by procedure similar to the above was subtracted in each case. The $E^{1\%}_{1cm}$ obtained for ribonuclease is 7.6 (λ_{max} = 278 mμ); 6.7 for BSA (λ_{max} = 278 mμ); and 25.4 for lysozyme (λ_{max} = 281 mμ). Yang (53) obtained $E^{1\%}_{1\ cm}$ = 6.7 for BSA (dry weight).

The refractive indices of the purine solutions were measured with a Zeiss Abbé refractometer. Refractive index corrections were made

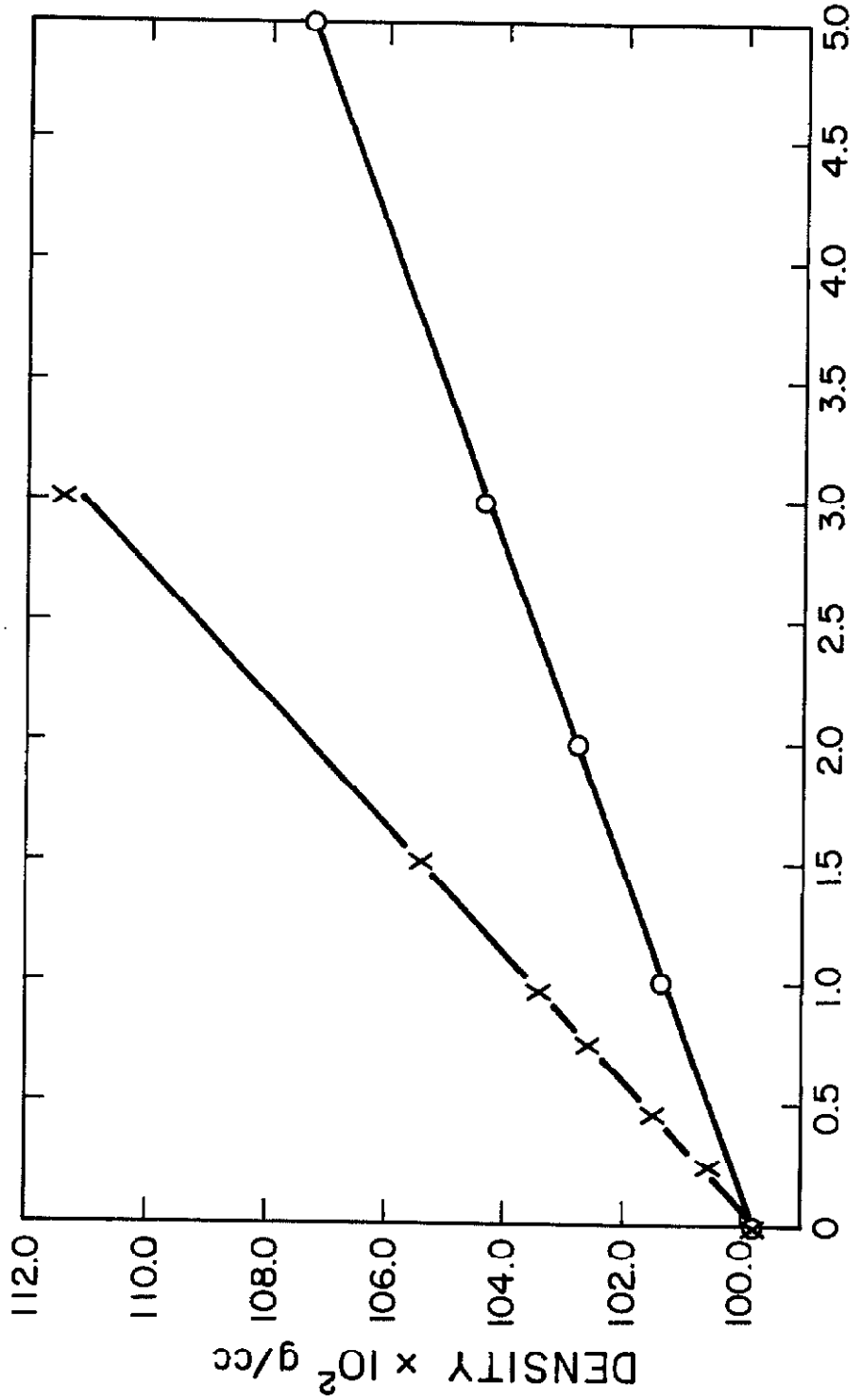


Figure 1. Plot of the density of purine (x——x) and urea (o——o) in water at 23-24°C versus the concentration of solute in moles per liter.

for calculating the specific rotations for the protein-purine solutions. This usually represents a small correction even at the highest purine concentration. The refractive index of purine at 4 molal concentration (25°C, 589 mμ) is 1.4080. The corrections were made with the rotation in water as reference, using the equation

$$\frac{n_w^2 + 2}{n_p^2 + 2} = \frac{(\alpha)_w}{(\alpha)_p}$$

where $(\alpha)_w$ and $(\alpha)_p$, respectively, represent the specific rotation in water and in purine solution, and n_w and n_p the corresponding refractive indices in water and in purine solution. The refractive index of purine as a function of purine concentration is shown in table 2.

Table 2. Refractive Index of Purine Solution versus Purine Concentration

Purine concentration in moles per liter of water	Refractive Index of Solution at 25°C, λ = 589 mμ
0.00 (water)	1.3326
0.75	1.3513
1.5	1.3701
3.0	1.4080

Solubility Measurements

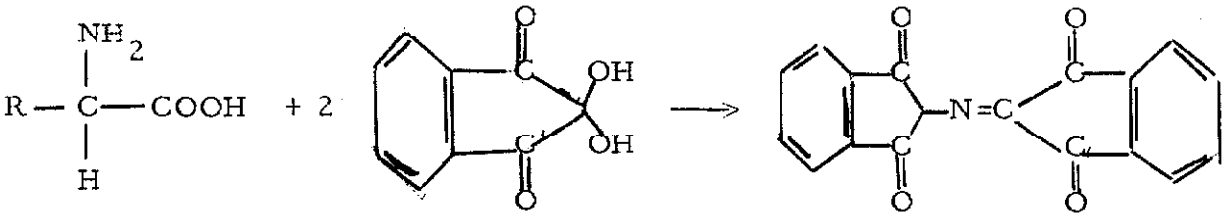
A known weight of amino acid, together with 1 ml of solvent, was sealed in a 10-ml glass ampule. The solvent was 0.1M K-phosphate buffer, pH 6.54, with or without purine of known activity. The sealed ampules were shaken in a 55°C water bath for 15 minutes. Under this

condition, the solution was supersaturated at the temperature (25°C) at which the solubility was measured. A single determination of the solubility required four ampules. Two of these were shaken in the equilibration bath (25°C) for 5 days. The other two ampules were stored in a refrigerator (4°C) for 24 hours before placing them in the equilibrium bath. The contents of the ampules were centrifuged for 1-2 minutes in a clinical centrifuge at room temperature (24-26°C). The supernatant was carefully removed and centrifuged at about 3,000 rpm for 5-10 minutes. The concentration of the amino acids was determined by the ninhydrin colorimetric method (Moore and Stein, 25; Amino Acid Analyzer, Beckman, AIM-2).

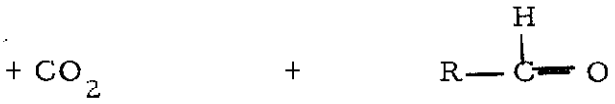
The ninhydrin reagent contains stannous chloride as reducing agent. Sodium acetate buffer (4M, pH 5.51) was prepared by dissolving 544 grams of Na-acetate $3H_2O$ in 400 ml of deionized water in a 1-liter flask. 100 ml of glacial acetic acid and deionized water were added to make the total volume equal to one liter. The pH can be adjusted with solid sodium hydroxide (0.5 gm of NaOH = 0.02 pH units). The ninhydrin reagent was prepared under nitrogen to eliminate the possibility of oxidation by oxygen. To prepare one liter of ninhydrin reagent, 750 ml of filtered peroxide free methyl cellosolve was added to a 2-liter reagent bottle equipped with a nitrogen supply line. 250 ml. of the 4M sodium acetate buffer was added, and the ninhydrin was dissolved in the solution. While ninhydrin was being dissolved, 0.4 gm of stannous chloride

was added. The mixture was stirred under nitrogen until a clear pale solution was formed. The nitrogen saturated solution was stored at 4°C protected from light.

The reaction between ninhydrin and amino acids can be represented as below:



- amino acid ninhydrin diketohydrindylidene-diketohydrindamine (DYDA)



carbon dioxide aldehyde

The DYDA has a purple color and an absorption maximum at about 570 mμ. The color is stable for over two hours, and it gives measurable color with about 0.1 μg of amino acid nitrogen/1 ml of solution.

The procedure for analysis used is similar to that described by Kabat et al. (17): 0.2 ml of the amino acid (0.5-4.0 μg/ml) was accurately pipetted into a 2-ml volumetric glass tube. 0.2 ml of the ninhydrin solution was added. The tube was sealed, first with aluminum foil, and then with parafilm wax. The sealed tube was heated for exactly 15 minutes in boiling water bath, cooled, and made to 2 ml with 50% ethanol.

The intensity of the color was read at 570 mμ. A standard curve was prepared from dilutions of a phenylalanine solution. The concentration of the phenylalanine was determined spectrophotometrically (figure 2).

The quadruplicate determinations of the amino acid concentrations are within 1-2% of each other. The values shown in table 4 are the averages for each quadruplicate analysis.

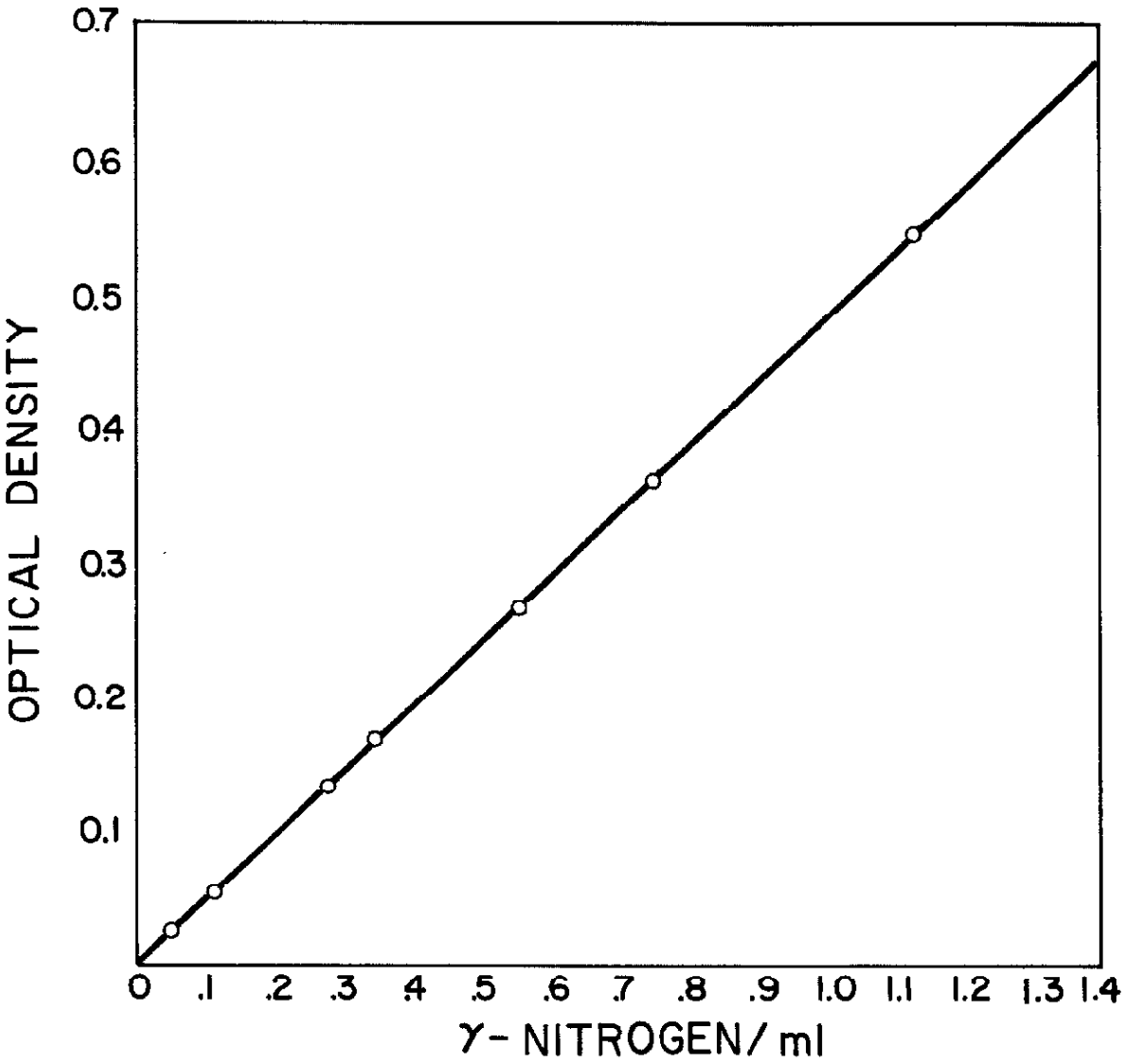


Figure 2. Standard curve for determining the concentration of amino acids by the ninhydrin colorimetric method. Phenylalanine was used in preparing the standard curve.

RESULTS

For the sake of simplicity in discussion, the following terms are defined:

Heating curve: Optical rotation of protein versus temperature measured during the course of increasing temperature.

Cooling curve: Optical rotation versus temperature profile measured during the course of decreasing temperature.

Helix coil transition temperature (T_m): The temperature which is at the midpoint of a heating curve, unless otherwise specified, between the temperature-insensitive zone of optical rotation in the lower temperature region (native state) and the temperature-insensitive zone of optical rotation at higher temperature region (denatured state). This point usually coincides with the region in which the change in optical rotation is most sensitive to temperature.

ΔT_m: The lowering of T_m at constant pH and ionic strength brought about by the presence of an interacting substance.

All the rotation measurements were made at 589 mμ. At lower wavelength, such as at 436 mμ, the higher concentrations of purine absorb appreciably. In addition, much of the earlier data on the optical rotation properties of the proteins studied here were collected at the higher wavelength region.

I. Effect of purine on the heating curve and denaturation

Three observations have been made:

A. In the presence of purine, the heating curves are shifted to lower temperature regions with little change in their shapes. These shifts can, therefore, be characterized by the corresponding shifts in $T_m(\Delta T_m)$ (figures 3, 4, 5).

B. The quantity ΔT_m is a linear function of purine activity up to an activity of about 0.25 m (concentration = 1.1 m). A similar plot of ΔT_m against molal concentration is not linear. The activity coefficient measurements of Ts'o et al. (48) do not extend above 1.1 m, and the points at higher purine concentration in figure 9 have been plotted with the assumption that the activity coefficient remains constant at concentration above 1.1 molal. The shape of the line beyond 0.25 m activity will be considered in the discussion.

C. The negative value of optical rotation at the temperature-insensitive region above T_m, i.e., the denatured state, is larger in the presence of purine than in the absence of purine. In other words, the curve reaches a higher plateau in the presence of purine than in the absence of purine (figures 3, 4, 5). The decrease in the negative rotation of the denatured state seems to increase with increasing purine concentration until a maximum is reached at higher concentration of purine (above 3 molal purine).

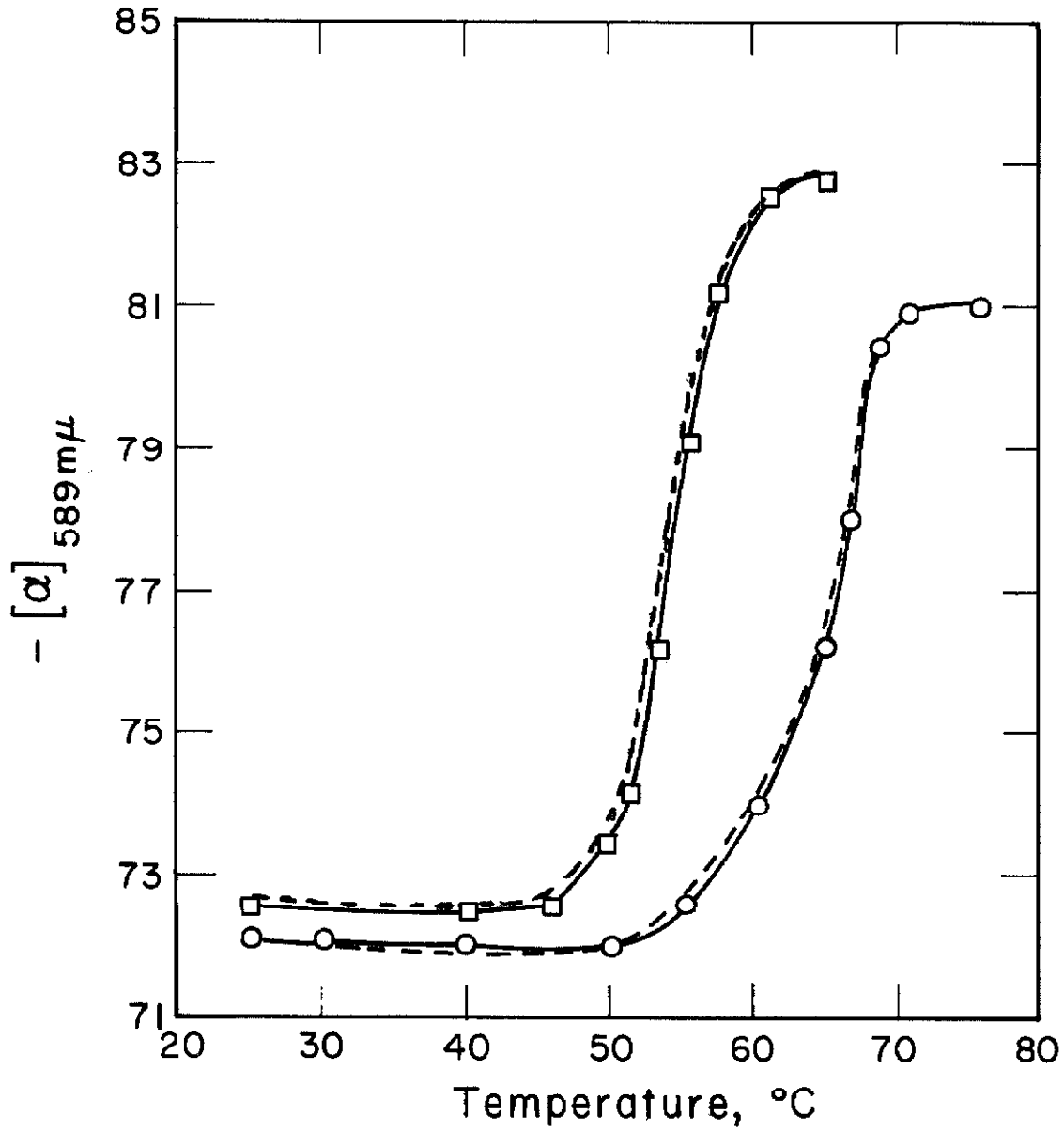


Figure 3. Profile of $-[\alpha]589$ versus temperature of ribonuclease in 0.1M K-phosphate, pH 6.54 (o——o) and in 1.1 molal purine (0.1M K-phosphate, pH 6.54) (□——□). The dotted lines represent the cooling profiles.

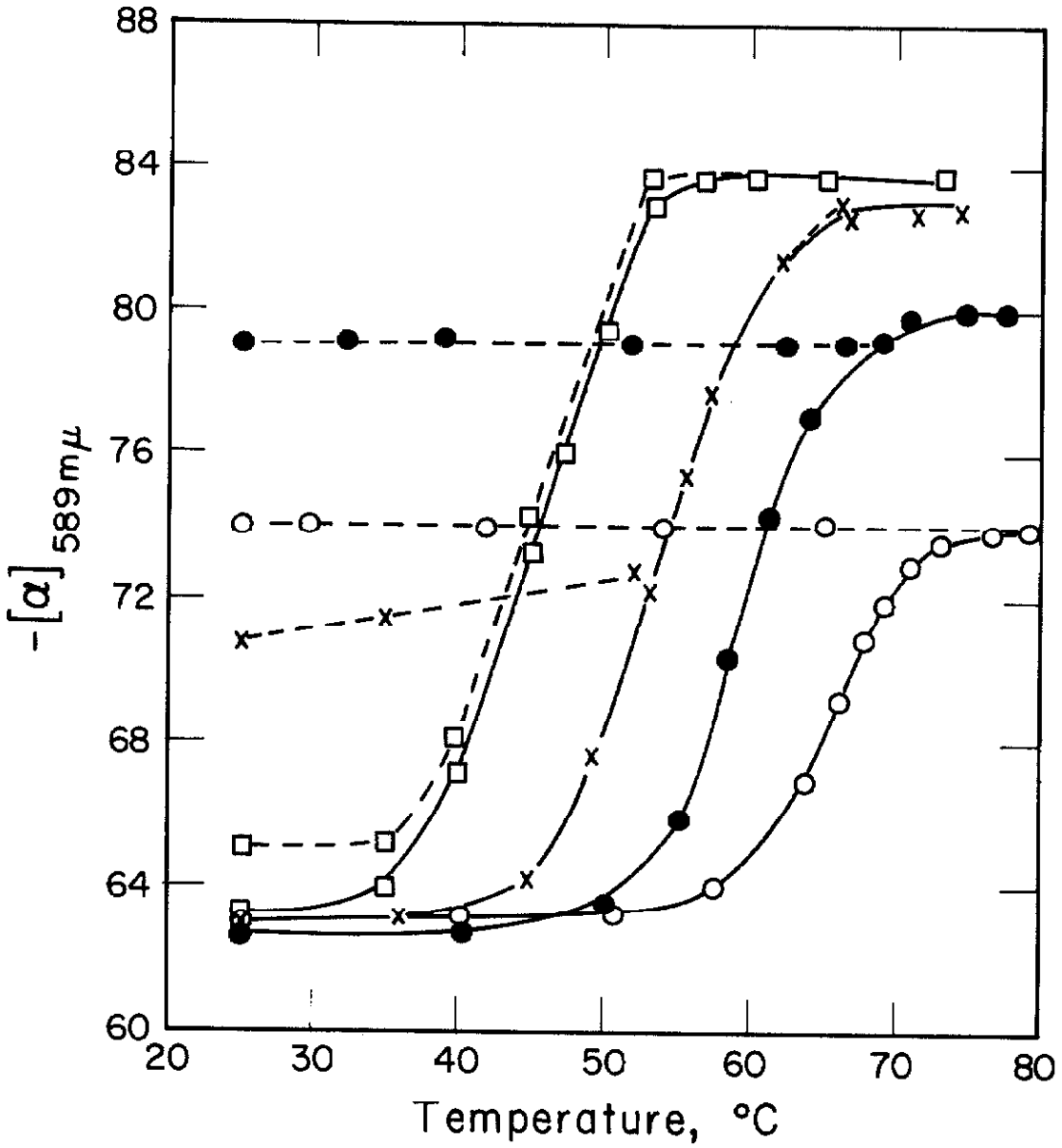


Figure 4. Profile of $-[\alpha]$ 589 versus temperature of BSA in 0.1M K-phosphate, pH 6.54 (o——o); in 0.8 m purine (●——●); in 1.7 m purine (x——x); and in 4 m purine (□——□). Purine was dissolved in 0.1M K-phosphate, pH 6.54. The corresponding dotted lines represent the cooling profiles.

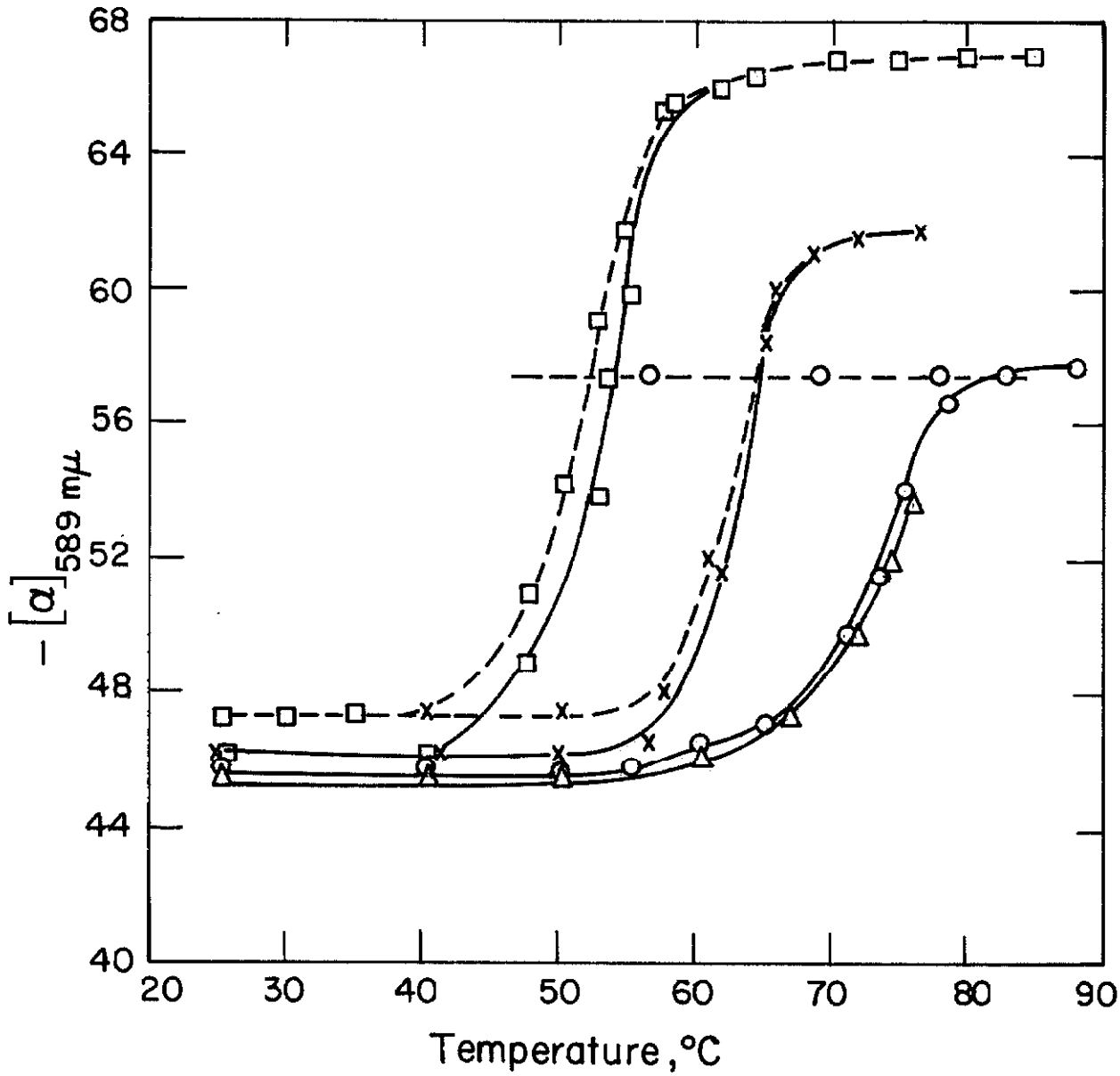


Figure 5. Profile of $-[\alpha]$ 589 versus temperature of lysozyme before addition of purine (Δ——Δ); in 0.8 m purine (x——x); and in 4 m purine (□—□), all buffered with 0.1M K-phosphate, pH 6.54. (o——o) represents the profile for a sample previously treated with purine, and the purine removed by dialysis at 4°C. The corresponding dotted lines represent the return profiles.

II. Effect of purine on the cooling curve and on denaturation

In the absence of interactant, ribonuclease is the only protein of those tested which yields a cooling curve superimposable on the heating curve, an indication of a rapid renaturation.

In the case of lysozyme (fig. 5), there is very little decrease in negative rotation as the solution is cooled from the denatured state at 85°C (0.1M K-phosphate, pH 6.5). Occasionally, the solution becomes turbid at elevated temperatures. Foss (12) observed a similar turbidity at a temperature (67°C) which is well below the denaturation temperature. He attributed this turbidity to the precipitation of impurities in the lysozyme rather than to the precipitation of lysozyme. Foss indicated that these impurities could be eliminated either by a short time heat treatment of the lysozyme solution at 90°C, or by adding a dilute solution to mercuric chloride. The supernatant from the heat-treated lysozyme solution could then be heated to 82°C without the appearance of turbidity.

Lysozyme solution that could be heated to 85°C without the appearance of turbidity was prepared by preheating the lysozyme solution to 85°C in the presence of purine, cooling, and removing the purine by dialysis. No turbidity formed in this solution on reheating to 85°C. However, they did sometimes become turbid on cooling to room temperature. In all these cooling curves in the absence of purine, whether or not turbidity appeared, the negative rotation was not at any time appreciably decreased from the value obtained at the highest temperature

region (85°C). Such irreversibility in rotation has been observed by Foss (12) for thermally denatured lysozyme in 0.1M potassium chloride. In the presence of purine, however (molal concentration, 0.3 to 4.0), the cooling curve is superimposable on the heating curve (fig. 5). This indicates that lysozyme can renature rapidly in the presence of purine. To test this conclusion, lysozyme was slowly heated to 80°C in 1.7 molal purine solution, and then rapidly cooled to 25°C. The purine was dialysed out in the cold and the soluble fraction, which constituted about 90-95% of the original lysozyme solution, was reheated to determine its T_m. As shown in figure 5, the T_m of the lysozyme solution so treated is essentially the same as that of the original solution before heating.

Similarly with BSA, there is very little decrease in negative rotation upon cooling from the denatured regions at 85°C in 0.1M K-phosphate, pH 6.5 phosphate buffer, in the absence of purine. This indicates irreversible denaturation as a result of heating above the T_m. In the presence of 4 molal purine, the cooling curve is superimposable on the heating curve. This is indicative of a rapid renaturation. Interestingly, as the concentration of purine decreases, say to 1.7 molal, only the upper part of the cooling curve simulates the heating curve. The lower part of the cooling curve deviates from the heating curve and exhibits a much higher negative rotation. This observation supports the idea that BSA renatures only partially in 1.7 molal purine. In the presence of 0.8 molal purine, the renaturation of BSA becomes hardly

noticeable. These results suggest that a higher concentration of purine is required for BSA than for lysozyme for the rapid renaturation process.

III. Comparison of the effect of purine and urea

A detailed study of urea denaturation of these proteins is clearly beyond the scope of this thesis. The intention here is merely to compare the effects of purine and urea on heating and cooling curves under a comparable set of conditions. There is no significant difference in the shapes of the heating curves of lysozyme in the presence or absence of urea, although the T_m changes (fig. 6). An entirely comparable result was found for ribonuclease. The comparison of effects of urea and purine on the ΔT_m is given in table 3. It can be seen that purine is usually two to three times more effective than urea. The kinetics of the denaturation of BSA in urea is complex, and therefore no simple comparison can be made. In 1 molal urea, however, the rotation of BSA remains essentially constant up to 49°C, and the shape of the transition is similar to that of BSA in the absence of urea up to 76°C. On cooling to 25°C, a slight but noticeable negative increase over the value obtained at the highest temperature (76°C) can be observed. In 2.2. molal urea, this additional increase in negative rotation becomes more noticeable. The same phenomena, i.e., the gradual time-dependent increase in negative rotation, can be observed if a BSA solution is left for a considerable length of time before cooling. These complications are typical of BSA denaturation by urea. The T_m reported at 1 and 2.2 molal urea

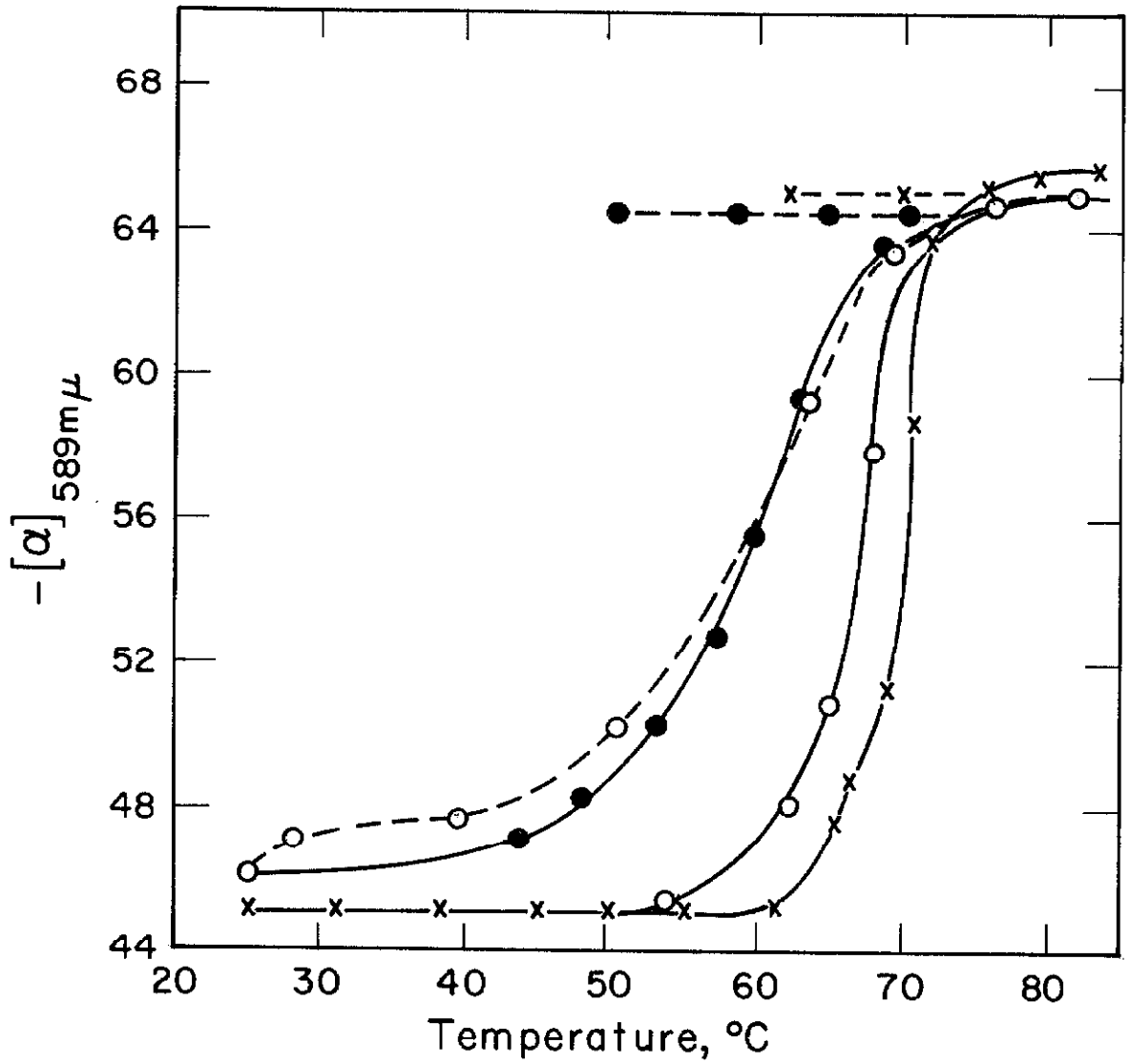


Figure 6. Profile of $-[\alpha]$ 589 versus temperature for lysozyme in 1.6 m urea (x——x); in 3.5 m urea (o——o); the dotted line which shows a decrease of $-[\alpha]$ with decreasing temperature is the first return profile; (•——•), second heating cycle 12 hours later; the return profile this time shows no change of $-[\alpha]$ with decreasing temperature.

Table 3. The Effects of Urea and of Purine on the T_m of Three Proteins

Proteins	T_m in buffer °C	Urea activity* (γ x m)	ΔT_m due to urea	Purine activity** (γ x m)	ΔT_m due to purine
Lysozyme	75.5	1.43	5.0	0.145	6.0
		2.78	8.5	0.231	10.5
		4.58	15.0	0.404	14.0
Ribonuclease	64	1.89	10°	0.145	6.0
		2.8	14°	0.231	9.0
				0.404	12.5
Bovine serum albumin (BSA)	66	0.925	5.0	0.231	6
		2.8	partially denatured at 25°C	0.404	11

* Scatchard et al. (32)

** Ts'o et al. (48)

concentration for BSA (table 3), however, refers to the middle of the transition obtained immediately after the plateau has been reached at elevated temperature. It does not take the time-dependent increase in negative rotation into consideration. Urea also tends to increase the negative value of rotation at the plateau region of high temperature. Kauzman (19) obtained a negative rotation of -74°C for BSA after it had been allowed to stand for 120 minutes at 60°C, and a value of -105°C for the same protein in 6.67M urea after 120 minutes. Foss (12) observed a similar increase in levorotation at the high temperature plateau region for lysozyme at higher urea concentrations. A similar increase can be observed as the concentration of urea is increased from 1.5M to 5M.

Under the condition tested, the cooling curve of ribonuclease in urea is also superimposable on the heating curve. However, the heat denaturation of BSA and lysozyme is not reversible in the presence of low concentration of urea (1 to 6.5 molal). Foss (12) observed the same irreversible denaturation in 3M and 5M urea.

Lysozyme solution becomes turbid on heating in 1.6 molal urea. In 3.5 molal urea, the cooling curve has a much lower T_m than the heating curve, but is essentially the same as the reheating curve carried out after 12 hours of storage at 25°C. After the second heating, the lysozyme solution becomes turbid on cooling (fig. 6). In 6.5 molal urea, again the cooling curve has a lower T_m than the heating curve, and the shape of the profile is broadened. After 12 hours at 25°C, the shape of the reheating curve appears to be sharpened, with the T_m lying between the heating and cooling curve (fig. 7).

IV. Increase of solubility of amino acids in the presence of purine

Interactions of amino acids with purine were investigated in an attempt to locate the site of interaction between proteins and purine. The solubilities of seven amino acids (glycine, alanine, leucine, histidine, tyrosine, phenylalanine, tryptophan tyrosine) in the absence and presence of purine were measured at various purine concentrations. The solubilities of glycine and of alanine were slightly decreased by 0.53 molal purine, and the solubilities of leucine and histidine increased

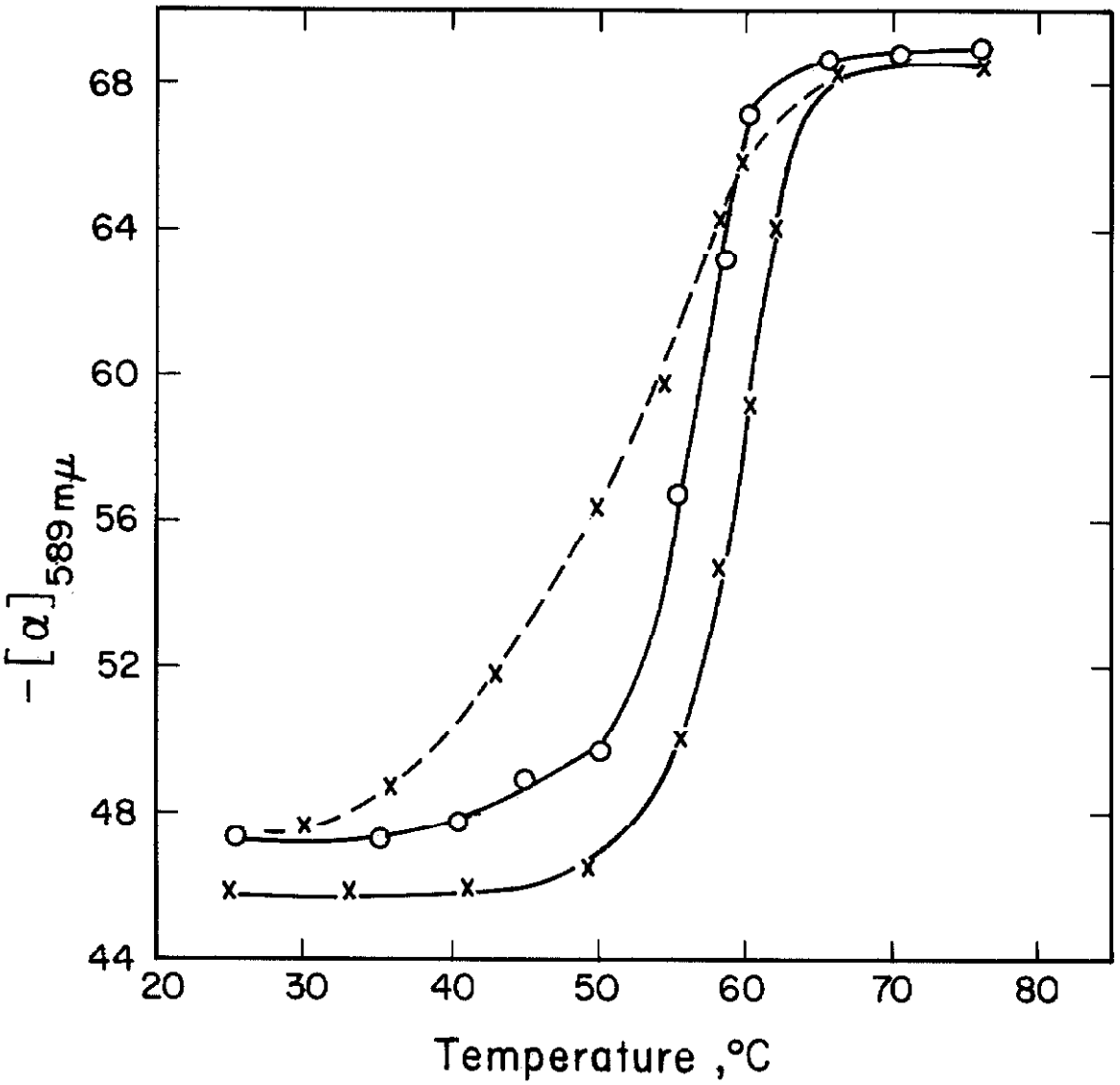


Figure 7. Profile of $-[\alpha]$ 589 versus temperature for lysozyme in 5M urea, 0.1M K-phosphate, pH 6.5, (x——x), and in the same 5M urea solution on second heating cycle 12 hours later (o——o). The dotted lines represent the return profile for the heating curves.

by approximately 10-20% (fig. 8 and table 4). The effect on the solubility of phenylalanine is larger by about 40%. The effects on tryptophan and on tyrosine are still larger, up to 300-400% in 0.53 molal purine (activity = 0.19). All changes in solubility are linearly related to activity of purine except for tryptophan . Interestingly enough, in 0.53 molal purine, the solubility of tryptophan is lower than in 0.4 molal purine. In 0.63 molal purine, the solubility is still further decreased (fig. 8). No conclusive explanation can be given of this observation. It can, however, be suggested that the purine-tryptophan mixture probably begins to form a more insoluble complex at higher purine concentrations.

Difficulties were encountered in the qualitative measurements of the solubilities of the poly-amino acids (polyalanine, polyphenylalanine, and polytryptophan) and the amino acid N-acetyl-amide derivatives (N-acetyl-L-tryptophan, and N-acetyl-L-phenylalanine) due to their low solubility in water. However, gravimetric measurements of the insoluble residue left when a known weight of the polymer or amino acid derivative is dissolved in a large volume of solvent, consistently show increased solubility for the aromatic amino acid derivatives and the polypeptides in 0.53 molal purine solution.

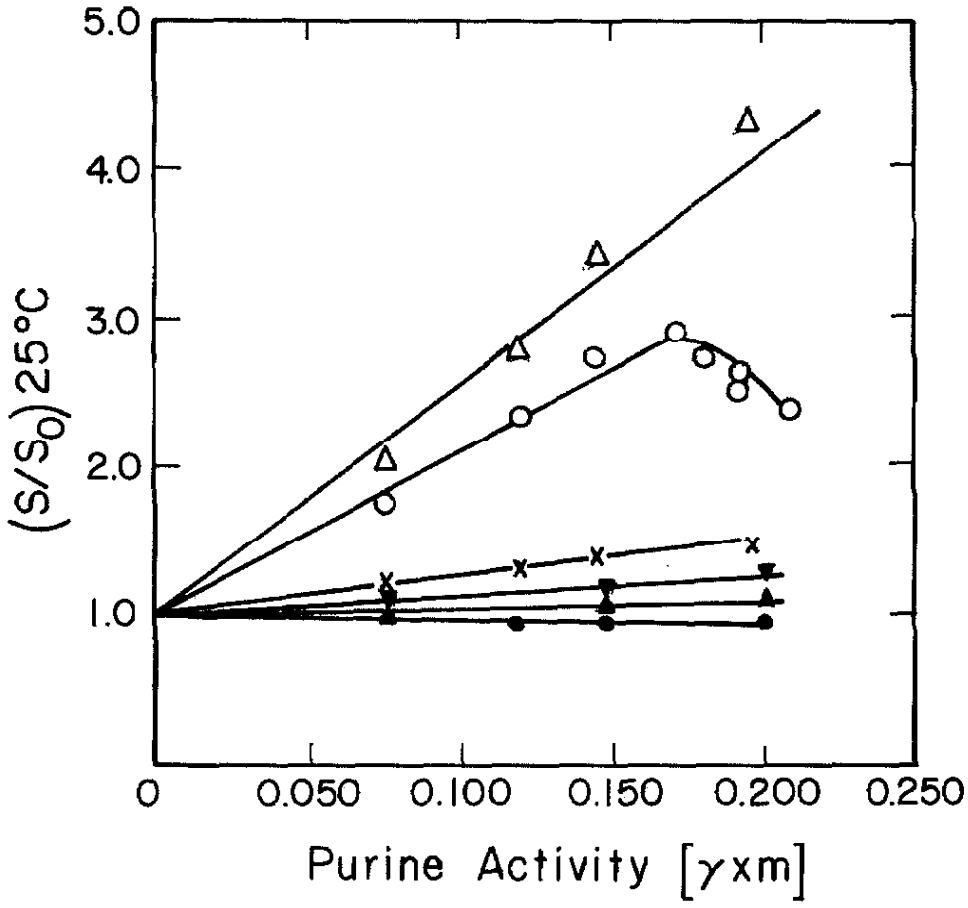


Figure 8. Plot of $\frac{\text{solubility in purine}}{\text{solubility in buffer}}$ (S/S_o) at 25°C, 0.1M K-phosphate, pH 6.54, versus the activity of purine (γ x m) for tyrosine (Δ), tryptophan (o), phenylalanine (x), glycine and alanine (●), histidine (▼), and leucine (▲).

Table 4. Solubility of Amino Acids in Purine

	Solubility gram/100 ml solvent 25°C	(S/S_o) 25°C	Purine activity (γ x m)
Tyrosine			
Buffer	0.0445	1.0	-
0.1 m purine	0.0912	2.05	0.073
0.2 m "	0.121	2.72	0.115
0.3 m "	0.149	3.35	0.144
0.5 m "	0.192	4.31	0.188
Tryptophan			
Buffer	1.201	1.0	-
0.1 m purine	2.090	1.74	0.073
0.2 m "	2.822	2.35	0.115
0.3 m "	3.311	2.75	0.144
0.4 m "	3.483	2.90	0.167
0.45 m "	3.180	2.65	0.177
0.5 m "	3.003	2.50	0.188
0.63 m "	2.942	2.45	0.198
Phenylalanine			
Buffer	2.895	1.0	-
0.1 m purine	3.474	1.20	0.073
0.2 m "	3.735	1.29	0.115
0.3 m "	3.995	1.38	0.144
0.5 m "	4.111	1.42	0.188
Glycine			
Buffer	25.12	1.00	-
0.2 m purine	24.12	0.96	0.115
0.3 m "	23.86	0.95	0.144
0.5 m "	23.86	0.95	0.188
Alanine			
Buffer	16.59	1.00	-
0.1 m purine	16.26	0.98	0.073
0.2 m "	16.09	0.97	0.115
0.3 m "	15.93	0.96	0.144
0.5 m "	15.76	0.95	0.188
Leucine			
Buffer	2.51	1.00	-
0.1 m purine	2.61	1.04	0.073
0.3 m "	27.2	1.08	0.144
0.5 m "	28.2	1.122	0.188

Table 4. Solubility of Amino Acids in Purine (continued)

	Solubility gram/100 ml solvent 25°C	(S/S_o) 25°C	Purine activity (γ x m)
Histidine			
Buffer	4.05	1.00	-
0.1 m purine	4.29	1.06	0.073
0.3 m "	4.74	1.17	0.144
0.5 m "	5.1	1.25	0.188

DISCUSSION

The results of the experiments will be discussed in three parts, (a) the effect of purine on the T_m of proteins, (b) the effect of purine on the solubility of the amino acids, and a simple comparison of this effect with that of urea, (c) the rapid reversibility of purine denaturation of proteins as compared with the more complex denaturation process which has been observed for urea. The above three features of the experiments will be used to deduce the possible mechanism of purine and urea denaturation of proteins in aqueous solution. The application of the findings will be briefly discussed in the last section of the discussion.

A. The Effects of Purine on the T_m of the Proteins

The plot of ΔT_m versus purine activity is linear up to 0.25-0.4 m activity (1-1.5 molal) (fig. 9). The slope $\frac{\Delta(\Delta T_m)}{\Delta \text{ Activity}}$ can be defined as the unit of effectiveness of purine in lowering the T_m of the proteins. The slopes for both lysozyme and ribonuclease are essentially the same, but slightly higher than that for BSA. Above 1.5 molal purine, the lines are no longer linear (the dotted line in fig. 9), but the entire curve may be considered to a first order approximation to consist of two linear regions, (a) and (b). The activity in the (b) region was calculated with the activity coefficient found at 1.1 molal (Ts'o et al., 48). If the difference in the slopes of (a) and (b) is entirely due to the use of incorrect activity coefficients, the difference between $\frac{\Delta(\Delta T_m}{\Delta \text{ Activity}}$ at the (a)

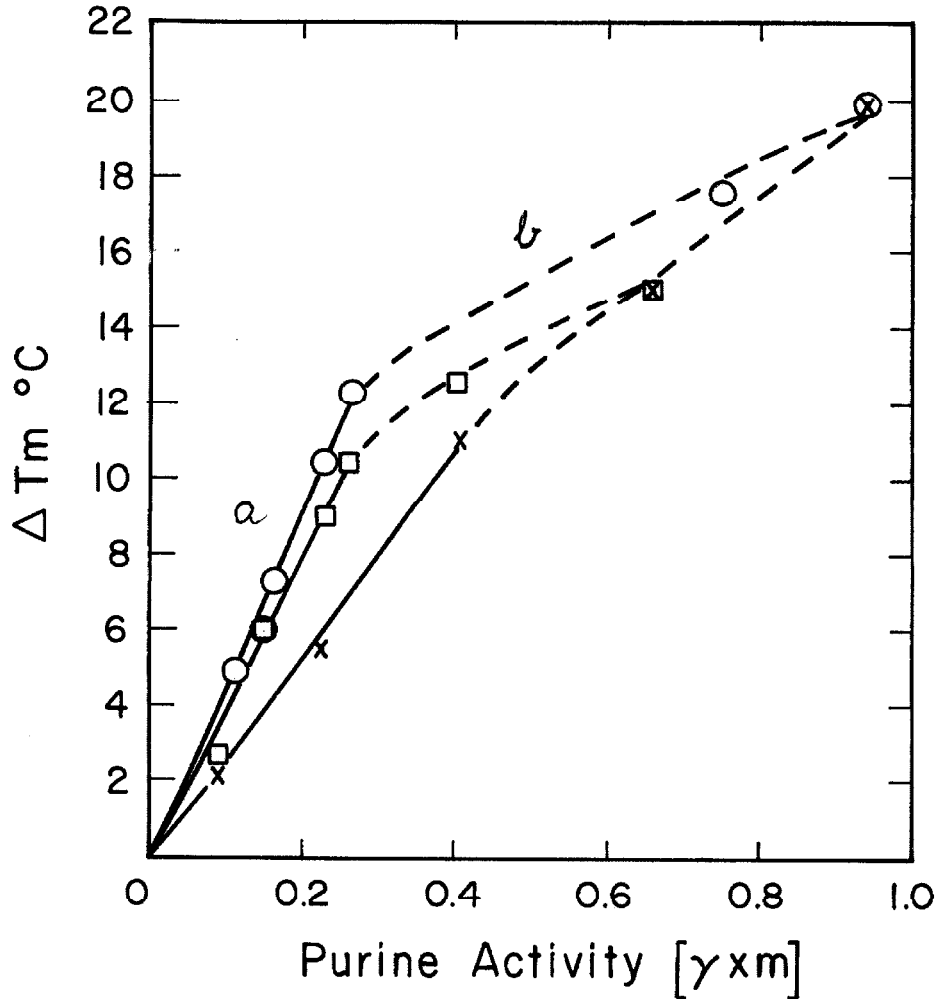


Figure 9. Plot of change in the transition temperature (ΔT_m) versus the activity (γ x m) of purine (0.1M K-phosphate, pH 6.54) for lysozyme (o), ribonuclease (□), and BSA (x).

portion and $\frac{\Delta(\Delta T_m)}{\Delta \text{ Activity}}$ at the (b) portion should be equal for the curves obtained with the three proteins. This can be seen from a simple treatment presented below:

The equation for the linear portion of the curves can be represented as: $y = A_n x + c$, where $\underline{x}$ is a variable and $\underline{c}$ is a constant. For the complete curves, including the non-linear portion, the deviation from linearity will be a linear function of $\underline{x}$, if the deviation from linearity is entirely due to the use of incorrect activity coefficients $\underline{x}'$ instead of the correct value $\underline{x}' - \Delta x$. Thus, one can write a general equation of the type: $y = A_n x - kx + c$ for any of the curves in figure 9. Taking the first derivative of the above equation, $dy/dx = A_n - k$. The value of the derivative of the equation in the (a) portion of the curve is A_n, and $A_n - (A_n - k) = k$. Since k is a constant at the same concentration, $\underline{c}$ in the (b) portion of all three curves, then it follows that the numerical value of $\underline{k}$ should be equal for the three curves under the above specified conditions.

Using the above approach, the difference between $\frac{\Delta(\Delta T_m)}{\Delta \text{ activity}}$ at (a) and $\frac{\Delta(\Delta T_m)}{\Delta \text{Activity}}$ at (b) gives a value of 1.82 for lysozyme, 1.69 for ribonuclease, and 0.31 for BSA at the same concentrations of purine in the (b) portion for the three curves. Thus, $\underline{k}$ is clearly a variable which, at least in part, should best be associated with the intrinsic properties

of the proteins. In other words, the deviation from linearity at higher purine concentration has to be attributed to some phenomenon other than the use of incorrect activity coefficient at higher purine concentrations. From the shapes of the curves, and from solubility data, it would appear as if at least part of the lowering of the effectiveness of purine at concentrations higher than 1.5 molal is due to the saturation of sites on the protein molecule.

B. Solubilization of Amino Acids by Purine and Urea

The solubility data give information about the nature of the protein side-chains which are presumably the reactive sites for purine. The data showed that the aromatic residues, such as tyrosine, tryptophan, phenylalanine, and histidine interact more with purine than with the side-chains of glycine, alanine, or leucine. This finding is consistent with other observations about the properties of purine discussed in previous sections:

(a) That the "polymerization" of purine in aqueous solution involves hydrophobic interaction and stacking, as indicated by its osmotic (Ts'o et al., 48) and nuclear magnetic resonance properties (Schweitzer, 35) in aqueous solution.

(b) That the interaction of purine with the polycyclic aromatic hydrocarbons is due to the formation of hydrophobic bonds (Booth et al., 1, 2).

(c) That the interaction of purine with the nucleic acids involves stacking of bases and formation of hydrophobic bonds similar to that found in the nucleic acid bases (Ts'o et al., 45).

Whitney and Tanford (52) have studied the effect of urea on the solubility of the amino acids. It is interesting to compare the effectiveness of purine with that of urea in enhancing the solubility of several amino acids. At equivalent activities, purine is 40-fold more effective than urea in solubilizing tyrosine, 12 times as effective in solubilizing phenylalanine, and over six-fold more effective in solubilizing leucine.

C. Reversible Denaturation of Proteins in Presence of Purine

The study of thermal denaturation of proteins in purine solution has revealed interesting properties of the action of purine. Besides lowering T_m, purine prevents an irreversible conformational change which occurs in the proteins when they are brought into the unfolded configuration at higher temperatures in the absence of purine. For instance, when lysozyme is heated at 80°C, it is irreversibly denatured in the absence of purine, but not in the presence of purine. The amount of purine required to obtain this rapid reversible denaturation differs among the three proteins studied. The difference may be expected to depend on the native conformation of the protein. On the one hand, purine facilitates the unfolding of the protein molecule, as evidenced by the lowering of T_m; on the other hand, purine prevents the unfolded

molecule from undergoing reactions that may result in irreversible conformational changes, and therefore keeps it rapidly renaturable upon cooling.

Suggested mechanism for purine-protein interaction

Any mechanism proposed for purine-protein interaction at this stage can only serve as a working hypothesis for future experimentation on this subject. This is because some of the physical and chemical properties of the proteins cannot as yet be defined in terms of meaningful universal physical-chemical parameters. In other words, each protein has certain physical-chemical properties which are an expression of the degree of its chemical complexity and conformational uniqueness. In order to formulate the tentative mechanism, however, it will be taken for granted that the individual peculiarities of the proteins are not very important in protein-purine interactions. This assumption is justifiable since the interaction of purine with proteins appears to involve specific side-chains on the protein molecule, and the degree of interaction appears to be a function of the number of interacting sites. In addition, it will be assumed that the interaction does not depend on the sequential distribution of these sites on the molecule. Thus, for our purpose, the proteins are treated as polymers in a specific conformation and with the interacting sites distributed at random on the macromolecule.

The lowering of the helix-coil transition temperature of such a polymer can be treated formally as if the interactant prefers to bind with the polymer in the coil, or unfolded state. The binding of the interactant

thus leads to a preferential stabilization of the unfolded state and facilitates the unfolding of the polymer. This proposal was first made by Peller (28) on theoretical grounds. It has been well substantiated by the work in this laboratory and elsewhere on the interaction of purine and nucleosides with nucleic acids (Ts'o et al., 44; Ts'o and Lu, 46; Ts'o et al., 47). Thus, the problem of reduction of T_m can be examined on the basis of interaction through specific binding.

Purine compared to urea, on activity basis, is 6-10-fold more effective in lowering T_m of proteins (table 3). Urea is probably one of the best hydrogen bond formers. It is very unlikely that the superiority of purine over urea in reducing T_m of protein is due entirely to a greater ability of purine to form hydrogen bonding with protein in aqueous solution. The above deduction is based on the analysis discussed in previous sections on why urea will not form strong hydrogen bonding in water. This notion is substantiated by the results of studies on the interactions of purine in solution. The aggregation does not appear to arise from the formation of hydrogen bonding between purine molecules in aqueous solution.

The mechanism of urea denaturation has been reviewed in previous sections. Apparently, urea breaks hydrophobic bonds by disrupting the hydration lattice around the hydrocarbon side-chain of protein molecules (Klotz and Stryker, 21; Kauzman, 18). This mechanism of urea denaturation has more experimental support than the hydrogen

bonding mechanisms originally proposed for urea denaturation. As mentioned previously, purine interacts with the aromatic side-chains in preference to the aliphatic side-chain. Furthermore, there appears to be a correlation between the superiority of purine over urea in its solubilizing influence on the aromatic amino acids and its reduction of the T_m of these proteins. These facts strongly indicate that hydrophobic interaction (purine aromatic side-chain overlap) is the most likely mechanism for the interaction between purine and protein, as well as for purine and nucleic acids.

The following concept of the interaction of purine and proteins emerges from a consideration of the results discussed so far. Purine binds to proteins through hydrophobic interaction, principally with the aromatic side-chains. This binding weakens the hydrophobic forces which hold the protein molecule in its native conformation. The binding of purine, however, also provides shielding for the reactive group exposed in the unfolded molecules. For instance, the bound purine may prevent the approach of water molecules to the exposed sites on the peptide chain, and thus prevent the formation of a random intra- and interpeptide hydrogen bonding. It may shield the labile -SH group and thus prevent it from a random exchange reaction. Without the protection of the bound purine, the subsequent reaction of the exposed groups of these polypeptide chains can lead to aggregation or other irreversible changes which prevent rapid renaturation to the original conformation.

This deduction leads to a clear distinction between the mechanism of purine denaturation and the mechanism of urea denaturation of proteins. All the evidence mentioned above points to the notion that purine combines directly with the side-chains through hydrophobic interactions, and thus weakens the hydrophobic forces of the polymers by its immediate action. On the other hand, all the current findings and concepts discussed above on urea denaturation point to the notion that urea interacts with the water layer around the polymer. It is conceivable that the interaction with the hydration layer involves the hydrogen bonding of urea with the water lattice around the protein side-chain. This would result, therefore, in the weakening of the hydrophobic forces through an indirect action. It is therefore not surprising that purine is much more effective than urea in reducing the T_m of both proteins and nucleic acids.

The kinetics of urea denaturation of proteins is complex with respect to temperature and urea concentration. Studies on urea denaturation of lysozyme shown above (figs. 6 and 7) clearly indicate the complexity of urea denaturation. The shape of the heating profile of the reheated solution is a function of time. The shape of the cooling profile is a function of urea concentration, and the minimum negative rotation attained on cooling from the high temperature-insensitive zone to room temperature (25°C) is also a function of time. Apparently, the renaturation of lysozyme in urea solution goes through a series of intermediate steps, some of which are rapid, and some others very slow. Because

of the slow steps in the renaturation process, secondary reactions involving interpeptide hydrogen bonding, --SH exchange, and perhaps interpeptide hydrophobic interactions, are able to take place before a complete renaturation can be attained. This may result in a partial renaturation of the proteins, depending on the extent of the interpeptide interactions. Since these are physical-chemical reactions involving the proteins, urea, and their solvents, the extent of the interpeptide interaction depends to a large extent on the concentration of the interactants, the solvent, and the temperature. Such complicated relationships were rarely observed for the purine denaturation of proteins. Its action, as compared to urea, is fast and simple.

Possible Applications of Findings

There are two broad implications of the above findings. From both experimental and theoretical grounds, it is clear that the secondary structure in nucleic acids is much more sensitive to the influence of purine than the secondary structure in proteins. The advantage of this difference has been magnified by a recent finding (Sander and Ts'o, 31) that nucleic acids in the coil form are much more sensitive to the effect of purine than nucleic acids in the helical form. After heating DNA or RNA in the presence of 0.5M purine to temperatures above its T_m, optical studies indicate that there is no secondary structure left in nucleic acids upon cooling. The purine is so dilute in this solution (0.5M) that it has no effect on the protein subsequently dissolved in it. It is possible,

therefore, to obtain an aqueous solution of protein and nucleic acid in which the secondary structure of the protein is intact, while the secondary structure of the nucleic acid is reduced to a minimum, such as will be obtained for the nucleic acids at elevated temperatures (95°-100°C).

The hydrophobic and stacking interaction of purine with the aromatic side-chains of proteins may prove to be of importance in the specific interaction between nucleic acids and proteins, such as in viruses and the ribosomes. The hydrogen bonding sites in the peptide backbone are not likely to offer any specificity in this interaction, since they represent a common property of proteins, and probably remain, for the most part, unexposed for such interactions.

SUMMARY

The effect of purine on the transition temperature (T_m) of BSA, ribonuclease, and lysozyme has been studied by optical rotation at 589 mμ. It is found that purine decreases the T_m of these proteins in proportion to the activity of purine in solution. In appropriate concentration of purine (higher required for BSA), the thermal denaturation of lysozyme and BSA is instantly reversible, a phenomenon not observable in the absence of purine. In comparison to urea on activity basis, purine is 6-10-fold more effective in lowering the T_m of the proteins. Purine (0.3 molal) increases the solubility of tyrosine (300%), of tryptophan (250%), of phenylalanine (40%), and of histidine (15%), but does not increase the solubility of glycine and alanine. Secondary structures of proteins appear to be much less sensitive to the influence of purine than those of nucleic acids. Purine may serve as a selective denaturant for nucleic acids in protein solution. A distinction between the mechanism of purine denaturation and urea denaturation has also been made.

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PART III

BINDING OF BASIC PROTEINS TO DNA

INTRODUCTION

It has been established beyond doubt that the DNA in the cell nucleus is a carrier of genetic information. It is known too that the cell nucleus contains a large amount of basic protein in addition to DNA and other variable chemical components. In most cells, only about ten per cent of the negative charges on the DNA are neutralized by inorganic cations (Peacocke, 27). This immediately leads one to speculate that there might be some kind of chemical interaction between basic proteins and DNA in the cell nucleus.

The above speculation raises many fundamental questions. Does the DNA-protein interaction have any specific biological function, and, if so, what kind? Does the interaction alter the basic structure of DNA, and, if so, how does this affect the biological functions of DNA? Does the interaction involve any specificity with respect to the DNA and the interacting proteins?

Questions of this kind and many more can be answered only if one knows (a) the chemical and other properties of the basic proteins, (b) the structure of the DNA before and after interaction with the basic protein, (c) the nature of the interaction between DNA and basic protein, and the nature of the complex formed as a result of such interaction. In this section, a very brief review of findings and speculations on these problems will be presented.

A. Protamines and Histones

The protamines are basic low molecular weight proteins (av. M. W.=10,000). Arginine constitutes over 90 per cent of their basic residues, and about 60-70 per cent of their total amino acid composition. The protamines do not contain tyrosine, tryptophan , methionine, or cystine. Because of their basic character, the protamines can form insoluble complex with several polyanions, including DNA. The amino acid sequence of the protamines is not known. From partial hydrolysis of a heterogeneous mixture of protamine, it has been suggested that the molecule consists of units having the composition MAA where M is a mono amino acid residue other than arginine, and A an arginine residue (Felix, 14). However, different authors disagree on this point (Ando et al., 1). From N-terminal amino acid analysis, and from the electrophoretic patterns of protamines in the presence of different cations, pH, and ionic strength, it has been concluded that the protamines are very heterogeneous, and probably aggregate to some extent in solution (Felix et al., 15; Ando et al., 1; Scanes et al., 31; Rauen et al., 29) The protamines are all in the extended β-conformation at neutral pH.

The histones, like the protamines, are basic proteins, but of a larger molecular weight (av. M. W. = 10,000-30,000). The basic residues of histone include lysine and histidine in addition to arginine. The histones are very heterogeneous, the components differing not only

with respect to molecular weight, but also with respect to amino acid composition. Acid extracted calf thymus histone can be fractionated into four distinct fractions by chromatography on Amberlite IRC columns in the presence of guanidium chloride solution (Stake et al., 30). The four principal fractions are termed: Ib (arg/lys ratio 0.1), IIb (arg/lys ratio 0.6), III (arg/lys ratio 1.4), and IV (arg/lys ratio 1.4). Each one of these fractions is non-homogeneous (Rasmussen et al., 28). Fractionation of histones can also be accomplished by other methods--such as by electrophoresis (Davidson et al., 9), or by alcohol precipitation (Gregoire et al., 16). In this manner, six or more fractions of histone have been obtained from calf thymus histones.

Optical rotatory dispersion measurements indicate that the heterogeneous histone mixture is not in the α-helical conformation in water. However, a film of histone cast from water shows some helical structure with a $b_o = -237$ (Bradbury et al., 5). Similarly, histone dissolved in ethylene chlorohydrin appears to increase in helical content which decreases with aging. Doty and co-workers (10, 11) have previously estimated an α-helical content of 25-40 per cent for histone in water from the values of its negative rotation in water and in concentrated urea solution, and also from optical rotatory dispersion studies. Recent investigations of Bradbury et al. (5),on the other hand, concluded that histone has no helical content in water.

Histones, especially fractions III and IV, tend to aggregate in solution. The details of the mechanism of aggregation are yet to be investigated. Like the protamines, the histones also bind to polyanions, and to several anions. All evidence points to the likelihood that the structure of histone on the polyanions (DNA) and on other solid surfaces is not the same as in aqueous solution.

DNA in Nucleoprotamine (DNP) and Nucleohistone (DNH)

Studies of Bradbury et al. (5) and of Zubay and Doty (36) clearly indicate that the DNA in DNP or DNH is double-stranded and in the B-configuration. In other words, the interaction of histone or protamine does not lead to any gross change in the structure of DNA as proposed by Watson and Crick. The DNA in DNP is very similar to the DNA in sodium or lithium deoxyribonucleate. Thus, the DNA in DNP or DNH has all the general properties of native DNA described in Part I of this thesis.

Nucleoprotamine and Nucleohistone

Nucleoprotamine occurs in the head of fish sperms. The DNA and protamine are bound together by electrostatic interaction. The bound protamine has no α-helical structure. According to Bradbury et al. (5), the polypeptide chain is wrapped around the DNA helix in a partly folded and partly extended form, with the folds occurring at the paired non-basic groups. The side chains of the basic residues in the

extended part of the protamine are stretched out at right angles to the main chain, and thus permit the arginine residues to combine, by electrostatic interaction, with the ionized phosphate group of the DNA. The extended polypeptide chain lies in the shallow groove of the DNA helix, which is inclined to the helical axis by about 50°.

Luzzati et al. (21) have studied the nucleoprotamine gel by means of X-ray diffraction. At concentrations of 5-50 per cent (wt. of DNA/wt. of gel), the nucleoprotamine-water system contains two phases, namely, the saturated solution and a paracrystalline phase. At higher concentrations, only the paracrystalline phase exists. The X-ray diffraction pattern of unfixed trout sperm head is identical to that of the paracrystalline phase from the gel water system. There is as yet no explanation for the phase transition in DNP. It is possible that the phase transition is a reflection of some physical-chemical changes.

DNP complex can be dissociated in high salt (2M NaCl), as anticipated from the electrostatic nature of the interaction between protamine and DNA. However, the complexing of protamine to DNA does not appear to increase the T_m of the DNA (Huang et al., 17).

Nucleohistone (DNH) is present in the nuclei of the somatic cells of plants and animals. Isolated DNH has been studied by several investigators. Its general properties can be summarized as below:

(a) The predominant force in the interaction appears to be electrostatic. Under certain conditions, it is possible to obtain soluble and fully dispersed DNH molecules in solution.

(b) The protein is evenly distributed on the DNA (Zubay and Doty, 36) and the ratio of DNA to protein in the isolated complex is around 1:1 (Zubay et al., 36); Verndreley et al., 34).

(c) The histone on the DNA (in DNH) has about 50 per cent α-helical content. This conclusion is drawn from the observation that a fraction of the polypeptide amide exchanges very slowly with D_2O (Bradbury et al., 5), and from the characteristic nature of the amide II I. R. band of the polypeptide.

(d) DNH can be dissociated in the presence of high concentration of salt (1-2M NaCl). This observation supports the notion that the interaction is electrostatic in nature. However, the dissociation does not start to occur until the concentration of the sodium chloride is above 0.3M, and it is only about 70 per cent complete in 1M sodium chloride (Bayley et al., 3).

(e) The E_p of DNH is about 10 per cent higher than its DNA (Bayley et al., 3). Furthermore, the T_m of DNH is higher than that of its DNA component under identical experimental conditions (Huang et al., 17).

(f) DNH has a great tendency to aggregate, especially at higher concentrations. The DNH-water system has been observed (Luzzati et al., 21) to undergo several phase transitions, depending on the weight ratio of DNA to gel. At low concentration of DNH, the solution is homogeneous. At higher concentration of DNH, a phase transition occurs.

At still higher concentrations, a third and a fourth phase transition may take place. Each phase can be characterized by its X-ray diffraction pattern, and each phase exists only within a small concentration range. However, two phases can exist in equilibrium with each other. The unfixed fowl erythrocyte nuclei DNH gives a diffraction pattern similar to that obtained after the first phase transition.

(g) No proposed detailed molecular structure of nucleohistone can be regarded as conclusive. Some authors suggest an extensive cross linkage for DNH in water (Luzzati et al., 21), while others suggest that the histone is wound around DNA in a very specific manner, with the α-helical portion of the histone accommodated in the larger groove of the DNA helix (Zubay et al., 36), and the DNA phosphate ion oriented to allow for adequate electrostatic interaction between the basic residues of the protein and the negative charges on the DNA (Bradbury et al., 5). In all of these proposed structures, the conclusions are based on indirect evidence. Even the X-ray data are not definitive enough in these studies to specify how histone is specifically attached to the individual molecules of DNA.

(h) The artificially reconstituted soluble DNH has some properties similar to those of soluble native DNH. The artificially reconstituted DNH has a higher T_m than its DNA component. Its U.V. spectrum and its activity as primer in DNA-dependent RNA synthesis is very similar to that of the native DNH (Huang et al., 17). Interestingly

enough, the electrophoretic mobilities of the DNH from the four histone components (Ib, IIb, III, and IV) are different from one another (Olivera, 26). This may be attributed to the differences in their amino acid composition. It may also be attributable, in part, to differences in their ability to completely neutralize the negative charges on the DNA due to differences in the sequence of the basic amino acid residues. The average sedimentation coefficient for the native DNH (soluble) is about the same as that of the artificially reconstituted DNH (Huang et al., 17).

A very fundamental but challenging question still remains to be answered. Is the isolated or reconstituted soluble DNH an artifact derived from the real complex as it exists in the cell nucleus? The answer to this question is not available. Several investigators (Dounce et al., 12, 13; Kirby et al., 19; Mirsky and Ris, 24) claim that it is, and that this artifact may not necessarily bear any relationship to the in situ DNH. Dounce and co-workers have isolated and characterized a residual basic protein which they claim is a part of the DNH complex in the cell nucleus, and is responsible for its gelation. DNAase and certain proteolytic enzymes dissociate this residual protein, which, presumably, is covalently bound to the DNH in the cell. Reasoning along this line, Zubay has postulated that the histones may not necessarily be specifically wound around the DNA in the cell. The histone, he suggested, is merely a connecting tissue for chromosomal DNA. Thus,

these authors believe that the soluble DNH is merely an artifact of preparation.

It appears to this author that these conjectures are based more on our ignorance than on our knowledge about the properties of nucleohistones. The residual protein, if it exists as defined, constitutes only about 5-10 per cent of the total basic proteins interacting with the DNA. From the data of Dounce and others, its interaction is rather localized, and from the data of Mirsky and others, the histones or protamines practically bathe the DNA in the nucleus. So far, no other positively charged molecular components have been found in the nucleus in excess of the histones or protamines. One will therefore be tempted to consider an extensive interaction between the histone or protamine and DNA of the nucleus. Whether or not this interaction involves an extensive cross linkage can be studied by in vitro experiments on the mixture in appropriate solvents. If the interaction is as specific as depicted by Peacocke and others, one might have to invoke the participation of specific short-range interactions other than, and in addition to, electrostatic ones, to explain this specificity. Thus, the specific interaction which has been associated with soluble DNH represents one kind of interaction which can occur in the cell between DNA and histone. The fact that this more specific type of interaction rarely occurs in the cell simply means that a third component within the cell acts to decrease the energy of interaction for the secondary short-range interaction

responsible, in part, for the more specific intermolecular interaction observed in the isolated soluble molecules. Thus, the unknown third component merely acts to enhance the more random electrostatic interaction at the expense of the other specific short-range interactions. These considerations indicate that studies of DNH, even if it is artifact, are an indispensable step toward understanding the nature and interactions of DNH and DNP within the cell nucleus. Indeed, such studies represent the mechanistic approach to the study of the interaction of DNA and histone in the cell. Whether or not such studies are carried out in the presence of all the ingredients present in the cell nucleus is not relevant to the problem at this initial stage. As a matter of fact, the mechanistic approach merely supplements the cellular approach, but it gives, in addition, more room for speculation about the relationship of DNA-histone interactions to the mechanisms involved in cellular function.

PURPOSE OF EXPERIMENTS

The apparent binding constants of protamine, poly-L-lysine, and histone fractions Ib and IV are studied by equilibrium dialysis. The binding constants are measured as a function of salt concentration. All binding constants are measured using a large DNA: protein ratio. In all cases, the ratio of protein to DNA is only varied between 1:11 and 1:9. The ratio of protein to DNA is maintained small and relatively constant for the following reasons:

(a) The apparent binding constant measured at measurable protein:DNA ratio is different from the intrinsic binding constant, and varies with the ratio of protein to DNA. At a small and relatively constant protein/DNA ratio, comparisons can be made of the apparent binding constants obtained under different experimental conditions.

(b) The interaction of the basic polypeptides and DNA in water often leads to rather complex phase transitions. At low concentration ratio of protein to DNA, the system remains homogeneous.

The method of analysis does not permit one to use extremely low concentrations of polypeptides, a situation which would allow one to obtain the intrinsic binding constants and at the same time eliminate the possibility of measurement in a two-phase system.

Measurements of binding constants for DNA protein interactions can give useful information about the nature of their interactions. If the

interaction is suppressed in the presence of neutral salt solution, it can be concluded that the charges on the interacting polymers are being shielded by the counterions from the neutral salts. This deduction will be particularly true if the extent of the inhibition of the polymer-polymer interaction by the salt is dependent on the valence of the interacting cations or anions. In other words, the inhibition obeys the double valence rule. Later in this thesis, this will be shown to be the case with DNA-basic protein interaction.

The cooperative nature of interacting molecules can often be deduced from data on their binding constants as a function of different ratios of the interactants. A plot of $\log \left(\frac{r}{n-r}\right) - \log C$ (where C = concentration of monomer and $\underline{n}$ and $\underline{r}$ are as defined below) against $\log \underline{r}$ will give a curve whose shape can give information about the cooperative nature of the interacting molecules (J. Bjerrum, 4). This approach is not possible with DNA-protein interactions because of the experimental and theoretical difficulties involved in calculating $\underline{n}$ and $\underline{r}$. However, the shape of the curve obtained when the apparent binding constants at different salt concentrations are plotted against the ionic strength of the medium can give information about the cooperative nature of the interaction. This point will be further discussed in later sections.

Finally, since the biological and certain physical-chemical properties of the protamines and histones appear to be dependent on their arginine/lysine ratio, an attempt is made to establish a correlation

between nk, the binding constants, and the arginine/lysine ratios for the different proteins studied.

EXPERIMENTAL

The dialysis tubing was purchased from Visking Co., Div. of Union Carbide Corp., Chicago. To increase its porosity, the cellophane dialysis bag (20/100) was treated with zinc chloride solution as follows (McBain and Stever, 22 and 23):

> The bottom of a clean rectangular dish was covered with a 65-67% solution of zinc chloride. The cellophane bag was laid flat in the solution. More zinc chloride solution (65-67%) was added to the dish to cover the cellophane bag. The bag was left immersed in the zinc chloride for 15-20 minutes at about 10°C, the temperature at which the swelling of the bag is maximum. The zinc chloride was carefully suctioned out, and the cellophane bag covered with distilled water at room temperature (22-25°C). The dish was rotated to speed up the diffusion of the zinc chloride. After a few minutes, the membrane was removed and washed free of zinc chloride with dilute solution of hydrochloric acid. The acid was washed off with water until the pH of the solution was above 5.0. The membrane was stored in 50% glycerol at 4.0°C. In presence of glycerol, the membrane retains its permeability for a long time, depending on the temperature at which it is stored.

Cellophane bags treated as described above are permeable to fully dispersed histone IV fraction. The untreated bag (20/100) has a 50% escape time of 27 hours for ovomucoid ($\bar{M}_W$ = 28,000) (Craig, 8). The zinc chloride treated bag was washed several times with a large volume of water at 4.0°C before using for the dialysis experiments.

Calf thymus DNA was purchased from Nutritional Biochemical Corp., N. J. Approximately 4 mg of DNA was dissolved per 1 ml of buffer (0.015M Na-citrate , 10^{-4}M EDTA, pH 6.6). About 100 ml of the mixture was shaken on a Burrell-Wrist Action shaker in the presence of one to two drops of chloroform (0.05-0.1 ml) for 48 hours. The viscous homogeneous solution was centrifuged at about 10,000 rpm for 10 minutes, and the supernatant re-centrifuged at 40,000 rpm for 12 hours to obtain DNA of large molecular weight. The pellet was dissolved in the buffer (60-70 OD/ml) and dialysed against a large volume of 1M sodium chloride for 12-16 hours in the treated cellophane bag, and finally against three exchanges of approximately 10 times its volume of buffer for two hours each. In this way, any protein contaminants in the DNA, which might dissociate into the solution at higher salt concentrations, are eliminated. The DNA concentration is then adjusted to about 3 mg per ml. An aliquot was denatured by heating in a sealed tube immersed in a bath at 92-95°C for 15 minutes. The boiled solution was rapidly cooled at 4°C. The fraction denatured this way is described hereafter as denatured DNA.

Poly-L-lysine was purchased from Pilot Chemical Company, Watertown, Mass. The average molecular weight of the sample used is 9,000. Protamine sulfate was purchased from Nutritional Biochemical Corp., N. J. The histone fractions were kindly provided by Prof. Bonner and Dr. Huang. Protamine sulfate , poly-L-lysine, and histone Ib were separately dissolved in the buffer (0.015M Na-citrate, 10^{-4}M EDTA, pH 6.6) in the amount of approximately 0.4-0.5 mg/ml. Histone IV was dissolved in 0.2N hydrochloric acid (0.5-0.6 mg/ml), and then brought to pH 6-7 with 2M sodium hydroxide. The protein solution (hist. IV) was dialysed against two exchanges of the buffer (50X the volume of the protein solution) to remove the excess salt. The protein solutions (protamine, poly-L-lysine, histones Ib and IV) were separately diluted 10X with the buffer containing the appropriate salt concentration. A known volume of each of the solutions was used directly for the binding experiments. All the other compounds were of reagent grade.

In the equilibrium dialysis experiments, 6 ml of the protein or polypeptide prepared as described above was added to a 12-ml Servall tube. One ml of the native or denatured DNA (approximately 3 mg/ml) was added to the zinc chloride treated bag, and the bag was tied at both ends without appreciable stretching. A large air bubble was left in the bag to allow thorough mixing during equilibration. The cellophane bag containing DNA was checked for leakage and then washed several times

with the buffer to get rid of any DNA solution not enclosed within the dialaysis bag. The DNA in the dialysis bag was immersed in the protein solution contained in the Servall tube. The tube was stoppered with a serum stopper. The stopper was held in place with a time tape. The tube was rotated at about one revolution per second for about 80 hours at 6-7°C.

To check if equilibrium was attained, histone fraction IV, which has the largest molecular weight of all the proteins used, was made to a concentration of about 15 microgram protein nitrogen per ml. Six ml of this solution was dialysed against 1 ml of the buffer in the dialysis bag. The concentrations of protein inside and outside the bag were checked after 70 hours of equilibration. The concentration of protein inside the bag was within 3-5% of the protein concentration outside the bag. This indicates that equilibrium is attained in 80 hours. To check this, the equilibration of protamine was carried out in 65 hours, instead of 80 hours, against native DNA. The binding constant obtained under this condition is within 10% of that obtained for 80 hours equilibration.

Two controls were set up. In one, 1.0 ml buffer was substituted for the DNA in the dialysis bag, and dialysed against 6 ml of the protein solution used in the equilibration experiment. In the other control, 6 ml of the buffer was substituted for the protein solution in the Servall tube and the buffer dialysed against 1.0 ml DNA solution in the dialysis tubing as described above. The first control allows one to determine the amount

of protein, if any, that sticks to the bag. This can be obtained by analyzing the protein concentrations inside and outside of the bag at the end of the equilibration. The difference between the total amount of protein concentration before and after equilibration gives the amount of protein that sticks to the bag. In all cases, this represents a small percentage (0-10%) of the initial protein amount. This control also allows one to check if equilibrium has been attained within the equilibration period. In all subsequent experiments, the analysis of the protein outside the bag was used for the calculation of the total protein concentration. The total concentration of any protein used was always between 9-11 μg protein nitrogen/ml. The second control allows one to analyze the amount of DNA or any material on the DNA which dialyses into the solution outside of the bag. The unbound protein material outside the bag was measured directly as described below, and corrected for materials which dialyse out of the bag (DNA) and gives ninhydrin reaction on hydrolysis. The correction is high but reasonably constant for all the experiments (10%$\pm$3% of total protein nitrogen added).

The physical characteristics of the complex in the dialysis bag and of the free protein in the Servall tubes were checked visually. In no case was the precipitation of the protein observed. At the concentration ratio of protein to DNA used, the solution of the complex within the bag appears homogeneous in all cases.

The concentrations of the DNA were determined spectrophotometrically using the extinction coefficient (E_P, 260 mμ) of 6.6×10^{-3} (Ts'o et al., 32) for the DNA in native form. The extent of denaturation of the denatured DNA was checked by determining the melting profile in the Beckman DK-2 ultraviolet spectrophotometer fitted with a modified temperature control device (Ts'o et al., 33).

The concentration of the protein was determined by the ninhydrin colorimetric method (Moore and Stein, 25). The protein was hydrolyzed in 6N HCl for 20-21 hours at 103°C in a sealed 5 ml ampule. The sealed tip of the ampule was filed off , and the contents of the ampule dried under nitrogen at about 60-70°C. The dried hydrolysate was diluted to 1-5 microgram nitrogen with a known volume of water. The concentration of the amino nitrogen was determined as described in Part II of this thesis.

In all cases, duplicate equilibration experiments were run at least twice. The results from two separate experiments were in all cases within about 10 per cent of each other. For each experiment, quadruplicate ninhydrin analyses were carried out. Each of the recorded values for $nk_{apparent}$ is an average from eight ninhydrin analyses. These exhibited a spread of about 10 per cent.

ANALYSIS OF DATA

In a polymer monomer interaction not involving charge neutralization, the extent of binding of the monomer (A) to the polymer (P) can be represented by the parameter r, where

$$r = \frac{\text{moles bound (A)}}{\text{moles total (P)}} \qquad \text{(Klotz, 20)}$$

Using the approach of Klotz (20), it can be shown that

$$r = \frac{nk\,(A)}{1 + k\,(A)} \qquad (1)$$

where k is the binding constant for a single binding site and n represents the number of independent binding sites on (P). Thus, nk is the binding constant for a polymer-monomer interaction, when the polymer (P) has n independent binding sites.

The above equation does not take the electrostatic effect into consideration. If the interaction of (A) and (P) is affected by the charges on (A) and (P), a modified equation of the type:

$$\frac{r}{(A)}\, e^{2wr} = kn - kr \qquad (2)$$

should be written for the binding of a monovalent monomer to a polyvalent polymer (Klotz, 20). w (in equation 2) $= \frac{N(z')^2 e^2}{2RTD}\left(\frac{1}{b} - \frac{K}{1+ka}\right)$ (all the terms are as defined in Part II of this thesis). The evaluation of w is often not possible due to the limitations of the data that are usually available.

The situation becomes more complex when the interaction is between two polyvalent polymers. The estimation of the statistical factor becomes difficult, since it is no longer equal to $\left(\frac{n - i + 1}{1}\right)$ estimated for neutral polymer-monomer interaction. In other words, equation 1 and equation 2 cannot be used to calculate the binding constants for a charged polymer-polymer interaction without making the following assumptions:

(a) It will be assumed that a comparative nk can be calculated for the interaction of all the proteins studied without taking the electrostatic effect into consideration. The assumption has no theoretical or experimental justification. It is made because the data for calculating $\Delta \bar{F}_{elec}$ in these reactions are not available.

(b) It will be assumed that a comparable set of data can be obtained by treating the polymer-polymer binding as a polymer-monomer binding. As in (a), assumption (b) has no strong theoretical or experimental basis. However, since the DNA-protein ratio is very large, it can be argued that the statistical factor is not as involved as it would be if the polymer:polymer ratio approached 1.0.

With the above two assumptions, the binding of basic protein to DNA can be represented by equation 1 above. With an additional assumption that the number of sites on the DNA occupied by proteins is small and negligible compared to the total number of available binding sites, a simplified equation can be written for nk.

$$nk = \frac{\text{(moles protein nitrogen bound)}}{\text{(moles unbound protein nitrogen)(moles total DNA phosphate/liter)}}$$

In the above analysis, $\underline{n} \cong 1.0$. Furthermore, a simple calculation from the data (Table 1) indicates that about 25% of the negative charges on the DNA are neutralized by the bound protamine at low ionic strength. Since all the measurements of $\underline{r}$ were made at a measurable concentration of proteins, the $\underline{nk}$ calculated from the above equation will be referred to as $nk_{apparent}$. Because of the several uncertainties in calculating $nk_{apparent}$, no significance is to be placed on its magnitude. However, comparisons can be made between the relative magnitude of $nk_{apparent}$ for the different proteins and polypeptides studied.

The nk for the binding of arginine and lysine have been determined by Jardetsky (18). The nk for lysine and arginine at pH 7.0, ionic strength = 5×10^{-4}, is 400 liter moles^{-1}.

RESULTS

The experimental results will be presented in four parts: (a) a comparison of the apparent binding constants obtained for native and denatured DNA, (b) variation of apparent binding constant with the type of basic polypeptide bound to DNA , (c) effect of sodium chloride on the apparent binding constants, and (d) the effect of magnesium chloride on the observed binding constants.

A. Binding constants in native and denatured DNA

The values of $k_{apparent}$ for native DNA are in all cases higher than those obtained for denatured DNA. The quantity $\Delta = 1 - \frac{nk \text{ d-DNA}}{nk \text{ n-DNA}}$ is a measure of the decrease in the values of $nk_{apparent}$ caused by substitution of denatured DNA (d-DNA) for native DNA (n-DNA) in the binding experiments. The calculated Δ for protamine-DNA binding is about 0.55, about 0.25 for histone IV-DNA binding, 0.05 for histone Ib-DNA binding, and about 0.15 for poly-L-lysine-DNA binding in 0.1M NaCl. It appears, therefore, that the affinity of the arginine-rich proteins for DNA is affected more than that of the lysine-rich fractions when the helical conformation of the DNA is reduced. The implication of this is not immediately obvious.

B. Variation of $nk_{apparent}$ with the type of polypeptide used for binding

Of the basic proteins-polypeptides studied, protamine binds most with native and denatured DNA. The apparent binding constant of

protamine with native DNA (see equation 1) is 1,600 ℓ/mole (fig. 1). The binding constant to denatured DNA is 700 ℓ/mole in the same salt solution. The arginine-rich histone IV also binds very well with DNA at lower salt concentrations (0.1M). The binding constants of histone IV to native DNA and denatured DNA are, respectively, 840 and 630 ℓ/mole (fig. 2).

In comparison, the $nk_{apparent}$ for poly-L-lysine and histone Ib (figs. 3 and 4) are much lower than those for the arginine-rich proteins (histone IV and protamine) under the same experimental conditions. The $nk_{apparent}$ for histone Ib-native DNA binding is about 220 ℓ/mole, and about 210 ℓ/mole for denatured DNA-histone Ib at pH 6.6, 0.1M sodium chloride. The $nk_{apparent}$ for poly-L-lysine-DNA is essentially the same as for histone-Ib-DNA (table 1) (240 ℓ/mole for poly-L-lysine-native DNA and 200 for poly-L-lysine denatured DNA). Thus, it appears as if the arginine-rich polypeptides bind more to DNA than the lysine-rich polypeptides.

Effect of sodium chloride on $nk_{apparent}$

The effect of sodium chloride concentration on the association constants for DNA and the several basic proteins is shown in figures 1, 2, 3, and 4. The midpoint of the profile of nk versus salt concentration is at about 0.45-0.5M sodium chloride for the protamine binding to both native and denatured DNA. The slope of the curve, which we will regard as representing a transition between free DNA and the

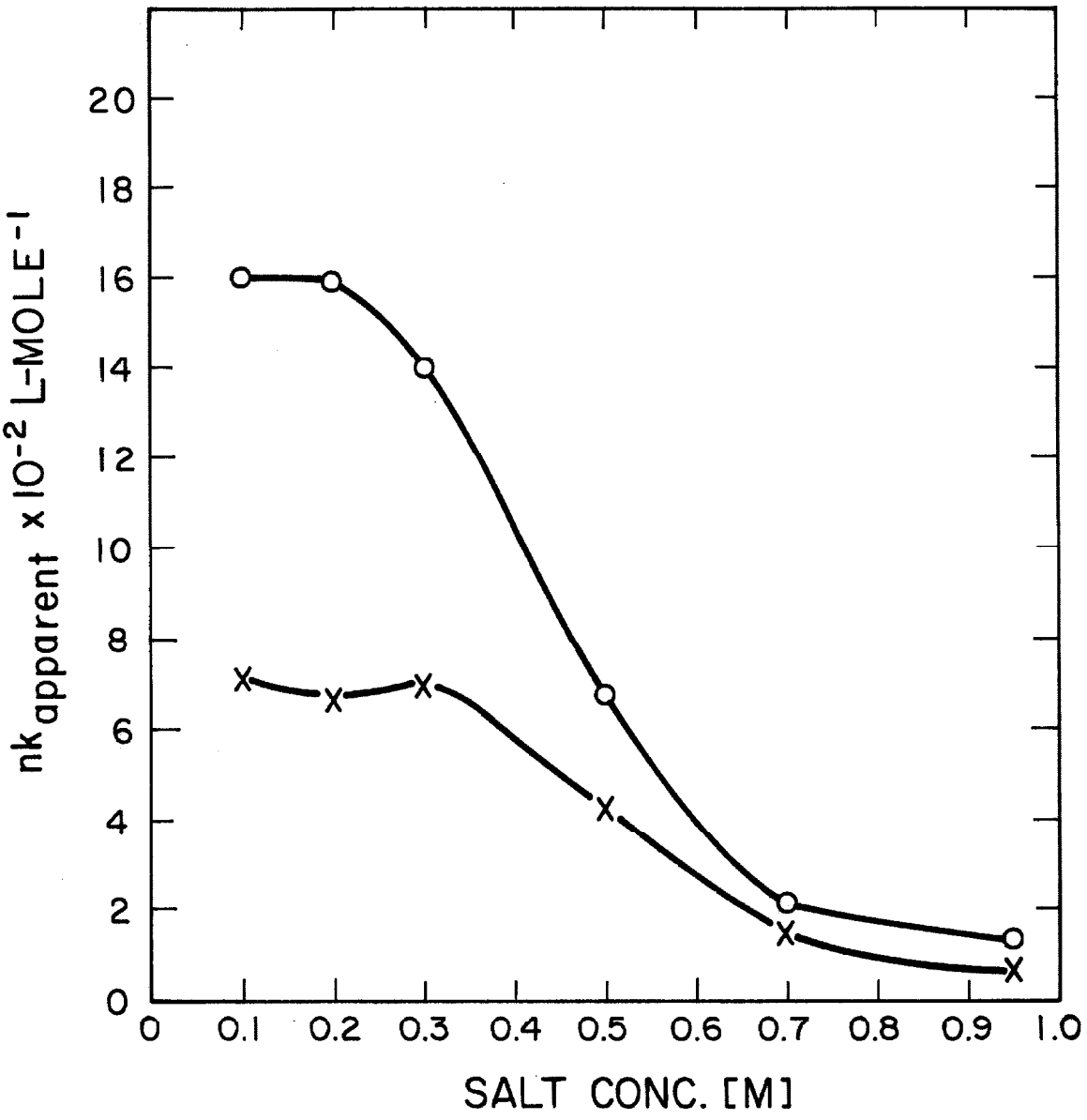

Figure 1. Plot of $nk_{apparent}$ of protamine to native DNA (o——o) and denatured DNA (x——x) versus sodium chloride concentration. All solutes were dissolved in 0.015M Na-citrate, 10^{-4}M EDTA, pH 6.6.

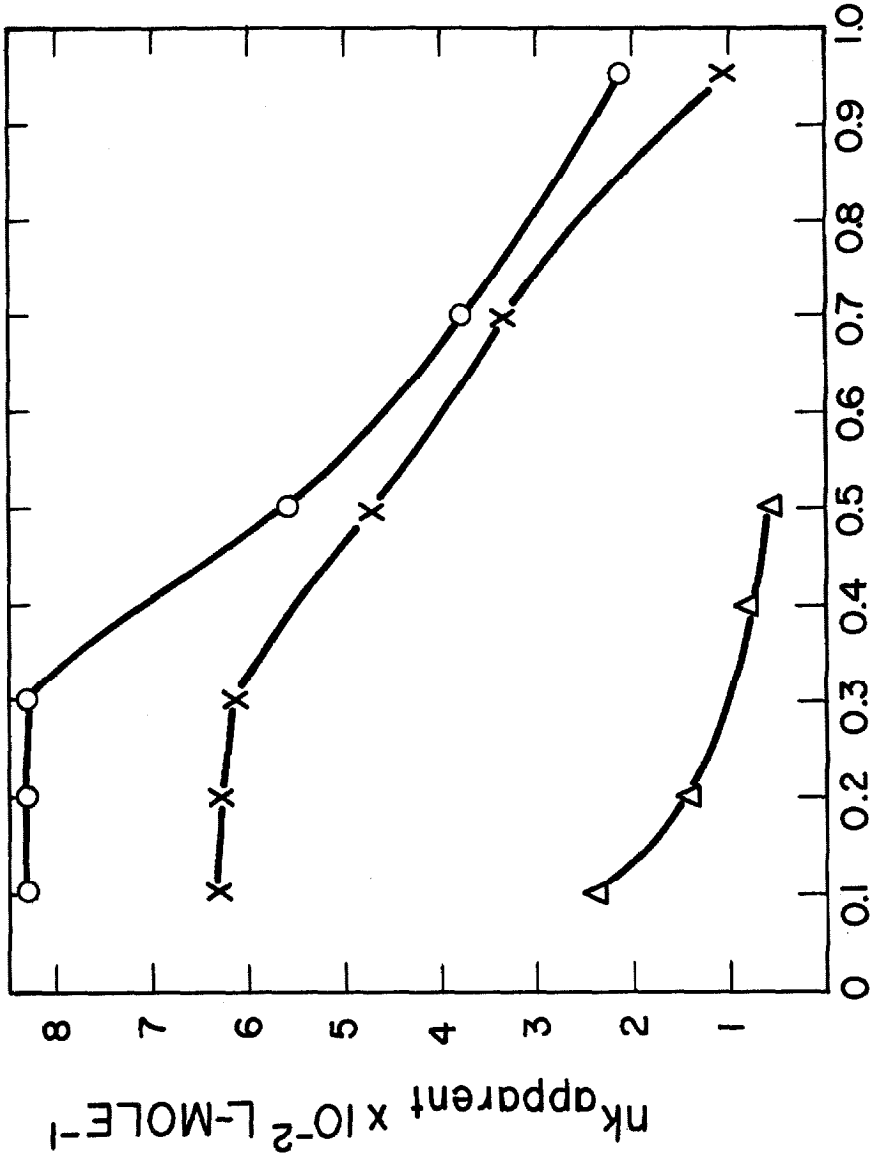

Figure 2. Plot of $nk_{apparent}$ of histone fraction IV to native DNA (o——o) and denatured DNA (x——x) versus sodium chloride concentration, and of $nk_{apparent}$ of histone fraction IV to native DNA versus magnesium chloride concentration (Δ——Δ). All solutes were dissolved in 0.015M Na-citrate, 10^{-4} M EDTA, pH 6.6.

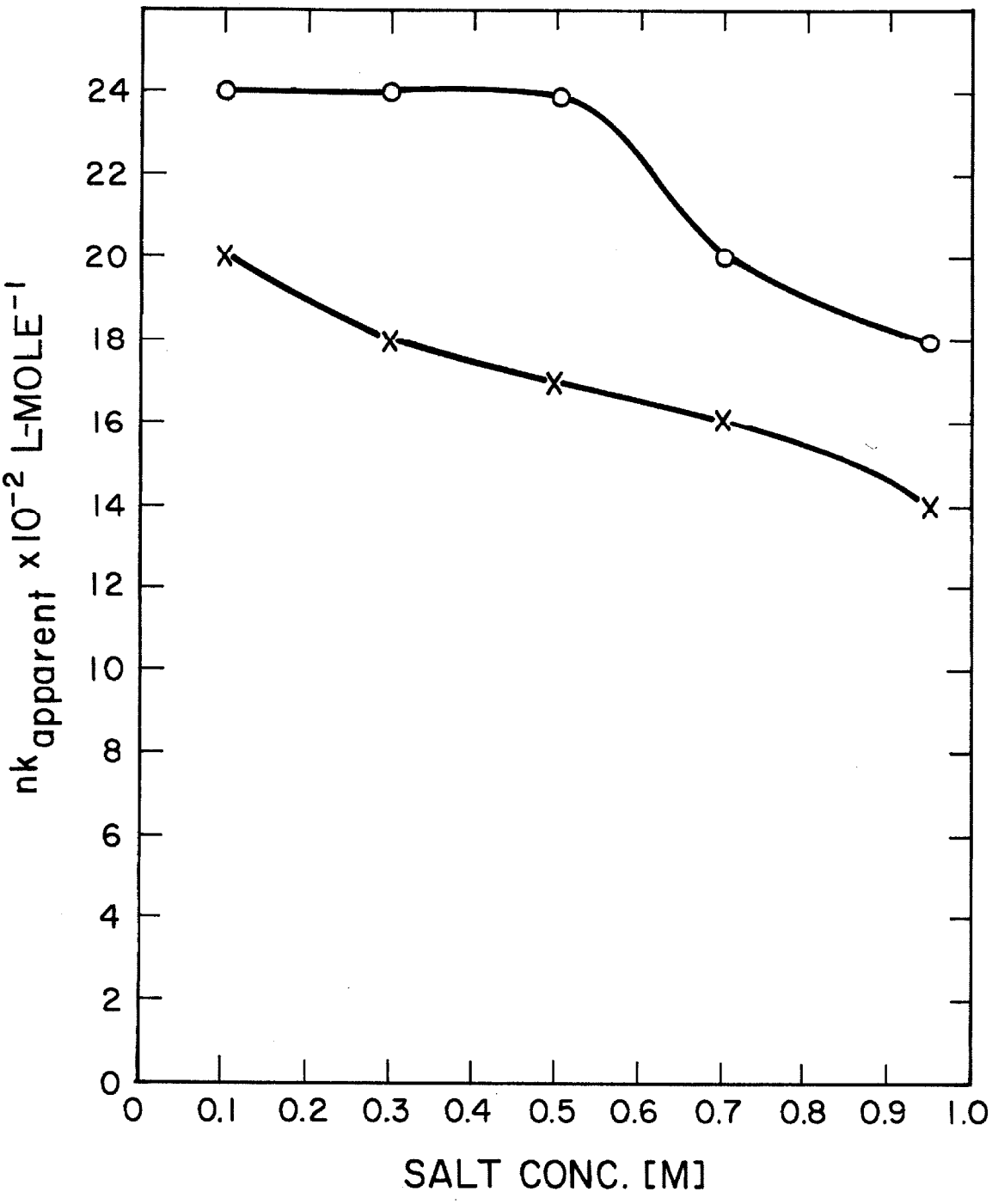

Figure 3. Plot of $nk_{apparent}$ of poly-L-lysine to native DNA (o——o) and denatured DNA (x——x) versus sodium chloride concentration. All solutes were dissolved in 0.015M Na-citrate, 10^{-4}M EDTA, pH 6.6.

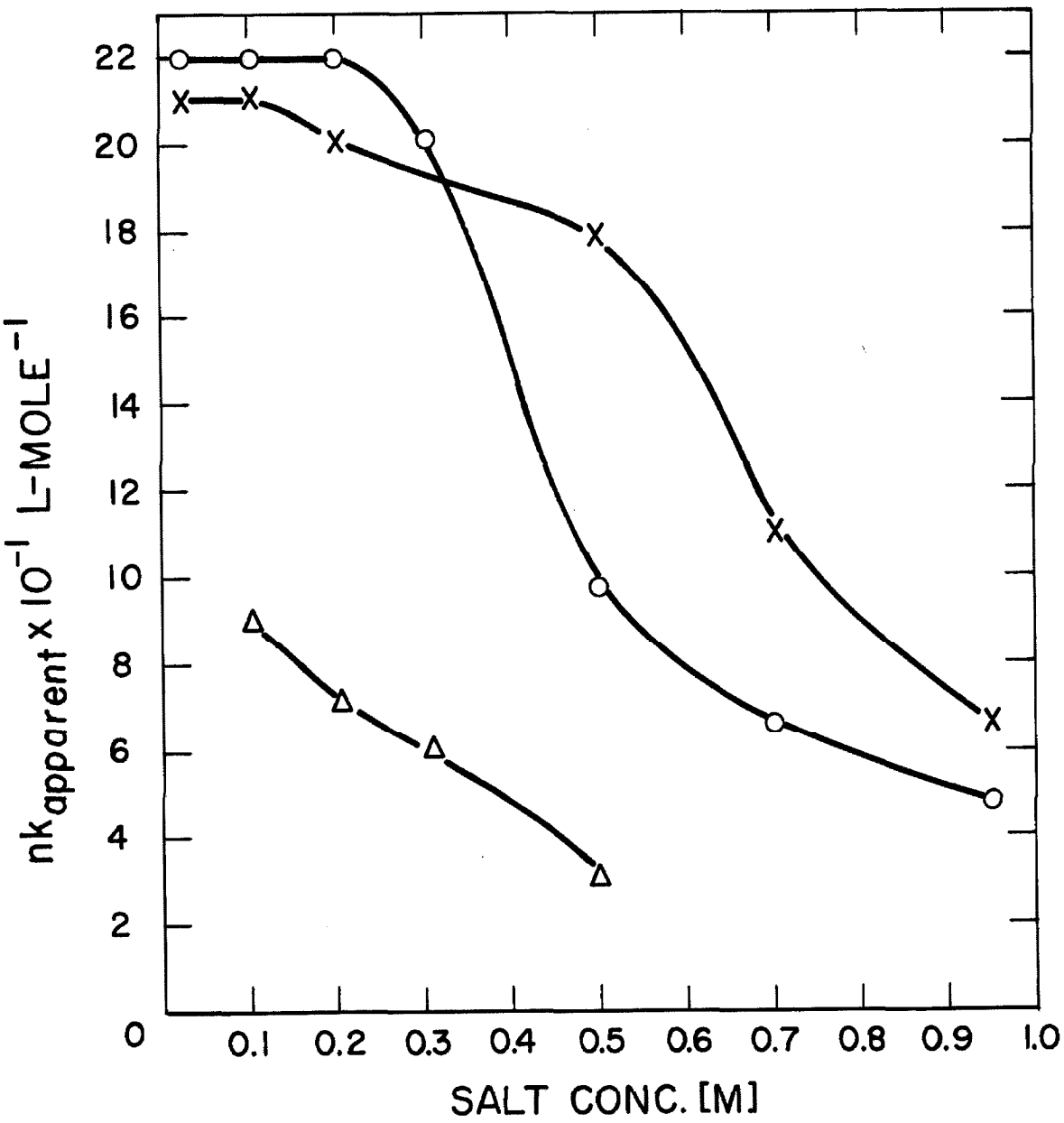

Figure 4. Plot of $nk_{apparent}$ of histone fraction Ib to native DNA (o——o) and denatured DNA (x——x) versus sodium chloride concentration, and of $nk_{apparent}$ of histone Ib to native DNA versus magnesium chloride concentration (Δ——Δ). All solutes were dissolved in 0.015M Na-citrate, 10^{-4} M EDTA, pH 6.6.

Table 1. Variation of Apparent Binding Constant with Salt Concentration

1. Protamine binding to native DNA (n-DNA) and denatured DNA (d-DNA)

Sodium chloride-molar concentration	$nk_{apparent}$-(n-DNA)	$nk_{apparent}$-(d-DNA)
0.10	16×10^2	7.0×10^2
0.19	16×10^2	6.6×10^2
0.31	14×10^2	7.0×10^2
0.50	6.8×10^2	4.2×10^2
0.70	2.2×10^2	1.5×10^2
0.95	1.4×10^2	0.6×10^2

2. Histone IV binding to n-DNA and d-DNA

Sodium chloride-molar concentration	$nk_{apparent}$-(n-DNA)	$nk_{apparent}$-(d-DNA)
0.1	7.8×10^2	6.3×10^2
0.19	7.8×10^2	6.2×10^2
0.31	7.8×10^2	6.2×10^2
0.50	5.6×10^2	4.7×10^2
0.70	3.8×10^2	3.4×10^2
0.95	2.1×10^2	1.0×10^2

3. Poly-L-lysine binding to n-DNA and d-DNA

Sodium chloride-molar concentration	$nk_{apparent}$-(n-DNA)	$nk_{apparent}$-(d-DNA)
0.015	2.4×10^2	---
0.1	2.4×10^2	2.2×10^2
0.31	2.4×10^2	1.8×10^2
0.50	2.4×10^2	1.7×10^2
0.70	2.0×10^2	1.6×10^2
0.95	1.8×10^2	1.4×10^2

Table 1. (continued)

4. Histone Ib binding to n-DNA and d-DNA

Sodium chloride-molar concentration	$nk_{apparent}$-(n-DNA)	$nk_{apparent}$-(d-DNA)
0.015	2.2×10^2	2.1×10^2
0.10	2.2×10^2	2.1×10^2
0.19	2.2×10^2	---
0.31	2.0×10^2	2.1×10^2
0.50	0.993×10^2	1.8×10^2
0.70	0.663×10^2	1.1×10^2
0.95	0.492×10^2	0.66×10^2

5. $MgCl_2$ effect on binding of histone IV and Ib to n-DNA

$MgCl_2$ concentration (molar)	$nk_{apparent}$ for DNA - hist. IV	$nk_{apparent}$ for DNA - hist. Ib
0.1	2.4×10^2	0.823×10^2
0.19	1.4×10^2	0.723×10^2
0.31	0.886×10^2	0.632×10^2
0.50	0.52×10^2	0.333×10^2

complex, is maximal between 0.3M and 0.7M sodium chloride. The maximum slope of the profile (fig. 1) for protamine binding to native DNA is higher than that for denatured DNA. The calculated $\frac{(nk_{apparent})}{(NaCl)}$ for native DNA around the midpoint of the profile is about 40, and the corresponding value for denatured DNA is about 15. The binding constants at the highest salt concentration are almost the same for native and denatured DNA.

The behavior of histone IV-DNA binding constants in sodium chloride concentrations (0.1-0.95M) is in certain respects similar to that of protamine-DNA (fig. 2) for both denatured and native DNA. In both cases, there is a sharp drop in $nk_{apparent}$ beyond 0.3M sodium chloride. However, the drop in nk for histone IV appears to be slightly more gradual within the salt concentration range of 0.3-0.95M. As in DNA-protamine binding, the slope $\frac{(nk_{apparent})}{[NaCl]}$ around the midpoint of the transition for histone IV-DNA binding is higher for the native DNA than for the denatured DNA. The midpoint of the transition is about 0.55M sodium chloride for both native and denatured DNA. At the highest salt concentration (0.95M), $nk_{apparent}$ for the native-DNA-histone IV complex is still over 200 ℓ/mole and about 100 ℓ/mole for the histone IV-denatured-DNA complex. In other words, at about 1M NaCl, the apparent binding constant is still about 25 per cent of the value at 0.1M sodium chloride, and still about as high as for histone Ib-DNA binding at 0.1M NaCl.

In the case of histone Ib-native DNA binding, the transition in nk as a function of sodium chloride is rather sharp. The midpoint of the transition is between 0.40-0.45M NaCl. At about 0.7M sodium chloride, the $nk_{apparent}$ begins to approach the minimum value obtained at 0.95M NaCl (about 50 ℓ/mole). The situation with the denatured DNA-histone Ib binding is more erratic. Because of the nature of the transition as recorded, an accurate midpoint of transition cannot be

accurately calculated. It appears, however, that the transition is spread over a greater salt concentration range than is the corresponding transition for the native-DNA-histone Ib binding. Poly-L-lysine-native DNA binding constants appear to be less sensitive to salt effect than histone Ib-DNA binding constants. A rather short but relatively sharp transition is observed between 0.5M NaCl and 0.95M sodium chloride. The midpoint of this transition is about 0.6M NaCl. The decrease in association constant as a function of salt concentration for poly-L-lysine-DNA binding is gradual over the whole range of salt concentration. Poly-L-lysine appears to bind relatively well to native and denatured DNA, even at 1M NaCl.

Effects of magnesium chloride on nk apparent

The variation of nk apparent of protamine DNA binding with magnesium chloride concentrations was not studied because of the complexity of the behavior of protamine fractions in the presence of different cations (Felix, 14).

The change of nk apparent with $MgCl_2$ concentration is shown in figures 2 and 4 for the DNA-histone complexes. For both histone Ib-native-DNA binding and histone IV-DNA binding, the effect of $MgCl_2$ at 0.1M is about as effective as 0.5M to 0.6M sodium chloride in decreasing $nk_{apparent}$. In histone IV-DNA binding, the tail of the $nk_{apparent}$ transition can still be seen. However, in histone Ib-DNA binding, the decrease in $nk_{apparent}$ is approximately linear with magnesium chloride concentration.

DISCUSSION

The observation that the association constant of the basic proteins and polypeptide is not affected by sodium chloride concentration up to about 0.3M is consistent with the previous observation of Bayley et al. (3). They observed that the dissocation of nucleohistone is not affected by sodium chloride up to 0.3-0.4M. The plot of per cent dissociation against molar concentration of NaCl, according to Bayley et al. (3) shows a gradual profile. The per cent dissociation per molar concentration of sodium chloride is about 50% at 0.4M NaCl and about 40% at 1.5M sodium chloride (Bayley et al., 3). On the other hand, the data on association constants of histone Ib and IV fractions (figs. 2 and 4) indicate that the nk apparent versus ionic strength profile goes through a rather sharp transition. This apparent contradiction probably arises from the fact that the midpoint of the transition is not the same for all the histone fractions. Furthermore, their apparent binding constants are not the same (figs. 2 and 4). If one added the curves obtained for histone Ib-DNA binding to histone IV-DNA binding (figs. 2 and 4), one would obtain a more gradual decrease in $nk_{apparent}$ as a function of increasing sodium chloride concentration. Thus, the reason why the per cent dissociation versus salt concentration curve of Bayley et al. (3) does not exhibit a sharp distinctive profile might very well be due to the greater heterogeneity of the histones.

The observation that sodium chloride and magnesium chloride are both effective in decreasing the binding constants of the basic proteins and polypeptides supports the notion that electrostatic interactions between the basic arginine or lysine residues and the phosphate ion on the DNA are of importance in the interaction of these polypeptides to DNA. The magnesium cation binds much more strongly to DNA than does the sodium cation (Cavalieri, 7; Zubay and Doty, 35). Cavalieri (7) has concluded that Mg^{++} binds strongly both to the phosphate and the amino groups of DNA. The greater effectiveness of magnesium cation in reducing nk apparent is thus consistent with the conclusion that arginine-phosphate or lysine-phosphate binding is the most important factor determining the interaction of histone to DNA.

The decrease in DNA-protein binding accompanying the denaturation of the DNA can be attributed to one or both of two factors, namely, (a) a decrease in the charge density of the DNA macro ion upon denaturation (Ascoli et al., 2), and (b) the destruction of the secondary structure of the DNA which specifically participates in the interaction. It is very difficult from the data to assess the relative importance of these two factors. It is the opinion of the author, however, that the determining step in the interaction is electrostatic, especially at a very low ratio of protein to DNA. For this, only the disposition of the charges on the macromolecules is likely to be of significance in determining the binding of any given protein or polypeptide. At higher

ratios of protein:DNA, the situation may become more complex and involve both factors.

The sharp decrease in $nk_{apparent}$ within a small range of concentration of sodium chloride is interesting. A similar curve has been obtained for gelatin-gum arabic interaction in the presence of calcium chloride (Bungenberg De Jong, 6). The complexing of basic proteins to DNA can be regarded as polyvalent polymer-polymer interaction. In other words, the complex formation is an example of complex coacervation.

Complex coacervation depends, among other things, on the pH and the ionic strength of the medium in which the reaction occurs. Its formation can be suppressed when a sufficiently high concentration of salt is present, and the suppression action of the salt appears to obey the double valence rule (Bungenberg De Jong, 6). In the case of DNA-protein interaction, it is the shielding of the polyvalent anions that is responsible for the inhibition of complex coacervation.

The binding equation used for calculating nk does not take the complex statistical factor of polyvalent binding into consideration. Several sites, n, on polymer (A) bind to another set of sites, i, on polymer (P) whenever one molecule of (A) reacts with another molecule of (P). The addition of a certain concentration of salt may displace a fraction of n sites and proportionally reduce the free energy of interaction of (A) with (P) without causing a dissociation of (A) from (P).

As the salt concentration is increased, the number of n or i is decreased until a critical point (n-j) or (i-1) is reached, at which point (A) dissociates from (P). Thus, as long as enough n sites are occupied by i sites, the complex will still appear to be intact. However, as soon as the interacting sites are reduced to a certain minimum, the complex falls apart. The concentration of salt required to attain this minimum interaction varies with the valence of the salt cation. In addition, the concentration of any one salt required to cause a maximum suppression of complex formation varies with the nature of the complex. This explanation suggests that the interaction of histone or protamine to DNA is dominated by a large entropy effect, and that the salt resistance should be very sensitive to temperature changes.

The above explanation can be used to explain the flat tops in the low salt concentration area in figures 1-4, as well as the sudden drop in nk between 0.3M and 0.7M sodium chloride. The secondary flattening of the curves (figs. 1-4) above 0.7M sodium chloride is more difficult to explain. One possible suggestion is that, at such high salt concentration, the secondary forces in the interaction (probably not electrostatic) predominate. The nature of this secondary interaction, if it exists, is not understood. However, it may well be that such a secondary interaction, superimposed on the electrostatic interaction, is important to the firmness of the binding of histone or protamine to DNA. One possibility is that hydrophobic interaction is involved in the

binding of these basic proteins to DNA. There is no reason why this kind of interaction may not be considered a likely possibility in DNA-protein interactions. However, there are at present no data to prove or disprove this notion.

It is interesting that histone IV has a higher nk apparent and requires more salt (NaCl) for half-dissociation than does histone Ib, even though the latter, and not the former, stabilizes DNA against thermal denaturation. Bradbury (private communication) has similarly observed that more salt is required to dissociate histone IV than histone Ib. Furthermore, Huang et al. (17) have shown that the binding of histone Ib to DNA inhibits DNA-dependent RNA synthesis, whereas histone IV and protamine do not significantly inhibit DNA-dependent RNA synthesis under the same experimental conditions. From this, one is tempted to speculate that histone Ib binds differently from histone IV to DNA. Possibly, histone Ib is able to fit into the larger groove of DNA, especially at higher histone Ib:DNA ratio. Thus, in addition to forming salt linkage, histone Ib can specifically fit into DNA grooves in a rather specific manner. On the other hand, histone IV merely forms electrostatic bonds with the DNA, and its contact with the DNA non-polar residue is at special points on the molecule.

In order to make the above speculation compatible with previous findings about the structure of native histone on DNA, one will have to assume that the α-helical content in histone fractions Ib and IV are

different. In fact, in order that histone Ib can form the type of complex postulated above, its conformation will have to be almost entirely in the extended β-form. Such a conformation is expected in histone Ib because of its high proline content. But since whole histone has over 50% helical content on the DNA molecule, it is reasonable to come to a speculative conclusion that histone IV must have a high percentage of α-helical content.

Proceeding from this deduction, the difference in behavior between histone Ib and histone IV with respect to the stabilization of helical DNA against thermal denaturation can be attributed to differences in their mode of interaction with the DNA. Inasmuch as the extent of binding is determined, especially at low protein:DNA ratio, by the electrostatic attractive interaction between the arginine or lysine residues and the phosphate ions, one will not necessarily expect to find a conclusive correlation between the magnitude of the binding constants, or the behavior of $nk_{apparent}$ in salt and the secondary interaction postulated above for DNA-histone Ib interaction.

The parameter Δ, defined in previous sections, can be used to justify the validity of this postulate only if data are available on the relationship of $\underline{r}$ (moles protein bound/mole total DNA) for the two forms of DNA. The value of $\underline{\Delta}$ for histone Ib-DNA complex will be much more sensitive to variations in $\underline{r}$ than that of histone IV-DNA complex. A single value of Δ at a single value of $\underline{r}$ is not very useful

in establishing the participation of the secondary structures of the DNA and protein in the interactions. The equilibrium dialysis method does not allow one to determine $\underline{r}$ at different concentration ratios of protein to DNA, since the complex forms a thick gel at higher protein concentration. Furthermore, the method of analysis of the protein does not allow one to work at a much lower concentration of protein. Knowledge of $\underline{r}$ as a function of concentration of interactants will be very useful in defining the nature of basic protein-DNA interactions.

SUMMARY

The binding of protamine, histone fractions Ib and IV, and poly-L-lysine to native and denatured DNA has been studied by equilibrium dialysis in different concentrations of sodium-chloride (0.1-0.95M) and magnesium chloride (0.1-0.5M). The binding constant of any one of the proteins to native DNA is higher than its binding constant to denatured DNA in 0.1M sodium chloride, pH 6.6. The arginine-rich proteins bind more to native or denatured DNA than the lysine-rich proteins under identical experimental conditions. The effect of sodium chloride on the binding constants of the complexes is similar to that expected of a polyvalent polymer-polymer interaction. Half-dissociation of the complexes occurs between 0.4-0.55M sodium chloride in all cases. Magnesium chloride is more effective than sodium chloride in reducing complex coacervation.

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