COMPLEXES OF SILVER (I)

WITH NUCLEIC ACIDS

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1965

(Submitted October 22, 1964)

ACKNOWLEDGEMENTS

To Professor N. Davidson I express my sincere appreciation. My education has been influenced not only by his guidance, but also by his example. Thanks also to the members of his research group. Their companionship, cooperation, and criticism have made this research a rewarding experience.

I am grateful also to Dr. J. Vinograd, Dr. J. Bonner, Dr. R.L. Sinsheimer, and Dr. J.J. Weigle for the use of each of their analytical ultracentrifuges. For a period, my research world revolved around these machines. I would like to express further thanks to Dr. J. Vinograd and the members of his group for teaching me the basics of ultracentrifugation.

My appreciation to the members of the Biology Division from whom I received many of my materials and advice on how to handle them.

A special note of thanks belongs to my wife, Judy. As well as providing a constant source of moral and spiritual support, she worked her fingers to the bone for me (typing this thesis).

Financial assistance from the National Institutes of Health and from the California Institute of Technology is gratefully acknowledged.

ABSTRACT

The equilibrium properties of the Ag (I) complexes of DNA have been studied potentiometrically and spectrophotometrically at ionic strength of 0.1 M ($NaClO_4$) and at pH's from 5.6 to 8.0. There are at least two different complexes which form at low silver ion concentrations. The strength of association of one complex (type I binding) is pH independent. The type I complexing reaction releases no protons from the DNA. A second complex (type II binding) is stronger at high pH. This complexing causes the release of approximately one proton per Ag (I) bound.

DNA which is rich in GC binds Ag (I) more strongly than does AT rich DNA. However, at pH's between 5.6 and 8.0 more protons are released from AT-rich DNA's.

It is proposed that the first complex (type I) involves only GC base pairs, with silver (I) adding to the sigma electrons of a ring nitrogen or interacting with the pi electrons. In the second complex (type II), Ag (I) replaces an N-1 (purine) to N-3 (pyrimidine) hydrogen bond, thus displacing one proton.

The complexing behavior of various polynucleotides, nucleotides and substituted purines was studied, and the DNA complexes are discussed in the light of these results.

A cesium sulfate equilibrium density gradient ultracentrifugation technique has been developed in which buoyant density differences between different DNA's are generated by the specific formation of their Ag (I) complexes. When Ag (I) is bound to the extent of one silver ion per DNA base pair, the buoyant density of either native or denatured DNA increases by about .17 g/cc.

Competition experiments in which Ag (I) was added to a mixture of two different DNA's resulted in specific binding of the Ag (I) by one of the DNA species. At equilibrium in the ultracentrifuge two widely separated DNA bands were observed. The buoyant density difference between these bands corresponded to the difference in Ag (I) binding by the two species of DNA.

A survey was done of various useful preparative separations based on this technique. One such preparative separation has been accomplished, and the separated components have been characterized.

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INTRODUCTION AND REVIEW

In recent years the important biological functions of the nucleic acids have been greatly clarified. The physical and chemical properties of these unique molecules must be studied in vitro, in order to gain a better understanding of their in vivo functions. This study of the Ag (I)-DNA complexes has two major purposes: 1.) Information about these complexes may provide some insight into the structure and properties of DNA in the absence of silver (I). 2.) The complexes may provide a tool which can be used to separate one DNA molecule from another, or to change the structure of DNA so as to make it more amenable to observation or experimentation.

DNA interactions with various metal ions (eg., Mg^{+2}, Mn^{+2}, Na^{+}, and Co^{+2} (1,2)) have been studied rather extensively, and these have been found to possess one common characteristic: the interaction is of a nonspecific electrostatic nature between the negative phosphate groups in the DNA backbone and the positibely charged metal ions. However, the Hg (II) interactions with DNA have been shown to be of a different variety (3,4,5). When $HgCl_2$ is added to a DNA solution, the intrinsic viscosity drops drastically, the UV absorption changes markedly, and the binding itself is remarkably strong. The exact nature of the Hg (II)-DNA complexes has not yet been determined, but it is known that, unlike most metal ions, Hg (II) is bound directly to the purine or pyrimidine bases. This is not surpising since, in general,

Hg (II) has a much higher affinity for nitrogenous bases than for oxygen ligands and would not be expected to bind very strongly to the phosphates in the DNA backbone.

Silver (I) is similar to mercury (II) in its complexing behavior, and with this in mind Yamane and Davidson began a study of the complexes of Ag (I) with DNA (6). The first indication that Ag (I) also interacts with the base moeities in DNA is that on complexing there is an ultraviolet absorption change. Another similarity is that silver (I) also is bound very strongly to DNA. However, unlike the case for Hg (II) binding, there is no important change in the viscosity of DNA solutions when Ag (I) is added. These complexes are different enough from the Hg (II) complexes to warrant an independent investigation.

Potentiometric pH-stat titrations of DNA with $AgNO_3$ were performed by Yamane, and these revealed some important equilibrium properties of the complexes. Portions of Yamane's data are pertinent to my work, and those parts will be included in the text of this thesis. His most important conclusion is that there are at least two different types of complexing between Ag (I) and DNA,

$$\text{Type I} \qquad Ag^+ + HB\text{-}PO_4^- \rightleftharpoons Ag(HB)^+\text{-}PO_4^- \qquad (1)$$

$$\text{Type II} \qquad Ag^+ + HB\text{-}PO_4^- \rightleftharpoons AgB\text{-}PO_4^- + H^+ \qquad (2)$$

where $HB\text{-}PO_4$ represents a nucleotide contained in a DNA chain. As stated by Yamane and Davidson (6), it is possible that each of the complexes is really a composite of two different types, one for GC base pairs and another for AT base pairs.

The major part of this thesis is devoted to a description of the chemical, physical, and structural characteristics of these complexes, both with regard to conditions of complexing and to DNA specificity.

I. EXPERIMENTAL

A. Materials

The nucleotides were the highest grade available from the sources indicated below and were not further purified. Thymidylic acid (5') (chromatographically pure) was from Mann Research Laboratories. Deoxycytidylic acid (5') (A grade) and the diammonium salt of deoxyguanylic acid (5') (A grade) were obtained from Calbiochem. Details as to the adenine derivatives can be found in section II-E.

Calf thymus DNA was purchased from Worthington in a fibrous form, while M. Lysodeikticus and E. Coli DNA were prepared using a modified phenol extraction.* All DNA solutions were dialized in Visking tubing against a.) 10^{-1} F EDTA, pH 8 for 4 hours, b.) frequent changes of 10^{-1} F $NaClO_4$ for 36-48 hours. The solutions used in the low ionic strength studies were further dialyzed against 10^{-3} F $NaClO_4$ for 36 hours. A Schmidt-Tanhauser test** done on each DNA sample showed less than 5% RNA, and a protein analysis (Lowry test** or OD_{260}/OD_{230}) showed less than 5% protein.

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* The technique which has been developed is described in appendix I.
** This technique is described in appendix II.
*** A procedure developed by H.H. Ohlenbush (private communication) from the original folin reagent analysis (7).

Cs_2SO_4 used in the ultracentrifuge studies was obtained from Penn Rare Metals. Of three preparations which we received from them, only one was pure enough to use directly in my studies. An attempt to purify the other two preparations was unsucessful. A second method for obtaining pure Cs_2SO_4 was to neutralize a solution of Cs_2CO_3 (from Penn) with reagent grade H_2SO_4 to a pH of 7 after boiling. The Cs_2SO_4 was then precipated by adding three volumes of spectral grade methanol. After three washings with this methanol, the solid was dried in a vacuum desiccator over $CaSO_4$. Solutions of these preparations at a density of 1.50 had an absorbance of about 0.1 at 260 mu.

Cacodylic acid, which was used as a buffer throughout the studies, was C.P. All other chemicals used were reagent grade, and all solutions were made in redistilled Puritas water.

B. Methods

Binding analysis.- The strength of Ag (I) binding was measured potentiometrically using a Ag/Ag half cell versus a standard calomel electrode as a measure of the concentration of Ag (I) free in solution. After adding a measured volume of standard silver nitrate solution, the amount of Ag (I) bound to the DNA could be calculated by subtracting the amount of free silver (found potentiometrically) from the total amount added.

A pH-stat titration was performed simultaneously with the potentiometric titration. The solution being titrated was not buffered, and when a small amount of $AgNO_3$ was added, the pH decreased. The amount of NaOH required to return the pH to its original value was then measured. This quantity is equivalent to the number of protons released in the complexing reaction.

The results of these measurements are expressed in terms of two quantities, r_b and $\frac{\Delta H^+}{\Delta Ag^+_{bd}}$. The quantity r_b is the ratio of moles of silver (I) bound to the moles of nucleotide phosphate present in solution. The quantity $\frac{\Delta H^+}{\Delta Ag^+_{bd}}$ is the number of protons released per silver ion bound when a small amount of silver (usually corresponding to $\Delta r_b \approx 0.1$) is added to the solution. It should be mentioned that large errors are incurred for weak binding, since r_b is derived from the difference $(Ag^+)_{added}$ minus $(Ag^+)_{free}$, and when there is only weak binding, these are approximately equal. Furthermore, $\frac{\Delta H^+}{\Delta Ag^+_{bd}}$ is even less precise, since it is a differential measurement. Details of the apparatus can be found in section II-E.

Except where indicated in the text, the conditions for the binding studies were as described below. DNA or synthetic polynucleotides were always at a concentration of 1-2 X 10^{-4} M (in phosphates). In the spectral studies the supporting electrolyte was 0.1 F $NaClO_4$ with 10^{-2} F cacodylate added to maintain the desired pH. The pH of an aliquot was always measured before and after each spectral titration, and these never differed by more than 0.1 pH units. Potentiometric pH-stat titrations were carried out in 0.1 F $NaClO_4$ with no added buffer.

Spectral measurements were all done on a Cary Model 14 spectrophotometer, and melting curves were done exactly as discussed by Dove and Davidson (1).

Ultracentrifugation. - All of the ultracentrifuge experiments were done using cesium sulfate to form the density gradient. The more usual choice of cesium chloride was not acceptable due to the insoluble AgCl which would form in competition with the Ag (I) - DNA. In general a concentrated stock solution of cesium sulfate in 0.1 F cacodylate buffer pH 8.0 was prepared, and an aliquot was diluted to an appropriate density using the same buffer. A small volume of concentrated DNA

solution (in 0.01 F Na_2SO_4) was added so that the final concentration was ca. 2 X 10^{-5} M in DNA phosphate.* This is about three times more concentrated than is recommended for density gradient work, but it was used so that the added Ag (I) would bind to DNA rather than to trace impurities. A small volume of silver sitrate solution was added last, and 0.65 ml of the final solution was put into a centrifuge cell using a glass syringe. The remainder of the solution (ca. 0.3 ml) was used to measure the index of refraction and the pH. The pH, which was measured using a Beckman one-drop electrode, was accurate to ± 0.1 pH units.

A number of standard solutions of Cs_2SO_4 in o.1 F cacodylate at pH 8.0 were weighed using a calibrated 0.250 ml micropipette, and their densities were then calculated. The index of refraction of each solution was measured, and a plot was made of index of refraction versus density.

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*In some experiments the DNA concentration was as high as 10^{-4} M in DNA phosphate. This resulted in very broad bands in the density gradient and a decrease in resolution of band position, but allowed a reasonable extimate of bound Ag (I) using UV absorption.

There is a linear relationship between index of refraction and density. The empirical equation which was used to calculate density from index of refraction is

$$\rho = 13.6986\, n_D^{25} - 17.3108 \qquad (3)$$

$$\text{for } 1.40 \leq \rho \leq 1.56$$

with 0.1 F cacodylate pH 8.0.

The equation derived by Hearst and Vinograd (8) is

$$\rho = 13.6986\, n_D^{25} - 17.3233 \qquad (4)$$

$$\text{for } 1.40 \leq \rho \leq 1.70.$$

In these equations, ρ is the density of the solution at 25°C, and is the index of refraction at 25°C for the sodium D line.

The difference between the two equations is due to the presence of cacodylate buffer in my solutions. The index of refraction versus density of a solution of my Cs_2SO_4 with no added buffer fits equation (4).

There were some experiments performed at pH 7.0 and at pH 5.6. In these cases, the density of each solution was measured by weighing.

After equilibrium was attained, any run could be stopped, a small amount of silver nitrate added directly to the cell, and the solution rebanded. In this way one DNA solution could be titrated directly in the cell, and the buoyant density versus amount of silver (I) added could be calculated.

The ultracentrifuge experiments were done in a Spinco model E ultracentrifuge at 25° C. at a speed of 44,770 RPM using ultraviolet optics. The majority of the runs were simultaneous double cell runs using a one degree side wedge window in one cell and an alternator to pick the desired image to be photographed. Both cells were 12 mm cells with either a 2° or a 4° sector centerpiece made of Kel F. The top window in each cell was a 1° negative window, which corrects the image displacement caused by refraction of the light as it passes through the Cs_2SO_4 solution. The normal radiation put out by the Spinco U. V. source was modified by a chlorine, a bromine, and a purple corex filter before entering the centrifugation chamber. This filters the output of the source to the extent that 99% of the resultant light is of wavelength 244-285 mu (9). The exposure time for all pictures was five minutes, with five minutes of development time also. All the negatives were traced on a Joyce-Loebel double-beam recording microdensitometer at a lever arm ratio of 7.5/1.

Band breadth and position generally became constant after 12-16 hours. However, in two runs the centrifugation was continued for 44 hours after apparent equilibrium, and there was no measurable change in band position or breadth.

The method used to calculate band density was the one suggested by Hearst and Vinograd (10). The limiting isoconcentration point is

$$r'_e = \left(\frac{r_b^2 + r_a^2}{2} \right)^{\frac{1}{2}} \qquad (5)$$

where

r_a = distance from rotor center to meniscus,

and r_b = distance from rotor center to bottom of cell.

To a good approximation r_e' is the distance at which the density at equilibrium corresponds to the original density of the solution (11).

Then the equation used to calculate the density at band center is

$$\rho_o = \rho + \alpha \omega^2 \bar{r} (r_o - r_e') , \quad (6)$$

where

$$\bar{r} = \frac{r_o + r_e'}{2},$$

r_o = distance from rotor center to band center,

ω = angular velocity in radians per second,

α = constant determined by the density gradient,

ρ = the original density of the solution,

and ρ_o = the density at band center.

The constant $\alpha = \frac{1}{r\omega^2}\left(\frac{d\rho}{dr}\right)$ was taken to be 1.403×10^{-9} cgs. Erikson and Szybalski (12) calculated this from a measurement of banding position of E. Coli DNA in two Cs_2SO_4 solutions of slightly different density. Assuming α is independent of solution density and that ρ_o is the same in both solutions, they used the measured distances and the measured average density of the solutions to solve two equations (equation (6) used once for each cell) in two unknowns (α and ρ_o). In a few cases α was calculated by this same method using my measurements. The values obtained varied between 1.4×10^{-9} and 1.7×10^{-9} cgs.

Ultracentrifuge errors.- The ultracentrifuge experiments have been done largely to show the possible applications of Ag (I)-DNA complexes in density gradient centrifugation; therefore, much of the potential precision and accuracy of the method has been sacrificed in favor of a wider range of conditions and a sampling of possible DNA separations. Some of the errors and their causes are enumerated below. With some effort these can be eliminated for any particular separation which one wishes to study in detail.

The major source of error for all the measurements is probably in the quality of the Cs_2SO_4. There is as yet no satisfactory commercially available Cs_2SO_4. It is either optically impure or chemically impure. Even the Cs_2SO_4which was made from Cs_2CO_3, as described in the materials section, was none too acceptable, inasmuch as it is somewhat variable in regard to UV absorbance, carbonate contamination, and possibly in regard to trace Ag (I) complexing impurities.

Choice of buffer is important, and the use of cacodylate at pH 8.0 is not very satisfactory. It has a pK_a at 6.2, and therefore the buffering capacity at pH 8.0 is very small. A more satisfactory buffer, although it has not been tested, is methyl arsonate ($CH_3AsO_3Na_2$) which has $pK_1 = 4.02$ and $pK_2 = 8.66$. This has three very desirable qualities; 1) It does not absorb UV light strongly, 2) it interacts only weakly with Ag (I), 3) it buffers well in the mildly alkaline region. There are very likely other buffers which fulfill these demands also.

The concentration of DNA which was used in these ultracentrifuge experiments was about 2×10^{-5} M (in phosphates). This concentration was too high to allow accurate ultracentrifuge analysis. At equilibrium in the ultracentrifuge the bands were somewhat broad, and band center was not well defined. From another point of view these solutions were too dilute. The UV absorption was too low to allow a good estimate of silver binding on the basis of spectral changes.

To deal satisfactorily with both of these problems would require high quality Cs_2SO_4. If an optically pure Cs_2SO_4 solution can be obtained, the UV absorption for 1×10^{-5} M DNA solutions can be used in order to measure r_b. This concentration would afford a significant improvement in the precision with which one can measure r_o.

A major error is introduced into the buoyant density calculations by using $\alpha = 1.403 \times 10^{-9}$ cgs. This was taken from a measure of the buoyancy density gradient made by Erikson and Szybalski (12). If the results from CsCl are at all indicative of the situation for Cs_2SO_4, the α which they obtained at $\rho = 1.426$ could be very different from the α at $\rho = 1.60$. For CsCl α varies from 5.05×10^{-10} cgs at $\rho = 1.20$ to 8.40×10^{-10} cgs at $\rho = 1.70$ (11). In order to be able to calculate densities accurately, a good

measure of α versus ρ should be made (10).

Another error in the calculation of buoyant density is introduced by neglecting pressure effects. However, Hearst, Ifft, and Vinograd (13) showed that density changes due to pressure effects are often small. They are probably insignificant as compared to the density changes which occur when heavy metals are bound.

II. BINDING - RESULTS AND DISCUSSION

A. Ag(I) - DNA Complexes

A direct study of the interaction of Ag (I) with DNA has been made using spectral titrations and potentiometric pH-stat titrations similar to those performed by Yamane (6). His preliminary work has been greatly extended by varying the pH and by changing the ionic strength. The postulate that there are two types of complex has been supported by these studies, and some postulates have been made as to the exact nature of these complexes.

Spectral. - For all DNA's studied there are significant spectral changes when Ag (I) is complexed, and the general character of the changes is the same in all cases under all conditions studied. The first small amount of silver (I) bound causes a decrease in the intensity at the absorption maximum (258 mμ). There is an isosbestic point at 267 mμ. As binding increases there is a shift in the maximum to

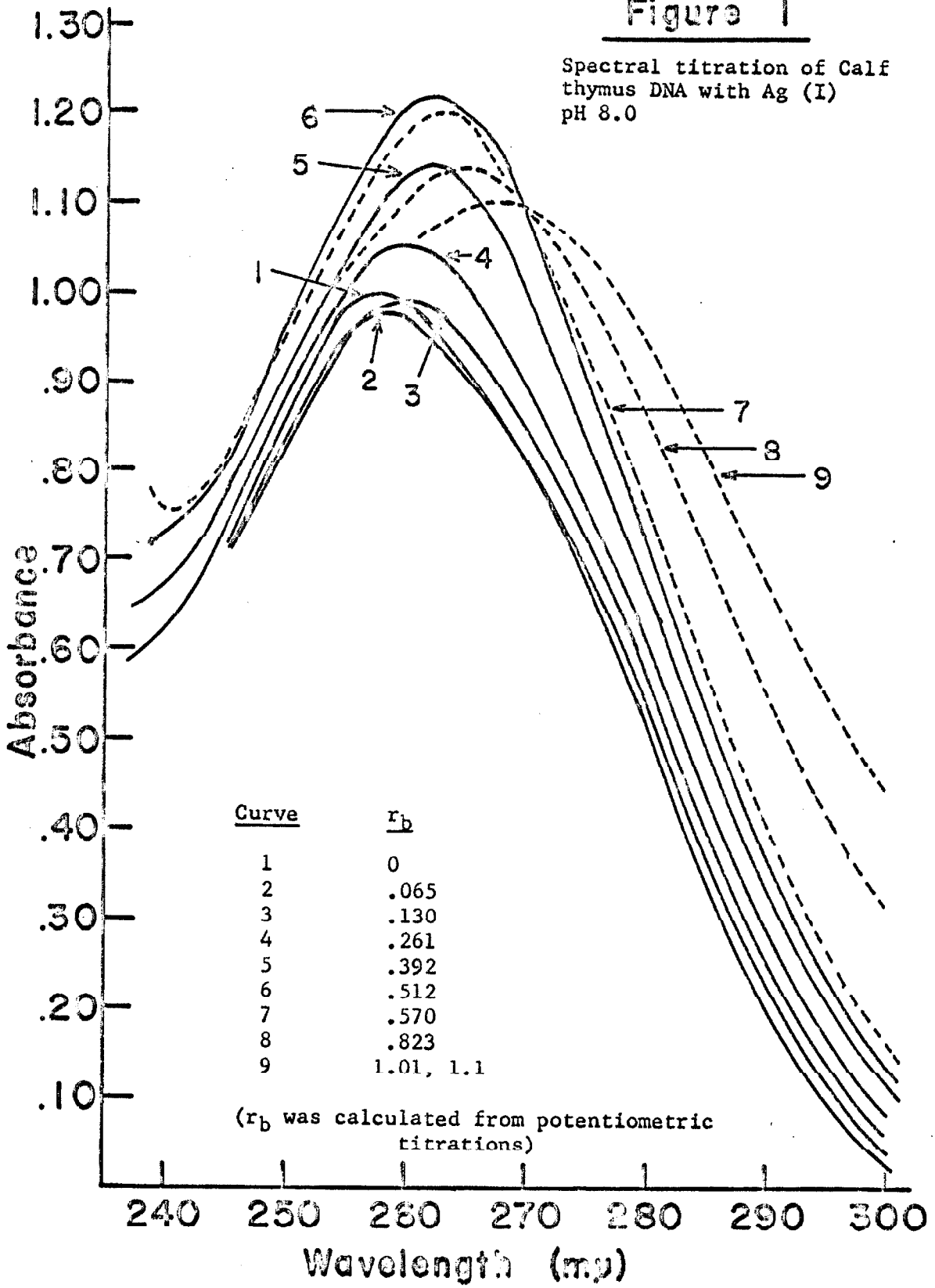
262 mμ. This is frequently simultaneous with a general increase in absorbance throughout the ultraviolet, which always occurs late in the titration but at r_b less than 0.50.

There is yet another kind of spectral change (above $r_b = 0.5$) which can only be observed at pH 8. At pH less than 8 there is a precipitate formed above $r_b = 0.5$, and spectral measurements become meaningless. A typical example of the spectral changes which occur is shown in figure 1. These data suggest that up to $r_b = 0.5$ (curves in solid lines) there are at least two and perhaps three complexes formed. Another complex forms for r_b greater than 0.50 (dotted lines). For simplicity all the binding above $r_b = 0.50$ has been neglected, and detailed analyses were done only on the first complexes.

The pH dependence of the spectral changes is shown in figure 2, where the absorbance at 260 mμ is plotted for Ag (I) titrations of Calf thymus DNA at three different pH's.

Figure 1

Spectral titration of Calf thymus DNA with Ag (I) pH 8.0



Curve	r_b
1	0
2	.065
3	.130
4	.261
5	.392
6	.512
7	.570
8	.823
9	1.01, 1.1

(r_b was calculated from potentiometric titrations)

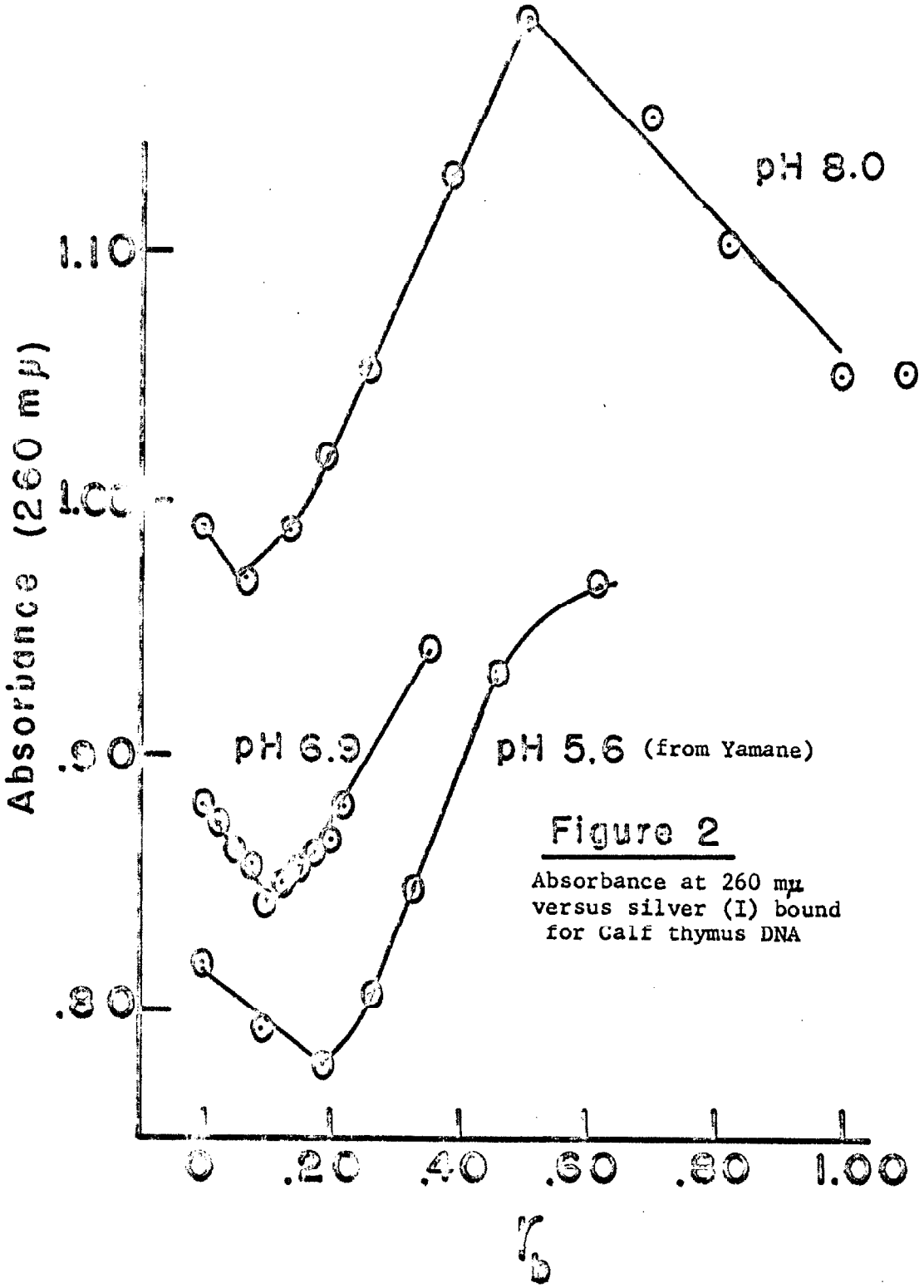


Figure 2

Absorbance at 260 mμ versus silver (I) bound for Calf thymus DNA

At pH 5.6 the minimum in absorbance is at $r_b = 0.20$. At pH 6.9 the minimum is at $r_b = 0.09$, and at pH 8.0 the minimum is at $r_b = 0.02$. These facts indicate that the first complex is more persistent at lower pH's. Potentiometric pH-stat data, which appear in the next sections, support this conclusion.

The compositional dependence of the spectral changes was studied at various pH's also, and figures 3,4, and 5 show the results of these experiments. The only obvious conclusion is that there are three different kinds of spectral change. The general order in which these appear is always the same, but the overlapping of the changes is variable with pH and with base composition.

The binding reactions are so complex (as will be demonstrated throughout the text) that it is fruitless to attempt a detailed analysis of complex formation using the spectral results.

Potentiomentric.- The potentiometric binding measurements give a good confirmation to the characteristics of the Ag (I) complexes which were indicated by the spectral results. Figure 6 shows the pH dependence of binding of Ag (I) to Calf thymus DNA in 0.1 F $NaClO_4$. The binding strength is essentially pH independent early in the titrations, indicating that the first complexing reaction does not release protons. Later in the

Figure 3

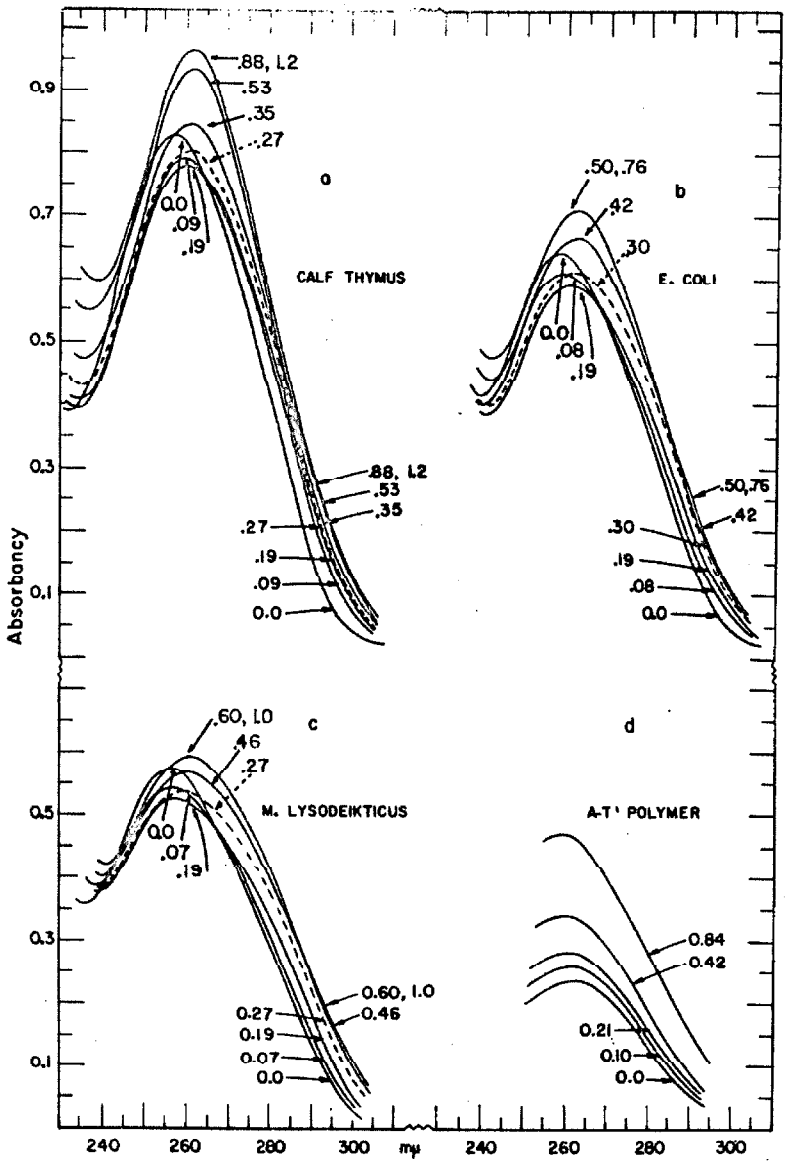


Fig. 2. Effect of Ag(I) on the ultraviolet absorption spectra of various DNA's. a, calf thymus (% GC = 42); b, *E. coli* (% GC = 50); c, *M. lysodeikticus* (% GC = 72); d, AT polymer (% GC = 0). Spectra taken in 0.10 *M* $NaClO_4$, pH 5.6–5.7, with increasing r_f. The actual concentrations of the DNA's can be calculated from the molar absorbancies given in the EXPERIMENTAL section.

Figure 4

Effect of Ag (I) on
UV absorption spectra

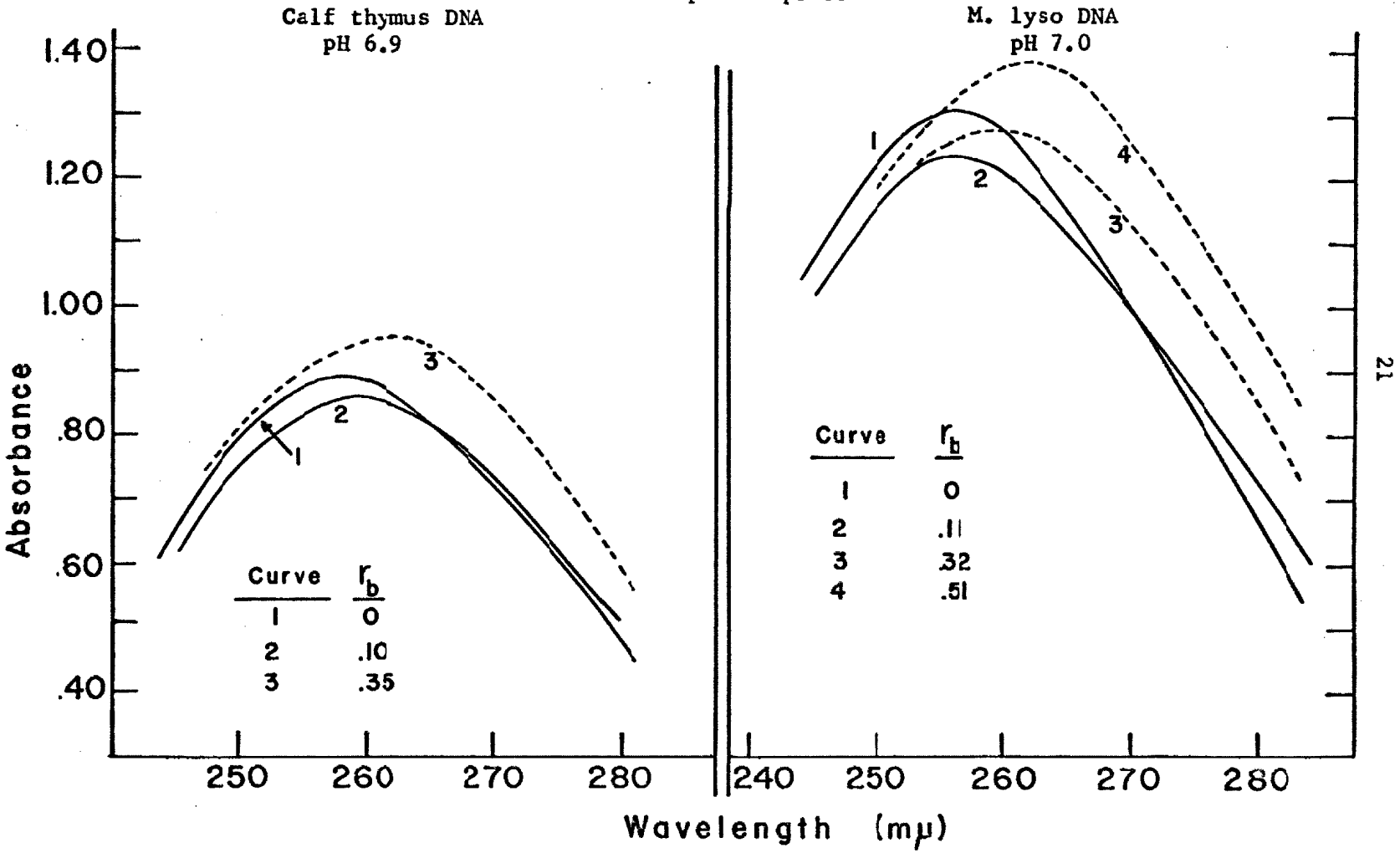
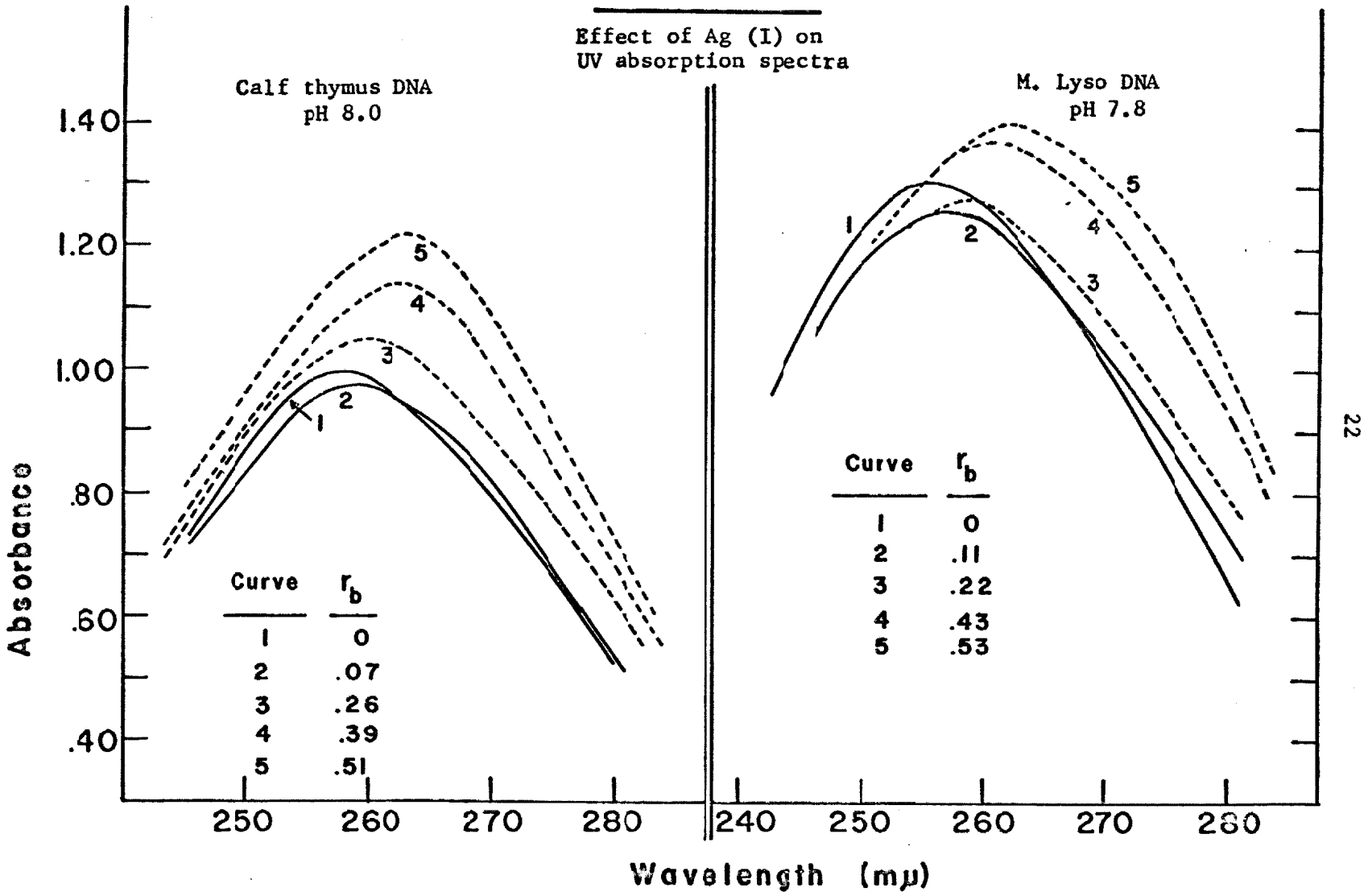


Figure 5

Effect of Ag (I) on
UV absorption spectra



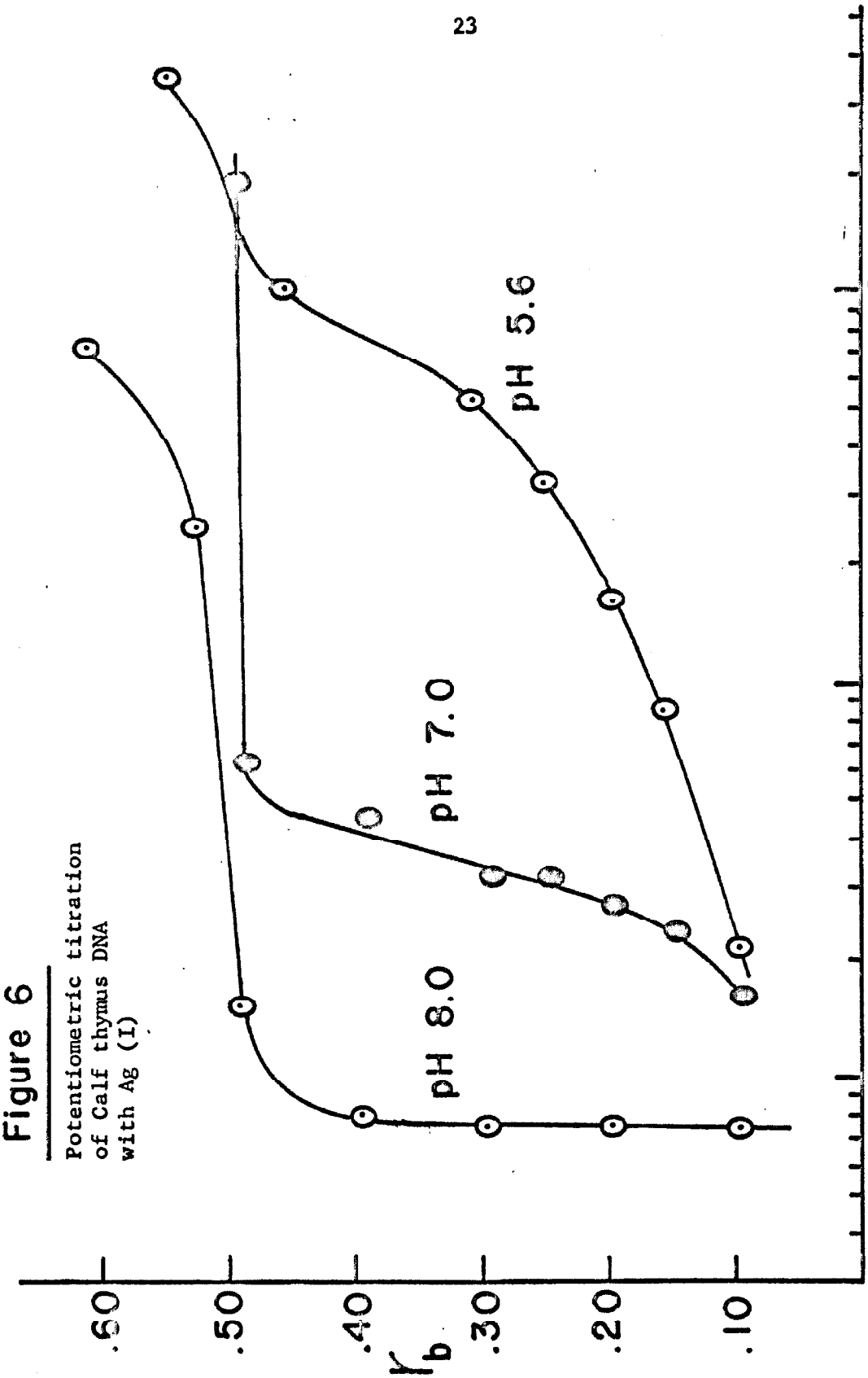


Figure 6

Potentiometric titration of Calf thymus DNA with Ag (I)

titration there is a definite pH dependence of binding, and then, finally, there is a plateau at $r_b = 0.50$. The strength of Ag (I) binding is approximately proportional to the hydrogen ion concentration, as would be predicted by equation (2). However, the situation is not as simple as equation (2) implies. The pH dependence of the binding increases as r_b increases. Furthermore, the slopes of the titration curves are greater at higher pH's.

The base composition dependence of binding is shown in figures 7, 8, and 9 for the various pH's. At pH 5.6 the early part of the titration is independent of base composition, while in the later stages, GC rich DNA binds more strongly. At pH 7 and 8, M. Lyso DNA binds Ag (I) more strongly than does T-4 DNA throughout the titration.

It should be noted that at $r_b = 0.4$ the separation between the various DNA's increases as pH decreases. This implies that there are two different pH dependent complexes, one which involves GC pairs and another which involves the AT pairs. The complex which involves the GC pairs is stronger than the AT complex in the entire pH region, but the pH dependence is stronger for the latter complex. It is possible that at pH 9.0 the strength of binding might be reversed, with Ag (I) complexed preferentially to AT rich DNA.

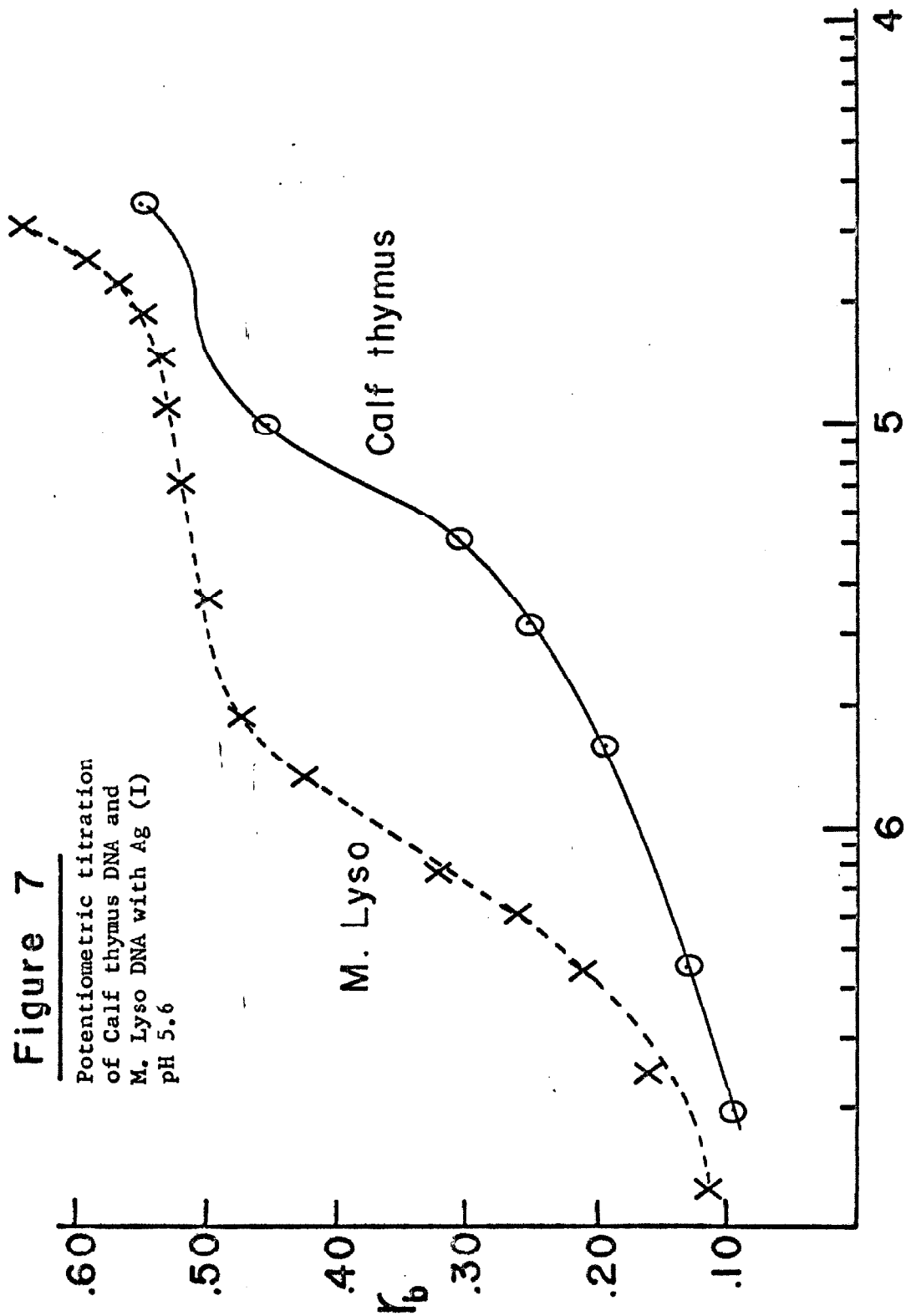


Figure 7

Potentiometric titration of Calf thymus DNA and M. Lyso DNA with Ag (I) pH 5.6

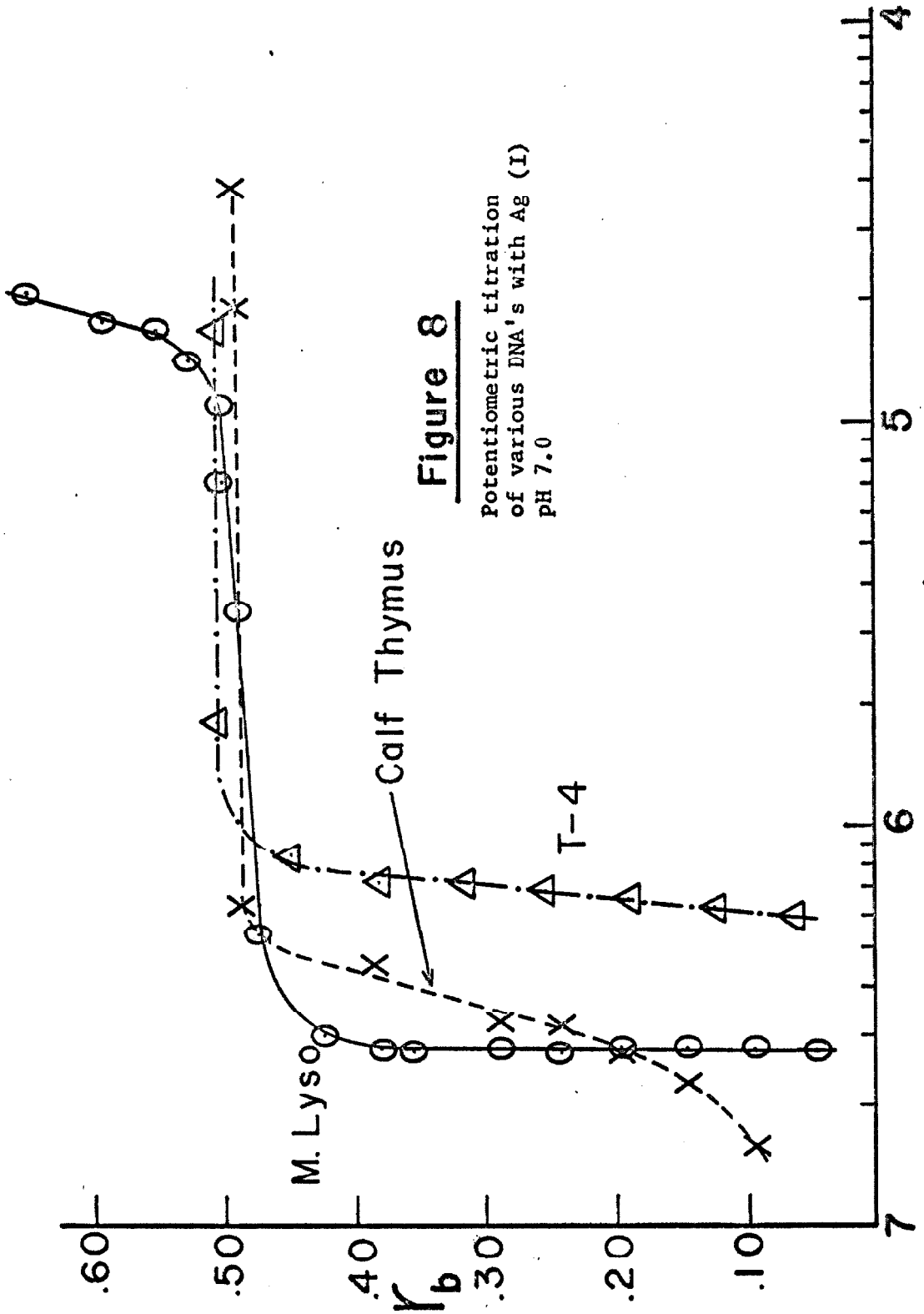


Figure 8

Potentiometric titration of various DNA's with Ag (I) pH 7.0

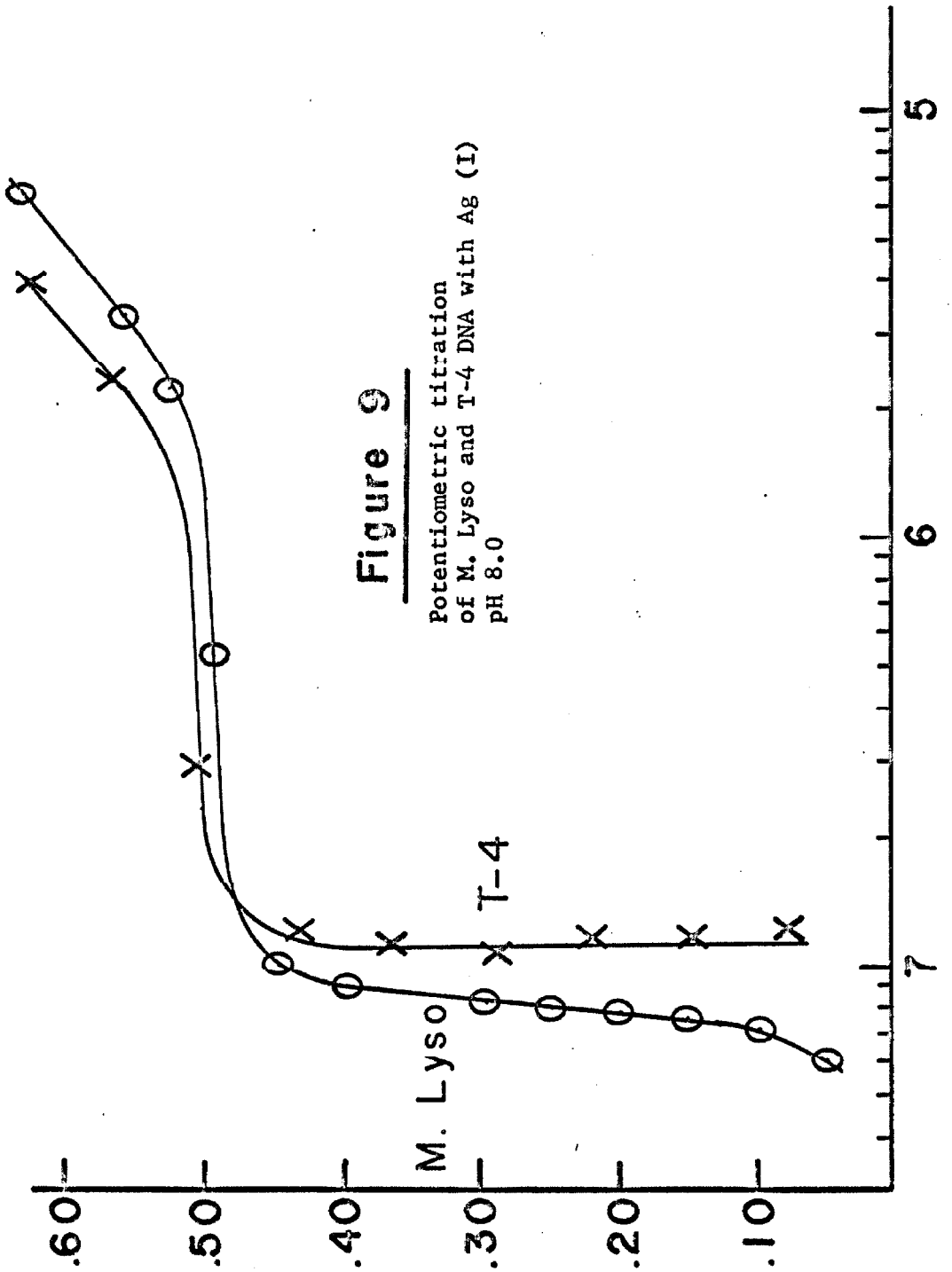


Figure 9

Potentiometric titration
of M. Lyso and T-4 DNA with Ag (I)
pH 8.0

The binding of Calf thymus DNA is stronger at low r_b than is expected from its GC contents, and its titration curve is also broader than that of the other DNA's (see figure 8). There are two possible explanations for these results. Calf thymus DNA is more heterogeneous in base composition than the phage or bacterial DNA's and the Ag (I) binding might be a measure of a true base composition heterogeneity. Another possibility is that the Calf thymus DNA was partially denatured in preparation. The extraction procedure for mammalian DNA is more drastic than that for bacterial or phage DNA, and the extraction procedure might introduce denatured regions. This would cause the titration curve to be broadened, with stronger binding at low r_b (see the section concerning denatured DNA).

Proton release. - The number of protons released per Ag (I) bound was measured in the same experiments as the binding, and the dependence of proton release on pH is shown in figure 10. At pH 5.6 there are no protons released early in the titration (the pH independent complex is formed), and up to 1.0 protons are released at high r_b's. Increasing the pH causes protons to be released earlier in the titration, since the pH dependent complexes become important earlier in the titration. Note that even at pH 8.0, where the first complex which forms is a pH dependent one, the early proton release is 0.8 (not 1.0).

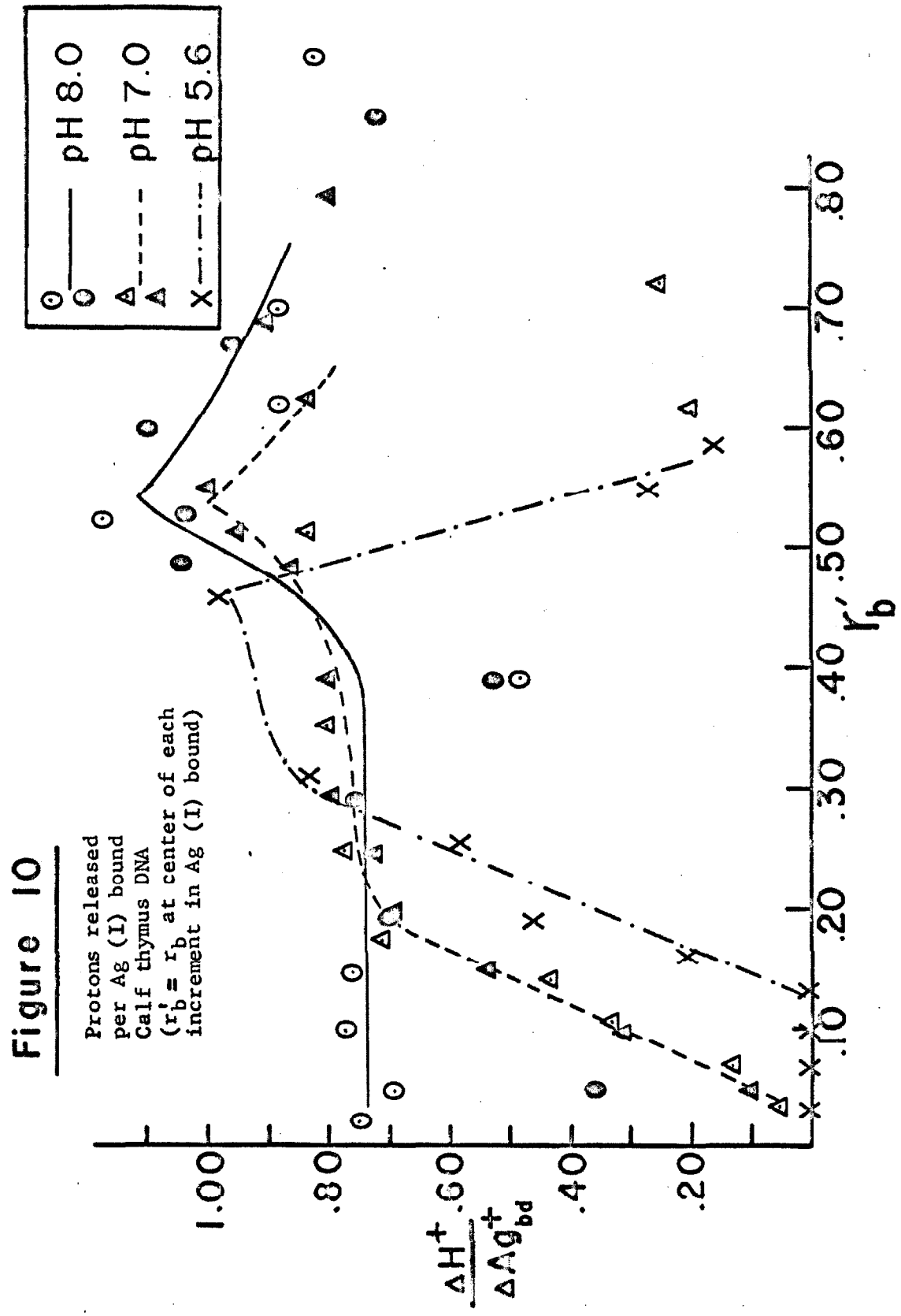


Figure 10

Protons released per Ag (I) bound
Calf thymus DNA
($r'_b = r_b$ at center of each increment in Ag (I) bound)

At all pH's M. Lyso DNA releases less protons than do the other DNA's (Figure 11). It is presumed that the GC dependent complex must release less protons (per silver (I) bound) than does the AT dependent complex. The possible nature of these complexes is discussed later.

Denatured DNA. - The Ag (I) binding and simultaneous proton release were measured for denatured DNA and compared to that of native. At pH 5.6 the binding of denatured Calf thymus DNA is somewhat stronger than that of native, with about the same numver of protons released (6). At pH 7.2 and pH 8.0, the initial silver (I) binding is much stronger to denatured DNA than to native. Furthermore, the titration curves for denatured DNA are considerably broader than for native. (Compare figures 8 and 9 with figure 12.)

Except for these two obvious differences, the Ag (I) binding characteristics of native and denatured DNA are very similar.

Analysis of binding curves. - Yamane and Davidson (6) calculated the association constants for the Ag (I)- DNA complexes using their pH 5.6 data. In order to perform this calculation, they made the following simplifying assumptions. They assumed two types of binding sites. The first kind of complexing saturates completely at $r_b = 0.20$, and the type II complexing occurs between $0.20 \leq r_b \leq 0.50$. They included a semi-empirical correction for interactions between Ag ions

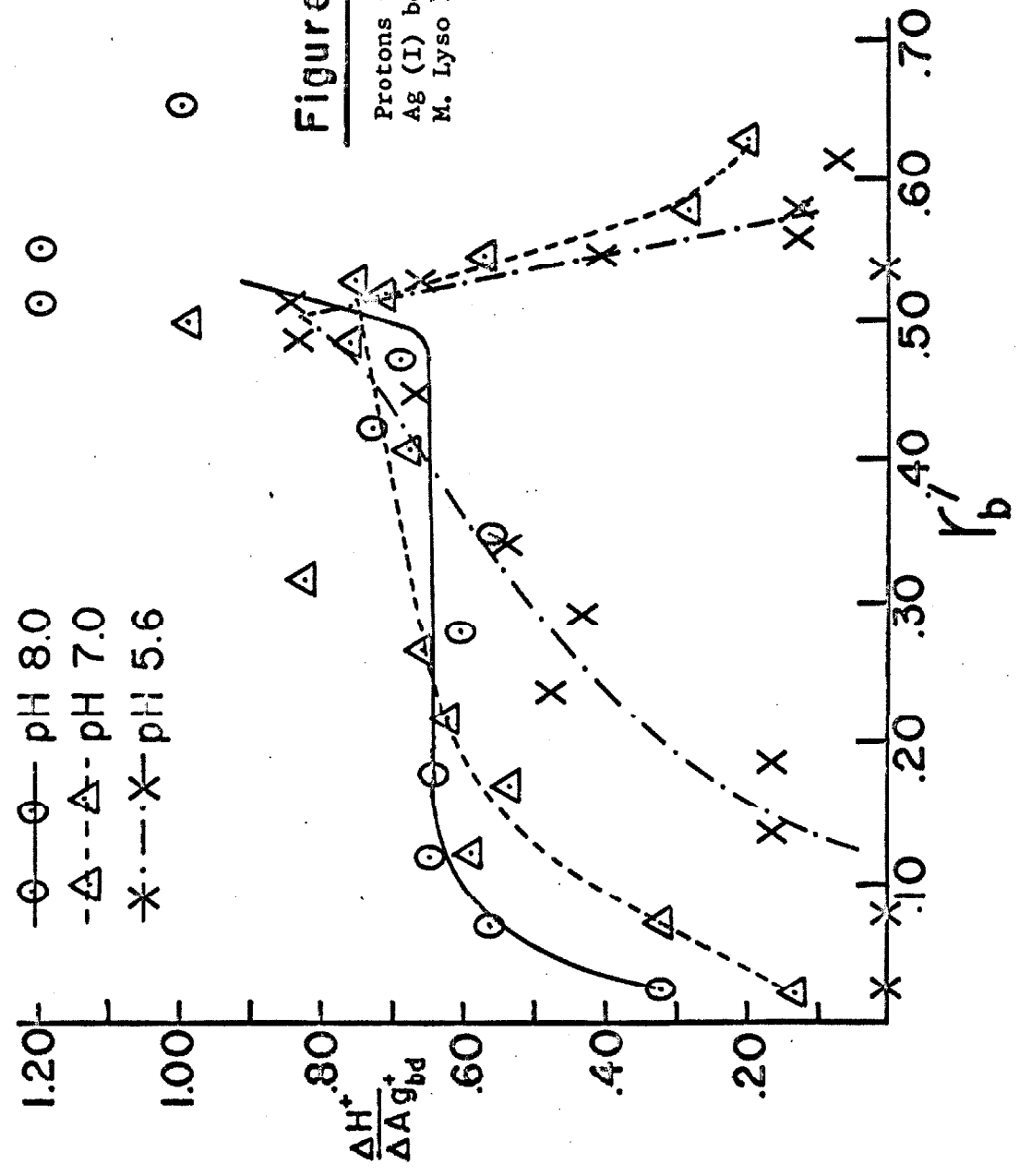
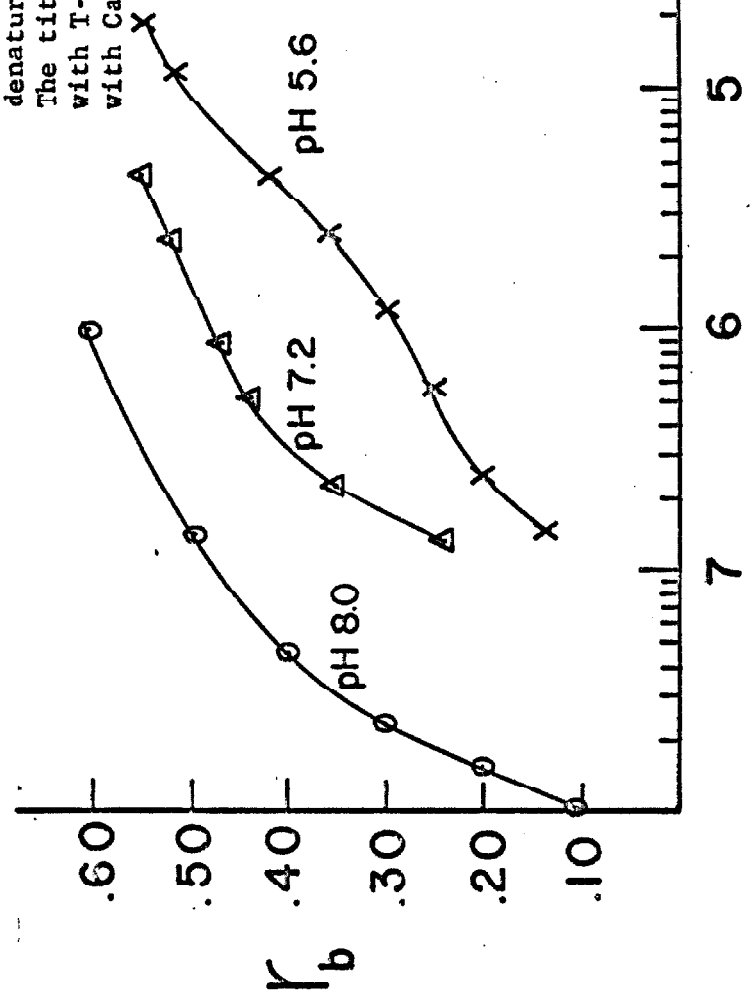


Figure II

Protons released per Ag (I) bound versus r'_b M. Lyso DNA

Figure 12

Potentiometric titration of denatured DNA with Ag (I). The titration at pH 8.0 is with T-4 DNA, the others are with Calf thymus DNA.



bound at neighboring sites. However, no provision was made for overlapping of the complexing reactions. The calculations gave fairly self-consistent results, so they felt their assumptions were justified. However, these assumptions cannot be extended to the pH 7.0 and pH 8.0 data.

It is expected that the separation between the two complexing reactions would decrease at higher pH, due to the fact that the weaker of the two complexes at pH 5.6 is formed in a reaction in which protons are released. Therefore, this reaction is more favorable at pH 8. On the other hand, the strength of type I complexing should be the same at pH 8 as at pH 5.6. This means that the two complexing reactions postulated by Yamane and Davidson will occur simultaneously. The binding data (Figure 6) and the proton release data (Figure 10) indicate that the postulate is correct.

The sharpness of the titration curves at pH 8.0 indicates that the binding is even more complicated than is implied above. At this pH, type II complexing has been enhanced to such an extent that it occurs at $(Ag^+) = 7.0 \times 10^{-8}$ (as indicated by the release of protons at $r_b = 0.05$). If type I binding is not pH dependent, it should be negligible until (Ag^+) reaches 2×10^{-7}. However, at $(Ag^+) = 1.5 \times 10^{-7}$ the binding curve already shows a plateau (at $r_b = 0.50$), and there is no further binding until much

higher Ag^+ ion concentration (ca. 10^{-5}). Also, the proton release data show that about 0.8 (not 1.0) protons are released per silver (I) bound in the entire region $0 < r_b < 0.50$. The implication is that type I binding is enhanced by the pH dependent binding such that at all pH's, type I binding occurs as one of the primary reactions.

All of these factors complicate the binding situation to such an extent that one cannot calculate association constants which are meaningful. However, an average association constant for the combined addition to type I and type II sites has been calculated. The equation for this is exactly the same as is usually given for the binding of small molecules by a polymer, including a correction for interactions between neighboring sites (4),

$$K\,(Ag^+)\,e^{-wr_b} = \frac{2\,r_b}{1 - 2\,r_b} \qquad (7)$$

where w is an interaction constant and K is the intrinsic binding constant for a site.

Taking the log of this

$$\log \left(\frac{2\,r_b}{(1-2r_b)\,(Ag)} \right) = \log K - 2.30\,wr_b. \qquad (8)$$

If the analysis is correct, a plot of the left side of equation (8) versus r_b will give a straight line of intercept log K and slope -2.30w. The data from titrations of M. Lyso and T-4 DNA at both pH 7.0 and 8.0 were used to make such a plot. The points in figure 13 are from these data. It is evident that the points do not trace good straight lines.

In order to evaluate the relevance of these plots, a similar plot was drawn for a theoretical titration. In this titration, the silver (I) binding is assumed to occur as a step function. The silver ion concentration increases with $r_b = 0$ until the first silver (I) is bound. The silver ion concentration then remains constant until r_b reaches 0.50, at which time (Ag^+) again increases with no change in r_b. For this case, equation (8) can be simplified to

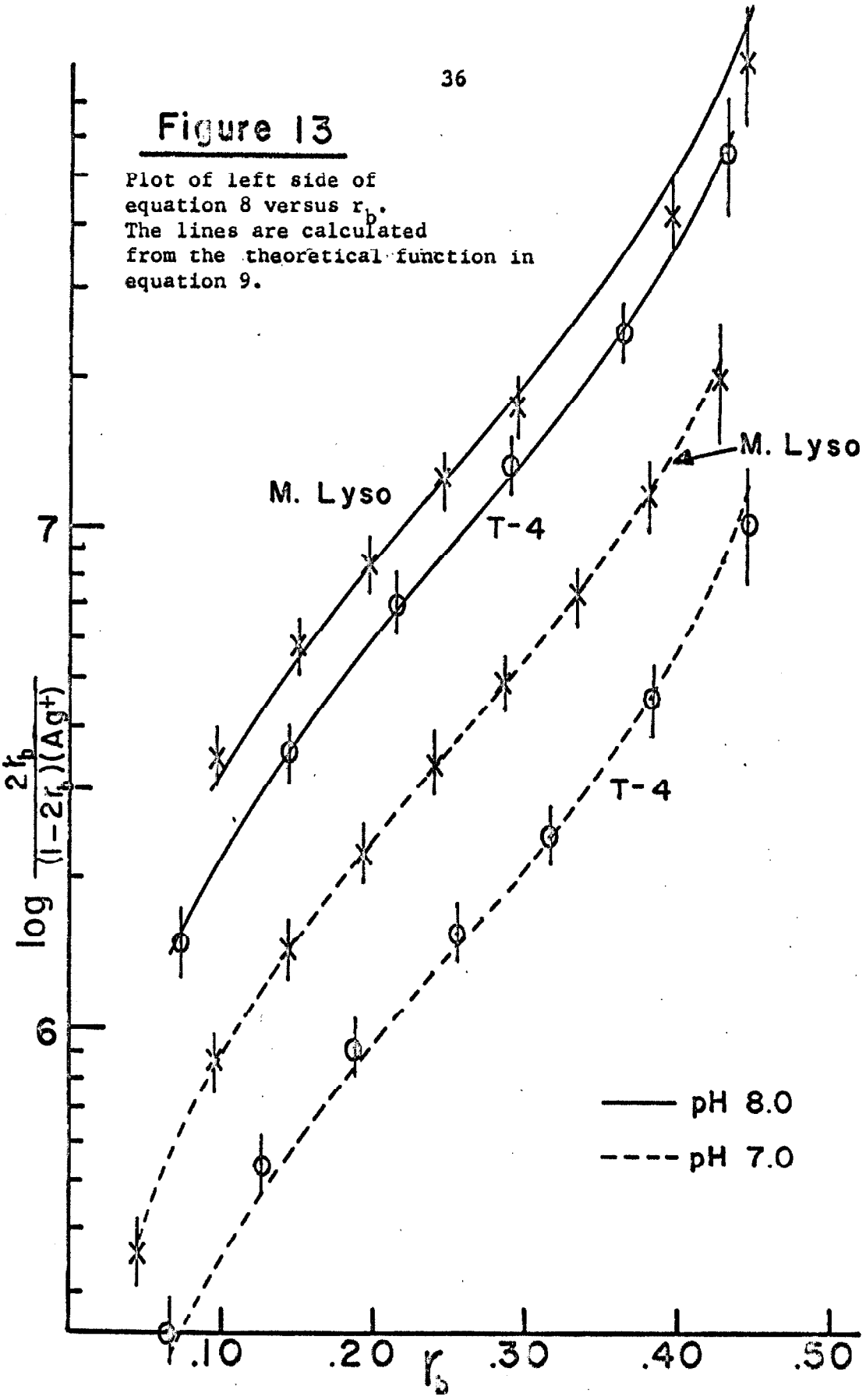
$$\log \left(\frac{2\, r_b}{1 - 2\, r_b} \right) = \log K' - 2.30\, w\, r_b \qquad (9)$$

where K' is a constant equal to $K(Ag^+)$. The lines in figure 13 are from this theoretical function.

It can be seen that, within the precision of the measurements, the points lie on the theoretical lines. Thus the analysis indicates that the titration curves are indistinguishable from a step function, and one is forced to the unlikely conclusion that the silver (I) binding is infinitely cooperative. Any calculation of w as a measure of the cooperativeness of the transition is therefore meaningless.

Figure 13

Plot of left side of equation 8 versus r_b. The lines are calculated from the theoretical function in equation 9.



Approximate binding constants can be taken from extrapolated intercepts, and these are equal to

	pH 7.0	pH 8.0
M. Lyso	4.0×10^5	2.0×10^6
T-4	1.0×10^5	1.1×10^6.

The conclusion, which should be obvious by now, is that an inspection of the binding curves is more informative than the numbers derived from this analysis. This binding analysis, along with the spectral results, does serve to demonstrate that the complexing is somewhat more complicated than Yamane and Davidson originally assumed (6). Therefore, the details of the stoichiometry cannot be discerned. The structures of some possible Ag (I)- DNA complexes are discussed in a later section.

B. Ag (I) - Synthetic Polynucleotide Complexes

The Ag (I) complexes with some synthetic polynucleotides were studied in order to throw some light on the DNA results. The three polymers studied were Poly U, Poly A and Poly dAT*, with the hope that these simpler polymers would give results which could be easily interpreted, and that the characteristics of these complexes could be extended to the DNA complexes. This has been the case only to a limited extent, inasmuch as the results are 1) not as simple to interpret as one might imagine at first glance, and 2) not easily extended to the DNA complexes. However, the results are interesting enough even in themselves to make the experiments worthwhile.

Polyuridylic acid. - As in the DNA complexes, the Ag (I) complexes of Poly U involve the nitrogenous bases rather than the sugar or phosphate groups in the backbone. The main indication of this is that the UV spectrum changes on adding Ag NO_3 to a Poly U solution, and therefore the pi system must be affected by the binding. The first silver ions bound cause a decrease

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* Poly U - polyribouridylic acid.
Poly A - polyriboadenylic acid.
Poly dAT - the alternating copolymer of deoxyadenylic and thymidylic acid.

in the absorbance, and the maximum shifts slightly to the red. Further binding causes an increase in the OD throughout the UV. One fairly precise titration was done at pH 8.0, and this allows a semiquantitative analysis of the results. A potentiometric titration was carried out in the usual way, except that at intervals an aliquot was taken for spectral measurements. A plot of the absorbance at two different wavelengths versus the Ag (I) bound (Figure 14) shows two fairly straight lines which intersect at $r_b = 0.4 - 0.5$, with a decrease in the slope of the second line as r_b approaches one. This is fair evidence for two sucessive complexing reactions, the first saturating at $r_b = 0.5$ and the second saturating at $r_b = 1$.

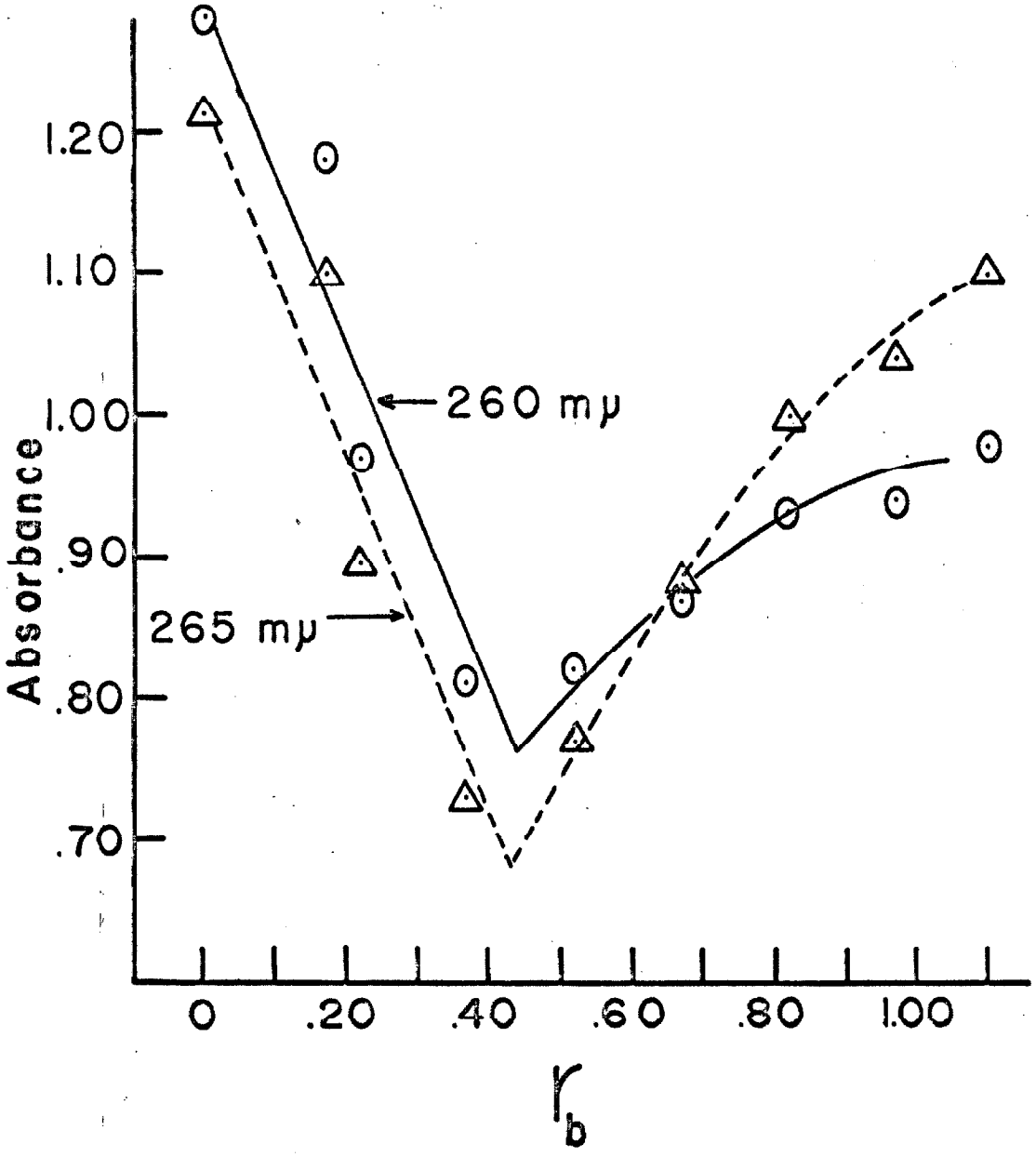
The potentiometric pH-stat data support this picture, and reveal even more about the nature of these two complexes. Figure 15 shows the titration curves for Poly U at three different pH's. It is clear that the early part of the titration is pH dependent, and the later part is almost independent of pH. Furthermore, at all pH's there is a plateau at $r_b = 1$.

Proton release data from the same experiments show very clearly the effects of two complexes forming (Figure 16). At pH 8.0 the two complexing reactions are separated; the first releases two protons per Ag (I) bound and saturates at $r_b = 0.5$,

$$\text{Complex 1} \qquad Ag^+ + 2UH \rightleftharpoons AgU_2 + 2H^+ \qquad (10)$$

Figure 14

Spectral titration of
Poly U with Ag (I)
pH 8.0



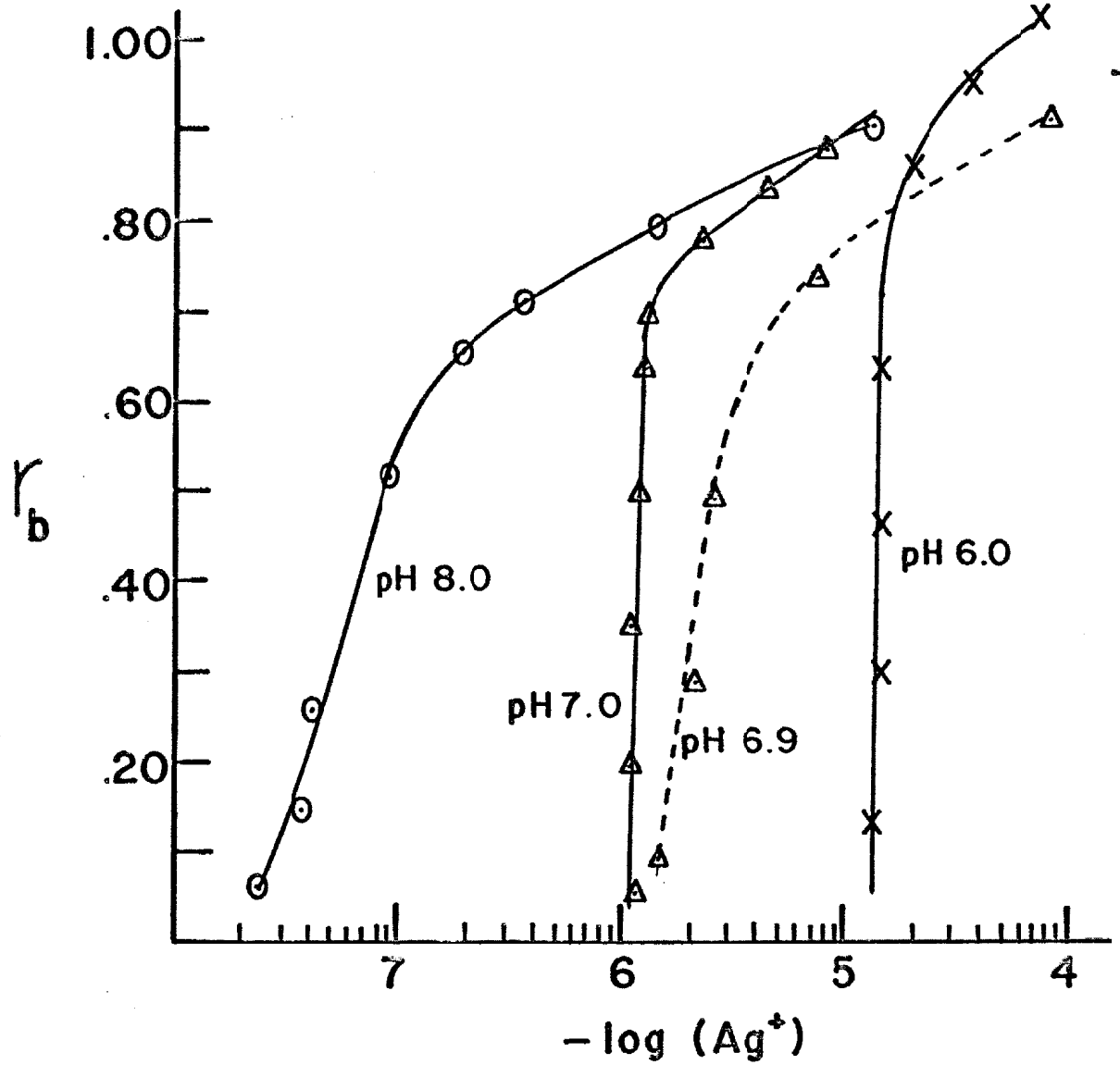
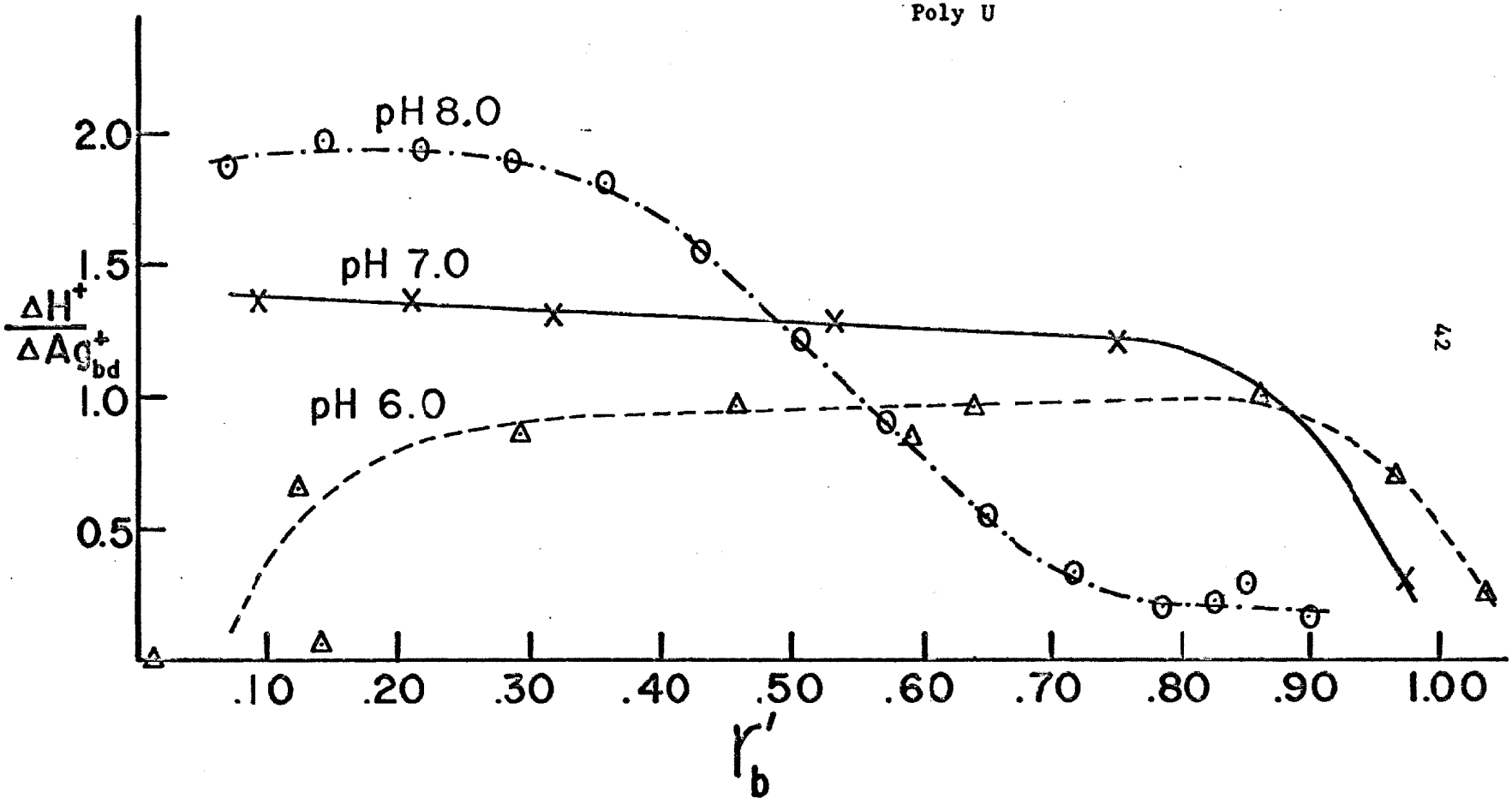


Figure 15

Potentiometric titration of Poly U with Ag (I), in 0.1 F $NaClO_4$ (solid lines) and 10^{-3} F $NaClO_4$ (dotted line).

Figure 16

Protons released per
Ag (I) bound
Poly U



the second releases no protons and saturates at $r_b = 1$. The only alternative for this is

$$\text{Complex 2} \qquad Ag^+ + AgU_2 \rightleftharpoons 2AgU \quad . \qquad (11)$$

At pH 7.0 the two reactions overlap somewhat, and at pH 6.0 complex 1 does not form at all ,

equation (10) + equation (11)

$$Ag^+ + UH \rightleftharpoons AgU + H^+ \quad . \qquad (12)$$

In these equations UH represents a uridine residue in a Poly U chain.

The integrated proton release data for the Poly U titrations at all three pH's support the interpretations offered. The ratio of total number of protons released to total number of uridines present is 1.06, 0.88, and 0.88 for pH 8.0, 7.0, and 6.0 respectively. These are all within experimental error of 1.0 proton released per uridine residue. This proton can only be from the nitrogen in the 3 position on the ring.

Several other experiments using the pH-stat method should also be mentioned. Thymidylic acid (5') was titrated at comparable conditions, and the binding is at least 1000 times weaker for this monomer than for the polymeric analogue (see section II-C). This result is difficult to explain simply because of the magnitude of the difference. The polyelectrolyte effect due to the charge on the phosphate groups has been ruled out by

the result shown in figure 15. Lowering the ionic strength 100-fold did not affect the binding strength significantly, and one would expect this to greatly enhance interaction between neighboring charges on the polyelectrolyte.

The only other reasonable explanation is that in the case of the polymer there is a second ligand which binds the Ag (I) and stabilizes the complex. This ligand must be either a second uridine residue (as in complex 1) or an oxygen (either in a sugar or a phosphate).

The really interesting result of Poly U - Ag (I) complexing is that at pH 8 the first complex forms intermittent bridges of N_3- Ag -N_3 along a chain (or between two chains), and that this affords the stability needed for the rest of the uridines in the chain (or chains) to "zip up" through hydrogen bonding. Evidence for an ordered configuration is contained in the melting curves which are displayed in figure 17. It is evident from this graph that there is an order-disorder transition that takes place, and that increasing amounts of Ag (I) bound (up to $r_b = 0.5$) afford better stability for the ordered regions.

The optical rotation of Ag (I) - Poly U solutions also changes with temperature. Table I contains a summary of the optical rotation results. These agree qualitatively with the absorbance results. However, the rotation measurements are less accurate than the UV absorption measurements. At pH 7.0 a visible precipitate

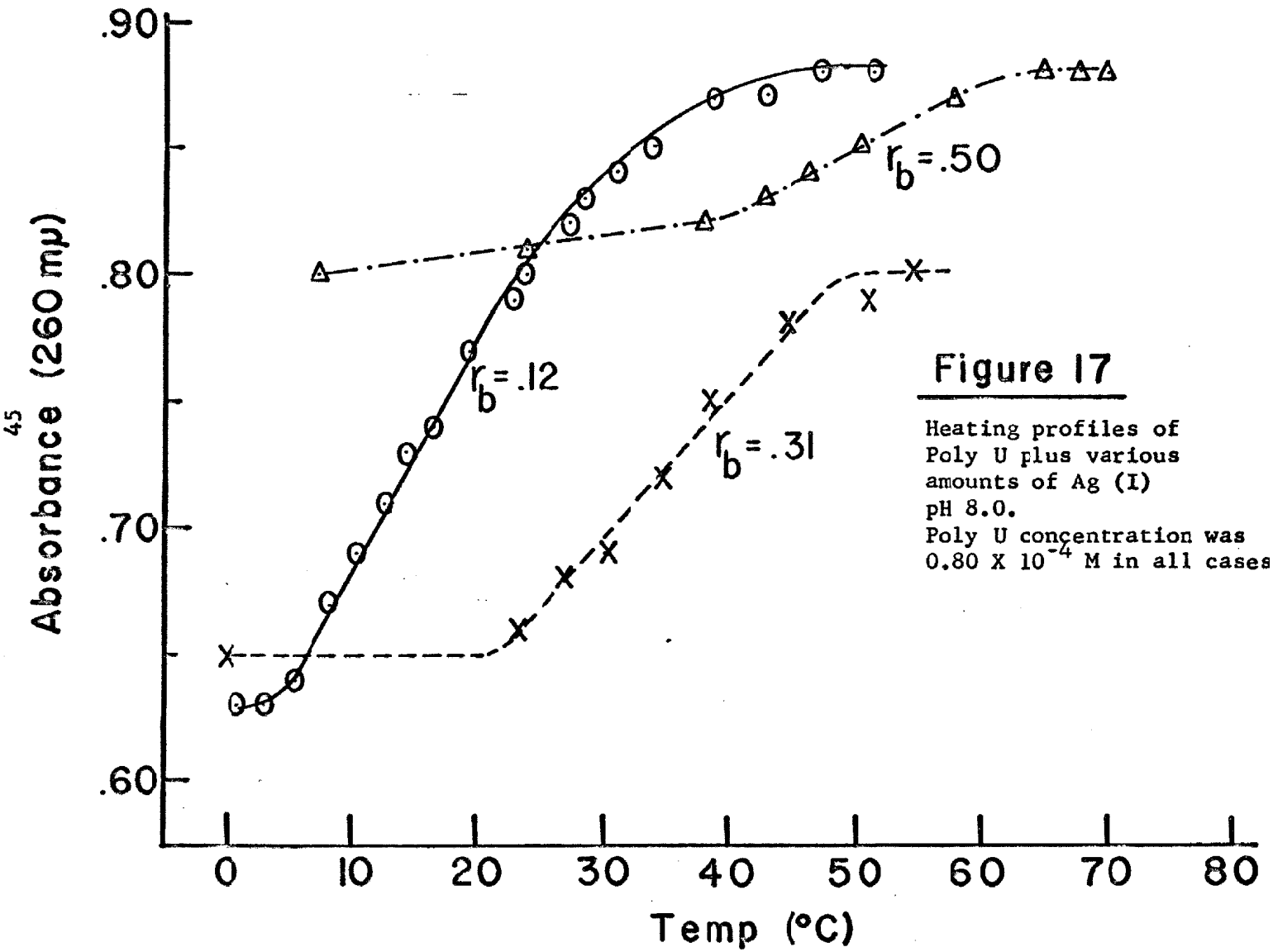


Figure 17

Heating profiles of Poly U plus various amounts of Ag (I) pH 8.0. Poly U concentration was 0.80×10^{-4} M in all cases

TABLE I

Melting of Ag (I) - Poly U pH 7.0 as measured by optical rotation.

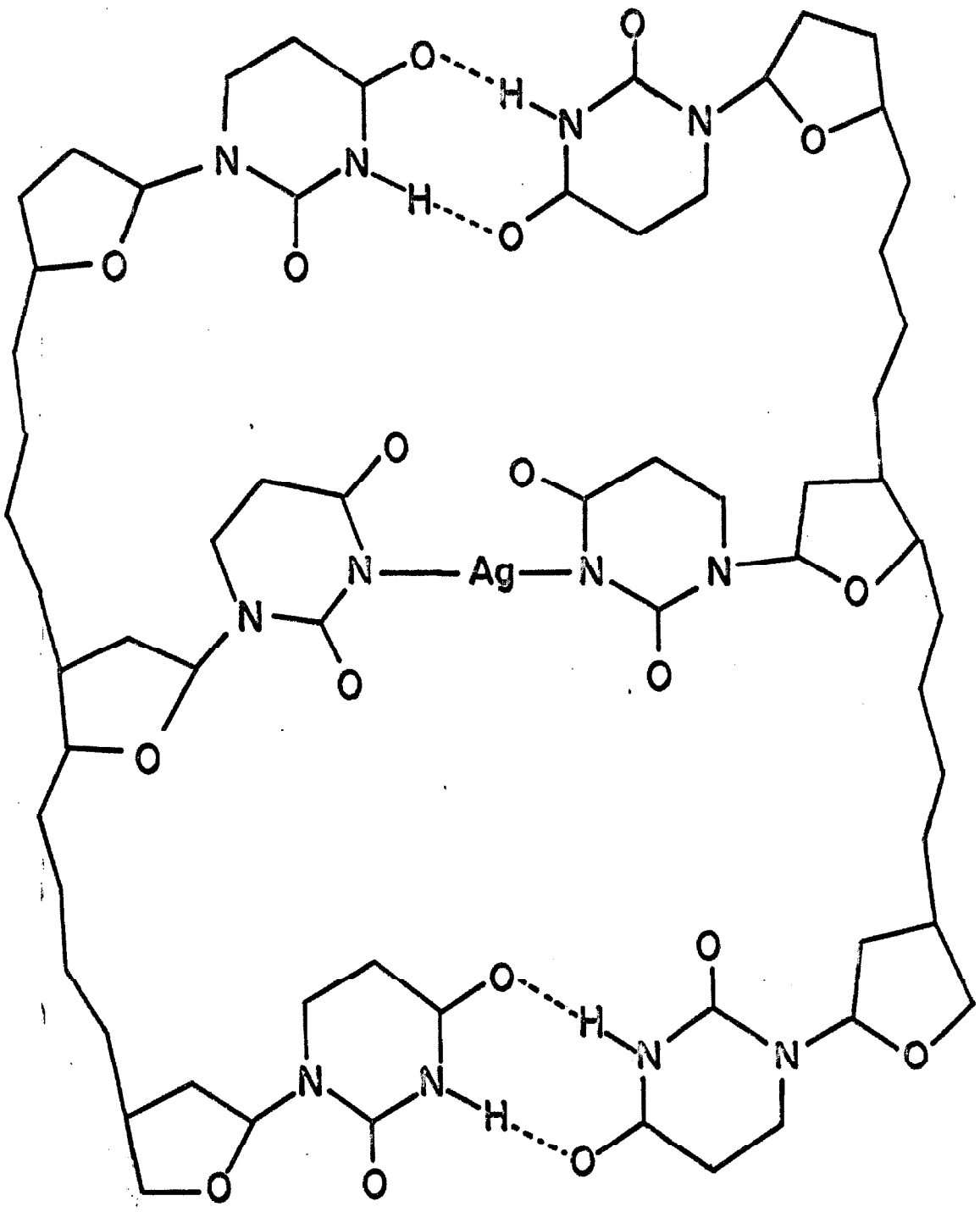
r_b	$[\alpha]_{436}$	Temp. (^oC)
0	80°	1
	40°	23
0.1	510°	1
	75°	22
0.5	260°	1
	75°	20

appears near $r_b = 0.5$ and at pH 6.0 this precipitate appears even earlier (perhaps with the first silvers bound). This does not interfere with the potentiometric pH-stat experiments, but light scattering makes the UV spectral changes obscure and renders the optical rotation almost useless. Since the rotation measurements were done at pH 7, the results for r_b above 0.5 are not reliable. However, the approximate agreement between UV melting curves and optical rotation melting curves at pH 7 implies that melting curves measured by optical rotation at pH 8.0 (where no precipitate forms) would agree well with the UV absorption results.

A reasonable model for the complexes which form is shown in figure 18. It is worth noting that this model agrees in detail with the melting curves. Over the temperature range used the Ag (I) binding strength probably does not change drastically, so that the total number of U-Ag-U and/or U-Ag bonds remains fairly constant. The hyperchromic change that occurs is due to the dissociation of the hydrogen bonded uridines, not the dissociation of the Ag-U bonds. This means that the total hyperchromic change should be greatest for small r_b where there are a large number of hydrogen bonded uridines (not Ag (I) bridged uridines). Of course these low r_b polymers should be the least stable due to the low number of silver (I)

Figure 18

The ordered structure of complex 1 of Ag (I) plus Poly U



bridges binding the chains together. Therefore, T_m should increase with r_b, as indeed it does (Figure 19).

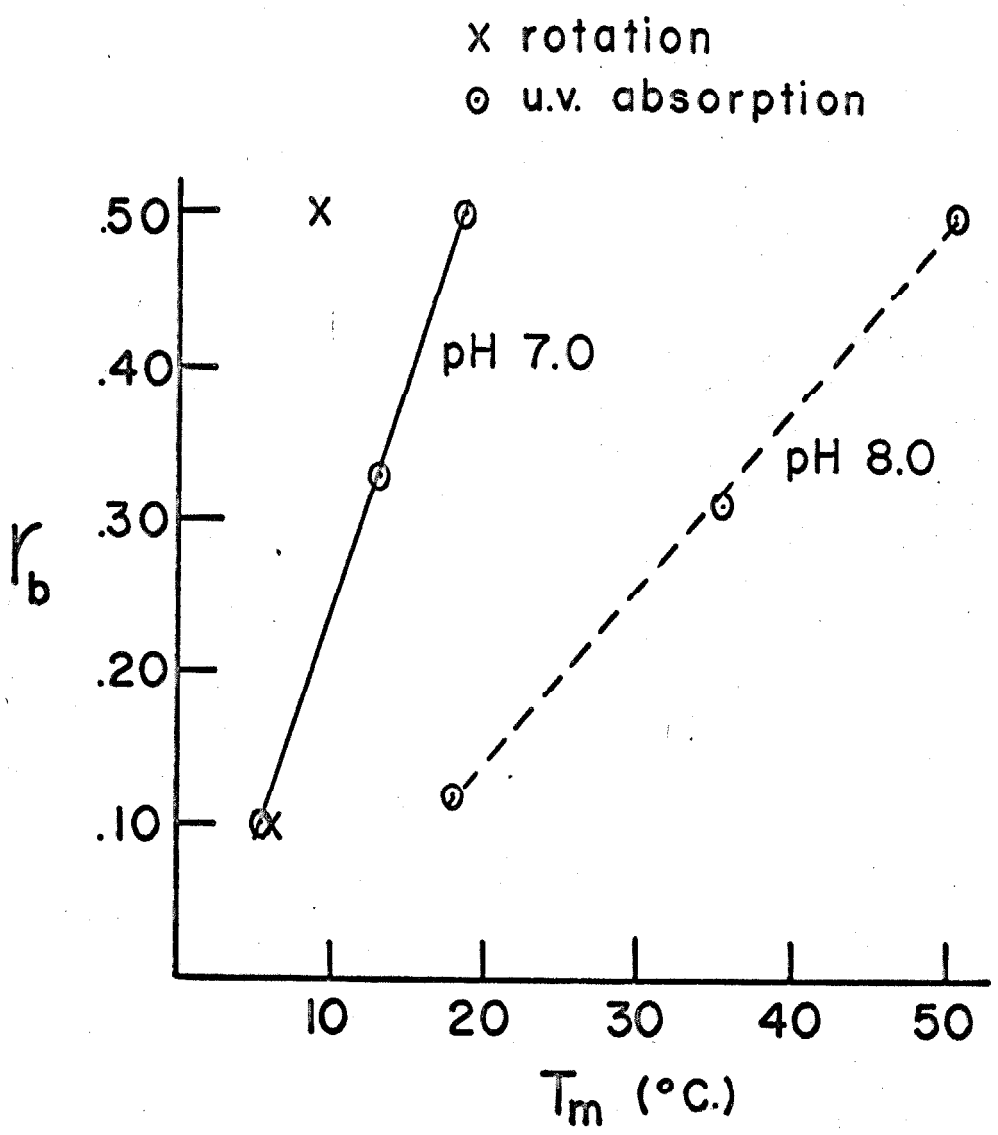
Yet a third relevant point is that above $r_b = 0.5$ there should be no hyperchromic change. All the N_3 nitrogens should be tied up (either in Ag-U or Ag-U_2, depending on r_b), and no hydrogen bonding would be possible. At $r_b = 0.70$, the absorption at 260 mμ changes negligibly between 2° C ($OD_{260} = .930$) and 95° C ($OD_{260} = .910$). One is amazed to find that, in fact, all the features of the assumed model fit the data.

Polyadenylic acid. - Because Poly A can exist either as a random coil or an ordered helix, the Ag (I) binding was expected to be more complicated than for Poly U (which was none too simple). Therefore, not much was done in trying to determine the details of the structure and properties of these complexes. Some enlightening data did come out of this, however, and they are contained in figures 20 and 21.

Figure 20 shows the Ag (I) titration curves of Poly A in the coil form (pH 7) and in the helix form (pH 5). It should be noted that, as with Poly U, the polymer binds much more strongly than the monomer, but not as strongly as DNA. There is somewhat of a plateau at $r_b = 1$, but the data are not very precise. Therefore, it cannot be said with certainty that the first complex is a one to one complex. To improve the accuracy of the titration, one would have to use

Figure 19

Melting temperature of Poly U in the presence of Ag (I), T_m vs. r_b



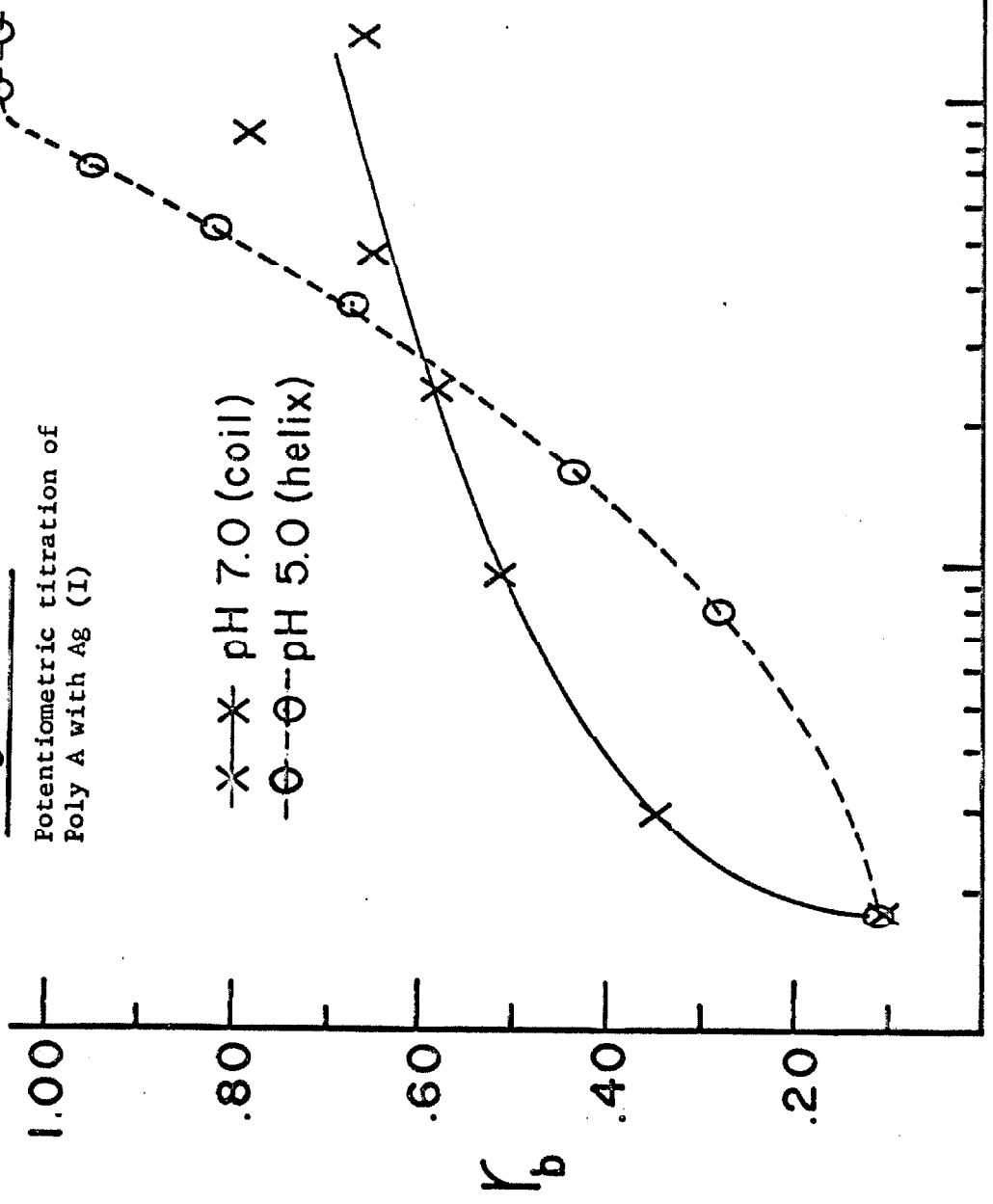
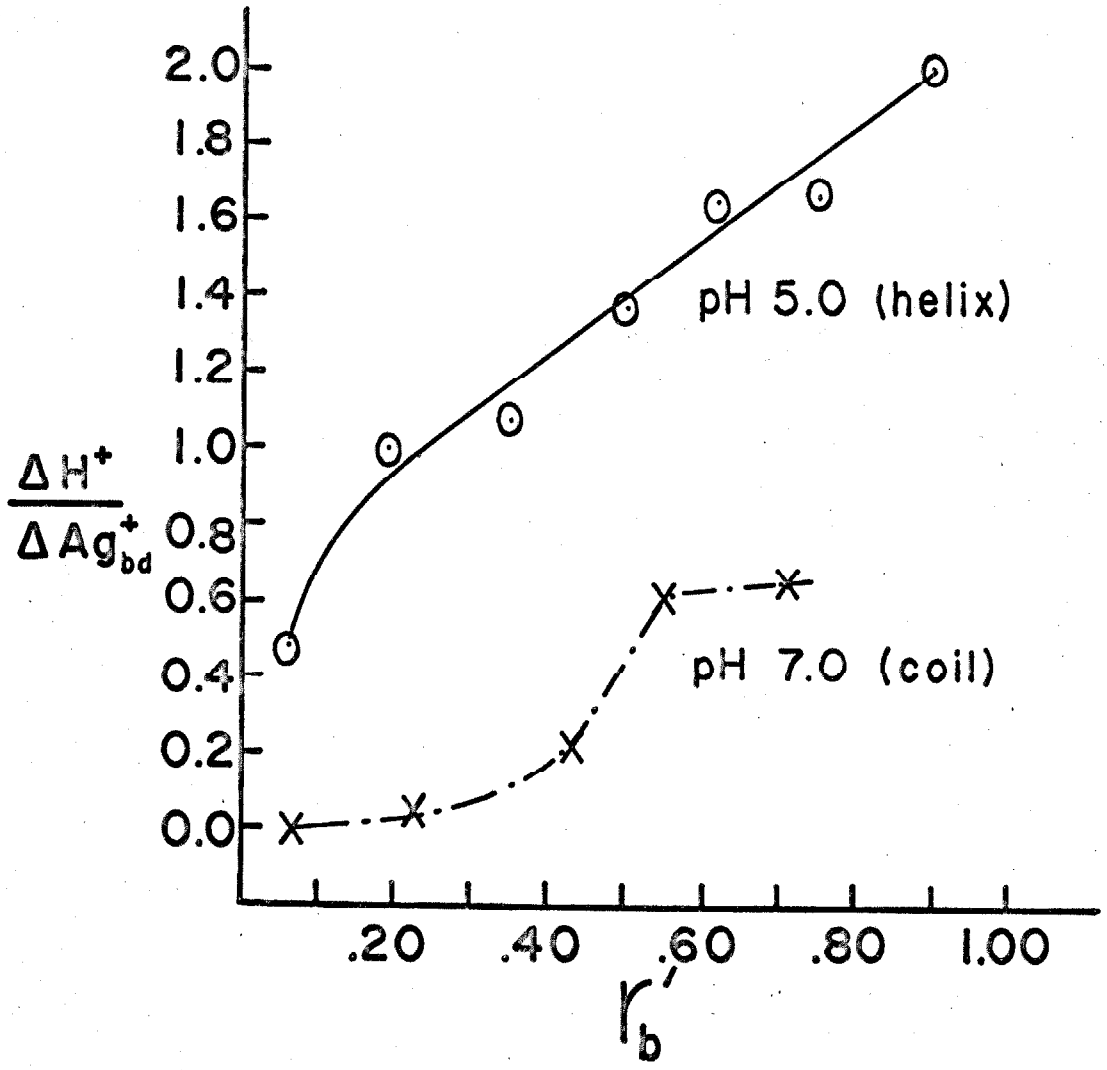


Figure 20

Potentiometric titration of Poly A with Ag (I)

Figure 21

Protons released per Ag (I) bound versus r_b' Poly A



ten times the concentration of Poly A. However, insufficient material was available at that time.

A most interesting result was obtained from the proton release measurements (Figure 21). For both the helix form and the coil form, there are protons released when Ag (I) is bound. This is to be expected for the helix form, since in order to form the helix, some of the adenosine residues must be protonated (14). It is therefore not at all surprising that the addition of Ag (I) to these residues drives off that proton. That the coil form also releases protons is somewhat surprising, for the only available protons are in the amino group. This becomes less surprising when it is found that the monomeric species, deoxyadenosine, also releases protons (see section II - E). Presumably, the same amino hydrogen is displaced when silver (I) is bound to either the deoxyadenosine or the coil form of Poly A. The spectral titration of Poly A with Ag (I) shows a small decrease in absorbance at the maximum with a slight red shift, and a fairly large increase in absorbance in the 300 mμ region. This is very similar to the spectral change which occurs when deoxyadenylic acid (5') complexes with silver (I), and also argues that the complexes are similar. To extend this analogy even further, it is likely then that an amino hydrogen might be displaced from the adenine residues in DNA, and this possibility

should be kept in mind when analyzing the DNA results.

Poly dAT. - Poly dAT is a double stranded helical alternating copolymer of deoxyadenosine and thymidine. It can be synthesized enzymatically (15), and a naturally occurring DNA which is very similar to it has been isolated (see section III - F). The rigid restrictions put on chemical interactions by the alternating double helix afford a possible easy explanation of the results.

Potentiometric pH-stat titrations and also spectral titrations were done on the synthetic copolymer by both Yamane (6) and me. Yamane's results at pH 5.6 show that there is only one kind of spectral change, an increase in absorption with no shift in maximum. Furthermore, he reports that there are no protons released when Ag (I) is bound to Poly dAT.

My results at pH 7.0 show that there is still only one spectral change, similar to that which Yamane shows. However, my potentiometric pH-stat data at pH 7.0 disagree with his proton release data. Unfortunately, a combination of circumstances make my results only qualitatively correct, and therefore, they are not presented in detail.

One problem is that a fine precipitate forms early in the titration, and the spectral results are obscured by this. Also, the potentiometric titration was performed on too small a volume to be quantitatively reliable.

It can be said with certainty that at pH 7.0 the binding to Poly dAT is weaker than to DNA (about like Poly A or Poly U at pH 7). A more important result is that there are protons released (at least one proton per Ag (I) bound early in the titration and perhaps less later). This result is incompatible with Yamane's results, and my feeling is that he simply did not dialyze out all of the buffer in which we received the Poly dAT.

The pH 7 results are in good agreement with the postulated complexes for DNA. The Ag (I) binding to Poly dAT is simply type II complexing. If this conclusion is extended to the DNA case, it indicates that, for DNA, type II complexing occurs with AT base pairs. This does not exclude the possibility that type II complexing also occurs for GC base pairs, but it does imply that type I complexing is reserved exclusively for GC base pairs.

Some care should be taken in extending the Poly dAT results to the natural DNA's. Many of the properties of Poly dAT have been found to be strikingly different from those of natural DNA's (16). These differences are probably due to the repeating sequence of nucleotides in the synthetic polymer. Such differences can also be of importance in the Ag (I) complexing behavior of Poly dAT as compared to the naturally occurring DNA's.

C. Ag (I) - Monomer Complexes

As in the case of the synthetic polynucleotides, a study of the Ag (I) complexes of purines, pyrimidines, nucleosides, and nucleotides, contributes only indirectly to the DNA story. One important result is shown in figure 22, which is a summary of some of the binding measurements. At pH 7 in 0.1 F $NaClO_4$, Ag (I) is bound at least 100 times more strongly to DNA than to any of the nucleotides. As was stated in regard to Ag (I) - Poly U binding, one cannot attribute this enhanced binding to a poly-electrolyte effect. In order to provide such strong binding, there must be some special feature (presumably stereochemical) in the way in which the nucleotides are arranged in a DNA molecule. More will be said about this in the discussion in section II - D.

Besides this cursory examination of binding strength of the nucleotides, a thorough study has been made of silver (I) binding adenine derivatives.

The most interesting conclusion from that investigation is that, with adenosine and 9-methyladenine, the very weakly acidic amino hydrogens can be displaced by silver ions. The complexes which are formed in the case of the adenine derivatives are ones which are sterically impossible in native DNA, but nonetheless

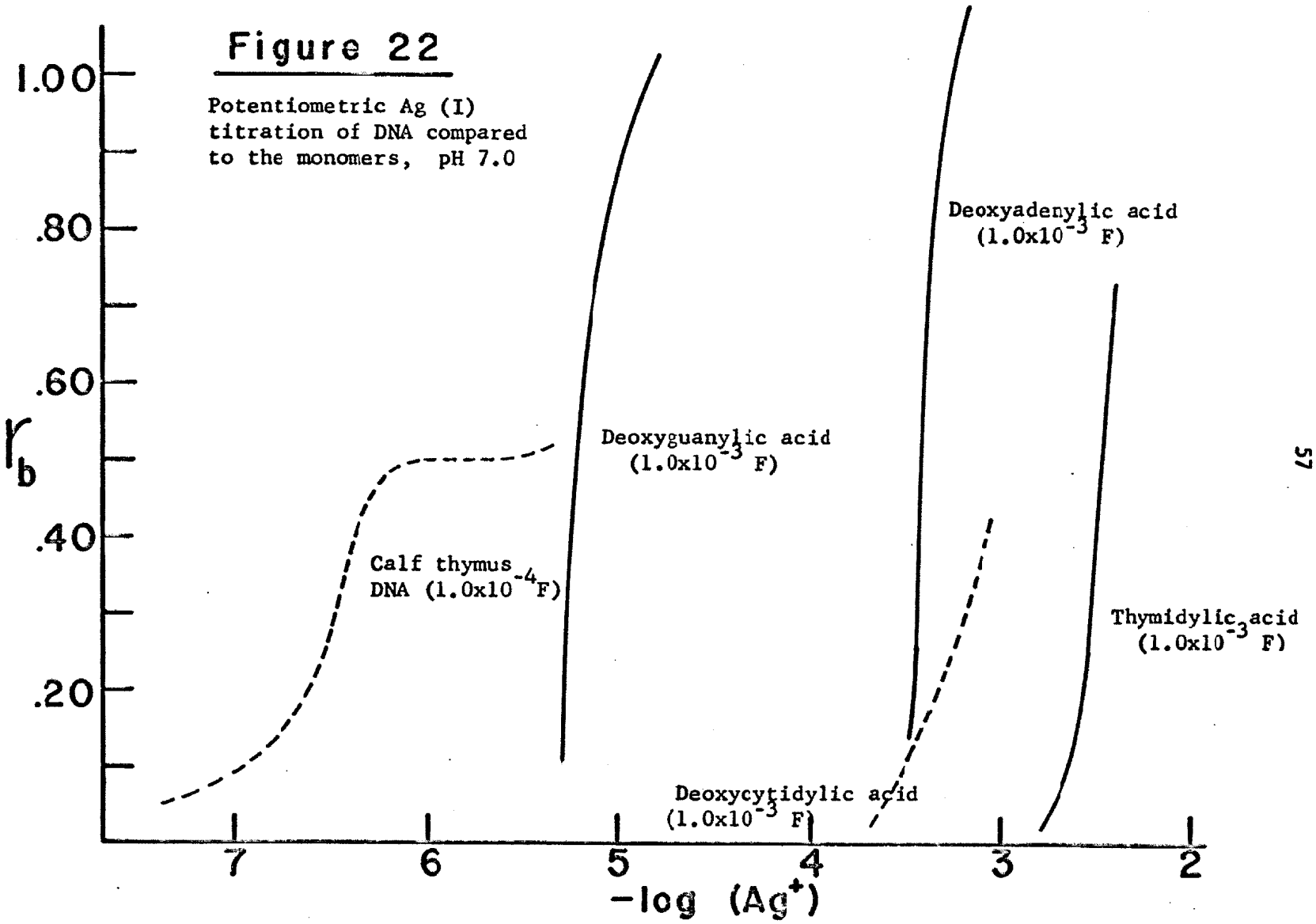


Figure 22
Potentiometric Ag (I) titration of DNA compared to the monomers, pH 7.0
1.00
.80
.60
.40
.20
r_b
Calf thymus DNA (1.0×10^{-4} F)
Deoxyguanylic acid (1.0×10^{-3} F)
Deoxycytidylic acid (1.0×10^{-3} F)
Deoxyadenylic acid (1.0×10^{-3} F)
Thymidylic acid (1.0×10^{-3} F)
7
6
5
4
3
2
$-\log (Ag^+)$

those results suggest that the amino hydrogens might be replaced by Ag (I) in DNA.

Section II - E contains the text of a publication concerning the Ag (I) complexes with adenine and substituted adenines.

D. Nature of the Ag (I) - DNA Complexes

All the data which have been presented for the Ag (I) complexes with DNA can be explained reasonably well using the Yamane and Davidson postulate that there are two basically different types of complexes (6),

$$\text{Type I} \qquad Ag^+ + DNA \rightleftharpoons AgDNA \qquad (13)$$

$$\text{Type II} \qquad Ag^+ + DNA \rightleftharpoons AgDNA + H^+ \qquad (14)$$

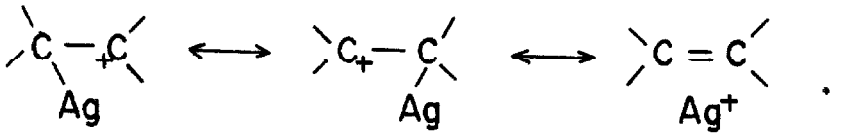
In order to satisfy all of the data, the complexes must be endowed with some special characteristics.

The type I complex is restricted to GC base pairs. The binding reaction releases no protons, but nonetheless this type of complexing is stronger at pH 8.0 than at pH 5.6. This is due to the influence of the type II complexing on type I complexing. Whenever the DNA structure is changed by the type II binding (what this structural change might be is discussed later), the type I site is put into a more favorable position for binding. Therefore, since type II binding is pH dependent, the effect is to make type I binding also pH dependent.

There are two reasonable hypotheses as to exactly where Ag (I) is associated to the GC base pair in the type I complex. Either it is complexed with the sigma electrons of the N-7 or N-3 of guanine, or there is some sort of pi complex formed between the silver (I) and an unsaturated region of either the

cytosine or the guanine (or both).

Pi complexes between silver (I) and various unsaturated systems have been postulated previously. Winstein and Lucas (17) used distribution studies to investigate silver complexes with a series of mono and diolefins. Their work indicated that the complexing was associated with the unsaturation, and they postulated that the Ag (I) shares the π electrons of the double bond,



Andrews and Keefer (18) used the same method to obtain formation constants for a series of silver complexes with aromatic substances. These form π-complexes, which for benzene (19) were shown to be somewhat localized in the region of a pair of carbon atoms. Table II is a summary of some of the constants which have been measured for Ag (I) binding with non nitrogen containing aromatics and with some aromatic nitrogen heterocycles.

TABLE II

Ag (I) binding constants for some aromatic systems.

Substance	log K_1^*	Reference
Benzene	.382	18
Toluene	.470	18
Napthalene	.467	18
Acridine	(2.20)	20
Quinoline	(2.05)	20
Isoquinoline	(1.98)	20
Pyridine	1.93	20

$$^{*}\quad K_1 = \frac{(Ag\ L)}{(Ag^{+})\ (L)}$$

The numbers in parentheses are really $\frac{1}{2}$ log $K_1\ K_2$ since only the product $K_1\ K_2$ was given. However, for a large number of silver (I) complexes $K_1 \approx K_2$.

The table shows that the aromatics which are obviously involved in only pi complexing (benzene, toluene, and napthalene) bind Ag (I) an order of magnitude less strongly than do the heterocycles. Secondly, the strength of binding of the heterocycles goes in the same order as the electron densities on their respective nitrogen atoms (21). This indicates that a nitrogen atom in an aromatic ring is a preferred site for silver (I) binding. Therefore, the ring nitrogens in the DNA bases should be the primary Ag (I) binding sites.

However, pi complexing might be tremendously enhanced by the stacking of the bases in the native DNA helix. A fact which seems to argue against pi complexing is that denaturation enhances type I binding. Yamane (6) found that at pH 5.6 and at r_b less than 0.2, neither native nor denatured DNA releases protons on binding Ag (I). This indicates that in the region $0 < r_b < 0.2$ type I binding occurs exclusively. His data show that denatured Calf thymus DNA binds Ag (I) more strongly (at $r_b = 0.10$, $(Ag^+) = 1.0 \times 10^{-7}$) than does native Calf thymus DNA (at $r_b = 0.10$, $(Ag^+) = 2.7 \times 10^{-7}$). The stacking advantage has been significantly decreased by denaturing the DNA, but the binding strength has been increased.

Superficially these observations argue that pi complexing is not involved. However, there is still a reasonable explanation which includes pi complexes. It is possible that there exists one configuration which is very favorable for pi interaction

in type I complexing and that the extra freedom which is afforded DNA by denaturation is enough to allow it to assume this configuration. The restrictions of Watson-Crick pairing would not allow this in the native structure, so that even though a good pi complex might be formed in the native helix, an even better one could be formed with denatured DNA.

The alternative hypothesis as to the nature of the type I complex is no more appealing. The N-3 and N-7 of guanine are the only nitrogens in a GC base pair which are not intimately involved in the Watson-Crick hydrogen bonding, and from which no protons will be released when Ag (I) binds. In terms of steric hindrance to the binding, either of these is a likely candidate. However, there is no good reason why type I Ag (I) complexing to the polymer should be 100 to 1000 times stronger than to the monomer. In the DNA structure there is no ligand which is held in a good position to form a second bond with a Ag (I) bound at either the N-7 or the N-3 of guanine. Therefore, type I complexing cannot be fully understood, and further work is needed to choose between the two postulates put forth.

One area of study which might be revealing is the strength of Ag (I) binding to dinucleotides. These might be regarded as miniature molecules of denatured DNA. The bases have the freedom of motion needed to assume a good configuration for pi

complexing, and strong binding of Ag (I) with no protons released could be an indication of the importance of pi complexing in type I binding.

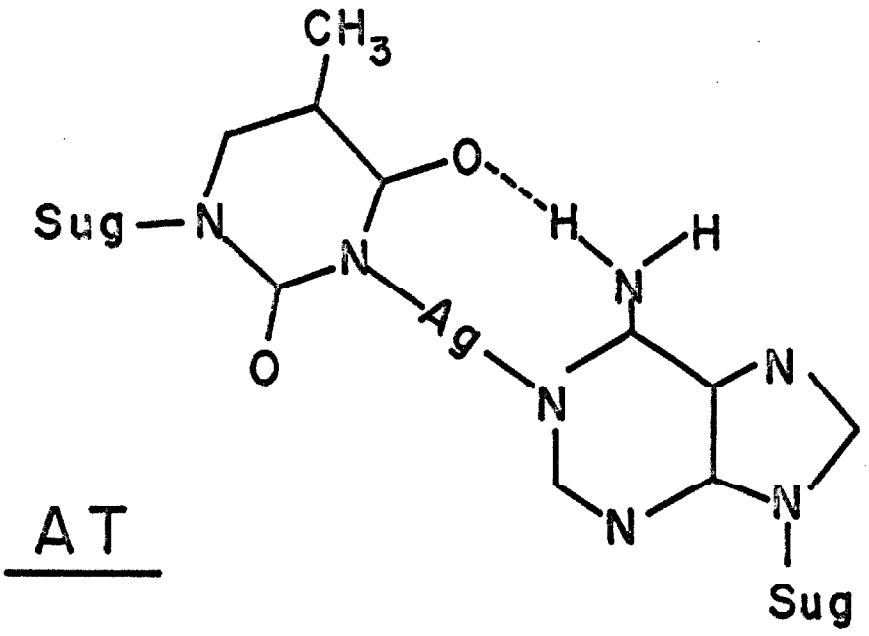
Type II complexing is more easily defined than type I. The Poly dAT results imply that it involves AT base pairs with the release of one proton per silver bound. However, there is evidence that GC pairs are also involved in a similar type of complexing.

The total number of protons released from M. Lyso DNA at saturation (r_b 0.50) is too great to be attributed to type II binding with only AT pairs. For M. Lyso DNA at pH 7.0, $r_b = 0.50$, the total number of protons released per base pair present is .62, and at pH 8.0 there are a total of .70 protons (per base pair) released by $r_b = 0.50$. Since only 28% of the base pairs are A-T, there must also be protons released from GC pairs. This demonstrates that type II complexing occurs for both AT and GC base pairs.

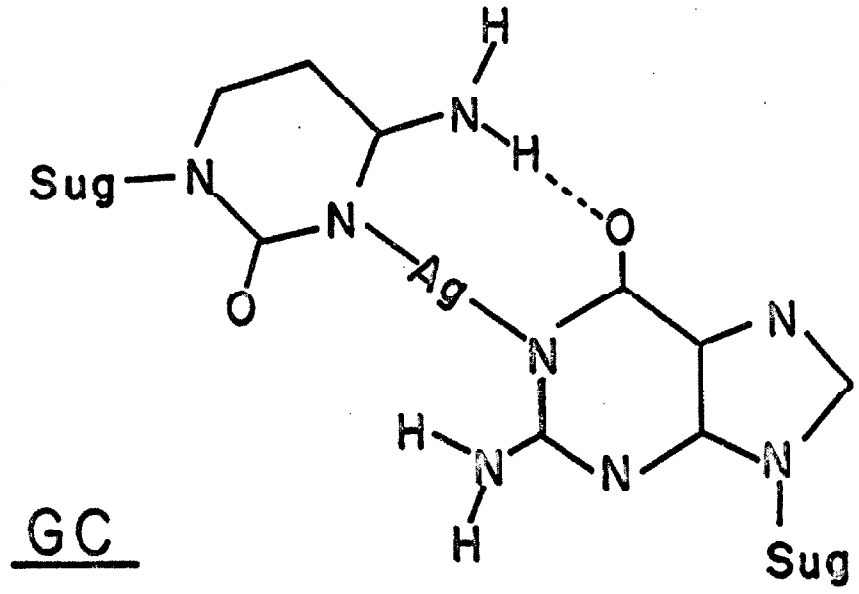
Likely structures for the type II complexes are shown in figure 23. These involve the replacement of one of the hydrogen bonds with a N-Ag-N crosslink. Corey and Wyckoff (22) calculated the Ag-N bond lengths in $Ag_2(NH_3)_4SO_4$ crystals to be 1.90 Å from X-ray diffraction measurements. In this crystal the silver is coordinated with two ammonia groups in a linear fashion, and the

Figure 23

Structures for type II complexing of Ag (I) with DNA



AT



GC

bond lengths should be nearly the same as in the crosslinking model postulated above. In the Watson-Crick structure the N-3 pyrimidine to N-1 purine distance is 3.0 Å, so there must be an increase of about 0.8 Å in this distance in order to accommodate the silver (I). This would cause a slight skew in the base pairing, (as shown in the sketch), which would result in an untwisting and a partial opening of the helix. The overall change must be relatively small since there is very little change in the visosity and sedimentation of DNA when Ag (I) is added. Since the binding for the polymer is so much stronger than for the monomers, we can presume that the energy gained by the formation of a crosslink is much greater than that needed to change the DNA secondary structure.

More evidence for the crosslinking is contained in the increase in T_m of DNA which is afforded by a small addition of Ag (I) (1). There is a large stability against heat denaturation of DNA when Ag (I) is bound only to the extent of $r_b = 0.2$, and simply on the basis of the thermodynamics of the complexing, this is unexpected. Denatured DNA binds Ag (I) somewhat stronger than native DNA, so addition of Ag (I) would be expected to destabilize the native form in favor of the denatured. However, if crosslinking occurred, one could easily visualize a way in which the melting temperature would be increased. The Ag (I)

bridges would hold the native strands together such that denatured regions would be small until a high temperature (relative to the melting temperature in the absence of Ag (I)). The Ag (I) complexes which should form with the denatured regions are thus being inhibited by the previous formation of the native Ag (I) complexes. Of course if Ag (I) were added to a mixture of denatured and native DNA, the Ag (I) would complex preferentially with the denatured DNA. This is demonstrated in the ultracentrifuge experiments (section III - C).

The Ag (I) - Poly U results argue that the silver (I) has a very strong tendency to form crosslinked complexes with the polynucleotides. The tendency is so strong that in this case it forces a random coil to assume a large degree of order. If this is possible, then it seems even more reasonable that an already ordered molecule can change its detailed structure (as in figure 23) to accomodate a similar complex.

Finally, the stronger type II binding by denatured DNA as compared to native DNA is also explainable using a crosslinking model. There is some type of Ag (I) bridge between two residues which can form in the denatured molecule but not in the native DNA. Such a bridge might be a G-Ag-G or a G-Ag-T, either of which is prevented in the native structure by the Watson-Crick restrictions, but can and does form in denatured DNA. As with

most of the results put forth here, this is not the only model which could explain this result. However, the fact that the characteristics of this model fit all of the observed properties argues well for its existence.

II - E

[Reprinted from the Journal of the American Chemical Society, 86, 2792 (1964).]

[Contribution No. 3075 from the Gates, Crellin, and Church Laboratories, California Institute of Technology, Pasadena, California]

Binding of Silver Ion by Adenine and Substituted Adenines

By Keith Gillen, Ronald Jensen, and Norman Davidson

Received February 15, 1964

Potentiometric, pH-Stat titrations reveal that silver ion forms insoluble compounds at pH values around 7 with 6-dimethylaminopurine, adenine, deoxyadenosine, and 9-methyladenine; and a soluble polymeric species with deoxyadenosine 5′-monophosphate. The binding by 9-methyl-6-dimethylaminopurine is much weaker. In the reaction with 6-dimethylaminopurine, a 1:1 compound is formed and the N-9 proton is displaced according to the reaction: $Ag^+ + BH \rightleftharpoons (AgB)_{ppt} + H^+$, $K = 5.0 \times 10^2\ M^{-1}$. With 9-methyladenine and deoxyadenosine, the reaction is: $3Ag^+ + 2BH + ClO_4^- \rightleftharpoons [Ag_3B_2 \cdot ClO_4]_{ppt} + 2H^+$, $K = [H^+]^2/[BH]^2[Ag^+]^2 = 1.37 \times 10^4\ M^{-3}$ and $6.08 \times 10^3\ M^{-3}$, respectively. The novel and unexpected result of this investigation is that with substances like adenosine and 9-methyladenine, the very weakly acidic amino hydrogens can be displaced by silver ions. With adenine, the reaction is complex; both N-9 and amino hydrogens are displaced. There is 1.5–2.0 Ag^+ bound per adenine and slightly less proton displacement. With deoxyadenosine 5′-monophosphate at pH ≥ 7, a soluble polymeric species is formed according to a reaction of the type: $2Ag^+ + BHP^{-2} \rightleftharpoons (1/n)[Ag_2BP]_n^{-n} + H^+$.

Introduction

Silver ion and mercuric ion form complexes with the nucleic acids and with polynucleotides which undoubtedly involve covalent bond formation with the purine and pyrimidine moieties, and most probably electron-pair bonds to σ-electron pairs of nitrogen atoms (although the possibility of π-bonding or some other exotic type of interaction is not absolutely excluded).[1-3] In order to understand these complexes more fully, we have investigated the reactions between silver ion and some monomeric adenine derivatives.

It is well known that silver ion forms precipitates with purines but not with pyrimidines in fairly acid solution.[4] (Although, so far as we know, the first measurement of a solubility product constant is that reported here.) A simple hypothesis as to the structure of the insoluble silver–purine complex is that it is a 1:1 compound, and a linear neutral polymer formed by replacement of the N-9 hydrogen atom of purine by silver, and then the formation of a second silver–nitrogen bond with one of the several basic nitrogen atoms of another purine molecule. In the sketch in Fig. 1, the polymerization involves the N-9 and N-7 nitrogen atoms. It will be shown that the reaction to form a 1:1 compound does occur, but it is not the only possible reaction for adenine derivatives.

If the N-9 hydrogen were the only replaceable hydrogen, adenine derivatives which are blocked in the N-9 position, such as 9-methyladenine or adenosine, would be unable to form such neutral linear polymers, and would either form conventional, soluble 2:1 complexes (analogous to $H_3N:Ag:NH_3^+$), or, conceivably, charged linear polymers, –Ads–Ag^+–Ads–Ag^+– (Ads being the neutral adenosine molecule).

The investigations reported here reveal that the predictions described in the previous paragraph are wrong. Silver ion can react with 9-methyladenine and with adenosine by displacement of one of the weakly acidic amino protons to form fairly insoluble compounds.

Experimental

Materials.—The adenine derivatives studied were the highest grade available from the sources indicated below. No further purification was attempted. The disodium salt of deoxyadenylic acid (5′) (A grade), deoxyadenosine (A grade), adenine (A grade), and 6-dimethylaminopurine (A grade) were from Calbiochem. 9-Methyladenine was supplied by the Cyclo Chemical Co.[5]; and 9-methyl 6-dimethylaminopurine was kindly furnished by Prof. Roland K. Robbins of the University of Arizona.[6]

(1) T. Yamane and N. Davidson, *J. Am. Chem. Soc.*, **83**, 2599 (1961).
(2) T. Yamane and N. Davidson, *Biochim. Biophys. Acta*, **55**, 609 (1962).
(3) S. Katz, *ibid.*, **66**, 240 (1963).
(4) A. Bendich, "The Nucleic Acids," Vol. I, E. Chargaff and J. N. Davidson, Ed., Academic Press, New York, N. Y., 1955, p. 124.
(5) Los Angeles 1, Calif.
(6) R. K. Robbins and H. H. Lin, *J. Am. Chem. Soc.*, **79**, 490 (1957).

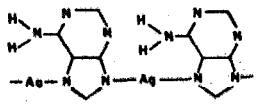


Fig. 1.—A possible neutral linear 1:1 polymer of silver ion and purine.

Physical constants from the literature or as determined by us for some of these compounds are: 6-dimethylaminopurine, λ_{max} 277 mμ (ε 1.56 × 10⁴) at pH 2 (data from Calbiochem and ref. 7), pK_a = 3.87 in H_2O,[7] pK_a = 4.53 in 0.1 *M* $NaClO_4$ (this work); 9-methyladenine, λ_{max} 262 mμ (ε 1.32 × 10⁴) at pH 7[8]; 9-methyl 6-dimethylaminopurine, λ_{max} 277 mμ (ε 1.64 × 10⁴) at pH 6, pK_a = 4.25 in 0.1 *M* $NaClO_4$ (this work).

Potentiometric pH-Stat Titrations.—This is the basic experimental measurement in this work, and makes it possible to measure the amount of silver ion bound and the number of protons released upon binding. Thus the equilibrium silver concentration and the unknown stoichiometric ratio m/β in the equation

$$\beta Ag^+ + H_nPu \rightleftharpoons [Ag_\beta{}^+H_{n-m}Pu]^{\beta-m} + mH^+ \quad (1)$$

are measured. The coefficient β has been determined by direct analysis of the precipitates, and by the shape of the titration curves. The method used in the titrations is essentially the same as used previously[5] but some important technical improvements have been introduced. The electrodes for measuring the free silver ion concentration are a silver wire and a Beckman saturated calomel electrode (s.c.e.). To prevent AgCl precipitation, the s.c.e. is separated from the solution being titrated by a salt bridge (a tube with a Beckman fiber junction) containing only the supporting electrolyte being used in the experiment. Since there should be no junction potential between the experimental solution and the salt bridge solution, the potential measured is as accurate as a s.c.e. allows.

The pH is measured using this same s.c.e. and salt bridge *vs.* a Beckman general purpose glass electrode. However, in standardizing the pH meter against a standard buffer, the salt bridge is not used. The ionic composition of the buffer and the salt bridge solution will in general be different, and a junction potential of several tenths of a pH unit can be generated.

The silver wire *vs.* s.c.e. potential was measured with a Leeds and Northrup K-2 potentiometer, using a Keithley Model 200B vacuum tube voltmeter as a null detector. This instrument has an input impedance of *ca.* 10^{14} ohms and worked well even with a shunt of 10^9 ohms (which was not however normally used). pH was measured with a Beckman Model 70 expanded-scale pH meter. In order to eliminate pickup in measurements like this it is essential to disconnect the potentiometer completely when measuring pH and to disconnect the pH meter completely when measuring the potential of the silver wire. A high impedance switch to do this was constructed. As an additional precaution, everything except the pH meter was enclosed in a Faraday cage. Potentials were reproducible to about 1 mv. (corresponding to an error of ±10% in $[Ag^+]$); pH could be read to 0.01 unit. The potential of the silver wire relative to the s.c.e. was given by

$$E\,(\text{mv.}) = 550 + 58.5 \log [Ag^+] \quad (2)$$

All solutions which were to be titrated were flushed with water-saturated argon for at least 30 min. before the titration and were kept under flowing argon during the titration. This expelled CO_2 to such an extent that the pH of an unbuffered solution would remain constant at 7 ± 0.05 for at least 3 hr. All pH-Stat titrations were done at 25 ± 1°.

Generally speaking, after each addition of silver, when precipitates were present, the pH and silver electrode potential become constant in about 30–60 min.

(7) S. F. Mason, *J. Chem. Soc.*, 2071 (1954).

(8) S. F. Mason, "CIBA Foundation Symposium on the Chemistry and Biology of Purines," G. E. W. Wolstenholme and C. M. O'Connor, Ed., J. and A. Churchill, Ltd., London, 1957, p. 66.

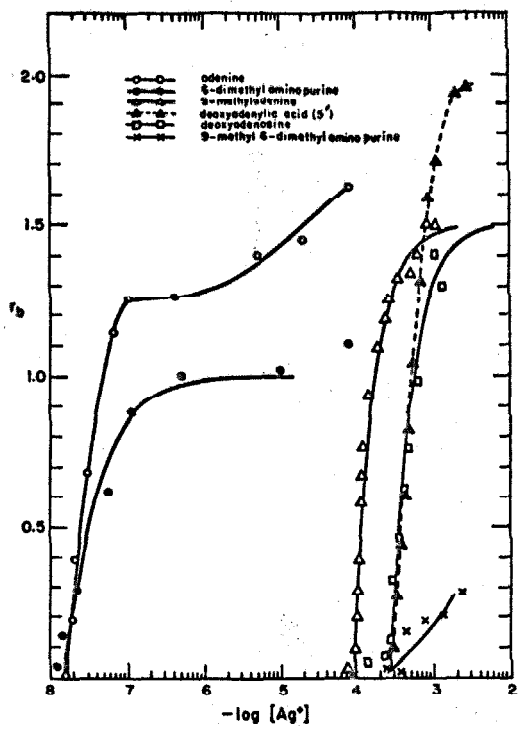


Fig. 2.—Binding curves for adenine derivatives and silver ion at pH 7 in 0.1 *M* $NaClO_4$ at 25 ± 1°. The initial purine concentration is always 10^{-3} *M*. The quantity r_b is defined as the moles of silver bound per mole of purine. For 9-methyladenine and deoxyadenosine the curves are theoretical curves for eq. 4 with $K = [H^+]^3/[Ag^+]^3[BH]^2 = 1.37 \times 10^4\ M^{-3}$ and $6.08 \times 10^3\ M^{-3}$, respectively. For 6-dimethylaminopyrine the curve is the theoretical curve for eq. 3 with $K = [H^+]/[Ag^+][BH] = 5.0 \times 10^3\ M^{-1}$.

Precipitate Analyses.—The ratio of protons displaced to silver ions bound is best measured by the titration method just described. The ratio of silver to purine in the precipitates formed is best measured by direct analysis. These analyses were done in the following manner. A deoxyadenosine or 9-methyladenine solution was buffered at the desired pH with cacodylate buffer (10^{-2} *F*). A specified amount of silver nitrate solution was added, whereupon a precipitate formed. At least 4 hr. was allowed for the system to equilibrate, after which the precipitate was separated from the supernatant either by centrifugation or filtration through a sintered glass filter. The precipitate was dissolved with 0.1 *F* $HClO_4$ and the ultraviolet spectra of the two solutions were taken. Assuming there was no soluble complex in either of these solutions, this immediately gave the amount of purine in the precipitate and free in the equilibrium solution. The sum of these always added to within 2% of the total purine present. The amount of silver in each solution was measured either by a direct potential reading using the silver wire electrode, or (in later, improved experiments) by titration with KSCN using the potential to determine the equivalence point. Using direct measurement of potential, the sum of the moles of silver in the two solutions was usually within about 10% of the total moles put into the system. By titrating with KSCN, the sum was within 1% of the moles of Ag^+ originally added.

Results

The results are displayed in the figures and tables which are, we hope, largely self-explanatory.

Figure 2 shows that the strongest binding is by the two purines, adenine and 6-dimethylaminopurine,

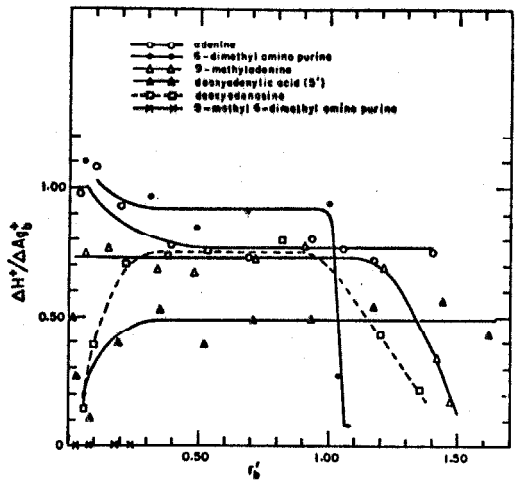


Fig. 3.—Proton release data at pH 7 with an initial purine concentration of 10^{-3} M. The quantity $\Delta H^+/\Delta Ag^+_b$ is the number of protons released per silver ion bound when a small amount of silver (usually corresponding to $\Delta r_b \approx 0.1$) is added to the solution under the given conditions. The horizontal coordinate (r_b') is the quantity r_b at the center of each increment in r_b.

which have an N-9 hydrogen atom. The three substances, 9-methyladenine, deoxyadenosine, and deoxyadenosine 5′-monophosphate (deoxy-AMP), which are N-9 blocked but have amino hydrogens, bind less strongly, but still very markedly. The binding by 9-methyl 6-dimethylaminopurine, with no N–H bonds, is weaker and just barely perceptible under the conditions used. In all cases where binding occurs, except with deoxy-AMP, a precipitate appears to form as soon as a significant amount of silver has been bound. The titration curves are quite sharp which is qualitatively more consistent with the formation of an insoluble precipitate or a soluble polymer than with the formation of a soluble low molecular weight complex.

It may be mentioned incidentally that the binding of Ag^+ by DNA and by poly-A at this pH is weaker than the binding by the N-9 unblocked purines but stronger than the binding by the other derivatives studied.[9]

The proton release data (Fig. 3) show that for 6-dimethylaminopurine, one proton is released per silver bound; Fig. 2 and 3 both indicate that the reaction stops with one silver ion bound per purine. The reaction is therefore

$$Ag^+ + (CH_3)_2N\text{-}C_5H_2N_4H \rightleftharpoons [(CH_3)_2N\text{-}C_5H_2N_4Ag]_{ppt} + H^+ \quad (3)$$

For 9-methyladenine and deoxyadenosine, there is about $0.75H^+$ released per Ag^+ bound. This is true at all pH values where reasonably accurate measurements can be made. (The binding of Ag^+ by deoxyadenosine is too weak at pH 6 for accurate measurements.) The proton release is approximately constant until $r_b > 1$ and then decreases toward zero at $r_b \approx 1.5$ (where r_b is the moles of silver bound per mole of purine). There is a fair amount of experimental error in these differential measurements.

(9) R. Jensen and N. Davidson, unpublished work.

The binding curves (Fig. 2) appear to level off at $r_b = 1.5$. We therefore propose as an idealized interpretation of the data that the reaction taking place throughout the range $0 \leq r_b \leq 1.5$ is

$$3Ag^+ + 2BH + ClO_4^- \rightleftharpoons [(Ag_3B_2)ClO_4]_{ppt} + 2H^+ \quad (4)$$

where BH is the neutral purine with H being an amino hydrogen. This predicts a constant displacement of 0.67 proton per Ag^+ bound up to $r_b = 1.5$. Reaction 4 conceivably could occur in stages, with a 1:1 precipitate and one proton displaced in the early states of the reaction, and an additional $0.5AgClO_4$ being bound later. The constancy of the proton release argues against this possibility. Furthermore, the precipitate analyses in Table I confirm the silver

TABLE I

PRECIPITATE ANALYSES FOR SILVER ADENOSINE AND SILVER 9-METHYLADENINE PRECIPITATES

Purine[a]	pH[b]	r_b	Ratio[c]	Method[d]	Silver lost, %[e]
dA	6.60	0.87	1.54	AgSCN	<1
dA	7.80	0.87	1.46	AgSCN	<1
9-MA	6.87	0.82	1.49	AgSCN	<1
9-MA	6.85	0.80	1.46	AgSCN	<1
dA	6.7	0.10	1.45	Pot.	15
dA	6.7	0.20	1.4	Pot.	25
dA	6.8	1.10	1.53	Pot.	4
dA	6.7	1.16	1.91	Pot.	2

[a] Deoxyadenosine (dA) or 9-methyladenine (9-MA). [b] pH of precipitation. All solutions 0.1 M in $NaClO_4$ and buffered with 0.01 F cacodylate. [c] Silver to purine ratio in precipitate. [d] Thiocyanate titration or direct potentiometric determination of silver concentration. [e] Silver found in precipitate and supernatant compared to silver added. The amount of purine found was always within 1% of that expected.

to purine ratio of 1.5:1 even at low r_b values; thus, reaction 4 is the main reaction occurring. (The most accurate silver determinations on the precipitate are done by the thiocyanate method. Unfortunately, due to an oversight, these were all done at fairly high r_b. However, we believe the analyses by the direct potential method are sufficiently accurate to justify the statements made above.)

The variation of the log of the equilibrium silver concentration with pH at fixed r_b may be represented by a straight line with slope 0.7 ± 0.1 (Fig. 4) in approximate agreement with the predictions of eq. 4. The predicted variation of the silver concentration with r_b at a fixed pH from eq. 4 is plotted in Fig. 2 for average values of the equilibrium constants. The results are in approximate agreement with the theoretical curves. The values of K computed from individual points along the curve however vary by more than 50%; hence, the agreement between theory and experiment displayed in Fig. 2 is not sufficiently good to be a strong argument for the correctness of eq. 4. The same remark applies to eq. 3 and the theoretical curve for 6-dimethylaminopurine.

The results with adenine (which has been less intensively studied) suggest that silver is bound by two consecutive but overlapping reactions

$$Ag^+ + BH_2 \rightleftharpoons [AgBH]_{ppt} + H^+ \quad (5a)$$

$$[AgBH]_{ppt} + xAg^+ + (x - y)ClO_4^- \rightleftharpoons [Ag_{1+x}BH_{1-y}(ClO_4)_{x-y}] + yH^+ \quad (5b)$$

where BH_2 is neutral adenine and $1 \geq x > y \geq 0.5$. The N-9 hydrogen is displaced in (5a) and the amino hydrogen from some of the purines in (5b). The proton release starts at unity and decreases with r_b; this is the principal evidence that the two reactions overlap. The subsequent binding reaction that follows (5a) has not been carefully studied because the results at high r_b are not very precise.

The integrated proton release data for the various substances (Table II) support the interpretations offered. When an excess of silver ion is added, one proton is displaced from deoxyadenosine, 9-methyladenine, and 6-dimethylaminopurine in agreement with eq. 3 and 4. From adenine 1.5 protons are displaced in approximate agreement with eq. 5.

TABLE II

TOTAL PROTON RELEASE IN THE pH-STAT TITRATIONS[a]

Purine	pH	Final r_b	Total H⁺ released / Total purine present
6-Dimethylaminopurine	7	1.1	0.90
	7	1.0	0.93
Deoxyadenosine	7	1.6	1.0
	7	1.4	0.85
	8	1.6	1.1
9-Methyladenine	7	1.6	0.94
	7	1.5	1.0
	8	1.5	1.1
Adenine	7	1.6	1.5
Deoxyadenylic acid	7	2.8	1.0
	7	2.1	0.91
	8	2.3	0.96
	8	2.7	1.1

[a] The conditions of the titrations are as described in the legends to Fig. 2 and 3.

With deoxyadenosine 5′-monophosphate, a precipitate is not observed until about $r_b = 2.3$ at pH ≥ 7 and $r_b = 0.3$ at pH 6. The proton release data are probably somewhat complicated by the fact that the phosphate group protonates with a pK of about 6.2. The indications are that above pH 7 a soluble polymeric species is formed according to a reaction of the type

$$2Ag^+ + BHP^{-2} \rightleftharpoons (1/n)[Ag_2BP]_n^{-n} + H^+ \quad (6)$$

where BHP^{-2} is the dinegative deoxyadenosine monophosphate anion. The precipitate at lower pH is presumably due to protonation of the phosphate groups.

Discussion

For the soluble monomeric complexes of a series of monofunctional nitrogen bases with silver ion, the equilibrium constants for the reactions

$$Ag^+ + B \rightleftharpoons BAg^+ \quad K_{BAg} \quad (7a)$$

$$H^+ + B \rightleftharpoons BH^+ \quad K_{BH} \quad (7b)$$

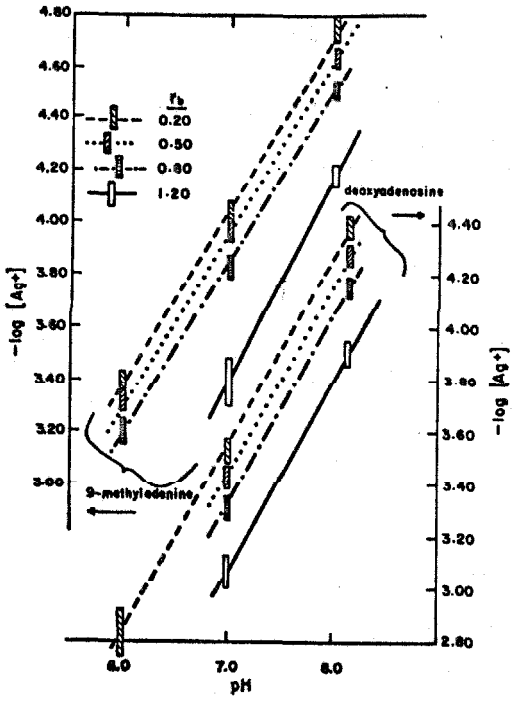


Fig. 4.—Variation of log [Ag⁺] with pH at several r_b values for deoxyadenosine and 9-methyladenine. The slopes of the straight lines are shown.

r_b	Slope (9-methyladenine)	Slope (deoxyadenosine)
0.20	0.68 ± 0 04	0.72 ± 0.06
0.50	0.66 ± 0.04	0.74 ± 0.07
0.80	0.65 ± 0.05	0.73 ± 0.07
1.20	0.79 ± 0.13	0.72 ± 0.09

fit the linear free energy equation[10]

$$\log K_{BAg} \approx 0.3 \log K_{BH} + \text{constant} \quad (8)$$

That is, for a series of acids BH, the equilibrium constant K_9, for the breaking of a nitrogen to hydrogen bond and the formation of a nitrogen to silver bond

$$Ag^+ + BH \rightleftharpoons BAg^+ + H^+ \quad K_9 = K_{BAg}/K_{BH} \quad (9)$$

is predicted to follow the equation

$$\log K_9 \approx -0.7 \log K_{BH} + \text{constant} \quad (10)$$

The weaker the acid, BH, the more is reaction 9 displaced to the left at a given pH and silver ion concentration.

The pK values of the N-9 hydrogens of adenine and 6-dimethylaminopurine are 9.8 and 10.5.[7] The amino hydrogens of adenine, adenosine, and 9-methyladenine are much less acidic. Thus, at first sight, it appears plausible that the N-9 hydrogen should be replaceable by silver at a moderate pH such as 7 but surprising that the amino protons can be displaced.

(10) R. J. Bruehlman and F. H. Verhoek, *J. Am. Chem. Soc.*, **70**, 1401 (1948).

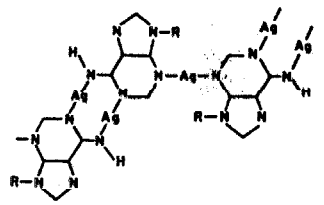


Fig. 5.—A speculation as to the structure of the silver 9-methyladenine (or silver adenosine) precipitate.

On reconsideration, however, this result may not be so surprising. Equation 10 is not expected to apply accurately to the substances studied here. They are not structurally very similar to the nitrogen bases which were correlated by eq. 8 in the original paper.[10] The formation of a precipitate and the necessity of comparing different reaction types (eq. 3 and 4) is an additional complicating factor. However, eq. 10 might be approximately applicable. Figure 2 shows that at pH 7 and at any fixed r_b around 0.5, the equilibrium silver ion concentration for displacing an N-9 hydrogen is about 10^{-4} of that for displacing an amino hydrogen. Take 10 for the pK_a of an N-9 NH bond; eq. 10 then predicts a pK_a of $10 + (4/0.7) \approx 16$ for an amino hydrogen. There are no measurements available, but our chemical intuition is not repelled by this estimate.

In part, the driving force of the reactions studied here is the formation of polymeric species, either soluble or as precipitates, due to the presence of several basic nitrogen atoms in a purine ring system. This is not a decisive factor however in making a displacement of an amino hydrogen possible. Simpson has observed proton displacement of amino hydrogens of purines and pyrimidines by the monofunctional methylmercury cation to give soluble complexes.[11] Eichorn and Clark[12] have presented evidence that mercuric ion can replace a proton from the amino group of adenosine on cytidine.

There remains the perplexing question of why the silver to purine ratio for amino binding (deoxyadenosine and 9-methyladenine) is 3:2 rather than 1:1. A number of speculations can be put forth but, in the absence of further evidence, it is not profitable to do so. However, we wish to record the suggestion displayed in Fig. 5. Here, two silvers are used in the way that hydrogens are used in the hydrogen-bonded base pairs of nucleic acids. This type of ring formation would provide an additional driving force for the displacement of the amino hydrogens. The uptake of an additional silver per base pair (by N-3 in the figure but it could just as well be N-7) is necessary in order to get an insoluble, polymeric species.

Acknowledgment.—This research has been supported by the Atomic Energy Commission (Contract AT-11-1-180) and the United States Public Health Service (GM-10991). R. J. is a Predoctoral Fellow of the Public Health Service and K. G. is the recipient of a summer undergraduate research grant from the California Foundation for Biochemical Research.

(11) R. B. Simpson, *J. Am. Chem. Soc.*, **86**, 2059 (1964).
(12) G. L. Eichorn and P. Clark, *ibid.*, **86**, 4020 (1963).

III. ULTRACENTRIFUGATION - RESULTS AND DISCUSSION

In the following sections, a method for the separation of different DNA's based on equilibrium density gradient ultracentrifugation of the Ag (I) - DNA complexes is described. The basis of this technique is the significant increase in buoyant density of a DNA molecule when it complexes with the heavy metal. Since Ag (I) is selective in its strength of binding to different DNA molecules, one can use this to be selective as to the density increase for differing DNA's. If a small concentration of Ag (I) is added to a mixture of different DNA molecules, some will bind a large number of the silver ions and become more dense than those which bind less (or none at all). This would then afford a method by which one could use silver ion selectivity to separate DNA molecules which otherwise have small or subtle differences.

Vinograd and co-workers are largely responsible for developing the density gradient technique. A good review of its applications and limitations is given by Hearst and Vinograd (10).

A. Native DNA

The buoyant density of native DNA at pH 8.0 increases quite markedly when $AgNO_3$ is added, and the increase in density is approximately proportional to the amount of silver (I) bound. Figure 24 shows a plot of buoyant density versus r_f for native E. Coli DNA. The quantity r_f is defined as the ratio of the total moles of Ag (I) added to the total moles of DNA phosphates present. It is apparent that there is a significant increase in density until about $r_f = 0.5$, and no further increase up to $r_f = 0.7$. This agrees with the titration data inasmuch as at pH 8.0 essentially all of the added silver (I) is bound up to $r_f = r_b = 0.5$ at which point there is no more binding until much higher silver ion concentration.

To be certain that all the silver (I) added is bound, an experiment was done using ca. 10^{-4} M DNA (in phosphates). Spectrophotometric observation showed that all the silver added was indeed bound, and the buoyant density in the ultracentrifuge agreed with the other density data. The E. Coli DNA results are replotted in figure 25 versus r_b assuming that all added silver (I) is bound up to $r_b = 0.50$. Figure 26 shows a similar plot for T-4 and for T-7 DNA.

Within the precision of these experiments, which is fairly poor, the density change for all these DNA's is the same. There

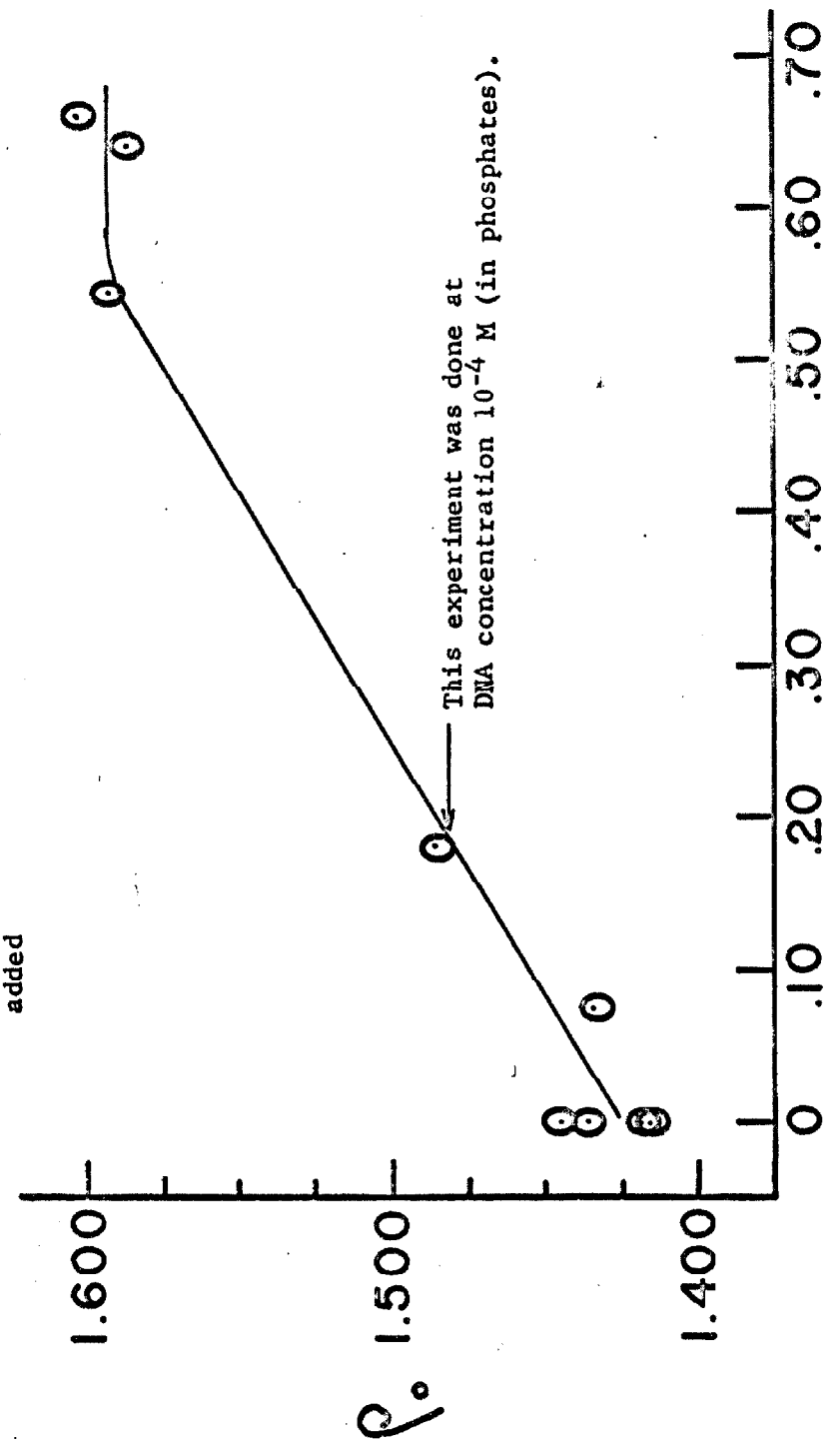


Figure 24

Buoyant density of native E. Coli DNA versus Ag (I) added

Figure 25

Buoyant density of native
E. Coli DNA versus Ag (I) bound

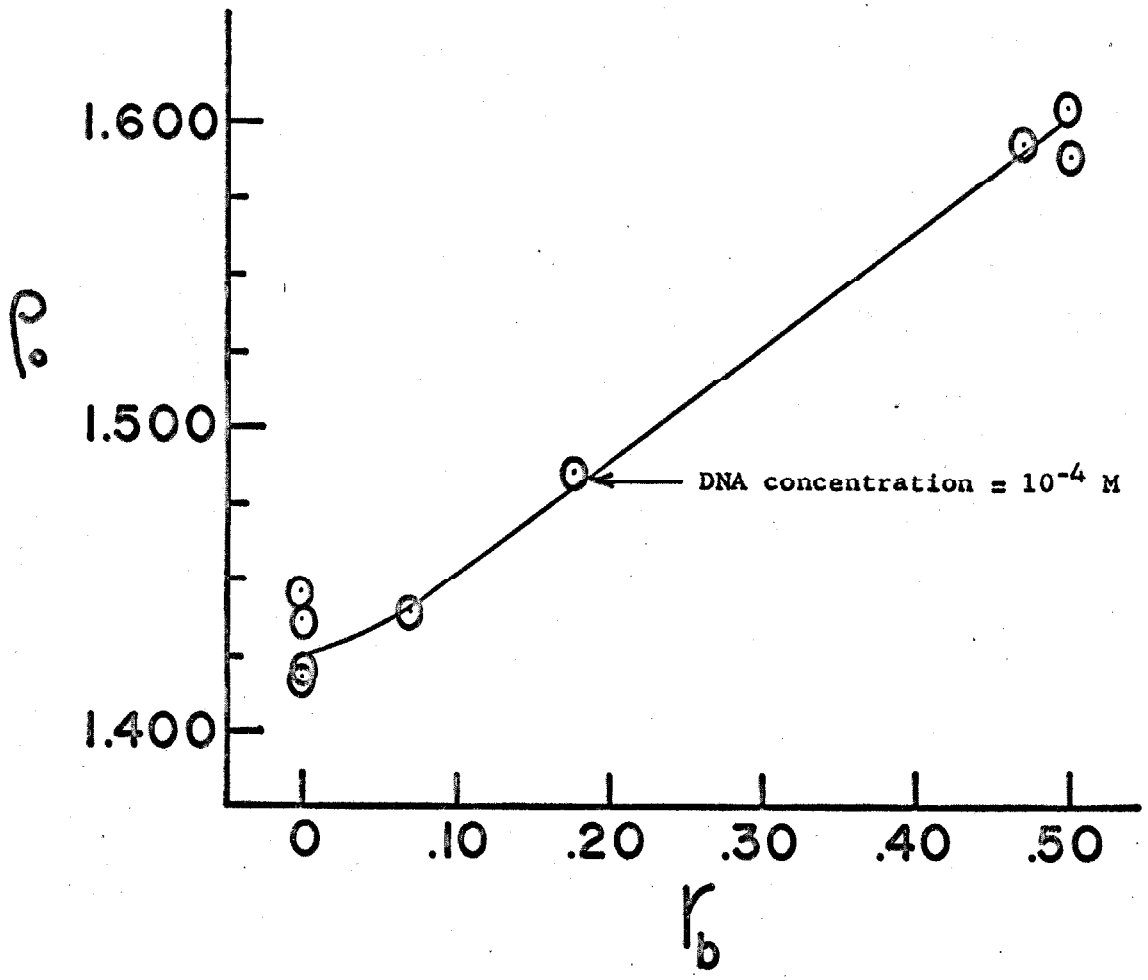
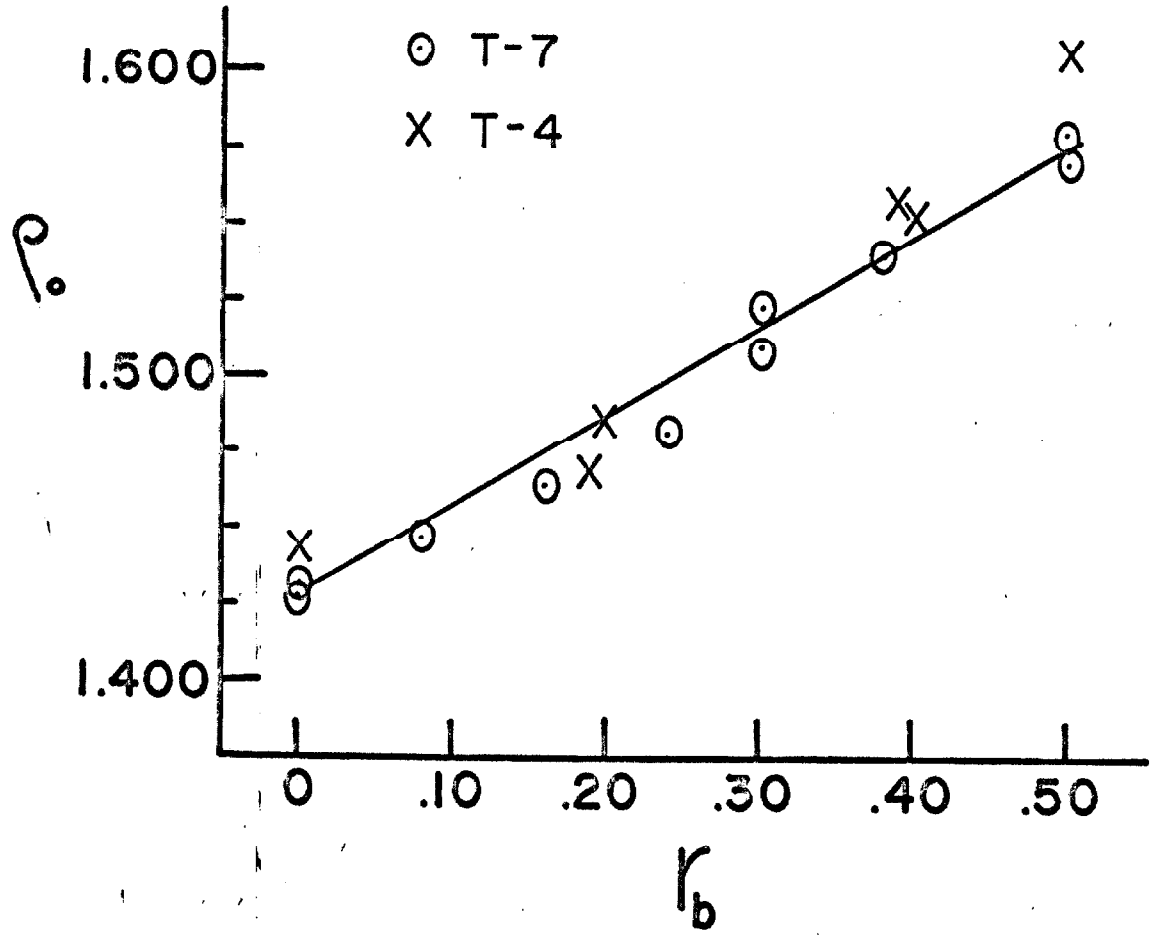


Figure 26

Buoyant density of native T-4 and T-7 phage DNA versus Ag (I) bound



is a similar increase in density for M. Lyso DNA, but only one experiment was done on it. The important result is that there is a large density change (about 0.175 density units at saturation) when native DNA binds Ag (I) at pH 8.0.

B. Denatured DNA

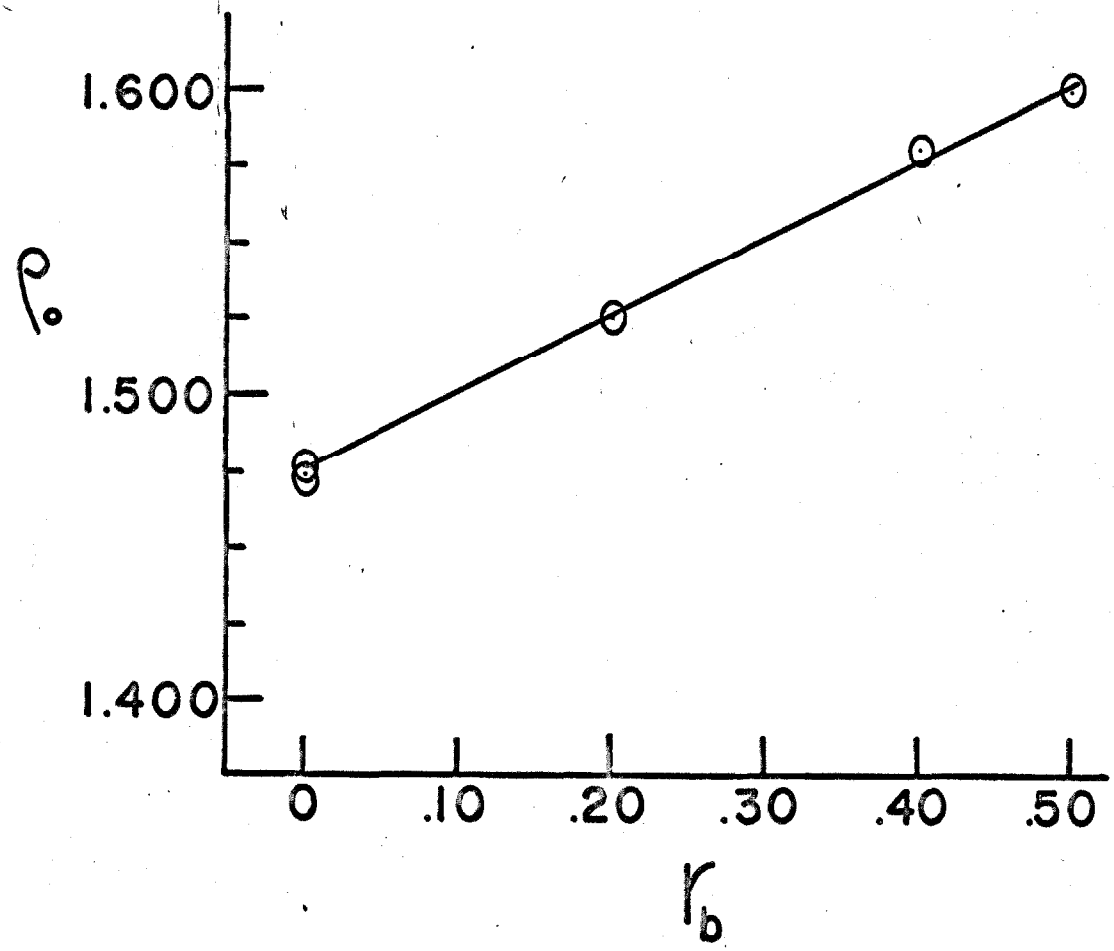
When denatured DNA is titrated with $AgNO_3$ in the density gradient, it increases in density much like native DNA. T-4 DNA was denatured by heating to 100° C. for 10 minutes (at ionic strengths 0.02 - 0.10 M) and was titrated with $AgNO_3$ in a Cs_2SO_4 solution in exactly the same manner as was done for the native material. Figure 27 shows that the gross result is very similar to that of the native DNA, but there is one major difference. The shape of the band which results when denatured T-4 DNA binds Ag (I) is strange and variable. Often the band has a skewed appearance, or even looks like multiple bands. These anomalies seem to be generally nonreproducible, and there seems to be no correlation between r_b and the shape of a band.

There are a number of possible explanations for these results which could be tested in a fairly systematic manner. There is evidence that T-4 DNA has a number of single chain scissions (23). When the DNA is denatured, the single chains separate and fall into pieces. If these pieces are not identical, a heterogeneous population of molecules would be present. This problem can be eliminated by using phage B-3 DNA, which has very few single chain breaks (23).

The denaturing conditions were very harsh ones and might well have caused chain scission or depurination with a resultant heterogeneous population. Milder denaturation by alkaline

Figure 27

Buoyant density of denatured T-4 DNA versus Ag (I) bound



treatment might result in more reasonable band shapes, and would in any case result in a better representation of whole single strands of denatured DNA.

A third explanation which should be mentioned is that the act of silver (I) binding might cause a population heterogeneity. Silver ions might be crosslinking two or more separate strands much in the manner postulated for the crosslinking in the native DNA helix. If this occurred, there could result a mixture of dimers, trimers, and higher aggregates which might well contain variable amounts of bound Ag (I). The heterogeneity in bound Ag (I) would result in an heterogeneity in buoyant density. It is possible, then, that the Ag (I) density gradient technique would not be useful for separations involving denatured DNA.

Nonetheless, the important result remains. When denatured DNA is titrated with Ag (I) in the density gradient, it increases in density much like native DNA.

C. Native plus Denatured DNA

One short experiment was done to demonstrate the competition between native and denatured DNA for Ag (I) binding. A mixture of native T-4 DNA and alkaline denatured T-4 DNA was put into Cs_2SO_4 solution at pH 8.0 such that each was at a concentration of 2.5×10^{-5} M, and this solution was centrifuged to equilibrium. Enough silver (I) was then added to make the solution 1.03×10^{-5} F in $AgNO_3$, and it was again centrifuged. If we assume that all of the Ag (I) is bound by the denatured DNA (as would be predicted on the basis of the titration data), then this would correspond to $r_b = 0.40$ for the denatured DNA. Figure 28 shows superimposed microdensitometer traces for the solution before and after addition of the $AgNO_3$. Correction has been made for the slight shift in position due to dilution of the Cs_2SO_4 which occurred when the $AgNO_3$ solution was added. The prediction made on the basis of the potentiometric results is borne out. The experiment shows that native DNA can be separated from denatured by a large difference in density using this method.

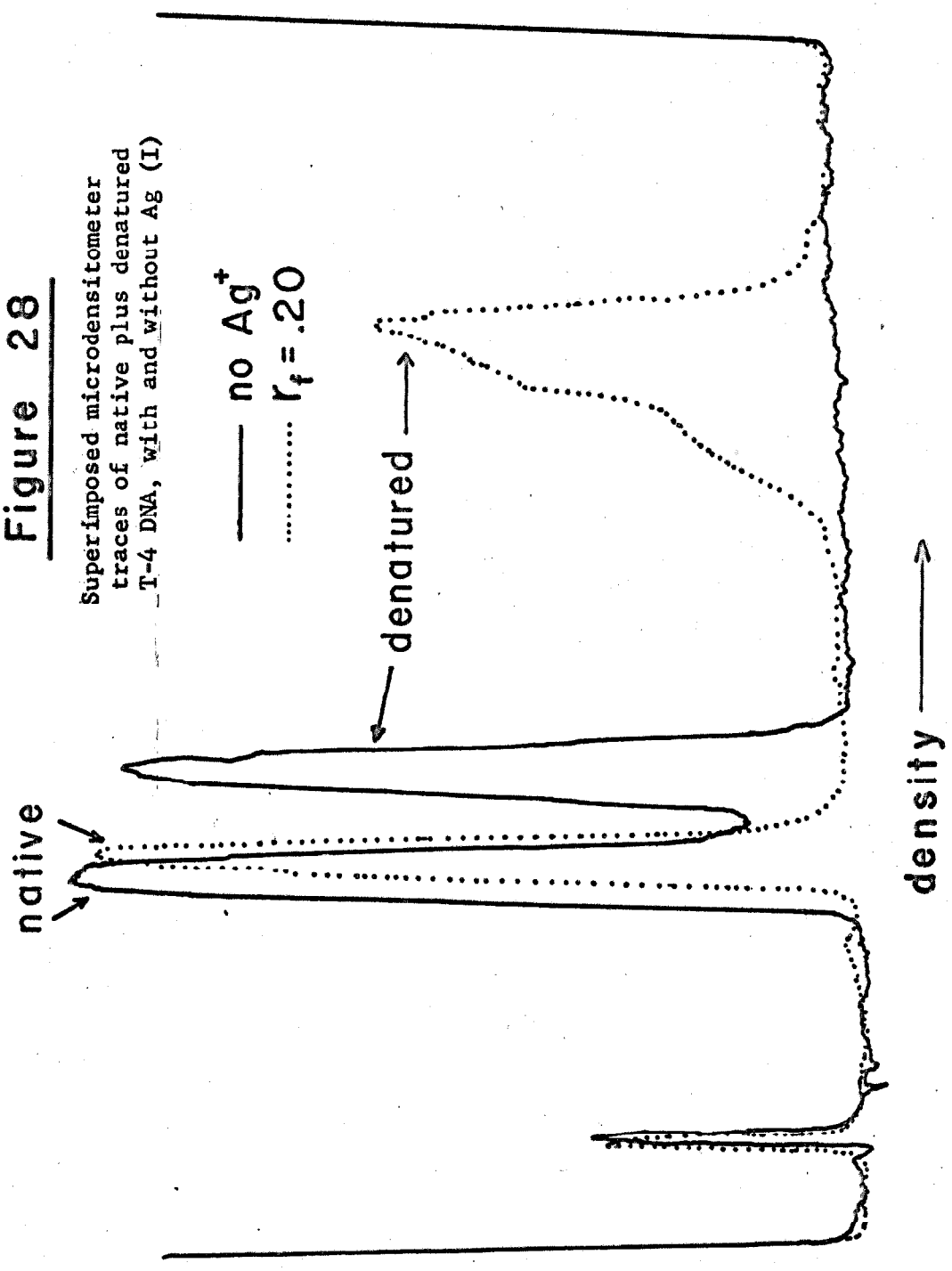


Figure 28

Superimposed microdensitometer traces of native plus denatured T-4 DNA, with and without Ag (I)

D. pH Dependence

The proton release results indicate that DNA releases less protons per silver bound at pH 5.6 than at pH 8.0. This means that at different pH's there should be a charge difference for DNA titrated with silver ions to the same r_b. The buoyant species in a density gradient must be neutral, so that the DNA band in a Cs_2SO_4 gradient is really CsDNA (neutral polymer) (10). After silver ions are bound to the DNA, the banding species must still be neutral. At pH 8.0 about one proton is lost per silver ion bound, so the total change in charge is zero. However, at pH 5.6 there are no protons released until $r_b = 0.20$, and since the banding species must still be neutral, one cesium ion must be lost for each silver ion bound. This would mean that there is no gain in mass when a silver ion is bound since for each Ag (I) (atomic wt. 107.9) bound one Cs (I) (atomic wt. 132.9) is lost. Assuming this is the only change in the buoyant species, there would be no density increase at pH 5.6, $r_b = 0.20$, and in fact there might be a slight decrease.

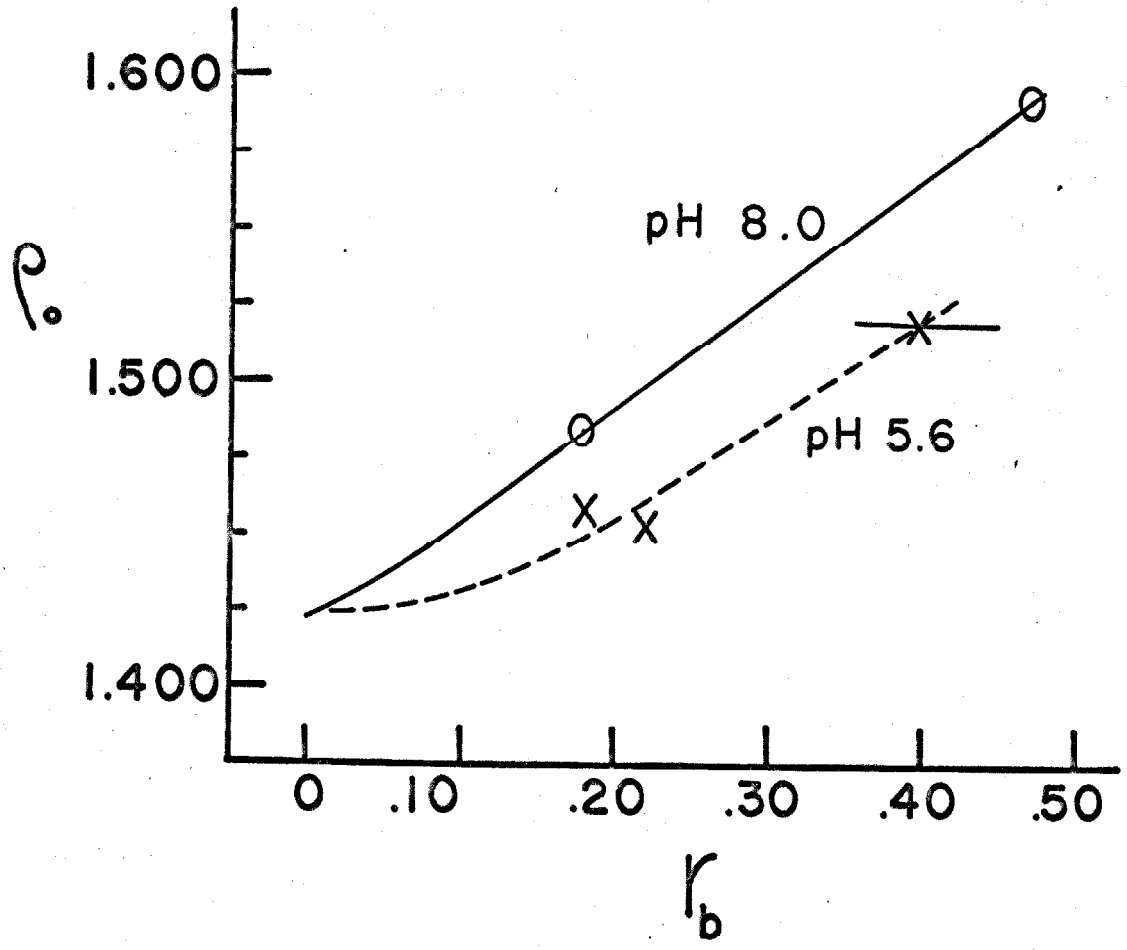
To test this prediction, native E. Coli DNA was titrated with $AgNO_3$ in Cs_2SO_4 at pH 5.6 (10^{-1} F cacodylate buffer). The concentration of the DNA was such (0.75×10^{-4} M) that the change in UV absorption could be observed and used as a measure of the

amount of silver (I) bound. This DNA solution was then centrifuged to equilibrium, and in figure 29 the density at pH 5.6 is compared to that at pH 8.0. The data for pH 8.0 are the same as in figure 26.

As is shown in the plot, at pH 5.6 and r_b close to 0.5 it is difficult to measure the amount of Ag (I) bound by using the UV absorption, since there is a slight preciptate formed. However, the data still indicate clearly that at comparable r_b's there is a smaller increase in density at pH 5.6 than there is at pH 8.0.

Figure 29

Buoyant density of native E. Coli DNA versus Ag (I) bound, at two different pH's. The r_b was measured using spectral shifts.



E. Ag (I) Binding Competition between DNA's of Different Base Composition

If we can push the titration results further, one would predict that if two different kinds of DNA were mixed in a dilute Ag (I) solution, the more GC rich of the DNAs would bind the silver ions preferentially. A number of experiments were done to show that this competition results in a large increase in density by the more GC rich of the two DNA's. Figure 30 shows a microdensitometer trace of a mixture of T-4 DNA and E. Coli DNA at a silver ion concentration such that neither DNA is at saturation. The concentration of the E. Coli DNA is twice that of the T-4 DNA, so the relative area under each peak identifies it (assuming that each peak corresponds to a unique kind of DNA). There is no doubt that the heavier band has the greater area, and therefore corresponds to the E. Coli DNA. The densities at which these two bands appear correspond to r_b for E. Coli of .35 - .40 and r_b for T-4 of .15 - .20. A similar experiment was done competing E. Coli DNA against M. Lyso DNA, with the E. Coli at twice the concentration of the M. Lyso. The result was that M. Lyso DNA bound the Ag (I) first, and the smaller peak appeared at the higher density.

A more complete set of competition data is shown in figure 31. Equal concentrations of T-4 and E. Coli DNA were mixed, and small aliquots of $AgNO_3$ were added to produce a titration

Figure 30

Microdensitometer trace of native E. Coli DNA plus native T-4 DNA

(T-4 DNA) = 1.10×10^{-5} F
(Coli DNA) = 3.20×10^{-5} F
$(Ag^{+})_{added}$ = 1.40×10^{-5} F

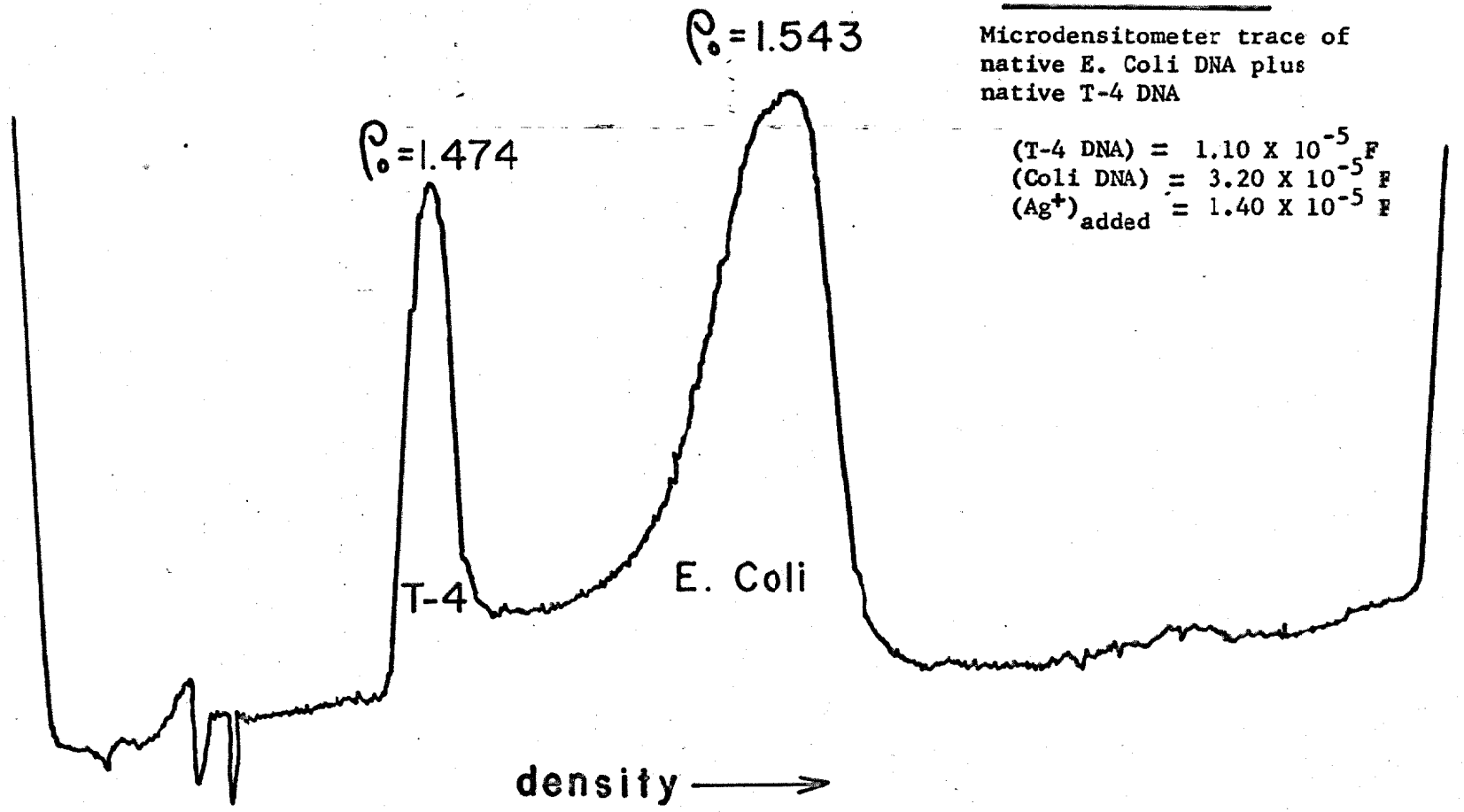
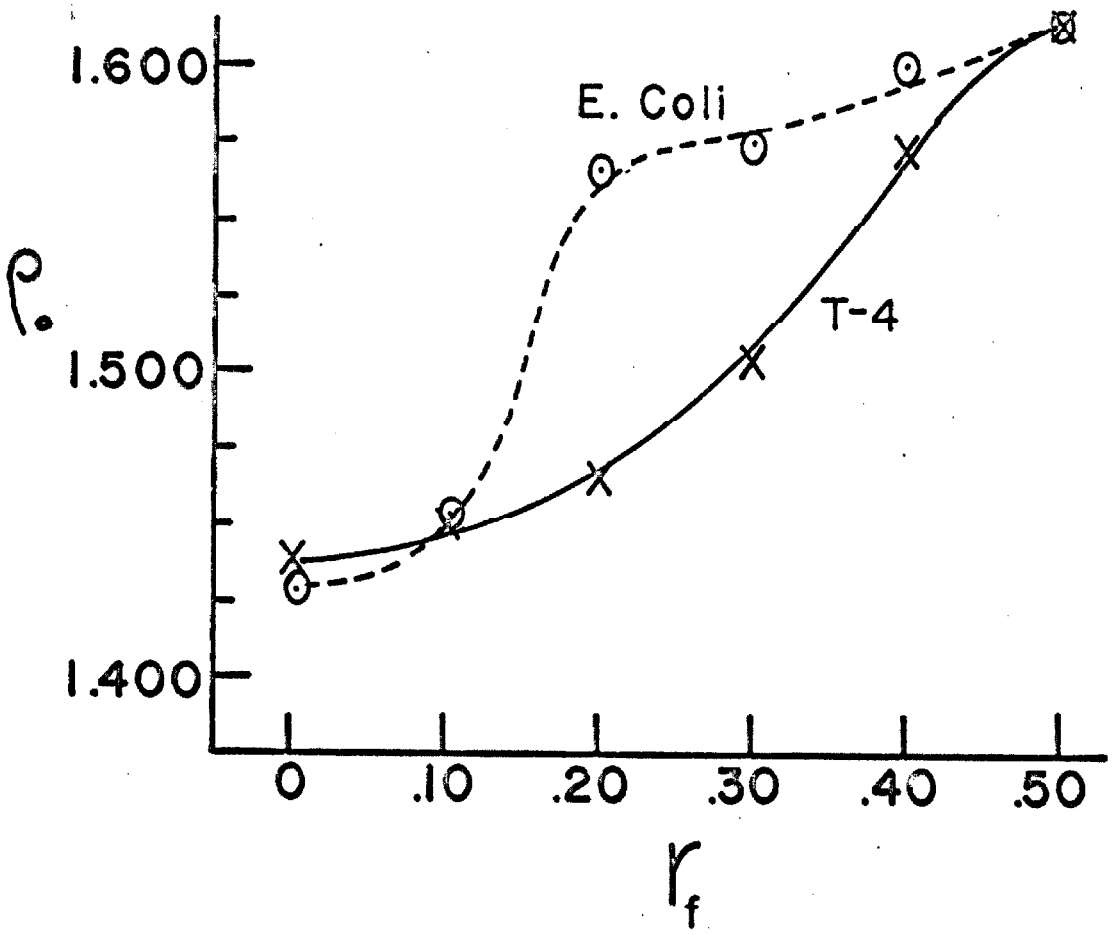


Figure 31

Competition of T-4 DNA with E. Coli DNA for Ag (I) binding.
Buoyant density of each band versus Ag (I) added.



curve for both DNA's in competition. Presuming the E. Coli DNA binds first (since there is no way to identify the bands here), the plot shows that it binds to about 8/10 of saturation before T-4 DNA begins to compete effectively. Furthermore, under optimum binding conditions, i.e., $r_f = 0.20$, the two bands are widely separated enough (0.10 density units) to allow an easy separation in a preparative ultracentrifugation.

F. A Preparative Separation

The GC specificity of Ag (I) binding has been applied to a separation of whole Crab DNA, and has shown that this technique can indeed be used preparatively. Sueoka and Cheng (24) and Smith (25) showed that crabs related to Cancer Borealis contain two very different components of DNA. They have shown that one component, which comprises 70 - 90% of the total DNA, contains about 40 mole percent guanine plus cytosine while the minor component (10 - 30% of the total) has only 3% GC. The latter fraction, which is called dAT, consists of an alternating copolymer of deoxyadenine and thymine that is very similar to the enzymatically synthesized Poly dAT (26). The only difference is the few base pairs which are GC instead of AT.

Sueoka and his co-workers have developed a procedure for separating the two fractions (27). However, it contains two faults; 1.) It is difficult to obtain large amounts of the dAT fraction uncontaminated by the other fraction (called simply crab DNA); 2.) It depends on heat denaturing and quick cooling the whole sample, and then presumes that the dAT regains its fully native state. It is not likely that the dAT can be fully native after all this, and the Ag (I) density gradient separation gives us an opportunity to demonstrate this.

A sample of whole crab DNA was obtained* and a silver (I) titration in the density gradient was performed on it. The results, which are shown in figure 32, clearly indicate that the crab DNA fraction binds all the added silver (I) until it becomes saturated, and that at r_f 0.35 - 0.45 there is a large density difference between the two bands.

A preparative separation was then attempted using 2 ml of OD = 2.3 whole crab DNA in an SW 39 rotor at 32,500 RPM. After allowing fifty hours for equilibrium (the longer time was due to the greater column length), the tube was dripped into ten fractions and the absorption at 260 mμ was measured for each fraction. Figure 33 shows a plot of the results. The peak to peak separation of the two bands is satisfactory (1 cm in the radial direction), but the peaks are broader than would be expected. This is probably due to collecting fractions which are too large, and to my inexperience in dripping the fractions.

Fractions 1 & 2 were pooled, as were fractions 5 & 6, and each of these was dialyzed against 0.1 F EDTA pH 8.1, then 0.1 F $NaClO_4$, and finally versus .01 F Na_2SO_4, 10^{-3} F cacodylate pH 8. Melting curves were done on each of the samples, and the

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* Isolated and purified by Jack Widholm.

Figure 32

Buoyant density of the two components of whole crab DNA versus Ag (I) added

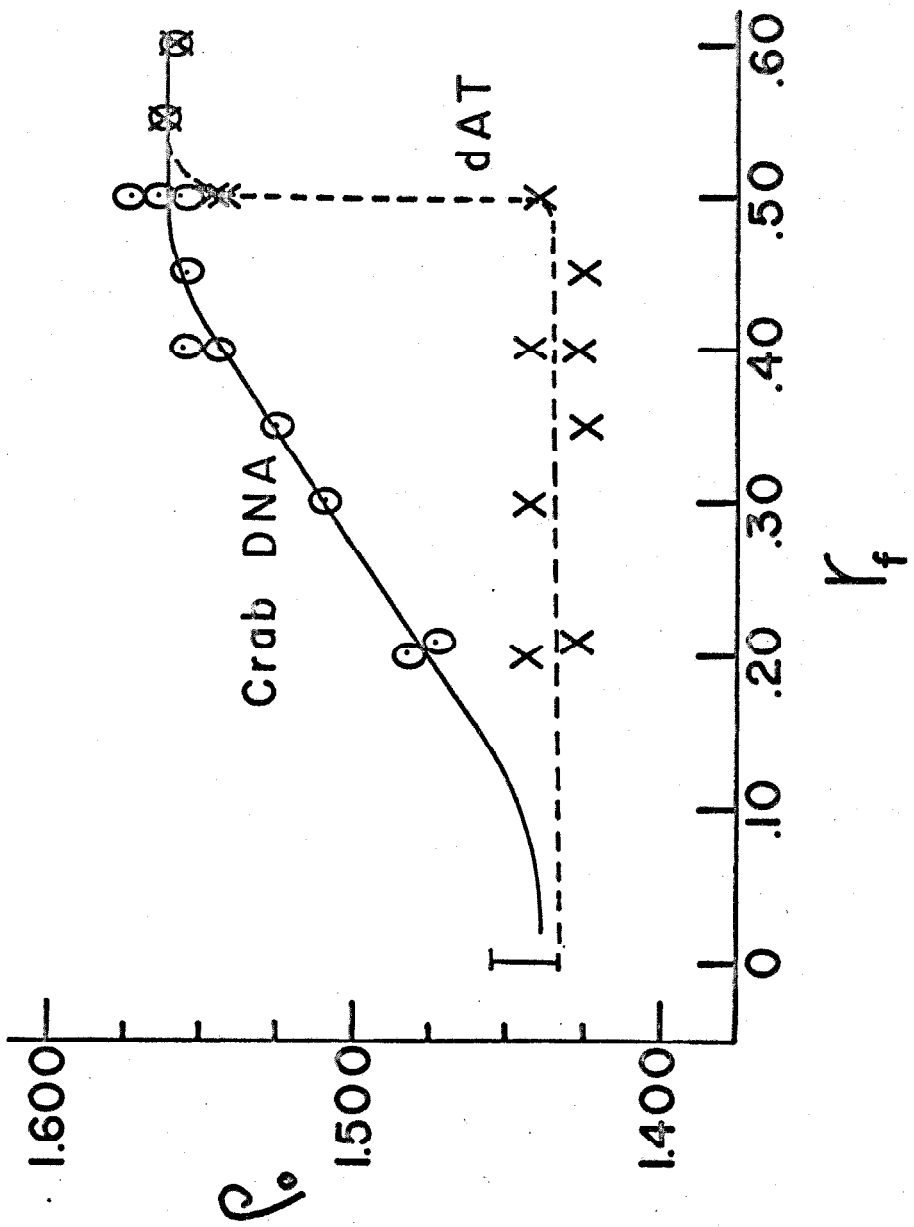
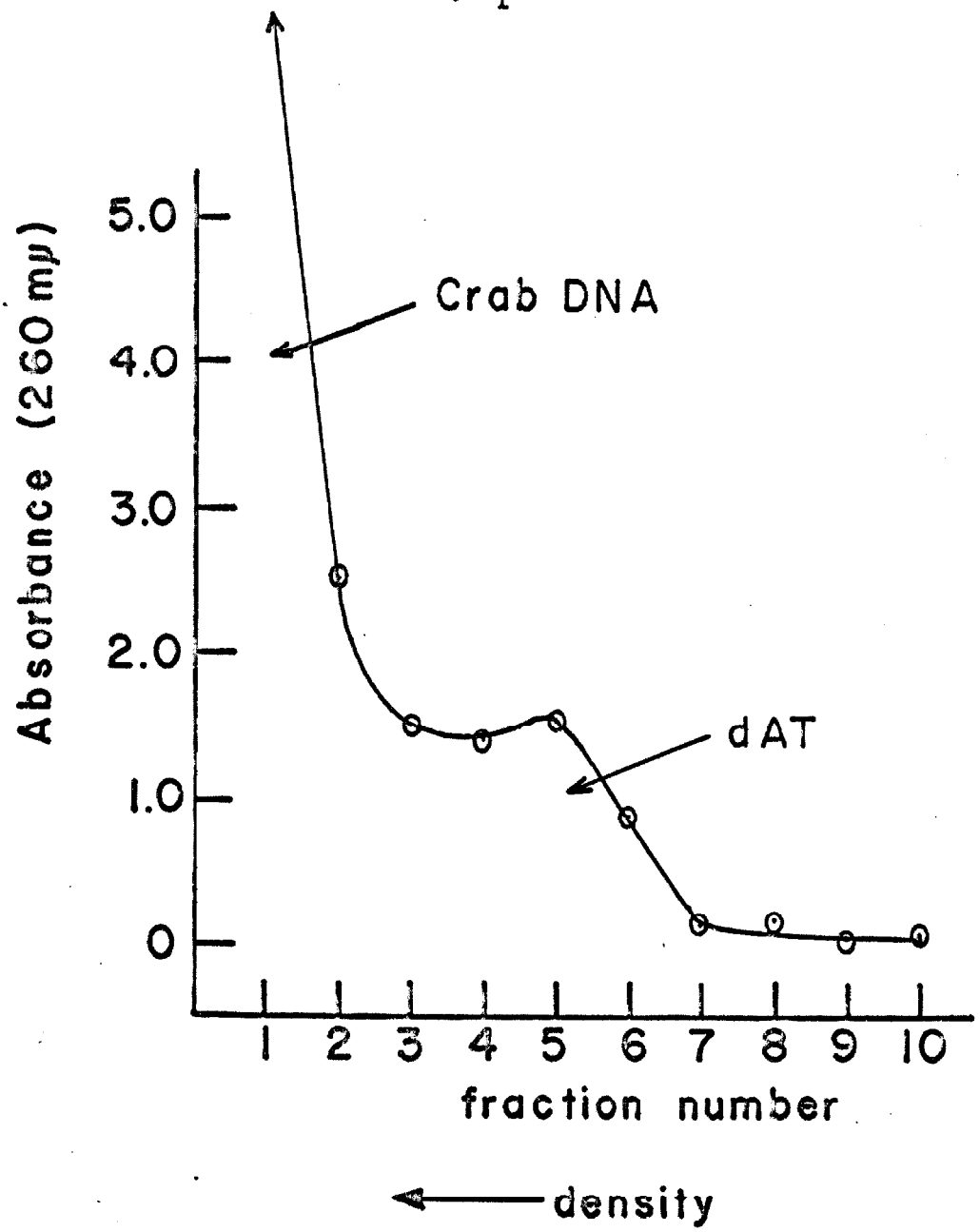


Figure 33

Preparative density gradient of the two components of whole crab DNA, $r_f = .35$



results are shown in figure 34. The most important observations are that there are two one-step melting curves, and that there is a large difference between their melting temperatures. However, the concentration of the dAT fraction was very low (OD = 0.3) and no more can be said about the properties of the dAT, using these data.

To study more carefully the characteristics of the dAT which can be separated in this way, a separation was accomplished using Hg (II) - DNA complexes. Simultaneous with my experiments, Dr. U.S. Nandi has been studying the Hg (II) complexes with DNA. Using procedures analogous to mine, he has shown that at pH 9 in a Cs_2SO_4 density gradient the dAT fraction of whole crab DNA binds Hg (II) preferentially, and it becomes more dense than the crab DNA fraction. He also performed a preparative separation based on this difference in density, and on dripping, obtained two well separated fractions of absorbance about 1 (dAT) and 5 (crab DNA).

After dialysis, very precise melting curves were done on the dAT fraction, and from these come some interesting conclusions. Figure 35 is a comparison of the melting behavior of the dAT fraction and the whole crab DNA. Again it is clear that the dAT sample is not contaminated by the crab DNA fraction, since it corresponds to the first step of the two step melting

Figure 34

Melting profiles of the separated components of whole crab DNA in .01 F Na_2SO_4, 10^{-3} F cacodylate pH 8.0 . These were separated in the preparative experiment shown in figure 33.

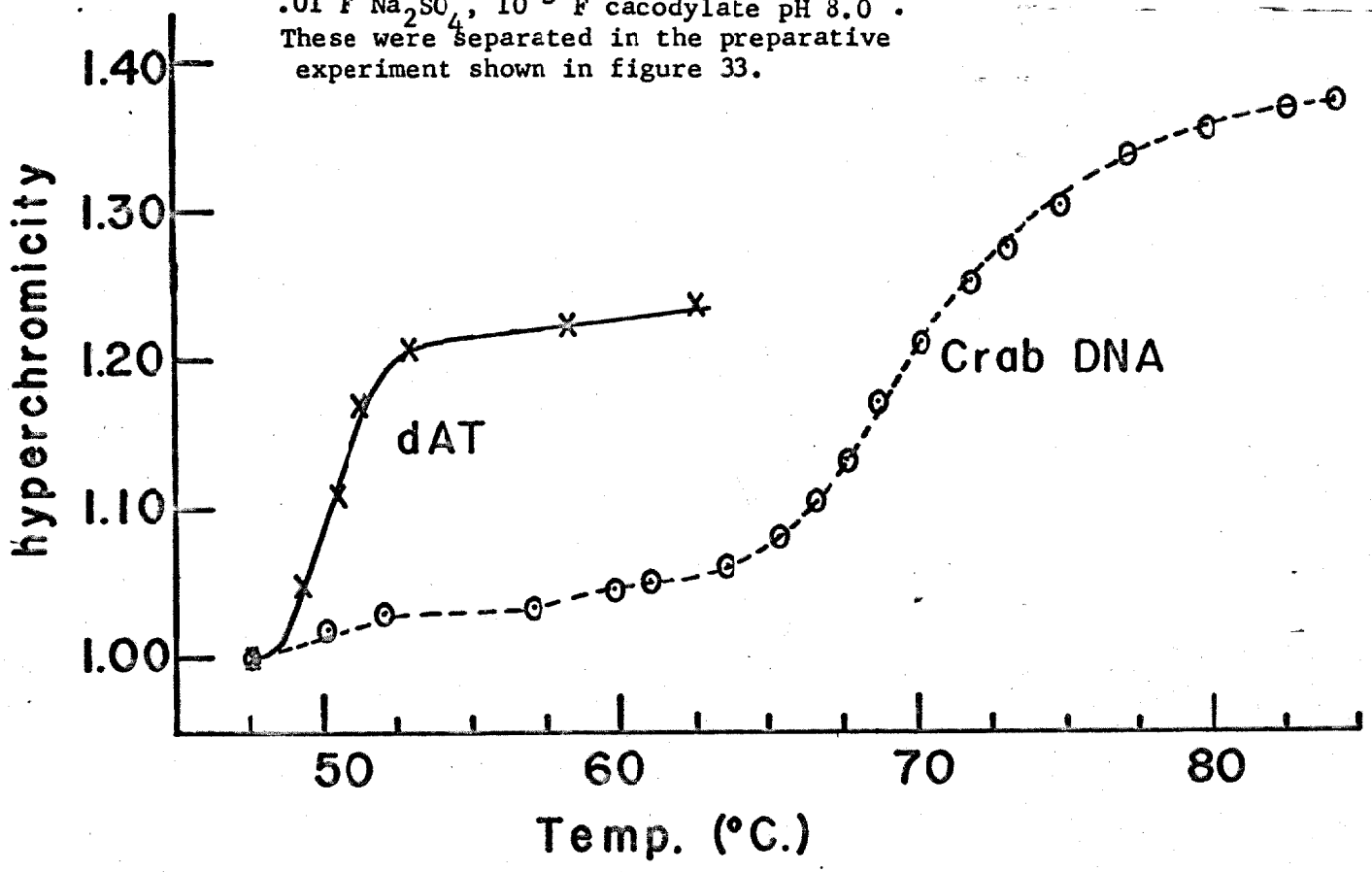
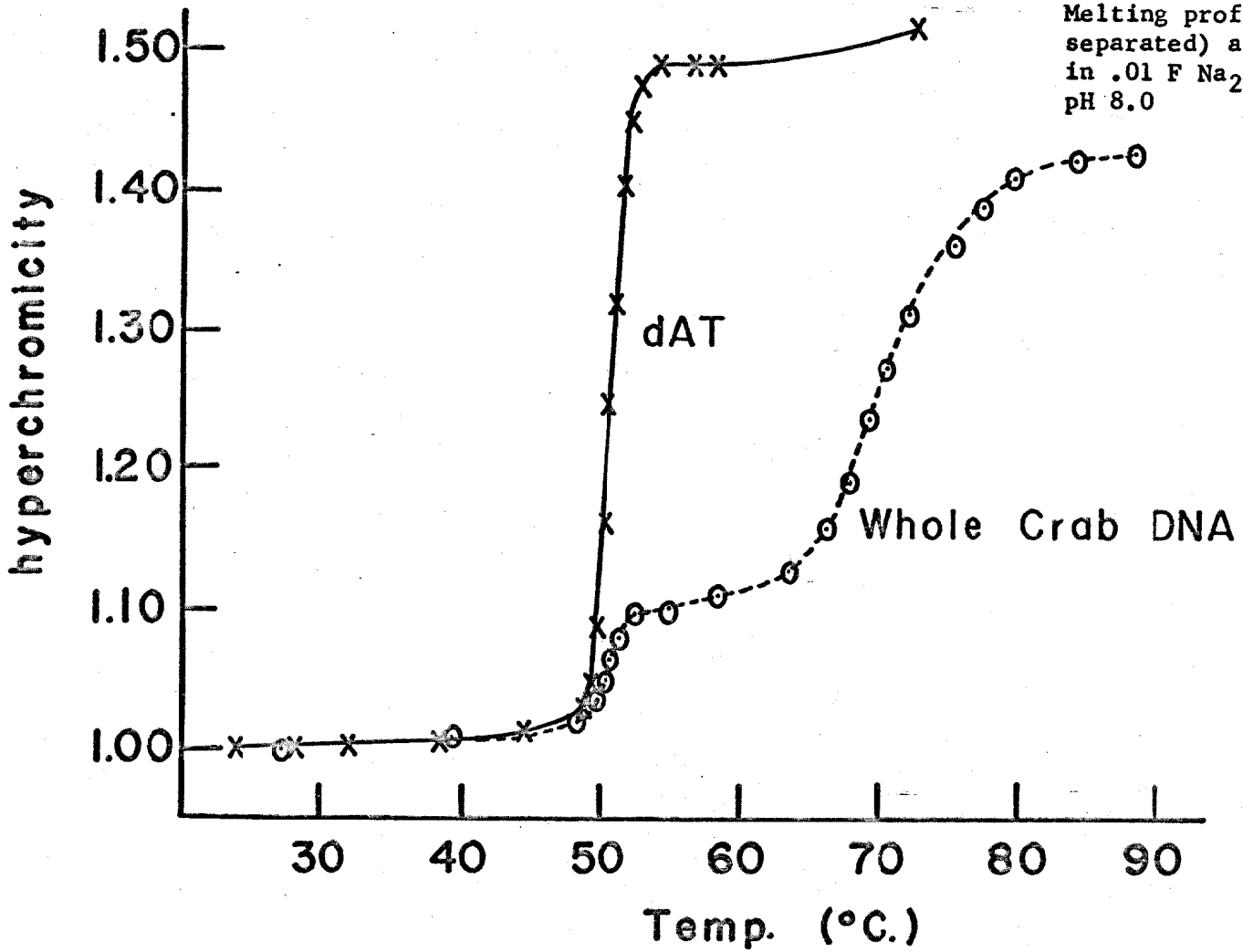


Figure 35

Melting profiles of dAT (Hg^{+2} separated) and whole crab DNA in .01 F Na_2SO_4, 10^{-3} F cacodylate pH 8.0



for whole crab. A repeated heating and cooling of the dAT fraction (Figure 36) shows that indeed there has been an irreversible change in the dAT during the first heating. The melting profile of this dAT after one heating (followed by rapid cooling) is identical to that of dAT which has been separated (by J. Widholm) using the method of Sueoka and Cheng (26). A summary of all the pertinent data is contained in Table III.

There is a decrease in melting temperature (about 6°C) and an increase in transition breadth (by about 3 fold) which is caused by the first heating and recooling of the dAT fraction. This is different behavior from synthetic Poly dAT (16), which has a totally reversible melting profile. The irreversible change is undoubtedly due to the few GC base pairs which occur in the crab dAT. Upon denaturation, the two strands of the polymer separate, and on recooling, the few G's and C's have a very small probability of relocating a complementary mate. The A's and T's can reform the hydrogen bonded structure, but for each G or C there will be an interruption in the helix. If at least three nuleotides are required for each non helical loop (28), then at least 9% of all the bases will be non helical. The result of this would be a decrease in the cooperativeness of denaturation (broadening the transition) and a decrease in the overall stability of the helix (decreasing T_m). Isolation of

Figure 36

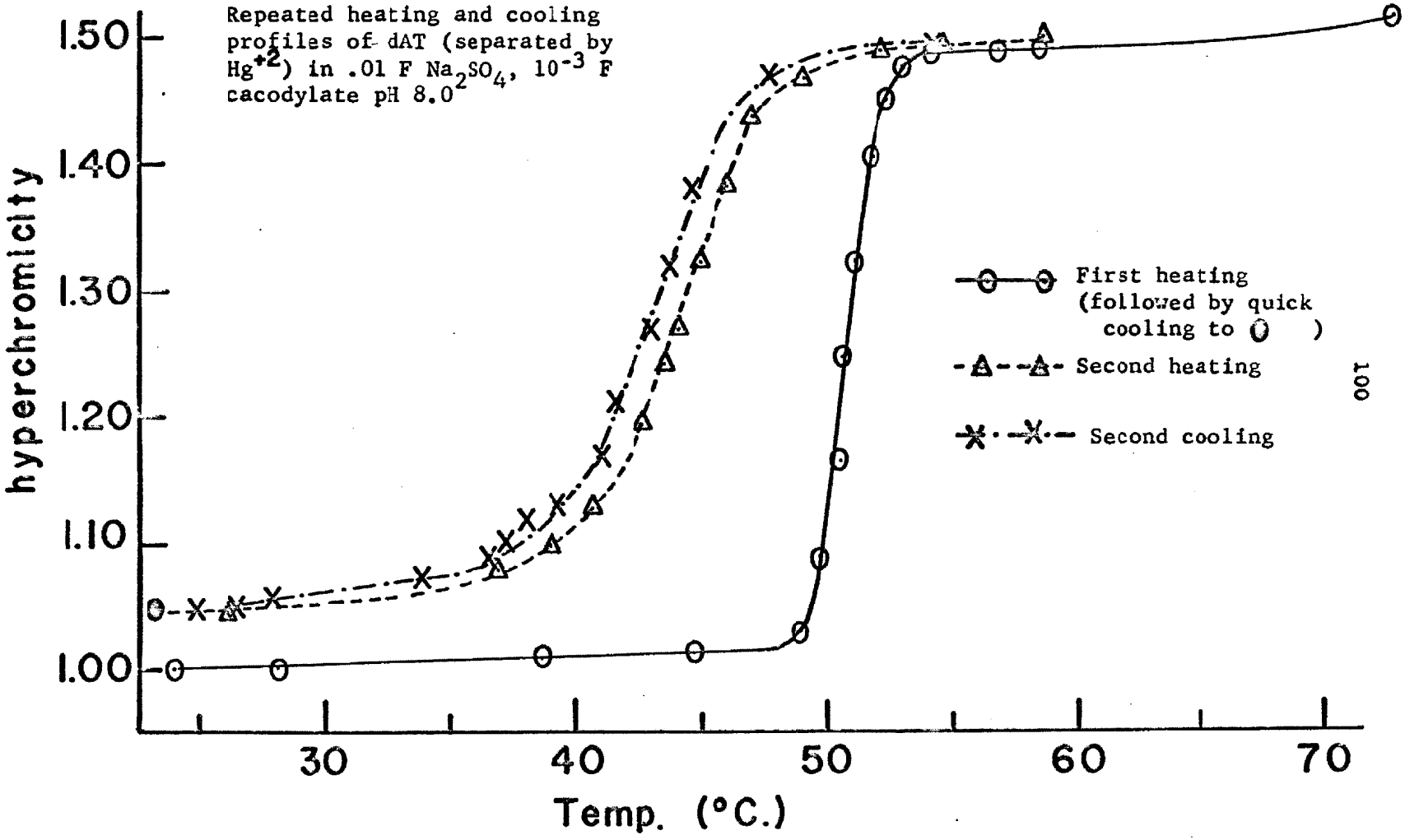

Repeated heating and cooling profiles of dAT (separated by Hg^{+2}) in .01 F Na_2SO_4, 10^{-3} F cacodylate pH 8.0
hyperchromicity
1.50
1.40
1.30
1.20
1.10
1.00
30
40
50
60
70
Temp. (°C.)
First heating (followed by quick cooling to 0)
Second heating
Second cooling
100

TABLE III

Sample	T_m (°C)	σ_T (°C)
dAT Sueoka method	43.3	2.8
dAT Hg (II) separated		
1st heat	50.6	1.1
2nd heat	44.1	3.0
dAT Ag (I) separated	50.5	1.
crab DNA		
Ag (I) separated	70	5
Whole crab DNA		
1st step	50	1.
2nd step	70	4.5

All these results are for melting in .01 F Na_2SO_4 10^{-3} F cacodylate pH 8,

fully native crab dAT based on the Ag (I) or Hg (II) density gradient technique will allow a thorough study of its properties, and this is being pursued by Widholm.

G. What Ag (I) Does Not Separate

In contrast to the previous results, this section describes two cases where Ag (I) does not separate the components.

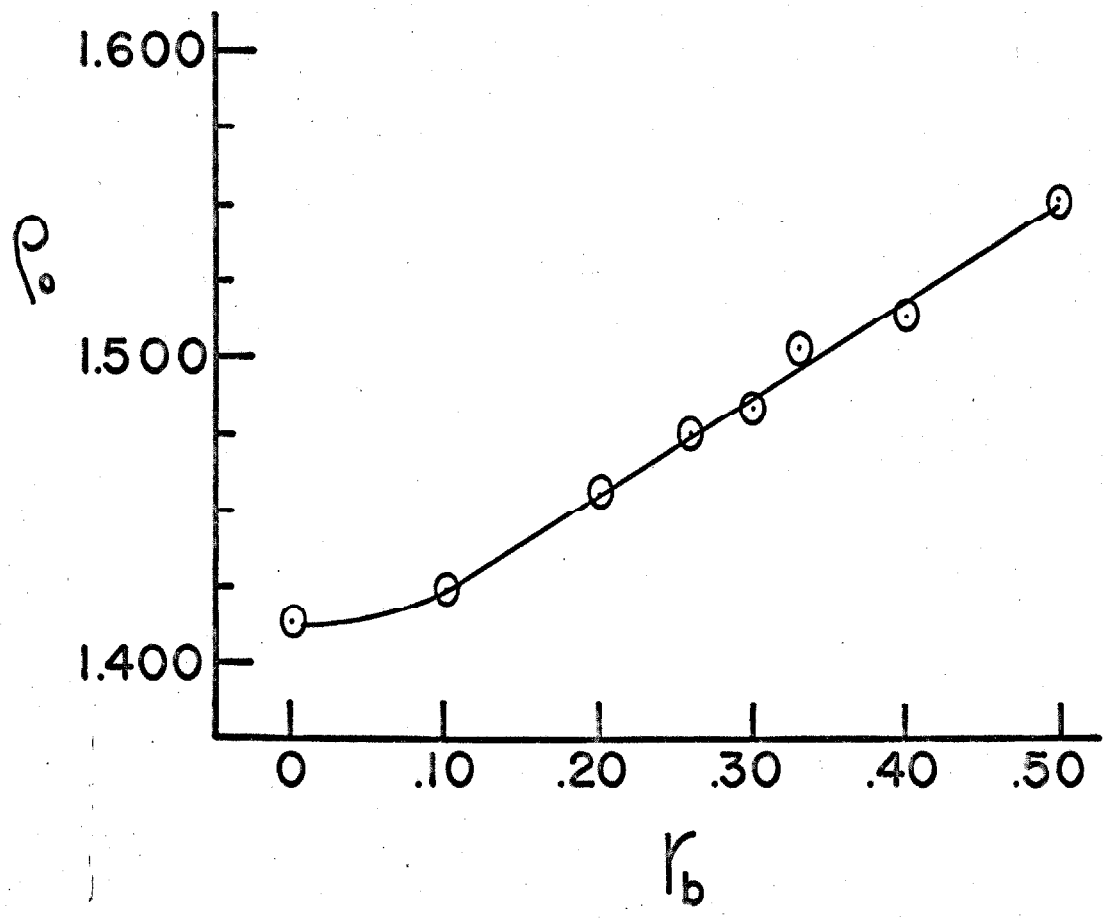
Sheared Lambda DNA. - A sample of native $\lambda\ b^+$ DNA which was sheared into half molecules by high speed stirring was received from Dr. P. Davison (MIT). There is good evidence (29, 30) that these halves are native, infective DNA with a difference in base composition between the two halves (GC content 54% versus 45%). Previous experiments by Dr. Nandi indicated that Hg (II) could separate the sheared λ DNA into two bands. This separation was achieved for both $\lambda\ b^+$ and λ dg(kindly provided by Professor D. Hogness of Stanford University). In the latter case Dr. Hogness showed that the bands separated by Nandi are indeed the two different halves of the DNA molecule carrying different genetic markers.

Two carefully executed experiments with Ag (I) at pH 8 show only one band with perhaps a shoulder which appears in the intermediate titration region (r_b = .10-.40). The change in density with silver (I) bound agrees fairly well with the change that was found with the other native DNA's (Figure 37). This result is puzzling since the previous GC competition experiments separated E. Coli DNA (50% GC) quite well from T-4 DNA (34% GC), and the GC compositional difference between the two halves of sheared DNA is nearly as large as between E. Coli and T-4 DNA (9% for λ halves, 16% for E. Coli versus T-4).

Hershey has reported that under most conditions sheared λ

Figure 37

Buoyant density of native sheared λb^+ DNA versus Ag (I) bound



DNA can "reanneal" again to a state which closely resembles the original DNA (31). Since about two weeks time elapsed between Nandi's successful separation and the Ag (I) separation attempt, it is probable that a "reannealling" had occurred. An attempt to separate the same sample of sheared λ DNA using Hg (II) was made about four weeks later and was also unsuccessful.

Denatured Lambda DNA. - It has been postulated that there is a compositional difference between the two separate strands of λ DNA, so that using denatured λ DNA one can identify two distinct bands at equilibrium in an alkaline CsCl density gradient (32). An attempt has been made to use the compositional specificity of Ag (I) binding to enlarge this difference in density so as to separate the strands using Cs_2SO_4 density gradient ultracentrifugation.

λb^+ DNA was denatured by alkaline treatment (pH 12.2) in 10^{-2} F Na_2SO_4 and reneutralized. The sample was then split into two aliquots to be run simultaneously in a double cell centrifugation. Besides the necessary Cs_2SO_4 and cacodylate, which was added to both samples, a small amount of $AgNO_3$ ($r_b = 0.10$) was added to one aliquot. At equilibrium the cell which contained no Ag (I) showed a single band at a buoyant density of $\rho_o = 1.450$, which is a reasonable density for denatured λ DNA (native + .034). The

aliquot with Ag (I), $r_b = 0.10$, was higher in density and had split into two bands which were separated by .009 density units (Figure 38). Even under the best conditions this is not a large enough density difference to allow a preparative separation, so this is a second case in which the Ag (I) density gradient technique failed.

This result cannot be attributed to an artifact (like the "reannealing" of sheared λ DNA), and is in fact not unexpected. The binding studies which have been done on denatured DNA (section II - A) show that even at pH 8 the titration is not very cooperative. The binding curves are quite broad when compared to those of native DNA, and one would expect that the titration of two single strands would overlap to a large extent. This means that, unless the composition of the strands is very different, they will both increase in density a comparable amount using the Ag (I) density gradient technique.

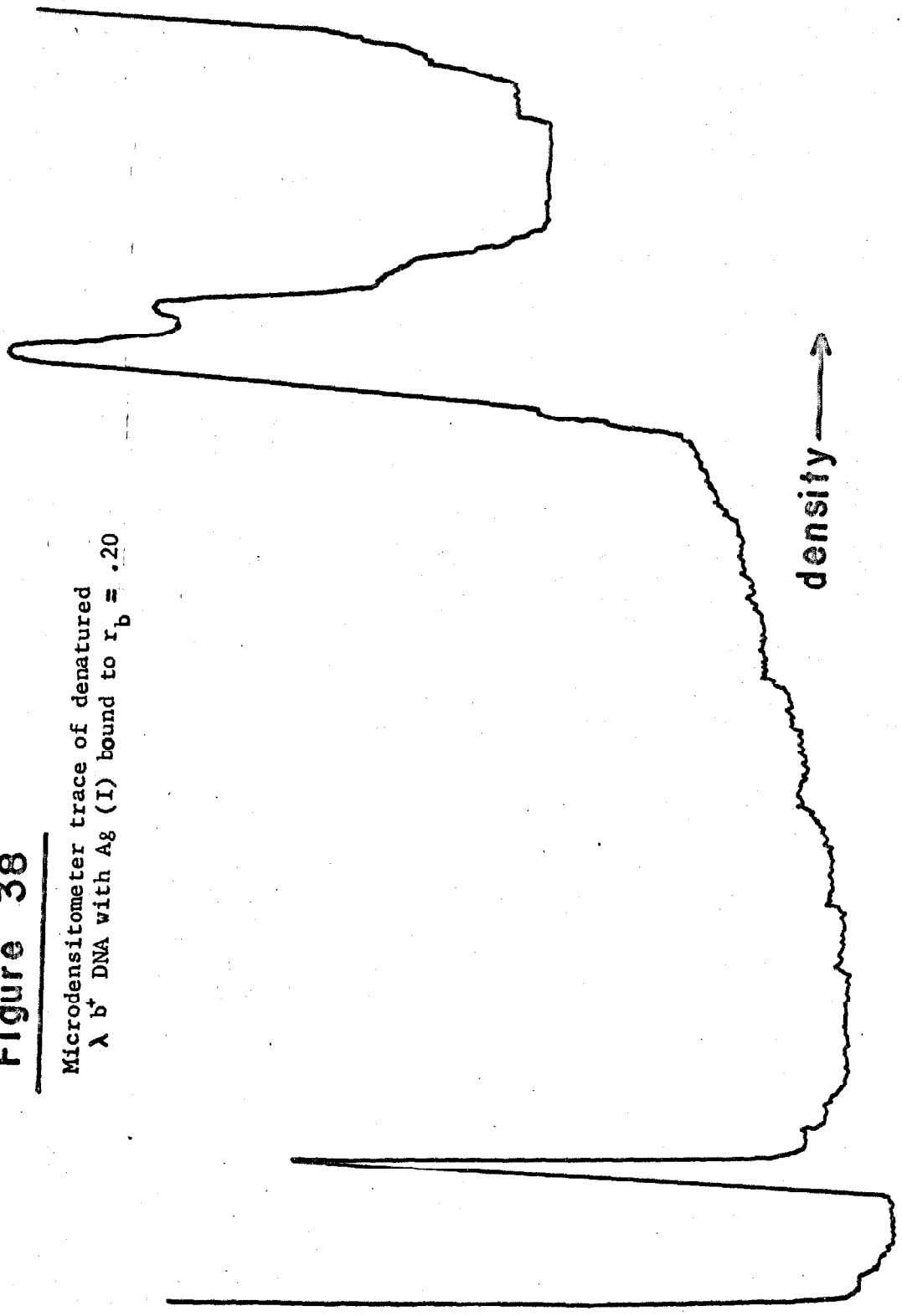


Figure 38

Microdensitometer trace of denatured
λ b^+ DNA with Ag (I) bound to r_b = .20

H. Reversibility of Ag (I) Binding

In order to use the Ag (I) complexing as a separation technique, there must be no irreversible changes introduced into the DNA by the complexing. A number of physical and biological tests were made to determine if there were any such changes in the DNA.

Cyanide was used as a Ag (I) complexing agent to reverse the binding of Ag (I) to DNA and form Ag $(CN)_2^-$. This was done by adding a small volume of 0.04 F HCN (made by adding $HClO_4$ to 0.04 F NaCN to pH 7 and buffering with pH 7 phosphate buffer) to an aliquot of an Ag (I) - DNA solution such that the (CN^-) was 10 to 100 times the total silver (I) formality. When this was done, the ultraviolet absorption spectrum always reverted to that of the original DNA, and in the ultracentrifugation experiments, the density of the resulting DNA was the same as that of the original DNA. These tests indicate that the changes which silver (I) induces in the spectrum and the buoyant density of DNA are totally reversible. It should be recalled that Yamane's data (6) show a very small change in viscosity and sedimentation velocity when Ag (I) is added to DNA, and although in these experiments the Ag (I) was still complexed to the DNA, they also demonstrate that there is no change in the physical state of the DNA molecules (eg, no denaturation).

A very sensitive biological test as to the integrity of the DNA was done using both single stranded DNA (ϕ X174) and double stranded DNA (phage λ). For each type of DNA a biological assay method has been devised (29,33). Using a culture of bacterial protoplasts, the purified phage DNA itself is able to infect the bacteria at a low but measurable efficiency. A number of the bacteria produce whole phage particles and lyse. A measure of the number of whole phages produced is then a measure of the original number of infective phage DNA molecules. This DNA infectivity has been shown to be very sensitive to physical and chemical changes in the DNA molecule (eg, strand breakage or depurination) (29,34), and is therefore appropriate as a test as to whether the Ag (I) complexing affects the DNA integrity.

The λ DNA was put into a solution comparable to one used in the centrifugation experiments (Cs_2SO_4 and 10^{-1} F cacodylate pH 8, $\rho = 1.5$) at a concentration of about 10^{-4} M (DNA phosphate). The ϕX174 DNA was put into 0.1 F $NaClO_4$ plus 0.1 F cacodylate pH 8.0 at a concentration of 5 X 10^{-5} M (DNA phosphate). Aliquots of each kind of DNA were taken and to them were added various amounts of Ag (I). These solutions were left at room temperature in the dark (to protect against photoreduction of Ag (I)) for at least 20 hours, after which they were diluted by a factor of at least 5000 with the appropriate solvent (29,33), and the infectivity

was measured. The infectivity of ϕX174 DNA was assayed by M. Yarus, and Dr. W.F. Dove performed the infectivity studies on λ DNA.

Tables IV and V show the results of these assays, and it is apparent that Ag (I) complexing affects the infectivity very little. The Ag (I) has not been removed from the solutions before the assay, but the dilution is such that the Ag (I) - DNA complexes must be dissociated. Even if the dilution were not sufficient, there are other Ag (I) complexing agents in the assay media (eg, 0.1 F Cl) which would dissociate the Ag (I) - DNA.

The infectivity of all the solutions (even the controls which contained no Ag (I)) is fairly low, and especially so for the ϕX174 DNA. The tables show infectivity relative to a good preparation of infective DNA, so the general inefficiency of infecting DNA in these tests cannot be responsible. No special precautions were taken to sterilize the glassware and solutions, and it is likely that small contamination from enzymes occurred. The long incubation period at room temperature would allow a small amount of enzyme to cause much degredation, especially to the ϕX174 DNA which is more sensitive to enzymes. However, this general decrease in infectivity does not affect the main conclusion.

Dove and Davidson showed that Ag (I) in solution with DNA

TABLE IV

λ DNA Infectivity

r_f	% Infectivity*
0	71
0.5	88
1.0	76
10	65

TABLE V

ϕ X174 DNA Infectivity

r_f	% Infectivity**
0	0.7
0.5	21
1.0	18
10	7.4

The original DNA concentration was 1.3×10^{-4} M and 0.5×10^{-4} M (in DNA phosphates) for λ and ϕ X174 respectively.

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* % Infectivity is the infectivity as compared to a standard λ DNA which has been carefully prepared by Dove and has a typical infecting efficiency.

** % Infectivity is the infectivity compared to a standard of ϕ X174 DNA which was carefully prepared by M. Yarus, and has a typical infecting efficiency.

does affect its transforming ability if left at an elevated temperature (75°C) for several hours (35). Therefore it is evident that even though Ag (I) does not irreversibly alter the chemical, physical, or biological properties of DNA at room temperature, it can cause a slow irreversible change at elevated temperatures.

I. Discussion of the Ultracentrifuge Results

The increase in buoyant density of DNA which results when silver ions are bound is largely a result of an increase in mass when the reaction

$$\text{CsDNA} + \text{Ag}^+ \rightleftharpoons \text{CsDNA - Ag} + \text{H}^+ \qquad (15)$$

occurs. However, this cannot account for the total density change which occurs. The net hydration of DNA can be calculated (8) using

$$\frac{1}{\rho} = \frac{V_3 + \Gamma' V_1}{1 + \Gamma'} \qquad (16)$$

where Γ' is the solvation parameter and is a measure of the grams of hydrated water per gram of neutral polymer (in this case CsDNA or CsDNA - Ag).

V_3 is taken to be the extrapolated specific volume at zero water activity for CsDNA (for Cs T-4 DNA, $1/V_3 = 2.12$ g/cc).

V_1 is the specific volume of the solvated water, which is taken to be one.

For T-4 DNA (12), using $\rho_0 = 1.443$,
then $\Gamma' = 0.720$.

One can easily calculate the increase in density of hydrated CsDNA which would occur if silver ion binding resulted in no volume change and no change in hydration.

$$\rho_0 \text{ Hydrated CsDNA - Ag} = \rho_0 \text{ Hydrated CsDNA} \left(1 + \frac{107\, r_b}{477\,(1 + \Gamma')}\right) \qquad (17)$$

This equation assumes that one proton is released per silver ion bound and would therefore apply as a theoretical density change for pH above 8. Decreasing pH would decrease the expected density change due to loss of one cesium ion for each silver ion that binds without displacing a proton. The density of complexed DNA using the above equation would then be $\rho_o = 1.540$ ($r_b = 0.50$) as compared to the experimental value at pH 8.0 of $\rho_o = 1.60$ (T-4 DNA at $r_b = 0.50$).

The natural conclusion is that one or more of the assumptions which was made is wrong. That one proton is released per silver bound is the most reliable of this assumptions since it is taken from direct experimental tests on solutions similar to those used in ultracentrifugation, and if anything, the direction of possible error here is such that the theoretical density change would be even smaller. The other assumptions concerning volume change and hydration change are probably interrelated, and in any case there is no a priori reason to believe that they must be valid. However, it seems unlikely that when the buoyant species binds Ag (I) the volume would decrease (which is what would be required to explain the large density increase).

On the other hand, a hydration change is not unexpected. Adding Ag (I) ions to DNA molecules probably does not decrease the DNA hydration. However, when Ag (I) is bound, the buoyant

species becomes more dense, and it then finds itself in a medium of higher salt concentration. There is a subsequent loss of hydrated water due to the decrease in water activity, and the buoyant density increases even more.

Hearst and Vinograd (8) have studied this phenomenon, and using their data, one can calculate theoretical hydration changes. T-4 DNA in a cesium sulfate solution of density 1.60 would have 7 less hydrated water molecules per DNA residue than it would at a density of 1.44. Using equation (17) above, the net hydration would change from 20 to 15 water molecules per DNA residue when r_b goes from 0 to 0.5.

The agreement is good considering the approximations made in the analysis. The total density change on Ag (I) binding is now accounted for. Part of the increase is due to addition of the mass of the heavy metal ions, and the rest of it is due to loss of solvated water.

The phenomenon of DNA losing hydrated water is a blessing to the preparative results. The density changes on binding Ag (I) are larger than would be expected, and this allows a better separation of different DNA's than would be expected. It is certain that native DNA's of different base composition can be separated by this technique, but how different these must be is still a question.

T-4 DNA (34% GC) can easily be separated from E. Coli DNA (50% GC), but DNA's with less compositional difference have not been tested. There is a good possibility that at pH 7 the titration differences are big enough to allow separation of two DNA's which differ by only 5% in base composition. One possible use for this sensitivity could be in isolating ϕX174 RF from the bulk Coli DNA.

As is demonstrated by the denatured λ DNA results, different kinds of denatured DNA cannot be separated nearly as easily. The compositional difference which would be required to accomplish a good separation is probably so large that other techniques would be more effective in separating single strands. Denatured DNA does separate well from native, and this technique would be satisfactory in separating double stranded polynucleotides (eg, DNA-RNA hybrids) from single stranded ones (non hybridized DNA).

In order to apply this method to a particular problem, the large gap in present knowledge can be filled by testing the various constituents of mixtures of interest using competition experiments. That this is not only possible, but also fairly easy, has been indicated by this survey of the applications of the technique.

APPENDIX I

PHENOL EXTRACTION PROCEDURE

Micrococcus Lysodeikticus was grown in 16 liters of Hershey Broth (Nutrient Broth 8 g/l, Bactopeptone 5 g/l, NaCl 5 g/l, glucose 1 g/l, pH 7.2 - 7.4) at 37° C for 10 hours to a final titer of 5-10 X 10^8 B/ml. The suspension was spun down in 1 liter batches and the total yield was 11 gm of wet cells. The cells were resuspended in 0.1 F NaCl, 0.1 F EDTA pH 8.0 (10 grams bacteria in 100 ml solvent), and lysozyme was added to a concentration of 0.4 mg/ml. Lysis began immediately and the cloudy suspension cleared noticeably. To complete the lysis the suspension was incubated for 60 minutes at 37° C, Duponol was added to a concentration of 20 mg/ml, and the solution was again incubated (60° for 30 minutes). Solid NaCl was added to make the solution 5 M in NaCl. At this point there were two phases visible, a white gelatenous phase (presumably denatured protein) and a clear viscous phase. These were separated by centrifugation (10 minutes at 20,000 RPM) in a Beckman Model L ultracentrifuge using a swinging bucket rotor. The protein phase was washed with a small volume of 4 M NaCl and after centrifuging again the wash solution was pooled with the original clear phase. The

total volume at this point was about 150 ml.

The DNA was collected on a glass rod by slowly stirring in 1.5 volumes of cold ethanol. The fibers were redissolved by leaving the glass rod overnight in the cold in a volume of 60 ml of 10^{-2} F NaCl, 10^{-3} F Tris pH 7.1. The resulting solution was made 1 M in NaCl and 6 ml of 0.2% RNAase solution was added.* The solution was then put at 37° C for 30 minutes. After cooling to about 4° C, 4 ml of 0.1 F sodium tetraborate pH 9.1 was added.

Fischer liquified phenol was twice equilibrated with an equal volume of 0.1 F borate buffer pH 9.1 and chilled. Equal volumes of this phenol and the DNA solution were shaken in the cold for 15 minutes, and the emulsion was centrifuged at 0° C for 30 minutes at 20,000 RPM. The aqueous phase was more dense than the phenol phase because of the concentrated NaCl, therefore the bottom of the cellulose tubes were punctured, and the lower phase was collected by dripping.

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* The RNAase solution was prepared by dissolving Pancreatic Ribonuclease in 0.15 F NaCl, .015 F citrate buffer pH 5.0. The solution was then incubated at 80° C for ten minutes to inactivate any DNAase present.

The entire phenol extraction was repeated, again collecting the lower phase. This solution was extracted six times with an equal volume of ether (in the cold), and the ether was bubbled off with dry nitrogen. The volume at this point was estimated to be 100 ml. The DNA was again ethanol precipitated and redissolved in 50 ml of 10^{-2} F NaCl, 10^{-3} F Tris pH 7.1. This final solution was placed in visking tubing for dialysis, first against 2 F NaCl, then against EDTA, and finally versus $NaClO_4$ as outlined under Materials. The final yield of purified M. Lyso DNA was about 50 ml of $OD_{260} = 1.6$ (ca. 4 mg).

E. Coli DNA was prepared by U.S. Nandi by an identical procedure, except that he received wet packed bacteria instead of growing and harvesting them himself. Also, E. Coli cells were lysed using only the Duponol treatment.

APPENDIX II

RNA ANALYSIS

A variation of the Schmidt-Tanhauser RNA analysis was developed in order to test a small amount of the DNA solutions used. This test is dependent on a difference in concentration measured by spectrophotometry and is therefore most sensitive for DNA concentrations of absorbance about one to two. Below is the procedure which was used and is recommended for similar studies.

To 0.40 ml of the sample to be tested add 0.17 ml of 1.0 N KOH. Incubate the solution at 37° C for 18 hours. Cool this solution along with an aliquot of 2.0 F $HClO_4$ to 0° C. Add 0.30 ml of the cold $HClO_4$ to the test solution and allow 30 minutes for precipitation. Be sure to keep the solution very close to 0° C during these steps. Transfer the solution to a precleaned (with 2 F $HClO_4$) micro centrifugation tube (about 1 ml volume) which is supplied by Beckman Spinco Division and fits into an adapter for an SW 39 rotor. Place this in a precooled rotor and centrifuge at 15,000 RPM for 15 minutes at 0° C. Remove the supernatant, warm it to room temperature, and measure the ultra violet absorption at 260 mu. A blank (using only solvent) should be run simultaneous to the test

sample, and the % RNA can then be calculated.

$$\% \text{ RNA} = \frac{2.18 \times 100 \times (\text{OD hydrolysate} - \text{OD blank})}{1.58 \times \text{OD original solution}}$$

The numerical factors correct for dilution and for the increase in UV absorption of RNA when it is hydrolized to the monomers.

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PROPOSITION I

Nirenberg and his co-workers (1), among others in the field of molecular biology (2,3), have postulated that a complex between DNA - RNA and ribosomes exists in cell free extracts of E. Coli. If this is so, then it is probable that in this system transcription of the genetic code from DNA to RNA is connected intimately to translation from RNA to protein.

Evidence for the existence of such a complex is in the characteristics of a fast sedimenting (100-200 S) component in these cell free extracts. This fast sedimenting component appears only when ribosomes and DNA are present, and when complementary RNA has been synthesized. This component disappears when either DNAase or RNAase is added.

Sucrose gradient sedimentation of the cell free extracts is a reasonable beginning in the study of the possible RNA - DNA - ribosome complexes, but more headway can probably be made using a different technique for separation and analysis. A good candidate for this technique is density gradient stabilized zone electrophoresis. In this laboratory an apparatus has been developed which can be used to study the electrophoretic properties of nucleic acids at concentrations less than .004% (4). This has been shown to be an effective tool in the analysis of

nucleohistones and chromatins as well as free nucleic acids (5).

If in fact there is a complex between DNA - RNA and ribosomes, zone electrophoresis should be a sensitive means by which to detect it. The mobility of free DNA is significantly greater than that of free RNA which in turn is greater than that of free ribosomes. In 0.01 F NaCl .001 F Tris pH 7.5 the mobilities are DNA = 2.17 X 10^{-4}, RNA=1.7 - 1.8 X 10^{-4}, ribosomes = 1.54 X 10^{-4} cm^2/volt sec. Even though the ribosome mobility (measured by B.M. Olivera) is probably not characteristic of whole ribosomes in a medium similar to cell free extracts, it nonetheless indicates that ribosomes move more slowly than the nucleic acids. Petermann and Hamilton have also reported that, in rat liver extracts, free RNA moves ahead of the ribosomes (6). Therefore, a complex between DNA - RNA and ribosomes will probably move significantly slower than either DNA or RNA. Even though it is generally not easy to predict the exact behavior of a particular species in electrophoresis, it seems clear that the complexing of ribosomes to a DNA - RNA hybrid should decrease the mobility.

The most definitive experiments on this proposed complex would involve radioactive labeling of the various components (DNA, RNA, or ribosomes). If all three of these components move together in the zone, the presence of a specific complex is supported.

Further experiments should be done in which competing non-homologous DNA, or nonhomologous RNA are added. If these are able to break up the complex, then one might suspect that a non-specific aggregation of the components has occurred.

An advantage which the electrophoresis has over the sucrose gradient sedimentation is that large quantities of material can be obtained once the electrophoretic conditions have been standardized. This would allow the accumulation of reasonable quantities of the complex so that some other experiments (such as UV melting, buoyant density, RNA, DNA, and protein analysis) could be done to characterize the complex.

One point which should be mentioned is the dissociation of ribosomes. In general, ribosomes dissociate to subunits unless there is Mg (II) present. Presumably this three component complex is also easily dissociated. In order to be cautious about dissociation, the electrophoretic medium suggested is 10^{-3} M $MgCl_2$ at pH 8.0 (Tris). This is similar enough to the sedimenting medium of Bryne et. al. (1) that there should be no gross change in the complex.

After preliminary experiments, it is suggested that dissociation of the complex be studied by changing the medium (pH, ionic strength, and Mg (II) concentration).

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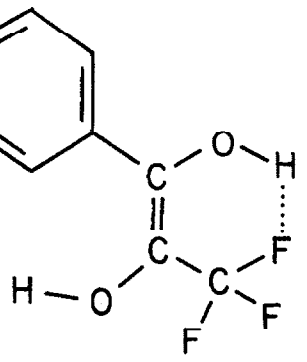
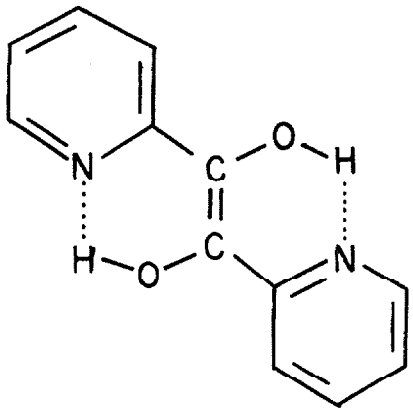
PROPOSITION II

The compounds related to benzoin which contain the group - COCHOH -, have been assumed to tautomerize to the enediol form, $-\overset{O}{\overset{|}{C}}\overset{H}{=}\overset{O}{\overset{|}{C}}-$. In general the benzoin form is much more stable than the enediols. However, some important exceptions have been found. One stable class of enediols is the so-called "chelated" enediols (1). There are apparently two requirements for the stability of these compounds: 1) the system must be highly conjugated with at least one aromatic ring being required, and 2) they must contain a good hydrogen bond acceptor.

An example of such an enediol is 1-phenyl-2-trifluoromethyl-1,2-ethenediol, which was isolated by Nes and Burger (2). They found that this compound forms a diacetate under conditions which might be expected to yield a monoacetate from a hydroxy ketone (3). This indicates that it is in the enediol form. Futhermore they were able to find no carbonyl bands in the IR spectra (in chloroform). They did find two bands which they felt were OH bands at 3617 and 3363 cm^{-1}. These were interpreted as being due to one normal hydroxyl and one hydrogen bonded hydroxyl. The stability of the compound was attributed to an intramolecular hydrogen bond as pictured on the following page.



On the other hand, there has been some evidence which argues that two "chelate" rings are required to stabilize the enediol structure. 1,2-Di- (2-pyridyl) - 1,2,-ethenediol in solution forms a monoacetyl derivative and a monourethane derivative, while as a solid it forms a diurethane (4,5). This has been interpreted to mean that in solution ketonization occurs as soon as one of the two "chelate" rings is broken. The two - ringed structure is pictured below.



The structures of these "chelated" enediols can be better understood by study of their NMR absorption. The 1-phenyl-2-trifluoromethyl-1,2-ethenediol can be studied in detail, since both H' and F^{19} absorb strongly. Due to the hydrogen bonding, proton resonance of one proton should be shifted downfield from normal O-H resonance.

If the hydrogen bonding is intramolecular as postulated, then dilution of a cyclohexane solution of the enediol should not change the position of the proton resonance. If there is intermolecular hydrogen bonding the proton resonance should shift upfield as more cyclohexane is added.

The fluorine resonance would also be affected by the intramolecular hydrogen bonding. Normal rotation of the trifluoromethyl group should be hindered in this molecule, and the fluorines will then be non equivalent. This would result in three fluorine bands instead of only one. As with the proton resonance, the position of these bands should be unaffected when various amounts of cyclohexane are added.

The proton resonance of 1,2-Di- (2 pyridyl) -1,2-ethenediol would also be of interest. The two OH protons should be equivalent if in fact this compound is in the enediol form (in cyclohexane). This means that there will be no chemical shift between the resonance peaks. They will in all probability be

split by spin coupling to one another. By varying the oscillator frequency (and the field strength) a chemical shift can be distinguished from spin-spin splitting.

Here again, a dilution experiment would distinguish between intramolecular and intermolecular hydrogen bonding.

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PROPOSITION III

Bolton and McCarthy have reported a technique which can be used to detect homologies in single strands of polynucleotides (1). This has been called the "agar gel" method of column chromatography. By adding high molecular weight denatured DNA to a 3% agar solution at 100° C. and slowly cooling, they have been able to trap this DNA in an agar gel. This gel is granulated and washed with salt solution at room temperature, and it is found that nearly all of the DNA remains fixed within the agar.

A "hybridization" can then be accomplished by adding a small amount of complementary single stranded polynucleotide (either RNA or denatured DNA) and incubating the whole mixture at 60° C. At this temperature the agar retains its gel-like character and the DNA remains trapped. After cooling to 25° C., the column is then washed with 2 X SSC*, and it is found that some of the c-RNA is not removed by this washing. The complementary material which remains can be washed off the column by decreasing the

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* SSC is an abbreviation for standard saline citrate, .15 F NaCl, .015 F citrate buffer pH 7.

ionic strength of the wash to .01 X SSC.

The material which remains through the washing with the 2 X SSC is termed the adsorbed material. They have shown that this material adsorbs because it has a sequence of bases which is homologous to a sequence in the trapped DNA. The incubation period allows these sequences to become matched, and a "hybrid" of RNA and DNA is formed which is trapped within the gel. To release this adsorbed RNA, the hybrid must be dissociated to the single strands again. The treatment with .01 X SSC accomplishes this. This agar gel technique has proven useful in identifying homologies between DNA - DNA single strands as well as DNA - RNA single strands (2,3).

It is proposed that this technique be adapted so as to use the specificity of the Ag (I) - DNA interactions to selectively adsorb free DNA to the trapped DNA. This adaptation will perform two services. It will confirm the postulate that Ag (I) forms crosslinks between the base moieties in two DNA chains, or it can serve as a separation technique which will select DNA strands on the basis of their guanosine content.

To accomplish the first objective, a very simple experiment can be performed. An agar gel column should be prepared as specified by Bolton and McCarthy (1). Silver nitrate solution is added to the column such that a reasonable amount of Ag (I) (say $r_b = 0.3$) is bound to the trapped DNA. A solution of

denatured non homologous DNA is then added. This DNA should be labelled with tritiated thymidine in order to allow easy assay of its exit from the column.

Assuming there is some sort of crosslinking by Ag (I), there will be three basically different complexes between DNA strands; 1) free DNA molecules can become linked to one another, 2) trapped DNA molecules can be linked to each other, or 3) the trapped DNA can be linked to the free DNA.

The first possibility would result in no adsorption to the column, and is therefore undesirable. To prevent this, the free DNA solution should be fairly dilute. Another method of preventing this is to choose the trapped DNA so that it binds Ag (I) strongly. If this is done, free DNA will be unable to compete effectively for the Ag (I), and there will be very little self linking in the free DNA.

The results in this thesis show that Ag (I) binds strongest to those kinds of DNA which are rich in guanosine and cytosine. From the relative strengths of the monomer complexes with Ag (I) (see section II - C), it can be inferred that G - Ag - G bridges are preferred over other possibilities. Assuming that this is the case, the DNA which is to be trapped in the gel should be high in guanosine, e.g., M. Lysodeikticus DNA (72% GC). Single strands of this material will then provide many sites to which

Ag (I) can coordinate.

There is little chance that there will be crosslinking between trapped DNA molecules. The strands are necessarily constrained to a large extent by the agar. This will prevent the formation of configurations which are conducive to silver (I) crosslinking. As a result, very few crosslinks will be formed between trapped DNA molecules.

The third possible complex is the one which is desired. It is felt to be the most likely of the three, since we have chosen conditions to repress self crosslinking. The experiment is designed to confirm the hypothesis that Ag (I) can form crosslinks, and that they are formed between the trapped DNA and the free DNA.

The next step in the experiment is to wash the column extensively with buffer, and assay the eluant. There should be little or no DNA detected. Finally, the column is washed with NaCN solution. This should complex all the Ag (I), and the adsorbed DNA will be released. The appropriate controls should be done, in which either the trapped DNA or the added Ag (I) is omitted. There should be good adsorption only when the column contains both Ag (I) and trapped single stranded DNA. If this occurs the above hypotheses have been confirmed.

In order to use this column technique for separations,

the experiment described above must prove successful. In the case that it does prove successful, one experiment which should be attempted is to separate the two strands of λ DNA. It has been postulated that there is a compositional difference between the two strands of λ DNA (4). However, no one has succeeded in preparatively separating these strands. An attempt was made (see this thesis section III - C) to use Ag (I) binding specificity to separate these in a Cs_2SO_4 density gradient. The density difference which was observed was too small to prove useful. However the fact that in that experiment two bands formed, indicates that silver (I) does bind one strand preferentially. Better advantage might be taken of this preferential binding, using the column procedure.

In this procedure, the column is prepared and the Ag (I) and denatured DNA are added exactly as before. However, more specific selectivity is needed here. Let us again analyze the possible complexes, this time on the assumption that the two strands of λ DNA are different. (For convenience they are labelled λ-1 and λ-2.) In this case, there will be four basically different complexes, 1) between free DNA strands, 2) λ-1 to trapped DNA, 3) λ-2 to trapped DNA, and 4) inter column crosslinking. Types 1 and 4 have been dealt with before, and no more needs to be said about them.

Strand separation depends on the difference in binding strength between type 2 and type 3. To exploit any difference, the complexing should be gradually weakened. One way to accomplish this is to slowly decrease the pH of the eluant. As the pH decreases the strength of silver (I) binding will also decrease (this thesis section II - A). The weaker of the two complexes (type 2 or type 3) will be released at a higher pH than the stronger.

A better method for selectively dissociating the complexes is to include increasing concentrations of a silver (I) complexing agent in the eluant. This complexing agent should bind Ag (I) weaker than does CN^- so that intermediate Ag^+ ion concentrations can be attained. A good possibility for this is imidazole.

Using either of the above elution methods there should be a selection on the basis of guanosine content. The majority of the crosslinks formed will link a guanosine in a trapped DNA chain to a guanosine in a free DNA chain. The DNA strands which contain high amounts of guanosine will be strongly adsorbed, and released last. For λ DNA this would mean that the complementary strands could be separated.

It should be mentioned that this separation does not depend on G-Ag-C being the preferred Ag (I) crosslink (even though it is probably the case). If Ag (I) selects for any one base (or two bases other than the Watson-Crick pairs), the single strands

of λ DNA might still be separated. A number of adsorption experiments using various DNA's will give an indication as to which base (or bases) are responsible for the separations.

The selectivity of Ag (I) binding to DNA can probably be used in other columns in much the same manner as described above. The column can be made up of polymer beads (many are commercially available) which contain a large number of basic nitrogens or sulfhydryl groups. These groups will then serve as the anchors to which the DNA strands can be tied using silver (I) as the linker. An investigation of the characteristics of these columns would involve a large number of empirical observations on their selectivity under varying conditions (pH, ionic strength, Ag (I) concentration, etc.).

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PROPOSITION IV

The text of this thesis (section III - F) shows that the dAT component from some Cancer species of crabs can be isolated in native form. This material has been shown to be a double stranded alternating copolymer of deoxyadenosine and thymine (1) interrupted by a few (about 3%) GC base pairs. We have further shown that the first heat denaturation of this polynucleotide results in an irreversible change in the characteristics and have postulated that this is due to the presence of the GC base pairs. On cooling, the adenines and thymines in the chains can easily find complementary bases and re-form a native-like structure. The guanines and cytosines, however, cannot easily find mates, and they remain in a nonhelical configuration.

It is proposed that this material be used to test two new methods for study of DNA structure, proton magnetic resonance and silicotungstate density gradient ultracentrifugation. Both of these techniques have been shown to detect denatured regions in helical polynucleotides, and this recooled crab dAT is a model of a slightly denatured polynucleotide.

Proton magnetic resonance. - McDonald, Phillips and Penman (2) have used a computer of average transients coupled to the output of an NMR spectrometer to obtain proton magnetic resonance

signals of some polynucleotides. This technique has been used in order to average out background noise and allow measurements on reasonably dilute solutions (ca. 10^{-2} M).

Their studies show two important results. For single stranded polymers, there are distinct signals in certain regions of the spectra, and they have made assignments for these peaks. Also, the integrated intensity in two regions of the spectra (-30 to -80 cps and -180 to -200 cps relative to HDO resonance) can be used as a measure of the amount of denaturation.

Helical nucleic acids produce no detectable signal in these measurements. This is presumably because the signals have been broadened to a large extent. Even though the integrated intensity is large, the intensity above background at any one point is undetectable. It has been shown (3) that the absorption lines of NMR signals are broadened by constraining the motions of the absorbing molecules. Helical nucleic acids rotate so slowly that their absorption signals are extensively broadened. Denatured regions in DNA chains gain enough freedom to significantly decrease this broadening, and their signals are sharp enough to be detected.

The use of recooled crab dAT in a series of proton magnetic resonance experiments would be interesting in regard to both of the above results. For heated and recooled crab dAT, there

should be an increase in intensity in the regions mentioned over that of native crab dAT. One can use this as a measure of the number of nonhelical bases in the polymer and correlate this with the number of guanines and cytosines present. This would not only be a test of the sensitivity of the technique in detecting small denatured regions, but would also determine the number of bases involved in each nonhelical region. Fresco and Alberts (4) have postulated that three nonhelical nucleotides are required to have a denatured "loop". The number of nonhelical bases in recooled crab dAT as measured by proton magnetic resonance would give an independent estimate.

There is still another observation which should be made in these experiments. McDonald et. al., have assigned a peak at +175 cps to the thymine methyl protons. This peak should be absent in native crab dAT. If in recooled crab dAT only the C's and C's are nonhelical, this peak should still be undetectable. However if the bases which neighbor the G's and C's are also nonhelical, there will be some nonhelical thymines and a signal will appear at +175 cps. The integrated intensity in this region will be another measure of the number of bases needed to form a nonhelical "loop".

Some care should be taken in attempting to correlate the results of these experiments with the number of nonhelical bases.

The formation of a number of small denatured regions will permit the helical regions a fair amount of freedom. It is possible that the helical regions will gain enough freedom to also contribute significantly to the NMR signals. The possibility of this phenomenon makes any interpretation of the results less convincing, but nevertheless the experiment is of interest. Futhermore, the silicotungstate ultracentrifugation will give an independent measurement of the number of nonhelical bases. Comparing the ultracentrifuge results with the NMR results will aid in interpreting the NMR spectral changes.

Silicotungstate ultracentrifugation. - Vinograd and Hudson (5) have investigated the buoyant density of various DNA's in a sodium silicotungstate gradient in the ultracentrifuge. They have found that at fairly low pH (ca 3-4) the buoyant density of denatured DNA is much greater than that of native. This is presumably because at this pH some of the bases are titrated in denatured DNA. Since the buoyant species must remain electrically neutral, a silicotungstate ion will be added with each proton. This results in a large increase in the density at which the DNA bands in the ultracentrifuge.

The details of the interaction are not fully understood as yet, but Hudson has demonstrated the effect of a small amount of denaturation on buoyant density in silicotungstate.

The DNA from phage λ can exist in two physically different forms, linear and cyclic. According to Hershey, Burgi, and Ingraham (6), the linear form contains a small single stranded region at each end. These single stranded ends are complementary and join to form the cyclic form.

In slightly acidic silicotungstate solution, the buoyant density of the cyclic form is 1.051 g/cc. This is the same as was found for native T-4 and T-7 DNA (5). The linear form of λ DNA had a buoyant density of 1.057 g/cc in the same solution. Vinograd and Hudson estimate that this corresponds to about one hundred nonhelical bases in the linear form of λ DNA. This is about 0.1% of the total bases present.

Recooled crab dAT would again serve as a model of slightly denatured DNA. In acidic silicotungstate, the buoyant density of this material should be much higher than that of native crab dAT. A calculation based on this increase in density would give an independent estimate of the number of nonhelical bases in the recooled crab dAT. As stated earlier, this would not only be interesting in itself, but would also aid in interpreting the NMR results.

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PROPOSITION V

In recent years Davidson and his coworkers have studied the properties of the Hg (II) complexes with DNA, and have devised a separation technique based on equilibrium density gradient sedimentation (1). This technique is analogous to the one described in the text of this thesis, with some minor changes (one of which is the use of Hg (II) instead of Ag (I)). With this technique they have been able to accomplish all that the Ag (I) equilibrium density gradient technique has and more. Since Hg (II) is much heavier than Ag (I), it was not unexpected that the density changes which occur when DNA binds Hg (II) are larger than when it binds Ag (I). This makes the Hg (II) density gradient technique a potentially more powerful tool for separations than the same technique using Ag (I). However, the binding of Hg (II) to DNA is not as cooperative as that of Ag (I). This means that two DNA's which differ in, for example, GC content are likely to bind Hg (II) simultaneously. It is likely that with Ag (I) one kind of DNA would bind to saturation before the second would bind at all. The lessor selectivity of Hg (II) decreases the density difference which can be generated between two unlike DNA's, and therefore the utility of the method is lessened.

It is proposed that the usefullness of Hg (II) complexing to DNA can be expanded by using band velocity sedimentation in a density gradient. Yamane and Davidson (2) showed that there is a large

increase in sedimentation coefficient of DNA when Hg (II) is bound. They measured an increase in sedimentation velocity of Calf thymus DNA from $S_{20,w} = 18.8$ with no added Hg (II) to $S_{20,w} = 47.6$ at $r_f = 0.5$ and to $S_{20,w} = 62.3$ at $r_f = 1.2$. The quantity r_f is defined as the moles of $HgCl_2$ added per mole of DNA phosphate present. This great increase in sedimentation velocity has been shown to be due to a collapse of the rod like structure and a subsequent decrease in the frictional coefficient. In band velocity sedimentation this increase in sedimentation velocity could provide a separation of different DNA's based on the decrease in hydrodynamic volume as well as the increase in density which occurs when Hg (II) is bound.

It should be mentioned that the large increase in sedimentation velocity which Yamane and Davidson observed was not due to nonspecific aggregation. If aggregation were occurring, the equilibrium density gradient separations would have revealed it. The experiments by Nandi and Wang (1), one of which is discussed in the text of this thesis (section III - F), demonstrate that there is no significant aggregation.

There are two reasonable ways to use the band sedimentation technique. One utilizes the analytical ultracentrifuge, and the other is done on a preparative machine.

The analytical technique is described by Vinograd et. al. (3), and some of its advantages over the preparative technique are outlined below. In the analytical ultracentrifuge, band sedimentation is a sensitive method for detecting components with different sedimentation rates (4). Homogeneous components with sedimentation velocities differing by only 10% can be observed to separate into two bands (5). This means that the technique should separate Hg (II) - DNA complexes which differ in the amount of bound Hg (II) by only a few percent. This is a significant increase in sensitivity over the equilibrium method.

Another consideration in using the analytical band sedimentation is the large amount of information which can be gained from each experiment. One can make the bulk Cs_2SO_4 solution of such a density that the DNA will not sediment all the way to the bottom. The density gradient then forms such that after a long period (20 hrs.) the DNA assumes its equilibrium buoyant density. In this way, one experiment would provide the sedimentation coefficient and the buoyant density for each DNA band which forms. This combined velocity and equilibrium experiment is, in practice, difficult. In order to be able to calculate both the sedimentation velocity and the buoyant density from the data obtained, DNA concentrations, Cs_2SO_4 densities, and

Hg (II) content must be chosen carefully. It is advised that this combined technique be used only when the separate techniques have been studied enough to be used routinely.

An interesting property of Hg (II) - DNA band velocity sedimentation is that "S on C" would probably be eliminated. Vinograd et. al. (3) pointed out that the leading side of a DNA band spreads due to the fact that the sedimentation velocity is concentration dependent. Those DNA molecules which find themselves in low DNA concentration (in the outside edges of a band) move faster than those at high concentration (in the center). This causes the leading molecules to move out faster, spreading this edge, and causes the trailing molecules to move faster, sharpening that edge.

For high molecular weight DNA (e.g., T-4 DNA) this intermolecular interaction is so large that resolution of two bands of different $S_{20,w}$ is very difficult. Hg (II) probably collapses the DNA structure enough to effectively eliminate "S on C". Separations involving large DNA molecules are then made possible using band sedimentation.

The preparative method would simply be an application of a sucrose gradient (or D_2O gradient) sedimentation as outlined by Martin and Ames (6). The only reason for mentioning this method is that it allows the DNA in each peak which is formed

to be characterized in more detail than does the analytical technique. A radioactive label can be affixed to one or more of a mixture of DNA's, and the kind of DNA responsible for each band in a sedimentation velocity experiment can be identified.

Using either of these methods opens an avenue of study which is not possible in the equilibrium density gradient. RNA is too dense to float in many of the media which can be used for equilibrium density gradient ultracentrifugation. Furthermore, the molecular weight of some interesting varieties of RNA are so low ($S_{20,w}$ = 2-4S) that the bands which would form at equilibrium are very broad (7). The Hg (II) interactions with RNA (either natural or synthetic) can be studied using the resulting change in sedimentation velocity.

Based on this method, useful separation techniques which involve various RNA's can be devised. One possibility is the separation of various different messenger RNA molecules on the basis of base compositional differences. The presently accepted hypothesis (8) is that messenger RNA which can be isolated from bacteria is a mixture of many different messengers,each of which corresponds to a different cistron or operon. It is likely that these vary in base composition, so that if there were a method by which one could select for this difference,

the various messages could be separated from one another. Mercury (II) would bind preferentially to those messengers which are rich in AT (1). This would greatly increase their sedimentation velocity, thus allowing a separation of the various messenger RNA molecules.

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