INTERACTION OF THE SILICOTUNGSTATE ANION WITH NUCLEIC ACIDS

AND

PREPARATION OF CLOSED CIRCULAR DNA FROM HeLa CELL MITOCHONDRIA

Bruce Hudson

Research Report
Submitted for Don Baxter, Inc.
Research Award
May 19, 1967

INTERACTION OF THE SILICOTUNGSTATE ANION WITH NUCLEIC ACIDS *

This report describes an investigation of the interaction of the silicotungstate anion with DNA in acidic solutions. The properties of equilibrium density gradients of solutions of sodium silicotungstate were determined. It was found that the silicotungstate anion binds to protonated sites in DNA with a subsequent increase in buoyant density. Denatured DNA binds silicotungstate at a higher pH than native DNA. Equilibrium dialysis measurements show that protonation must precede binding of the silicotungstate, and that the mechanism of binding between the protonated species and the anion is not simply counterion binding as required by electrical neutrality. A "local insolubility" mechanism is proposed for the binding. The application of this system to the investigation of the properties of DNA, particularly the detection of short single stranded regions in molecules, has been explored. The DNA from λ bacteriophage has been investigated with the hope of separating the linear form (with single stranded ends) from the two circular forms (hydrogen bonded and covalently closed.) This study was troubled by the decyclization of the hydrogen bonded rings in the low salt banding medium, and by difficulties in the preparation of closed circular λ DNA.

Introduction Equilibrium sedimentation in a density gradient has proved to be a very useful technique for the isolation and characterization of nucleic acids. A variety of inorganic salts have been used as solutes in the density gradients, generally cesium or lithium salts. The requirements for a banding solute are (1) solubility of the nucleic acid in the solution, and (2) sufficient solubility of the salt that densities greater than the buoyant density of the nucleic acid can be obtained. There are also two secondary requirements. The solution should be clear in the region around 365 mμ so that the absorption optical system can be used. And the system should have as high

* This research was done under the direction of Dr. Jerome Vinograd.

a resolving power as possible so that small differences in buoyant density can be detected.[1] The usual choice of banding solvent is a concentrated aqueous solution of CsCl.

One application of this method has been the investigation of the alkaline buoyant density titration of DNA in CsCl. As the unionized hydrogens of DNA are removed by base, cesium is bound as a counter ion and the buoyant density increases. Single stranded DNA undergoes a sharp transition at pH 10.8. Double stranded DNA undergoes an extremely sharp transition at pH 11.5. This transition is presumably a cooperative denaturation resulting in strand separation.[1]

In a study of the hydration of DNA by Hearst and Vinograd,[2] the banding solute lithium silicotungstate ($Li_4[SiO_4 \cdot 12WO_3]$) was used to obtain buoyancy at very high levels of hydration. The very high molecular weight of the silicotungstate anion means that solutions of a salt of the anion have a high density at low concentrations, and thus have high water activities. The hydration of DNA is increased very greatly with increasing water activity in this region of high water activity. The water activity of a lithium silicotungstate solution at the buoyant density of DNA is almost 1.0. The buoyant density of DNA at pH 7 in CsCl is about 1.7 gm cm^{-3}, while in sodium silicotungstate it is 1.05 gm cm^{-3}. Because of this high hydration this system has a very poor resolving power, and even very dilute solutions of the silicotungstate anion are opaque in the ultraviolet. The interesting and potentially useful property of the silicotungstate anion is its high mass. By analogy with the alkaline buoyant density titration in CsCl,

the acidic buoyant density titration of DNA in silicotungstate should proceed via protonation of the bases followed by counter-ion binding of silicotungstate. Because of the high mass of the anion this should result in a large density change. Also, because the titration will move the nucleic acid into a region of lower water activity, the dehydration will cause a further increase in density. This system should therefore be very sensitive to the presence of single stranded regions in DNA, which will protonate sooner.

The Silicotungstate Anion and its Equilibrium Density Gradient

The silicotungstate anion is a member of a class of compounds of tungsten and molybdenum known as 12-heteropoly anions. These anions have a Keggin[3] structure consisting of a central tetrahedron bearing the formal charge which is surrounded by twelve tungsten-oxygen or molybdenum-oxygen octahedra which share apices. The overall diameter of the anion is 12.1 Å. The heteropoly acids are very strong acids, highly dissociated electrolytes, and very stable within a prescribed acidic pH range.[4] The cage structure with a central electronegative element results in a very low surface charge density. The silicotungstate anion is probably the most stable of the heteropoly anions. This species is stable up to a pH of about 4.5. The base hydrolysis reaction leads to other heteropoly species, especially 11-, 10-, 6-, and perhaps 5-silicotungstates, plus free tungstate.[5] The reaction is very slow and results in the release of H_3O^+ ions so a neutralized solution of a heteropoly acid shows a slow drift of pH with time. Solutions at pH's slightly higher than pH 4.5 are probably stable for some

time. The hydrolysis reaction is effectively irreversible so neutralization of the acid must be done with care to avoid high local pH's.

The refractive index of solutions of silicotungstic acid and partially neutralized solutions was determined as a function of density. The result is

$$n_D^{25} = 0.1167\,\rho + 1.2159$$

$$\rho = 8.5690\,n_D^{25} - 10.4190$$

for $n_D^{25} < 1.410$, $\rho < 1.700$. This result does not depend on the pH of the solution. The density as a function of concentration was also determined. This result is

$$\rho = \rho_s + 2.45\,m$$

for $\rho < 1.80$, m in moles/liter. A value is given in the literature of $\rho = \rho_s + 2.46\,m$. The value of the constant depends somewhat on the pH, and increases with pH as expected.

Density gradients were generated in a preparative ultracentrifuge at 25 Krpm, in an SW39 rotor at either 30°C or 6°C. Polyethylene tubes containing 3 ml of solution were used and drops collected with a standard dripping device. The density of each sample was determined by measurement of refractive index. The results for one initial density are shown in figure 1. This density gradient was compared with the results of Johnson, Kraus, and Scatchard[6] who investigated the extended Debye-Huckel theory of ionic activity coefficients by analysis of equilibrium density gradients. They find a distance of closest approach of 7.6 Å which agrees fairly well with the crystallographic value of 7.0 Å (comparison is not simple because of the high charge type and the

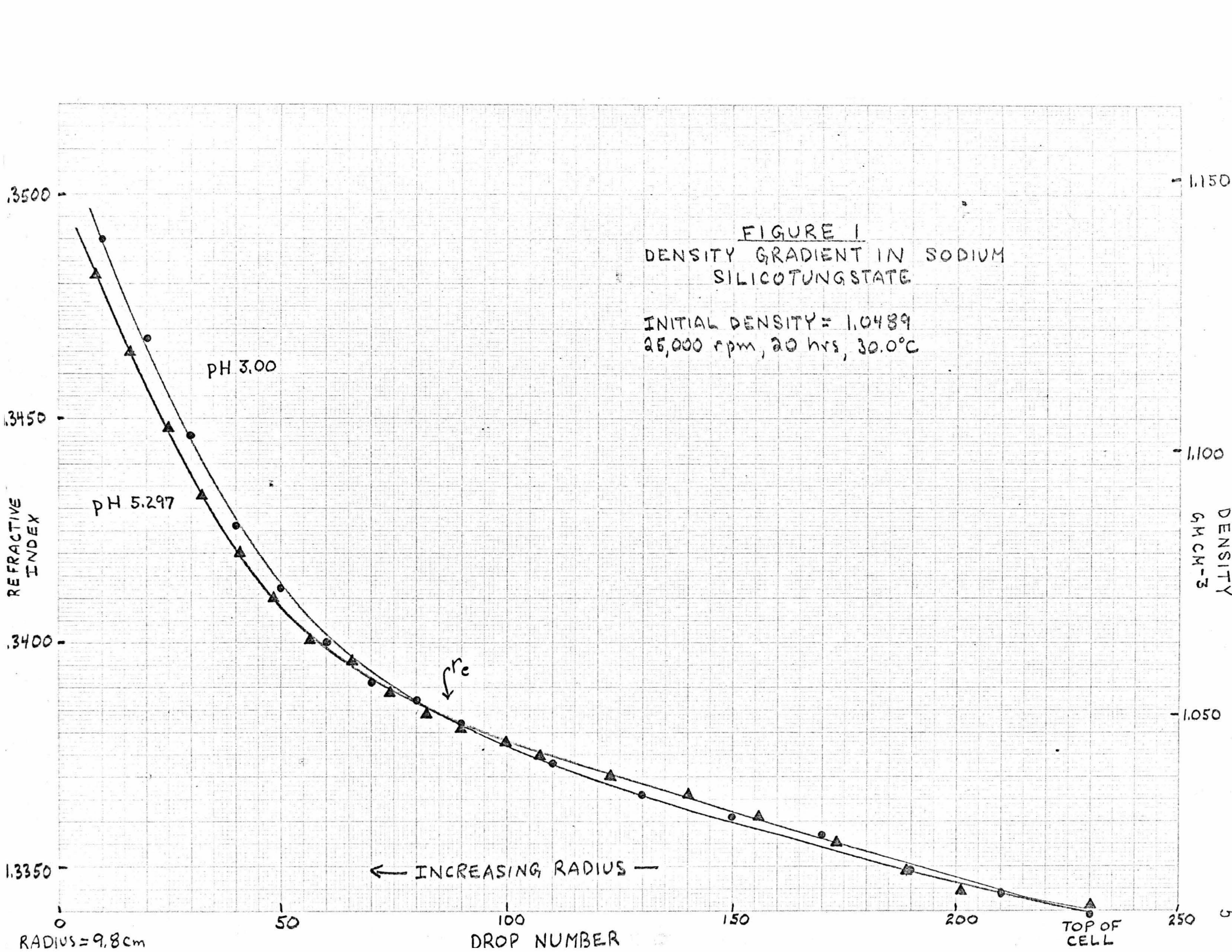


FIGURE 1
DENSITY GRADIENT IN SODIUM SILICOTUNGSTATE
INITIAL DENSITY = 1.0489
25,000 rpm, 20 hrs, 30.0°C
pH 3.00
pH 5.297
r_e
← INCREASING RADIUS —
REFRACTIVE INDEX
DENSITY GM CM-3
1.3500
1.3450
1.3400
1.3350
1.150
1.100
1.050
0
50
100
150
200
250
TOP OF CELL
RADIUS = 9.8 cm
DROP NUMBER

very large difference in sizes of the H_3O^+ and silicotungstate anion.) This parameter and the known value of $\bar{v}$ are used to calculate the density gradient at r_e, the radius at which the original uniform density occurs, in this system from the relation

$$d\rho/dr = (d\rho/d\ln a)(1 - \bar{v}\rho)(M\omega^2 r/RT)(2/n+m)$$

for an n:m electrolyte. $(d\rho/d\ln a)$ is the term given by the extended Debye-Huckel theory. In this case $\rho = 1.049$, and $T = 30^\circ C$. The result is $(d\rho/dr)_{CALC.} = 2.926 \times 10^{-2}$ gm cm^{-4}. The experimental result is 2.976×10^{-2} gm cm^{-4}. Gradients generated at 6°C had a smaller slope. The gradient at r_e as a function of initial density were determined as shown in figure 2. Densities from almost 1.0 to 2.5 can be generated in a 3 ml (~3 cm) column.

The buffer concentration in these density gradients was generally 2×10^{-2} M sodium acetate. Acetate buffers well in the region of interest (pH 3.5 - 5.0). The buffer concentration was kept low so that the ionic strength would be due primarily to the silicotungstate. Under these conditions significant pH gradients are generated in the column. These pH gradients were measured for solutions with several pH's. In each case the starting density was 1.049, the same as that used for the density gradients in figure 1. These pH gradients are shown in figure 3. These pH gradients are probably generated by the migration of the lighter H_3O^+ and Na^+ ions with the heavier silicotungstate anions. If there were no voltage gradient in the column, the density and pH gradients should be parallel when expressed in the same logarithmic manner. According to this model, if the concentration of silicotungstate (which effectively determines the density) is c(r), then $[H_3O^+] = \alpha c(r)$, where $\alpha = [H_3O^+] / [\text{silicotungstate}] = \text{constant}$.

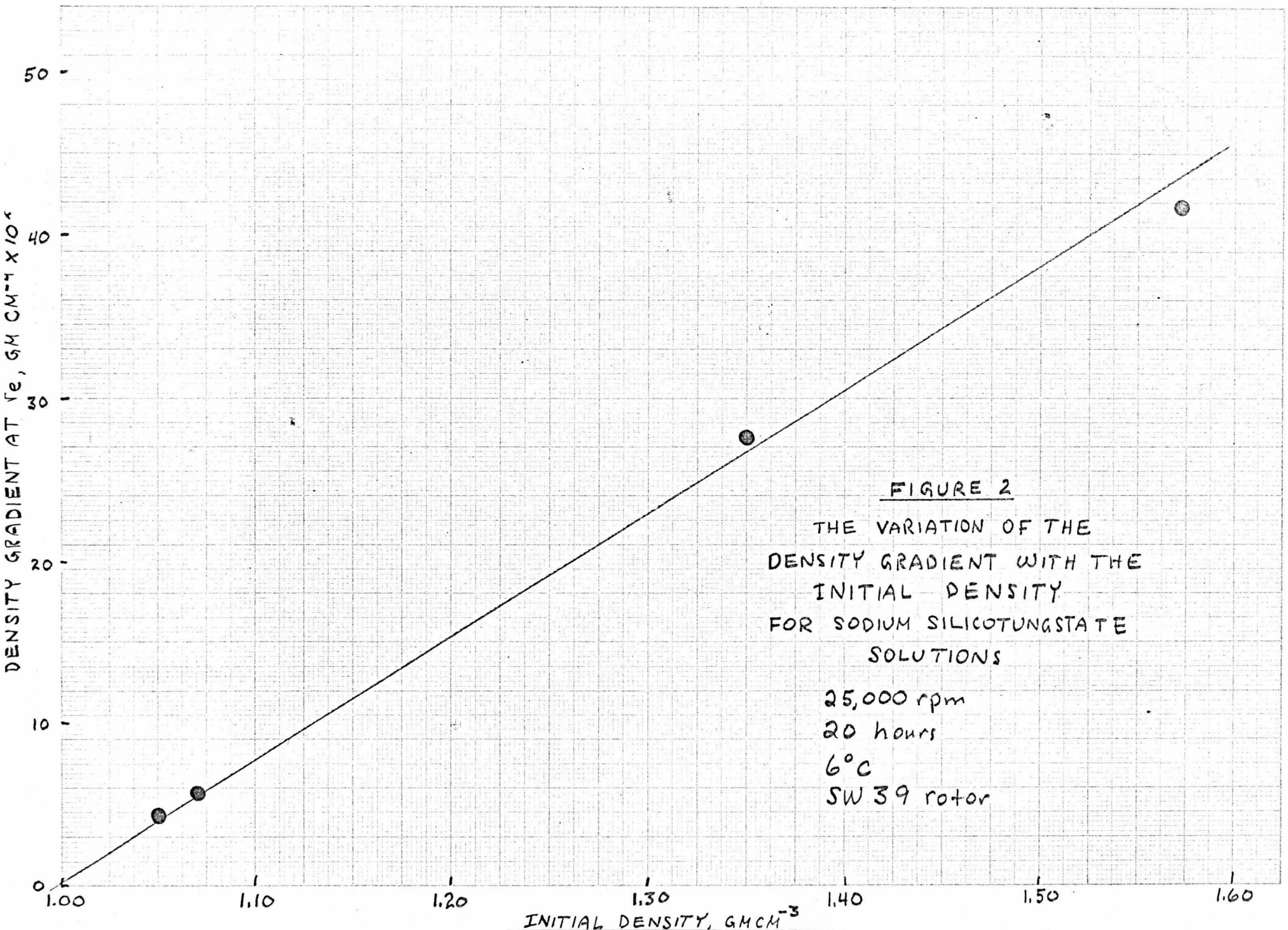


FIGURE 2

THE VARIATION OF THE DENSITY GRADIENT WITH THE INITIAL DENSITY FOR SODIUM SILICOTUNGSTATE SOLUTIONS

25,000 rpm
20 hours
6°C
SW 39 rotor

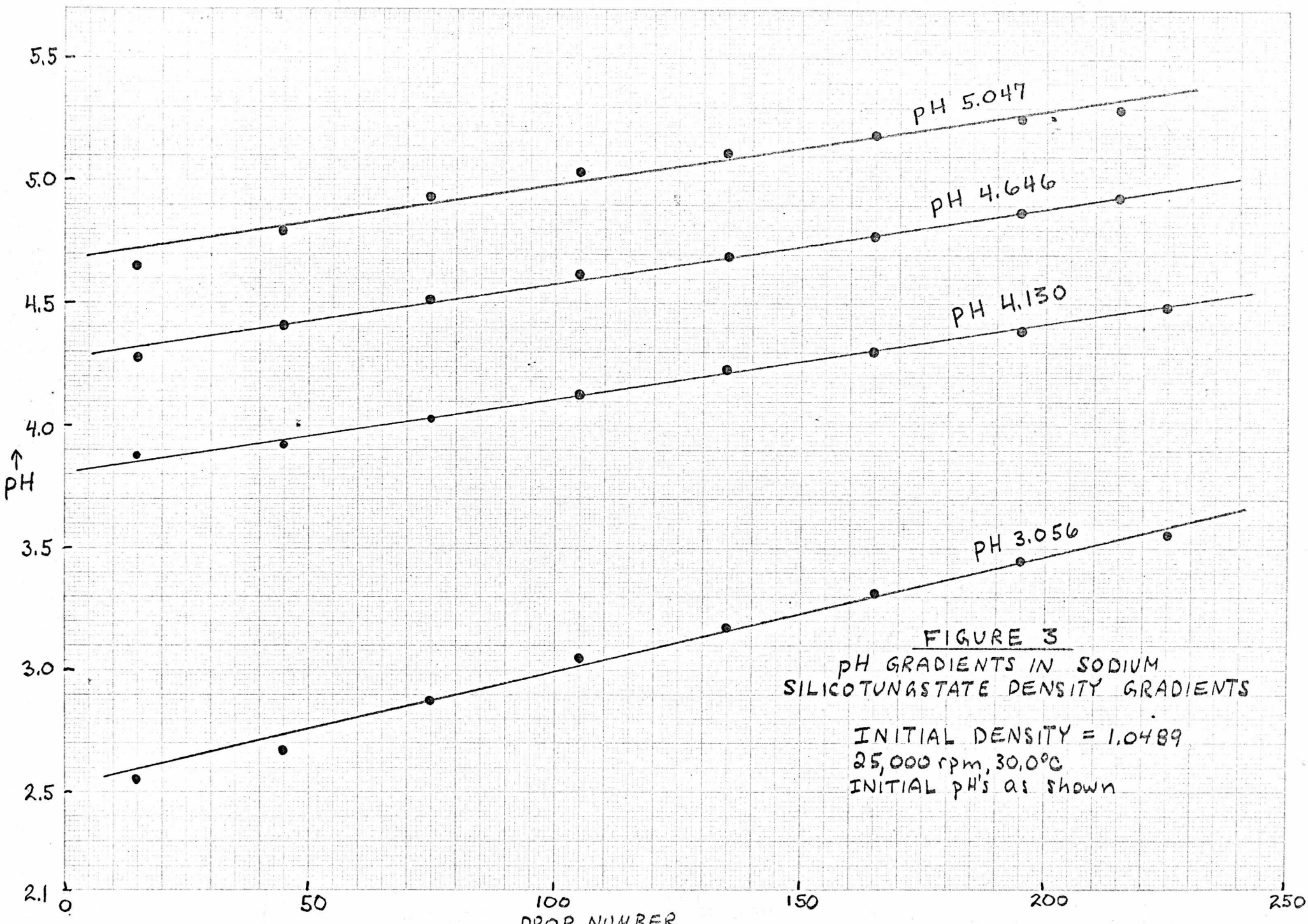


FIGURE 3

pH GRADIENTS IN SODIUM SILICOTUNGSTATE DENSITY GRADIENTS

INITIAL DENSITY = 1.0489
25,000 rpm, 30.0°C
INITIAL pH's as shown

The rest of the charge is compensated for by sodium ions whose concentration would be $[Na^+] = (4 - \alpha)c(r)$. The ratio $[H_3O^+]/[Na^+] = \alpha/(4 - \alpha)$ is assumed to be a constant because there should be very little centrifugal redistribution of these light species. Thus, if pc = -log silicotungstate ,

$$pH = -\log(\alpha c(r)) = -\log\alpha + pc.$$

This predicts that the plots of pH and pc vs. r should be parallel. It does not imply that these plots should be straight lines. These plots are shown in figures 4 and 5 for the extreme pH's of interest. There is a considerable error in the determination of the silicotungstate concentration from the refractive index at these low concentrations because of the large correction for the solvent. A better method would be to determine the concentration by measuring the UV absorbance. These preliminary results do indicate, however, that this simple model accounts for the observed pH gradient.*

The near linearity of the logarithmic plot of concentration vs. radius is also interesting. The expression for sedimentation equilibrium is

$$d\ln a/dr = (\text{const.})(1-\bar{v}\rho)r.$$

But

$$d\ln a/dr = (d\ln c/dr)(d\ln a/d\ln c) = (d\ln c/dr)c(d\ln a/dc).$$

The term $d\ln a/dc$ was claculated from the extended Debye-Huckel theory and found to be very nearly a constant. Thus

$$d\ln c/dr = (\text{const.})(1 - \bar{v}\rho)(r/c).$$

Since $d\ln c/dr$ is approximately constant, a plot of $(1 - \bar{v}\rho)/c$ vs. $1/r$ should be linear. If $\bar{v}$ is assumed constant, the linearity

---

* pH gradients in the alkaline CsCl system have been proposed as a possible explanation of the extremely sharp observed transitions.

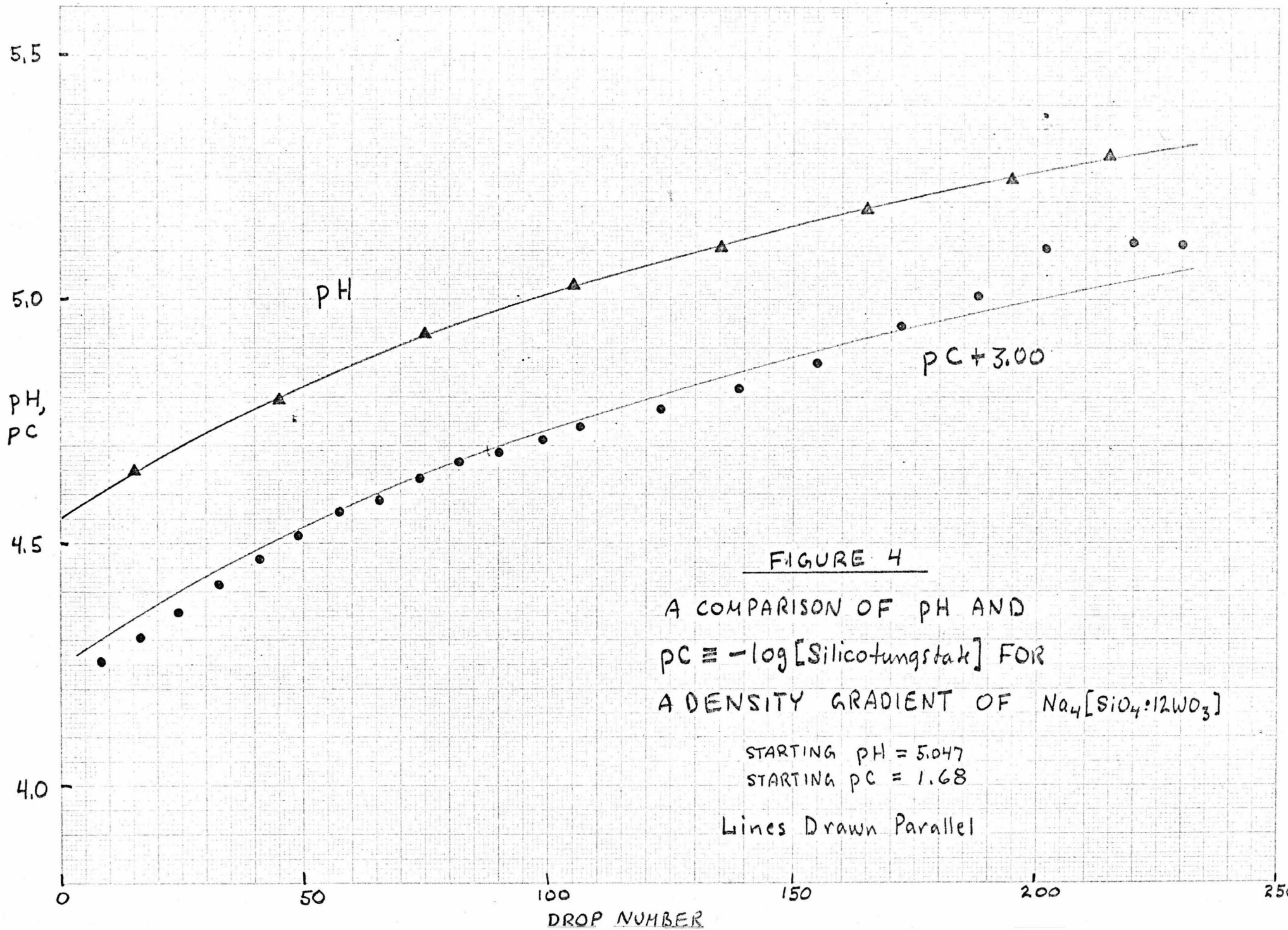


FIGURE 4

A COMPARISON OF pH AND
pC ≡ −log [Silicotungstate] FOR
A DENSITY GRADIENT OF $Na_4[SiO_4 \cdot 12WO_3]$

STARTING pH = 5.047
STARTING pC = 1.68

Lines Drawn Parallel

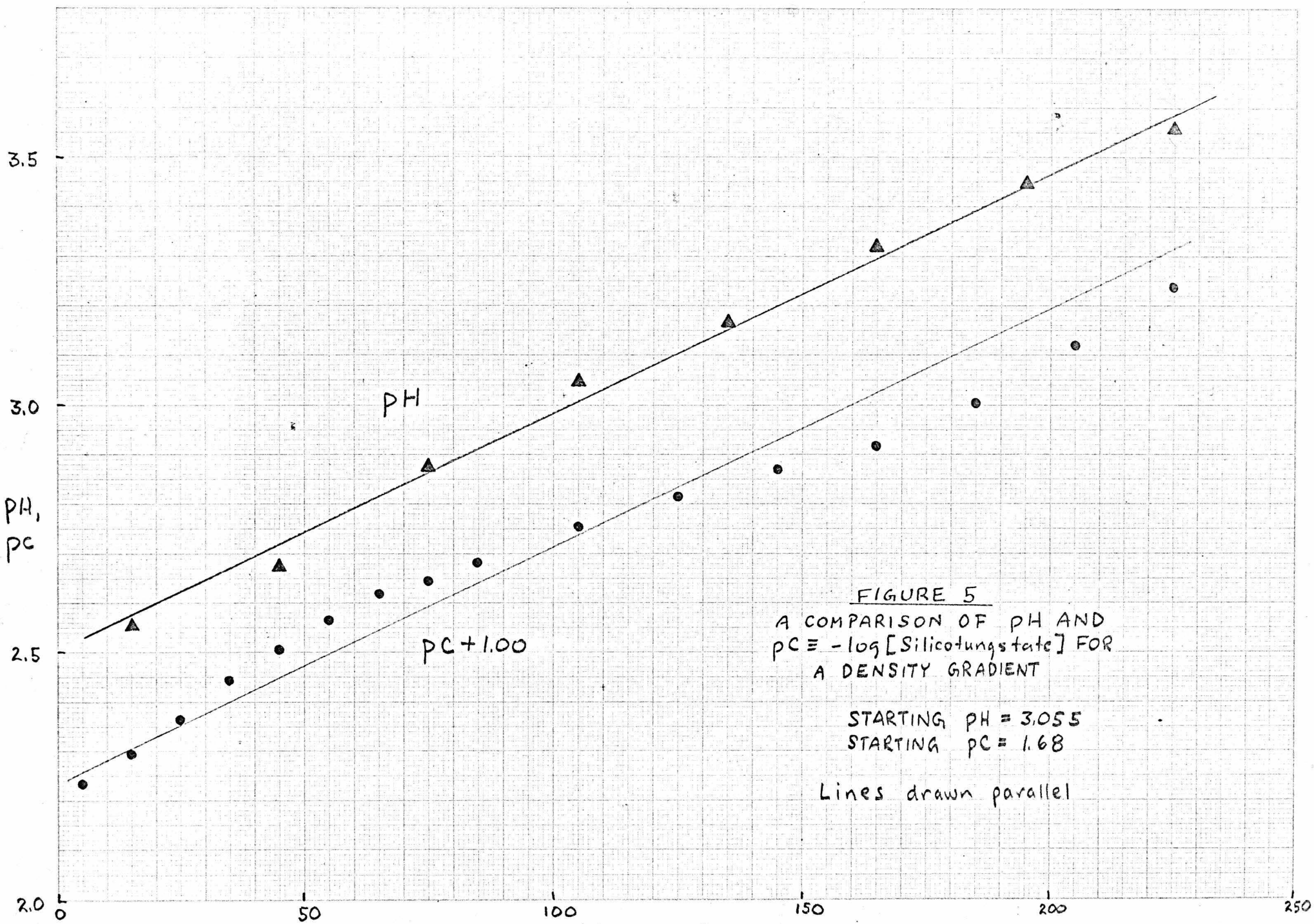


FIGURE 5

A COMPARISON OF pH AND $pC \equiv -\log[\text{Silicotungstate}]$ FOR A DENSITY GRADIENT

STARTING pH = 3.055
STARTING pC = 1.68

Lines drawn parallel

of ρ with c implies that 1/c vs 1/r should be linear. This plot is shown in figure 6. This explains the linearity of the pH gradients since they are directly related to the density gradient.[7] Silicotungstate gradients are quite different from CsCl gradients where the concentration is roughly linear in radius.

The spectrum of the silicotungstate anion in the UV is shown in figure 7. This spectrum happens to be very similar to that of DNA or the free bases.

Experiments with Nucleic Acids Radioactively labeled 18S and 28S ribosomal RNA obtained from the laboratory of Dr. Giuseppe Attardi was used to study the possible use of silicotungstate as an RNA banding medium. The pH was kept as high as possible and was generally around 4.5. In all of a series of 21 experiments using starting densities ranging from ρ = 1.05 to ρ = 1.99 (ρ_{max} of about 2.6) the RNA was on the bottom of the tube. The radioactive counts were distributed over a considerable portion of the tube, however, indicating that precipitation had not occurred. (Precipitation results in very narrow bands.)

An experiment with native and denatured T-4 DNA was preformed using the analytical ultracentrifuge and schlieren optics. One cell contained T-4 DNA which had been denatured by boiling, while the other contained a mixture of native and denatured material. Both solutions were ρ = 1.046 and pH 4.4. The cell with the mixture of DNA's contained a band at ρ = 1.051, while the cell containing only denatured DNA had no band. The value of 1.051 for the density of native DNA agrees well with the

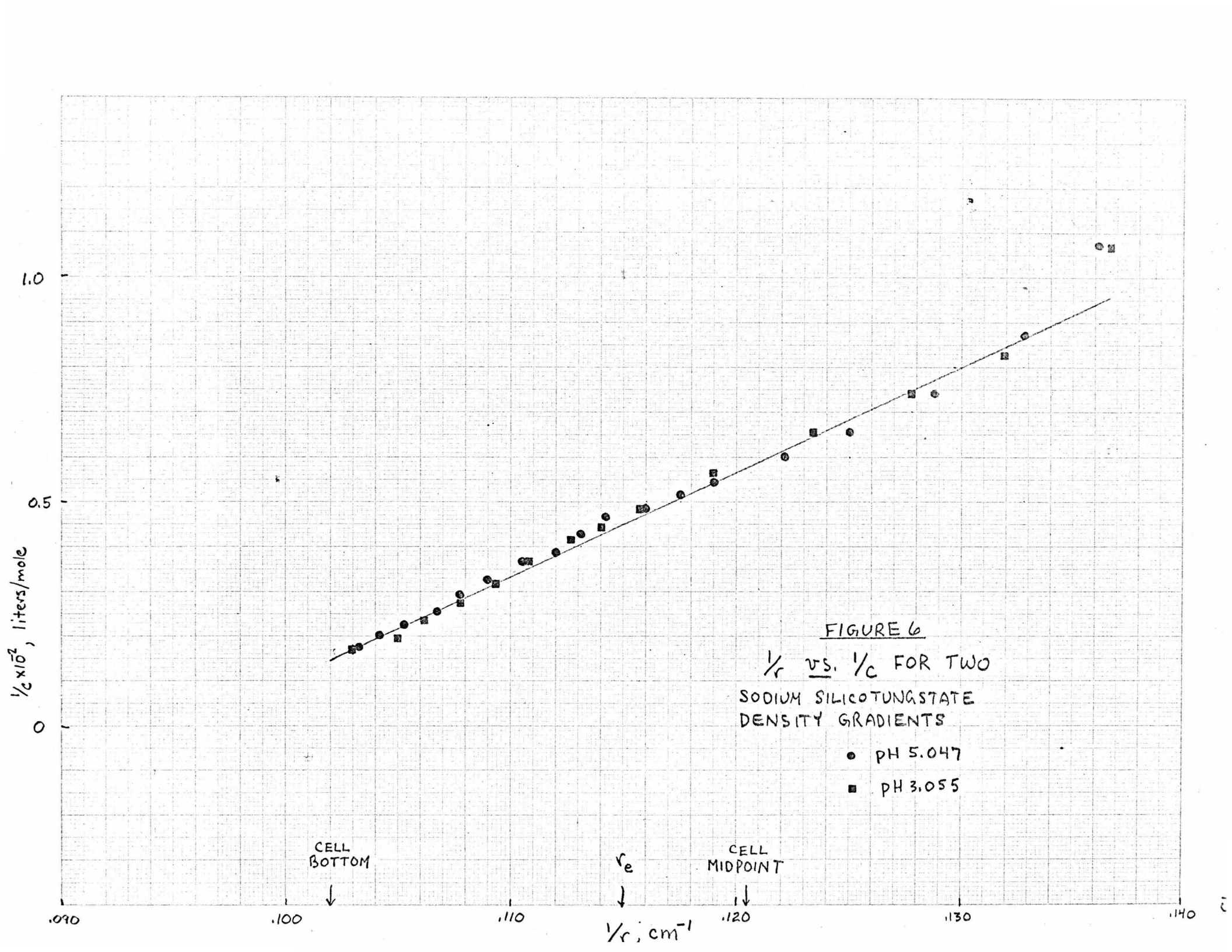
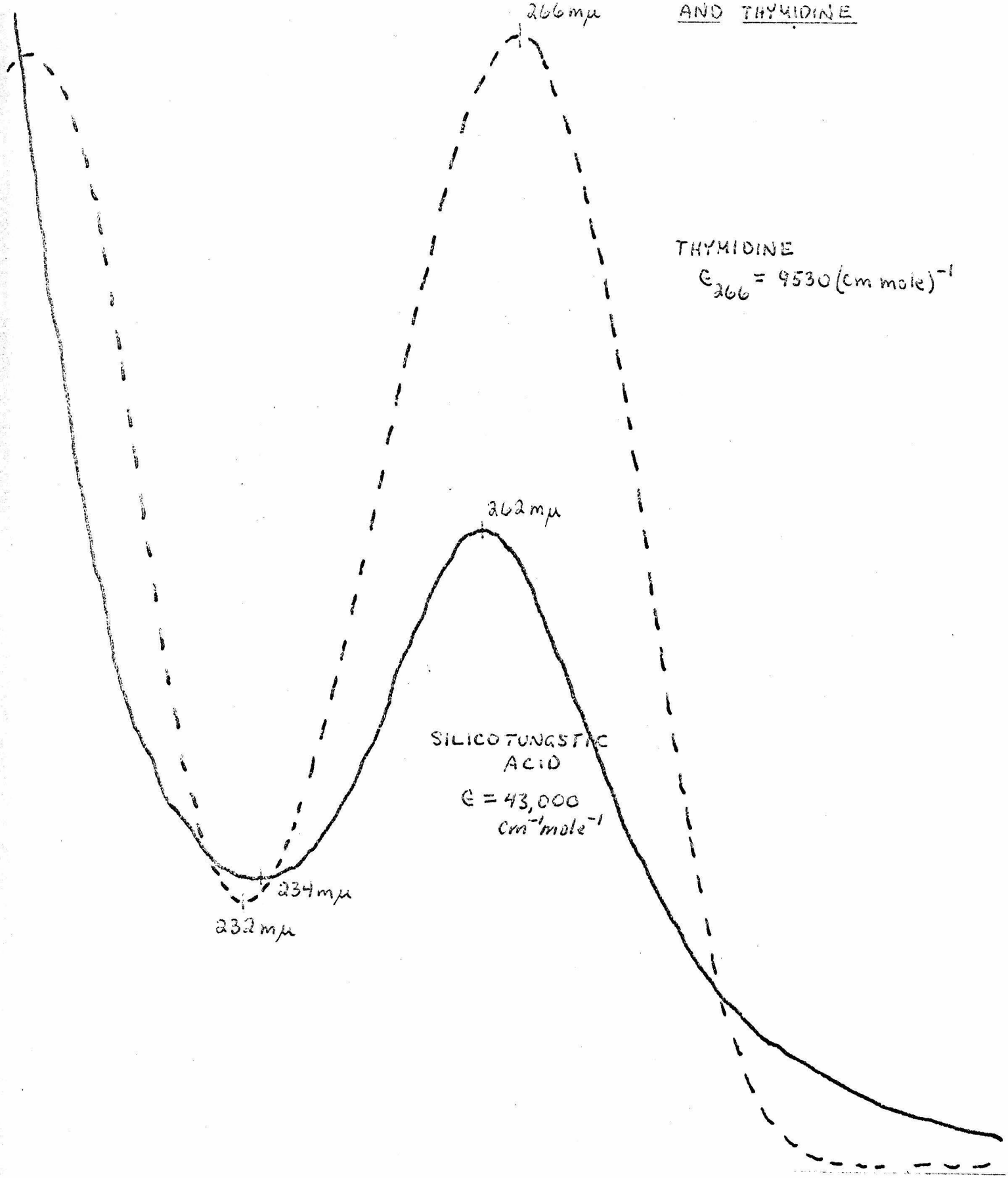

1.0
0.5
0
1/c x10^-2, liters/mole
.090
.100
.110
.120
.130
.140
1/r, cm^-1
CELL BOTTOM
re
CELL MIDPOINT
FIGURE 6
1/r vs. 1/c FOR TWO SODIUM SILICOTUNGSTATE DENSITY GRADIENTS
pH 5.047
pH 3.055

FIGURE 7

THE SPECTRA OF SILICOTUNGSTIC ACID AND THYMIDINE



measurements of Janet Morris in this laboratory.[8] It differs from the value of Hearst[2] because of a different buffer concentration.

An experiment was performed in the preparative ultracentrifuge using P^{32} labeled, native, linear λ DNA at a variety of pH's. At pH 4.17 there was a peak at a density of about 1.05, but at pH's 3.59 and 3.05 the material was at the bottom of the cell. These are starting pH's and not the pH's at band peak. Further experiments of the same type show that at pH = 4.027 there is a light band while at pH 3.678 it is much denser. These experiments have also been performed by Ponzy Lu in this laboratory.[9] He found that at pH 3.80 the density of the DNA was 1.65, while at pH 4.00 it was about 1.07. He also investigated the density change of denatured λ DNA, and found that it could be banded only at pH's of about 5, where it was very light ($\rho = 1.05$.) In all cases the bands had normal widths indicating that there was no precipitation.

Thus, at pH's between pH 4.0 and 4.5 this system easily separates native and denatured DNA. Because of this property it should be possible to separate completely double stranded DNA from DNA which contains short single stranded regions. The linear form of λ DNA contains a short single-stranded region at each end which is now known to be about 10 nucleotides long.[10] The linear DNA can be formed into hydrogen bonded circles by heating and slowly cooling a dilute solution of linears in 0.6M NaCl.[11] Dilute solutions favor cyclization over polymerization. The rather high salt concentration seems to be necessary to stabilize the circles

by lowering the phosphate repulsion energy. A series of experiments were preformed to try to separate the linear and circular forms of λ DNA in silicotungstate density gradients. One experiment with P^{32} labeled DNA gave two peaks with areas roughly corresponding to the initial concentration ratio and with a separation of about 0.006 gm cm^{-3}. This experiment was preformed at a pH of about 4.3. Attempts to repeat this experiment have, however, been unsuccessful. The difficulty is almost certainly the instability of the hydrogen bonded rings under these conditions. The ionic strength of these solutions is only about 0.25, and it was shown that the rings were unstable at $\mu = 0.10$. Also, since pH's less than 4.0 seem to cause denaturation of the DNA (although this has not really been shown), the low pH probably causes small regions of local denaturation which could open the circle. This difficulty could be avoided by use of the covalently closed circular λ DNA formed during the eclipse period of infection.[12] Attempts in this laboratory to prepare purified, labeled, closed circular λ DNA were unsucessful, however. Newer methods for the isolation of closed circular DNA[13] and methods for the enzymatic closure of linear DNA into covalently closed circles[14] would probably have greater success.

These studies indicate that the silicotungstate ion binds to native DNA at pH's lower than about 4.0 and to denatured DNA at slightly higher pH's. By analogy with the alkaline titration of DNA in cesium chloride, a reasonable mechanism would be protonation of a ring or amino nitrogen in DNA followed by the

inclusion of a silicotungstate anion in the solvation shell as a counterion. No specific binding between the positive site and the counterion is required. Electroneutrality of the buoyant thermodynamic species requires that the solvation shell of the macromolecule must be large enough that a number of ions sufficient to neutralize the charge of the macromolecule are included in the shell. In the case of an alkaline buoyant density titration in CsCl there is an increase in solvation which partly compensates for the density increase due to Cs^+ binding. In the case of the sodium silicotungstate system, however, it would seem more reasonable that the increased positive charge on the molecule due to protonation would be compensated by the removal of one of the sodium ions from the solvation sphere. The silicotungstate anion has an extremely low charge density and would not be expected to bind to a positive charge. In fact, a molybdenum anolog of silicotungstate, $H_4[PO_4VO \cdot 11MoO_3]$, has been shown to be almost completely dissociated in dilute solutions.[4] The expulsion of an Na^+ ion from the solvation shell could explain the increased density. It seems more probable, however, that there is some specific binding between the protonated bases of DNA and the silicotungstate anion.

In order to test this hypothesis a dialysis equilibrium experiment was performed. Calf thymus DNA at a high concentration was denatured by boiling, dialysed to remove short segments of DNA and they dialysed against a dilute solution of silicotungstic acid at pH 5.2 in the cold for 24 hours. There was no detectable decomposition of the anion in this time. The DNA

was placed in a flask to which was added a stirring bar and a dialysis bag filled with a top such that the contents of the bag could be changed or removed. The bag was filled with the same silicotungstate solution used for dialysis. The pH of the system was then lowered by the addition of concentrated HCl. After equilibrium had been reached, the concentration of the silicotungstate ion in the bag (where there was no DNA) was greatly lowered. The experiment was continued by further lowering the pH and by repeating the operation with other flasks.

The following model of this system is proposed. It is first assumed that at pH 5.2 there is little if any binding of silicotungstate to the DNA, based on the results of Lu. Even a considerable amount of binding of silicotungstate to the DNA at this pH would not affect the evaluation of the experiment significantly. As the pH is lowered, the bases are protonated. It is assumed that there is only one pK for the bases. This would be the case if only one base were involved or if the pK's of the reacting bases are about the same, as is probably the case. This reaction is

$$H^+ + B \underset{}{\overset{K_1}{\rightleftharpoons}} HB^+$$

The next step is the formation of a complex between the protonated base and the silicotungstate anion

$$(HB)^+ + (ST)^{-4} \overset{K_2}{\rightleftharpoons} (HB)(ST).$$

This reaction is assumed to be 1:1. The mass actions equations for these reactions plus the equation for the conservation of silicotungstate anion leads to the equation

$$(1/H)(K_1 K/k) + (K_2/k) = c_2/(c_1 - c_2)$$

where k is a known constant of the system involving the volumes

of the bag and flask and the concentration of DNA, H is hydrogen ion concentration, c_2 is the concentration of free silicotungstate ion, c_1 is the original concentration (at pH 5.2), and K_1 and K_2 are the equilibrium constants as given above. Thus a plot of $1/H$ vs. $c_2/(c_1 - c_2)$ should be linear with an intercept which gives K_2 and a slope which gives K_1K_2. This plot is shown in figure 8. Since $k = 1.228 \times 10^{-3}$ this plot says that the pK for protonation of the bases is $pK_1 = 3.73$ which seems reasonable.* The resulting dissociation constant for the protonated base-silicotungstate complex is 1.5×10^{-4}. Notice that it is not necessary to convert the absorbances of the heteropoly to concentrations since the extinction coefficient cancels. The factor k could be off by a factor of 3/4 to 1/4 if only 3, 2 , or 1 of the four bases protonate. If 3 of the bases protonate the pK would be 3.95, 2 bases would yield 4.12. The number of reactive bases cannot be obtained from this experiment. The Donnan effect in this system has not been analysed quantitatively, but it can be seen that this would probably decrease the apparent binding constant. The linearity of the plot shows that the reaction between protonated bases and silicotungstate is one to one. This would seem to be a reasonable result because the probability of getting two protonated bases together must be fairly small. At a pH of 3.682 the absorbance in the dialysis bag increased with time.and the spectrum changed slightly. This indicates that depurination was occuring slowly at this pH and 5°C. This sets a lower limit to studies of this system.

This experiment proves that there is a mechanism of binding

* Magnetic resonance experiments by S. I. Chan and coworkers give a value of the pK of 3.80. (S. I. Chan, private communication).

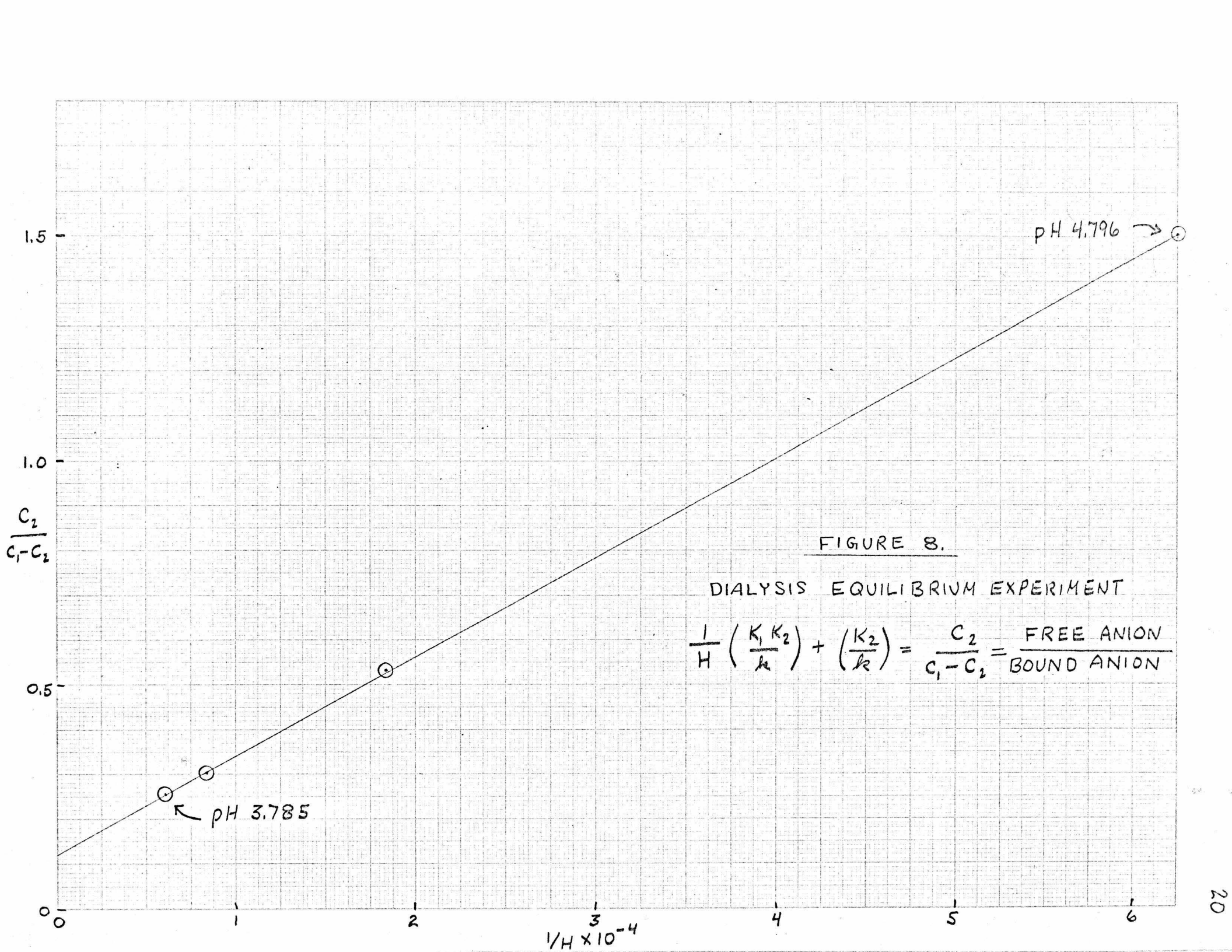


FIGURE 8.

DIALYSIS EQUILIBRIUM EXPERIMENT

$$\frac{1}{H}\left(\frac{K_1 K_2}{k}\right) + \left(\frac{K_2}{k}\right) = \frac{C_2}{C_1 - C_2} = \frac{\text{FREE ANION}}{\text{BOUND ANION}}$$

between protonated bases of DNA and silicotungstate which differs from simple counterion binding. Chloride ion would serve as a better partner in an ion pair. Precipitation of the silicotungstate-protonated base complex is another possible explanation. This seems unlikely, first, because of the band width in density gradients mentioned above, second, because no precipitate was observed in extremely concentrated mixtures of DNA and silicotungstate, and third, because the concentration of silicotungstate is three orders of magnitude lower than that of the bases of the DNA. It would seem unlikely that one silicotungstate every 1000 bases~~pairs~~ would cause precipitation.

An explanation for the binding phenomenon can, perhaps, be inferred from the solubility behavior of the heteropoly acids in general. The solubility properties of all heteropolies are nearly identical. The acids and sodium salts are very soluble in a wide variety of rather nonpolar organic solvents such as alcohols (including n-dodecanol), dioxane, and ethyl ether.[15] This is unusual for a strong electrolyte. The lithium and sodium salts are very soluble in water, the potassium salt somewhat less so, and the rubidium and cesium salts are very insoluble. In fact all large cations precipitate the heteropoly anions. These include tetralkyl ammonium, pyridinium and similar organic cations. Measurements of the variation of $\bar{v}$ with the charge type of the heteropoly anion indicate that it lowers the density of a layer of water surrounding it, similar to the iceberg effect of hydrophobic bonding. We are lead to the hypothesis that the binding of the silicotungstate anion to protonated bases in DNA is due to the formation of a locally insoluble complex which is

kept in solution by the high solubility of the remainder of the polymer. As a simple case, pyridine and the free bases of DNA form precipitates with silicotungstic acid, while the nucleosides (base plus sugar) do not. Thymidine forms a soluble yellow complex at high concentrations. This proposed picture of the complex may be somewhat similar the action of soap on organic compounds in that the driving force of complex formation is the higher entropy of the complex because the components disrupt the structure of water. There is the further similarity that the solubility of the comples depends on the presence of a distant charged group.

PREPARATION OF CLOSED CIRCULAR DNA FROM HeLa CELL MITOCHONDRIA

Radloff, Bauer and Vinograd have described[13] the isolation of closed circular DNA from the mitochondria of the human cervical cancer cells known as HeLa cells. Electron microscopy of these closed circles has indicated that most of these closed circles are 5 microns in length and quite uniform. About 10% of the molecules, however, were multiples of this basic unit, primarily doubles. These doubles were pinched in the middle, giving the appearance of joined singles. It has been shown in this laboratory by David Clayton that open doubles occur in human leukemia cells. The author is presently involved in the preparation of double length mitochondrial DNA from HeLa cells in sufficient quantities that some of its physical properties can be determined. The basic question is how the double length molecules are related to the singles and what is their biological significance. The open doubles in leukemia mitochondrial DNA were first discovered by modifying the Kleinschmidt electron microscopy procedure. These modifications are being used by the author for the first HeLa preparations. Mitochondrial circles are shown in figure 9. Larger quantities of the mitochondrial circles are being prepared by the repetitive processing of increasing volumes of HeLa cells by the methods of Radloff et. al. It is hoped that the doubles can be separated from the singles by velocity methods.

FIGURE 9

CLOSED CIRCULAR MITOCHONDRIAL DNA FROM HeLa CELLS

1. Vinograd, J., and J. E. Hearst, Prog. Chem. Org. Nat. Prod., Ed., L. Zechmeister, Springer-Verlag, Vienna, 1962, Vol. XX.

2. Hearst, J. E., and J. Vinograd, Proc. Natl. Acad. Sci., 47, 825 (1961)

3. Keggin, J. F., Nature, 131, 908 (1933); Proc. Roy. Soc. (London), A144, 75 (1934)

4. Hallada, C., G. A. Tsigdinos and B. Hudson, J. Inorg. Chem., in press.

5. Tsigdinos, G. A., Heteropoly Compounds of Molybdenum and Tungsten, Climax Molybdenum Company, Bulletin Cdb-12a

6. Johnson, J. S., K. A. Kraus and G. Scatchard, J. Ph. Chem., 64, 1867

7. It should be possible to analyse the pH and density gradients of sodium silicotungstate theoretically starting from the equation of sedimentation equilibrium and the extended Debye-Huckel theory of the activity coefficient. This has so far not been possible because of mathematical complications. The results of figures 3-6 are probably a coincidental result of the high mass of the silicotungstate anion.

8. Janet Morris, notebook number 1261

9. Ponzy Lu, notebook number

10. Wang, J. C. and N. Davidson, J. Mol. Biol, 15, 111

11. Hershey, A. D., Burgi, E. and Ingraham, L., Proc. Nat. Acad. Sci., 49, 748 (1963)

12. Bode, V. C. and Kaiser, A. D., J. Mol. Biol., 1965, 14, 399

13. Radloff, R., W. Bauer and J. Vinograd, unpublished results.

14. Gellert, M., Proc. Natl. Acad. Sci., 57, 148 (1967)