THE INTERACTIONS OF BASIC PROTEINS AND DNA

I ELECTROPHORESIS OF THE NUCLEIC ACIDS

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III STUDIES OF THE ELECTROPHORESIS AND MELTING BEHAVIOUR OF NUCLEOHISTONES

IV THE DISSOCIATION OF HISTONE FROM CALF THYMUS CHROMATIN

Thesis by

Baldomero Marquez Olivera, Jr.

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Abbreviations Used In This Thesis

A°	angstrom unit
A	absorbance
Ac	acetate
C/D	cytochrome to DNA, mass ratio
d-AMP	deoxyadenylic acid
d-AT	polydeoxyadenine-thymine, alternating co-polymer
d-ATP, d-CTP, d-GTP, d-TTP	deoxynucleoside triphosphates of the four DNA bases
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
EDTA	ethylenediamine tetraacetate
F	formal, formality
GuCl	guanidinium chloride
%H	per cent hyperchromicity, at 2600 A° unless otherwise specified
H/D	histone to DNA mass ratio
MW	molecular weight
NH	nucleohistone
poly A	polyadenylic acid
poly U	polyuridylic acid
Q	coulombs
RNA	ribonucleic acid
RNAase	ribonuclease

Abbreviations - Cont'd.

S	sedimentation coefficient in Svedbergs
T_{10}, T_m, T_{90}	temperature at which 10%, 50% and 90% (respectively) of the total absorbance increase on denaturation has been achieved
UV	ultraviolet light

PREFACE

One of the major problems in writing this thesis was to organize the material so that it might make some sense to a reader and yet keep the total length of the thesis within reasonable limits. The work described in this thesis is made up of different projects which are loosely related to one another. I have chosen to divide the thesis into four parts. Each part is written as an independent unit, and may be read without reading the preceding sections of the thesis.

It was felt that different sections might be of interest to different readers. Each section therefore has an independent set of reference, figure and table numbers.

Another problem was how to deal with the literature of interest. I have made no attempt to review any previous literature, and instead refer the reader to various reviews on the different subjects. In general, I have followed the practice of putting down reviews as references instead of the primary literature since in many cases, a general view of the results of a number of different workers is more pertinent than the specifics of each investigation.

In the last three sections of the thesis, the experimental method used, the results and the discussion of the results are all integrated in the text since it would have been cumbersome to discuss each separately. The text is written so that it is not at all necessary to refer to the figures for a general view of the results. However, a lot of the experimental details are put into the legends to the

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figures, and many details can only be obtained by examination of the various figures. This is especially true of the last section where a close scrutiny of the photographed gels is necessary to have some feel for how good the evidence is for each point made.

I

ELECTROPHORESIS OF THE NUCLEIC ACIDS

Abstract

A zone electrophoresis apparatus using ultraviolet optics has been constructed to study nucleic acids at concentrations less than 0.004%. Native DNA has a mobility about 15% higher than denatured DNA over a range of conditions. Otherwise, the electrophoretic mobility is independent of molecular weight, base composition or source. DNA mobilities change in the expected way with pH but the fractional change in mobility is less than the calculated change in charge. A small decrease in mobility accompanies an increase in ionic strength. RNA's from various sources have mobilities slightly lower than denatured DNA except for s-RNA which travels slightly faster. The important considerations governing the mobility of nucleic acids appear to be the nature of the hydrodynamic segment, and the binding of counterions. The differences between electrophoresis and sedimentation stem from the fact that all random coil polyelectrolytes are fundamentally free draining in electrophoresis.

Baldomero M. Olivera, Jr.

The Interactions of Basic

Proteins and DNA

II

THE CYTOCHROME C/DNA COMPLEX

Abstract

The basic protein, cytochrome c, has been complexed to DNA. Up to a cytochrome:DNA mass ratio of 2, a single type of complex is formed. Dissociation of this complex occurs between 0.05F and 0.1F NaCl. The complexing of cytochrome to DNA causes a slight increase in the melting temperature of the DNA, and a reduction of the electrophoretic mobility proportional to the decrease in net charge. Above a cytochrome:DNA mass ratio of 2.5, a different type of complex is formed. The results suggest that complexes such as are formed in the Kleinschmidt technique of electron microscopy would not exist in bulk solution and are exclusively film phenomena.

III

STUDIES OF THE ELECTROPHORESIS AND MELTING BEHAVIOUR OF NUCLEOHISTONES

Abstract

Electrophoresis studies on reconstituted nucleohistones indicate that the electrophoretic mobility for these complexes is a function of the net charge of the complex. The mobility is therefore dependent on the charge density of the histone complexing the DNA, as well as on the histone/DNA ratio. It is found that the different histones affect the transition from native to denatured DNA in different ways. It appears that histone I is exchanging quite rapidly between DNA molecules in 0.01 F salt, while histone II is irreversibly bound. Histone III-IV enhances the capacity of non-strand separated denatured DNA to reanneal. Studies on native nucleoproteins indicate that there are no gene-sized uncomplexed DNA regions in any preparations studied.

IV

THE DISSOCIATION OF HISTONE FROM CALF THYMUS CHROMATIN

Abstract

Calf thymus nucleoprotein was treated with varying concentrations of NaCl. The identity of the histones associated and dissociated from the DNA at each salt concentration was determined by gel electrophoresis. It was found that there is no appreciable histone dissociation below 0.4 F NaCl. The lysine rich histones dissociate between 0.4 and 0.5 F NaCl. Their dissociation is accompanied by a marked increase in the solubility of the chromatin. The moderately lysine rich histones dissociate mainly between 0.8 and 1.1 F NaCl. There are two arginine rich histone components: the first dissociates between 0.8 F and 1.1 F NaCl, but the second class is the very last to be dissociated from the DNA (dissociation beginning at 1.0 F NaCl). By 2.0 F NaCl, essentially all the histones are dissociated.

The properties of the extracted nucleoprotein were studied. The electrophoretic mobility increases and the melting temperature decreases as more histones are dissociated from the DNA. A comparison with the dissociation of histones from DNA in $NaClO_4$ shows that to dissociate the same class of histones, the concentration of NaCl required is twice that of $NaClO_4$.

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I

ELECTROPHORESIS OF THE NUCLEIC ACIDS

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Electrophoresis of the Nucleic Acids*,†

BALDOMERO M. OLIVERA, PETER BAINE, and NORMAN DAVIDSON, *Department of Chemistry, California Institute of Technology, Pasadena, California*

Synopsis

A zone electrophoresis apparatus using ultraviolet optics has been constructed to study nucleic acids at concentration less than 0.004%. Native DNA has a mobility about 15% higher than denatured DNA over a range of conditions of pH and ionic strength. DNA's from different sources have closely similar mobilities. A study of a molecular weight series of DNA indicates that the mobility is constant in the molecular weight range of 2.5×10^5 to 1.3×10^8. DNA mobilities change in the expected way with pH but the fractional change in mobility is less than the change in charge calculated by titration curves. A small decrease in mobility accompanies an increase in ionic strength. RNA's from various sources have mobilities slightly lower than denatured DNA except for s-RNA which travels slightly faster.

INTRODUCTION

Previous studies of the electrophoretic properties of nucleic acids in homogeneous solution (as contrasted with electrophoresis on a supporting medium) have been carried out in a conventional Tiselius apparatus using schlieren optics.[2–7] This normally requires concentrations greater than about 0.1% (corresponding to A_{260} values greater than 40). As regards the properties of sedimentation and viscosity, the hydrodynamic behavior of DNA at these concentrations is very different from that at infinite dilution, and it is at least conceivable that the electrophoretic behavior would also be different.

The electrophoretic properties of nucleic acids are of fundamental interest for an understanding of the structures of these polyelectrolytes in solution and conceivably of practical interest for various separations. We have therefore constructed a zone electrophoresis apparatus with U.V. scanning optics for concentration measurement and using a sucrose density gradient for convective stability. The optical system works well for nucleic acid concentrations corresponding to A_{260} values of 0.2–1.0 (0.001–0.004% weight concentration).

* Contribution No. 3108.

† A preliminary report of this work has appeared elsewhere.[1]

EXPERIMENTAL

The Electrophoresis Apparatus

The basic features are shown in Figures 1 and 2. The important part of the apparatus consists of three concentric quartz tubes about 25 cm. long. The innermost, with an internal diameter of 1.0 cm., is the electrophoresis column; the second is a cooling jacket through which water at 0°C. is usually pumped, and the outer is a vacuum jacket for thermal insulation and to prevent condensation of water vapor.

Platinum electrodes are used in the electrode compartments. Mixing of the electrolyte from these compartments with the supporting electrolyte

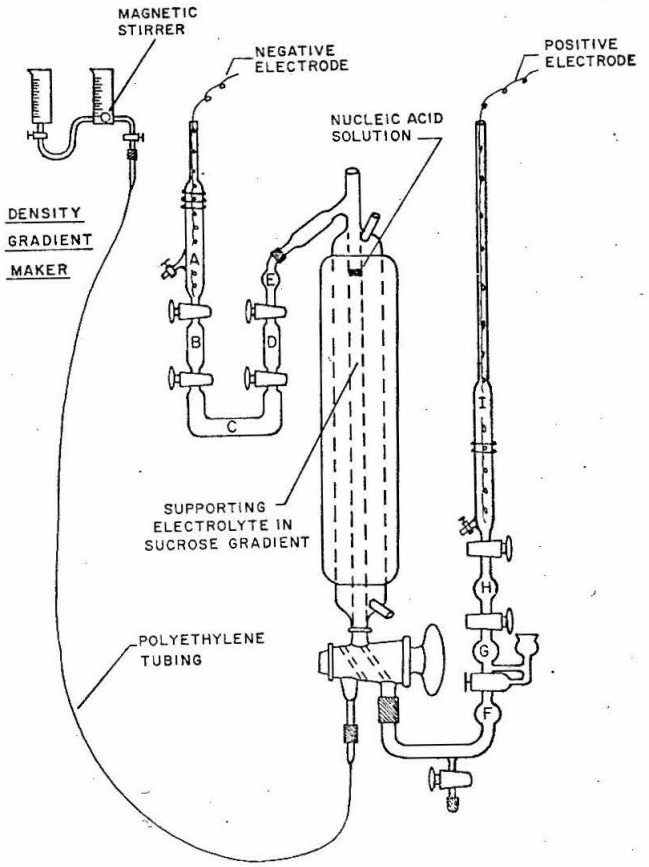

Fig. 1. Electrophoresis apparatus. For details of the central tube and optical system, see Figure 2. When the composition in the central tube was 0.01*M* NaCl, 0.001*M* Tris, pH 7.5, 0–20% sucrose, the composition of the other compartments was: A and I, 0.1*M* NaCl, 0.2*M* Tris; B, D, and H, 0.2*M* NaCl, 0.02*M* Tris, 5% sucrose; C and G, 0.2*M* NaCl, 0.02*M* Tris, 15% sucrose, F, 0.01*M* NaCl, 0.001*M* Tris, 25% sucrose.

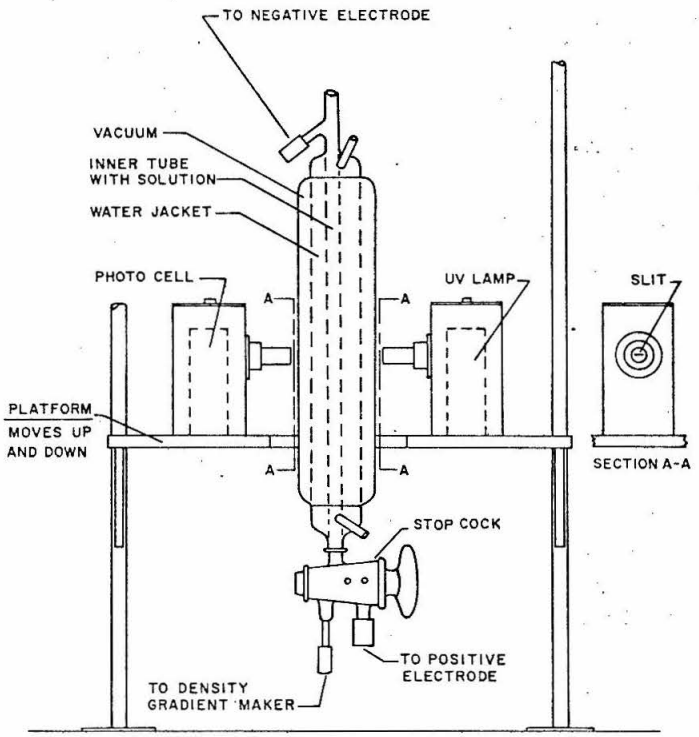

Fig. 2. Further details of electrophoresis apparatus.

in the electrophoresis column is prevented by the intermediate compartments that contain buffer, supporting electrolyte, and suitable sucrose concentrations. Typically, the electrolyte and connecting compartments have a salt and buffer concentration ten times higher than that in the electrophoresis column to decrease the voltage drop.

An approximately linear density gradient between 5 and 20% sucrose (with supporting electrolyte at a uniform concentration) is run in through the bottom stopcock, and extends over a distance of 25 cm. A narrow nucleic acid zone in 3% sucrose plus electrolyte is layered on top and then covered with electrolyte (0% sucrose).

The U.V. optical system consists of a low pressure 15 W mercury resonance lamp (GE FG445-X) and a U.V. sensitive phototube (RCA 935). The photocurrent of *ca.* 10^{-8} amp. passes through a 5×10^6 ohm resistor; the signal is amplified by a Keithley Model 151 microvoltmeter and used to drive a recorder. The lamp and photocell compartments have aligned horizontal slits, 1×5 mm.; this is then the size of the sheet of light which passes through the electrophoresis tube. The lamp and photocell are on a moving platform, and the complete tube can be scanned in 8 min. A scanning speed of $^1/_6$ of the above is also available.

About 80% of the light from the lamp is in the ultraviolet, mostly at 2537 A. and is strongly absorbed by nucleic acids. For ideal operation, the nucleic acid zone should have an absorbance of 0.2 to 1.0. Below this it is difficult to distinguish true absorption from background noise, mainly due to irregularities in the quartz tubes and to air bubbles. Quantitative measurements of concentration at high absorbance are inaccurate because of the nonabsorbed visible light. Thus, the photometer is very primitive, but adequate for the initial investigations reported here.

The supporting electrolyte used in most of the work is 0.01M NaCl, 0.001M tris buffer, pH 7.5, where the tris is about 80% in the ionized form (the pH measured at room temperature). The average resistance in the central tube (area $\approx$ 0.80 cm.^{2}) is 2850 ohms cm.$^{-1}$. With this supporting electrolyte, the applied voltage across the entire apparatus was usually 600 v., the voltage drop across the central column was 360 v., the current was 4 ma., and the maximum power dissipation was 0.08 w. cm.$^{-1}$. There was noticeable convective disturbance of the boundary for a power dissipation of 0.3 w. cm.$^{-1}$.

The charge passed through the unit was measured with an Electro-Methods, Ltd. Low Inertia D.C. Motor and Counter Unit (type 913L) shunted by a 700 ohm precision resistor.[8] This is a coulometer with a calibration of 5.28 counts per coulomb.

The column is normally scanned at intervals such that the zone has moved 1 cm. (about 10 min. in the standard medium).

Calculation of Mobility

The mobility u, is the velocity per unit field:

$$u = \frac{v}{E} = \frac{v}{IR} = \frac{vt}{IRt} = \frac{d}{QR} \tag{1}$$

where v is the velocity of the macroion, E is the electric field, I is the current, R is resistance per unit length, t is time, d is the distance the macroion migrates, and Q is the number of coulombs that have passed through the solution. The mobility is then calculated from the slope of a plot of d vs. Q, and an independent measure of R.

Electrophoresis occurs in a sucrose gradient with a constant concentration of supporting electrolyte but a varying viscosity. If the effect of the sucrose were only the classical hydrodynamic one of changing the viscosity and thus the friction coefficients of the ions,

$$u_0 = u\eta/\eta_0, \; R_0 = R\eta_0/\eta \tag{2}$$

where subscript zero refers to properties in water plus supporting electrolyte, with no sucrose. This assumption—that the sucrose or other neutral component used to provide the density gradient has the same fractional effect on the mobility of all ions—predicts that d/Q should be constant as the nucleic acid migrates down the tube.

This prediction and eq. (2) do not hold accurately. The measured relative resistivities of 0.01M NaCl, 0.001M tris, pH 7.5, in varying percentages of sucrose are compared to the relative viscosities below.

% Sucrose	$[\eta/\eta_0]_{T=0°}$	$[\eta/\eta_0]_{T=20°}$	$[R/R_0]_{T=0°}$
0	1	1	1
5	—	1.14	1.11
20	2.13	1.94	1.67

Thus, the effects of sucrose on the small ions that constitute the supporting electrolyte are more complex than predicted by eq. (2). Furthermore, it is observed that the d/Q slope for the DNA samples studied here typically decreases by about 12% between 5% sucrose and 20% sucrose. The mobilities reported here are calculated by taking the limiting slope of d/Q in 5% sucrose from experiment and increasing it by 3% to extrapolate to 0% sucrose. The value of R_0 is then used in eq. (1).

Experimentally, it has been found that the ratio d/Q is the same at 1–2°C. (the usual steady-state temperature in the electrophoresis tube) and at 8°C.; thus we use R_0 at 0°C. and report mobilities at 0°C.

MATERIALS

The T-2 DNA was a gift from Dr. D. Crothers. It was prepared by the phenol extraction method.[9] A molecular weight series was prepared by shearing in a high-speed mixer.[10] Molecular weights were estimated by him from viscosities[11] and the relation[12] $[\eta] = 1.45 \times 10^{-6} M^{1.12}$. T-4 phage was a gift from Professor W. Dreyer and the DNA was prepared by phenol extraction.[9] *E. coli* DNA was a gift from Dr. U. S. Nandi, pea DNA from Dr. R. C. Huang, and the *M. lysodeikticus* DNA was from Dr. J. Vinograd. The 5′-deoxyadenylic acid was from Calbiochem, Lot 2570. Calf thymus DNA was dissolved directly from Worthington stock 599. The polyuridylic acid from Miles Laboratories was either dissolved directly or phenol-extracted and dialyzed against supporting electrolyte. The MS-2 and TMV RNA were gifts of J. Strauss, Dr. E. Carusi, and Professor R. L. Sinsheimer. All ribosomal RNA's and the Hela 4s RNA were gifts from Dr. P. C. Huang and Professor G. Attardi. The yeast s-RNA was a gift from Dr. G. L. Cantoni and had been purified on a DEAE column. The liver s-RNA was from the Nutritional Biochemical Corporation, Control No. 3066.

All chemicals used were reagent grade. Water for making solutions was redistilled from commercial distilled water.

All DNA's described as denatured were either heated slowly in a heating cell for the Cary spectrophotometer[13] to about 10° above T_m, or immersed in boiling water for a few minutes. Either treatment was followed by quenching in ice water.

Conductivities

Conductivities of solutions were measured at 1000 c.p.s. with an Industrial Instruments Conductivity Bridge, Model RC 16B2, using a cell with smooth platinum electrodes and a cell constant of 0.926 cm.$^{-1}$. The measurements were reproducible to 1%.

Sonication

A T-4 DNA solution in a nitrocellulose test tube was sonicated in a 9000 c.p.s. Raytheon Sonicator Model No. S102A at full power for 5 min. Before sonication, argon was bubbled through the DNA solution to remove dissolved oxygen.

Titration of DNA

DNA at a concentration of 1 mg. ml.$^{-1}$ in a medium of 0.011M NaCl (to give an ionic strength similar to that used in electrophoresis) was titrated with 0.01M HCl. The titration was done at 4°C. with the Beckman Model 76 pH meter using a special low impedance glass electrode for low-temperature measurements kindly provided by the Beckman Company.

RESULTS

DNA; Effect of Denaturation and of Molecular Weight

Samples of varying molecular weight, both native and denatured, prepared from high molecular weight T-2 DNA by high-speed mixing and from T-4 DNA by sonication were studied. Molecular weights of the fragmented DNA's were estimated from the relations[12]

$$s = 0.063M^{0.37} \tag{3}$$

$$[\eta] = 1.45 \times 10^{-6}M^{1.12} \tag{4}$$

The results are presented in Table I. One of the main results is that over a molecular weight range from 2×10^5 to 1.3×10^8, there is less than a 2% variation in electrophoretic mobility, although the sedimentation coefficient changes by a factor of six. [Under these circumstances, there is no point in worrying about the accuracy of eqs. (3) and (4), or whether the T-2 and T-4 DNA's as obtained by phenol extraction were 100% intact or consisted partly of half-molecules as suggested by the observed s and $[\eta]$ values.]

As shown in Table I, the electrophoretic mobility of the monomer unit of DNA, 5′-deoxyadenylic acid, at a pH of about 5 where it has a charge of −1, is 50% that of high molecular weight single-strand DNA's. Indeed, even with a charge of −2 (pH 7.5), the monomer is 16% slower than the polymer which has a charge of −1 per monomer unit. Thus, the independence of mobility on degree of polymerization does not extend all the way down to monomers. This general characteristic of electrophoresis of

TABLE I
Electrophoretic Mobilities of DNA's

DNA	Mol. wt., $\times 10^{-6}$	$[\eta]$, dl. g.$^{-1}$	$S_{w,20}$, $\times 10^{13}$	u^a, cm.^{2} v.$^{-1}$ sec.$^{-1}$, $\times 10^4$ (native)	(denatured)
T-2[b]	130[c]		42[h]	2.18	1.85
T-2[e]	2.5	21.7[d]		2.16	
T-2[e]	0.45	3.2[d]		2.13	1.87
T-2[e]	0.26	1.7[d]	7.4[h]	2.13	
T-4	130[c]	240[f]	37, 69[g]	2.18	1.86
T-4	1.2		11.1[h]	2.16	1.86
E. coli	1.2		11	2.16	1.88
Calf thymus	11	90[f]		2.17	1.90
Pea				2.17	
d-5′ AMP^{-2}[i]					1.58
d-5′ AMP^{-1}[j]					~0.95

[a] Supporting electrolyte: 0.01*M* NaCl, 0.001*M* Tris, pH 7.5.
[b] From Dr. D. Crothers.
[c] Assumed.
[d] Measured by Crothers.
[e] Sheared by high-speed mixing by Crothers, molecular weights from eq. (4).
[f] Measured by H. Ohlenbusch in this laboratory with a rotating cylinder viscometer.
[g] Measured in this laboratory on two different samples at 36,000 r.p.m. and $A = 0.10$ (37), and at 30,000 r.p.m. and $A = 0.5$ (69).
[h] Measured in this laboratory.
[i] at pH 7.5.
[j] at pH 5. Calculated from runs at pH 4.5 and 7.5 and the titration curve.

mono- and polynucleotides was recognized some time ago by Markham and Smith, on the basis of paper electrophoresis experiments.[14]

The third important result is that all the DNA's studied have the same mobility, irrespective of base composition or source (animal, plant, bacterial, or viral).

Last, but not least, the mobility of denatured DNA is 15% lower than that of native DNA. Although not very large, this difference may be practically useful. A mixture of native and denatured DNA can be separated quite readily; a typical run on such a mixture is shown in Figure 3.

An electrophoretic anomaly should be mentioned here. Native T-4 DNA of high molecular weight gives in certain runs a minor artifact peak that travels faster than the main native DNA peak. This has happened only in the low salt medium used for most of the experiments (0.01*M* NaCl, 0.001*M* Tris, pH 7.5); in 0.1*M* salt it has never been observed. It also seems to be related to high molecular weight; when a T-4 DNA preparation that gives this anomalous peak is sonicated, the sonicate does not, under identical conditions give two peaks. (Calf thymus, pea, and *E. coli* DNA have never shown this behavior.) This fast peak (10–15% faster than the true native DNA peak) often seems to travel like a real electrophoretically homogeneous component; that is, a plot of the distance traveled against the number of coulombs approximates a straight line.

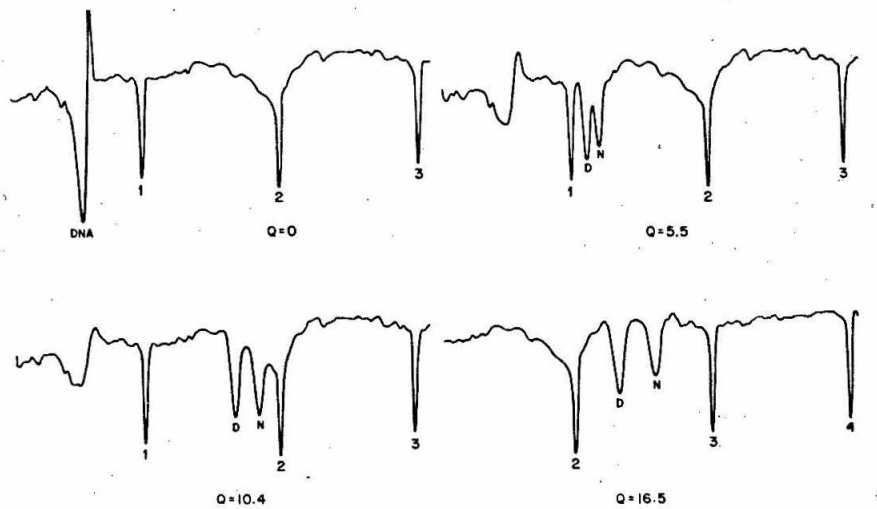

Fig. 3. Results of an electrophoresis run on a mixture of native and denatured T-4 DNA in 0.01*M* NaCl, 0.001*M* Tris. Distance down the electrophoresis tube is the horizontal coordinate on each recorder display. Decreasing photocurrent is a vertical deflection downward. There are calibration markers every 3.0 cm. along the tube. Total charge passed is given as Q (coulombs). (The current was about 4 ma. or 0.24 coulombs min.$^{-1}$). In the upper left record, the DNA band is to the left of marker 1. A schlieren peak due to the sharp refractive index gradient is also present in this tracing. At the end of the run the DNA has moved to the extreme right and resolved into its two components, denoted by D and N. (To aid in roughly estimating absorbances of the peaks, it may be noted that the heights of the photo signals due to the markers correspond to an absorbance of about 0.3.)

Two facts show it is an artifact: As it runs down the column, the peak seems to "grow" and if the electrodes are reversed, the peak does not travel up the column with the mobility that it came down with, but rather with the mobility of normal native DNA. In other words, passing an equal number of coulombs after reversing the field does not result in the anomalous peak going back to its position at the beginning of the experiment.

We believe this anomaly is probably due to some sort of electro-osmotic effect resulting in migration of annular zones close to the wall, but we have no detailed explanation. For practical purposes it can be avoided by working at higher salt with the extremely high molecular weight DNA's.

DNA, Variation with pH

Decreasing the pH of the medium results in protonation of DNA, mainly of cytosine and adenine bases.[15] Denatured DNA protonates much more readily than native DNA and cytosine somewhat more readily than adenine. Thus one might expect that, at acid pH's, the electrophoretic separation between native and denatured DNA would be improved and some fractionation on the basis of base composition would be achieved.

As shown in Table II, neither of these hopeful predictions is confirmed by experiment. The mobility of both native and denatured DNA decrease at acid pH; however, the fractional mobility change is less than proportional to the change in formal or titration charge due to protonation and is about the same for native and denatured DNA even though the latter binds more protons. For example, at pH 4.10, the number of protons bound by

TABLE II
Comparison of Titration Charge and Electrophoretic Mobility of Nucleic Acids

Sample	pH	Mobility	Ratio of mobility to that at pH 7.5	Titration[a] charge
C.T.,[b] native	7.50	2.17	1.00	−1.00
C.T., native	4.10	1.96	0.90	−0.75
C. T., denat.	7.50	1.90	1.00	−1.00
C. T., denat.	4.10	1.71	0.90	−0.52
T-4, native	7.50	2.18	1.00	−1.00
T-4, native	4.57	2.04	0.94	−0.89
T-4, native	4.10	1.95	0.90	−0.82
T-4, denat.	4.57	1.73	0.93	
T-4, denat.	4.10	1.68	0.90	
M. lyso, native	4.10	1.95		
M. lyso, denat.	4.10	1.65		
poly-U	7.50	1.86		−1.00
poly-U	4.10	1.82		−1.00

[a] Determined by titration at 4°C. as described under methods; charges calculated assuming −1 per phosphate at pH 7.5 and adding the number of protons bound.
[b] Calf thymus DNA.

native and denatured calf thymus DNA are 0.18 and 0.48 per monomer unit, respectively, but in both cases, the mobility is 90% of that at pH 7. Native and denatured samples of the GC-rich DNA, *M. lysodeikticus*, have the same mobilities in acid, as do the other DNA's. It is comforting that poly-U, which does not bind protons significantly, shows only a slight change in mobility.

Qualitatively similar results, in which the titration charge changed more than did the mobility, and in which the per cent mobility change was about the same for native and denatured DNA in spite of significant differences in the titration charge, were observed in the alkaline region.

Effect of Ionic Strength

Our results on the effect of varying the ionic strength are displayed in Table III. There is a small increase in electrophoretic mobility with decreasing ionic strength.

With these data in hand, it is possible to compare the present results with those obtained earlier in a Tiselius apparatus, usually at nucleic acid

TABLE III
Variation of Mobility with Ionic Strength

Medium	DNA	u, cm.^{2} v.$^{-1}$ sec.$^{-1}$, $\times 10^4$ native	denatured
0.1M NaCl,	Calf thymus	1.51	1.33
0.01M Tris	T-4	1.50	1.25
0.01M NaCl,	Calf thymus	2.17	1.90
0.001M Tris	T-4	2.18	1.86
0.004M NaCl,	Calf thymus	2.34	2.06
0.0004M Tris			

concentrations of 0.1%. A detailed listing of all the data recorded in the literature is not desirable, but generally speaking, the earlier results give about 10–15% higher mobilities in 0.1M salt and 30% higher in 0.01M salt.[2,4,6] (The values in ref. 5 are unusually high.) The important conclusion is that the mobility is only slightly dependent on nucleic acid concentration, but that the discrepancies are more marked at low salt concentration, where the DNA concentration (in equivalents per liter) is comparable to the supporting electrolyte concentration.*

Dr. J. Vasilevskishas measured the mobility of DNA in 0.1M $NaClO_4$ in a D_2O density gradient at 8°C. at the same DNA concentration as used here, but in a different zone electrophoresis apparatus. His results, corrected to water at 0°C., agree with those in 0.1M NaCl reported in Table III.[16]

Studies with RNA

These results are reported in Table IV. The transfer RNA's, which have more helical content than other RNA's, have a higher mobility. The RNA's without much secondary structure have a lower mobility (1.7–1.8 $\times 10^{-4}$) than does denatured DNA (1.88); transfer RNA has a lower mobility (1.95) than does native DNA. The difference between DNA and RNA has been noted before.[4]

Considerable boundary spreading was observed in some of the RNA samples. For example, a commercial calf thymus RNA preparation (NBC 9622) showed a mobility range from 1.50 to 2.00 $\times 10^{-4}$. That this spreading was due to true electrophoretic inhomogeneity was confirmed by sharpening of the peak upon reversal of the current. The Hela 4s RNA was also heterogeneous, even though it was taken directly from a zone centrifuge

* Note added in proof. The recent results of Ross and Scruggs (manuscript in preparation) with a Tiselius apparatus at fairly low DNA concentrations ($\sim 1.6 \times 10^{-3}M$) also give higher mobilities than reported here: 0.10M NaCl, 1.85 $\times 10^{-4}$; 0.05M NaCl, 1.99 $\times 10^{-4}$; compared to our values of 1.50 $\times 10^{-4}$ and 1.72 $\times 10^{-4}$ (extrapolated). Costantino, Liquoroi, and Vitagliano [*Biopolymers*, **2**, 1 (1964)] have measured electrophoretic mobilities in a Tiselius apparatus. They report mobilities about 15% greater than those reported here. They also observe that denatured DNA has a mobility about 12% less than that of native DNA.

TABLE IV
Mobilities of Different RNA's[a]

RNA	Mobility cm.²/v.-sec. × 10^4
Viral	
MS2	1.74 (1.64–1.81)[b]
TMV	1.80
Ribosomal	
Hela 18S	1.74
Hela 28S	1.69 (1.62–1.72)
Rat liver	1.77 (1.68–1.84)
Transfer	
Yeast	1.99 (1.91–2.07)
Hela 4S	1.92 (1.76–2.03)
Liver	1.97 (1.47–2.07)
	1.59[c]

[a] All in a medium of 0.01*M* NaCl, 0.001*M* Tris, pH 7.5.
[b] Range of mobilities for which concentration is greater than half of zone maximum.
[c] This preparation resolved into two peaks, this slower peak is probably made up of degradation products.

preparation. However, the viral RNA's and the yeast 4s RNA prepared by Cantoni gave reasonably sharp zones. Data indicating these features are included in the table.

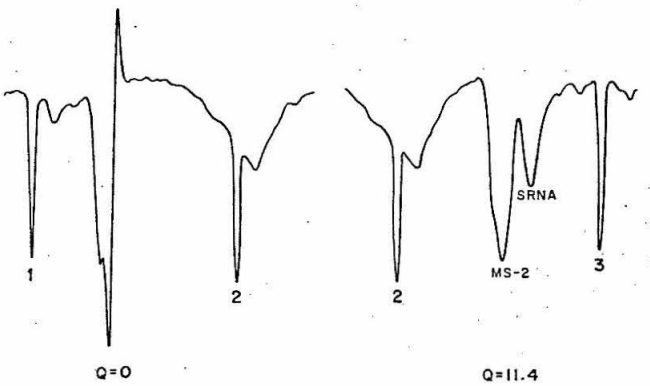

Fig. 4. Separation of yeast s-RNA and MS2 RNA. At the beginning (left side tracing) of the run the RNA was between markers 1 and 2 and a schlieren peak is noticeable. After moving 3 cm., the peaks are well resolved.

That the difference in mobility between yeast s-RNA and MS2-RNA is significant, and not experimental error, is confirmed by running a mixture and observing a separation into two zones. Such an experiment is displayed in Figure 4.

DISCUSSION

The theory of the electrophoretic migration of random coil polyelectrolytes has been discussed by several authors.[17] The Hermans theory is one of the simplest to discuss.[18] We regard native DNA as a random coil polyelectrolyte with a segment length of the order of 1000 A. (300 base pairs).[19] The effective radius is then about 1 μ and 0.3 μ for molecular weights of 10^8 and 10^7, respectively. The Hermans theory regards the polyelectrolyte as a porous gel bead immersed in a supporting electrolyte. The important quantities may be taken as: z, the charge density per segment; f, the friction coefficient per segment; R, the radius of the bead; λ, a hydrodynamic shielding parameter; and κ, the inverse Debye radius. Since $1/\kappa = 30$ A. in 0.01M NaCl, the parameter $\kappa R >> 1$ under practical conditions. The dimensionless parameter λR measures whether the bead is free draining ($\lambda R << 1$) or impermeable ($\lambda R >> 1$). For the large DNA's, it appears that $\lambda R > 1$; however, $\kappa R > \lambda R$. Under these circumstances, the formula for the mobility is very simple.

$$u = \frac{z}{f} \tag{5}$$

That is, for electrophoresis (but *not* for sedimentation), the polymer is free-draining and the mobility of the molecule is the same as the mobility of a segment.

This model correctly predicts that the mobility is independent of molecular weight, even when the sedimentation coefficient does vary with molecular weight. However, in its most naive and straightforward application, eq. (5) does not explain the fact that the electrophoretic mobility does not change proportionally with the titration charge as the pH is changed. In the Hermans theory, the polyelectrolyte gel bead is treated as a uniform smeared out charge distribution. To understand qualitatively the effect of addition of protons to the polymer, we must add the idea of site binding. In this picture, there is a certain fraction of counter ions, sodium ions in this case, tightly bound to the negative phosphate groups of the polymer chain. The charge z in eq. (5) is not -1 per phosphate but a smaller number due to the tight sodium ion binding. When protons are added to the bases, some of the sodium ions are released from the phosphates. Thus the change in charge is less than the number of protons bound. This explanation of the observations is intrinsically plausible, but it does not seem practicable to use it for quantitative calculations.

From a practical point of view it is disappointing that electrophoresis cannot be used to fractionate or analyze DNA's on the basis of size or titratability by acid or base. However, the separation between native and denatured DNA is useful. Furthermore, we have recently observed that the nucleohistones differ among themselves and from DNA in electrophoretic mobility. Thus to some extent binding of appropriate counter-

ions can be used to influence the mobility of nucleic acids, and there is a class of separations and analyses for which electrophoresis is useful.

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FURTHER DISCUSSION ON THE ELECTROPHORESIS OF THE NUCLEIC ACIDS

In the year since the preceding paper was published, some results have come to light which suggest explanations for the various phenomena that were described. One of the most puzzling results was the difference in mobility between native and denatured DNA. In view of the insensitivity of electrophoresis to large changes in molecular weight or fairly large changes in formal charge, it was surprising that such a large change in mobility should come about because of denaturation.

The results with crab d-AT that have helped to explain this phenomenon are shown on table V. A thorough discussion of the structure of renatured crab d-AT, with all the evidence for such a structure has recently been published (1). The most important thing to bear in mind for this discussion is that renatured crab d-AT which is 93% native by the criterion of absorbance measurements has a mobility that is closer to denatured than to native DNA. As we hope to show, this result implies that the change in mobility for denatured DNA is not due to any intrinsic chemical difference between native and denatured DNA, but rather is a consequence of the hydrodynamics of random coils.

This becomes clearer if we compare native DNA or d-AT with renatured crab d-AT. As was mentioned in the discussion in the preceding paper, we may regard DNA as a random coil polyelectrolyte with a segment length of 1000 A° (700 A seems to be a more accurate number) (2). However, renatured crab d-AT has guanine or cytosine

residues out of register on the average once every 15 base pairs. This out-of-register base would provide a "joint" in the helical structure thru which there is considerable rotation possible. We may no longer look at renatured d-AT as a random coil with 200 base pair segments--hydrodynamically, it may be considered as a random coil with segments approximately 30-60 base pairs long.

A single kink from an out-of-register base would not constitute a universal joint, but a combination of 2-4 probably approximates it. Since the Hermans theory predicts that the electrophoretic mobility of any random coil is equal only to the mobility of a single segment, what we are comparing between native d-AT and renatured d-AT is really the mobility of a 200 base pair unit versus the mobility of a 30-60 base pair unit. It is entirely conceivable that the smaller unit will have a mobility 12% slower than the bigger segment.

In the case of denatured DNA, because the Watson-Crick structure has collapsed, the intrinsic nature of the segment we are interested in has changed. For a given molecular weight, the random coil of denatured DNA is somewhat more compact than that of native DNA and the radius of gyration smaller.(3). We know that the length of, and number of bases in the segment of interest can be deduced from the radius of gyration of the random coil. As is discussed in detail in proposition 4 of this thesis, the number of bases in a hydrodynamic segment can be calculated from recent sedimentation data.(4). It is proportional to the inverse square of the sedimentation coefficient and the average distance between the bases in denatured DNA. From these calculations,

we find that denatured DNA has a segment which is of the order of 40-50 bases in 0.01 F NaCl. The denatured DNA segment so computed is of course not a Watson-Crick helix but a single chain.

The Hermans theory explains all the behaviour we have observed if we combine it with the segmented random coil picture. All native DNA's have the same mobility because no matter what the molecular weight or base composition, electrophoresis is sensitive only to the nature of the segments of the coil, and all native DNA's have segments 200 base pairs long. Electrophoresis is therefore also insensitive to molecular weight changes in denatured DNA. However, when we compare the mobility of native DNA with renatured crab d-AT and denatured DNA, we are really comparing the mobilities of a 400 base (200 base pair) Watson-Crick structure segment, a 60-120 base (30-60 base pair) Watson-Crick segment, and a 30-50 base single chain segment. It is conceivable that these segments should have the mobilities found. For any random coil polymer, therefore, the electrophoretic mobility does not change with a gross change in length but only with alterations in the nature of the hydrodynamic segments.

It may also be enlightening to consider the pH behaviour of DNA in somewhat more detail than we did in the paper. The explanation given there for the pH behaviour did not give a complete picture.

We treat DNA, as mentioned previously, as having a certain fraction of Na ions bound to each phosphate. Therefore, the charge that the mobility of DNA is dependent upon is not one negative charge per phosphate but less than one because of the bound Na ions. If we

protonate the bases, we are introducing a positive charge fairly close to the phosphates so that, say, the nearest phosphate is now ion-paired and has no need to attract Na ions. The fractional Na ion that it was holding is thus released. If this were the only effect, then the mobility should still be proportional to the formal charge, since the mobility of the DNA we originally measured is that of DNA with Na counterions.

There is however a secondary effect: protonating a base not only releases the Na ion held by the phosphate nearest it but also affects the binding of Na ions by neighboring phosphates. The fractional Na ions that these phosphates hold is diminished by the neutralization of their neighbor. This is a well known polyelectrolyte effect on counter ion binding. If for example we compare the mobilities of 5'-deoxy-adenylic acid that has a double negative charge on its phosphate with the mobility of the same substance with a single negative charge (see table I of the paper), we find that the doubly charged molecule does not have double the mobility. This is because each charge attracts a higher fraction of Na ions than a single isolated negative charge: the two neighboring charges help each other bind Na ions. If we lower the pH so that we have protonated one of the negative charges, we not only release the fractional Na ion that was bound by the charge that we protonated, but we also lower the fraction of a Na ion that the un-neutralized charge binds. Our net charge has thus fallen by considerably less than half, and so has the mobility. The same thing is true for DNA. Since we are protonating bases fairly randomly along the DNA

chain, we are not only affecting the phosphates nearest the bases, causing the fraction of a Na ion that each holds to be released, but we are also lowering the fraction of a Na ion that the neighboring phosphate groups attract. Thus the mobility does not change as the formal charge, because it is affected by this change in Na ion binding.

With this simple picture of why the DNA mobility does not change proportionally to the formal charge on protonation, we may make the prediction that if there were some way of neutralizing phosphate groups that were adjacent to each other and leaving long stretches of phosphate groups completely uncomplexed along the DNA helix, we would get the mobility proportional to the formal charge. This is because under these conditions, only the charges actually neutralized release their Na ions, the remaining charged phosphates remaining in an unchanged electrostatic environment and therefore keeping the same number of Na ions bound per phosphate.

Such conditions are approximated when we bind positively charged proteins to DNA. As we shall see in the next section, under those conditions, our predictions are borne out.

Table V

ELECTROPHORESIS EXPERIMENTS ON d-AT

Medium: 0.01 F NaCl, 0.001 F tris, pH 7.5

Material*	Mobilities x 10^4 cm^2/volt-sec.
(1) Crab D-AT, isolated by Hg-Cs_2SO_4 centrifugation	2.16
(2) Crab D-AT, isolated by heating and cooling whole crab DNA, then column chromatography	1.98
(3) Preparation (1), heated to 95° and quickly cooled	1.92
(4) Whole crab DNA, contaminated with some RNA	2.18, 1.79
(5) Preparation (4), heated to 56° in DSC	2.18, 1.92, 1.76
(6) Synthetic d-AT	2.21
(7) Synthetic d-AT, heated to 95° and quickly cooled	2.18

****

Thus our mobility assignments are:

"Native" crab d-AT	2.16-2.18
"Renatured" crab d-AT	1.92-1.98
Synthetic d-AT, "native" or "renatured"	2.18-2.21

*The preparation of all of these materials is described in N. Davidson et al, Proc. Nat. Acad. Sci. U.S.A., 53, 111 (1965).

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II

THE CYTOCHROME C/DNA COMPLEX

THE CYTOCHROME C/DNA COMPLEX

INTRODUCTION

The DNA of all higher organisms is found associated with basic proteins, mainly histones. Such complexes are obviously of great interest. Any study of these complexes is complicated by the fact that the histones are a heterogeneous assortment of proteins which are rather badly behaved in solution. It may be useful therefore to thoroughly study the interaction of DNA with a single homogeneous well characterized basic protein and compare this with what is known about histone -- DNA interactions.

We have chosen to study the oxidized cytochrome c-DNA complex for the following reasons:

1. The primary structure of cytochrome c is known (1).
2. The absorption spectrum of cytochrome c makes it easy to assay.
3. The protein is readily obtainable in an extremely pure form.
4. Such complexes may be pertinent to the Kleinschmidt technique (2) in electron microscopy.

MATERIALS:

Horse heart cytochrome c was a gift of Prof. R. Dickerson and was purified by E. Margoliash. The spectrum corresponds very well to the published data for oxidized horse heart cytochrome c (3). To check for complete oxidation, some of the stock solution was dialyzed against 0.001F $K_3Fe(CN)_6$; this caused no change in spectrum, indicating that all the cytochrome was in the oxidized form. The presence of

dimers and higher oligomers was checked for by taking the ratio of the absorbance of reduced cytochrome (reduced by adding a few grains of solid $Na_2S_2O_4$) at 5500 A° to that of oxidized cytochrome c at 2800 A°; a ratio of 1.19-1.26 is considered acceptable (4). The ratio for the stock tested was 1.24.

The DNA used in all cases was commercial Worthington calf thymus DNA. All chemicals unless otherwise specified are reagent grade.

The dialysis tubing used was treated (5) in the following way: it was first boiled in 0.02F EDTA, pH 8.0, then in 1F NaCl, next in distilled H_2O and lastly in the medium it was to be dialyzed in. Between each boiling, the interior of the tubing was washed thoroughly with water. This treatment was found to get rid of all impurities that absorbed light above 2100 A°. Visking tubing #8 was used in all cases because it was found to be strongest. Dialysis was done with 100x the volume of the desired medium and at least three changes. The dialysis tubes were knotted in such a way that there was always a bubble inside; the container was then stoppered so there was a bubble in the outside solution also. The stoppered container was clamped on to a motor that slowly rotated it; in this way, the bubbles inside and outside the dialysis bag gave continuous mixing.

THE FORMATION OF THE COMPLEX: ESTIMATION OF COMPOSITION

The complex was formed by mixing together cytochrome c and DNA in a medium of high ionic strength (1F NaCl or higher in which the cytochrome does not bind the DNA) and dialyzing into 2.5×10^{-4}F EDTA,

pH 8.0 (the buffer is made by titrating the disodium EDTA to pH 8.0 with NaOH). At this very low ionic strength, the cytochrome is completely bound to the DNA. The cytochrome to DNA mass ratio (henceforth abbreviated as C/D) was estimated using the published spectral data of Margoliash and Frowirt (6). The cytochrome is estimated by its strong absorbance at 4100 A°. A correction is necessary, however, for the absorbance of the cytochrome at 2600 A°. The resulting formula for the cytochrome to DNA mass ratio (assuming that 50 micrg./ml. of calf thymus DNA has an absorbance at 2600 A° of 1) is:

$$\frac{C}{D} = \frac{2.44\ A_{4100\ A^\circ}}{A_{2600\ A^\circ} - \frac{A_{4100\ A^\circ}}{5.04.}}$$

Mass ratios were calculated before and after complex formation, in all cases.

It was found that for low coverages ($C/D < 2$) the estimate by this formula before complex formation agreed to within 3% of the estimate after the complex was formed. At high coverages, the estimate after complex formation came out consistently lower than before. However, if high salt (2F NaCl) is added to the complex, causing it to dissociate, then the calculated C/D agrees very well with the estimate before complex formation. It therefore appears that at high C/D's, characteristic spectral changes take place. At low coverages however, no spectral changes can be detected on binding to DNA. The visible absorption spectrum (from 5000 to 3500 A°) of the cytochrome/DNA in 2.5×10^{-4}F EDTA, pH 8.0 is identical to that of free cytochrome in

this medium. Spectral changes take place when cytochrome is in 8 F urea or 5 F guanidinium, the most prominent feature being a hypochromicity of the shoulder at 3600 A° with respect to the Soret peak at 4100 A°. This would indicate that the cytochrome when complexed to DNA is, by this crude criterion, substantially native or at least not denatured in the same way as it is in urea or guanidinium. The visible spectrum of cytochrome at all ionic strenghs studied was unchanged, and only in the two denaturing solvents was there any measurable effect.

We will give a brief description of how these complexes behave as they are formed by dialysis into low salt. The DNA concentration used in forming the complex varied from 0.1- 1.0 mg./ml.

In general, as long as the cytochrome to DNA ratio is low (C/D less than 2), there are no changes that can be observed as the DNA and cytochrome are dialyzed into low salt. There is occasionally a slight opalescence in the initial dialysis, but this always disappears and the final solutions are clear.

If the cytochrome to DNA ratio is greater than 2.5, when the mixture of the protein and DNA are first dialyzed into low salt, a very cloudy solution or precipitate results. For a C/D of between 3 and 4 a precipitate appears in all cases and most of the cytochrome is in the precipitate, as the supernatant is colorless. On continued dialysis however, the cytochrome c is observed to go back into solution. This takes a long time and it is important to have thorough mixing within the tube; in dialysis bags with no bubble or very small

bubbles, the precipitate always takes longer to dissolve than in bags with large bubbles. Eventually all the precipitate redissolves and a clear solution remains. Complexes have been made with cytochrome/DNA ratios of up to 5.40. The higher the C/D, the harder to handle--complexes with C/D's above 3.5 tend to precipitate out on to the surface of glass containers, or may become very cloudy on standing.

Except for the highly covered complexes, the resulting cytochrome c-DNA complex is quite stable and has been kept without degradation for periods up to two weeks. The spectrum of the complex should always be checked before use, however. In two cases, complexes were made and rather fast degradation was observed (within 5 days). This was detected by progressive changes in the UV absorption spectrum of the complex while standing in the refrigerator. The most prominent feature of the change was a shift of the maximum of the Soret band from 4100 A° to lower wavelengths. This degradation seems to occur only to the cytochrome when complexed on to the DNA (preparations of the same protein stock were simultaneously kept in the refrigerator with no signs of degradation). The causes of this degradation in certain preparations are not understood, and are possibly due to some contaminant in the DNA stock used or some subtle change in the conditions used in the formation of the complex. Unless otherwise noted, all experiments were performed on complexes in which no detectable degradation had occurred.

THE BINDING OF CYTOCHROME C TO DNA

Although the behaviour of cytochrome and DNA on dialyzing into low salt strongly implies that at low salt cytochrome is binding DNA, there are more direct proofs for this:

1. The cytochrome travels with the DNA on sedimentation. In 0.01F NaCl, 0.001F and pH 7.5 and at 2.5×10^{-4}F EDTA pH 8.0 for a complex with C/D = 1.72, pelleting the DNA pellets the cytochrome with it.

2. The cytochrome travels with the DNA in electrophoresis. The cytochrome, being positively charged would normally travel towards the negative electrode. In zone electrophoresis experiments which we will discuss later, the cytochrome c/DNA complex went towards the positive electrode; this could be ascertained both by eye (the colored band could be seen) and by following the band with the electrophoresis optical system at both 2600 and 4100 A°. In 0.01F NaCl, 0.001F tris, pH 7.5 no cytochrome could be detected moving towards the negative electrode.

We may therefore conclude that at these low ionic strengths, a tightly bound complex of cytochrome c to DNA is formed. We then ask the question: in what ionic strength range is there appreciable dissociation of the cytochrome from the DNA? To answer this, the amount of cytochrome free and bound is measured by sedimentation or electrophoresis experiments at various ionic strengths.

The sedimentation is performed by having a uniform concentration of the cytochrome/DNA complex and the supporting electrolyte of

interest in a sucrose gradient. Sedimentation is done in an SW39 swinging bucket rotor at 33,000 RPM for 22 hours or under equivalent conditions. The supernatant is then fractionated and spectra are taken. The fractions are all pooled together and the spectrum of the whole supernatant also taken. The pellet is dissolved in 4F NaCl (in which the complex readily dissociates) and an absorption spectrum taken of it. From the spectra, the amounts of cytochrome free and bound are determined: the amount free being that amount in the supernatant and the amount bound being that sedimented with the DNA.

Though sedimentation is an easy method for determining binding of DNA, it is complicated by the fact that the cytochrome alone has a finite sedimenation coefficient (S= 1.8) (7), and in the course of the run, sediments to a certain extent. Therefore in all these determinations the concentration of the cytochrome in the supernatant was not constant but had a fairly steep gradient from the top to the bottom of the tube at the end of the run. Although one may estimate that under the conditions of centrifugation, the gradient becomes significant probably only after most of the DNA has pelleted (8), it is difficult to exactly predict how large the effect on the measured binding constant would be. For this reason, electrophoresis experiments were also performed.

Binding constants are determined by electrophoresis in a manner entirely analogous to the sedimentation experiment. Cytochrome c/DNA complex of a constant concentration is run in a gradient from 7-17% sucrose in the desired supporting electrolyte. Above this gradient is

a gradient with no cytochrome c/DNA from 0-5% sucrose (and the same supporting electrolyte). The positive electrode is connected to the bottom, the negative to the top. When the voltage is applied, the DNA travels downwards and the cytochrome up. There therefore results a region of free cytochrome c with no DNA. The concentration in this region is determined by actually collecting samples and taking spectra, and is the amount of cytochrome free. This experiment is illustrated in figure 1.

The results of these experiments are shown on table I. Though the numbers are somewhat rough, and the starting concentrations not identical, the general picture is clear. The sedimentation and electrophoresis values agree well enough to support the following general conclusions:

1. The complex dissociates over a rather broad salt concentration range (0.05-0.10F NaCl); it is not as sharp a dissociation as one might expect from the complexing of two poly ions.

2. Native and denatured DNA seem to bind equally strongly.

THE MELTING BEHAVIOUR OF DNA WHEN COMPLEXED TO CYTOCHROME

Since we have been able to compare the binding of native and denatured DNA to cytochrome c and have found that they are about the same, it is interesting to see what effect cytochrome would have on the melting of DNA. Since the cytochrome c binds native and denatured DNA equally strongly, if the transition from native to denatured DNA as well as the binding of cytochrome c were governed merely by equilibrium considerations, then the transition from native to denatured DNA

Figure 1

A diagrammatic representation of how the amount of cytochrome bound to DNA is measured by electrophoresis. In the experiments that were actually performed, the medium was 0.07 F NaCl, 0.007 F tris, pH 8.0. The cytochrome/DNA is originally present in constant concentration in the 7-17% sucrose gradient. After applying the electric field as shown, the free cytochrome has moved upwards into the 0-5% sucrose gradient, while the DNA has moved downwards. The solutions are dripped down, and the region of free cytochrome analyzed for spectrally. It is thus possible to calculate the % cytochrome free.

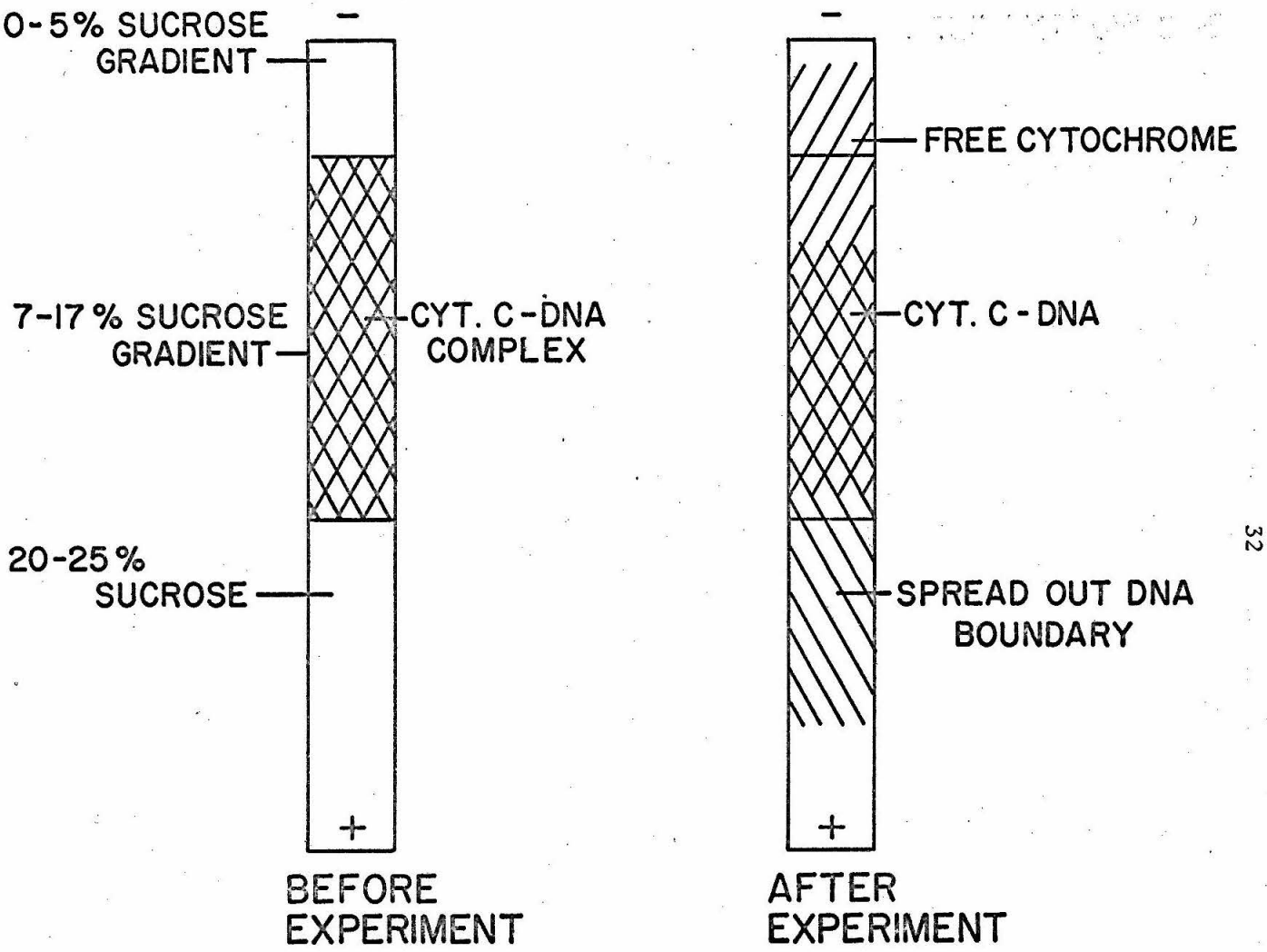

0-5% SUCROSE GRADIENT
7-17% SUCROSE GRADIENT
20-25% SUCROSE
CYT. C-DNA COMPLEX
−
+
BEFORE EXPERIMENT
FREE CYTOCHROME
CYT. C-DNA
SPREAD OUT DNA BOUNDARY
AFTER EXPERIMENT

Table I

IONIC STRENGTH DEPENDENCE OF
THE BINDING OF CYTOCHROME C TO DNA

Medium pH 8.0 F NaCl	F tris	Method[b]	DNA[c] Concentration Micrograms/ml.	C/D	% Free[d]
Native DNA[a]					
.05	.005	S	116.5	1.27	15±3
.06	.006	S	111.5	1.24	30±3
.07	.007	S	116.5	1.27	45±2
.07	.007	E	83.5	1.03	51
.07	.007	E	81.0	1.22	54
.09	.009	S	107.5	1.33	65±3
.12	.012	S	113.0	1.25	80±5
Denatured DNA					
.07	.007	S	68.2	1.27	49
.07	.007	E	68.2	1.27	53

a) Worthington calf thymus DNA

b) S= Sedimentation, E= Electrophoresis

c) Assuming A_{260} of 1 = 50 micrograms/ml.

d) The ± values are the uncertainty in a single experiment, arising from mass balance discrepancies.

should be relatively unaffected by complexing cytochrome on the DNA.

Melting curves were obtained by using the Gilford model 2000 multiple sample absorbance recorder of Prof. J. Vinograd. This has a heating unit with a constant heating rate of 2/3°C per minute. The solvent used was 2.5×10^{-4}F EDTA, pH 8.0.

Up to a C/D of 2, the behaviour is relatively straightforward. The melting profiles for this range of C/D's are shown on figure 2. A tabulation of the midpoints of the transition, T_m, as well as of the transition widths is shown on table II. It is clear that up to a C/D of 2, as one complexes on more cytochrome to the DNA, T_m's and transition widths both increase. The hyperchromicities on denaturation remain normal (34-38%).

We find that in this range, the T_m increases to 10°C above that of uncomplexed DNA. Though this is a modest increase compared to that shown by some of the histone/DNA complexes, it seems significant enough to say that complexing cytochrome does have an effect on the melting temperature of DNA. Because cytochrome binds equally well to native and denatured DNA, we expected no rise in T_m, and this result implies that melting cannot be explained by simple equilibrium considerations.

The melting profiles of the very highly covered complexes are anomalous and rather complex. Melting curves for a set of very highly covered complexes are shown on figure 3. Very high hyperchromicities result on heating, (over 50%) and the absorbance increase consists of two distinct steps, a relatively sharp initial increase (which has a

Figure 2

Heat denaturation curves of cytochrome c/DNA complexes of low coverage. The medium is 2.5×10^{-4}F EDTA, pH 8.0 (prepared by titrating the disodium EDTA salt to pH 8.0 with NaOH). The heating rate used was 2/3°C per minute, and the experiments were done on the Gilford multiple sample absorbance recorder. The hyperchromicity is computed with respect to the DNA absorbance at 2600 A°, the absorbance of cytochrome at this wavelength being corrected for.

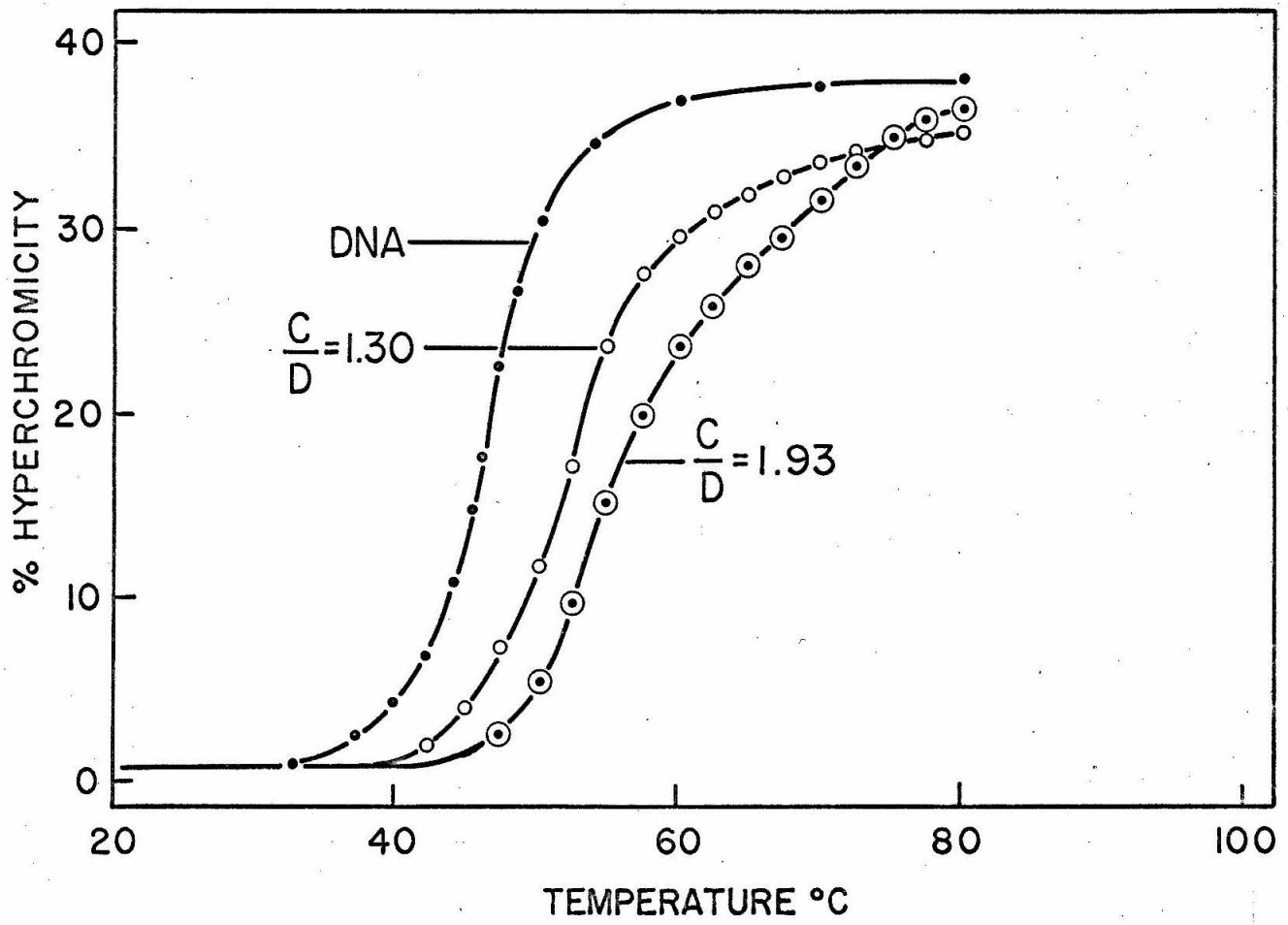

40
30
20
10
0
% HYPERCHROMICITY
DNA
C/D=1.30
C/D=1.93
20
40
60
80
100
TEMPERATURE °C

Figure 3

Heat denaturation curves of the very highly covered cytochrome c/DNA complexes. The conditions are identical to those used in figure 2. The hyperchromicity is with respect to DNA at 2600 A°, the cytochrome absorbance at this wavelength having been corrected for.

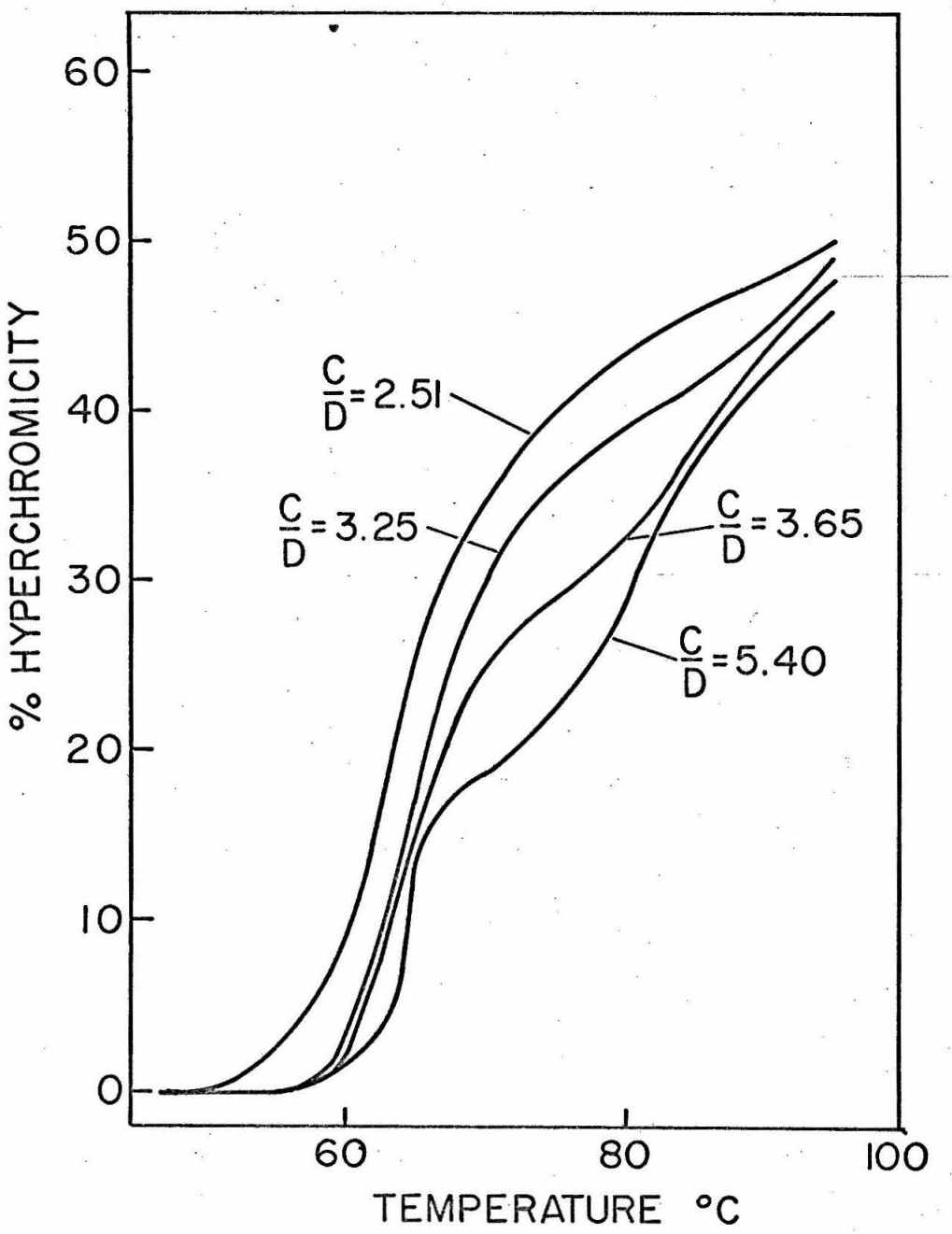

60
50
40
30
20
10
0
% HYPERCHROMICITY
C/D = 2.51
C/D = 3.25
C/D = 3.65
C/D = 5.40
60
80
100
TEMPERATURE °C

Table II

MELTING EXPERIMENTS ON CYTOCHROME C/DNA COMPLEXES

LOW COVERAGE

C/D	T_m	$T_{90}-T_{10}$	%H
0	47.5	13.3	37
1.10	52.9	18.8	34
1.30	53.1	20.3	35
1.53	54.8	25.0	36
1.93	57.8	26.1	38
2.06	58.8	27.8	41

HIGH COVERAGE

C/D	T 18% H	T_m, 1st Step[a]	%H (at 90°C)	% 1st Step[a]
0	47.3	47.5	37	100
2.06	59	58.8	41	100
2.51	62.5	63.8	51	80
3.25	65.8	65.9	49	70
3.65	66.5	65.3	48	60
5.40	68.5	64.6	46	40

a) The first step is defined as the melting up to the inflection point (denoting a minimum $\frac{\Delta A}{\Delta T}$ on the melting profiles).

T_m of 63°-66° in all complexes of C/D greater than 2.5) and a very much broader increase in absorbance at very high temperatures. Even above 90°C there is a substantial increase in absorbance. This is not due to the cytochrome alone, as cytochrome itself gives negligible changes in absorbance at 2600 A° in 2.5×10^{-4}F EDTA, pH 8.0 on heating.

The absorbance increase of the first step decreases with increasing coverage, so that by the time we get to a C/D of 5.40, the initial sharp transition results in a hyperchromicity of only 19%, or 40% of the total hyperchromicity exhibited at 90°C. The rest of the absorbance increase takes place very gradually at high temperatures as described.

The curves on figure 3 suggest a biphasic transition, and as the coverage is increased, the second phase is increased at the expense of the first. All the curves on figure 3 are denaturation profiles of very highly covered complexes that were prepared all together. We believe that these complexes are generally not highly aggregated (most had no appreciable scattering) but such complexes are difficult to make as one has to dialyze for several days to redissolve the precipitates that originally form. The melting behaviour of only one other set of highly covered cytochrome c-DNA complexes has been studied and these were samples in which there were still precipitates present when the complex was obtained. The precipitates were sedimented off in a clinical centrifuge. Such preparations showed a generally similar behaviour, (a sharp step and a more gradual transition at higher temperatures), but the details of the melting curves differed slightly (in T_m and hyperchromicity at 90°C).

We have not really investigated the highly covered complexes sufficiently, and any explanation offered for their behaviour is necessarily of a somewhat speculative nature. The high hyperchromicity suggests that the denatured state of the highly covered cytochrome c/DNA complexes is a structure that has the bases very unstacked. Anomalously high hyperchromicities on denaturation have also been observed when DNA is denatured in nonaqueous solvents (9). Perhaps the denatured DNA, when complexed with very large amounts of cytochrome is in a state that is similar to denatured DNA in a nonaqueous solvent. It might be possible that at high C/D's, the bases in denatured DNA are interacting with non-ionic groups of the cytochrome, but at low C/D's, they do not. It is conceivable that this base-cytochrome interaction would only come about if there were enough protein to interact with both the phosphate groups and the bases: at low C/D's, the main interaction would be between the positively charged side chains of the protein and the phosphates.

Another interesting feature is that increasing the C/D above 2 does not stabilize the double helix against melting appreciably more. All of the highly covered complexes start to melt at approximately the same temperature. The first steep initial rise in absorbance has approximately the same T_m for all complexes above a C/D of 2.5. This implies that above a C/D of 2 or 2.5, the cytochrome that gets complexed to native DNA does not interact as strongly with the phosphate groups and does not screen out charge repulsions as does the cytochrome at low coverages. From the melting behaviour, we

would picture the cytochrome as complexing the double helix such that there is maximal interaction between the phosphates and positive side chains up to a C/D of 2 or 2.5. Above this, steric considerations force the cytochrome to bind the DNA at less favorable sites.

THE ZONE ELECTROPHORESIS OF CYTOCHROME C/DNA COMPLEXES; STOICHIOMETRY

The binding studies on cytochrome c indicate that for a sample of moderate coverage (C/D= 1.72) there is essentially no free cytochrome that can be detected at either 0.01F NaCl, 0.001F tris pH 7.5 or 2.5×10^{-4}F EDTA, pH 8.0. This therefore make it feasible to do zone electrophoresis experiments and measure the mobilities of the cytochrome c/DNA complexes.

It has been previously shown that for native DNA, the mobility in a given medium is completely independent of either molecular weight or base composition (10). Thus electrophoresis is a technique that would be sensitive only to the effect of the protein on the DNA.

It has also been shown in this thesis and by a number of other workers (11) that when DNA is protonated by lowering the pH, the mobility does not decrease proportionally to the formal charge. The fractional formal charge at low pH's (taking pH 7.5 DNA as basis) can be calculated by subtracting from unity the fraction of protons per phosphate group added to the bases at any pH. If this is compared to the ratio of the mobility at the low pH to the mobility at pH 7.5, the % mobility is always seen to decrease much less rapidly than the corresponding calculated % charge. In a sense adding a basic protein

to DNA is analogous to titration. For any complex, we can calculate the net formal charge (with uncomplexed DNA as basis). For cytochrome c/DNA this is particularly easy because the ratio of cytochrome to DNA can be determined with great accuracy, and the amino acid composition is known. For the cytochrome c used (oxidized equine heart), there are 15 acidic (and therefore negative) groups and 23-25 positively charged groups (depending on how many histidines are positively charged) (12). There are thus 8-10 net positive charges per molecule of MW 13,000. DNA has one negative charge per monomer unit of MW= 340. The % formal charge for any complex is therefore:

$$\% \text{ formal charge} = 1 - \frac{C/D\ (340)n}{13{,}000}$$

wherein the net positive charge per cytochrome molecule is a number between 8 and 10.

By taking mobilities of cytochrome-DNA complexes, we may therefore compare the % mobility with the % formal charge, as is done on figure 4. The actual mobility values are shown on table III.

Up to a cytochrome/DNA ratio of around 2, the % mobility is seen to decrease linearly with C/D and is consequently proportional to % formal charge. This is in marked contrast to the situation when we protonate DNA.

Protonation and neutralization of charge by the addition of a polymer are intrinsically different in one particularly significant

Figure 4

A plot of the % charge of cytochrome/DNA complexes calculated from the amino acid compos-tion of cytochrome and the % charge obtained by taking the ratio of the mobility of the complex to the mobility of uncomplexed DNA versus the cytochrome/DNA ratio. The value of n for each line is the net positive charge per cytochrome molecule assumed. The different types of points are sets of complexes made up with different DNA and protein stocks. The dotted circles indicate that these mobilities are final mobilities and that the mobility of the complex was changing as it first moved (see ure 5). The data for this table are shown on table III. The medium for electrophoresis experiments was 0.01 F NaCl, 0.001 F tris, pH 7.5. The important result is that the electrophoresis charge falls on a straight line plot up to C/D= 2, but it corresponds to an n= 11. The most acceptable n from the amino acid composition is 8. This implies a difference in relative counter ion binding of the cytochrome and DNA.

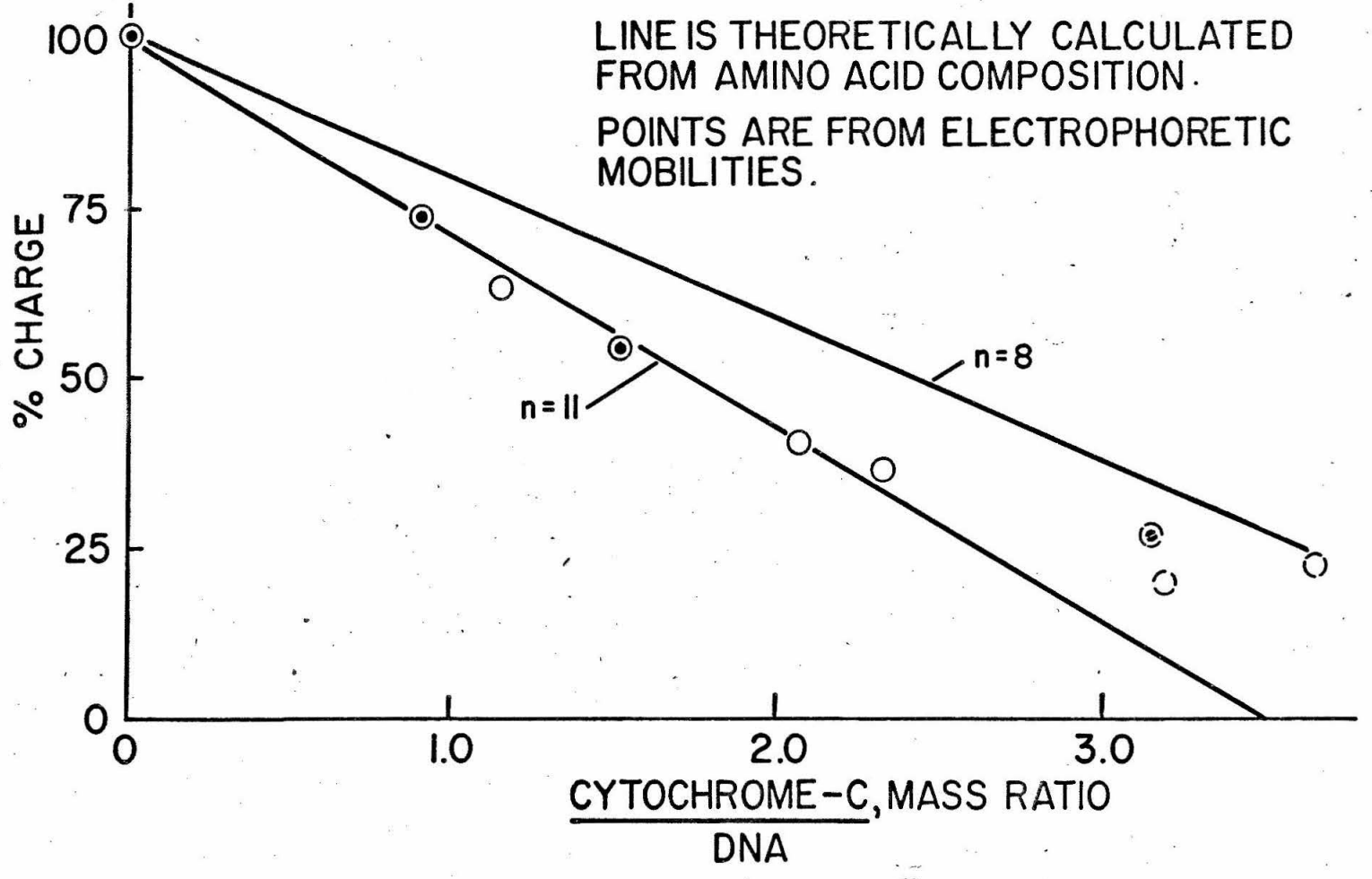

LINE IS THEORETICALLY CALCULATED FROM AMINO ACID COMPOSITION.
POINTS ARE FROM ELECTROPHORETIC MOBILITIES.
n=8
n=11
% CHARGE
100
75
50
25
0
0
1.0
2.0
3.0
CYTOCHROME-C, MASS RATIO
DNA

Table III

ELECTROPHORESIS EXPERIMENTS WITH CYTOCHROME C/DNA COMPLEX

C/D[a] (Before Dialysis)	C/D[a] (After Dialysis)	$M \times 10^4$ cm.^{2}/volt-sec.	% M[a]
0	0	2.18	100
0.92	0.92	1.61	73.9
1.17		1.37	63.2
1.50	1.53	1.16	53.3
2.06		.866	39.8
2.40	2.32	0.76	34.8
3.25		0.398	18.2
(3.62)[b]	(3.15)[b] (4.00)	0.56	25.8
3.70		0.47	21.6

a) M = mobility.

b) This complex originally had a C/D = 3.62. When dialysis was stopped, the supernatant had a C/D = 3.15, but some precipitate with C/D = 4.00 remained undissolved.

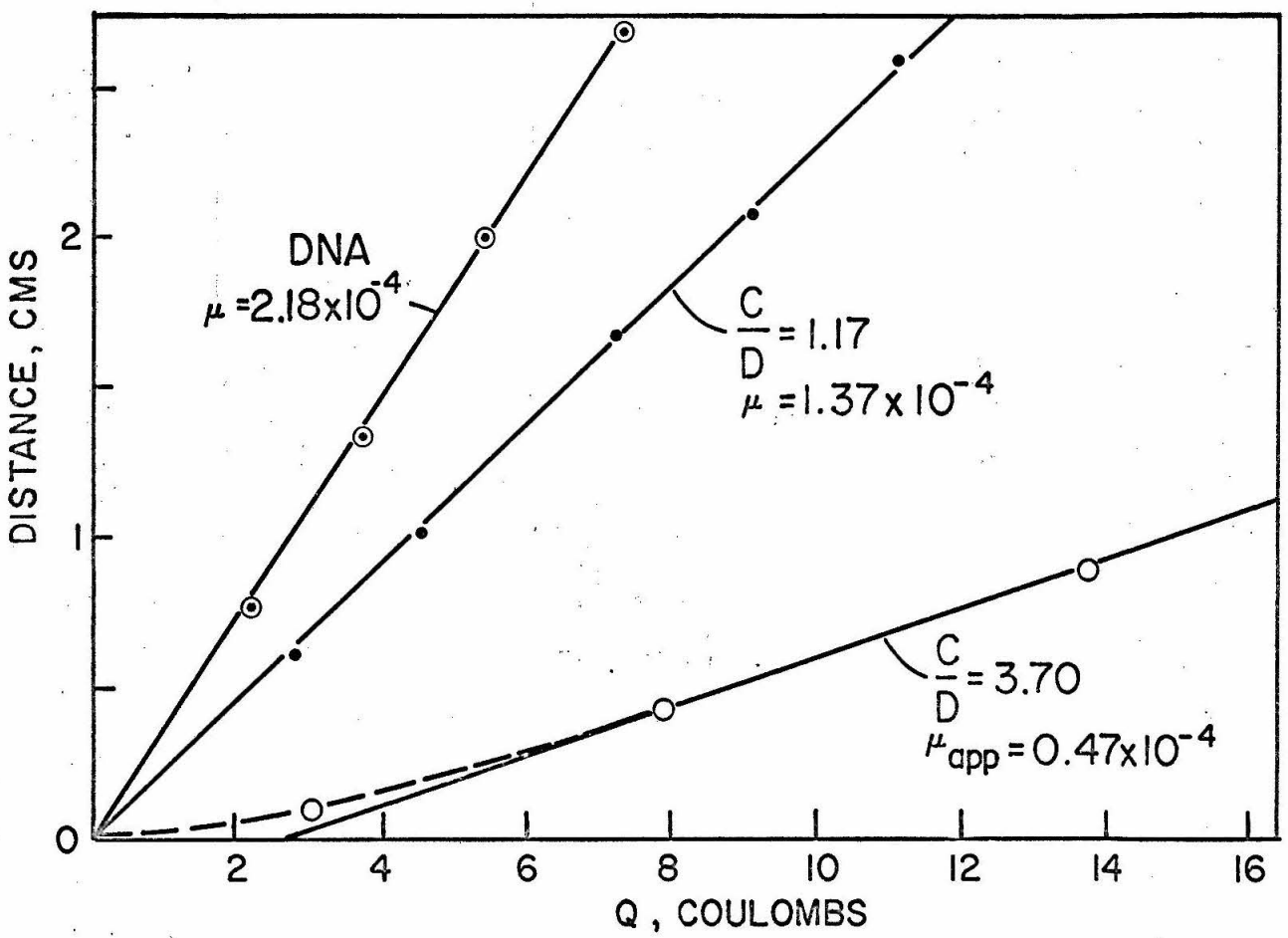
respect. On protonation we are adding single charges fairly randomly: it is conceivable that protonation of one base results in the release of a disproportionate number of Na^+ ions. The protonation of a base may not only release the Na^+ ion held by the nearest phosphate group but affects the binding of Na^+ ion by adjacent phosphates as well. On the other hand, for protein/DNA complexes, we are adding blocks of positive charges and leaving fairly long stretches of DNA uncomplexed also. The Na binding of these uncomplexed stretches is thus unaffected by the presence of the protein and the net result is that the electrophoretic mobility is proportional to formal charge.

The other striking result from the electrophoresis experiments is the fact that for highly covered complexes ($C/D > 2.5$), the mobility ceases to be proportional to the charge. It must be mentioned however that for these very high coverages, the mobility changes as the complex moves through the solution until it reaches a constant value, and it is this constant value that is used in table III. For $C/D < 2$, the mobility appears to be constant at all times. Plots of the distance travelled versus the number of coulombs passed are shown in figure 5 for C/D's of 1.17 and 3.70 to illustrate this. It is clear that initially the high covered complex travels very slowly and gradually goes faster until it reaches a constant mobility.

The logical conclusion from all this is that at low coverages, all the cytochrome is bound with negligible dissociation. At higher coverages, some of the cytochrome becomes less tightly bound and its dissociation in the electrophoresis medium becomes significant.

Figure 5

A plot of the distance travelled down the electrophoresis column versus the number of coulombs that have been passed through. The important point is that the DNA and cytochrome/DNA complex of low coverage yield straight line plots, but the highly covered cytochrome/DNA complex does not. The straight line drawn for this last complex is based on points that are outside the drawing. The apparent mobility given (which is also tabulated in table III) is for the straight line portion of the plot. The medium for these experiments is 0.01 F NaCl, 0.001 F tris, pH 7.5.


DISTANCE, CMS
0
1
2
2
4
6
8
10
12
14
16
Q , COULOMBS
DNA
$\mu = 2.18 \times 10^{-4}$
$\frac{C}{D} = 1.17$
$\mu = 1.37 \times 10^{-4}$
$\frac{C}{D} = 3.70$
$\mu_{app} = 0.47 \times 10^{-4}$

Therefore the complex loses this loosely bound cytochrome as electrophoresis proceeds (causing an increase in mobility) until all the cytochrome is tightly bound and the mobility is constant.

This explanation of two types of binding is defensible from the molecular point of view. Cytochrome complexes the DNA and is generally tightly bound in 0.01F NaCl, but as more cytochrome is added, some molecules can no longer come into as close contact with the DNA charges as previously. This is conceivable if one remembers that at this point there is about 2.5 times as much cytochrome as DNA in the complex. Any additional cytochrome may thus be considerably more loosely held (with Van der Waals forces and H binding perhaps playing a proportionally more important role than electrostatic forces), and will be slowly dissociated as the complex moves in electrophoresis. This occurs until only the intimately held cytochrome remains.

These observations may be tested by doing sedimentation experiments on complexes of various coverages. For a complex with C/D = 1.72, less than 4% of the cytochrome was found free in solution in either 2.5×10^{-4}F EDTA, pH 8.0 or 0.01F NaCl, 0.001F tris pH 7.5. For a complex of C/D = 3.50, less than 4% of the cytochrome was found free in 2.5×10^{-4}F EDTA but about 10% was found free in 0.01F NaCl, 0.001F tris. Thus in contrast to the low C/D complex there seems to be a detectable amount free. The accuracy of these last experiments is not very great because the particular complexes studied had 2-5% of the cytochrome degraded into a more easily dissociated form. This degradation was described in the section on the formation of the complex.

A FEW COMMENTS ON THE KLEINSCHMIDT TECHNIQUE

The Kleinschmidt technique (13) of mounting DNA on electron microscope grids consists of mixing DNA (1 microgram/ml.) with 100 microgram/ml. of cytochrome c in a medium of 1 F NH_4Ac. This is then layered carefully over a solution of 0.15 F NH_4Ac so that a film forms. The grid is placed over the film and is raised so that some of the film sticks to it. After drying the grid it is found that the DNA can be seen as filaments 80 A° in diameter under the electron microscope.

The one thing that seems to be clear from our studies on cytochrome c is that the complex we are studying is not the Kleinschmidt complex. In the first place, the 80 A° diameter suggests a C/D of about 20 for the Kleinschmidt complex. Our studies would indicate that such a complex probably does not exist in bulk solution at 0.15 F NH_4Ac. The grids can apparently be prepared successfully even if the bulk solution on which the film is layered is as high as 0.4 F NH_4Ac (14).

We believe that the Kleinschmidt complex is exclusively a film phenomenon. It would be reasonable to believe that the binding of DNA would be much stronger to a cytochrome surface film than to cytochrome in bulk solution. This picture of the differences between the complex in bulk solution and the Kleinschmidt complex is reenforced by the observation that complexes of reasonably high coverages in bulk solution (C/D greater than 5) have a marked tendency to go out of solution and form surface films on glass. One may also observe that in the early stages of making the complexes that the bubbles within the dialysis tubing are all very nicely coated with films of cytochrome/DNA, making the bubbles remarkably stable.

1. A review of the properties of cytochrome c is found in: S. Paleus and K. G. Paul in The Enzymes, ed. by P. D. Boyer, H. Lardy and K. Myrback, Academic Press, New York, 1963, p. 97.

2. A. K. Kleinschmidt, D. Lang, D. Zachers, and R. K. Zahn, Biochem. Biophys. Acta, 61, 857 (1962).

3. E. Margoliash and N. Frohwirt, Biochem. J., 71, 570 (1959).

4. This is a criterion devised by E. Margoliash, personal communication to Prof. R. Dickerson's group.

5. This is a modification of a procedure devised by R. Jensen.

6. loc. cit. ref. 3.

7. E. Margoliash and J. Lustgarten, J. Biol. Chem., 237, 3397 (1962).

8. K. E. Van Holde and R. L. Baldwin, J. Phys. Chem., 62, 734 (1958).

9. T. T. Herskovits, Arch. Biochem. Biophys, 97, 474 (1962).

10. See the first section of this thesis.

11. See Pp. 217-218, D. O. Jordan, The Chemistry of the Nucleic Acids, Butterworths, London, 1960.

12. loc. cit. ref. 1

13. loc. cit. ref. 2

14. J. Wetmur, personal communication.

III

STUDIES ON THE ELECTROPHORESIS AND MELTING BEHAVIOUR OF NUCLEOHISTONES

STUDIES ON THE ELECTROPHORESIS AND MELTING BEHAVIOUR OF NUCLEOHISTONES

RECONSTITUTED NUCLEOHISTONES

INTRODUCTION

In this section we will first consider artificially made ("reconstituted") nucleohistones. By studying the melting behaviour and electrophoresis of these model nucleohistones, we hope to learn more about the interactions between histones and DNA.

One might, however, misconstrue the meaning of the word reconstituted, and infer that the complexes that we will describe are very similar to the complexes in the chromosome, or to the native nucleohistones extracted from chromatin. It should be made clear at this point that there are vast differences between these artificially made complexes and the natural nucleohistone complexes. As a matter of fact, to be candid, the results of this study seem to have less bearing on the true histone/DNA complex within the cell than was hoped.

One of the main reasons is in the way the histones are isolated and purified before they are recomplexed on to the DNA (1). Whole calf thymus histone is first extracted from the nucleoprotein by a fairly high acid concentration (0.2F H_2SO_4). The different histone fractions are then isolated by chromatography through an Amberlite-IRC column. The eluting solvent is guanidinium chloride (for histone III-IV, concentrations of up to 40%) which is a protein denaturing agent. It is really too much to expect that histones which have undergone such a severe treatment would not have changed in some way.

In addition, there is the fact that the chromosomal non-histone proteins are entirely missing. These undoubtedly play an important role in the organization of the histone/DNA complex in the chromosomes.

However, as will be seen, there are interesting differences in the way that the various histones behave when complexed to the DNA. These may indeed have some bearing to histone function and binding in the native complex, but the reservations we have presented against making any impetuous conclusions should be borne in mind throughout the remainder of this discussion.

The last thing that should be mentioned in introduction is that the histone fractions we will be using to complex the DNA do not consist of a single molecular species. The dominant species always have at least minor contaminants. The histone literature is full of woeful cries about how tremendously complex the histones are: my feeling is that in all probability, these fractions are purer than is believed. The old gel electrophoresis runs showed very complex patterns (2), but if the histones are run in gels with urea, the patterns look simpler. (We will use the nomenclature of Luck (3) throughout this thesis for the different histone fractions.) As will be described in the next section, about 90% of histone I and 95% of histone II seem to travel as single components on gel electrophoresis. Histone III-IV shows a more complex pattern and is made up of at least two major components, but a lot of the gel electrophoresis bands in this fraction may be due to aggregation of the two major components. Therefore, while we are not dealing with single, homogeneous proteins

Table I

DIFFERENCES BETWEEN HISTONE FRACTIONS

Fraction	Mole % lys[a]	arg	his	pro	glu[b]	asp[c]	amide NH_3	MWx10^{-3}[i]
I[d]	25.8	2.8	0.3	8.9	4.4	2.5	1.4[g]	10±2
II[e]	13.5	7.9	2.8	4.7	8.7	5.6	2.7[g]	16±2
III-IV[f]	9.9	12.8	1.6	4.0	4.5	10.1	4.5[h]	30

Amino acid data from: K. Murray in The Nucleohistones, ed. by J. Bonner and P.O.P. T'so, Holden Day Inc., San Francisco, 1964, p. 23.

a] includes methyl lysine

b] includes glutamic acid and glutamine

c] includes aspartic acid and asparagine

d] average of Ia and Ib

e] composition of IIb

f] average of III and IV

g] computed from the data of J. M. Luck et. al., J. Biol. Chem., 235, 2801, 1960.

h] from the data of D.M.P. Phillips and E.W. Johns, Biochem. J., 72, 538 (1959) for the f_3 fraction which is equivalent to III-IV.

i] There is considerable disagreement about molecular weight values especially for the III-IV fraction. For I and II, most reports are in the ranges given. For a survey of measurements, see reference 6 of this section.

in these studies, we are probably not dealing with extremely heterogeneous mixtures of 20 or so proteins either.

Some of the differences between Amberlite column fractions are shown on Table I. Only the charged amino acids and proline are tabulated. For the purposes of the discussion here, the important point is that the various purified fractions do have different amino acid compositions, and different molecular weights.

The early experiments described in this section were done in cooperation with Dr. Ru-chih Huang.

HISTONE II/DNA

Histone II is the histone present in greatest amounts within the calf thymus nucleus (about 50% of all histone), (4) and we shall consider its behaviour first. In many respects it is a "typical" histone: it has an average amino acid composition (5) (moderately lysine rich) and it seems to be intermediate in molecular size (MW = 16,000) (6) between the two other major histone fractions.

The histone/DNA complex was prepared as has been described by Bonner, Huang and Murray (7). For low coverages, it was found that satisfactory results were obtained by mixing the histone and DNA at high salt and directly dialyzing into 2.5×10^{-4}F EDTA, pH 8, as has been described for the cytochrome/DNA complex. However, for more highly covered complexes, the method of Huang et. al. should be followed: if the reconstitution is not done by a gradual dialysis as they describe, then histone rich aggregates form, making the bulk of

the DNA much poorer in histone. If the histone/DNA ratio is higher than about 1.10 (absorbance 2200 A°/absorbance 2600 A° greater than 1.15), then precipitates always result. We have not succeeded in getting homogeneously covered complexes with coverages above this.

We will make an approximate estimate of the histone/DNA ratio by spectral methods. We will assume that the histone II has negligible absorbance at 2600 A°, and recent estimations of histone absorbance at 2200 A° show that 100 micrograms/ml. of whole histone gives an absorbance of 0.92 (8). By taking the ratio of the absorbance at 2200 A° to the absorbance at 2600 A°, we may therefore estimate what the histone/DNA mass ratio is. This ratio (hereafter called 2200/2600) is related to the mass ratio (which we will call H/D) in the following way:

$$H/D = (2200/2600 - 0.65) \times 2.17$$

(assuming that an absorbance at 2600 A° of 1 for DNA is equivalent to 50 microgram/ml.).

This simple assay method, while admittedly approximate gives results which for the very small amounts of material that are involved, are just as good as the conventional Lowry method. Some care must be taken to be sure the dialysis bags are clean (this has been described in the previous section), and the assay is rather inaccurate for solvents which absorb moderately at 2200 A° (EDTA, for instance). But in general it gives consistent and reproducible results.

ELECTROPHORESIS (9):

The electrophoresis data for the histone II/DNA complex are shown on table II. We see that the mobility decrease is proportional to the amount of histone complexed, and there is a rough correlation between the % mobility and the % formal charge, as we might have expected from the cytochrome c results. We had previously concluded that the % mobility was not proportional to the % formal charge (9): this erroneous conclusion was arrived at probably partly because of inaccuracies in the assay, but more importantly because the complexes studied may not have been homogeneously covered and a very small proportion of the DNA had a very disproportionate amount of the histone. Thus the mobility was faster than would have been expected since most DNA molecules had less histone than was calculated from the assay.

The other result of electrophoresis studies is that for histone II/DNA, there is no exchange of histone molecules between DNA strands. Once complexed on a DNA strand, a histone II molecule apparently does not exchange at all in 0.01F NaCl, 0.001F tris, pH 7.5. A mixture of histone II/DNA and uncomplexed DNA may be run on the electrophoresis column and the two components resolve completely from each other. The complexed and uncomplexed DNA may be mixed together for as long as 10 days, and there is still no appreciable exchange. In figure 1, a separation of two histone-DNA complexes from uncomplexed DNA is shown. The mobility differences are such that all three components are clearly resolved. As can be seen from the figure, all the bands are very sharp. The width of the band on electrophoresis of a histone

Figure 1

Results of an electrophoresis run on a mixture of DNA, and two separately reconstituted histone II/DNA samples, one of low coverage (H/D about 0.7) and the other of high coverage (H/D about 1.05). The medium is 0.01F NaCl, 0.001 F tris, pH 7.5. The distance down the electrophoresis tube is the horizontal coordinate on each recorder trace. The distance between markers is 3.0 cms.

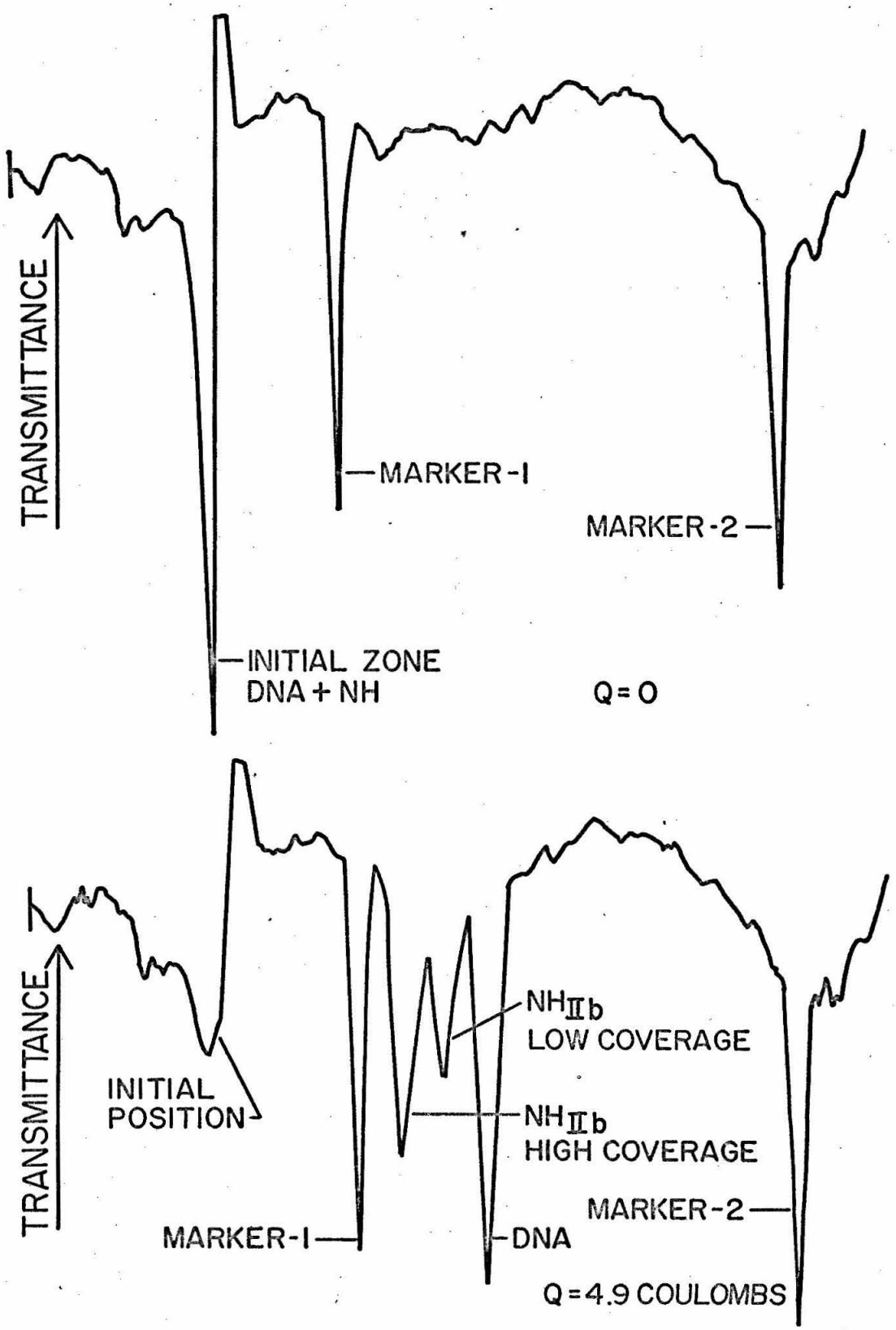

TRANSMITTANCE
MARKER-1
MARKER-2
INITIAL ZONE
DNA + NH
Q = 0
TRANSMITTANCE
INITIAL
POSITION
NH IIb
LOW COVERAGE
NH IIb
HIGH COVERAGE
MARKER-1
DNA
MARKER-2
Q = 4.9 COULOMBS

Table II

MOBILITIES OF RECONSTITUTED NUCLEOHISTONES

Material	H/D	Mobility x 10^4 in cm^2/volt-sec.	% charge	% mobility
DNA	0	2.18	100	100
Histone I/DNA	0.37	1.71	74	78
	0.8	1.30	45	59
Histone II/DNA	0.30	2.05	89	94
	0.45	1.86	83	85
	0.70	1.75	74	80
	0.90	1.58	67	73
	1.05	1.48	61	68
His. III-IV/DNA	0.70	1.90		
Mixtures				
NH Ib + DNA	0.8	1.89 [single peak]		
NH Ib + DNA		1.82 $\pm$0.10 [single broad peak]		
NH Ib + DNA	0.37	2.02 [single peak]		
NH II + DNA	0.70	1.75, 2.18 [two distinct peaks]		
NH II + DNA	1.05	1.46, 2.18 [two distinct peaks]		
NH II + NH I	1.05 0.37	1.40, 1.66 [two distinct peaks]		

II/DNA complex is therefore an indication of the heterogeneity of coverage.

MELTING BEHAVIOUR

Melting curves of DNA in 2.5×10^{-4}F EDTA, pH 8.0 (prepared by titrating disodium EDTA with NaOH) covered with various amounts of histone II are shown on figure 2. Though the melting behaviour of reconstituted complexes has been studied before (10), the low ionic strength makes the melting profiles more sensitive to H/D. It is clear that complexing histone on the DNA stabilizes the native structure. If we compare these melting curves with those of the cytochrome c/DNA complex, we find that histone II is a much better stabilizer of native DNA than is cytochrome c. A cytochrome c/DNA complex with a C/D of 1.54 has a very much slower mobility than histone II/DNA complexes of H/D's of 0.70 or 0.90 (1.16×10^{-4} vs. 1.75 and 1.58×10^{-4}) which means that the overall complex has a lower charge in the cytochrome case. If all other things were equal, we would expect the repulsions between strands to be diminished more in the complex with the lower mobility, and therefore it should have the higher melting temperature. This is in fact not the case, the histone II complexes having higher mobilities but higher melting temperatures. This implies (though it is by no means the only explanation) that the histone II charges are much more efficiently used in shielding the repulsive phosphates on opposite strands from each other. This would agree with our model of the cytochrome charges being distributed along the surface of the protein and therefore only a few could come into really intimate contact with

Figure 2

Heat denaturation curves of histone IIb/DNA complexes in a medium of 2.5×10^{-4}F EDTA, pH 8.0. The estimation of the histone/DNA ratio is done spectrally, as is described in the text.

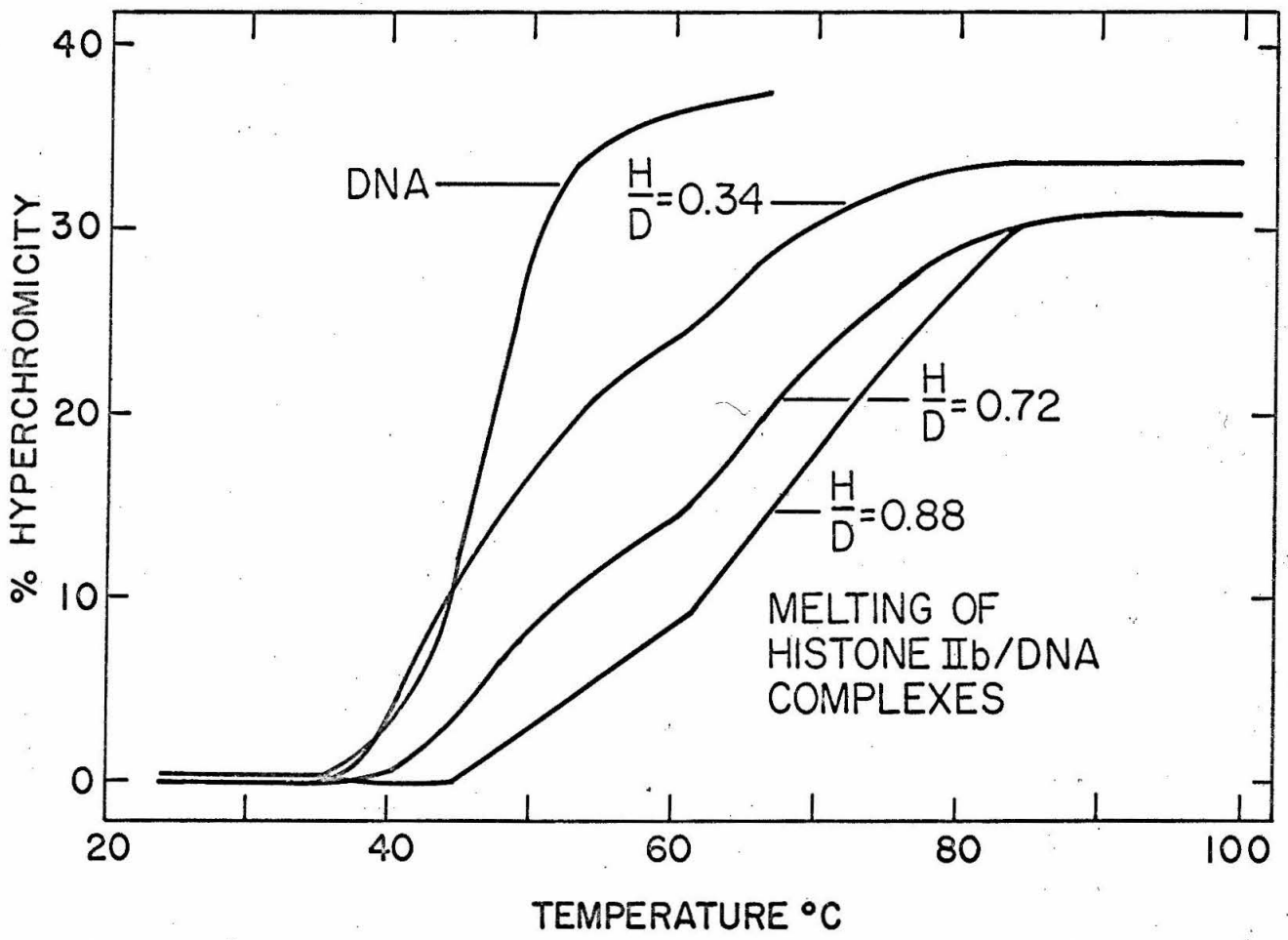

40
30
20
10
0
% HYPERCHROMICITY
DNA
H/D=0.34
H/D=0.72
H/D=0.88
MELTING OF
HISTONE IIb/DNA
COMPLEXES
20
40
60
80
100
TEMPERATURE °C

Figure 3

Heating-cooling curves for the histone II/DNA complex, in a medium of 2.5×10^{-4}F EDTA, pH 8.0. The heating rate was 2/3°C per minute, but the cooling rate was faster and non-linear (the heated cell was merely allowed to stand with water at room temperature circulating around the cell compartment). Another sample with about twice as much coverage was done; the same general features are found, except that the denaturation is shifted to higher temperatures.

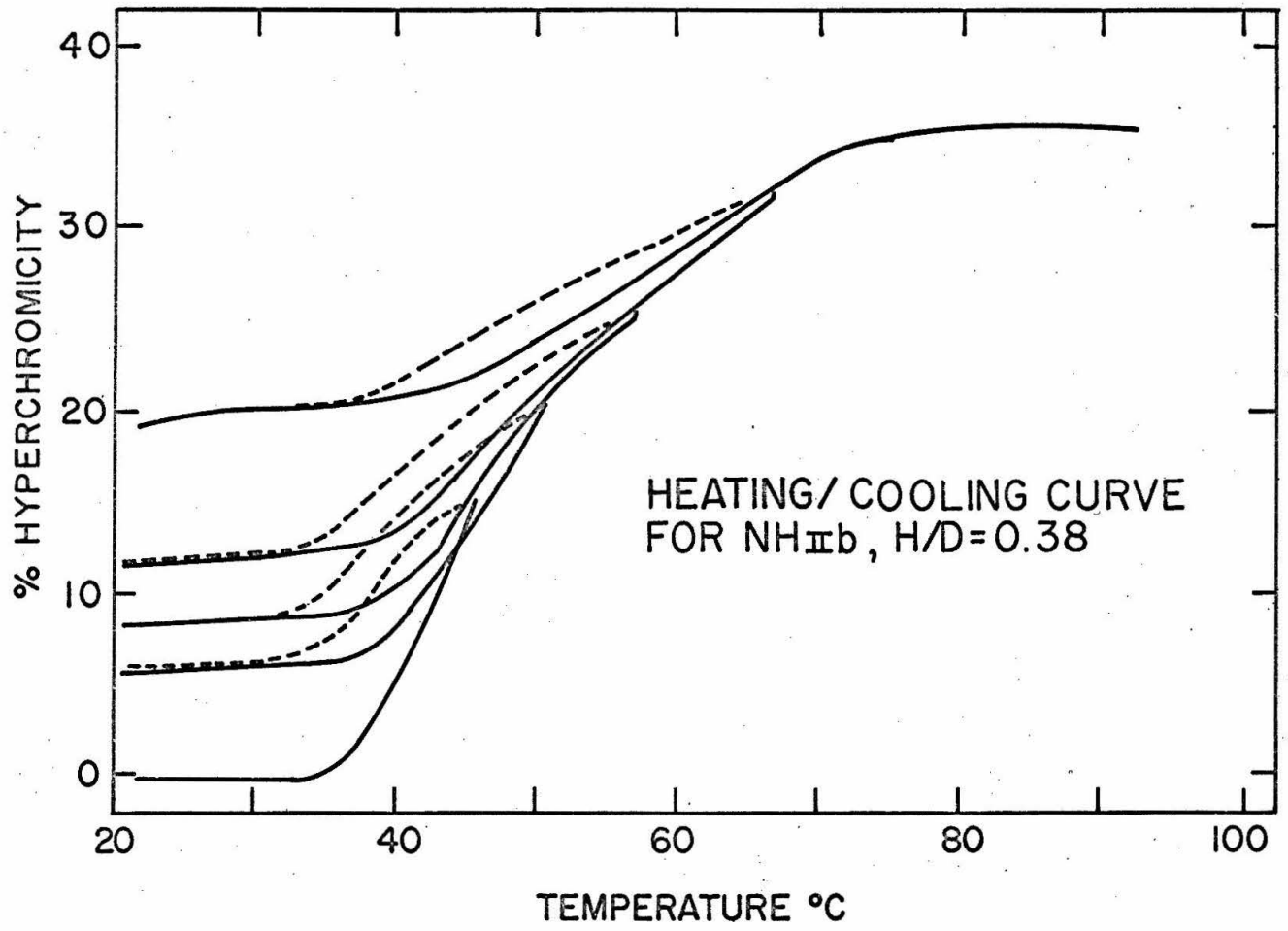
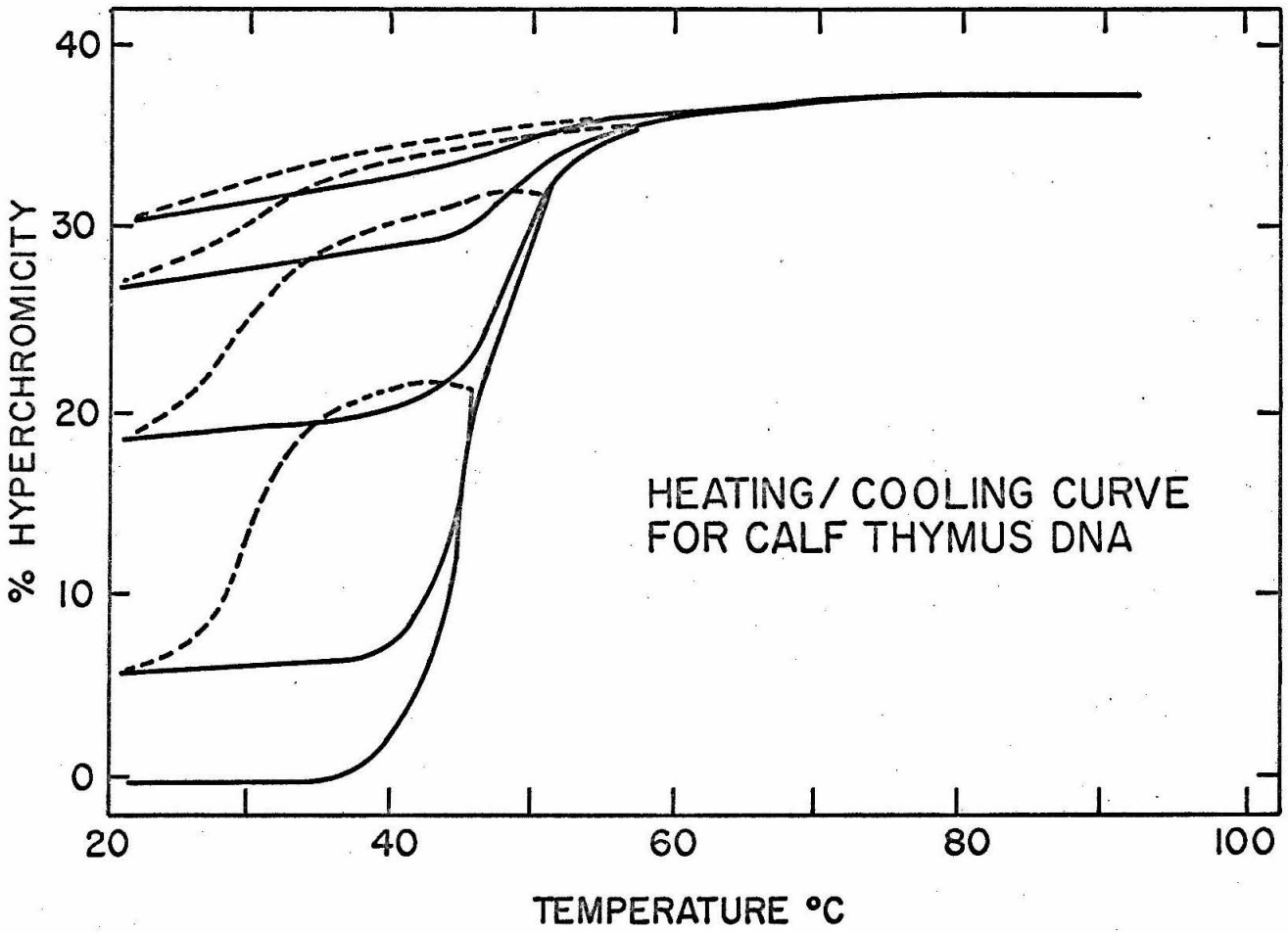

40
30
20
10
0
% HYPERCHROMICITY
20
40
60
80
100
TEMPERATURE °C
HEATING/ COOLING CURVE
FOR NHIIb, H/D=0.38

Figure 4

Heating-cooling curves for calf thymus DNA. The conditions in this experiment are identical to those described in figure 3.


40
30
20
10
0
% HYPERCHROMICITY
20
40
60
80
100
TEMPERATURE °C
HEATING/ COOLING CURVE
FOR CALF THYMUS DNA

the phosphate groups on the DNA, making the cytochrome a poor stabilizer. Since histone II on the other hand has as its function the complexing of DNA, it is entirely possible that the positive groups are arranged in the primary structure of the histone in such a way that the distance between positive amino acids is approximately equal to the distance between phosphate groups. In this way, a very large proportion of the positive charges on the protein is in intimate contact with the phosphate groups, and the shielding per given amount of positively charged protein is very efficient compared to cytochrome.

An experiment that might shed light on the mechanism of the denaturation of the complex is shown on figure 3. Here, the complex is heated to a certain temperature below the temperature of complete denaturation, then cooled, then heated again-- this process being repeated in the denaturation temperature range until the complex is completely melted. There are two major conclusions from the experiment: first is that the histone II does not at all enhance the reannealing of DNA. If anything, it has the opposite effect: on cooling the solution after the complex has been melted part way, the complex has less tendency to go back to its original absorbance than does uncomplexed DNA (see figure 4). This would imply that either the histone/DNA denaturation is an all or none phenomena so that denaturation is very fast once started and at any point along the melting curve above the T_m almost all the hyperchromicity is due to strand separated molecules which are incapable of renaturing, or more likely, most molecules are at any point along the melting curve only partly

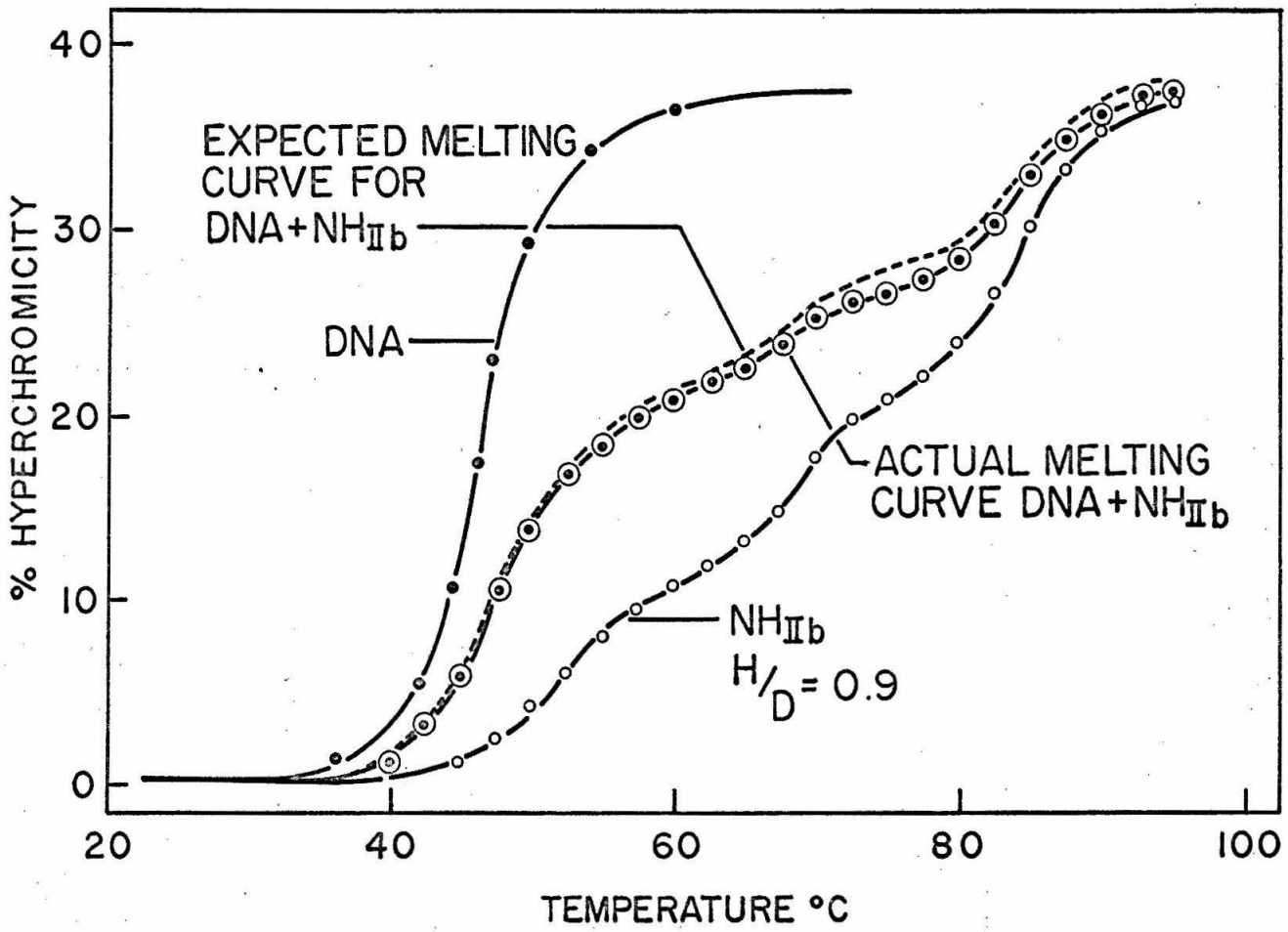
denatured, but when we cool the solution, the portions of the DNA that have denatured are incapable of renaturing because the single strands are wrapped around histone II molecules such that reannealing would be difficult. The latter explanation would imply that the histone is bound to the denatured DNA in a relatively irreversible way.

The other observation we can make about the heating-cooling curve is that the cooling curve is not very greatly displaced from the heating curve, which is quite a contrast to uncomplexed DNA (again see figure 4). In other words, the hysterisis loop in the heating-cooling-heating cycle is much narrower than it would be for uncomplexed DNA. I believe that this implies that the regions of the complex which do reanneal on cooling are very small regions of the DNA, much smaller than the regions which reanneal in uncomplexed DNA. If this is the correct interpretation, then these regions are probably regions along the double helix that are not complexed by protein, but which are bordered by regions which are more densely complexed with protein and hence have not melted. Such regions would be expected to reanneal, and would tend to reanneal close to the temperature at which they separated. Once the region that is complexed with histones melts, however, the DNA strand that is melted might wrap all around the histone in some unspecific way, making it difficult to reanneal the strands.

We can also support our contention that at low salt, histone II does not exchange between DNA molecules by doing a melting curve. Figure 5 shows the melting profiles of DNA, histone II/DNA, and what

Figure 5

Melting curves of: DNA, a histone IIb/DNA complex (H/D= 0.9), a 3:2 mixture of the NH and the DNA (theoretically calculated), and an actual 3:2 mixture. The calculated and actual melting curves are identical. Medium is 2.5×10^{-4}F EDTA, pH 8.0.


40
30
20
10
0
% HYPERCHROMICITY
EXPECTED MELTING CURVE FOR DNA+NHIIb
DNA
ACTUAL MELTING CURVE DNA+NHIIb
NHIIb
H/D = 0.9
20
40
60
80
100
TEMPERATURE °C

melting profile we would expect if the two components were melting independently, as well as the actual melting profile of the mixture. As we see, the calculated and actual melting curves of the mixture are very similar, implying that the two components must have melted independently. This must mean that even at fairly elevated temperatures, there is essentially no histone II exchange.

HISTONE I/DNA

Histone I, the histone fraction richest in lysine, is about 30% of the total histone in the cell nucleus (11). The reconstituted histone I/DNA complex is made in exactly the same way as the histone II complex. However it is more difficult to obtain highly covered complexes that are not very turbid. To get a complex with an H/D of 0.7, we resorted to the expedient of heating the extremely turbid sample to 50°C for 10 minutes. There was some residual turbidity but this solution was used in that form.

The H/D was roughly estimated in the same way as for histone II, by using the extinction coefficient for whole histone at 2200 A°. The estimate is probably poorer for this complex than for the histone II/DNA complex, because this fraction has an amino acid composition that deviates more from that of whole calf thymus histone.

ELECTROPHORESIS:

The results of the experiments on histone I/DNA are shown on table II. As is the case for the histone II complex, the added histone decreases the mobility of the DNA, and this effect is dependent on how much histone is present.

The electrophoretic behaviour of this complex is distinctly different from the histone II/DNA complex in one respect: there seems to be complete exchange of histone I between DNA molecules in a few minutes. Thus, if a mixture of histone I/DNA complex and uncomplexed DNA are run in the electrophoresis column, there is never any separation of the two original components. Instead, all the DNA molecules travel with an intermediate mobility. We have never succeeded in getting any resolution at all of DNA from a histone I complex, even when the mixing was done just before the mixture was put on the electrophoresis column.

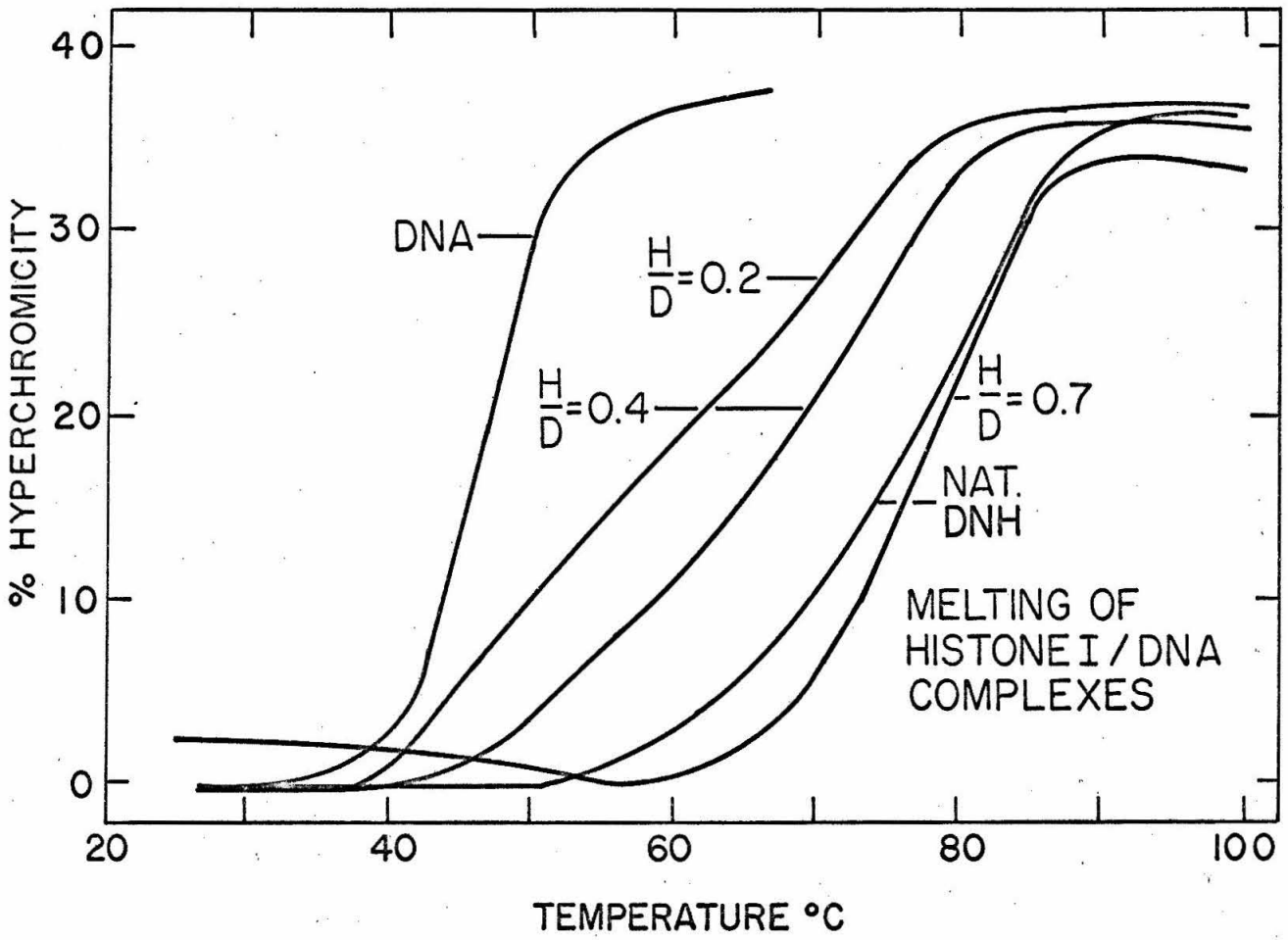
The only time we have been able to get a histone I/DNA complex to resolve from DNA is if the DNA is already appreciably covered with histone II. Thus, if a histone I/DNA complex with H/D = 0.37 is mixed with a histone II/DNA complex (H/D = 1.05), then we get two components on electrophoresis. This would tend to show that there is an actual exchange of histone I between DNA strands and the failure of DNA that is uncomplexed to separate from DNA that is complexed with histone I is not due to mere cross-linking of different DNA strands into aggregates which travel with intermediate mobilities.

MELTING BEHAVIOUR

Melting curves of various histone I/DNA complexes are shown on figure 6. A small amount of histone causes a great broadening in the melting curve and a very substantial rise in T_m. An interesting observation is that as the histone/DNA ratio gets higher, the melting curve becomes sharper once again. For the complex with an H/D = 0.72 the melting transition is sharper than for native nucleohistones.

Figure 6

Heat denaturation curves of histone I/DNA complexes. The medium was 2.5×10^{-4}F EDTA, pH 8.0. The heating rate was 2/3°C per minute. Calf thymus nucleohistone (native) is drawn in for comparison. It will be noted that the highly covered histone I/DNA melting curve is sharper than that of the native nucleohistone.

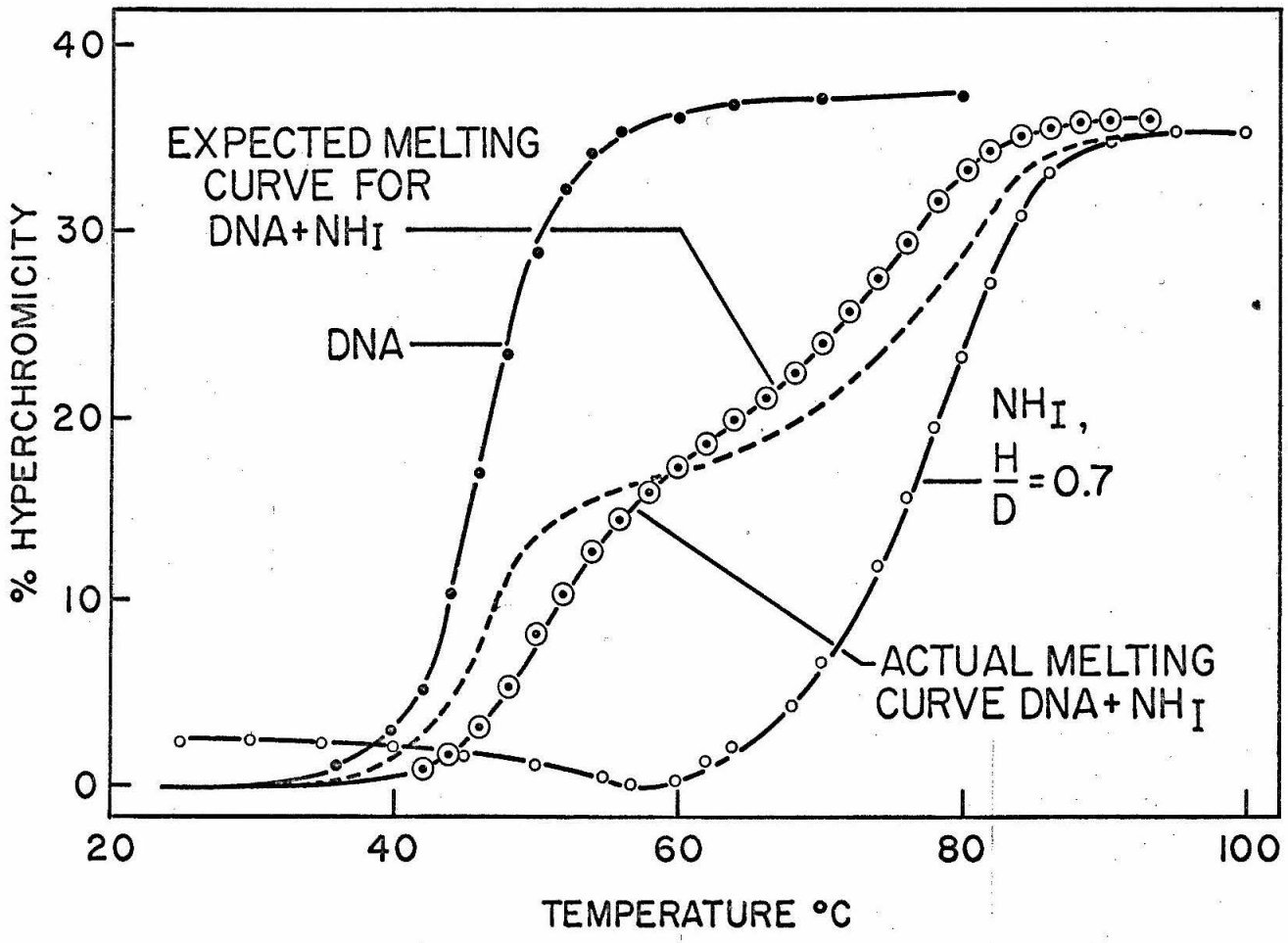

40
30
20
10
0
% HYPERCHROMICITY
20
40
60
80
100
TEMPERATURE °C
DNA
H/D=0.2
H/D=0.4
H/D=0.7
NAT. DNH
MELTING OF HISTONE I / DNA COMPLEXES

The broadness of the transition when the H/D is low might be explained by saying that the binding of histone I to native DNA is probably stronger than it is to denatured DNA, and as a region denatures, some of the histone I complexed to it dissociates and complexes on still native regions stabilizing them even more. A very little histone I causes a substantial rise in the T_m and a great broadening of the profile. We expect the transition to get sharper as we increased the coverage because there would be no more low melting regions at high coverage. Thus, above 68°C, the complex with an H/D of 0.7 has just barely started to melt, the complex with an H/D of 0.38 has halfway more to go, and the complex with an H/D = 0.18 has about 30% more to go. The idea we propose is that at this temperature, all the native DNA left in each has a coverage of about H/D = 0.7. In the case of the complexes of originally lower coverage a large fraction of the histones that was complexed on to the DNA that has now denatured has transferred to the DNA which is still native.

The melting curves also show the exchange of histone I, which is apparently fast even in 2.5×10^{-4}F EDTA, pH 8.0. In figure 7, the melting profiles of calf thymus DNA and the same DNA complexed with histone I are shown. A profile of what one would expect if each component were melting independently in a 1:1 mixture is also drawn in. The actual melting profile of such a mixture is shown-- it clearly deviates from the expected curve. The DNA which was uncomplexed now seems complexed, since it melts at a somewhat higher temperature, and the material which melted as nucleohistone melts at a somewhat lower temperature. This demonstrates that there has been exchange between

Figure 7

Melting curves of DNA, a histone I/DNA complex (H/D= 0.7), a theoretically calculated 1:1 mixture, and the actual melting curve for such a mixture. Medium is 2.5×10^{-4}F EDTA, pH 8.0 and the heating rate is 2/3°C per minute.


40
30
20
10
0
% HYPERCHROMICITY
EXPECTED MELTING
CURVE FOR
DNA+NHI
DNA
NHI,
H/D = 0.7
ACTUAL MELTING
CURVE DNA+NHI
20
40
60
80
100
TEMPERATURE °C

the complexed and uncomplexed DNA. The fact that the second half of the transition deviates from what we would expect for a nucleohistone transition shows that the process we postulated to explain the broad melting curves on low histone I coverage, and the sharpening of the transition when we increase the coverage, cannot be taking place in a perfectly ideal way. If all the histones on denatured DNA transferred to the native DNA, the second half of the transition would be identical to the "theoretical" curve. The fact that it deviates shows that after we have melted half of the DNA, the histones which were on the denatured DNA transfer to the native DNA only partially.

HISTONE III-IV/DNA

Of all the different purified histone/DNA complexes, this is the one that we have studied least. It is very difficult to prepare in the way that we have described for the two other complexes, and on hindsight, it is almost certain that the complexes as prepared involve complexes of histone aggregates with DNA. HIstone III-IV is notorious for aggregating at high salt (12) and in the reconstitution procedure, the DNA and histone are mixed together at high salt. If we attempted to sediment off aggregates by spinning at 20,000 RPM for half an hour in the SW 39 rotor, the DNA which remained always had extremely low coverages (and the higher the initial H/D, the lower the yield).

We have never been able to get any histone III-IV/DNA complexes to go any slower than 1.90×10^{-4} cm^2/volt-sec. in electrophoresis measurements, although the protein/DNA ratios of some of the preparations were at least 0.7, and from the charge, we would expect a much slower

mobility. This is probably due to the very inhomogeneous coverage of the DNA, and the bulk of the DNA is probably hardly covered with histone at all, while some portions of strands are complexed on to large aggregates.

The melting curves of the histone III-IV/DNA complex are shown on figure 8. Complexing the histones causes an increase in the T_m of the DNA, but the change in T_m is much smaller than for the two other histone fractions. This is again probably due to the inhomogeneous coverage as a result of aggregation.

A rather interesting behaviour is shown by histone III-IV/DNA complexes if we do heating-cooling experiments in the way described for histone II/DNA. These are shown in figure 9. The main feature is that there is practically complete reannealing when the solution is cooled, even if the DNA had been heated to temperatures considerably above the T_m. This behaviour is very different from uncomplexed DNA and histone II/DNA complexes. A comparison of the behaviour is made in table III. It therefore appears that even when we have heated a complex so that 90% of it is denatured, none of the strands has yet separated, and they all renature on cooling so that the solution goes back to its original absorbance. It will also be noted that the heating-cooling hysterisis loops are quite wide, just like uncomplexed DNA but quite unlike histone II. The other feature is that when we melt the DNA that we have reannealed, it melts exactly as it did originally, showing that all the histone that was complexed on the DNA originally is still complexed to the DNA after denaturation and

Figure 8

Heat denaturation curves of histone III-IV/DNA complexes. The medium was 2.5×10^{-4}F EDTA, pH 8.0 and the heating rate was 2/3°C per minute. Calf thymus native nucleohistone is drawn in to illustrate that for the highly covered complex, there is substantial melting at temperatures in which nucleohistone is completely melted.

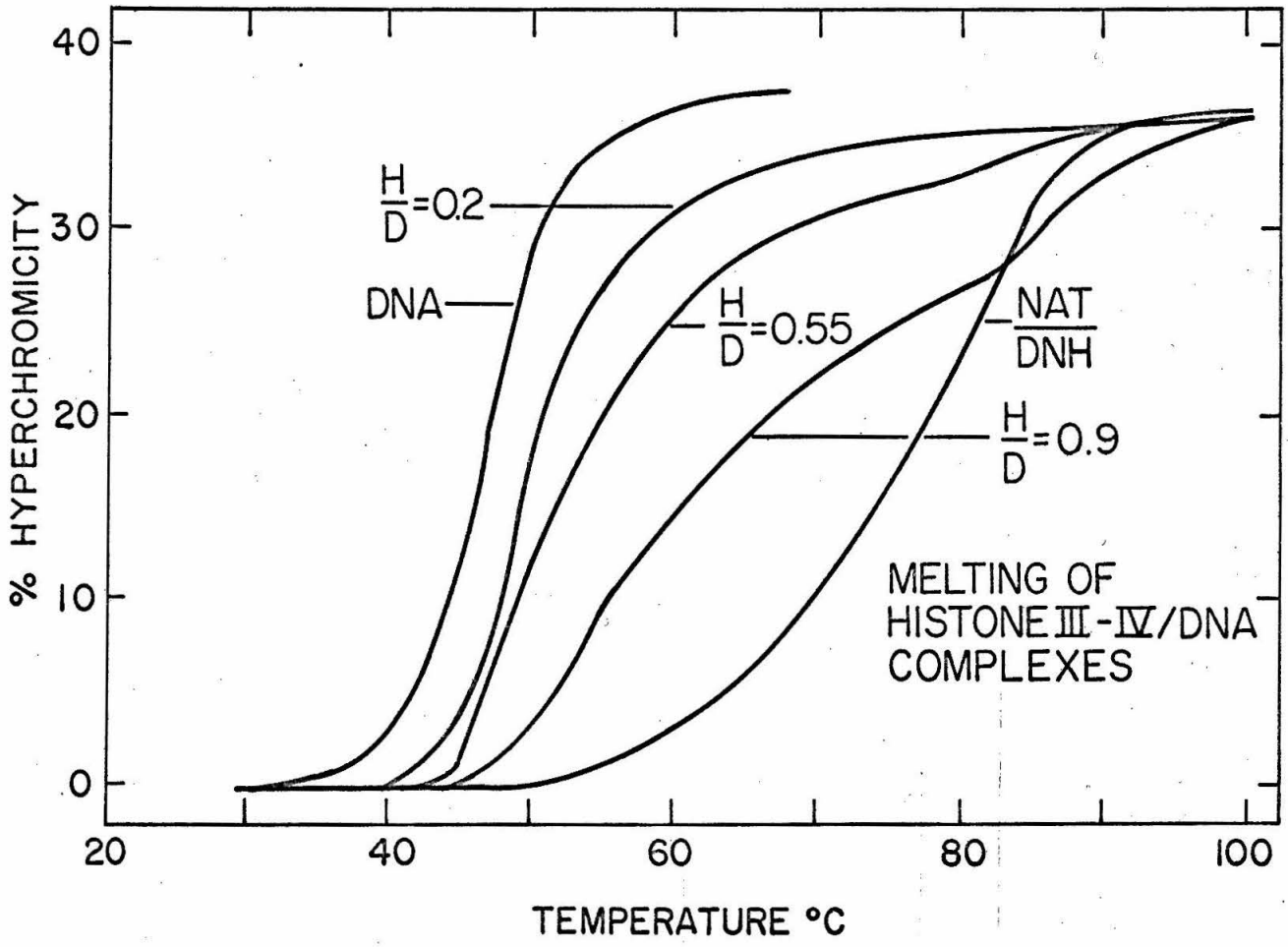

40
30
20
10
0
% HYPERCHROMICITY
20
40
60
80
100
TEMPERATURE °C
H/D=0.2
DNA
H/D=0.55
NAT/DNH
H/D=0.9
MELTING OF
HISTONE III-IV/DNA
COMPLEXES

Figure 9

Heating-cooling curves for histone III-IV/DNA complex of H/D= 0.18. The conditions were identical to those described in figure 3. Two other samples (more highly covered than this one) were done, and they showed the same striking recovery on cooling.

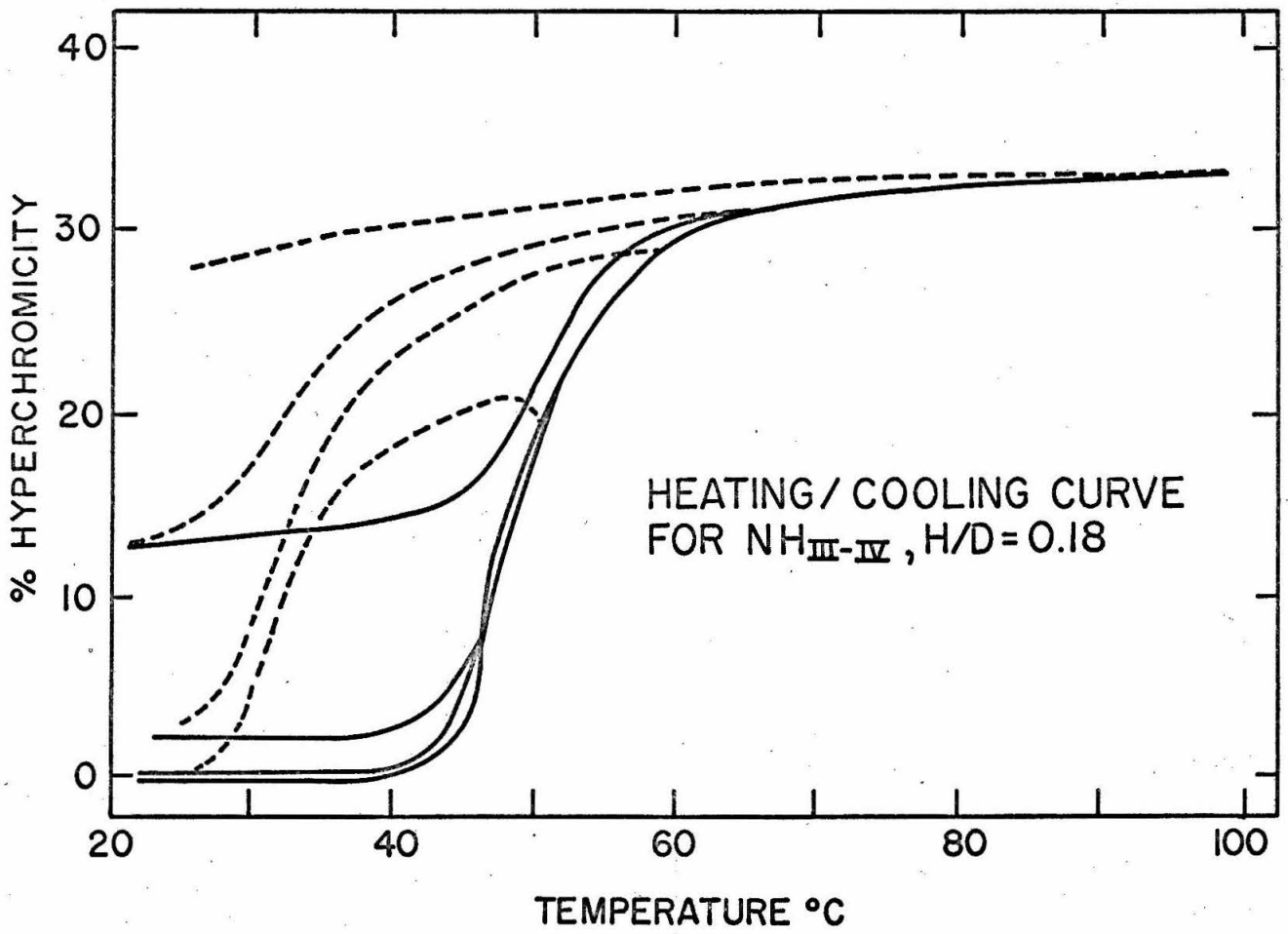

40
30
20
10
0
% HYPERCHROMICITY
20
40
60
80
100
TEMPERATURE °C
HEATING / COOLING CURVE
FOR NH III-IV , H/D = 0.18

Table III

HEATING-COOLING EXPERIMENTS ON NUCLEOHISTONES

Material	% Melting	% Recovery	$T_{max.}$ [°C]
DNA	60	72	46
	87	50	51
	96	35	58
	100	23	97
Histone III-IV/ DNA	63	100	50
	89	92	58
H/D=0.12	94	58	66
	100	14	100
Histone II/ DNA	43	60	46
	58	58	51
H/D=0.19	72	54	58
	100	20	97

reannealing. We shall see that this is not the case for some of the native salt extracted histone/DNA complexes.

I believe that the best explanation for all the effects we have observed is to say that the DNA is complexed to aggregates of histone III-IV of various sizes. A few DNA molecules are complexed to very large aggregates which have most of the histone. Most DNA molecules have considerably less histones. They may be complexed on to mainly smaller aggregates also. It would seem that since the hysteresis loops are broad in the heating cooling experiments, fairly long regions of DNA renature on cooling-- we might imagine that the average complex that we are studying consists of long strentches of uncomplexed DNA with a few aggregates per molecule, the aggregates cross linking both strands. The long uncomplexed regions melt, but there is never any strand separation, since the strands are always held together by the histone aggregates. Thus when the solution is cooled, there is perfect reannealing. However, if we heat above 60°, then even regions which are complexed by the aggregates start to denature, and the recovery starts to become very much poorer. An observation in support of these ideas is that in all the melting curves of this complex, the T_m's are relatively low, yet there is still melting at very high temperatures, even for very low coverages. This would be expected in our model since we say that most of the DNA is not covered with histone at all and hence melts low, but the DNA that is covered with aggregates would be expected to melt at very high temperatures. Perhaps in certain cases denaturation might not even be possible until the aggregate dissociates.

This would account for the fact that the histone III-IV complex is still melting above 90°C, a temperature when even the native nucleohistone is completely melted.

This picture may sound very pessimistic, but I believe that unfortunately, it is consistent with what is known about the behaviour of this histone fraction and it does explain all the facts.

NATIVE NUCLEOHISTONES

In this section we will consider the electrophoretic and melting behaviour of native nucleoproteins with one major objective in mind: to find out if there are uncomplexed regions of DNA in various native nucleoprotein preparations.

Studies (13) on the melting behaviour of native nucleoproteins have of course been done before by a large number of workers but these studies have generally been done at an ionic strength of about 0.01. At this supporting electrolyte concentration, the difference in temperature required to denature pure uncomplexed DNA and fully complexed nucleohistones is not great (about 10°C). It was thought that by lowering the ionic strength, one could make this difference much greater and could decide more clearly whether or not DNA existed in the purely uncomplexed form. We have carried out our studies in a medium of 2.5×10^{-4}F EDTA, pH 8.0 (prepared by titrating Na_2EDTA with NaOH to pH 8.0, thus, [Na] is about 7.5×10^{-4}F). In this medium, the T_m of pure DNA (calf thymus) is 47°C. The T_m of calf thymus nucleohistone remains very high however (76°C) since a DNA molecule complexed by positively charged proteins shows much less sensitivity to the

ionic strength of the surrounding medium in its denaturation behaviour.

Electrophoresis is another suitable technique for deciding whether there is uncomplexed DNA in nucleoprotein preparations, since it is shown in the preceding section that nucleohistones have much lower mobilities than uncomplexed DNA. We would therefore expect all molecules of uncomplexed DNA to separate from the nucleohistone portion of our chromatin preparations on electrophoresis.

MATERIALS:

The nucleoprotein preparations were all gifts of various members of Professor James Bonner's group. The pea nucleoproteins were prepared by Dr. Ru-chih Huang. The rat liver chromatin was a gift of Dr. K. Marushige, onion chromatin of Dorothy Tuan, rat tumor of Dr. M. Nicolson, and various calf thymus nucleoprotein preparations of D. Fambrough and D. Tuan. A good review of methods of preparation of these various nucleoproteins is about to appear in Methods in Enzymology (14).

ELECTROPHORESIS

The electrophoresis studies were carried out in the usual supporting electrolyte (0.01F NaCl, 0.001 F tris, pH 7.5). The mobilities that were obtained from various preparations are shown on table IV.

The most noteworthy thing about the electrophoresis experiments was that all the nucleoproteins travelled as a single peak. The peaks were somewhat broader than DNA but certainly did not show any

Table IV

ELECTROPHORESIS OF NATIVE NUCLEOPROTEINS

Material	Mobility[a] cm^2/volt-sec x 10^4
Nucleohistones[b]	
pea embryo	1.29±0.02
pea bud	1.27
calf thymus	1.35±0.03
Chromatins[b]	
pea bud	1.25
pea cotyledon	1.53±0.09
pea flower	1.32
calf thymus	1.31
rat liver	1.19
rat tumor	1.14
tobacco cell	1.15

a] All values are averages. If over three different preparations were examined, the ± values show the range within which all measurements fell.

b] For plant tissues, there are definite differences between nucleohistone and chromatin as described in J. Bonner and R.C. Huang, J. Mol. Biol., 6, 169(1963). For animal tissues differences are less well defined.

bimodal distribution of mobilities-- there was no detectable material which travelled with the mobility of pure uncomplexed DNA. We may thus conclude that the electrophoretically independent units (which are undoubtedly complexes with molecular weights greater than 10^6) are more or less homogeneously covered with protein.

The only exceptions to the statement that all preparations travelled as a single homogeneous peak were the pea nucleoproteins. Pea flower, pea bud and most strikingly pea cotyledon chromatin all seemed to be contaminated with an impurity that migrated slower than the nucleoprotein itself (a mobility of 1.00×10^{-4} cm.^{2}/volt-sec. or less). This impurity contains little or no DNA (in a CsCl density gradient, no DNA band could be detected). If pea cotyledon chromatin was sedimented twice through a sucrose gradient, the resulting highly purified nucleoprotein was found to be free of the contaminant. Electrophoresis itself might therefore serve as a promising purification tool for such contaminated nucleoproteins.

As seen from table IV, the mobilities of all the preparations fall in the range of $1.25 \pm 0.10 \times 10^{-4}$ cm.^{2}/volt-sec. Rat liver and tumor chromatin, as well as tobacco cell chromatin have somewhat slower mobilities than the other preparation, and they are known to have very large amounts of non-histone protein (15). Pea cotyledon chromatin however, consistently shows a mobility above this range. It has recently been demonstrated by D. Fambrough (16) that very young pea cotyledons lack histone I and start synthesizing them as they grow older. This would satisfactorily explain why this nucleoprotein has a higher

mobility than all the others. An absence or deficiency of histone I which has the highest positive charge density would result in a measurable increase in mobility. The mobilities of native nucleoprotein therefore reflect in a qualitative way the degree to which they are complexed by proteins.

MELTING CURVES

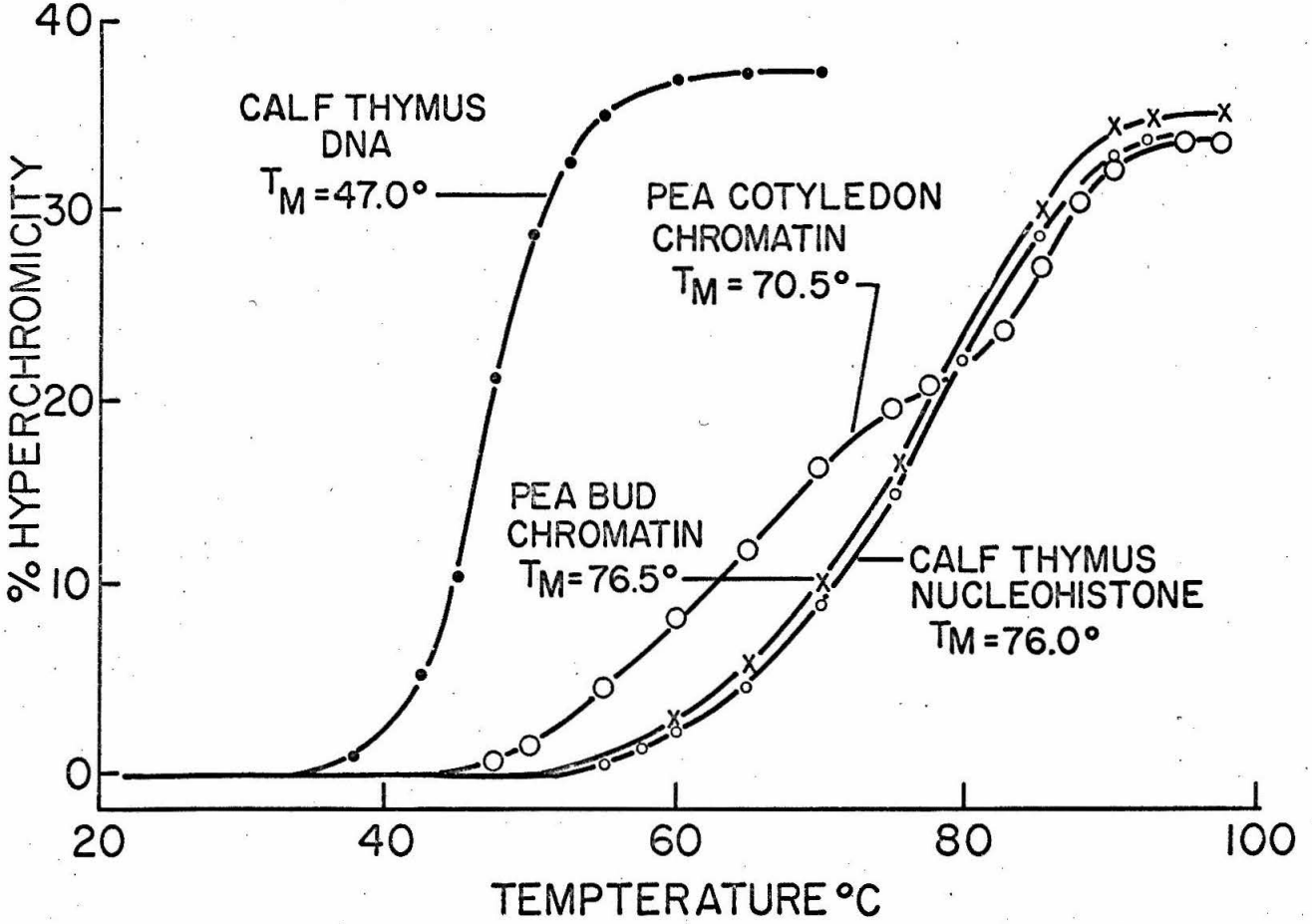
The melting curves were carried out in the Gilford Model 2000 multiple sample absorbance recorder. The medium is 2.5×10^{-4}F EDTA, pH 8.0. The results are shown on table V, in which the T_m's, as well as transition widths and % hyperchromicities are tabulated for the various nucleoproteins.

The melting profiles of calf thymus nucleohistone, pea bud and pea cotyledon chromatin are shown on figure 10. It will be noted that the pea bud chromatin gives a single monophasic transition. The pea cotyledon transition is broader, but even in this preparation, there is not much material that can be said to be melting like pure DNA. It must therefore be concluded from these experiments that there are no very long, independently melting DNA regions. Studies on denaturation suggest that gene-sized regions (500 base pairs long), if uncomplexed by histone, should melt independently of other regions on the same molecule (17). From these studies we can say that no uncomplexed DNA regions 500 base pairs long can be detected in these nucleoprotein preparations.

It will be noted that once again, pea cotyledon chromatin is dif-

Figure 10

Melting curves of some native nucleoprotein preparations. The medium was 2.5×10^{-4}F EDTA, pH 8.0 and the heating rate was 2/3°C per minute. All the other native nucleoprotein preparations studied showed melting curves similar to the pea bud and calf thymus preparations drawn in here. The unsheared calf thymus chromatin gave a melting profile essentially identical to that of the sheared calf thymus nucleohistone shown in this figure.


40
30
20
10
0
% HYPERCHROMICITY
CALF THYMUS
DNA
T_M = 47.0°
PEA COTYLEDON
CHROMATIN
T_M = 70.5°
PEA BUD
CHROMATIN
T_M = 76.5°
CALF THYMUS
NUCLEOHISTONE
T_M = 76.0°
20
40
60
80
100
TEMPTERATURE °C

ferent from the other preparations, this time in having a somewhat lower T_m. This agrees nicely with the discovery that this preparation lacks the histone I fraction.

Table V

MELTING OF SOME NATIVE NUCLEOPROTEINS

Material	T_m	$T_{10\%}$	$T_{90\%}$	$T_{90}-T_{10}$	%H
Pea bud nucleohistone	75±2*	58±4	87±0	29	34.0
Onion chromatin	73	55	85	33	30
Calf thymus nucleohistone	76	61	86	25	35
Calf thymus chromatin	77	62	87	25	33.8
Pea cotyledon chromatin	68±3	49±3	84±2	35	33.4

* The ± values indicate the range within which all values fall when three preparations or more were used.

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1. The purification of histones is described by K. Murray in The Nucleohistones, edited by J. Bonner and P. O. P. Tso, Holden Day, Inc., San Francisco, 1964, pp. 15-35.

2. See for example. H. J. Cruft in The Nucleohistones edited by J. Bonner and P. O. P. Tso, Holden Day, Inc., San Francisco, 1964, p. 72.

3. loc. cit. ref. 1.

4. H. Ohlenbusch, Ph.D. Thesis, California Institute of Technology, 1966, p. 153 and p. 337.

5. loc. cit. ref. 1, p. 24.

6. For a review see D. M. P. Phillips, Progr. Biophys. Biophys. Chem., 12, 213 (1962) pp. 240-241; K. Murray, Ann. Rev. Biochem. 34, 209 (1965) p. 227.

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IV

THE DISSOCIATION OF HISTONES FROM CALF THYMUS CHROMATIN

THE DISSOCIATION OF HISTONES FROM CALF THYMUS CHROMATIN IN NaCl

Of all native nucleoprotein complexes, calf thymus chromatin is the best studied and the easiest to prepare. In cooperation with Dorothy Tuan, a study of the behaviour of this nucleoprotein in NaCl has been undertaken. So far, the only other salts that have been used for the selective extraction of histones are $NaClO_4$ (1) and guanidinium chloride (2). Both have rather special effects on proteins and nucleic acids. A study of histone dissociation in NaCl would more nearly show pure ionic strength effects.

NaCl has in fact been used by a number of workers for histone dissociation (3) (most recently by Giannoni and Peacocke who concluded that the histones that are dissociated below 1FNaCl are richer in lysine than the whole calf thymus histone) (4). These early studies did not have the advantage of the elegant technique of polyacrylamide gel electrophoresis to fully characterize what histone fractions were being extracted.

PREPARATION OF CALF THYMUS CHROMATIN

The method used to prepare calf thymus chromatin is essentially the same as a Zubay-Doty (5) procedure, with modifications by Chalkley and Maurer (6). For each set of experiments, about 40 g of calf thymus tissue were cut, and homogenized in a waring blender to isolate nuclei. The medium used for homogenization was 0.25 F sucrose, 0.005M $MgCl_2$, 0.005F tris, pH 7.35. The homogenization was carried out by first suspending the tissue in 100 ml. of the above medium and increasing

the volume by 100 ml. each time the voltage is increased. The tissue is first homogenized at 50 volts for 2 minutes, then at 60 volts for 1 minute followed by 1 minute at 70 volts, and finally 80 volts for 30 seconds.

The resultant suspension is then filtered through 2 layers, then 4 layers of gauze, followed by filtration through 2, then 4 layers of miracloth. The filtrate is then centrifuged at 3500 RPM for 20 min. in a Servall centrifuge. The pellet is hand homogenized in the same medium as previously, then it is homogenized in saline EDTA (0.075F NaCl, 0.024F EDTA, pH 8.0). At this point we have fairly pure nuclei, in a fine suspension.

The nuclei are lysed by vigorously homogenizing in 0.01 F tris, pH 8, then stirring for 30 min. The chromatin is then layered on 1.7 F sucrose, and spun down at 22,000 RPM for 3 hours in a model L centrifuge using the SW 25 swinging bucket rotor. The chromatin pellets down into the sucrose and swells to form a clear gel. This material is what shall be referred to as chromatin.

Since this material is very aggregated and difficult to work with, it is generally sheared into what is called nucleohistone or sheared chromatin (the terms used interchangeably here). This is prepared (7) by dialyzing the chromatin against DSC diluted four times with redistilled water, and shearing the chromatin in a small waring blendor for 90 seconds at 80 volts, 20 ml. at a time. This material is then centrifuged for 30 minutes at 10,000 RPM in the Servall

centrifuge. The supernatant of such a sedimentation is the nucleohistone. The pellet may be resuspended and sheared again, to increase the nucleohistone yield. Unless otherwise specified, all the salt extraction experiments we will describe were done on the sheared nucleoprotein.

THE HISTONES OF CALF THYMUS NUCLEOPROTEIN: IDENTIFICATION BY GEL ELECTROPHORESIS

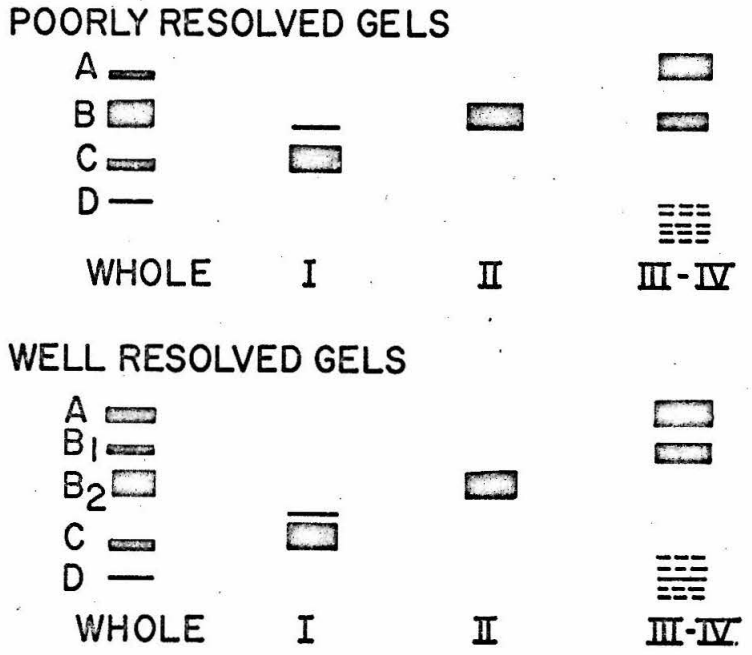
As we have discussed in the preceding section of this thesis, the histones in the calf thymus nucleus can be divided into a number of distinct fractions. Though various methods for isolating these fractions have been devised, all studies (8) agree that there are three major fractions: the lysine rich histones (lys/arg greater than 5, and in the Luck terminology, histone I), the moderately lysine rich histones (lys/arg slightly over 1, histone II in this discussion) and the arginine rich histones (lys/arg less than 0.8, histones III-IV).

The most convenient way to characterize the various histone fractions is by acrylamide gel electrophoresis. We will use this method in these studies. The gel electrophoresis is carried out as has been described by Riesfeld, Lewis and Williams (9), and refined for histones by Fambrough and Fujimura (10). Their refinement consists of using 10 F urea in the gels, thus giving very much sharper bands.

The identity of the different polyacrylamide gel bands from whole calf thymus histone is established by comparing the pattern of whole acid extracted histone with the pattern shown by the well characterized fractions purified on an Amberlite IRC column (11). Typical band

Figure 1

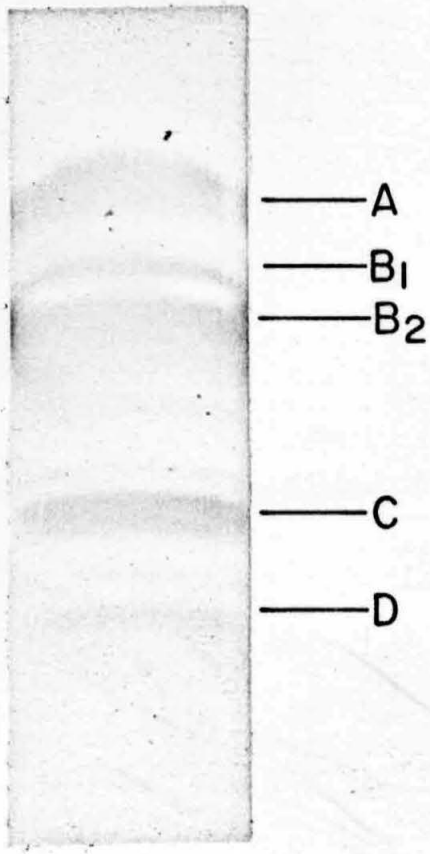
Here is a representation of the results of running whole histones and the various Amberlite-IRC column-purified fractions on polyacrylamide gels. The D band, though represented by a single band in whole histone pattern, is actually a series of faint bands. The III-IV pattern shows D bands, and many more which move slower. From these gels, we identify the bands as follows: A = one of the histone III-IV components, B_1 = the other III-IV component, B_2 = histone II, C = histone I. The D bands seem to be mostly histone III-IV components. The direction of migration is upwards. Dotted lines represent very faint bands.

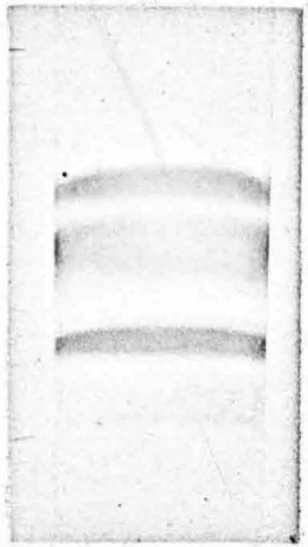


IDENTIFICATION OF BANDS IN GEL ELECTROPHORESIS

Figure 2

The figure shows two typical gels of whole histones. The gel on the left has been run 2 1/2 hours at a current of 4 ma., and maximum resolution has been obtained. The other gel, run for 1 hour at 3 ma. per tube does not show the same good resolution. The bands are labeled as is described in the text. About 50 micrograms of protein were applied initially. The direction of migration is upwards.


A
B1
B2
C
D


A
B(B1 + B2)
C
D

patterns for whole histone are shown in figures 1 and 2. There are three major bands that can be detected under all conditions (which we have designated, for the purposes of this discussion, A, B and C). There is in addition a group of slower bands which is much fainter (collectively designated the D components). In favorable gels, the B band resolves into two components, a faster minor one which we call B_1 and a slower major component (B_2).

We may summarize the identity of these bands as follows: the A band is for a protein which is a component of the III-IV (arginine rich) fraction, the B_1 band another III-IV component, the B_2 band is histone II and the C band the main component of histone I. The D bands seem to be mostly histone III-IV components, though it appears that histone I has a band in this region as well. B_1 and B_2 are not generally resolved and the resultant fused B band has considerably more histone II than III-IV. Histone I has a minor component that contributes very slightly to the B band. Compared to the other components, this component is negligible and can only be detected in fairly overloaded gels. A diagrammatic representation of the gels of whole histone and of the purified fractions is shown on figure 1.

If column purified histone I is run on the gels, it is found that over 90% of the protein travels in band C. There are in addition, two very much fainter bands, one with the mobility of band B and another with a mobility intermediate between B and C. These faint bands contain at most 10% of the histone I fraction, if the intensity of the bands is any indication of the amount of material in them. Histone

I is divided into two subgroups on the basis of the Amberlite-IRC column behaviour (11), which have been called Ia and Ib. We have run both subgroups on the gels and have found that they give identical patterns except that histone Ia seems to have a very faint band that moves in the D region.

Histone II travels almost exclusively in a single rather broad band, B. In gels with very good resolution, it can be shown that the histone II component of B is the B_2 band. In the particular standard we used, there were faint traces of the A band, which is probably due to some contamination by histones III-IV.

The histone III-IV fraction gives an extremely complex band pattern, as is shown on figure 1. There are two major bands (corresponding to A and B_1 in our whole histone band pattern), a pair of bands going slower than band C (in the D region), and a series of still slower pairs of bands. These last bands are regularly arranged, and their intensity decreases with decreasing mobility. These regular pairs of bands are probably not separate proteins but merely aggregates of the major bands. Their regular spacing and decreasing intensity suggests this, and such bands are generally not seen in the whole histone pattern, especially if the material has been freshly acid extracted. Apparently, dimers, trimers and higher aggregates form in the process of purifying this fraction and lyophyllizing it. On the other hand, the two major bands and the D bands are always present.

We will, in the discussion that follows, be concerned with the

components that travel in the A, B_1, and C bands which make up over 90% of the total histone. There is some variation in the apparent relative intensities of the bands in gels of histones from different calf thymus nucleoprotein preparations. The A band is darker than B_1 in most patterns that we have studied, but the reverse is true in gels from a few preparations. The biggest variation is in the intensity of the C (histone I) band. This is a function of how long the chromatin is left standing as a sucrose pellet before it is sheared. The longer it is left standing, the greater the amount of histone I that remains with the pellet material on shearing, and the lower the histone I yield from the soluble nucleohistone.

SOLUBILITY OF NUCLEOHISTONE IN NaCl

Nucleohistones have long been known to be insoluble at all but very high concentrations of NaCl (12). In the course of these studies, we have observed that above 0.1F NaCl and below 0.5 F, the chromatin is greatly aggregated (by observing either visual precipitates or measuring scattering at 3500 A°).

As a first step in salt extraction of histones, the chromatin is dialyzed against three changes of NaCl of the desired concentration. In 0.3 F NaCl, essentially no (less than 2%) DNA can be detected in the supernatant after spinning in a clinical centrifuge for 5 minutes. In 0.4 F NaCl, a very characteristic gel like precipitate (quite like $Al(OH)_3$ or DNA when it is starting to dissolve) is formed. At this salt at a starting DNA concentration of 100 micrograms/ml., the unsheared chromatin is completely precipitated but around 30% of sheared chromatin

goes into solution.

There is a very dramatic change in solubility between 0.4 and 0.5F NaCl solutions. The nucleoprotein goes into complete solution at 0.5F NaCl. The rate of solution is slow, and the dialysis is crucial.

This behaviour has previously been observed by Frick (13) in his very precise and comprehensive studies on a nucleoprotein preparation, that was unfortunately, at least partly reconstituted (it had been exposed to 1F NaCl). His results showed that although the nucleoprotein is soluble in pure water, it is essentially insoluble between ionic strengths of 0.02 and 0.35. Between 0.35 and 0.51, he found a striking increase in solubility.

The behaviour that we have observed was consistent and reproducible for every preparation of nucleoprotein studied. As we shall show, the increase in solubility of the nucleoprotein parallels the dissociation from the DNA of one class of histones.

SALT EXTRACTION

All extraction steps were carried out between 4-6°C. Due to various transfer operations, however, short exposures to room temperature were unavoidable.

The chromatin or nucleohistone was dialyzed against at least three changes of NaCl of the desired concentration. Original concentrations of 50-200 micrograms/ml. of DNA were used. The dialysis was carried out as has been described in the second section of this

thesis.

After dialysis, (and in the case of 0.3 and 0.4F NaCl, removal of the precipitates by sedimentation in a clinical centrifuge for 5 minutes), the nucleoprotein is pelleted by sedimentation in an ultracentrifuge for the appropriate length of time. SW 25 and 39 (or 50) rotors, as well as the #40 rotor for the Spinco model L or L_2 centrifuges have all been used with satisfactory results. Using the SW 50 rotor, 45,000 RPM for 15 hours was found to pellet all the DNA down at all salt concentrations; for salt concentrations below 1F NaCl, 22,000 RPM in an SW 25 rotor for 48 hours is sufficient. A layer of 1.7 F sucrose is added to the bottom so that the pellet does not pack too tightly. After sedimentation, supernatants and pellets are separated. For 0.3 and 0.4F NaCl, the original precipitates on dialysis are combined with the pellets of the ultracentrifuge run. The pellets are washed with redistilled water to remove all salt. The rate of solution of DNA is slow, so washing results in negligible loss. The pellets are redissolved in water by adding to the pellet an equivalent volume of water, allowing a gel to form, then doubling the volume with redistilled water. It is possible in this way to effect complete solution.

The histones were isolated from the pellets by acid extraction with 0.2 F H_2SO_4, sedimenting off the precipitated DNA, then alcohol precipitation of the histones (alcohol/H_2O = 4 by volume, 2 days in the freezer).

The histones were isolated from the supernatants of the centrifuge runs by first exhaustively dialyzing against 1 F acetic acid to get rid

of the NaCl, then lyophyllizing. The histones are then dissolved in 10 F urea, which dissolves them completely. Gel electrophoresis was then carried out as has been described.

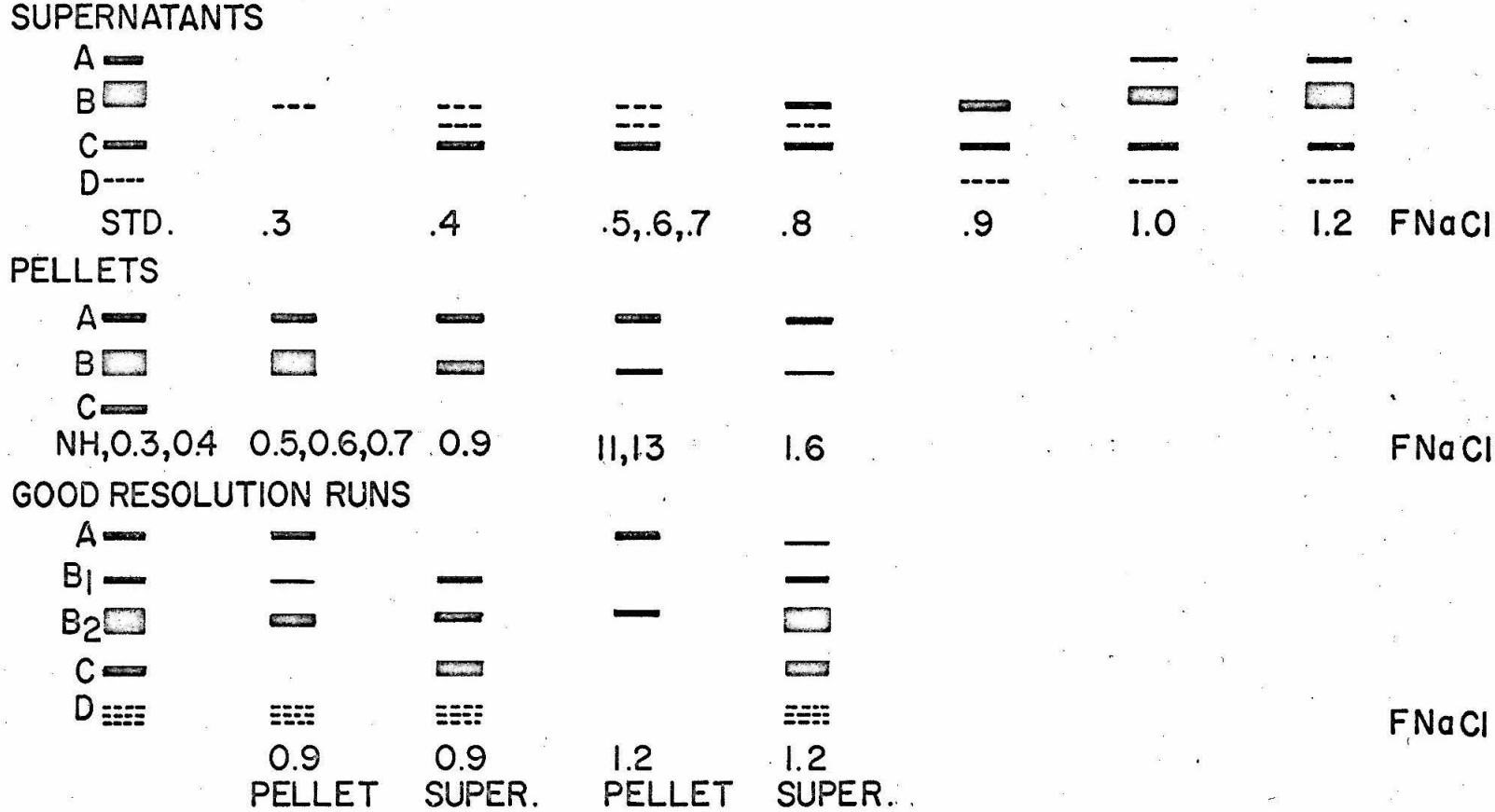
RESULTS OF SALT EXTRACTION: GEL ELECTROPHORESIS OF PELLET AND SUPERNATANT HISTONES

We identify which histones are associated to, and which are dissociated from the DNA at a particular salt concentration by gel electrophoresis of the histones from the pellet and supernatants of centrifuge runs at the salt concentration of interest. In the case of the pellet (DNA-associated) histones, we look for the diminution or disappearance of the various histone bands as we go to higher and higher salt. Bands that disappear from pellet gel patterns should appear in the supernatant gels. However, there is a possible complication. By acid extraction, we select for the histones and do not extract any non-histone protein. Since we are extracting with salt, non-histone proteins may also be extracted and remain in the supernatant, and will thus appear on the gels. It is possible therefore to find new bands when running the supernatant proteins.

A summary of the results of gel electrophoresis runs on the supernatants and pellets of sedimentation runs at various NaCl concentrations are shown on figure 3 and table I. The estimation of the amounts in the pellets and supernatants shown in table I was done visually, and though this is admittedly rather qualitative, it will be noted that in most cases bands are either completely present or absent. Where the decision is not so clear cut, averages were taken

Figure 3

A representation of the results of the gels from the supernatants (dissociated histones) and pellets (DNA-associated histones) of salt-extraction centrifuge runs. Since most gels were poorly resolved, this is how they are drawn here. A set of well resolved gels is drawn in for 0.9 and 1.2F NaCl, to illustrate that the B_1 component of histone III-IV dissociates ahead of the A component. An actual photograph of similar patterns for 1.0 F NaCl is shown on figure 11.



DIAGRAMMATIC REPRESENTATION OF RESULTS

Table I

GEL ELECTROPHORESIS OF SUPERNATANTS AND PELLETS OF SALT EXTRACTION CENTRIFUGE RUNS[a]

	Bands									
	A		B_1		B_2		B		C	
% Of Average[b] Preparation	10		10		50		(60)		25	
	% In Pellet And Supernatant[c]									
	A		B_1		B_2		B		C	
F NaCl Used For Extraction	P	S	P	S	P	S	P	S	P	S
0.3	100	0					95	5	100	0
0.4	100	0					95	5	80	20
0.5	100	0					95	5	0	100
0.6	100	0					95	5	0	100
0.7	100	0					95	5	0	100
0.8	100	0					85	15	0	100
0.9	95	5	40	60	50	50			0	100
1.0	80	20	10	90	20	80			0	100
1.1	70	30					15	85	0	100
1.2	60	40	0	100	15	85			0	100
1.3	60	40					10	90	0	100
1.6	30	70					5	95	0	100
2.0	5	95					1	99	0	100
4.0	0	100					0	100	0	100

a] Pellet histones are associated to the DNA at the salt indicated, supernatant histones are dissociated.

b] Estimated assuming that the amount of protein is proportional to the intensity of the band.

c] P=pellet, S=supernatant. The table should be read as follows: in 1 F NaCl (to take an arbitrary example), 20% of component A, 90% of B_1, 80% of B_2 and 100% of C are dissociated from the DNA and hence are found in the supernatant. In absolute amounts and arbitrary units, A=2(20% of 10), B_1=9, B_2=40 and C=25.

of several estimates usually involving more than one set of gels. It was felt that we did not have sufficient control of the amount of protein initially put on the get to justify gel traces on a densitometer. We have instead taken photographs of important gels to illustrate essential points in the following discussion.

Our results show that histone I (band C) is dissociated at the lowest salt concentration (between 0.4 and 0.5F NaCl). Histone IIb (B_2) and one component of histone III-IV (band B_1) are dissociated from the nucleoprotein next (dissociation starting at 0.8F NaCl). The component of histone fraction III-IV which gives rise to the A band is the very last to be dissociated. It is possible to dissociate histone I without any significant dissociation of other fractions. However, the dissociation range of histone II and the two components of III-IV overlap to a considerable extent.

We see that histone I is removed at the lowest ionic strength by the very striking disappearance in the gel patterns of histones from the pellets of band C between 0.4 F and 0.5 F NaCl. In some samples the pellet C band disappears completely in 0.5 F NaCl, while in other runs a much reduced band remains (figures 4 and 5). It is not clear whether this variability in behaviour is because of variations in the nucleoprotein preparations or in the extraction procedures, but in all cases, the greatest change in binding occurs in this narraw salt range. The only bands that can be detected from supernatants of runs between 0.5 F and 0.7 F NaCl are those of the histone I fraction. Apparently the fastest faint band of histone I is extracted first,

Figure 4

Gel patterns of histones acid extracted from: a) nucleohistone, b) nucleohistone treated with 0.3F NaCl, c) with 0.4 F NaCl, d) with 0.5 F NaCl. Extracted nucleohistone was separated from dissociated histone by sedimentation in an SW 25 rotor at 22,000 RPM for 48 hours. Note the disappearance of the histone I band in pattern d). The intensities of the bands vary from gel to gel because of variations in initial amounts of protein, and only relative intensities of the various bands in each gel are important.

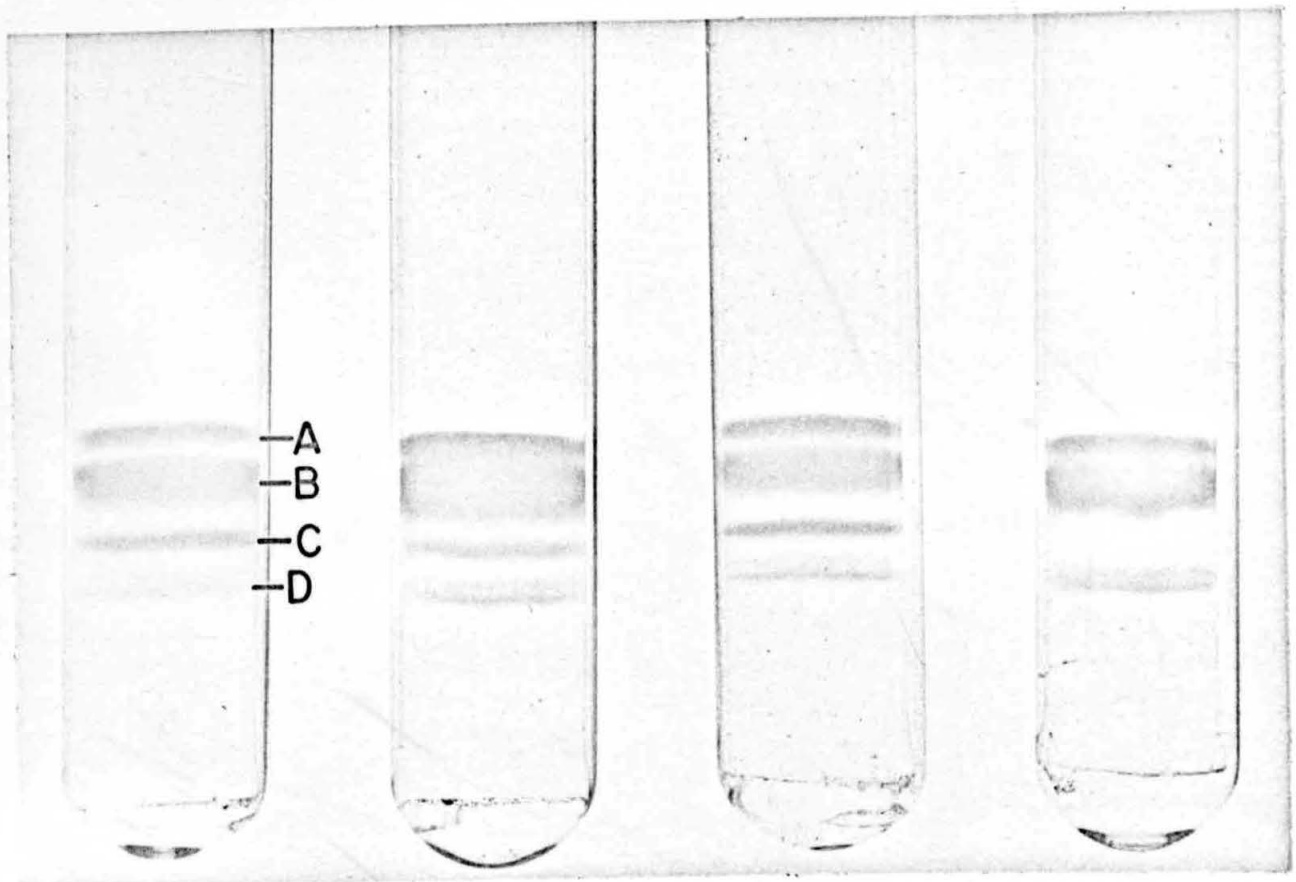
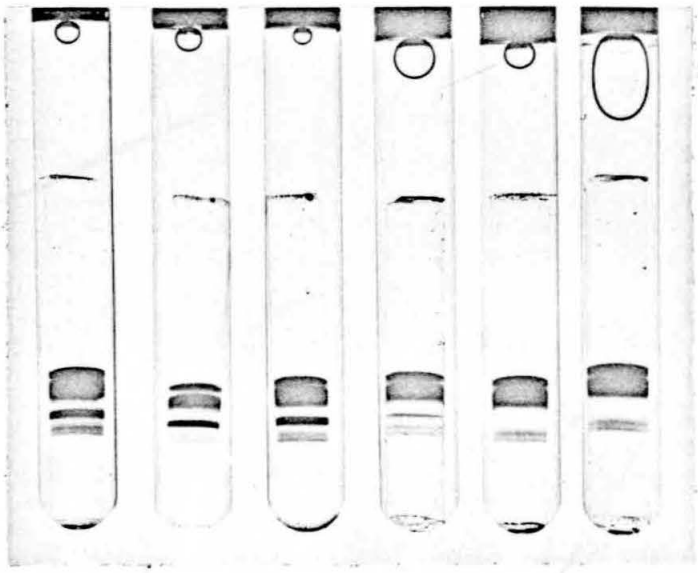

A
B
C
D
a.
b.
c.
d.

Figure 5

Gel patterns of histones acid extracted from: a) nucleohistone, b) nucleohistone treated with 0.3 F NaCl, c) nucleohistone treated with 0.4 F NaCl, d) with 0.5 F NaCl, e) with 0.7 F NaCl, f) with 0.8 F NaCl. Though the histone I band in pattern d is much weaker than in a, b, or c, it is detectable (in contract to gel c of figure 4). In e and f it is absent. The absolute intensities of the bands are unimportant because of variations in starting material. There has been a slight variation in the shrinking and swelling of these particular gels.



a. b. c. d. e. f.

since in the 0.3 F supernatant, band C is missing but this very faint band is present. By 0.4 F NaCl, band C is present in the supernatant, and the gel patterns of the supernatants from 0.5-0.7 F NaCl look pretty much like the pattern of column purified histone I.

The removal of histone I parallels a sudden increase in solubility of chromatin. In 0.3 F NaCl, histone I is completely bound to the DNA and the chromatin is insoluble. In 0.5 F NaCl, the histone I is dissociated and the chromatin is completely soluble (in the concentration range used). It is not clear whether this sudden increase in solubility is due to the increased repulsion between DNA strands when histone I is dissociated, or whether histone I has some special properties that cause aggregation of the nucleoprotein. There is support in the literature for the latter possibility (14).

In 0.4 F NaCl, the histone I begins to be extracted and most of the nucleoprotein is precipitated in a gel-like precipitate as has been described. A fraction of the sheared chromatin (30%) is in solution however, in a relatively non-aggregated form (no appreciable scattering at 3500 A°). There is no difference that can be detected in the histone content of the nucleoprotein in solution and in the precipitate; they appear to have equivalent amounts of histone I. It might have been imagined that the reason some of the nucleoprotein dissolves is because the histone I on it has dissociated. Therefore it would be different structurally from the nucleoprotein in the precipitate. However, as far as can be detected, there are no gross differences between the dissolved and precipitated nucleoprotein. Our assay is rather crude

and would not be able to detect subtle changes.

There is no extraction of histone II or III-IV at 0.7 F NaCl or below. Detectable dissociation begins at 0.8 F NaCl (see figures 6, 7 and 8). Most of histone II is extracted by 1.0 FNaCl. A look at the supernatant bands shows that at 0.8 F NaCl, band B is still much lighter than band C, but darker than would be the case if only histone I were present (see figure 7). In 0.9 F NaCl supernatants B_2 and C are of approximately equal intensities and in 1.0 F NaCl supernatant, the B_2 band is much darker than the C band, indicating that there is much more histone II in the supernatant than I. Correspondingly, the bands from the pellet histones show the B_2 band to be getting lighter with respect to the A band.

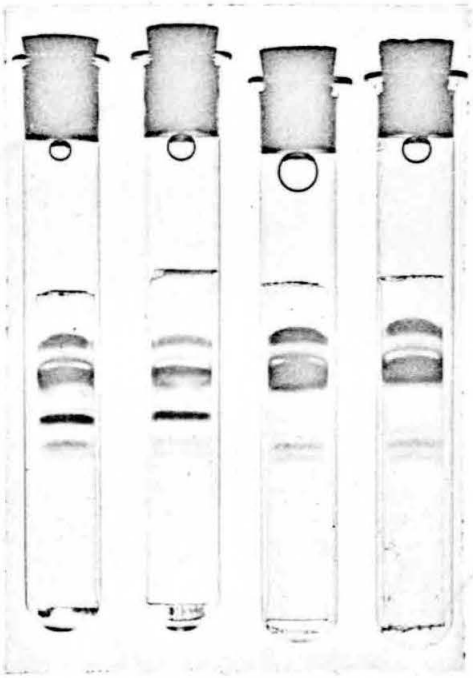
Figures 9, 10 and 11 show gels of histones associated to and dissociated from DNA in 0.9, 1.0 and 1.2 F NaCl. Resolution of the II and III-IV components (B_2 and B_1) of the B band was achieved. It appears from this set of gels that band B_1 extracted into the supernatant with and perhaps ahead of histone II (band B_2).

On the other hand, band A histone seems to be extracted later than either B_1 or B_2. In the 0.9 F supernatant, the B_1 band is very much darker than the A band, which is present only as a faint trace. By 1.2 F the two intensities are more nearly comparable (figure 9). Conversely, in the pellet, A and B_1 are of similar intensity in 0.9 F NaCl but the B_1 component is absent in 1.2 F NaCl pellets (figure 10).

These results definitely indicate that at or above 1.2 F NaCl the

Figure 6

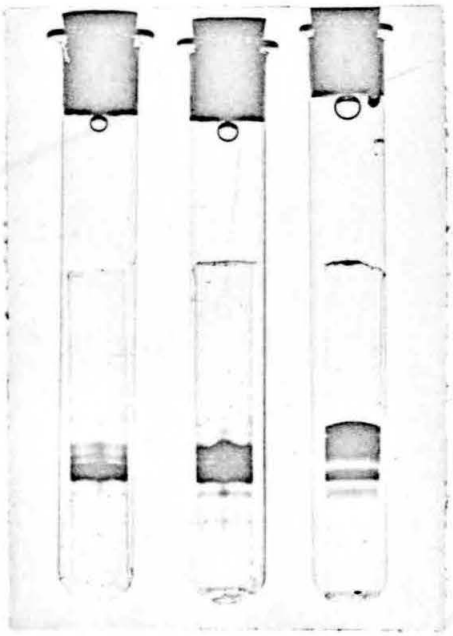
Well resolved gel patterns of histones acid extracted from: a) nucleohistone, b) nucleohistone treated with 0.3 F NaCl, c) with 0.7 NaCl, d) with 0.8 F NaCl. The extracted nucleoprotein was separated from dissociated histone by sedimentation in a #40 rotor, at 36,000 RPM for 36 hours. In c and d, the histone I band is missing, but all other bands appear to be present.



a. b. c. d.

Figure 7

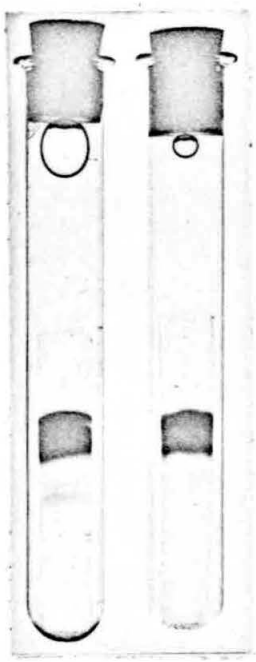
Gel patterns of the histones salt extracted by: a) 0.7 F NaCl, b) 0.8 F NaCl. The c) pattern is a standard, showing histones acid extracted from nucleohistone. The dissociated histones were obtained from the same sedimentation run as the DNA-associated histones in figure 6. Patterns a and b are the supernatant histones, while patterns c and d of figure 6 are the histones extracted from the pelleted nucleoprotein. It will be noted that while in figure 6, no difference can be seen between patterns c and d, patterns a and b show that though only histone I seems to be extracted by 0.7 F NaCl, some B band histones are being extracted by 0.8 F NaCl.



a. b. c.

Figure 8

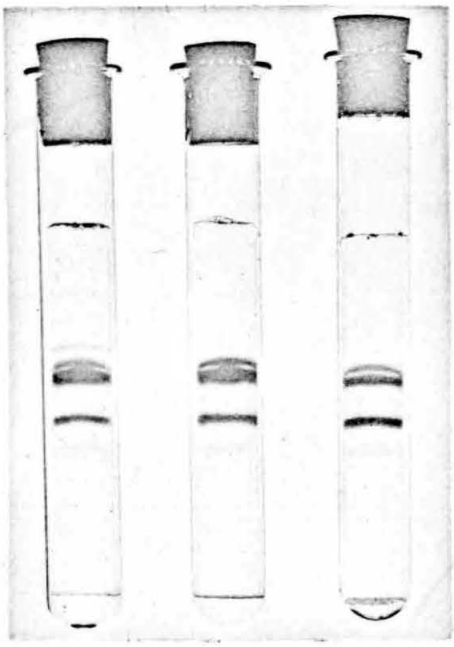
Gel patterns of histone acid extracted from: a) nucleohistone treated with 0.8 F NaCl, b) nucleohistone treated with 1.3 F NaCl. The slower moving band is much darker in the 0.8 F NaCl extract, but in the 1.3 F NaCl extract the faster moving (A) band material is darker. Thus the slower moving (B) band material is extracted at a lower salt than the fast-moving (A) band material. The histone I (C) band is not present in these patterns since it is completely extracted at these salt concentrations. Sedimentation performed at 39,000 RPM in SW39 rotor for 24 hours.



a. b.

Figure 9

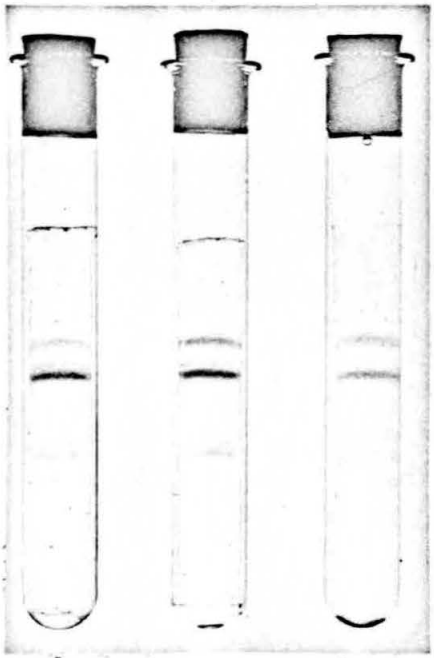
Gel patterns of histone extracted by: a) 1.2 F NaCl, b) 1.0 F NaCl, c) 0.9 F NaCl. In these gels, the B band has been resolved into its III-IV (B_1) and II (B_2) components. There are variations in the total amount of protein applied on the gel (not correlated to the amount of total histone extracted), so only the relative intensities of each band are important. Sedimentation to separate the dissociated histones from the nucleohistone was done in an SW50 rotor, 40,000 RPM, for 18 hours.



a. b. c.

Figure 10

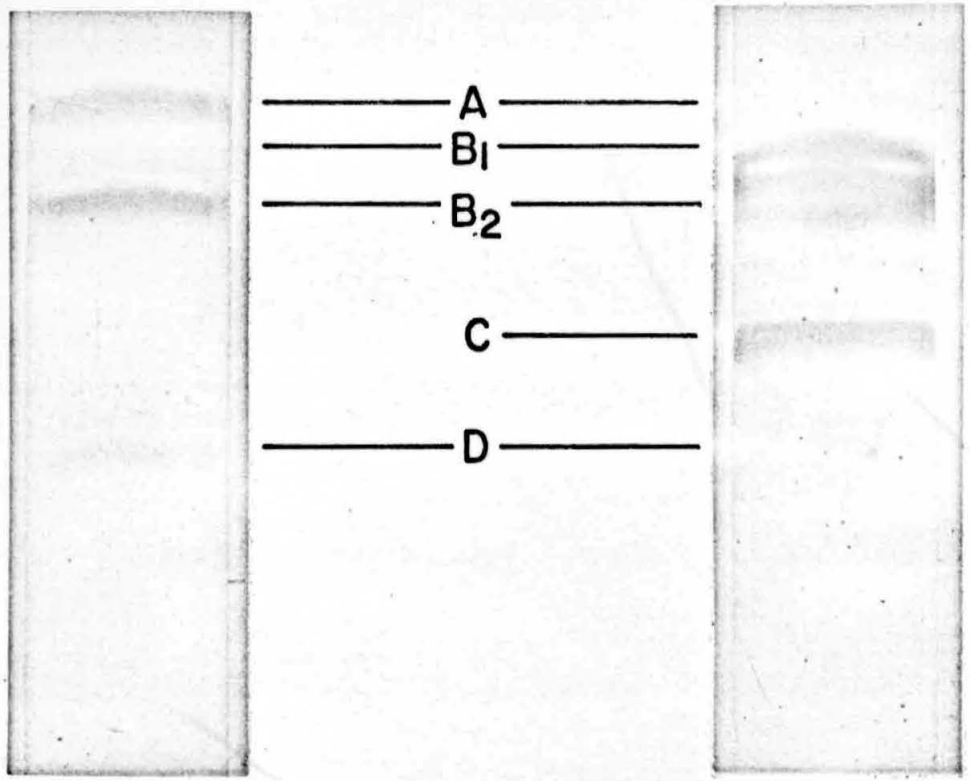
Gel patterns of histones acid extracted from: a) nucleohistone treated with 0.9 F NaCl, b) nucleohistone treated with 1.0 F NaCl, c) with 1.2 F NaCl. The sedimentation was done at 40,000 RPM for 18 hours, in an SW50 rotor. These correspond to the pelleted nucleohistone of the sedimentation run, while the patterns in figure 9 are the histones that have been extracted at each salt. Only the relative intensities of the bands are important.



a. b. c.

Figure 11

A comparison of the gel patterns of histones extracted from, and associated to DNA at 1.0 F NaCl. These are the same as the b gels in figures 9 and 10. The amount of histone applied in the unextracted histone case was somewhat disproportionately low, so if one wanted to make a comparison of the amounts, the bands in the gel on the left should be made about 50% darker. We see that all of histone I is extracted, and most of histone II (B_2 band) and the B_1 component of III-IV. The A component is still associated to the DNA. This salt concentration is especially interesting because it has long been used in the preparation of nucleohistones. These gels show that most of the histones dissociate from the DNA under these conditions.



UNEXTRACTED HISTONE PATTERN

EXTRACTED HISTONE PATTERN

SALT CONCENTRATION 1 F Na Cl

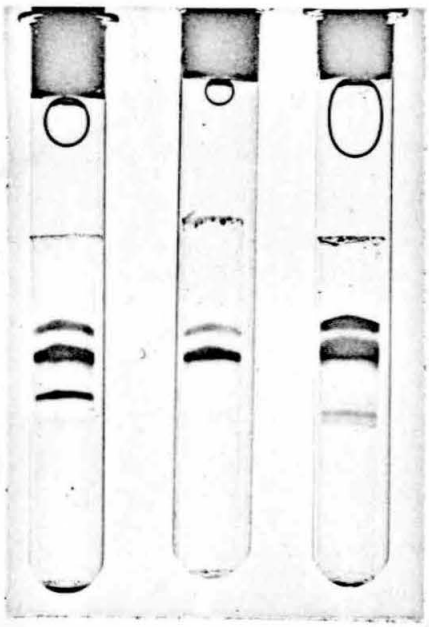
B band material (see figure 8) that we see in poorly resolved gel patterns of DNA-associated histones is almost all histone II. At these high salt concentrations, about equal amounts of histone II and III-IV are left on the DNA, all of the III-IV arising from the component that travels in the A band on gel electrophoresis. However, histone II is present in much greater amounts than the A component of histone III-IV in the original nucleohistone, so at 1.2 F NaCl, less than half of A but about 80-95% of B_2 (histone II) is dissociated. These experiments do not permit us to definitely say that the B_1 component of histone III-IV is dissociated at a somewhat lower salt than histone II. This is because gel electrophoresis will allow us to detect 5-10% of histone II with certainty, but not 10% of the B_1 component of histone III-IV, as it is present in a much smaller total amount.

We will make a few remarks about the D bands. These slow moving bands can, in favorable cases, be resolved into at least three components. At least two of the bands seem to be III-IV components, and perhaps one is a I component. It is possible that non-histone components also give bands in this region. The 4 F NaCl extract shows some D bands that are not present in whole acid extracted calf thymus histone.

Since all these D components make up 5% or so of the total, it is difficult to detect them. We will limit ourselves to saying that though the supernatant gel patterns show D bands at salts as low as 0.4F NaCl, D bands can still be detected in pellets that have been treated with salt concentrations above 1 F NaCl. A serious attempt

Figure 12

Gel patterns of histones acid extracted from a) unsheared chromatin treated with 0.3 F NaCl, b) with 0.5 F NaCl, c) with 0.8 F NaCl. Sedimentation is a #40 rotor at 36,000 RPM for 36 hours. Different amounts of proteins were put on the gels, and only relative intensities of bands in each gel should be compared.



a. b. c.

to distinguish the differences in salt extractability of the various D band components has not been made.

Lastly, it appears that the dissociation properties of sheared and unsheared chromatin are identical. Figure 12 shows gels of histones from salt extracted unsheared chromatin. Histone I dissociates at the same salt concentration (between 0.4 and 0.5 F NaCl) for sheared and unsheared chromatin. We have not studied the dissociation of the unsheared chromatin above 0.8 F NaCl, but up to this salt concentration, there is no difference.

THE EXTRACTION OF NUCLEOHISTONE BY ELECTROPHORESIS

Though the results of our experiments on the extraction in NaCl and separation of the DNA and dissociated proteins by sedimentation of the DNA seem fairly clear cut, another method which gives different results has recently been used to separate extracted from unextracted histones (15). In this scheme, the nucleoprotein is suspended in a NaCl solution of the desired concentration, and after thorough mixing, an equal volume of ethanol is added. This precipitates all the DNA as well as some histones, presumably those associated on to the DNA. Histones are not insoluble at these concentrations of ethanol, and therefore any dissociated histones are left in solution. This treatment gives results that are distinctly different from the results we have described. Histone III-IV is found to come off at concentrations as low as 0.4F NaCl, and by 0.7F NaCl, all the histones appear to be dissociated. The differences are explainable in two ways. One might say that although a certain histone is associated on the DNA at a

given salt concentration, the addition of an equal volume of alcohol may cause the histone to dissociate. Alternatively however, it is possible that in the sedimentation experiments, a given histone is dissociated at a certain ionic strength but because of aggregation phenomena, the dissociated histone sediments down with the DNA and we find it in the pellet.

To decide on which of the two alternatives is correct, we have performed an electrophoresis experiment on nucleohistone in a medium of 0.5 F NaCl, 0.05 F tris, pH 7.5. From the sedimentation results, we expect that at this salt concentration all the histone I would dissociate as we run the nucleohistone through the column, but histones II and III-IV should travel with the DNA. On the other hand, if histones III-IV are almost completely dissociated from the DNA at this salt concentration as the alcohol precipitation experiments suggest, then we would expect to find these fractions to be missing from the DNA after the nucleoprotein has been run a reasonably long distance down the column.

We have therefore performed the experiment by running a 3 cm. wide band of nucleohistone of an absorbance of about 2 at 2600 A° for a distance of 7.5 cms. down the column. In our apparatus this took 24 hours at a current of about 25 milliamperes. The band (which had spread out to 5 cms in this time) was collected and the DNA was sedimented down at 45,000 RPM in an SW 50 rotor in the model L-2 centrifuge for 15 hours. As a control, the same preparation of nucleohistone which had been dialyzed into 0.5 F NaCl, 0.05 F tris,

pH 7.5 and diluted to the same concentration as the nucleohistone that had been run on the electrophoresis column was spun down with it in another bucket. The pellets were then redissolved in water: after they had resuspended, they were extracted with 0.2 F H_2SO_4 in the usual way and the histones alcohol precipitated. The gel pattern of the histones from the nucleoprotein which had been subjected to electrophoresis was identical to the gel pattern from the nucleohistone which had merely been sedimented down in 0.5 F NaCl, 0.05 F tris, pH 7.5: they both showed the histone II and III-IV bands but no histone I band.

This result conclusively shows that at this salt, histones II and III-IV are bound to the DNA and our separation of the extracted nucleoprotein from the dissociated histones by sedimentation is a true mirror of which histones are bound. The alcohol precipitation of the DNA must therefore change the medium so that additional histones dissociate when the alcohol is added.

ELECTROPHORESIS EXPERIMENTS ON EXTRACTED NUCLEOPROTEIN

As we have shown in earlier sections of this thesis, DNA complexed on to histone has a considerably lower mobility than uncomplexed DNA. The elctrophoretic mobility is an excellent independent criterion for the extent of dissociation of histones from the DNA. We have therefore measured the mobilities of the nucleoproteins that have been extracted with various concentrations of NaCl.

After the chromatin has been dialyzed against a certain concentration

of NaCl and the nucleoprotein pelleted down by sedimentation, the resulting salt extracted nucleoprotein is redissolved in H_2O and its electrophoretic mobility measured in our usual medium (0.01 F NaCl, 0.001 F tris, pH 7.5).

The results of these experiments are shown on table II and figure 13. The very striking feature of the results is the two step rise in the mobility as the salt concentration used to extract histones is increased. The first abrupt rise in the mobility parallels the removal of histone I. The second more gradual increase reflects the removal of histones II and III-IV. The results reenforce our belief that histone I binding changes most radically between 0.4 and 0.5 F NaCl, and that histones II and III-IV are not appreciably extracted until above 0.8 F NaCl. A plot such as figure 13 is therefore equivalent to a histone dissociation curve.

It will be noted that the % rise in mobility upon removal of histone I is rather disproportionate to the amount of histone I present in native calf thymus nucleohistone (20-30%). This is entirely consistent however with the fact that histone I has a much higher positive charge density than other histones (16), and thus slows down the mobility of the DNA more. It was seen in the section of this thesis about reconstituted histone/DNA complexes that for the same histone to DNA mass ratio, the histone I complexes had lower mobilities than other complexes.

An important observation is that all salt extracted nucleoproteins

Table II

MOBILITIES OF NaCl EXTRACTED CALF THYMUS NUCLEOHISTONES

F NaCl Used For Extraction	Preparation 1	Preparation 2[a]	Preparation 3[b]
	(mobility, cm^2/volt-sec. x 10^4)		
Unextracted Nucleohistone	1.38	1.37	1.35
0.27	1.38		
0.30		1.40	1.41
0.40		1.48	1.42
0.45	1.53		
0.50		1.69	1.58
0.60		1.72	1.68
0.70			1.69
0.90			1.79
1.0			1.90
1.2			1.96
1.8	2.04		
4.0		2.07	

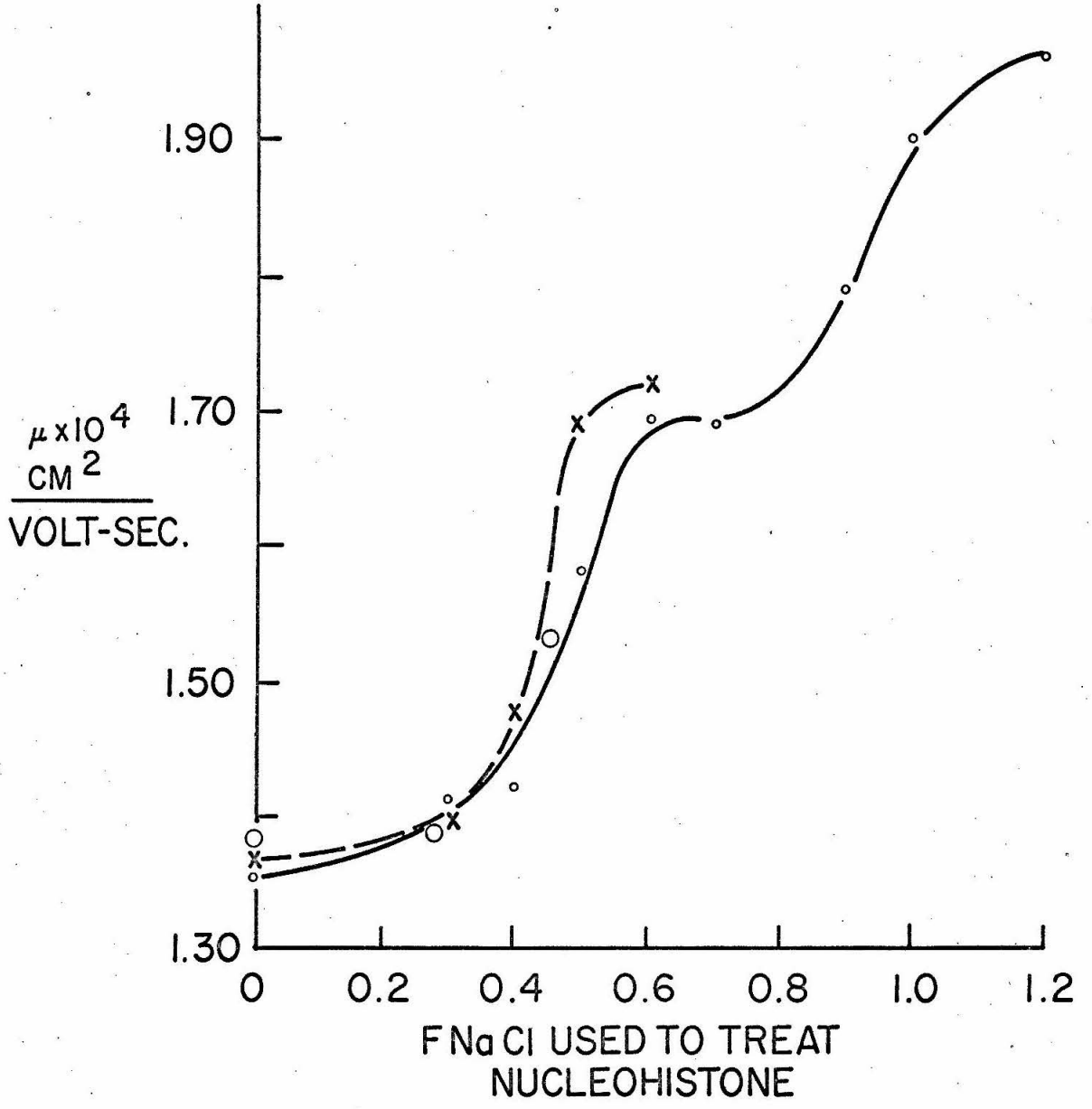
a] The histones of this preparation are shown on figure 4.

b] The histones associated and dissociated from the DNA at various NaCl concentrations for this preparation are shown on figures 5,9,10 and 11.

Medium: 0.01 F NaCl, 0.001 F tris, pH 7.5.

Figure 13

A plot of the electrophoretic mobility vs. the salt concentration with which the calf thymus nucleohistone preparation was treated. Points are shown for three different preparations. All electrophoresis experiments were done in 0.01 F NaCl, 0.001 F tris, pH 7.5. In all cases, the extracted nucleoprotein is obtained by dialyzing against the specified salt concentration, separating the undissociated nucleoprotein from dissociated histone by sedimentation, and redissolving the pelleted nucleoprotein in water.


1.90
1.70
1.50
1.30
μ x 10^4
CM^2
VOLT-SEC.
0
0.2
0.4
0.6
0.8
1.0
1.2
F Na Cl USED TO TREAT
NUCLEOHISTONE

travel as a single, if somewhat broad, peak. In general the peaks seem to be appreciably broader than either DNA or the unextracted nucleohistone, but there is never any evidence for a bimodal distribution of mobilities. There does not seem to be a detectable amount that moves like uncomplexed DNA or like unextracted nucleohistone; rather the electrophoretically independent units seem to have a uniform distribution of mobilities about a certain value. When we salt extract nucleohistone with intermediate concentrations (below 1F) of NaCl, we are therefore not getting molecules of free DNA that might move independently in an electric field.

It will be noted that the 4 F NaCl extracted chromatin still does not have the mobility of DNA. It is not clear why this is so: we have never been able to acid extract from the pellet of a 4 F NaCl centrifuge run any detectable histone (in 2 F NaCl, faint traces of histones may be discerned). We feel that the best explanation is that at these very high salts, some histone which has dissociated tends to sediment down with the DNA (since in these experiments the whole centrifuge tube is filled with the nucleoprotein at the salt of interest, except for a small layer of 1.7 F sucrose at the bottom, and the histones do have finite sedimentation coefficients). This histone, along with some non-histone protein, ends up in the pellet, and recomplexes the DNA when the pellet is redissolved in H_2O-- we would have to say however that such recomplexed histone is not acid extracted. There is some evidence that this is true. We have recomplexed salt extracted histone back on to a large excess of DNA, and have failed to recover by acid

extraction the histone so complexed. At any rate, if the pellets of such runs are resedimented at salts above 2 F NaCl, the resultant pellet does move with the mobility of DNA. The recomplexing of pelleted dissociated histones to the DNA could obviously be avoided if we used a band sedimentation or if we resedimented all samples. It was decided that the amounts required for histone characterization justified doing the experiments the way they were done. Moreover, if all the pellets were resedimented, the time required to resediment and again dissolve the nucleoprotein would cause an unpredictable amount of degradation, and since the nucleoprotein is known to degrade fairly rapidly, it was felt that it was safer to do the experiment by a single sedimentation. As it is, because of all the steps involved in histone extraction, the electrophoresis experiment could not be run less than four days after the nucleoprotein was prepared (though the salt extraction was always begun as soon after the nucleohistone was made as possible).

THE MELTING BEHAVIOUR OF THE EXTRACTED NUCLEOPROTEIN

Melting curves of the extracted nucleoproteins were also obtained. These were done in the usual medium of 2.5×10^{-4} F EDTA, pH 8.0. The results are shown in figure 14 and table III.

The general features are what one would expect: as one extracts more histones with higher salt, the melting temperature starts to become lower. As is seen in the figure, the removal of histone I by extraction with 0.5 F NaCl causes a 10°C fall in the T_m of the nucleohistone (66.4° vs. 76°C). It will be noted that the early part of the melting curve has been displaced to a lower temperature, but the high

Figure 14

Melting profiles of salt extracted nucleoprotein in 2.5×10^{-4}F EDTA, pH 8.0 (prepared by titrating the disodium salt to pH 8.0). The melting curves were done on the gilford multiple sample absorbance recorder.

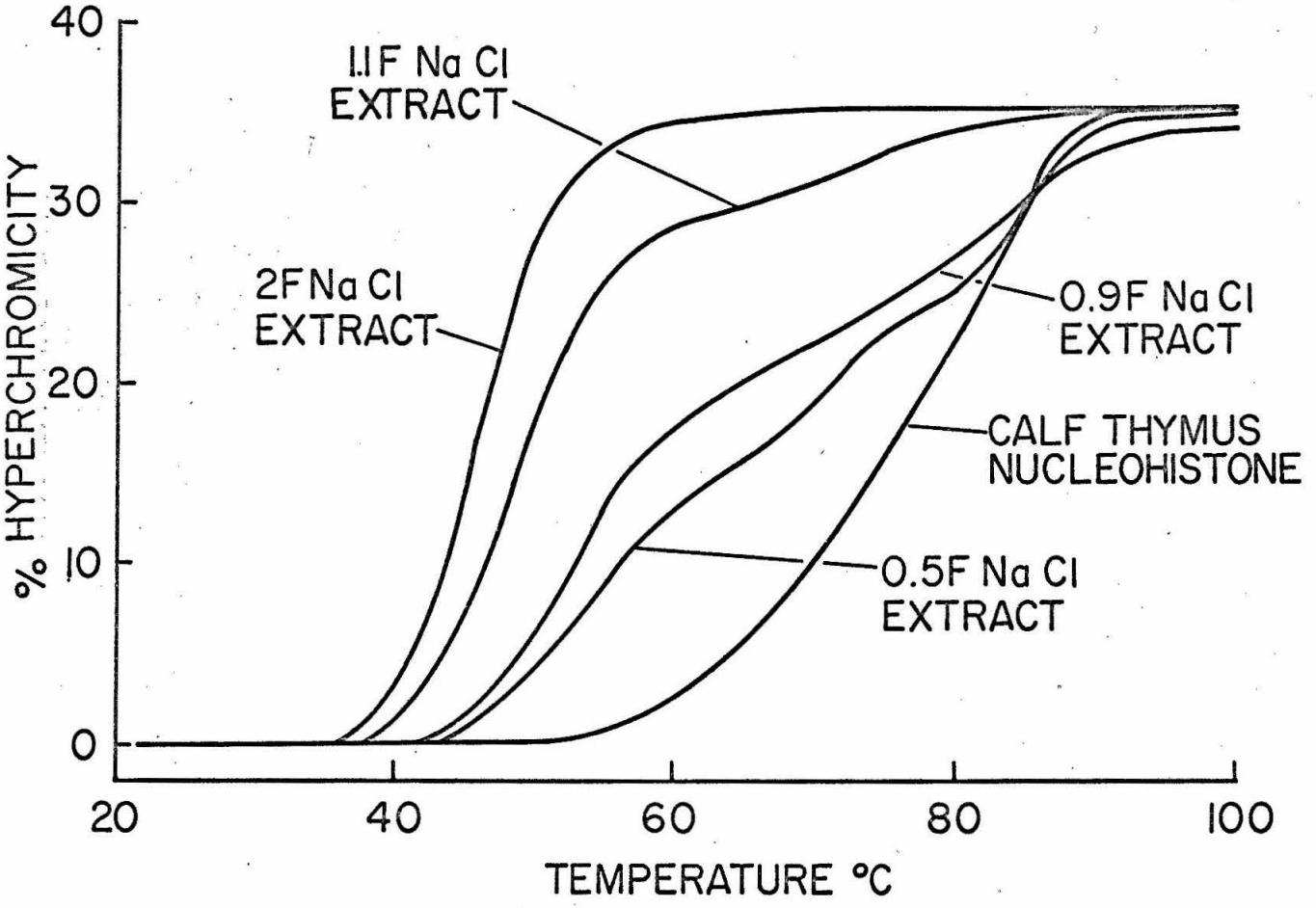

40
30
20
10
0
% HYPERCHROMICITY
1.1F Na Cl
EXTRACT
2F Na Cl
EXTRACT
0.9F Na Cl
EXTRACT
CALF THYMUS
NUCLEOHISTONE
0.5F Na Cl
EXTRACT
20
40
60
80
100
TEMPERATURE °C

Table III

MELTING OF NaCl EXTRACTED CALF THYMUS NUCLEOHISTONES

F NaCl Used For Extraction	T_m	$T_{10\%}$	$T_{90\%}$	$T_{90}-T_{10}$	%H
		(^oC)			
Untreated Nucleohistone[a]	76	60.2	86	25.8	35
0.4[a]	71.5	52.7	86	33.3	35
0.5[a]	66.4	48.8	86	37.2	36
0.9	60	47.2	86	38.8	35
1.1	48.8	41.3	67.5	26.2	35
2.0[b]	46.5	40.2	53	12.8	35
DNA[b]	47.5	40.2	53.5	13.3	37

Medium: 2.5×10^{-4}F EDTA, pH 8.0

a] The corresponding gels showing histones associated to the DNA for these samples are on figure 4.

b] The differences between 2.0 F NaCl extracted nucleoprotein and commercial Worthington calf thymus DNA are probably not significant. The values are within the scatter found for different commercial preparations.

temperature part remains very similar: about one third of the melting takes place above 80°C. The general shape of the curve does not change in the nucleoprotein that has been extracted by 0.9 F NaCl, where some histone II has been removed. The T_m has gone down by another 6°C though.

A very much more drastic change takes place in the melting profile of nucleoprotein which has been extracted with 1.1 F NaCl. At this salt concentration almost all of histone II and about half of III-IV has been extracted. The nucleoprotein now has a melting profile that is much more similar to DNA than to the nucleohistone, and a T_m of 48.8°C. Nucleohistone that is extracted with 2 F NaCl melts essentially like uncomplexed DNA.

The interpretation of these melting curves is difficult, but the results seem to indicate that the different histones are not uniformly distributed along the DNA, nor are certain regions of the DNA completely covered with exclusively one class of histone while another long region is covered by another fraction. The extremely broad melting curves obtained when histone I has been removed from the native nucleoprotein indicates either that the DNA must be very heterogeneously covered with histones, or that the histones that are left can transfer from denatured regions to double-helical regions of the DNA.

If we take that fraction of the melting curve below 55°C to indicate the fraction of the nucleoprotein which is uncomplexed or very lightly complexed to protein (DNA is 93% melted at this temperature, while native nucleohistone is less than 3% melted), we find that

removal of I and a little II by 0.9 F NaCl extraction results in 37% melting as DNA, while extraction in 1.1 F NaCl causes 85% to melt like DNA. If we examine how much of the transition takes place above 70°C as a measure of how much of the DNA is still almost completely complexed (the uncomplexed DNA melting is complete, but native nucleohistone has 70% more to go), we find for the 0.5 F NaCl extract, 45% of the transition is above 70°C, 37% of the 0.9F extract and only 9% of the 1.1 F extract. When we remove histone I, we thus see that 25% is DNA like, and 45% is nucleohistone-like (note that by this crude criterion only 70% of native nucleohistone is nucleohistone-like). The rest is presumably somewhat intermediate.

This interpretation is artificial and naive, but because of the lack of any other indications as to the relative histone distribution, it is used as a qualitative tool. Numerous flaws can be thought of: if two DNA double-helices were cross linked in two different and widely separated places by some histones for instance, and all the DNA in between were completely uncomplexed, such a configuration might not melt until a relatively high temperature because the histones would prevent the DNA from unwinding. We would in our crude assay count such a region as "nucleohistone", when it clearly would not be. Also, it is possible that there are really no fully covered regions in some of the salt extracts, but as the DNA is denatured, histones transfer from the denatured regions to the still native regions causing them to be fully covered and to have a high melting temperature. This would not therefore give us the real distribution of the histones in the unheated

sample. Heating-cooling experiments indicate that such histone transfer does occur.

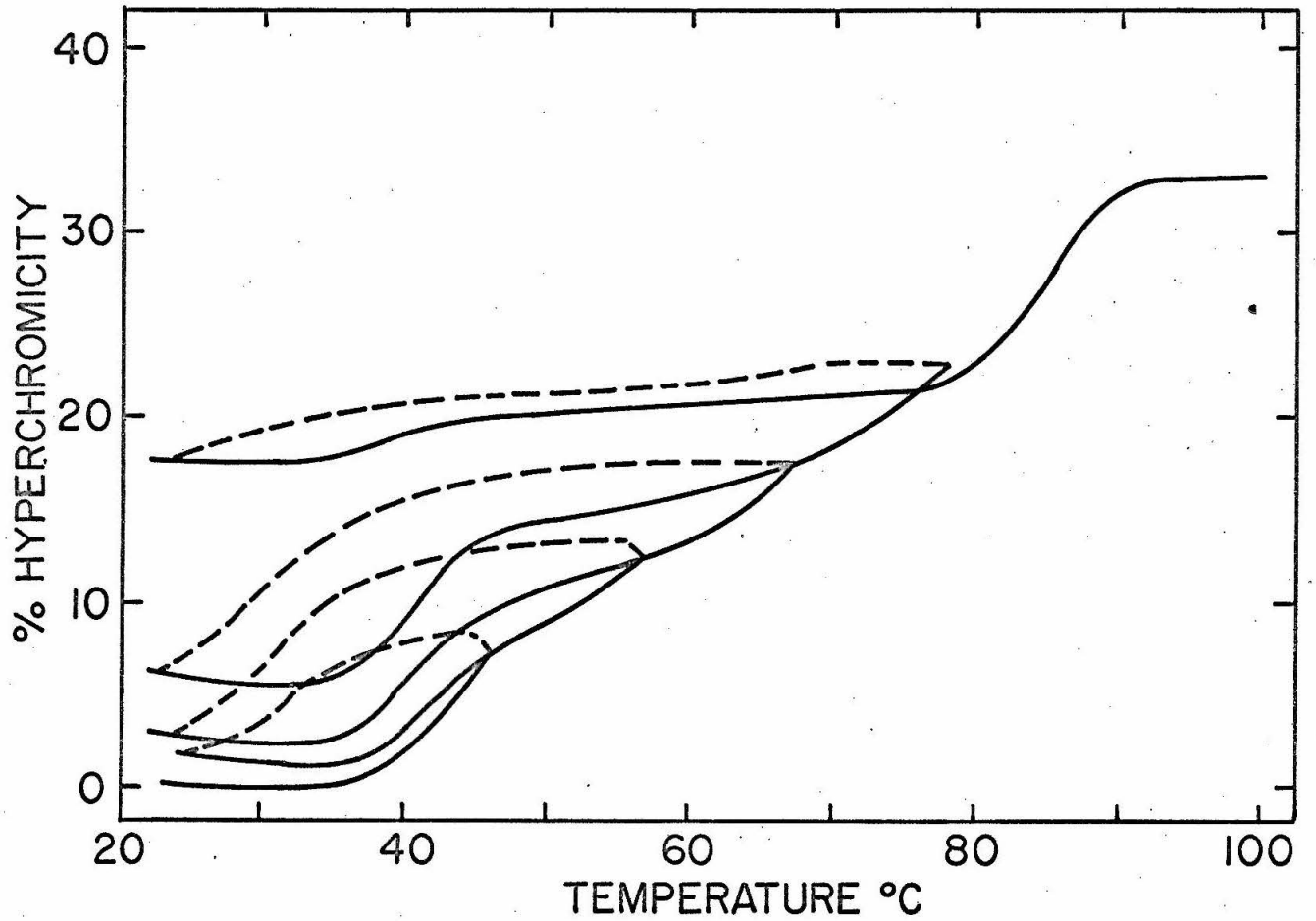
Figure 15 shows a heating cooling experiment on pea bud nucleohistone that was extracted with 0.36 F $NaClO_4$. The important result from this experiment is that if such a preparation is partially denatured, then allowed to reanneal, the reannealed native regions when reheated denature at a lower temperature than they did originally. The logical conclusion is that the histones which were complexed on to the DNA that denatured were dissociated from it when denaturation occurred, and did not recomplex back to it when the DNA was permitted to reanneal, probably because the histones had complexed irreversibly to other still native regions. Thus when such a reannealed preparation is reheated, then reannealed portions of the DNA, now uncovered by histone, melt at a temperature lower than they did originally. This property is not peculiar to salt extracted pea nucleohistones: calf thymus nucleoprotein shows the same behavior (17).

SOME RESULTS WITH $NaClO_4$; A COMPARISON OF NaCl AND $NaClO_4$

The use of $NaClO_4$ in the extraction of histones from nucleoprotein has recently been described in by H. Ohlenbusch (18). Most work done in cooperation with him is described in his thesis in sufficient detail so that discussing those results here is unnecessary. However, a number of experiments done independently of him are tabulated here. In order to fully compare the NaCl and the $NaClO_4$ extraction systems, we will also reconsider certain results that were included in his thesis.

Figure 15

Heating-cooling curve of pea bud nucleohistone extracted with 0.36F $NaClO_4$. The solid lines are heating curves, the dotted lines the cooling curves. The heating rate was constant, at 2/3°C per minute. The cooling rate was not constant, the chamber simply being allowed to cool by circulating water at room temperature around it after it had been heated to a given temperature. The important result from this experiment is that after heating and cooling, the reannealed DNA melts at a lower temperature than it did originally. If we follow the fourth heating curve and continue on heating until the melting was completed (ignoring the fourth cooling curve), we see a very marked two step transition, the first step corresponding to the melting of uncomplexed DNA. This would not have been the result if we had heated without any cooling. The medium is 2.5×10^{-4}F EDTA, pH 8.0.


40
30
20
10
0
% HYPERCHROMICITY
20
40
60
80
100
TEMPERATURE °C

Some of the electophoresis and melting curve results with $NaClO_4$ extracted nucleoprotein are shown in table IV. In general, these extracted preparations are less well defined than similar preparations in the NaCl studies. At the time these experiments were done, we were not using gel electrophoresis to characterize the histones, so it was not possible to do melting, electrophoresis, and histone characterization studies all on the same preparation.

A number of calf thymus nucleoprotein preparations were studied (see Ohlenbusch's thesis), but we have tabulated results only with those preparations which when untreated with salt had mobilities between 1.30- 1.40×10^{-4} cm.^{2}/volt-sec., as this was the range within which all the preparations used in the NaCl studies fell. Results with some pea nucleoproteins are also shown, in table V.

It is clear that the mobilities of nucleoprotein extracted with $NaClO_4$ are higher than the mobilities of nucleoprotein extracted with equal concentrations of NaCl. For instance, the two calf thymus preparations extracted with 0.5 F $NaClO_4$ had mobilities greater than 2.00×10^{-4}, while in the case of NaCl, even the 1.0 F extract had a mobility of only 1.90×10^{-4} cm.^{2}/volt-sec. Such results show that $NaClO_4$ is a more effective extracting agent than NaCl.

A comparison of the electrophoresis results for NaCl and for $NaClO_4$ is made in figure 16. The mobility data for the $NaClO_4$ extracts does not show the two step rise in mobility with increasing salt concentration of extraction that is exhibited by the NaCl data. This is probably

Table IV

MOBILITIES OF $NaClO_4$ EXTRACTED CALF THYMUS NUCLEOHISTONES

F $NaClO_4$ Used For Extraction	Prep. A[a]	Prep. B[b]	Prep. C[c]
	(mobility, cm^2/volt-secx10^4)		
Unextracted Nucleohistone	1.32	1.35	1.33
0.20	1.38		
0.27			1.44
0.40	2.01	1.92	
0.45			1.91
0.50	2.05	2.01	
1.00		2.16	

a] The salt extraction for this sample was done by centrifugation of a zone of nucleoprotein through a sucrose gradient in the $NaClO_4$ concentration of interest, and collecting the nucleoprotein before it was pelleted.

b] The salt extraction in this set was performed by sedimenting the nucleohistone twice through the medium of interest. H. Ohlenbusch performed the sedimentation runs.

c] These measurements are entirely by H. Ohlenbusch.

Table V

EXPERIMENTS ON $NaClO_4$ EXTRACTED PEA NUCLEOPROTEINS[a]

F $NaClO_4$ Used For Extraction	Pea Cotyledon Chromatin	Pea Bud Nucleohistone			
	Mobility[b] cm^2/volt-sec x 10^4	Mobility[b]	T_m[c]	%H	RNA SYNTHESIS[d] CPM
Unextracted Nucleohistone	1.53	1.27	77.2	34.7	100
0.36	1.83	1.60	65.6	33.6	455
0.45			53.7[e]	34.0	
0.54	1.91	2.03	45.2	33.2	895
0.72		2.16			1340
DNA (calf thymus)		2.18	47.5	37	1010

a] These nucleoproteins were gifts of Prof. J. Bonner and Dr. R. Huang.

b] Medium: 0.01 F NaCl, 0.001 F tris, pH 7.5.

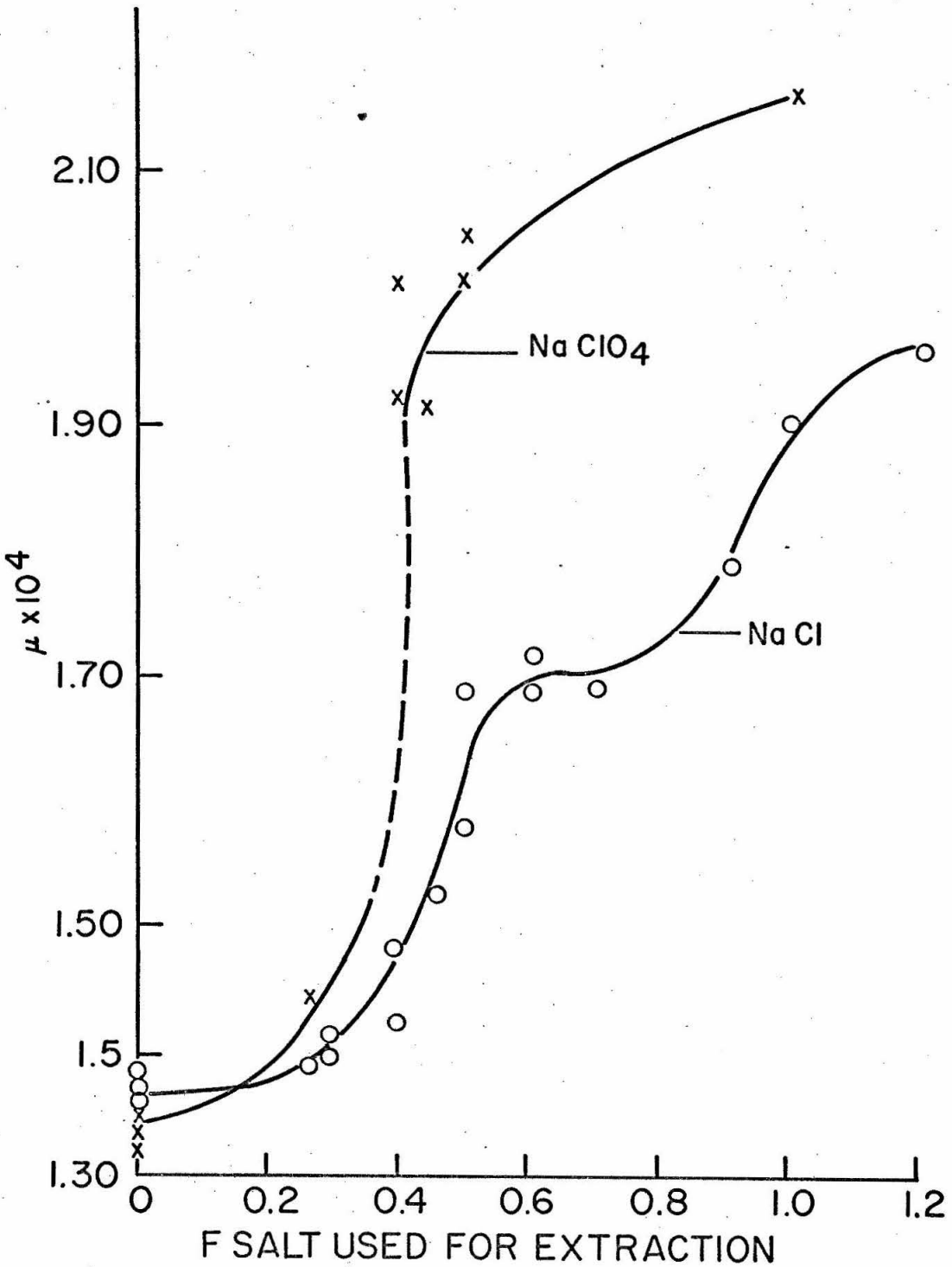
c] Medium: 2.5×10^{-4} F EDTA, pH 8.0.

d] These experiments were done by Dr. R. C. Huang. The background counts have been subtracted out.

e] A similar sample which was $NaClO_4$ extracted by electrophoresis through a medium of 0.4 F $NaClO_4$, 0.004 F tris, pH 7.5 had a T_m when melted of 49°C.

Figure 16

A plot of the electrophoretic mobility vs. salt concentration with which the calf thymus nucleohistone preparation was treated. The NaCl points are the same as in figure 13. The electrophoresis medium is 0.01 F NaCl, 0.001 F tris, pH 7.5. The $NaClO_4$ data are for several preparations (see table IV). We have drawn a dotted line in the $NaClO_4$ curve because there is a possibility that there is a plateau region in this curve between 0.27 and 0.45 F $NaClO_4$ as there is between 0.5 and 0.8 F NaCl.


2.10
1.90
1.70
1.50
1.5
1.30
μ x 10⁴
Na ClO4
Na Cl
0
0.2
0.4
0.6
0.8
1.0
1.2
F SALT USED FOR EXTRACTION

simply due to an insufficient number of points. Studies in which the extracted and unextracted histones were characterized by Amberlite IRC chromatography demonstrate that although the salt concentrations required are different, the order of removal of the histones by increasing concentrations of NaCl and $NaClO_4$ is the same. (Certain fine points, such as whether the two components of the III-IV fraction are extracted separately in $NaClO_4$ as they are in NaCl are not established.)

Melting studies of NaCl and $NaClO_4$ extracted nucleoprotein also suggest that the latter salt extracts histones at lower concentrations. Ohlenbusch (18) found that in a medium of $3x10^{-4}$ Na ion, a calf thymus nucleohistone preparation that had been extracted with 0.48 F NaCl had a T_m of 68.7°C; if 0.45 F $NaClO_4$ were used for extraction, the T_m was only 49.5°C.

Another proof that $NaClO_4$ is much more effective in extracting histones from DNA than is NaCl is the sedimentation studies of K. Marushige (19). He found that slightly sheared chromatin treated with 0.5 F NaCl had a sedimentation coefficient (S about 35) intermediate between that of unextracted chromatin (S = 46) and the DNA (S = 17) that could be extracted from it. On the other hand, if the chromatin were extracted with 0.5 F $NaClO_4$, the resultant extract had a sedimentation coefficient (S about 22) that was almost the same as of DNA, showing in agreement with the other results, that this concentration of $NaClO_4$ extracts nearly all the histones, while only histone I is extracted by 0.5 F NaCl.

All these different extraction studies were carried out at varying temperatures and under somewhat different sedimentation and (or) electrophoresis conditions, so they are in some cases not strictly comparable. However, it appears from the data that different extraction conditions yield comparable results, suggesting that despite slight changes in conditions from experiment to experiment and from worker to worker, the same salt concentration extracts the same histones. All studies support the conclusion that to extract a particular histone from DNA, the concentration of $NaClO_4$ necessary is approximately half that of NaCl.

This striking difference between the ability of the two salts to extract histones from DNA is explainable in two ways. First, we might say that dissociation of histone from DNA occurs at a lower salt concentration when perchlorate is the anion because perchlorate is more strongly bound to the positively charged groups on the histone than is the chloride anion. There is support for this in the literature: it has been shown that bovine serum albumin tightly binds approximately twice as many perchlorate ions per molecule as it does chloride ions (20). Another possible explanation is the effect of the perchlorate ion on water. It is known that perchlorate ion changes the aqueous medium such that it solubilizes many non-polar compounds, while the chloride ion tends to make the same compounds less soluble (21). Ions which cause a salting in effect of non-polar solutes into water have been called hydrophobic agents. This has been used to explain why presence of high concentrations of $NaClO_4$ lowers the melting temperature

of DNA (22).

In all probability, the two effects described above are interrelated. This possibility has been put forward by Cann (23) who used it to explain the peculiar effects of acidic perchlorate solutions on the solubility of albumin. He hypothesized that perchlorate ions near the surface of the proteins would tend to solubilize non-polar groups, "opening up" the protein somewhat. This would then permit some perchlorate ions to go into hydrophobic regions of the protein previously inaccessible to water and be complexed on to positively charged groups in the interior of the protein. Thus, more perchlorate ions would be tightly bound than the more highly hydrated chloride ions. This type of explanation seems entirely applicable to histones bound to DNA. We would expect from such reasoning $NaClO_4$ would dissociate histones from DNA at lower concentrations. The only surprising thing is the rather large magnitude of the effect.

REFERENCES

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Appendix 1

Modifications in the Electrophoresis Apparatus

The optical system of the electrophoresis apparatus described in the first section of this thesis was modified by Thomas Burke. The new optical system makes it possible to scan at any wavelength desired.

A spectrophotometer and photomultiplier were substituted for the simple mercury lamp and photocell arrangement first used. A Bausch and Lomb spectrophotometer with a deuterium lamp and a monochromator is used as the light source. The spectrophotometer is mounted on the same platform as the old mercury lamp, in such a way that the slit is horizontal. An I-P28 photomultiplier is used to measure the amount of light passing through the apparatus.

The normal working conditions are such that if one scans the electrophoresis column with 2600 A° light, the lamp current is 0.7 ampere and the photomultiplier voltage 430 volts. The output of the photomultiplier circuit under these conditions is in the 100 mv. range and a Model G-14 Varian recorder is used to keep a record of the scans.

The modified apparatus was used for most of the experiments in this thesis. The wavelength was generally set at 2600 A°. A few experiments were done at 4100, 2200 and 2300 A°. For these wavelengths, the photomultiplier voltage was merely adjusted until an output that could be conveniently used by the recorder was obtained.

Appendix 2

MISCELLANEOUS ELECTROPHORETIC MOBILITY RESULTS

Though the results tabulated here do not fit in well with the organization of the rest of the thesis, it is felt that they should be recorded. In general, the mobilities given are based on a single experimental measurement. Mobilities are given in units of [$x10^{-4}$ cm^2/volt-sec.] in all cases.

The supporting electrolyte concentrations used for the different experiments:

Medium A	0.01 F NaCl, 0.001 F tris, pH 7.5
Medium B	0.01 F $NaClO_4$, 0.0005 F borate, pH 9.0
Medium C	0.01 F NaCl, 0.001 F carbonate, pH 9.6

Material	Medium	Mobility
Nucleic Acids		(mobilities towards anode)
Native CT DNA	B	2.25
	C	2.40
Denatured CT DNA	B	1.93
	C	2.13
d-ATP[a]	A	2.13
d-GTP	A	2.19
d-TTP	A	2.22
d-CTP	A	2.07

Appendix 2 (cont.)

Material	Medium	Mobility
Histones[b]		(mobilities towards cathode)
Whole calf thymus	A	1.10,1.40
	C	0.82,1.03
Thymus histone, extracted by pH 1.9	A	1.41
	C	1.06
Nucleoproteins		(mobilities towards anode)
E. coli ribosomes	A	1.54
Histone I/denatured DNA (A_{2600}=0.31, A_{2200}=0.55)	A	1.11
HeLa cell unfractionated metaphase chromosomes (contaminated with whole nuclei)[c]	A	1.15(very broad peak)

a] The value of 2.13 for ATP with a charge at this pH of -4 is to be compared to the results with AMP, which with a charge of -1 had a mobility of 0.95 and with a charge of -2 had a mobility of 1.58. Poly A has a mobility of 1.94 (Tom Burke, unpublished results.)

b] The histones are a gift of D. Fambrough. All of the histone peaks are very broad on electrophoresis; the mobilities given are peak maxima.

c] A preparation by J. Hubberman.

PROPOSITION I

THE SLOWLY EXCHANGING AMIDE HYDROGENS IN PROTEINS

ABSTRACT

It is proposed that the technique of hydrogen exchange should not be used to measure the helical content of proteins. The experimental work on synthetic polypeptides (which is considered the main experimental evidence that amide hydrogens in helices are slowly exchanging) is questionable because of aggregation problems. Recent studies indicate that it is possible to have a high helical content and yet for rapid hydrogen exchange to occur. An examination of both synthetic polypeptide and protein hydrogen exchange data shows that there is no simple way to dependably correlate the % helix of a protein to the results of a hydrogen exchange experiment.

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Ever since the technique of hydrogen exchange was applied to proteins, it has been widely believed that hydrogens in alpha helices exchange slowly, while non-alpha helix amide hydrogens exchange rapidly. The number of slowly exchanging amide hydrogens has often been used to calculate the helical content of a protein (1). X-ray diffraction studies on proteins have shown that hydrophobic forces also play an extremely important role in protein structure (2). In recent years, it has been felt that hydrogens in hydrophobic regions would also be slowly exchanging, though the feeling persists that the helical content of the molecule is the major determinant (1).

Klotz and Franks have recently questioned this viewpoint (3). They studied the exchange rate of N-methylacetamide and found that the activation energy for the exchange reaction is about 20 Kcal. One of the major proofs that slowly exchanging hydrogens are in alpha helices was that the activation energy for the slowly exchanging hydrogens was 20 Kcal. (enough, in the original interpretation, to break several hydrogen bonds so that exchange could occur). The discovery that in monomeric methyl acetamide the same activation energy is required belies the interpretation, and suggests instead that this is the intrinsic activation energy needed in the exchange of an amide hydrogen. The same authors also had suggestive evidence that under conditions where association of N-methylacetamide occurred so that the amide hydrogen was hydrogen bonded, there was no significant change in rate (compared to the non-hydrogen bonded amide exchange). From this data, they felt that slowly exchanging amide hydrogens were a poor measure of helical content.

With this suggestive experimental evidence, it is perhaps pertinent to re-examine the hydrogen exchange data and ask the following questions: Do helical hydrogens really exchange more slowly than non-hydrogen bonded hydrogens? Can the differences in rates of exchange be used to estimate the helical content of a polypeptide or protein?

On examining the literature for hydrogen exchange in polypeptides, one finds that the major experimental evidence that would answer the above questions are studies on synthetic polypeptides by Elliot and Handby (4) and by Blout and co-workers (5). These studies if taken at face value would tend to indicate that helical hydrogens do exchange

much more slowly than non-helical hydrogens, the difference being very great. It takes a few minutes to achieve complete exchange of coil homopolymers but over 20 hours to get complete exchange of helical, hydrogen bonded hydrogens. However, on examining these studies more carefully, one finds that there is one complicating factor that is not critically evaluated: aggregation. Unfortunately, in both studies, one would expect that as the homopolymer got more helical, it would also become more insoluble.

Elliot and Handby (4) studied polybenzyl- L- glutamate, and found that if this polypeptide were first dissolved in deuterated dichloro-acetic acide (DCA) (which destroys the alpha helix), and then diluted to 10% DCA with chroroform, the amide hydrogens had all exchanged. If the reverse were done, that is the polypeptide first dissolved in chloroform, then diluted with DCA until the solution were 10% in deuterated DCA (a treatment which is known to preserve the alpha helix), there were hydrogens which had not exchanged and took several days to exchange. Unfortunately, this polypeptide is definitely known to associate in chloroform (6): it is therefore conceivable that the hydrogens are exchanging slowly not because they are in alpha helices, but because they are inaccessible to solvent due to aggregation phenomena. This clearly would not happen if the polypeptide were dissolved in the deuterated DCA first (a solvent in which the poly-peptide is known to be monomolecular) (6). Under those conditions, all the amide hydrogens exchange rapidly.

Similar objections can be raised against the experiments of Blout on polyglutamic acid. This polypeptide exists as a random

coil at pH's above 5, but as an alpha helix at lower pH's (7). It is found that as one lowers the pH, the polypeptide also gets progressively more insoluble, and though these workers used 1:1 water/dioxane to increase solubility, it is possible that the slowly exchanging hydrogens do not come about because of alpha helix formation but because of aggregation, and consequent inaccessibility of solvent. Due to uncertainties of this sort, these two studies cannot really be taken as reliable indications of how fast helix hydrogens exchange as opposed to non-helix hydrogens. One would have to do the appropriate molecular weight determinations in each solvent used to come up with a clear-cut interpretation.

Recent studies by a group of Japanese workers (8) have been suggestive. They studied the behaviour of polyglutamic acid at different pH's in water-- thus they are essentially repeating the Blout experiments in pure water. Because they used a tritium method in measuring exchange, they have more accurate data at much shorter times. Once again, aggregation is a complicating factor: though they claim that their solutions at low pH (the helix form) are clear when used, they concede that the solutions become turbid after standing several hours. We expect the effect of aggregation would be to slow down the rate of exchange, and if the aggregates are big enough, perhaps to prevent exchange of some hydrogens altogether. We could then look at the data of these workers as representing the slow limit of the exchange rate of the amide hydrogens.

Their results are revealing in two respects: first, it seems

that all the hydrogens at a given pH exchange with a single first order rate constant. This is a rather surprising result. We might have expected that at a pH where the polyglutamic acid is known to be 70% helical and 30% coil-like, we would have seen two classes of hydrogens, such that 30% exchanged very fast but 70% exchanged much more slowly. In fact all the hydrogens are exchanging at the same rate. The second feature is that this rate is really quite fast: the half life for the example cited above is 2.5 minutes.

At lower pH's where one would expect that aggregation would be more important, there was a great deviation from first order kinetics. The curve could not be represented by two first order terms either. Thus in a solution where according to optical rotatory dispersion (ORD) measurements the polypeptide was 100% helical, 10% of hydrogens exchanged in ten minutes, but 50% had not exchanged after an hour. The slowly exchanging hydrogens were thus exchanging much more slowly than would have been expected from the initial rate of exchange. This is most likely due to aggregation which would tend to support the contention that the two previous investigations mentioned had the same problem. A sample which had a helical content of 88% showed the following behaviour: 50% of the hydrogens had exchanged in 10 minutes, but 10% had not exchanged after an hour. The rapidly exchanging hydrogens are not coil hydrogens because then we would have expected only 12% to exchange rapidly. The 10% that are unexchanged after one hour are almost certainly in aggregated regions: we would on the basis of the initial rate, have expected only 0.2% to remain at this time. In solutions of

somewhat higher pH in which the helical content is still substantial, the hydrogen exchange does follow first order kinetics all the way down to 2% or so.

On the basis of these results, the authors suggested a mechanism of H exchange in this system. They imply that a static helix-coil model is insufficient. If by the criterion of ORD, the molecule is 70% helical, this does not mean that 70% of the amide hydrogens are always hydrogen bonded, while 30% are not. Rather, it means that an amide hydrogen is in the hydrogen bonded form 70% of the time, and in the non-hydrogen bonded form 30% of the time. Thus all amide hydrogens are equivalent, and exchange at a single rate. The authors suggest that the rate of exchange while the hydrogen is not hydrogen-bonding is so much faster than the rate while it is, that the rate in which all the hydrogens exchange can be correlated to the helical content. It should be emphasized that this rate is fast compred to what most people would call slowly-exchanging hydrogens. Therefore if polyglutamic acid with 70% helix (by ORD) were examined by hydrogen exchange in the classical way, by this criterion it would have a negligible helical content, since none of its hydrogens would be classified as "slowly exchanging."

Thus, the studies on synthetic polypeptides do not, contrary to what is commonly believed, indicate that amide hydrogens in random coils exchange too rapidly to measure, while helical amide hydrogens

exchange with half lives of hours or days. If a helical region were undergoing fast oscillation between H bonding and non-H bonding, though the time average helical content of such a region might be quite high, the hydrogens would still exchange at a fairly fast rate. If the helical region were in hydrophobic surroundings, it might not oscillate at all between H bonded and non-H bonded configurations, thereby exhibiting very slow exchange.

But what is the situation in real proteins? It is conceivable that in all real proteins, alpha helical regions are surrounded at least partially by other hydrophobic regions of the protein, causing all hydrogens in the helix to exchange slowly. We will now examine the H exchange data for three well-studied proteins: myoglobin (and hemoglobin), ribonuclease and lysozyme. All of these proteins have been studied by optical rotatory dispersion (ORD), and to varying extents, by X-ray crystallography. A comparison of the H exchange data with the data from other methods should give some indication of how dependable the technique is in measuring the helical content of proteins. The data is compared in table 1.

It seems clear that there is no unequivocal correlation between helical content and the H exchange data. The H exchange data are extremely variable; the value one obtains depends on what time one chooses to use as a cut-off point (so that hydrogens unexchanged at that time are regarded as being in non-coil regions). For ribonuclease

for instance, Englander (9) decided that the helical hydrogens were those that had a half life of 160 minutes. In the hemoglobin and myoglobin studies, all hydrogens unexchanged after 3 minutes were called helical amide hydrogens (ref. a of table); clearly, from the table, the two criteria are quire different. Perhaps not surprisingly, the criterion used in each case gave the closest agreement with the % helix estimated by other methods.

The pH of the experiment is also an important factor. For lysozyme, there are no detectable conformational changes between pH 2 and 12. However, the number of unexchangeable hydrogens changes radically between pH 3 and 6 (ref. e of table). It therefore appears that besides the fact that different workers have been using different time scales, the number of slowly exchanging amide hydrogens is affected by pH, solvent and temperature without any accompanying change in protein conformation. Though in the discussion sections of papers dealing with H exchange, it is often concluded that there is rough agreement between % helix and % hard to exchange amide hydrogens, an actual comparison of the data such as in table 1 shows that the agreement is really much poorer than it would seem on a cursory reading of the papers involved. Recent studies on non-globular proteins show even poorer correlations between hydrogen exchange and ORD. Hartshorne and Stracher recently examined several muscle proteins with widely varying helical contents (10). No correlation between helical content and number of slowly exchanging hydrogens was found.

Thus one of the proteins they studied had between 87-100% helical content by ORD measurements, but at pH 5.7 only 22% of its hydrogens were slowly exchanging. Another protein with 8% helical content under the same conditions had 18% of its hydrogens slowly exchanging.

In conclusion, the synthetic polypeptide studies, so often quoted as evidence that the method of hydrogen exchange can be used to estimate helical content, do not appear to give any credible support to this belief. In addition, a close comparison of actual H exchange data of some of the most intensively studied proteins shows that the method cannot be used in any simple way to estimate the helical content.

Table 1

Protein	pH	% Helix on the Basis of Unexchanged H's at: 3 min.	10 min.	1 hr.	24 hrs.	% Helix by X-ray Cryst.	ORD
Myoglobin[a]	6.5	50	43	27	10	75-80	75-80
	3.1	38	11	0			22-35
Hemoglobin[a]		60	50	32	14	75-80	75-80
Ribonuclease	4.7		34-46[b]				
	4.5		35[d]		12[d]		17[c]
Lysozyme[e]	3.2			70	60		
	3.9			55	48	40[f]	35
	6.3			20	10		
	4.5-5		45		15		

a) The data for hemoglobin and myoglobin are either from, or referred to in Beychok, S., C. de Loze and E. R. Blout, J. Mol. Biol., 4, 421 (1962).

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PROPOSITION II

THE BINDING OF T_1 RNAase TO DNA

T_1 RNAase is a guanine-specific ribonuclease which cleaves RNA into guanosine 3' phosphates and oligonucleotides terminating in guanosine 3' phosphates. It has been purified so that it gives a single component by electrophoresis and chromatography, it shows no DNAase activity or non-specific RNAase activity, it is extremely stable and can stand extremes of pH and temperature without appreciable loss of enzymatic activity (1).

It is proposed that the binding of this RNAase to DNA be studied, not only so one might learn more about non-electrostatic enzyme-specific binding of the nucleic acids, but also with a view towards practical separations of denatured DNA's of different guanine contents by either density gradient centrifugation or electrophoresis.

Felsenfeld and Von Hippel have shown that while pancreatic RNAase binds native DNA in a manner that is typical of positively charged proteins, it binds denatured DNA more strongly, presumably because denatured DNA is much more like its substrate, RNA, than is native DNA (2). It seems reasonable to believe that T_1 RNAase will bind denatured DNA in a similar manner.

A simple system in which one could study the binding of T_1 RNAase is crab d-AT. Native crab d-AT either should not bind the RNAase at all, or if it did, only very weakly. Renatured crab d-AT (3), which presumably has cytosine and guanine residues out of register provides a substrate with a limited number of sites that should bind much more

strongly. The advantage of this binding substrate is that we may be able to compare the binding strengths of the denatured regions in the renatured crab d-AT with cytosine out of register, with those in which guanine is out of register. Because of the enzyme's specificity, those regions with guanine should bind measurably stronger.

By comparing the difference in binding of native crab d-AT to the binding of the cytosine out-of-register regions in renatured crab d-AT, we may have some idea of the relative importance of the secondary structure of the substrate. We may also determine whether the binding involves the active site of the enzyme. This can be tested for by enzymatic tests (as has been done in the case of chymotrypsin-DNA complexes). The importance with respect to binding of the base could be evaluated by the difference in binding of cytosine and guanine out-of-register regions. This should clarify whether T_1 RNAase requires a single polynucleotide configuration for binding, or whether it requires both a single polynucleotide configuration and a guanine residue.

The results of this study should be specially interesting because the enzyme has a net negative charge (4), and one would therefore expect that any measurable binding would occur as a result of its being an enzyme with a substrate that is structurally similar to denatured DNA. We would not expect any non-specific electrostatic type binding: in this respect it is different and perhaps more interesting than is pancreatic RNAase which is positively charged. There is some indication in the literature that the base specificity of binding we would hope for should occur: Sato and Egami (5) have shown that guanylic acid

(specially 2' or 3') binds the enzyme at pH 5.0 much more strongly than the monophosphates of the other bases.

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PROPOSITION III

MEASUREMENT OF EQUILIBRIUM CONSTANTS BY GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis is a technique that has been recently developed which gives separations comparable to any previously used for proteins (1). It is possible to gel the acrylamide under a wide variety of conditions, and the gel itself is insensitive to changes in pH or temperature (2).

It is proposed that this method may be used to advantage for the easy measurement of the equilibrium constants between DNA and small cationic substances, most especially dyes.

The method we propose consists of preparing one gel of a certain concentration of polyacrylamide, and simultaneously preparing an identical gel except that the gelling medium has DNA. The DNA concentration may be varied within reasonable limits, but the polyacrylamide must be present in a high enough concentration so that the DNA cannot move through the resultant gel.

If we then electrophorese through the two gels a small enough molecule so that the exchange rate between the DNA and the cationic binding agent is fast compared to the rate of electrophoresis, the mobility of the binding agent in the gel without DNA is related to the mobility in the gel with the DNA (u and $\bar{u}$ respectively) by the following relationship:

$$\bar{u} = \frac{Ku}{(P) + K}$$

where K is the dissociation constant for the binding agent and the DNA phosphate, and (P) is the effective concentration of DNA phosphate in the gel (3). By a simple measurement of two mobilities, an equilibrium constant in any medium and at any temperature may be determined.

Let us examine our basic assumptions. First, it is necessary that the exchange between free and bound counterion be very fast compared to the rate of electrophoresis. This assumption is probably fairly true for most small molecules excpet for dyes which intercalate: under those conditions, the exchange might be much slower. It is possible that when two types of binding are present such as is believed to be the case for acridine (4), the dye may separate into two components, or into a regularly moving component, and a component that travels much slower and gets smeared out on the gel.

An implicit assumption is that the gel must act as a completely inert medium, which is more questionable. Control experiments have to be done to determine the extent of adsorption. It should be possible to correct for adsorption, even if it should occur to a significant extent.

Though the extent to which these complications may interface cannot be evaluated now, this method where applicable, will be easiest of all to use to determine equilibrium constants. As the gels are now run (16 samples at once), it would be possible to measure the binding constants of 8 dyes simultaneously in four short hours. To determine the binding of metal ions would in principle be just as easy, except that

to detect how far the metals have moved in the gels, one would probably need a radioactive metal ion present.

It will be noted that for materials which bind DNA very irreversibly (as the histones do for instance), the DNA/polyacrylamide medium may also be used to advantage. For histones we could construct a DNA/acrylamide plug, electrophorese the histones into the plug at low salt (causing them to be bound irreversibly to the DNA), then increase our buffer ionic strength stepwise so that one class of histones is released at one time. If the ionic strength is raised to 0.5 F NaCl for instance, then histone I will be dissociated, and will be the only histone to travel through our plug.

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PROPOSITION IV

THE HYDRODYNAMIC SEGMENT OF DENATURED DNA

ABSTRACT

The number of bases in a hydrodynamic segment of denatured DNA is calculated from recent published sedimentation data. A reasonable estimate for the segment in 0.01 F NaCl would be 40-50 bases, and 10-15 bases in 1 F salt. Electrostatic repulsions appear to be the major factor in stiffening the denatured DNA chain at low salt. It is suggested that additional stiffness is provided by base stacking and by hydration. It is also suggested that the sedimentation behaviour of denatured DNA at neutral pH's and high ionic strengths is somewhat anomalous because of random intramolecular base pairing.

****

In the course of interpreting the results of electrophoresis experiments with native and denatured DNA, it became necessary to try to estimate the length of the hydrodynamic segment of denatured DNA. Recent reliable sedimentation data has been published (1) in which old uncertainties about aggregation and random intermolecular hydrogen bonding in denatured DNA were eliminated. One could therefore calculate segment lengths from S values with confidence. It was found in the course of making these calculations that certain not-very-obvious considerations were involved, and for this reason a thorough (though qualitative) discussion of the hydrodynamics of denatured DNA in solution is given here.

The derivation of the parameters that are important for calculating how many bases are in a denatured DNA hydrodynamic segment is shown in table I. Here it is assumed that both native and denatured DNA are impermeable random coils. The equation, which is derived in a straightforward way, shows that the number of bases that is in a hydrodynamic segment can be calculated if we know the molecular weight (M), the sedimentation coefficient S, and the average distance between the bases, l. We find that the number of bases in a hydrodynamic segment is proportional to M/l^2S^2. The number of bases in a segment that we calculate is therefore extremely sensitive to the sedimentation coefficient and to the distance between bases. Unfortunately the latter quantity is an unknown entity: it is a parameter that would vary with the nature of, and ionic strength in the solvent. In its most extended form, the denatured DNA bases could be about twice as far apart as they are in a Watson-Crick helix.

If one takes the excellent data of Studier (1) for denatured DNA in neutral and alkaline solutions at different ionic strengths, it is possible to calculate how many bases would be in a hydrodynamic segment as a function of the average distance between the bases. The results of such calculations are in table II. If we take the 700 A° (2) segment length of native DNA as basis (and assume it is invariant with ionic strength), we find that the hydrodynamic segment has four times as many bases in 0.01 F NaCl as in 1 F NaCl for DNA in an alkaline medium, assuming no change in the average distance between bases. This is a rather poor assumption, and the change is therefore somewhat less than

Table 1a

THE CALCULATION OF THE NUMBER OF BASES IN A SEGMENT OF DENATURED DNA

The meaning of the various symbols:

f	the friction coefficient
R_G	the radius of gyration
η	viscosity
b	length of a hydrodynamic segment, A°.
σ	number of segments per molecule
ρ	the density of the segment, MW units/A°.
M	the molecular weight
l	average distance between bases
m	the number of bases per segment.
S	the sedimentation coefficient in Svedbergs.

The subscript N denotes native DNA, the subscript D denatured DNA.

$$f = 6\pi\eta(0.665)R_G \qquad R_G = \left(\sigma\frac{b^2}{6}\right)^{1/2}$$

$$\frac{S_N}{S_D} = \frac{M_N/M_D}{f_N/f_D} = \frac{M_N\, b_D\, \sigma_D^{1/2}}{M_D\, b_N\, \sigma_N^{1/2}} \qquad \sigma = \frac{M}{\rho b}$$

$$\frac{b_N}{b_D} = \frac{\rho_N/\rho_D \quad M_N/M_D}{(S_N/S_D)^2} \qquad 2b_N = l_N m_N \qquad \frac{\rho_N}{\rho_D} = 2\frac{l_D}{l_N}$$

$$b_D = l_D m_D$$

$$\frac{M_N}{M_D} = \frac{4\, m_N/m_D}{(l_N/l_D)^2(S_N/S_D)^2}$$

Table 1b

Some calculations from the results of table 1a

For Studier's data in 0.01 F salt (alkaline pH):

$S_N/S_D = 35/20 = 1.75$

$M_N/M_D = 2 \qquad m_N = 400$

$$m_N/m_D = \frac{4\ (M_N/M_D)}{(S_N/S_D)^2(l_N/l_D)^2} = \frac{(4)(2)}{(1.75)^2(l_N/l_D)^2}$$

Thus: $$m_D = \frac{400(1.75)^2}{8(l_D/l_N)^2} = (l_N/l_D)^2 152$$

Similarly, we find for 1 F salt (alkaline medium):

$$m_D = 38(l_N/l_D)^2$$

Results of Calculations

l_N/l_D	l_D	Alkali Results		Neutral Denatured DNA
		0.01 F	1.0 F	0.01 F
	A°	(m_D, bases per segment)		m_D
2	6.8	38	9.5	25
1.8	6.1	47	12	31
1.6	5.4	59	15	39
1.5	5.1	67	17	45
1.4	48	77	19	51
1.3	4.4	90	22	60
1.2	4.2	105	26	70
1.1	3.7	138	36	92
1.0	3.4	152	38	101

four fold. If we take the sedimentation coefficient at neutral pH in 0.01 F salt, we find that if we assume the average distance between bases is 6.8 A°, the segment would be 25 bases long, but if we had assumed an average distance of 3.4 A°, the segment would have been calculated to be made up of 100 bases. Probably, a reasonable average distance in this low ionic strength is about 5 A°, in which case we would say the hydrodynamic segment in this salt has between 40 and 50 bases.

The major weakness of any calculation for the hydrodynamic segment of denatured DNA is that an uncertainty exists with respect to the distance between the bases, which is not fixed as it is in native DNA. Also, the DNA random coils are not completely impermeable. This is shown by the molecular weight dependence of S. For native or alkaline denatured DNA, S is proportional to less than $M^{1/2}$.

Denatured DNA in neutral solution, has S values that are abnormally high, and do not give an accurate reflection of the intrinsic flexibility of the molecule. The reason for this is probably random intramolecular hydrogen bonds at high salt (Studier eliminated the possibility of intermolecular hydrogen bonds being important in giving large S values). Thus, the denatured DNA segment length cannot be calculated directly from an S value in say 1 F NaCl, pH 7: the reason why the DNA sediments so fast is not only because the single chain has become more flexible but because the coil as a whole becomes more compact due to random hydrogen bonds. If one wished to calculate what the segment length of denatured DNA in high salt were, it would

be better to either measure on S value of formaldehyde denatured DNA or calculate from the number of segments in denatured DNA in alkali. It is reasonable to assume that if the alkali denatured DNA has a segment with one fourth as many bases in 1 F NaCl as in 0.01 F NaCl, the same would be true in neutral solution. Since the DNA presumably is nearly free of random hydrogen bonds in 0.01 F salt, we can then calculate that if it has 48 bases in the low salt, a hydrodynamic segment would have a dozen bases in 1 F salt. This would imply that most of the inflexibility of denatured DNA is due to charge repulsion: once the charge repulsions are screened, it loses most of its stiffness. The change in flexibility as a function of ionic strength is mirrored by the sedimentation coefficient in alkali (or in formaldehyde denatured DNA), but not by neutral denatured DNA. However, the value of 12 bases per segment (which is probably a low estimate) indicates that the denatured DNA is still more stiff than one might expect from the nature of a single polynucleotide chain. It is proposed that this residual stiffness, present after most of the charge repulsions have been screened out, is due to two related causes: base stacking and hydration. If there were very much base stacking, then the bonds in the single chain would be constrained from rotating, resulting in a stiffer chain. Another possible factor is hydration. The presence of a water "cage" around the single polynucleotide chain would make it less flexible.

The studies of Hamaguchi and Geiduschek (4) indicate that in 7 F $NaClO_4$, DNA bases are probably less stacked and any water cage effect is also diminished. It is proposed that in this solvent, the DNA would

have a smaller segment length and be much more flexible. This could in principle be confirmed by determining the sedimentation coefficient of denatured DNA in this solvent, but the simplest confirmatory test for this hypothesis would be to do a light scattering experiment of a reasonably low molecular weight DNA, and determine its radius of gyration in both NaCl and $NaClO_4$. This type of measurement should reveal whether the factors we mentioned are what make denatured DNA as inflexible as it is. An interesting sidelight is that in the Hamaguchi and Geiduschek paper, there are indications that in $NaClO_4$, native DNA would also have a segment length considerably shorter than its value in a more normal solution. These workers indicated that there was a marked fall in viscosity of the native DNA which was not caused by any collapse in the secondary structure. This solvent might therefore present a means to vary the intrinsic bendability of a double helix.

From the above discussion, we can make certain predictions about the sedimentation behaviour of non-hydrogen bonded homopolymers. If poly U or some other homo ribo- or deoxyribohomopolymer which was incapable of forming random hydrogen bonds were used in an S versus ionic strength study, the behaviour of such a homopolymer at neutral pH should not resemble the behaviour of denatured DNA at neutral pH, but should be more like the behaviour of denatured DNA in alkali. We would expect a two-fold change in sedimentation coefficient in going from 0.01 F NaCl to 1 F NaCl, instead of a four fold change as is the case for denatured DNA at neutral pH's. The data available show that a hundred-fold change in ionic strength causes even less than a two-

fold change in S value for poly-U (5). For purine homopolymers (such as poly A in neutral or alkaline pH's) stacking would become more important as we increased the ionic strength. While the chain would tend to become more flexible because repulsions between phosphates will have decreased as we increase the ionic strength, this will be at least partly compensated for by increased stacking which would stiffen the polynucleotide chain. We would therefore expect for such polymers that the S value would be more nearly constant with ionic strength, and would show considerably less than a two-fold change as the ionic strength were changed from 0.01 F to 1 F. This might be a good method for evaluating stacking interactions in purine homopolymers, and would in principle anyway, be a basis for the possible separation of DNA strands which had different purine contents.

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PROPOSITION V

THE GUANIDINIUM ION AND THE STRUCTURE OF WATER

It is proposed that the guanidinium ion should be a strong structure-of-water breaker. The reasons for this on the basis of the Frank "flickering cluster" theory of the structure of water are discussed. Nuclear magnetic resonance experiments are suggested to test the prediction.

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The influence of ions on the structure of water is a subject of great importance about which relatively little is known. Much of the confusion results from the fact that the ions exert several different effects on water simultaneously, and most experiments only see the average result of all these effects: it is therefore very difficult to ferret out the relative importance of each.

In a proposition of this sort, it is all important to define what is being discussed. We will be examining the effect of ions on the structure of water. By this, we mean to ask: when an ion is dissolved in water, is the net result an increase or a decrease in the order of the water? A slightly different way of putting it (with a somewhat different but closely related meaning) is: when we add a particular ion to water, do more, or do less of the water molecules become hydrogen bonded? Therefore, even if an ion "destroyed the structure of water", if it rebuilds an ordered, if different structure around itself, we shall say that such an ion has increased the structure of water.

Although much confusion remains, it appears that most cations are structure making (1). Those which are somewhat structure breaking are generally weakly so. In contrast, some anions are very strongly structure breaking (the perchlorate ion, for instance). In this proposition, we will suggest that the guanidinium ion is a uniquely structure breaking cation.

Most of the ideas for this proposition are based on the ideas in the excellent book by Kavanau (1). We will take as our basis for the structure of water the Frank (2) "flickering cluster" model. This seems to be the most easily adapted model for explaining solute effects on water, and it has also been subjected to a statistical mechanical treatment (3) that gives results superior to any other existing model. In this view, most of water at reasonably low temperatures exists in ordered, ice-like clusters. These clusters are not very long lived, hence the term "flickering." They do have a significant lifetime however, existing at least 100 times as long as a molecular vibration. There is always some water between clusters that has no ordered structure. Clusters tend to form and break cooperatively: if a few molecules start to tetrahedrally hydrogen bond, they tend to induce their neighbors to join the cluster. On the other hand, if one member of a cluster breaks its hydrogen bonding, then the whole cluster tends to fall apart. The evidence for this view of the structure of water is outlined in Kavanau's book.

On this basis, the effect of solutes may be at least qualitatively

explained. Ions tend to destroy the clusters, because they orient the water dipoles. There are two types of ions in this respect, "structure breaking" ions, and "structure making" ions. Structure breaking ions destroy the clusters, but do not reorient a significant amount of water so that a new ordered structure of water is formed. In this class would be included the larger monovalent cations and anions. Structure making ions are those which destroy the original structure of water, but which have such a strong orienting power that a new ordered water structure builds around them. The quaint term "soft ice" has been coined for such oriented water. The presence of such an ion might therefore actually result in greater ordering of the water, which is why the ion is called structure making. Small cations, OH^-, F^- and multivalent ions are structure making.

The effect of organic solutes is more complicated and has been subject to controversy, but the predominant view seems to be that organiz molecules stabilize the clusters. This is because the main force that destroys ice-like clusters are disruptive influences propagated from the edge of the clusters. Ice-like clusters are stabilized by organic molecules at their edges, because such molecules are in general relatively inert in the production or transmission of such disruptive influences. Furthermore, the presence of a solute of this sort tends to lower the energy of a quadruply-bonded water molecule with respect to more incompletely bonded water molecules. All these things result in a non-polar organic molecule being surrounded by ice-like clusters, which are known as Frank-Evans icebergs.

Many very large anions are very strongly structure breaking, examples being the perchlorate and trichloroacete ions. However, the most structure breaking cations currently cited are the large alkali ions, but there is some debate as to whether these ions are structure breaking at all. The larger cations that can be made as derivatives of ammonia (such as the tetrabutylammonium ion) function as net structure makers, because though they are large and therefore from this point of view would tend to destroy clusters, the fact that they have large organic non-polar groups actually makes them stabilize clusters due to factors explained above.

It is proposed that the guanidinium ion should prove to be one of the strongest structure of water breaking cations. The guanidinium ion is planar with the N-C-N bonds being 120°. The $-NH_2$ groups are somewhat constrained from rotating because the C-N bonds have some double bond character. The ion would tend to hydrogen bond water molecules and orient them to a certain extent in a way consistent with its three fold symmetry. The diameter of guanidinium in the crystal (4) is 2 A°: it is generally felt that any ion larger than 1.6 A° would have no strong orienting effect. By this criterion alone guanidinium would be expected to be structure breaking. However, it is reasonable to suggest that the structure breaking capacity would be increased by the fact that the ion hydrogen bonds neighboring water molecules in such a way that it would be almost impossible for them to be part of a cluster. Therefore, here we have a big non-spherical ion that would tend to orient water such that if it were in or

adjoining a cluster, it would cause breakdown of the cluster.

It should be possible to experimentally test this hypothesis. Observation of the near-infrared region and the effect of the ion on the water peaks in this region would theoretically be an excellent way of evaluating how structure breaking guanidinium is (provided one accepts the assumptions of Buijs and Choppin) (5). Unfortunately, this will be a difficult experiment to carry out since the guanidinium ion itself probably absorbs significantly in this region making it difficult to assess what changes take place in the water bands.

A more feasible way would be to look at the nuclear magnetic resonance of the water protons. Solutes have two effects on the resonance of the water protons (6). If a solute causes greater disordering of water, and a breakdown of its hydrogen bonded structure, a shift of the water proton resonance to higher field is observed. If, on the other hand, the water is oriented strongly around the solute, there is a polarization effect and the proton resonance of the water is shifted downfield. Although a simple measurement of the field position of the water protons yields information as to the net structure-making or structure-breaking effects of any solute, the studies of Chan and co-workers (7) now make it possible to at least qualitatively separate the two effects. In their work, the effect of solutes on the water proton resonance in mixed solvents (such as acetone/water or acetonitrile/water) is measured, and since in such solvents the water doesn't have very much structure to begin with, the polarization effect is what is unambiguously observed. Therefore,

by this method, it is possible to tell whether, for example, an ion which didn't induce any net shift in the water proton resonance were merely inert, not polarizing any water and not breaking down any clusters, or whether it were polarizing some water molecules strongly and at the same time breaking down enough clusters so that the two effects cancelled out. It is predicted from the considerations above that the polarization effect for guanidinium would be negligible, but that the disordering effect would be very large.

A large part of the interest in the guanidinium ion stems from its use in protein chemistry, mainly as a protein denaturing agent. The structure breaking effect of water that we speak of in this proposition may not be adequate to explain the strong denaturing effect of guanidinium: the concentrations employed are so high that the simple considerations we have discussed may not apply. It is probable however that a large part of its denaturing action may be because of its solvent structure breaking capacity. The discussion we have made here would mainly pertain to guanidinium concentrations 1 F and below, and are not necessarily applicable to the 5 F concentration routinely used in protein work.

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PROPOSITION VI

AGAR AND AGAROSE

Agar is a natural polysaccharide from algae that has long been known but only rather recently has some understanding of its structure come about. The major interest in agar is in its widespread use as a gel.

It is probably unique as a gelling agent. Gels with an agar concentration as low as 0.5% will form stiff gels that do not liquefy until a temperature over 90°C (1). No other common gelling agent forms stiff gels stable to these high temperatures at such low concentrations.

Much of the difficulty in understanding the molecular nature of agar comes about because it is in reality a mixture of two distinct polysaccharides, agarose and agaropectin. The situation is quite similar to starch: agarose appears to be a linear polysaccharide (like amylose) while agaropectin is a complex substance, analogous to the amylopectin of starch (2). The work of Araki and Hirasei (3) has contributed to an understanding of the structure of agarose; the structure of agaropectin remains unknown. Apparently it is agarose that gives agar its unique gelling properties.

Agarose is composed of a basic disaccharide unit, agarobiose, which is beta-D galacto pyranose (linked beta-D (1-4)) and 3.6 anhydro-L-galactose (linked alpha-L-(1-3)). There is no evidence for branching. No end groups could be detected by hydrolysis of the

methylated agarose, indicating the polysaccharide has an extremely high molecular weight (3). The above structure of agarose causes a dilemma. Why is agarose such an efficient gelling agent? It was long believed that the reason agar gelled was because it was an acidic polysaccharide with semi-ester sulfate groups about once every 10 residues. These sulfate groups were then cross linked by calcium ions. Thus an agar gel was believed to be a network with extensive calcium-sulfate cross links (4). Agarose has been purified so it has no calcium ions, and trace amounts of sulfuric acid residues and yet it gels at lower concentrations that either agaropectin or the original agar from which it was derived. As far as can be detected, all the sulfate residues in agar are an integral part of the agaropectin component. The sulfate cross link explanation for the gelling behaviour is therefore not valid (2).

It is difficult to imagine why a linear polysaccharide such as agarose should form a gel at all at such low concentrations. It has been suggested that gelling takes place because of hydrogen bonding (2). One would imagine that any regular hydrogen bonding along the disaccharide units such that the linear chains would be hydrogen bonded to one another would cause insolubility of the polysaccharide. There seems to be nothing in the disaccharide that would give hydrogen bonding stable enough to hold up a gel at 90°C. From its basic structure, there is no compelling reason for agar to have different gelling properties from other linear polysaccharides.

We are therefore left in the rather uncomfortable position of having rejected all explanations that have been so far offered for the gelling action of agar. It is proposed that the simple linear structure that has been suggested for agar is inadequate to explain the gelling action. On close examination of Araki's papers, it is found that on hydrolysis, agar yields mostly the neutral products to be expected of the linear structure and the repeating disaccharide unit. However, on chromatography of the hydrolysis products, it was found that there was a very small amount of an acidic component (about 2%). Araki dismissed this acidic component as being present in such small amounts that it was structurally insignificant (3).

In considering the gelling properties of agarose, it would seem that because of the paucity of explanations for gelling on the basis of the linear model, one must look for explanations in the components not analyzed. While 2% may not seem very important from the structural point of view, it might be of paramount importance for the gelling behaviour.

Since the acidic components of agarose have not been analyzed to any extent, the possible identity of the cross linking agent has to be considered an open question. A plausible cross-linking model will be considered here, but it should be emphasized that there are many other possibilities, and the experimental evidence available does not permit a really definite proposal.

Hirasei has presented evidence that 2% of the disaccharide residues in agar contain a pyruvic acid residue (5). Araki believes that this pyruvic acid is present mainly in the agaropectin component of the agar (6). However, he presents no chemical evidence for this and considering that he stated that he did not analyze the 2% acidic component of agarose, it would seem that the presence of 2% cannot be eliminated.

It is conceivable that the pyruvic acid residue is the cross-linking agent in agar. Hirasei has shown that this pyruvic acid is found in the hydrolysis products bound to the galactoside residue in the 4, 6 position by a cyclic acetal linkage. The carboxylic acid group is free. It is possible therefore that the linear chains of agarose are cross-linked by pyruvic acid residues that occur rarely along the chain (not over 2% of disaccharide units have them), and the linkage is through an ester linkage in the carboxylic acid group of pyruvic acid, and a cyclic acetal linkage to the 4, 6 positions of the galactoside residues of the agarose. The cyclic acetal is acid labile (7) which may help explain why agar does not gel in acid media.

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