PART I

INTERACTION OF DNA AND HISTONE IN NUCLEOHISTONE

PART II

DORMANCY ASSOCIATED WITH REPRESSION OF GENETIC ACTIVITY

Thesis by

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In partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

(Submitted March 15, 1967)
1967

ACKNOWLEDGEMENTS

To Professor James Bonner, my thesis adviser, I wish to express my deep gratitude for his patient and inspiring guidance through my graduate studies at the California Institute of Technology. Special thanks are also due to Professor Norman Davidson and Dr. Roger Chalkley for their encouragement and constructive criticism in the preparation of this thesis, and to Dr. Ru-chih C. Huang, Dr. Baldomero Olivera, Professor Jerome Vinograd and Professor Robert Sinsheimer for many helpful suggestions.

I am also grateful for the financial support of an Institute assistantship and of the Arthur McCallum fellowship, which has made progress toward the doctorate possible.

The personally enriching experience of having been a member of the "Bonner Coffee Club" and a sharer in its gay spirit and comradeship will be a memory to be treasured always.

Finally, I wish to extend my warmest affection and gratitude to Mrs. Jean Charnley, who knew the cure for everything, from homesickness to examinationitis.

PREFACE

The genetic material of living organisms is present in the cell not as chemically pure DNA, but as DNA-protein complex. A major fraction of the protein complexed to the DNA of higher organisms belongs to a class of small, basic proteins — the histones. This complex of DNA with histone, nucleohistone, can be isolated from calf thymus as a chemically defined entity of DNA, histone and non-histone proteins, which possesses a sedimentation coefficient of approximately 30-60S. The DNA in nucleohistone is of sedimentation coefficient about 15S which is large enough for approximately 10 genes. The template activity of such nucleohistone for in vitro RNA synthesis is less than that of free DNA. Full activity is restored by removal of associated histones by salt extraction. These facts suggest that the histones function as regulators of gene activity. Thus the structure and function of nucleohistones may be basic for an understanding of differentiation and therefore a subject of central interest in molecular biology. To understand how nucleohistone functions, studies of its structure, the distribution of the various kinds of proteins among different DNA molecules, and of the mode and effects of interaction between the components are helpful. The following chapters, grouped into two parts, are aimed at achieving some understanding of the above matters.

Part I, which consists of three chapters, is a study of the effects on both components of interaction between DNA and histones in native nucleohistone. The first chapter is devoted to the study of selective dissociation of histones from nucleohistone by treatment with various

concentrations of NaCl. As a result of such treatment, native nucleohistone is separated into two components, the dissociated histones and the partially dehistonized nucleohistones with various histone to DNA ratios. The second chapter consists of an optical rotatory dispersion study of the histone fractions. The structural changes of the histones when they complex with DNA is considered. The third chapter is a study by optical rotatory dispersion of native nucleohistone and of partially dehistonized nucleohistones, and concerns the conformational changes in DNA as a result of histone binding or dissociation.

Part II, which is also divided into three chapters, is a study of the biological expression of the chromosomal material, chromatin or nucleohistone; i.e. a study of the relationship between dormancy of buds, a common biological phenomenon in the plant world, and template activity for in vitro DNA dependent RNA synthesis of chromosomal material isolated from dormant tissues. The tenability of the hypothesis of gene repression as the cause of dormancy is tested. The division of part II into three chapters is according to the different plant materials used. The first chapter concerns dormant potato tubers; the second chapter, onion bulbs; and the third chapter, dormant corms of gladiolus.

ABSTRACT

PART I

INTERACTION OF DNA AND HISTONE IN NUCLEOHISTONE

Chapter 1 SELECTIVE DISSOCIATION OF HISTONE FROM NUCLEOHISTONE

With increasing concentration of NaCl solution, an increasing amount of histone is dissociated from dissolved nucleohistone. The dissociated histone fractions are identified by gel electrophoresis. The lysine rich histone fraction I is dissociated from nucleohistone in the range 0.3-0.5 F NaCl; slightly lysine rich histone II in the range 0.8-1.6 F NaCl; arginine rich histone III+IV in the range 0.9-1.6 F NaCl. The results suggest that both electrostatic and non-electrostatic interactions contribute to the strength of binding between DNA and histones.

Chapter 2 OPTICAL ROTATORY DISPERSION STUDIES ON HISTONES

The optical rotatory dispersion spectra of histones free and in reconstituted nucleohistone (in which histone is complexed to DNA) are recorded. By the criterion of optical rotatory dispersion at wavelengths below 220 mu, histone I is the least helical of the histones, histone II the most helical. The helicity of DNA-bound histones in reconstituted nucleohistone is greater than that of free histones, but the order, histone II most helical and histone I least, is still preserved.

Chapter 3 OPTICAL ROTATORY DISPERSION STUDIES ON THE DNA OF NATIVE NUCLEOHISTONE AND OF PARTIALLY DEHISTONIZED NUCLEOHISTONES

The conformation of DNA in native nucleohistone is altered by the DNA-histone interaction. The dissociation of histone I does not produce

significant conformational change in DNA of nucleohistone but removal of histone II and of histone III+IV bring about changes which cause the conformation of DNA in nucleohistone to resume virtually that characteristic of free DNA. The possibility of DNA supercoiling in nucleohistone is discussed.

PART II

DORMANCY ASSOCIATED WITH REPRESSION OF GENETIC ACTIVITY

Chapter 1 THE DORMANCY OF POTATO BUDS

Chromatin of the buds of dormant potato tubers is almost totally incapable of the support of DNA-dependent RNA synthesis in the presence of added exogenous RNA polymerase. The chromatin of non-dormant buds of potato tubers (in which dormancy has been broken by treatment with ethylene chlorohydrin) is highly effective in the support of DNA-dependent RNA synthesis by added exogenous RNA polymerase. It is therefore concluded that the genetic material of the buds of dormant potato tubers is largely in a repressed state, and that the breaking of dormancy is accompanied by derepression of the genetic material.

Chapter 2 THE DORMANCY OF ONION BULBS

The chromosomal material of non-growing and non-dividing onion buds possesses template activity in support of in vitro DNA-dependent RNA synthesis. If we define dormancy not only by the absence of visible growth and mitotic division, but also by the lack of ability to direct in vitro DNA-dependent RNA synthesis, potato buds are then dormant but onion buds are not. And the block to onion bud growth must lie some where else than in the repression of genetic material.

Chapter 3 ISOLATION OF GLADIOLUS CHROMATIN

Gladiolus corms contain very little isolatable chromatin material and the isolated chromatin is highly contaminated by the presence of starchy material.

Table of Contents

PART I

INTERACTION OF DNA AND HISTONE IN NUCLEOHISTONE

PART II

DORMANCY ASSOCIATED WITH REPRESSION OF GENETIC ACTIVITY

PART I

INTERACTION OF DNA AND HISTONES IN NUCLEOHISTONE

Chapter 1

SELECTIVE DISSOCIATION OF HISTONES FROM NUCLEOHISTONE

I. INTRODUCTION:

Nucleohistone, the main chromosomal component of a wide variety of plant and animal cells (1), is a complex of protein with DNA. While studies of the intact nucleohistone have yielded valuable results, analytical investigations in which the nucleohistone is taken apart will not only supply new material (chemical derivative of NH) for analysis but also shed light on the mode of interaction of its components.

Because of the ionic nature of the protein-DNA interaction in NH(2), salt solutions provide such an analytical tool for NH dissociation. By variation of the ionic strength of the salt solution, the extent of NH dissociation can be varied. NaCl (3), $NaClO_4$(4), and guanidinium hydrochloride (5) have been used for dissociating protein from NH. NaCl was chosen for this study because ClO_4^- and the guanidinium ions have effects on the macromolecule other than the desired ionic one (6).

NaCl has been used by other workers for dissociation of NH(3) (7). The present study, however, is more complete because of the availability of polyacrylamide gel electrophoresis which makes possible the identification of histones dissociated from and these still associated with NH.

The following work on NaCl dissociation of Calf thymus nucleohistone was done in collaboration with Dr. Baldomero Olivera.

II. MATERIALS AND METHODS

PREPARATION OF CALF THYMUS NH

The preparation method is essentially that of Chalkley and Maurer (8) which lies in an initial isolation of nuclei. For each set of experiments about 30 g of frozen calf thymus tissue was minced and homogenized in a Waring blender, first at 50 volts for 2 minutes in 100 ml of grinding medium (0.25 M sucrose, 0.005 M $MgCl_2$, 0.005 M tris PH 7.3) with 1 ml of octanol to minimize foams; followed by 1 minute at 60 volts, with 100 ml more of grinding medium; then for 1 minute at 70 volts, adding another 100 ml of grinding medium and finally at 80 volts for 30 seconds. The homogenate was filtered through 2 layers and then through 4 layers of gauze; followed by 1 layer, then 2 layers of miracloth. The filtrate was centrifuged at 3500 rpm for 10 minutes in a Servall centrifuge. The pellet was next dispersed in grinding medium with 5 strokes at 50 volts in a Potter-Elvehjen homogenizer attached to a motor and powerstat. This was repeated once. The resultant nuclei were relatively free of cytoplasmic contamination as could be seen under the microscope. (To make them visible the nuclei were dyed with aceto-carmine). The nuclei were then washed twice with 0.15 M NaCl + 0.003 M $MgCl_2$ and twice with saline-EDTA (0.075 M NaCl, 0.024 M Na_2EDTA pH8). The washed pellet was dispersed in 0.01 M tris pH 8 with 20 strokes at 110 volts to lyse the nuclei. After having been stirred in the cold room for 30 minutes and sheared in a metal (stainless steel) Virtis container for 6 minutes at 30 volts, the very thick chromatin solution was layered in 5 ml portions on top of 25 ml of 1.7 M sucrose in cellulose nitrate tubes. The upper half of the contents of each tube was

stirred to form a rough gradient. The tubes were then centrifuged at 22,000 rpm in a SW 25 rotor for 2 hours. The gelatinous pellets (purified chromatin) were homogenized into 0.01 M tris pH 7.3 with 10 strokes in the homogenizer at 110 volts. The concentration of the purified chromatin solution was adjusted to about A260 mu = 20. The chromatin solution was then dialyzed against 20 volumes of 0.01 M tris pH 7.3 for 12 hours. To make NH from the purified chromatin solution, the chromatin was sheared in the metal Virtis container at 85 V for 90 seconds, then centrifuged at 10,000 rpm for 30 minutes in a Servall centrifuge. The supernatant is the soluble nucleohistone. The yield of this material from purified chromatin solution is from 40-80%. Less aggregated purified chromatin solutions usually give higher yields of NH.

SALT EXTRACTION OF HISTONES

5 ml of NH solutions (ca. 150-250 ug DNA/ml) were dialyzed in separate stoppered test tubes against the required concentration of NaCl, which was renewed every 3 hours for 3 times. The dialyzates were then layered on top of 1 ml of 1.7 M sucrose and centrifuged at 39,000 rpm for 24 hours in a SW 39 rotor. The top 4 ml of the supernatants (dissociated protein) was pipetted off. The intermediate layer was discarded and the pellets (partially dissociated NH's) which were usually the last 0.3 ml were dissolved in 0.01 M tris pH 7.3. Both the supernatant and the pellet were dialyzed against 3 changes of 0.01 M tris pH 7.3 for 12 hours to remove NaCl and sucrose.

NH in 0.3 F and 0.4 F NaCl produced white precipitates which were centrifuged off in a clinical centrifuge for 5 minutes. The NH

left in solution was then centrifuged in the SW 39 rotor for 24 hours at 39,000 rpm. The resulting supernatant, dissociated protein, was separated from the pellet as above and the pellet combined with that from the earlier centrifugation. It will be shown below that the 2 pellets are of identical composition.

SEPARATION OF HISTONE AND NONHISTONE PROTEINS FROM NH

Histone and nonhistone proteins were separated for analysis according to their solubilities in acid and alkali. To extract histones (10), one volume of 1 M H_2SO_4 was added to 4 volumes of NH solution and after 30 minutes the residue was centrifuged off at 12,000 rpm for 30 minutes in a Servall centrifuge. Histones in the supernatant were precipitated by adding 4 volumes of cold 100% alcohol. Precipitation was complete after 24 hours at -13°C. The precipitate was washed with ethanol and collected by centrifugation at 12,000 rpm for 30 minutes.

Nonhistone protein is defined as acid insoluble protein which is subsequently soluble in 1.0 N NaOH. The acid insoluble residue was dissolved in 1 N NaOH overnight at room temperature. The supernatant of this 1 N NaOH extract after centrifugation for 30 minutes at 12,000 rpm was used for nonhistone protein determination by the Lowry procedure.

DETERMINATION OF DNA IN NH

Optical absorption of DNA at 260 mu on a Hitachi Perkin-Elmer U.V. spectrophotomer was employed. The absorption spectrum (360 mu-210 mu) of DNA exibits a peak at 260 mu, while that of associated protein is featureless (ascending rapidly below 240 mu) in the same region.

Absorb nce of NH at 260 mu is a measure of DNA quantity since absorbence of protein in NH is only 2-3% that of DNA at this wavelength. One mg of commercial Sigma NaDNA (calf thymus) in 0.01 M tris pH 7.3 in one ml gives A_{260mu}=21 by phosphorus analysis (11).

$$\text{ug DNA} = \frac{A_{260mu} \text{ of NH}}{47.6} \times 10^3$$

DETERMINATION OF PROTEIN IN NH

Absorbence of protein at 230 mu was used for estimating the protein content of partially dissociated NH's. The NH solutions were diluted to about A_{260mu}=5 and centrifuged at 35,000 rpm for 15 minutes in a SW 39 rotor to remove any scattering particles. After appropriate dilution, the absorbences at 230mu, 260mu, 320mu were recorded (1 cm light path). The absorbence at 320 mu which is a measure of the sample scatter was usually one hundredth of that at 260 mu. The relation of absorbence to weight of histone was determined in the following way:

Samples of acid and of 4 F NaCl extracted histone were subjected to determination of total Nitrogen content by a standard Nessler procedure (I thank Dr. Justine Garvey for her counsel). Since the amino acid composition of thymus histone is known (2) the weight of histone in the sample can be computed from its Nitrogen content. 100 ug/ml of acid or 4 F NaCl extracted histone solution has an absorbence of 0.425±0.002 at 230 mu.

$$\text{ug protein} = \frac{A_{230mu}^{\text{NH}} - A_{230mu}^{\text{DNA in NH}}}{A_{230mu} \text{ of 1 ug protein}}$$

$$= \frac{A_{230mu}^{NH} - \frac{A_{260mu}^{NH}}{2.4}}{0.425 \times 10^{-2}}$$

$$\left(\frac{A_{260mu}^{DNA}}{A_{230mu}^{DNA}} = 2.4 \text{ for Sigma calf thymus NaDNA} \right)$$

The procedure of Lowry et. al. (13) was also used for histone and nonhistone protein determination. 4 F NaCl extracted protein in 1 N NaOH was employed to obtain the standard curve. Presence of DNA in the reaction mixture does not interfere with the color development.

POLYACRYLAMIDE GEL ELECTROPHORESIS

In preparation for gel electrophoresis, undissociated histones were acid extracted, alcohol precipitated and redissolved in 10 M urea. Dissociated histones were isolated by first dialyzing the histone solution exhaustively against 1 F acetic acid to remove the NaCl, then lyophilizing and finally redissolving in 10 M urea. The urea solutions were electrophoresed in polyacrylamide gel (10) and the resulting histone bands dyed and fixed with amidoschwarz.

The bands were identified by comparison with the gel electrophoresis patterns of acid extracted, Amberlite column separated histone fractions (10). (Fig II)

Gels were scanned using a Canalco Model E. Microdensitometer. The different histone bands have similar binding constants for amidoschwarz (14), so comparison of areas under the peaks gives a qualitative estimation of relative histone amount in the band. (Fig III and IV)

III. RESULTS AND DISCUSSION:

COMPOSITION OF NH:

Calf thymus NH, isolated as above, contains virtually no RNA, less than 1% as much as DNA (15), and consists principally of DNA, histone and nonhistone proteins. The possibility that there is a third protein component which is neither acid nor alkali extractable is ruled out by the fact that ug histone/ug DNA plus ug nonhistone/ug DNA (first number of 7th column in Table II) is very close to ug protein/ug DNA (first number of 5th column in Table I) estimated by $A_{230\ mu}$ which is a measure of total protein content.

Protein to DNA ratio of NH varies from 1.52 to 1.71 (Table I) with the present two preparations. This is due to variation in the content of nonhistone protein. Histone to DNA ratio of 1.2 is quite constant; the ratio however drops as the NH ages.

Histones can be divided into 3 main classes I, II and III+IV, according to their order of elution from the standard Amberlite column (10). The first main component eluted is the lysine rich histone I of lys./arg. ratio $>$ 9. Following this is the relatively argine rich histone II, lys./arg. ratio= 2. The last fraction is the argine rich histones III+IV, lys./arg. ratio $<$ 1. (Because of the lack of resolution of histone III and IV fractions on the Amberlite column and their similar amino acid composition, they are classified as one component-histone III+IV.)

It is not yet known whether nonhistone protein is heterogeneous; The fact that it is dissociated from DNA over a wide range of salt concentration would seem to indicate great heterogeneity.

TABLE I

Protein content of NH by $A_{230m\mu}$ and $A_{260m\mu}$

Partially Deproteinized NH's from various F. of NaCl	260/230		ug prot./ug DNA		% of prot. remained on DNA	
	(1)	(2)	(1)	(2)	(1)	(2)
0 (whole NH)	1.43	1.35	1.52	1.71	100%	100%
0.6	1.61	1.61	1.09	1.08	72%	64%
0.8	1.66	1.66	0.92	0.93	60%	54%
0.9	1.68	1.73	0.88	0.79	59%	46%
1.0	1.8		0.69		45%	
1.2	1.81	1.87	0.68	0.58	45%	34%
1.6	1.92	2.07	0.53	0.32	35%	19%
2	1.99	2.09	0.43	0.31	28%	18%
4	2.1	2.15	0.3	0.24	19%	14%

Footnote:

i) (1) and (2) are different preparations of NH.

ii) The difference of ug/ug DNA between (1) and (2) for partially deproteinized NH's is due to preparation technique. After centrifugation to separate the partially deproteinized NH from dissociated histones, the pellets from 0.9 F to 4 F NaCl are not well defined as NH, 0.6 F or 0.8 F NaCl pellets, so it is very easy to contaminate these pellets with supernatant histones. Preparation (1) gave higher recovery but poorer quality of NH's in the pellets. Preparation (2) gave lower yield but cleaner samples. Later discussions will be referred to preparation (2) only.

TABLE II

CONTENTS OF HISTONE AND NONHISTONE PROTEIN OF NH'S BY LOWRY PROCEDURE

Partial NH's	r his/r-DNA	% of histone remained by Lowry	% of histone remained by gel bands (ii)	r N-his/r-DNA	%of N-his. remained	r prot/r DNA (r his/r DNA + r N-his/r DNA)	% prot remained on DNA
NH	1.21	100%		0.55	100%	1.76	100%
0.6	0.83	69%	70%	0.26	47%	1.09	62%
0.8	0.74	61%	60%	0.23	42%	0.97	55%
0.9	0.5	41%	44%	0.26	47%	0.76	43%
1.2	0.188	16%	18%	0.24	44%	0.428	24%
1.6	0.021	2%	10%	0.21	38%	0.231	13%
2	0.06	0.5%	5%	0.18	33%	0.186	11%
4	0.01	0%	0%	0.17	31%	0.18	10%

Footnote:

i) Partial NH's are from the same preparation as (2) in Table I, so a comparison of the two methods can be made.

ii) Baldomero Olivera, p-116 Ph.D. Thesis, Div. of Chem. California Institute of Technology.

EXTENT OF DISSOCIATION OF HISTONE AND NONHISTONE WITH VARYING SALT CONCENTRATION

Extent of protein dissociation can be evaluated by A_{230mu} (last column in Table I) and the procedure of Lowry et. al. (last column in Table II). The two methods of estimating protein content agree well between 0.6 F and 1.0 F NaCl. Above 1.0 F, A_{230mu} method gives somewhat higher values (4-10%). This is probably due to the imperfect yield of histone and nonhistone extraction for the Lowry determination. Since over 60% of associated protein has already been removed at 1 F NaCl, the quantity of protein which remains for study is 50 to 100 ug and a small loss of material thus becomes more significant in this salt range. The A_{230mu} determination of total protein is preferable because it does not involve any additional experimental manipulation. But this method does not differentiate histone protein from nonhistone protein.

The extent of histone dissociation can be estimated by the relative densities of histone bands in polyacrylamide gels as well as by the Lowry procedure (3rd and 4th columns of Table II). The methods agree satisfactorily except for 1.6 F and 2 F NaCl extractions. In these cases the band patterns in the gels of dissociated histones look very much like those of whole histone and the method is not sensitive enough to pick up any differences.

From 0 to 0.6 F NaCl, about two thirds of the dissociated protein (40%) is histone, and the rest nonhistone protein. From 0.6 F to 1.6 F NaCl, more than 90% of the dissociated protein (40% of the total protein) belongs to the histone class. There is very little nonhistone dissociation in this range of salt concentrations. At salt concentrations

higher than 1.6 F, practically no protein (3%) is dissociated because 98% of the histone has been dissociated and most of the remaining nonhistone stays with DNA even after 4 F NaCl treatment (2nd, 5th, and 8th columns in Table II).

Histone dissociation is gradual with increasing salt concentration. (Fig I) Between 0 and 0.6 F NaCl, 30% of total histone is dissociated. Gel electrophoresis shows that only histone I (and all of it) is removed in this range of salt concentrations. The slope of the dissociation curve (Fig. I) is the sharpest between 0.8 to 0.9 F NaCl. By 2 F NaCl over 99% of histone is dissociated.

More than 50% of the nonhistone protein is dissociated by 0.6 F NaCl. The proportion of nonhistone protein is almost the same for 0.6 and 4 F NaCl extracted NH's. At salt concentrations higher than 1.2 F, in which most of the histones are dissociated, nonhistone becomes the major type of DNA-associated protein (Fig. I).

IDNETIFICATION OF DISSOCIATED HISTONES BY GEL ELECTROPHORESIS

Detailed study of the gels (16) and their densitometer tracings (Figs III and IV) shows that Histone I is dissociated mainly between 0.4-0.5 F NaCl, and is completely removed by 0.6 F NaCl (Fig IIId). Histone II dissociation begins at 0.8 F NaCl (Fig IIIi, e; Fig IVb). Up to 1.6 F NaCl there are still traces of undissociated Histone II (Fig IVf). Histone III+IV starts to dissociate at 0.9 F (Fig IVb,c). At 1.6 F NaCl in which over 95% of histones have already dissociated (Table II), Histone III+IV becomes the major type of associated histone (Fig IVf). There are no gel electrophoretically-detectable histones in the 4 F NaCl extracted NH in agreement with the Lowry determination

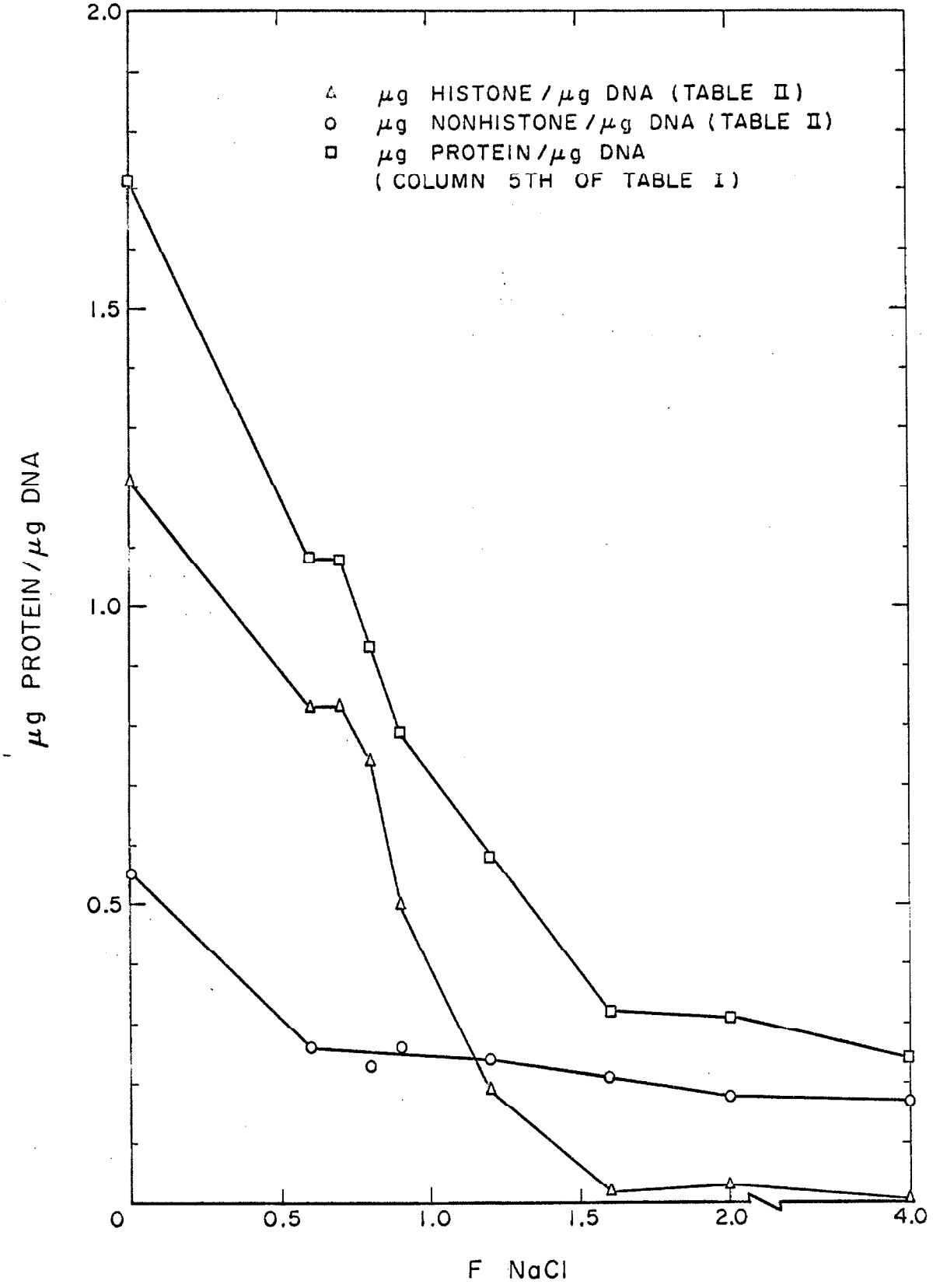

2.0
1.5
1.0
0.5
0
0.5
1.0
1.5
2.0
4.0
μg PROTEIN / μg DNA
F NaCl
Δ μg HISTONE / μg DNA (TABLE II)
o μg NONHISTONE / μg DNA (TABLE II)
□ μg PROTEIN / μg DNA
(COLUMN 5TH OF TABLE I)

Fig. I

THE GEL ELECTROPHORESIS BAND PATTERN OF HISTONES

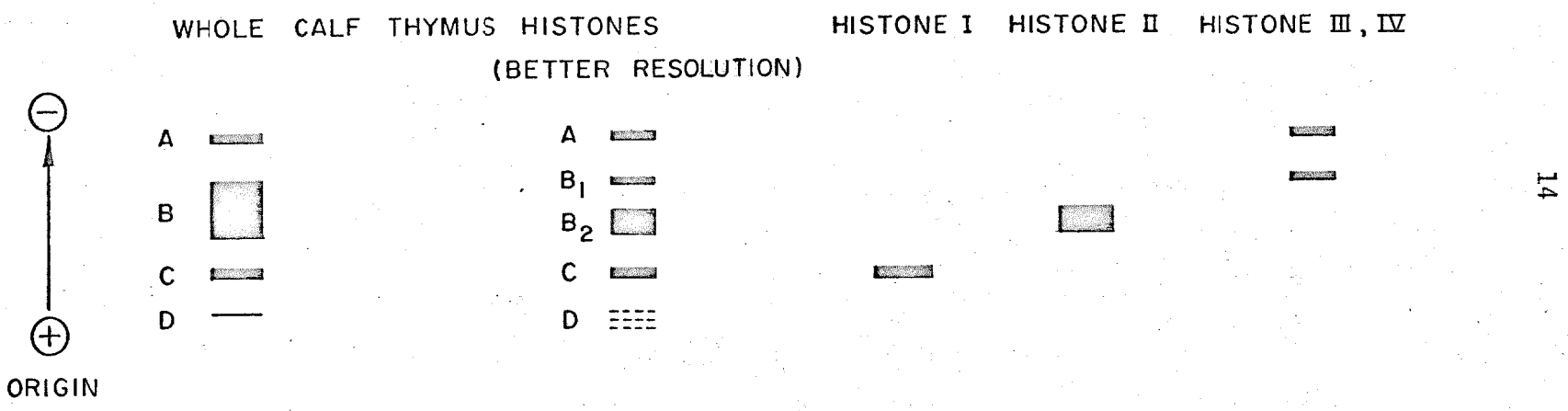


Fig. II

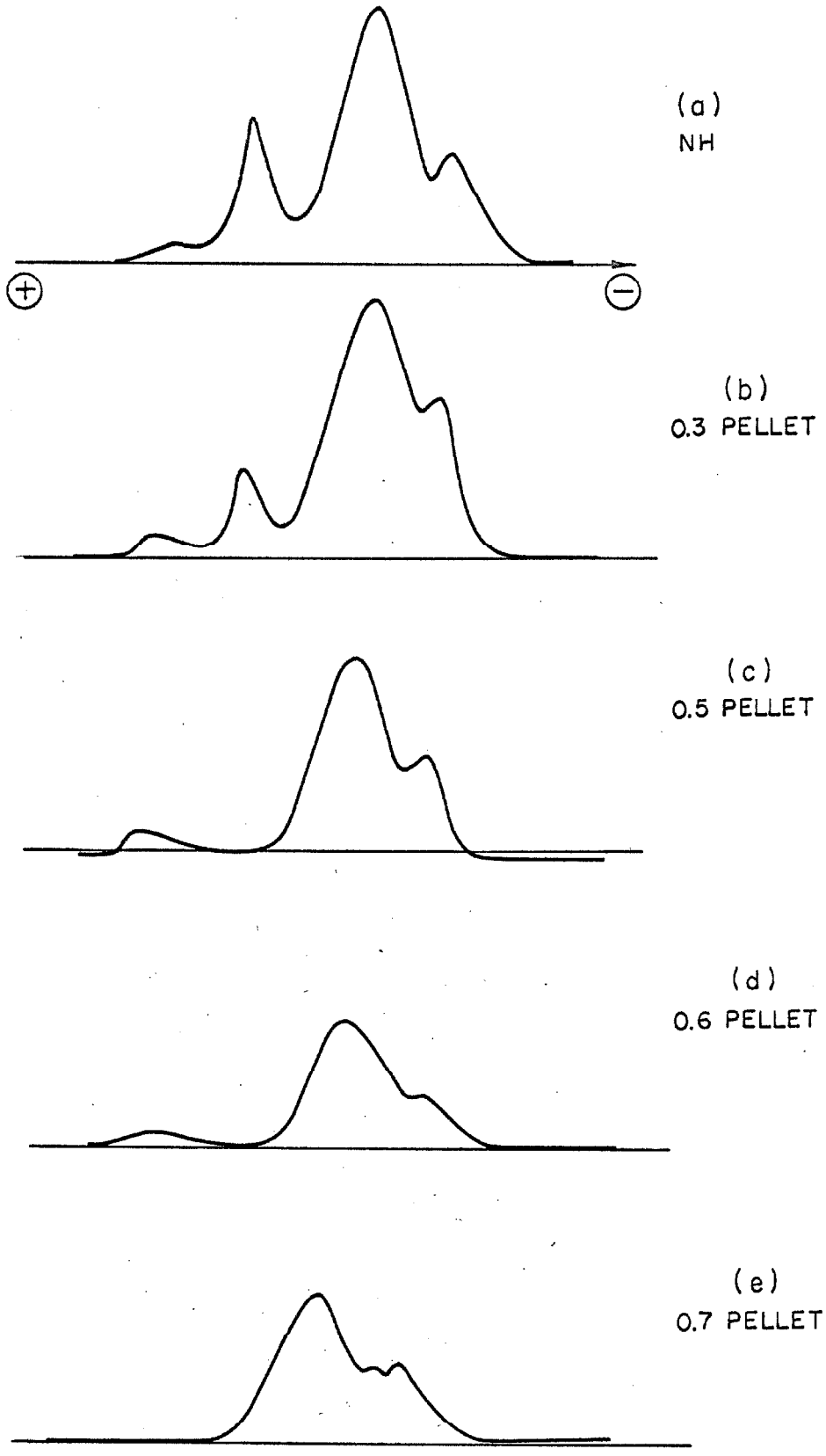


Fig. III

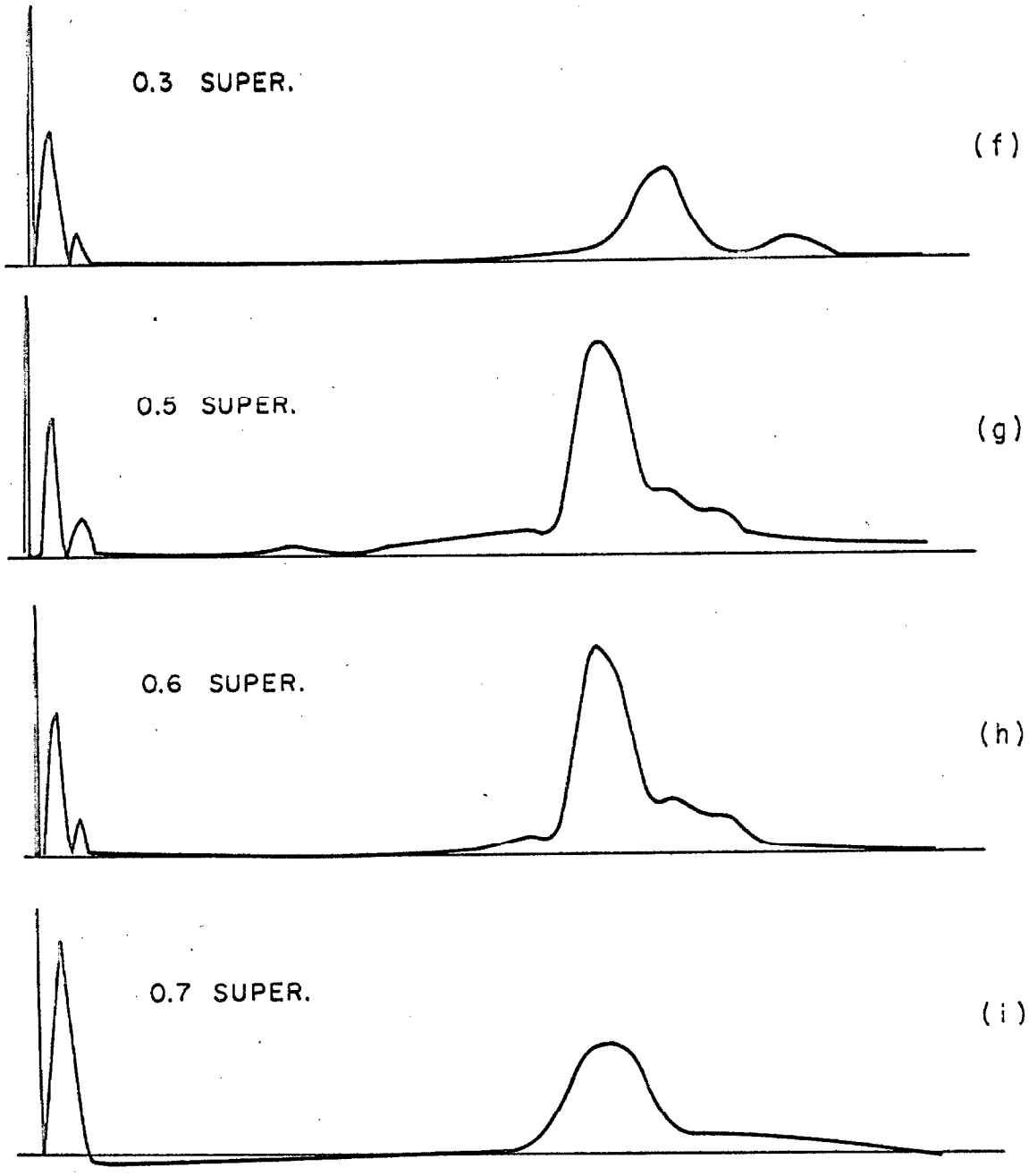


Fig. III

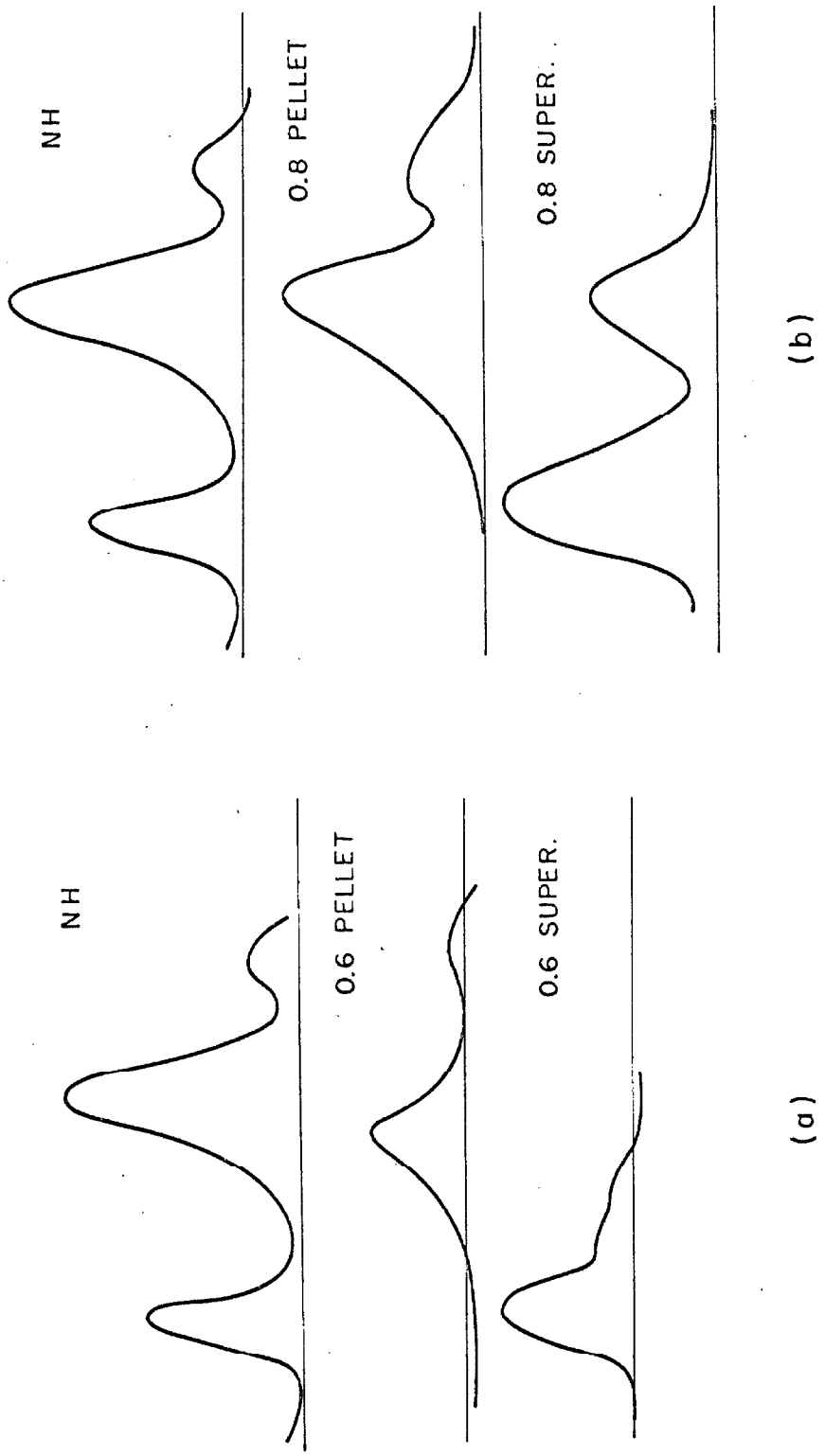


Fig. IV

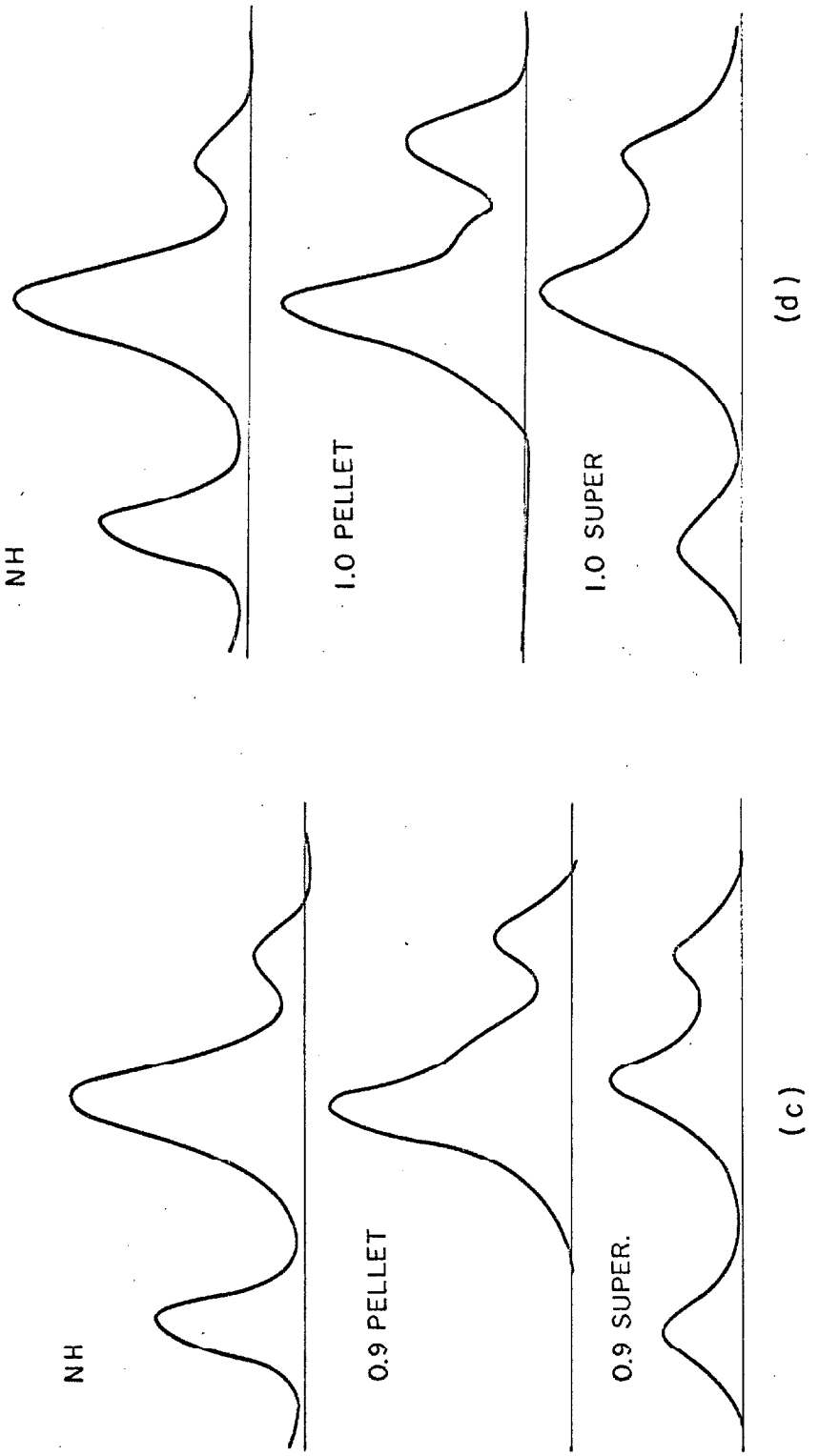


Fig. IV

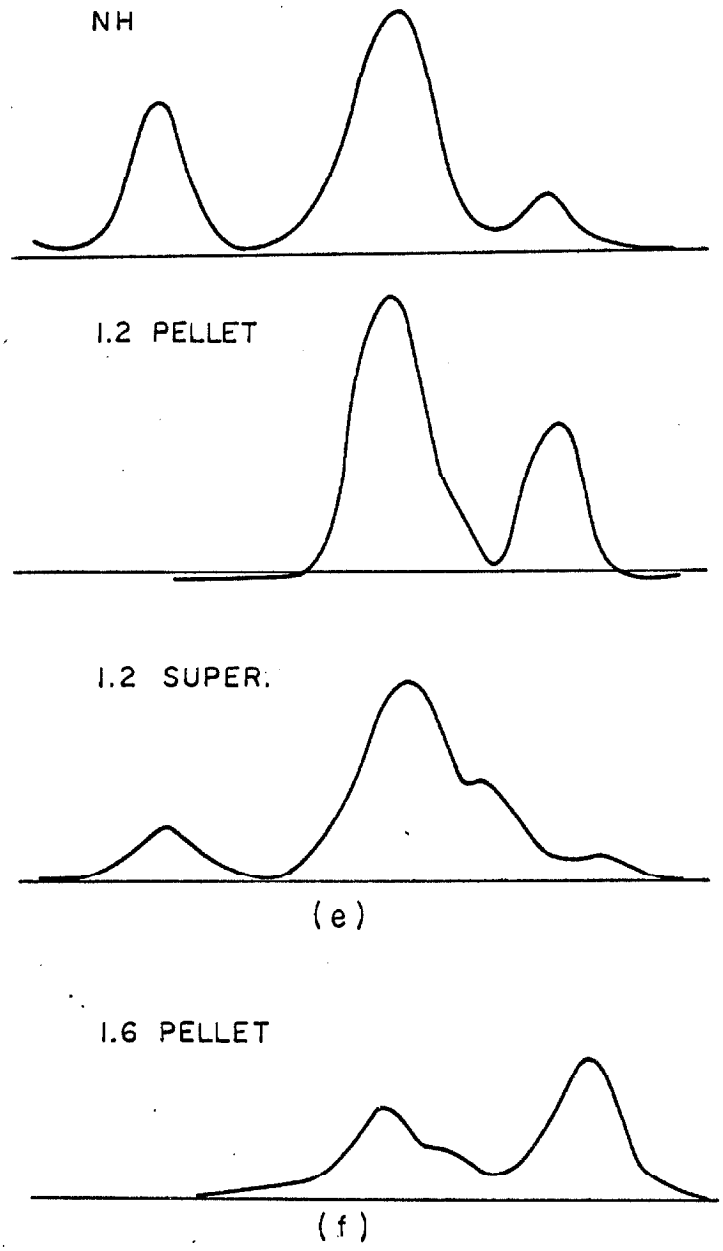


Fig. IV

results. (Table II).

It is possible to argue (5) that histone III+IV might be dissociated at lower salt concentrations than is histone I, but that because of its tendency to aggregate (17), is sedimented together with partially dissociated NH and thus appears to be still associated with the pellet. To settle this question, free boundary electrophoresis (18) as well as sedimentation was used to separate any dissociated histones from partially dissociated NH. This method utilizes the differences in charge density of different molecular species. Since it is positively charged in the medium used (0.5 F NaCl, 0.05 M tris pH 7.5) dissociated histone remains at the origin in the electrophoresis apparatus, while partially dissociated NH migrates (with a certain mobility) toward the cathode. Thus if histone III+IV was dissociated at lower salt concentrations than histone I, histone III+IV as well as histone I should be missing from the partially dissociated NH after its purification by electrophoresis in 0.5 F NaCl. The gel electrophoresis histone patterns of the free boundary electrophoretically purified NH and of that prepared only by centrifugation were identical--histone II and III+IV were present but histone I was missing. The above experiment demonstrates conclusively that histone III+IV is not dissociated at lower salt concentrations than histone I. The reported order of histone dissociation is thus established beyond doubt.

That histone I possesses higher charge density than histone II and III+IV is evident from: i) the amino acid composition of the histones (the number of basic amino acid residues minus the number of acidic amino acid residues is an indication of charge density of the histone) (19). ii) the lower electrophoretic mobility of reconstituted

NHI (prepared by artificial reconstitution of histone I with DNA, in which part of DNA charges are neutralized by those of the histone I molecules) as compared to the mobility of NHII or NHIII+IV of the same histone coverage (same weight ratio of histone to DNA) (20). Hence, so far as electrostatic interaction is concerned, histone I should be more firmly bound to DNA than the other histones and electrostatic interaction alone does not explain why histone I is dissociated at an ionic strength lower than that required to dissociate histone II and histone III+IV. It is probable that forces other than electrostatic one exist between the relatively arginine rich histone II, the arginine rich histone III+IV and DNA, which strengthens their binding to DNA; or that the lysine and proline rich histone I has such a conformation that a close binding of histone I to DNA is unfavorable. The fact that sodium desoxycholate at concentrations lower than 0.1F preferably dissociates histone II and III+Iv from NH (21) suggests that nonelectrostatic force plays a role in the binding of histone II and histone III+IV to DNA. Optical rotatory dispersion study on histones in the following chapter, however, lends evidence to the second possibility. It thus seems that the combination of the unfavorable conformation and the lack of interaction other than electrostatic force between histone I and DNA causes histone I to be the first histone dissociated.

SOLUBILITY OF NH IN NACL SOLUTIONS

NH is soluble in 0.01 M tris PH 7.3. When the ionic strength of the solvent is increased the solubility of NH decreases. At 0.3 F and 0.4 F NaCl, over 80% of NH is precipitated out of the solution. The precipitated NH (collected by centrifugation for 5 minutes in the clinical centrifuge approx. 1000 xg) and the soluble NH (collected subsequently

from the supernatant of 1000 xg centrifugation by centrifugation at 39,000 rpm (approx. 130,000 xg) for 24 hours; the resulting supernatant being the dissociated protein solution.) exhibit similar protein contents, both about 10% less than the original NH. That some kind of histone is dissociated at this salt concentration is evident from the presence in the dissociated protein of a gel electrophoreses band of mobility similar to that of histone I. (Fig. IIIf) Above 0.4 F NaCl the NH becomes soluble again. This enhanced solubility with increasing ionic strength parallels the dissociation of histone I. In 0.6 F NaCl, histone I is completely dissociated, and NH is completely soluble. That this increased solubility is indeed due to removal of histone I rather than to some other effect of 0.6 F NaCl on NH was confirmed by the solubility in 0.3 F NaCl of 0.6 F NaCl extracted NH (19). There is also the possibility that the removal of nonhistone protein may cause the increased solubility. However, the above possibility is not discussed in this section because we do not know enough about the properties of nonhistone protein to evaluate this suggestion critically.

There are two possible ways to explain the insolubility of NH at 0.3 F NaCl and its complete solubility at 0.6 F NaCl, if we assume that the removal of histone I is the important effect.

Histone I is known to have a peculiar ability, not possessed by other histones to bind DNA reversibly. Thus, DNA and NHI (an artificial DNA histone I complex) when electrophoresed together, give, instead of separate DNA and NHI bands, only a single band with a mobility intermediate between those of DNA and NHI (22). In 0.3 F NaCl, while some histone I is dissociated (Fig. IIIf), exposing regions of free DNA, some

histone I is still associated. At this stage of histone I dissociation, in which histone I is busy shifting from molecule to molecule, linking stretches of exposed DNA, there is a possibility that extensive cross linking by histone I may ensue, resulting in the precipitation of NH. At 0.6 F NaCl, when the cross linking histone I is removed completely, NH becomes soluble again.

Alternatively, the insolubility of NH at 0.3 F NaCl may be due to an enhanced attraction and decreased repulsion between the individual NH molecules, when the ionic strength of the solvent is increased from 0.01 to 0.3 (salting out). The solubility of NH in 0.6 F NaCl may result from the increased electric repulsion between the partially dissociated NH molecules due to their increased charge density as a result of complete dissociation of the postively charged histone I.

Both of the above alternatives have some experimental support but neither has sufficient to exclude the possibility of the other. In order to establish the true mechanism more experiments are apparantly needed.

REFERENCES:

(1) Bonner, J., et. al. Methods in Enzymology, Vol. VIII

(2) Akinrimisi, E. O., P. 162 Ph.D. thesis, Div. of Biology, California Institute of Technology. 1964

(3) Peacock, G., Biochim. Biophs. Acta 55, 943 1962

(4) Ohlenbusch, H., Ph.D. thesis, Div. of Chem. Calif. Inst. of Tech. 1965

(5) Chalkley, R., Methods in Enzymology, Vol. VIII

(6) Hamaguchi, K., and E. P. Geiduschek, J. Am. Chem. Soc. 84, 1329 1962

(7) Ohba, Y., Biochim. Biophs. Acta 123, 78 1966

(8) Chalkley, R., and R. Maurer, Proc. Nat. Acad. Sci. 54, 498 1965

(9) Olivera, B., P. 24 Ph.D. thesis, Div. of Chem. Calif. Inst. of Tech. 1966

(10) Fambrough, D., and J. Bonner, Biochemistry 1966

(11) Kindly performed by Lawrence M. White, Chemist, Fruit Lab U.S. Dept. of Agriculture.

(12) Rasmussen, P. S., K. Murray and J. M. Luck, Biochem. 1, 79 1962

(13) Lowry, O. H., N. J. Rosebrough, L. Farr and R. J. Randall, J. Biol. Chem. 193, 265 1951

(14) Fujimura, F., Personal communication

(15) Shih, T., Personal communication

(16) loc. cit. (9) P. 103

(17) Cruft, J., C. M. Mauritzen, and E. Stedman, Proc. Royal Soc. London 148, 21 1958

(18) loc. cit. (9) P. 139

(19) Ohlenbusch, H., B. Olivera, D. Tuan, and N. Davidson, J. Mol. Biol. in press.

(20) Olivera, B. M., R. C. Huang and N. Davidson, Berichte der Bunsengesellsehaft fur physikalische Chemie 68, 802 1964

(21) Smart, J., Personal communication

(22) loc. cit. (9) P. 80

Chapter 2

OPTICAL ROTATORY DISPERSION STUDY OF HISTONES

I. INTRODUCTION:

Histones can be separated by column chromatography and gel electrophoresis into a number of components (6 or 7). These are small molecules of molecular weight below 50,000 and are relatively homogeneous as indicated by end group analysis (1) (2). The free and fractionated histones can be artificially put back onto DNA without influencing much the DNA structure. Thus, with a proper method of investigation, we can study the structures not only of free histones but also of DNA-bound histones.

Optical rotatory dispersion (ORD) is a powerful tool for examining the conformation of proteins or polypeptides in solution. Recent improvements in the spectropolarimeter have enabled the Cotton effects of proteins to be accurately recorded. These contain information of a more subtle variety than ORD spectra in the visible region. Proteins or polypeptides possessing different conformations exibit different Cotton effects (3) (4) (5). It is therefore possible to study the structure of a protein with unknown conformation by comparing its ORD spectrum in the Cotton effect region with ORD spectra of protein or polypeptides whose conformations are known from other methods of investigation.

The present chapter is devoted to an application of the technique of ORD to the study of histones. The structure of histones and alterations in their conformation when complexed with DNA will be discussed.

It is to be emphasized that in this chapter we compare the ORD of free histones at two different salt concentrations and of histones in reconstituted NH (not in native NH). The ORD of the histone component is calculated from the observed ORD of the reconstituted NH by subtracting the ORD of the same amount of native DNA. Thus, the interpretation involves the assumption that the conformation of the DNA and its ORD is unchanged by association with histone in reconstituted NH. Some justification for this assumption will be presented later.

It should also be mentioned at this point that, in the next chapter, we shall present evidence to support the view that the conformation and ORD of the DNA in native NH is different from that of free DNA or of the DNA in reconstituted NH.

II. MATERIALS AND METHODS:

ISOLATION OF HISTONES

i) Acid extraction:(1) Purified chromatin (see previous chapter for its preparation) was stirred on ice and one-fourth volume of cold 1 N H_2SO_4 slowly added. After 30 minutes of stirring, the suspension was centrifuged at 17,000 xg for 20 minutes. The histone sulfate was precipitated from the supernatant by adding 4 volumes of cold absolute ethanol, washed and collected by centrifugation. The precipitate was dissolved in the desired solvent and used for ORD measurement.

ORD spectra were also measured on the acid extracted, subsequently Amberlite column fractionated histones. To fractionate the histones into their components (1), histone sulfate was dissolved in 8% guanidinium chloride, applied to an Amberlite Gc-50 column and eluted with a linear gradient of buffered 8% to 13% guanidinium chloride, followed by 40% guanidinium chloride. The first main component eluted is the histone I, followed by histone II, and finally histone III+IV. The different fractions of histones were dialyzed against cold 0.1 M acetic acid to remove guanidinium chloride and lyophilized. Histone solutions for ORD measurement were freshly prepared before usage.

The author thanks Mr. Douglas Fambrough for the acid extracted, Amberlite column franctionated histones used for this study.

ii) Salt extraction: The procedures of selective dissociation of histones described in the previous chapter can be applied to the salt extraction of histone on a preparative scale. Nucleohistone was dissolved in the required salt concentration and the dissociated protein freed from residual NH by centrifugation at 39,000 rpm in an SW 39 rotor.

The dissociated protein was dialyzed with three changes of 10x volumes of 0.01 F tris pH 7.3.

PREPARATION OF RECONSTITUTED NH:

The procedure is essentially the reverse of the dissociation of NH with NaCl. Soluble DNA-histone complex (reconstituted NH) can be prepared by mixing DNA and histone in a solution of high salt concentration and then gradually dialyzing to lower the salt concentration. Commercial Sigma calf thymus NaDNA and acid extracted, column fractionated calf thymus histones were used. For complex formation, equal volumes of solutions of DNA (200 ug/ml) and of the desired histone (100 ug/ml) were mixed in 2 F NaCl. The mixture was next dialyzed successively, for 4 hours each, against 25 volumes of 0.4 F, 0.3 F, and 0.15 F NaCl; and finally for 10 hours against 3 changes of 0.01 F tris PH 7.3. Any precipitate was centrifuged off at 10,000 rpm for 30 minutes to yield reconstituted NH in the supernatant. The final histone to DNA ratio of the reconstituted NH is usually 10-20% higher than the input histone to DNA ratio. It is very difficult to reconstitute the DNA-histone III+IV complex with a high histone to DNA ratio because of the tendency of histone III+IV to aggregate in moderate concentrations. But it is possible to prepare histone III+IV-DNA complex under the present experimental condition of low histone to DNA ratio and low histone III+IV concentration.

ABSORPTION CONSTANTS OF FREE DNA AND DNA COMPLEXED TO HISTONES:

Absorption constant is here defined as the ratio of A_{260mu} to A_{600mu}, where A_{260mu} is the absorption at 260 mu of DNA and A_{600mu} is a

measure by diphenylamine reaction of the DNA quantity. It is well known that the optical absorption of DNA at 260 mu is conformation dependent. Thus, any difference in magnitude of absorption constant at 260 mu of DNA complexed to histone and that of free DNA indicates conformational change in the DNA induced by the presence of histones. (The contribution of histone to A_{260mu} is less than 1%)

Determination of DNA quantity was by the modified diphenylamine reaction of Burton (6). The material under examination (DNA and reconstituted NH) was first hydrolyzed in 0.5 N $HClO_4$ at 70°C for 15 minutes. Triplicate samples of 0.2 ml of the hydrolysate were mixed with 0.4 ml of Burton's modified diphenylamine reagent. The mixture was incubated at 37°C for 18 hours. The optical absorbance at 600 mu of the mixture was measured on a Hitachi Perkin-Elmer U.V. spectrophotometer. Control experiments have shown that the presence of histones in the reaction mixture does not interfere with the final color development. The U.V. absorption at 260 mu of DNA and the reconstituted complex was recorded using the same spectrophotometer.

SPECTROPOLARIMETRY:

ORD measurements were made with a Jasco ORD recorder calibrated against camphor sulfonic acid. A high concentration of histones is required to obtain rotation in the visible region. However histone solutions at such concentration tend to aggregate and yield turbid solutions. Below 210 mu, the noise level of the instrument is high. Therefore, rotations were recorded with dilute histone solutions (50-75 ug/ml), and only in the region 300 mu to 210 mu which is the cotton effect region of proteins. All measurements were made at 30°C-31°C, in an atmosphere of nitrogen, with a scale setting of $\pm$50 m°, in a metal

cell (1 cm light path) with removable quartz windows. The cell assembly was not dismantled during the change of solution or solvent. Before loading the sample solution, the cell compartment was rinsed with the sample solution. Unless otherwise stated, the solvent used is 0.01 F tris pH 7.3. The base line was obtained with the solvent proceeding each ORD measurement.

The data are represented in terms of specific rotation $[\alpha]$

$$[\alpha] = \frac{\text{ROTATION OF SAMPLE IN DEGREES X 100}}{\text{CON. OF HISTONE IN GM/100ML X LIGHT PATH IN DM}}.$$

Histone concentrations were calculated from U.V. absorption measurements at 230 mu, using the relationship A_{230mu} (100 ug histone/ml)=0.425. A freshly prepared histone solution has very low A_{320mu} (ca. 0.005) and a ratio of $A_{230\ mu}$ to $A_{260\ mu}$ greater than 7. As histone solutions age, $A_{320\ mu}$ increases, indicating increased sample scattering and the ratio of $A_{230\ mu}$ to $A_{260\ mu}$ drops. For ORD measurements, only samples of low $A_{320\ mu}$ and high ratio of $A_{230\ mu}$ to $A_{260\ mu}$ were used. The concentration of DNA-bound histones were determined as described in the previous chapter.

COMPUTATION OF THE ORD SPECTRA OF DNA BOUND HISTONES:

The ORD spectra of DNA-bound histones are computed from the reconstituted NH's. For example, for the case of DNA-bound histone I:

$$[\alpha]_{\text{DNA-bound HI}} = \frac{[\alpha]_{\text{NHI}} - F_{\text{DNA}} \times [\alpha]_{\text{free DNA}}}{F_{\text{HI}}}$$

where $[\alpha]_{\text{NHI}}$ and $[\alpha]_{\text{free DNA}}$ are the specific rotations at wavelength of reconstituted NHI and free DNA; F_{DNA} and F_{HI} fractions of DNA and histone I in the reconstituted NHI. $[\alpha]_{\text{DNA-bound HII}}$ and

$[\alpha]_{\text{DNA-bound HIII+IV}}$ can be similarly calculated. Thus, this analysis is based on the assumption that the contribution by DNA in the ORD of reconstituted NH is that of free DNA.

COMPUTATION OF THE ORD SPECTRUM OF DNA-BOUND WHOLE HISTONE:

The ORD spectrum of DNA-bound whole histones is calculated from the ORD spectra of the component histones according to the formula:

$$[\alpha]_{\text{whole histone}} = [\alpha]_{HI} \times F_{HI} + [\alpha]_{HII} \times F_{HIII} + [\alpha]_{HIII+IV} \times F_{HIII+IV}$$

where $[\alpha]_{\text{whole histone}}$, $[\alpha]_{HI}$, $[\alpha]_{HII}$, $[\alpha]_{HIII+IV}$, are the specific rotations for whole histone, DNA-bound histone I, II and III+IV respectively. Whole histone is here defined as the acid extractable protein from NH. F_{HI}, F_{HII}, $F_{HIII+IV}$ are fractions of histone I, II and III+IV, in whole histone, estimated by measuring the relative areas of the densitometer

tracings of the gel electrophoresis bands of the whole histone, (F_{HI}= 0.26, F_{II}= 0.53, F_{III+IV}= 0.21).

III. RESULTS:

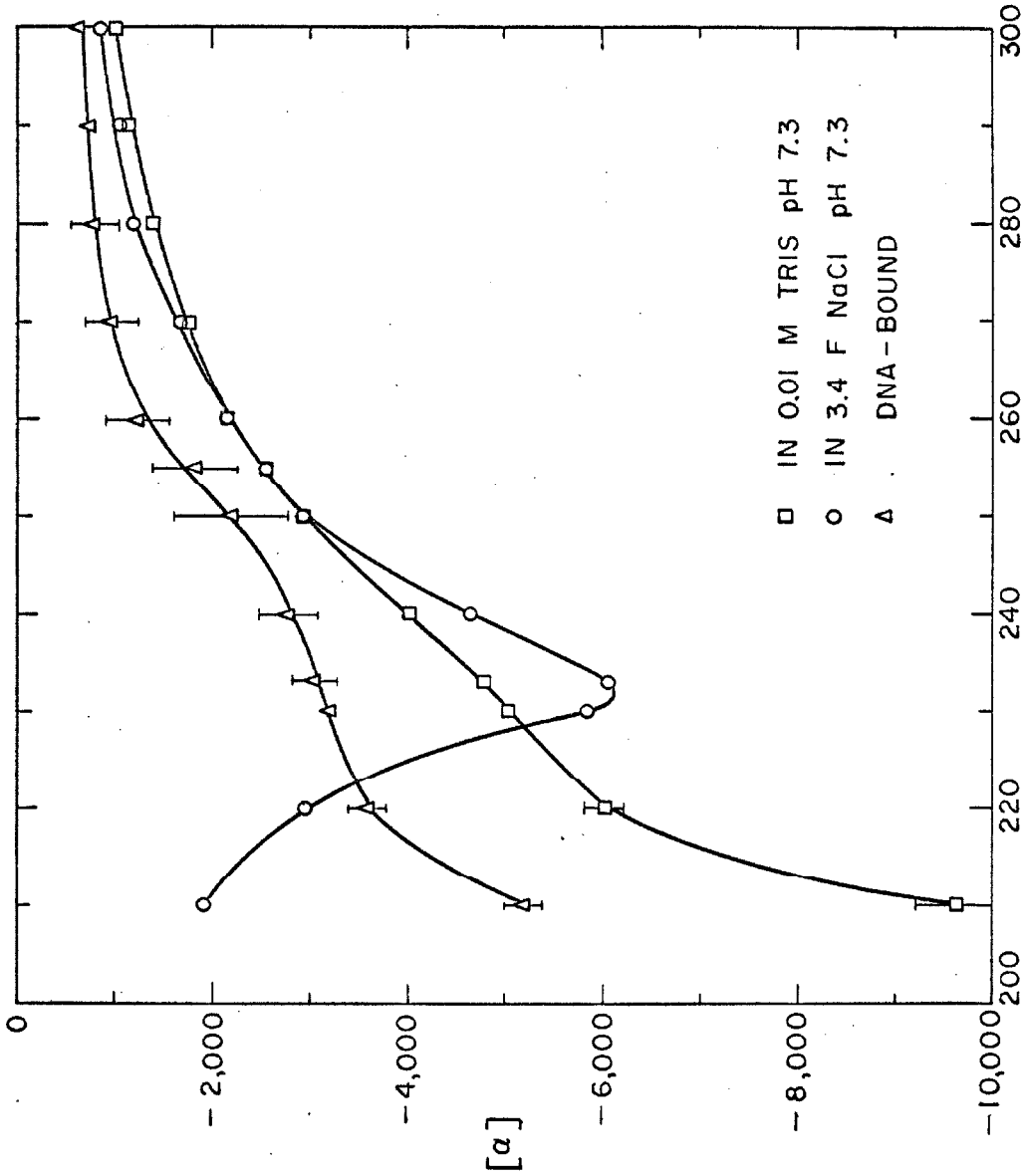
ORD SPECTRA OF ACID EXTRACTED HISTONES:

Histone I: The ORD of histone I (Fig Ia) in 0.01 F tris pH 7.3 is featureless in the region of 300 mu to 210 mu. It resembles the ORD pattern of polyglutamic acid at PH 8.1 whose conformation approximates that of a random coil (Fig II). In 3.4 F NaCl pH 7.3, however, the rotation of histone I is drastically changed. A trough of -6040° appears at 233 mu. In this solvent the ORD spectrum bears more resemblance to that of the helical polyglutamic acid at pH 4.3 (Fig. II).

Histone II: The ORD spectrum of histone II in 0.01 F tris PH 7.3 has a trough of -4200° at 233 mu and a plateau in the region of 220 mu to 210 mu (Fig. Ib). The ORD of histone II in 3.4 F NaCl differs from that in 0.01 F tris in having a steeper slope below 280 mu, a deeper trough of -7200° at 233 mu, a crossover point at 224 mu, and a magnitude of rotation of +14,000° at 210 mu (Fig. Ib).

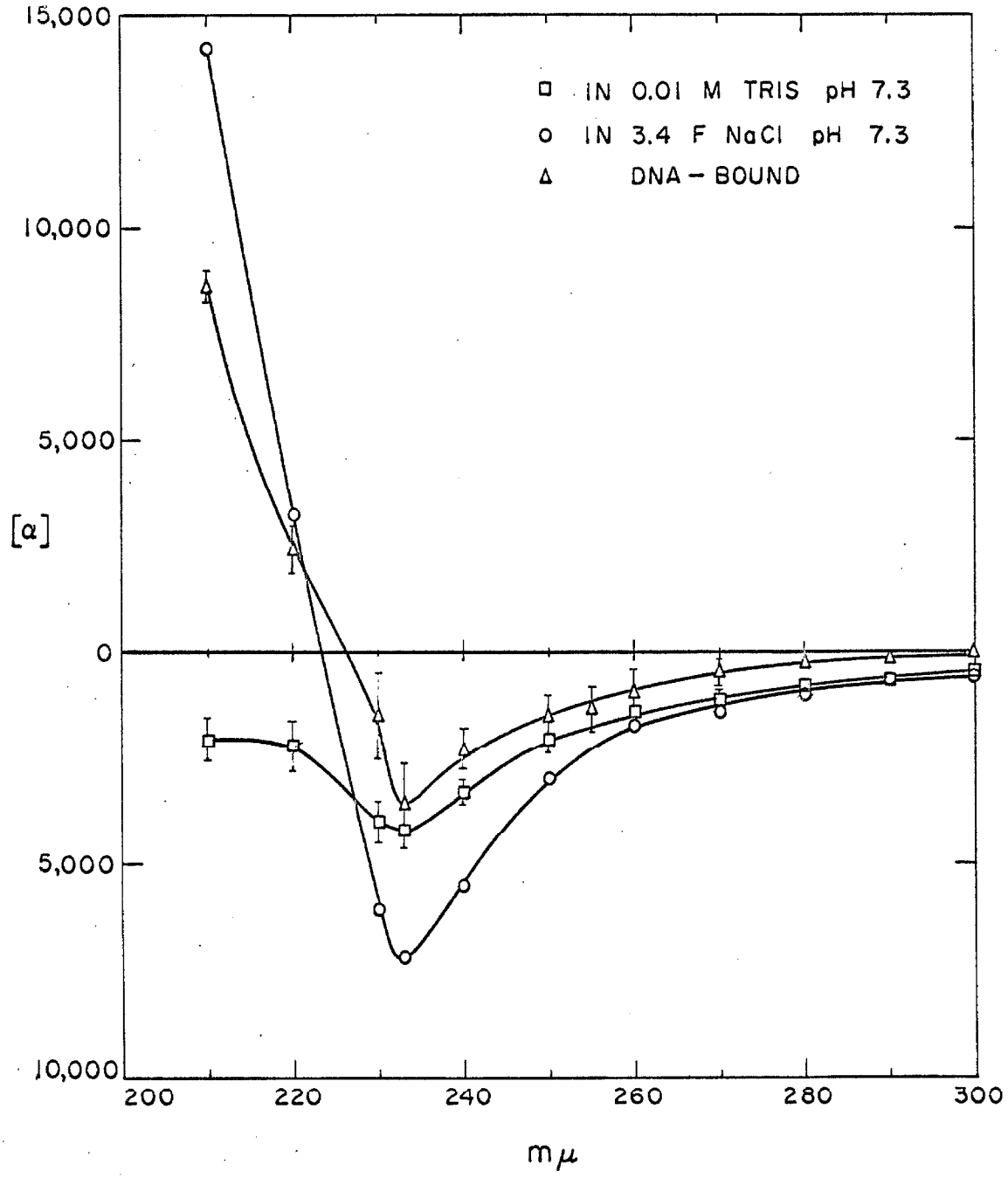
Histone III+IV: The ORD spectrum of histone III+IV is different from both that of the histone I and the histone II; it has a shallow trough of -3500° at 233 mu, a crossover point at 215 mu and a positive rotation of +1500° at 210 mu (Fig. Ic). Histone III+IV is the only free histone in 0.01 F tris which possesses positive rotations above 210 mu. The noise level of the ORD of histone III+IV in 3.4 F NaCl is extraordinarily high, probably due to aggregation of histone III+IV in this solvent. The ORD spectrum for this case is, therefore, not presented.

The changes in the ORD spectra of histones with ionic strength



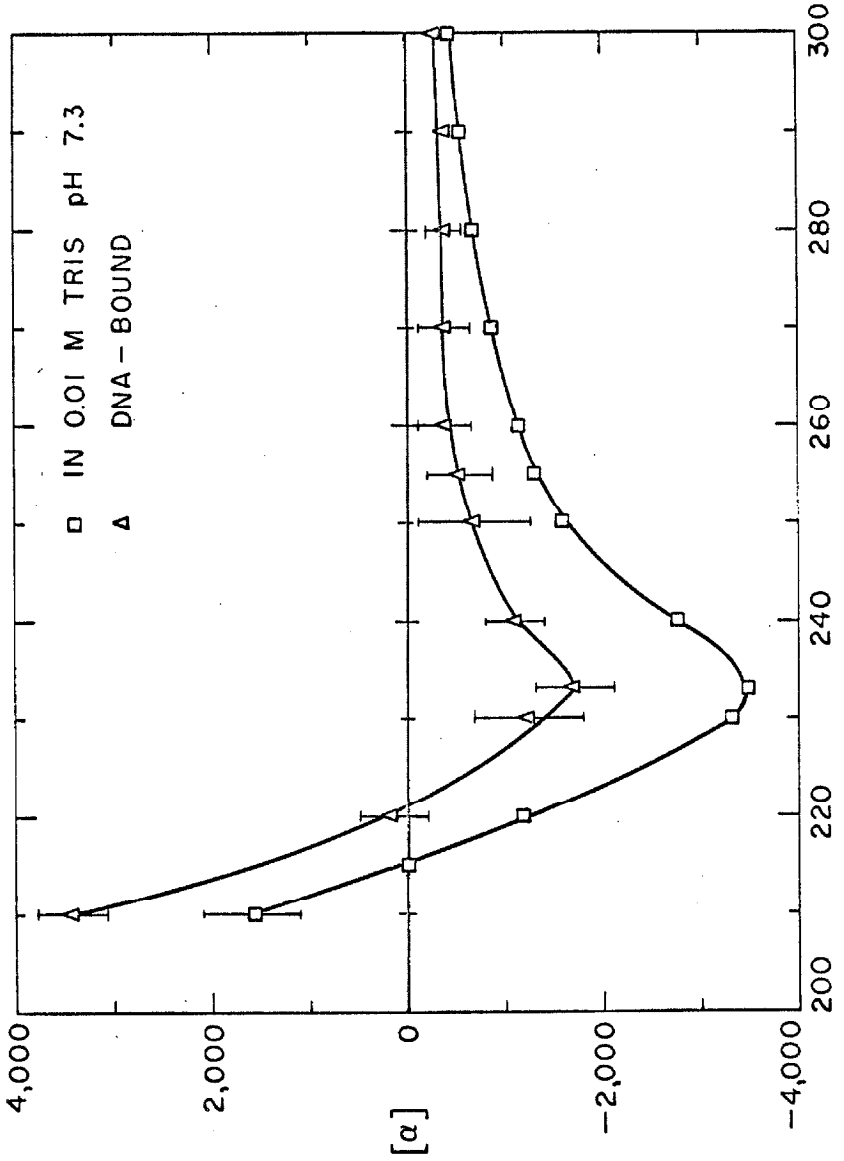
THE ORD SPECTRA OF HISTONE I

Fig. Ia



THE ORD SPECTRA OF HISTONE II

Fig. Ib



THE ORD SPECTRA OF HISTONE III+IV

Fig. Ic

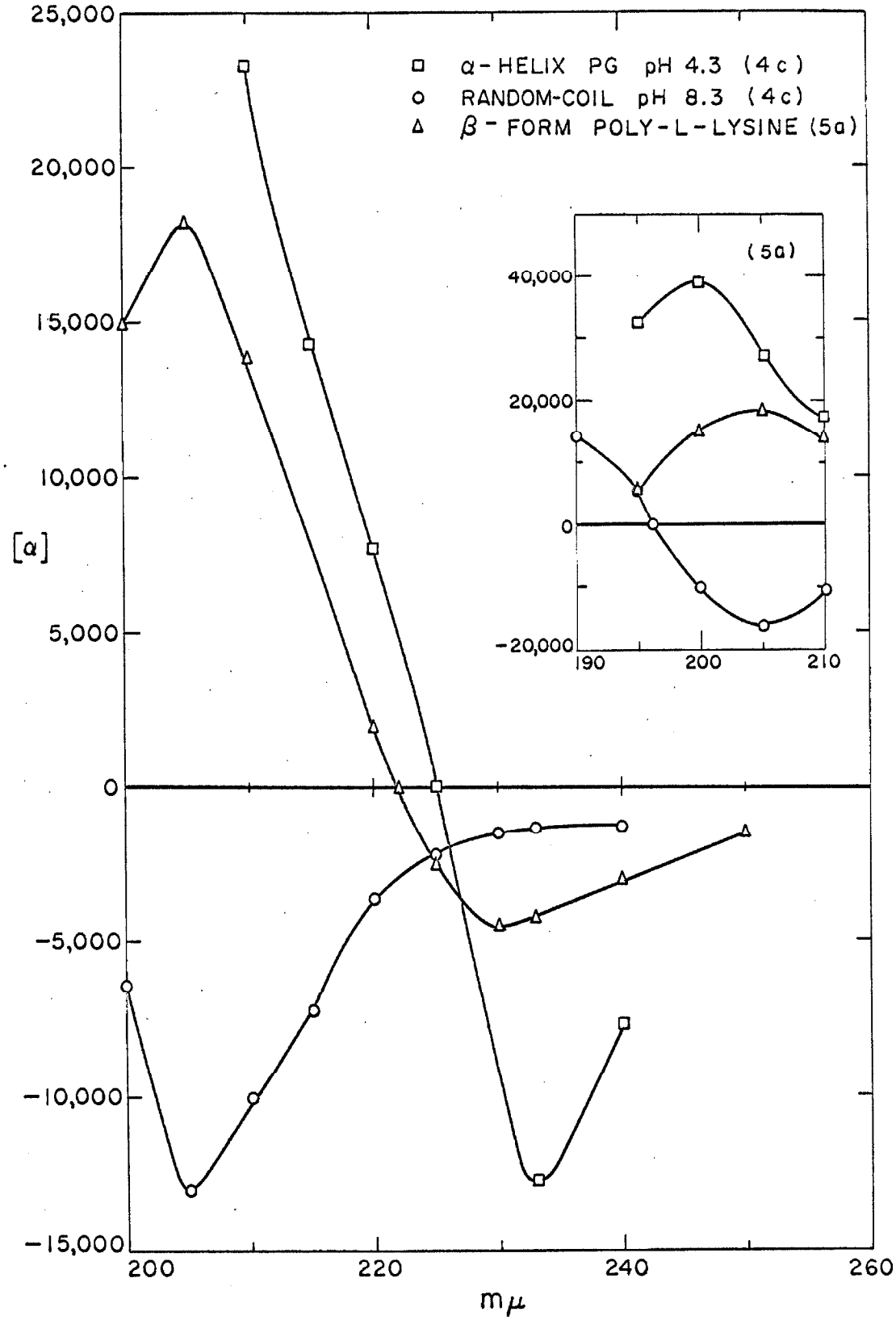

□ α-HELIX PG pH 4.3 (4c)
○ RANDOM-COIL pH 8.3 (4c)
△ β-FORM POLY-L-LYSINE (5a)
(5a)
25,000
20,000
15,000
10,000
5,000
0
-5,000
-10,000
-15,000
[α]
40,000
20,000
0
-20,000
190
200
210
200
220
240
260
mμ

Fig. II

are reversible. Thus, when histone solutions in medium of high ionic strength were dialyzed back to 0.01 F tris PH 7.3, the ORD reverted to the pattern originally found in 0.01 F tris.

There are variations in the magnitude of rotation with different preparations of histones. These are small (±100°) in the region 300 mu to 260 mu. Below 260 mu, however a maximum variation of ±400° has been occasionally observed.

COMPARISON OF THE ORD SPECTRA OF ACID EXTRACTED AND SALT EXTRACTED HISTONES:

Two methods have been used to extract histones from nucleohistone. Acid extraction, followed by column chromatography yields separated fractions of Histone I, II, III+IV. The salt extraction of histones by different concentrations of NaCl, being milder, yields, on the other hand mixtures of non-histone protein and histones I, II, III+IV. The relative amounts of each histone extracted increases with increasing concentration of NaCl according to the above order. Thus, while 0.6 F NaCl extracts only the non-histone protein and histone I, extraction with 4 F NaCl yields a mixture of non-histone protein and all of the histones present in the NH.

One may enquire whether the two methods of extraction yield histone preparations with comparable optical properties. The ORD spectrum of the acid extracted, unfractionated whole histone is compared with that of 4 F NaCl extracted histones (containing about 15% non-histone protein) in Fig. III. It can be seen that both the shapes of the spectra and the magnitudes of the rotations are similar for the two preparations.

A composite ORD spectrum for whole histone may be calculated

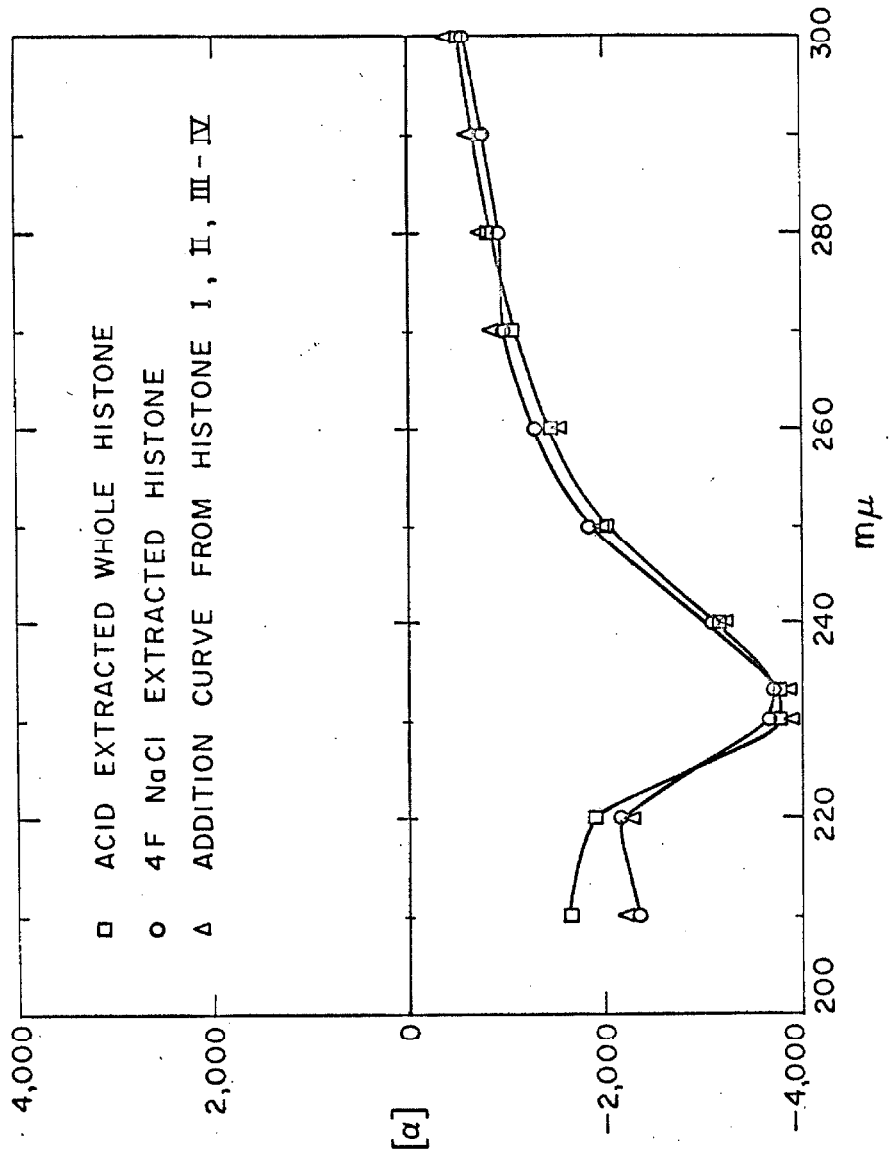

□ ACID EXTRACTED WHOLE HISTONE
○ 4F NaCl EXTRACTED HISTONE
△ ADDITION CURVE FROM HISTONE I, II, III-IV
4,000
2,000
0
−2,000
−4,000
[α]
200
220
240
260
280
300
mμ

Fig. III

from those of its components according to the formula:

$$[\alpha]_w = [\alpha]_I \times 26\% + [\alpha]_{II} \times 53\% + [\alpha]_{III+IV} \times 21\%$$

where $[\alpha]_I$, $[\alpha]_{II}$, $[\alpha]_{III+IV}$ and 26%, 53%, 21% are the specific rotations and relative amounts of histones I, II, III+IV in the NH respectively. This composite spectrum when compared with those of acid and 4 F NaCl extracted histones in Fig. III is again very close to the observed spectra.

The similarity in the ORD spectra of acid and salt extracted histones and the fact that the effect of high concentration of NaCl on the ORD spectra of histones is reversible indicates that in-so-far as optical rotatory properties are concerned, both the acid and salt extracted histones are similar.

THE ORD OF DNA-BOUND HISTONES:

The ORD spectrum of DNA-bound histone is computed from the difference spectrum between the ORD of reconstituted NH's and that of DNA in reconstituted NH. Since the conformationally sensitive absorption constants at 260 mu of both the free DNA and the DNA in the reconstituted NH are identical (Table I), and the X-ray diffraction patterns of both are also similar (7), it is reasonable to assume that the conformation of DNA in the reconstituted NH is the same as that of free DNA. The ORD spectrum of free DNA may therefore be used for the above computation.

TABLE I

DETERMINATION OF ABSORPTION CONSTANTS AT 260 mu OF DNA BY DIPHENYLAMINE REACTION

SAMPLE	$A_{260\ mu}/A_{600mu}$
Free DNA	2.74±0.03
DNA in reconstituted NHI	2.70±0.01
DNA in reconstituted NHII	2.74±0.03
DNA in reconstituted NH III+IV	2.69±0.09

The ORD spectra of DNA-bound histones calculated as described above are different from those of free histones. The trend of the differences in optical rotations at shorter wavelengths (220 mu-210 mu) for all classes of histones is in the direction of the changes induced in free histones by high ionic strength. That these differences are not due to the mere presence of DNA in the solution, but are due to the interaction of histone with DNA will be discussed in the next chapter.

DNA-bound histone I: The ORD spectrum of histone I is featureless even when the histone is complexed to DNA. But the slope of descent of the curve is more gradual and the absolute values of the rotations are smaller than those of free histone I (Fig. Ia).

DNA-bound histone II: Binding to DNA of histone II induces changes in both shape and magnitude of the ORD spectrum (Fig Ib). A trough of -3300° at 233 mu is present. Its depth is less than that of free histone II. There is a crossover point at 226 mu and a magnitude of rotation at 210 mu of +8500°.

DNA-bound histone III+IV: The shape of the ORD spectrum of bound histone III+IV (Fig Ic) is similar to that of free histone III+IV, but the bound histone exhibits a shallower trough of -1700° at 233 mu, a crossover point at 230 mu, and a rotation at 210 mu of +3500°.

The ORD spectra for histones bound to DNA drawn in Fig I are average values of three measurements. Fluctuation in magnitude is represented in Fig I by a bar whose length is proportional to the fluctuation . It can be seen that variations in $[\alpha]$'S of bound histones are more pronounced than the ones of free histones in 0.01 F tris and 3.4 F NaCl, especially around the absorption peak of DNA.

IV. DISCUSSION:

The ORD spectrum of a protein contains information about the molecular structure of the protein in solution. A chromophore of a protein or polypeptide, either inherently or as a result of interaction with its environment is asymmetric, it will exhibit Cotton effects associated with the specific spatial arrangement of the chromophore. An approach to correlate the observed Cotton effects with the molecular structure of proteins and polypeptides is to determine first, the ORD spectra of model compounds of known conformations. One then attempts to construct from these ORD spectra of the model conformations a weighted sum which is identical with the ORD spectrum of the unknown structure. The unknown structure is thus considered to consist of a mixture of the model conformations in proportions equal to their respective weights. The model compounds used for this purpose are generally synthetic homopolypeptides whose conformations are known. The ORD spectra of the three most commonly encountered conformations, α-helix, random coil, and B-form are reproduced in Fig. II. (B-form here refers to the conformation exhibited by poly-L-lysine in alkaline solution). These ORD patterns are different from one another. The ORD spectra of both the α-helix and the B-form have a trough at ca 230 mu, a crossover point at 222 mu to 225 mu and a peak below 210 mu. Similar in shape as they are, the amplitudes of the peaks and the depths of the trough for the B-form are, however, much smaller than for the α-helical form. Yet, because of the shallow trough of the B-form, it has a higher ratio of $[\alpha]_{210\ mu}$ to $[\alpha]_{233\ mu}$. The ORD spectrum of the random coil, on the other hand, is comparatively featureless above 210 mu. The greatest difference in

magnitude of optical rotation for the three forms occurs at wavelengths below 220 mu. Because of the greater degree of rotations of histones at wavelengths below 220 mu, variations are comparatively small and readings of optical rotations in this region are quite reproducible. The difference in magnitude of optical rotation at wavelengths below 220 mu can thus be utilized to calculate the relative amounts of the three structures in a protein whose ORD spectrum is known. To simplify the calculation, we will here consider only mixtures of two conformations, namely of α-helix and random coil and of B-form and random coil.

The content of α-helix present in a mixture of α-helix and random coil is calculated from the following equation:

$$\alpha\text{-helical content\%}_{(210\ mu)} = \frac{[\alpha]^{protein}_{210\ mu} - [\alpha]^{random\ coil}_{210\ mu}}{[\alpha]^{\alpha\text{-helix}}_{210\ mu} - [\alpha]^{random\ coil}_{210\ mu}} \times 100$$

where $[\alpha]^{rand.coil}_{210\ mu}$ $[\alpha]^{\alpha\text{-hel}}_{210\ mu}$ are the specific rotation at 210 mu of random coil and α-helix respectively, and are known quantities. $[\alpha]^{protein}_{210\ mu}$ can be obtained from the measured ORD curve of the protein.

Substitution of $[\alpha]^{\alpha\text{-hel.}}_{210\ mu}$ by $[\alpha]^{B\text{-form}}_{210\ mu}$ in the above equation yields the content of B-form for the case of a mixture of B-form and random coil.

It is evident from the above that there are many assumptions implicit in this procedure for determination of structural content. The rotational parameters for structural determinations (i.e. $[\alpha]^{\alpha\text{-hel.}}_{210\ mu}$ $[\alpha]^{B\text{-form}}_{210\ mu}$ $[\alpha]^{rand.coil}_{210\ mu}$) are obtained from synthetic homopolypeptides (polyglutamic acid and polylysine etc.) in aqueous solution at specified pH. The successful application of these rotational parameters for estimation of the structural content of proteins should then meet the following require-

ments:

1) The absence of side chain cotton effects. Cotton effects in the U.V. region of the side chain chromophores, such as tryptophane (8), tyrosine (9), and sulfhydryl group (10) of proteins overlap with and thus modify that of the backbone peptide bonds. The backbone peptide bonds, in these instances, are no longer the only source of optical activity contributing to the rotatory parameter.

The calf thymus histones, with the exception of histone I, which has a high proline content, have insignificant amounts of sulfhydryl groups and few aromatic amino and proline residues (Table II). Interference by side chain Cotton effects in this case is probably not serious.

2) The absence of end effects, i.e. the rotatory parameter should be independent of the number of residues in a given structured segment for residues located within that segment. The calf thymus histones probably contain a mixture of model structures, for instance, random coil and α-helix or B-form (or even other as yet unknown structures). It is unlikely that in a solution of histones some molecules have only α-helix and others only random coil. Rather, each histone molecule probably possesses some residues in one structure and some in the other. Thus the effect on the rotatory parameter of end residues and the distribution of the number of residues in each structured segment may be significant. Until the primary and secondary molecular structures of histone molecules are established, it is impossible to assess the importance of this effect.

TABLE II

THE AMINO ACID COMPOSITIONS OF CHROMATOGRAPHIC HISTONE FRACTIONS.

Amino Acid	Calf Thymus* Ib	Calf Thymus* IIb	Calf Thymus* III+IV
Lys	26.2	13.5	9.7
His	0.2	2.8	1.9
Arg	2.6	7.9	11.9
Asp	2.5	5.6	5.0
Thr	5.4	5.2	6.7
Ser	6.5	7.0	4.6
Glu	4.3	8.7	10.4
Pro	9.1	4.7	4.2
Gly	7.3	8.2	8.6
Ala	24.2	11.5	11.6
Val	4.1	6.7	5.9
Met	0.1	0.8	1.3
Ileu	1.2	4.5	5.3
Leu	5.0	8.6	8.9
Tyr	0.7	3.0	2.2
Phe	0.6	1.3	2.5

*Taken from Rasmussen et. al., 1962

3) The absence of non-conformational rotatory effects: The ORD of histones were recorded in dilute tris buffer, yet those of the model compounds were made in aqueous solution with different buffer system. In applying the rotational parameters of the model compounds for estimation of histone structure, it is assumed that no rotatory effect is generated by the change of the solvent. This is not an unreasonable assumption since the refractive index of water should not be significantly changed by the dilute buffer system.

It has been reported that aggregation affects the ORD of helical polypeptides (11). Precaution must therefore be taken to insure that the sample used does not aggregate under the experimental condition. The histone solutions employed for the present ORD studies were all fresh solutions of very low concentration. Aggregation in this case should not be serious and indeed is probably absent entirely. Possible aggregation of the model compounds used and ensuing effects on the standard ORD spectra were not investigated, since the results will mostly be considered relative to one another.

With the above assumptions in mind, Table III is constructed. In 0.01 F tris, histone I possesses virtually no ordered structure (3% α -helix in a mixture of α-helix and coil; or 5% of B-form in a mixture of B-form and coil), which is not unreasonable considering the high proline content of histone I.

TABLE III

ESTIMATION OF THE CONTENTS OF α-HELIX OR B-FORM IN THE HISTONES BY THE SINGLE WAVELENGTH METHOD

SAMPLES	α-HELIX% IN A MIXTURE OF HELIX AND COIL		B-FORM% IN A MIXTURE OF B-FORM AND COIL	
HISTONE I IN	210 mu	215 mu	210 mu	215 mu
0.01 M tris	3%	0%	5%	0%
3.4 F NaCl	26%	23%	36%	33%
DNA-histone complex	17%	13%	23%	20%
HISTONE II IN				
0.01 M tris	26%	24%	35%	35%
3.4 F NaCl	74%	75%	100%	100%
DNA-histone complex	57%	59%	79%	86%
HISTONE III+IV IN				
0.01 M tris	37%	34%	51%	49%
3.4 F NaCl				
DNA-histone complex	42%	42%	58%	61%
UNFRACTIONATED HISTONES IN				
0.01 M tris	27%	26%	38%	37%
3.4 F NaCl				
DNA-histone complex	41%	41%	56%	59%

Free histone III+IV has the highest content of ordered structure among the free histones (37% α-helix or 50% B-form). The same conclusion concerning nonhelicity of histone I and greater helical content of histone II, III+IV has also been reached by Jirgensons et. al. (12) using a Moffitt plot of ORD data in the 250 mu to 350 mu spectral zone.

When the ionic strength of the solvent is increased from 0.01 F tris to 3.4 F NaCl, there is an accompanying enhancement in the content of ordered structure for all classes of histones. The increment in ordered structure for histone I is from 3-5% in 0.01 F tris to ca. 30% in 3.4 F NaCl; for histone II, from ca 30% in 0.01 F tris to 75-100% in 3.4 F NaCl.

The contents of ordered structures calculated from specific rotations of DNA-bound histones at 210 and 215 mu (Table III) are intermediate between those in 0.01 F tris and in 3.4 F NaCl, which suggests that the effect of DNA binding on histone structure is to simulate the effect of increasing ionic strength. DNA-bound histone II is the most ordered of the three classes of bound histones.

If we calculate the content of ordered structure of histones from specific rotations in 220 to 240 mu region, which is not done here on account of the pronounced fluctuation of measurements in this region, the result will be different, because the specific rotations of the DNA-bound histones in 220 to 240 mu region are, instead of being intermediate between those in 0.01 F tris and 3.4 F NaCl, are the smallest in magnitude compared to those in 0.01 F tris and 3.4 F NaCl. It is thus possible that i) the assumption that the conformation of DNA in the reconstituted NH is the same as that of free DNA is inappropriate, or

ii) B-structures which possess a shallow trough around 230 mu rather than α-structure, are the major ordered structure in DNA-bound histones. Since the absorption constants of DNA in reconstituted NH's are the same as that of free DNA (Table I), the first possibility, in lack of any other experimental support, is not discussed here. The question of whether B-structure is the major form of the ordered region of the DNA-bound histones can be tested by extending the ORD spectra of histones into 190 to 200 mu region, where the coil form has small rotations and the differences in the specific rotations of α and B forms (Fig II) are considerably greater than that in the longer wavelength region. This is not done in the present study because of the sizable noise level compared to the degree of rotation in the 190 to 200 mu region probably resulting from the very low sample concentration (optimal for measurement in this region) and the low level of light source output.

In the chapter on selective dissociation of histones by NaCl solution, it was pointed out that despite the higher electric charge density of histone I, it is the histone which is dissociated at the lowest ionic strength. It was suggested that the early dissociation of histone I before the other histones may be due to its conformation which may inhibit a close binding to DNA. The ORD study in this section seems to support the above hypothesis. From Table III, it can be seen that histone I when bound to DNA is still in a highly coiled form, yet DNA-bound histone II and III+IV possess comparatively more helical structure. It is thus possible that some of the charges of histone I, are buried inside the molecule and the coil portions of the histone I molecule could bind DNA by fitting into the space of DNA grooves. As a result of this, not every positive charge on the histone I molecule may find the DNA counter ion. The contradicting observations of high electric charge density and early dissociation may hence find explanation in the reduced electrostatic attraction between DNA and histone I molecules, which in turn is a result of the globular conformation of histone I. On the other hand, DNA-bound histones II, III+IV, due to their high helical conformation may be able to bind DNA longitudinally (histones staying side by side with DNA or wrapping around DNA in a superhelix). This linear disposition of histone II and III+IV charges on DNA molecule may cause firmer binding, and give rise to dissociation at higher NaCl concentrations.

It is interesting to observe that even though histone I is the least firmly bound histone by the criterion of salt dissociation, it, however, is the most effective of all histones in inhibiting DNA-dependent RNA

synthesis (1) and in stabilizing DNA against thermal denaturation (1). These experiments again suggest that the mode of histone I binding to DNA may be different from that of histones II, III+IV. Turnover study of histones (13) demonstrates differential behavior of histones, namely, histone I, histone II as well, is synthesized only when the associated DNA is undergoing replication. Combining the above observation with the postulate that histone I binds to DNA by fitting into DNA grooves, and histones II, III+IV by assuming longitudinal binding, the question naturally following is whether the different mode of histone binding to DNA would bestow different biological roles on different classes of histones. The answer to the above question is as yet unknown and awaits future experiments to explore.

REFERENCES:

(1) Fambrough, D., and Bonner, J. (1966) Biochem. 5 2563.

(2) Stake, K., Rasmussen P. S., Luck, J. M. (1960) J. Biol. Chem. 235 2801.

(3) Proteins:

a) Jirgensons, B. (1965) 240 1064 J. Biol Chem.

b) Harrison, S. C. and Blout, E. R. (1965) J. Biol. Chem. 240 299.

c) Yang, J. T. and Samejima, T. J. (1963) J. Biol. Chem. 238 3262.

(4) Synthetic polypeptides:

a) Shecter, E. and Blout, E. R. (1964) Proc. Nat. Acad. Sci. 51 695.

b) Goodman, M. and Felix, A. M. (1964) Biochem. 3 1529.

c) Jirgensons, B. (1966) J. Biol. Chem. 241 150.

(5) a) Sarkar, P. K. and Doty, P. (1966) Proc. Nat. Acad. Sci. 55 981.

b) Fasman, G. D. and Potter, J. (1967) Bioch. Biophy. Res. Comm. 27 209.

(6) Burton, K. (1955) Biochem. J. 62 315.

(7) Pardon, J. and Richards, B. (1967) In preparation.

(8) a) Kronman, M. J. (1965) Biochem. Biophy. Res. Comm. 19 227.

b) Fasman, G. D., Landsberg, M. and Buchwald, M. (1965) Can. J. Chem. 43 1588.

(9) a) Fasman, G. D., (1964) Biochem. 3 1665.

b) Beychok, S. and Fasman, G. D. (1964) Biochem. 3 1675.

(10) a) Coleman, D. L. and Blout, E. R., (1966) Federation meeting.

b) Beychok, S. (1965) Proc. Nat. Acad. Sci. 53 999.

(11) a) Tominatsu, Y., Vitello, L., and Gaffield, W. (1966) 4 653.

b) Yang, J. T. and McCab, W. J. (1965) Biopolymers 3 209.

(12) Jirgensons, B., Hinlica, L. S. (1965) Biochim. Biophy. Acta 109 241.

(13) Chalkley, G. R. and Maurer, H. R. (1965) Proc. Nat. Acad. Sci. 54 498.

Chapter 3

OPTICAL ROTATORY DISPERSIONS AND OPTICAL ABSORBANCE STUDIES ON THE DNA OF NATIVE NH AND PARTIALLY DEHISTONIZED NH.

I. INTRODUCTION:

Electron microscopic study of chromatin (1) demonstrates that it is composed of fiber (or fibres), each 150Å in diameter for the case of calf thymus chromatin (2), The fibre consists of a trypsin sensitive sheath and a DNase sensitive core 23Å in width, a dimension compatible with a single DNA molecule. Partial digestion of the protein sheath results in a springing out of the core material, which suggests that the total length of the material in the core is considerably greater than the length of the chromatin fibre before digestion. Super coiling of DNA is probably involved so that the DNA can be packed into the much shorter chromatin fibre. The NH molecules, produced by shearing of chromatin, are fragments of chromatin fibres, the DNA of which is also believed to be a single DNA molecule (3).

It is our purpose in this chapter to infer information about the conformational changes for DNA between free DNA and DNA in native nucleohistone or in salt-extracted nucleohistones. In order to use ORD for this purpose, we assume that the ORD of histones in native nucleohistone are the same as those calculated in the previous chapter from the ORD spectra of reconstituted NH.

The possibility of DNA being supercoiled in native NH is discussed.

II. MATERIAL AND METHODS:

PREPARATION OF NH;

Calf thymus NH was isolated as described in chapter one.

CALF THYMUS DNA:

Three kinds of DNA were used: commercial Sigma NaDNA, 4 F NaCl DNA and DNA isolated by the Sevag procedure.

i) Preparation of 4 F NaCl extracted DNA: This has been given in detail in the chapter on salt dissociation of histone from NH.

ii) Preparation of DNA by the Sevag procedure (4): 20 ml of NH solution was shaken with 12.5 ml of 5 F NaCl, 7.5 ml of 1% SDS and 40 ml of chloroform-isoamylalcohol(24:1) for 30 minutes. The solution was next centrifuged for 30 minutes at 10,000 rpm in a Servall centrifuge. The DNA solution above the denatured protein skin is carefully pipetted out. The solution was then poured slowly into 2X volume of cold absolute alcohol and the DNA threads wound out on a glass rod. After dissolving the DNA in 0.01 F tris PH 7.3, the process was repeated twice more.

EXTINCTION COEFFICIENTS OF DNA, FREE, IN NATIVE NH, AND IN NH'S TREATED WITH VARIOUS CONCENTRATIONS OF NACL SOLUTION:

i) Measurement of $A_{260\ mu}$: Absorption at 260 mu of DNA and of various NH samples was recorded on a Hitachi-Perkin-Elmer U.V. spectrophotometer. The sample for optical density measurement is always adjusted to around $A_{260\ mu}=0.5$. For samples which scatter light at nonabsorbing region (320 mu), centrifugation at 35,000 rpm in SW 39 rotor was employed to reduce scattering to $A_{320\ mu}=0.005-0.006$ which is the amount of scattering a DNA solution of comparable concentration possesses. The yield of material in the supernatant is generally 60%-80% of the starting quantity.

ii) Determination of DNA quantity: This is done by two methods:

a) Diphenylamine reaction: The procedure has been described in the previous chapter and shall not be repeated here. Optimal concentration of DNA for this reaction is around 75 γ/ml ($A_{260\ mu}$=1.5.)

b) Phosphorus determination: The general scheme is the same as that of Chen's (5). Modification of the reagents used here is to adjust the final pH of the reaction mixture to optimal color developing range.

The reagent is prepared by mixing 3 volumes of 1 N H_2SO_4, 1 volume of 2.5% ammonium molybdate and 1 volume of 10% ascorbic acid and is prepared fresh before use.

0.5 ml of sample (ca. 200 ug/ml of DNA) (in triplicates) was mixed with 0.25 ml of concentrated H_2SO_4 (ca. 36 N). The mixture was digested, first over a very small bunsen flame for 30 minutes. In order to avoid splatter, another burner was used to drive off the water vapor on the side of the test tubes. After the initial 30 minutes, the flame was slowly turned up. During the next 30 minutes of heating with a larger flame, the solution gradually turns dark, after which the solution is refluxed for 15 more minutes. Heating is then discontinued and two drops of 60% $HClO_4$ added in each tube. The solution is heated for ca. 10 minutes until it is discolored. The solution in each tube is next diluted with water to 2 ml. Two tenths ml of this solution (triplicates) is mixed with 0.8 ml water and 1 ml reagent. After warming in a 37°C water bath for 1.5 hours, the mixture is cooled to room temperature and $A_{820\ mu}$ recorded.

The standard curves for both the diphenylamine reaction and phosphorus determination were constructed from Sigma NaDNA whose chemical composition (6) and extinction coefficient at 260 mu are known (Chapter 1).

SPECTROPOLARIMETRY:

The procedure is the same as described in the previous chapter for histones. Optimal concentration for DNA is $A_{260\ mu}$=0.5.

CALCULATION OF NH SPECTRUM:

The optical rotation of NH is calculated according to the following formula:

$$[\alpha]_{NH} = \frac{\alpha \times 100}{c \times d}$$

Where α is the optical rotation in degrees of the sample, c is the concentration of NH (DNA and protein) in g/100 ml, and d is the light path of the sample cell in decimeter.

CONSTRUCTION OF ADDITION ORD SPECTRA:

For the purpose of observing the ORD effect of the protein-DNA interaction in NH, an addition ORD curve is calculated from the ORD spectra of physically separated DNA and protein according to the following formula:

$$[\alpha]_{add} = [\alpha]_{DNA} \times F_{DNA} + [\alpha]_{prot.} \times F_{prot.}$$

where F_{DNA} and $F_{prot.}$ are fractions of DNA and protein in NH and $[\alpha]_{DNA}$ and $[\alpha]_{prot.}$ are specific rotation of 4 F NaCl extracted DNA and 4 F NaCl extracted protein.

COMPUTATION OF THE ORD SPECTRA OF DNA IN NH AND NH'S TREATED WITH VARIOUS CONCENTRATIONS OF NACL:

The ORD spectrum of NH consists of contribution from the ORD of histones and the ORD of DNA. The ORD spectrum of histones in NH is computed from DNA-bound histone I, II and III+IV (obtained from reconstituted NH) according to the following formula:

$$[\alpha]^{\lambda}_{\text{histone in NH}} = [\alpha]^{\lambda}_{DHI} \times F_{I} + [\alpha]^{\lambda}_{DHII} \times F_{II} + [\alpha]^{\lambda}_{DHIII+IV} \times F_{III+IV}$$

where $[\alpha]$ histone in NH is the specific rotation at wavelength λ of histones in NH or in dehistonized NH's and $[\alpha]^{\lambda}_{DHI}$ $[\alpha]^{\lambda}_{DHII}$, $[\alpha]^{\lambda}_{DHIII+IV}$ are the specific rotations at the same wavelength of DNA-bound histone I, histone II, histone III+IV, F_I, F_{II}, and F_{III+IV} are the fractions of each histone component of the total histones in either NH or dehistonized NH's.

The ORD spectra of NH and NH's treated with various concentration of NaCl solution can be recorded directly from the spectropolarimeter. So, the ORD spectrum of DNA in various preparation of NH can be computed:

$$[\alpha]^{\lambda}_{\text{DNA in NH}} = \frac{[\alpha]^{\lambda}\ \text{NH} - [\alpha]^{\lambda}\ \text{histones in NH} \times F_{\text{prot. in NH}}}{F_{\text{DNA in NH}}}$$

where $F_{\text{prot. in NH}}$ and $F_{\text{DNAin NH}}$ are fractions of protein and DNA in NH.

III. RESULTS:

EXTINCTION COEFFICIENTS AT 260 mu OF DNA IN NATIVE NH AND IN NH'S TREATED WITH VARIOUS CONCENTRATIONS OF NACL SOLUTIONS:

The extinction coefficients determined by phosphorus analysis and by diphenylamine reaction are presented in Table I. It can be seen that there is a close agreement between extinction coefficient measurements of DNA based on phosphorus analysis and deoxyribose analysis.

ORD SPECTRA OF DNA:

The ORD spectrum of 4 F NaCl extracted DNA measured in 0.01 F tris (Fig I_a) has a peak at 290 mu, a crossover point at 274 mu, a trough at 260 mu, another cross-over point at 247 mu and a plateau between 230 mu and 220mu. The specific rotations are:

$[\alpha]_{290\ mu}$=+2450°, $[\alpha]_{260\ mu}$=-2630°, $[\alpha]_{230\ mu}$=+4450°, $[\alpha]_{210\ mu}$=+9300°.

The ORD spectrum of Sevag DNA (Fig I_a) differs from that of 4 F NaCl extracted DNA in that the 220-230 mu plateau has become a weak

TABLE I

MOLAR EXTINCTION COEFFICIENTS OF DNA AS FUNCTION OF HISTONE-DNA INTERACTIONS

SAMPLES	Molar extinction coefficient $E(p) \times 10^{-3}$	
	based on phosphorus analysis	based on deoxyribose analysis
Native NaDNA (Sigma)	6,815±0,05	6,815±0,05
Denatured NaDNA (Sigma)	9,280±0,05	
Native NH	7,725±0,165	7,561±0,099
0,6 FNaCl ext, NH	7,679±0,09	7,635±0,149
0,8 F NaCl ext, NH		7,11±0,174
0,9 F NaCl ext, NH		7,083±0,075
1,2 F NaCl ext, NH		6,989±0,05
1,6 F NaCl ext, NH	6,887±0,074	6,815±0,149
4 F NaCl extracted DNA	6,675±0,1	6,815±0,05

* Calculation based on E(p) of native NaDNA and 36% hyperchromicity observed when DNA is denatured by heat,

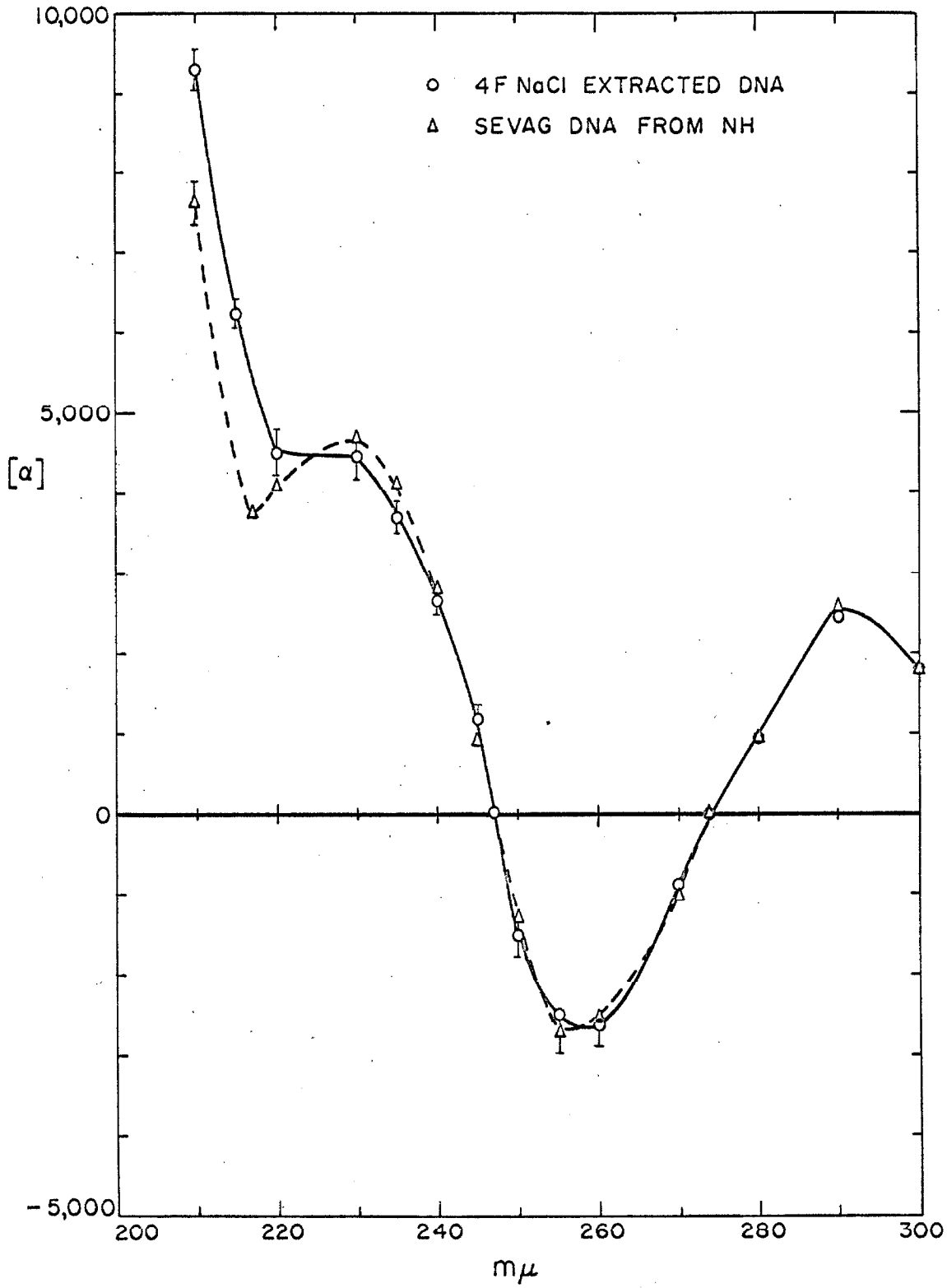

10,000
5,000
0
-5,000
[α]
200
220
240
260
280
300
mμ
o 4F NaCl EXTRACTED DNA
Δ SEVAG DNA FROM NH

Fig. Ia

maximum at 230 mu and minimum at 217 mu. The specific rotations at the extrema are: $[\alpha]_{290\ mu}$=+2600°, $[\alpha]_{255\ mu}$=-2700°, $[\alpha]_{230\ mu}$=+4700°, $[\alpha]_{217\ mu}$=+3750°. The specific rotation at 210 mu is +7620°.

ORD spectra of Sevag DNA and 4 F NaCl extracted DNA were also recorded in 4 F NaCl (Fig. I_b). The shapes of the curves are similar to that of Sevag DNA in 0.01 F tris with differences in the magnitude of the peaks and troughs. The extrema (for both 4 F NaCl extracted and Sevag DNA) are: $[\alpha]_{290\ mu}$=+1360°, $[\alpha]_{255\ mu}$=-1950°, $[\alpha]_{230\ mu}$=+5000°, $[\alpha]_{217\ mu}$=+3820°. The specific rotation at 210 mu is +7530° for 4 F NaCl extracted DNA. The cross-over point occurs at 279 mu and 248 mu.

THE ORD SPECTRA OF NH:

NH in 0.01 F tris (Fig II_a) features two peaks (290 mu, 240 mu) and two troughs (254 mu, 235 mu). The cross-over points are at 280 mu and 228 mu. The specific rotations at the extrema are: $[\alpha]_{290\ mu}$=+650°, $[\alpha]_{254\ mu}$=-1450°, $[\alpha]_{240\ mu}$=-780°, $[\alpha]_{235\ mu}$=-1020°. The specific rotation at 210 mu is +7000°.

In Fig. II_a there is also shown an addition spectrum which is a weighted sum of the ORD spectra of 4 F NaCl extracted DNA in 0.01 F tris and 4 F NaCl extracted protein in 0.01 F tris. The addition curve has only one peak at 290 mu and one trough at 260 mu. The cross-over points are at 280 mu and 234 mu. The specific rotations at the extrema are greater than that of the native NH, $[\alpha]_{290\ mu}$=+810°, $[\alpha]_{260\ mu}$=-1920°; but $[\alpha]_{210\ mu}$ =+3220° which is smaller than that of the native NH. The peak at 240 mu and trough at 235 mu found for native NH are missing from the addition curve.

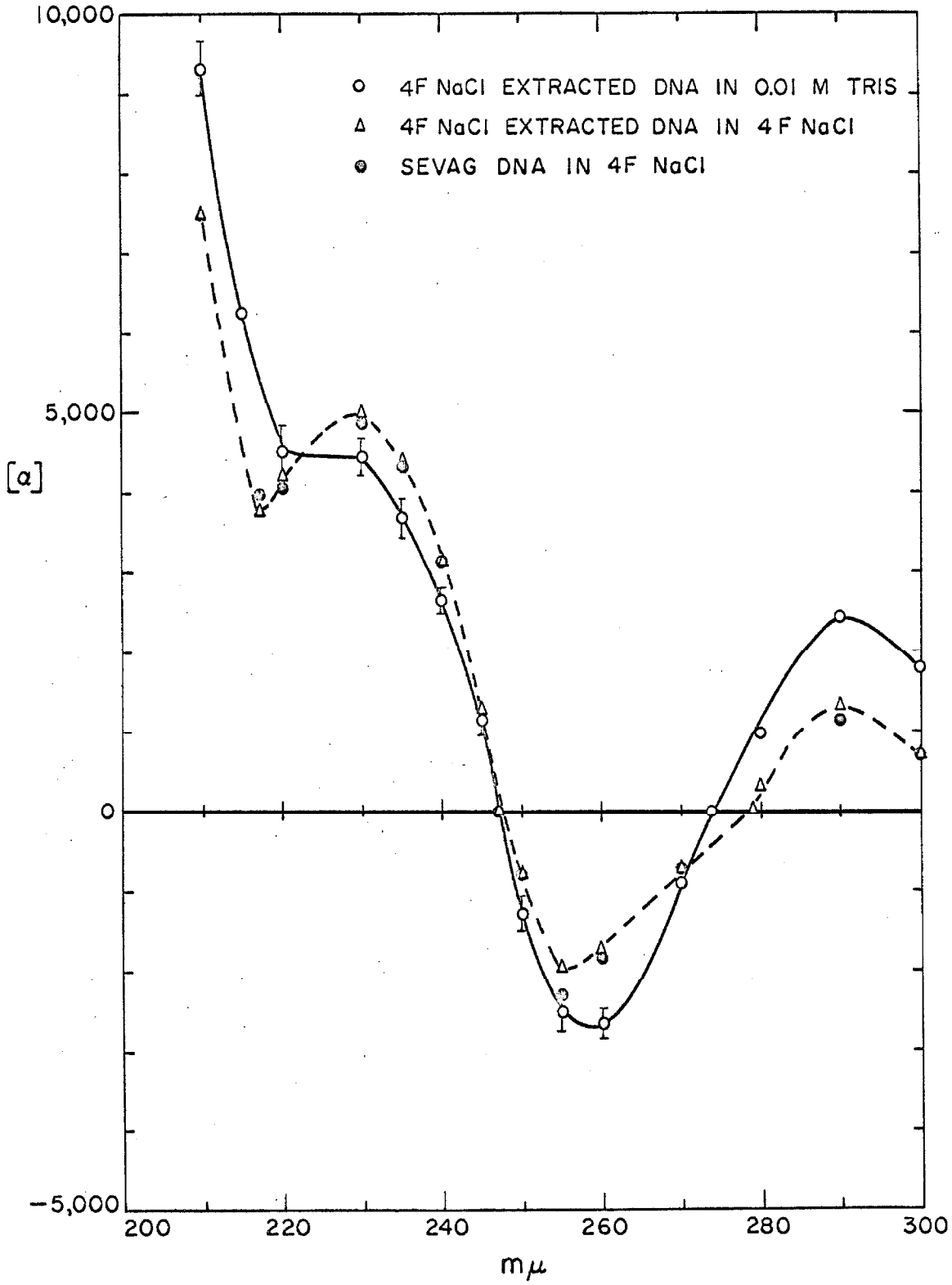

10,000
5,000
0
−5,000
[α]
200
220
240
260
280
300
mμ
4F NaCl EXTRACTED DNA IN 0.01 M TRIS
4F NaCl EXTRACTED DNA IN 4 F NaCl
SEVAG DNA IN 4F NaCl

Fig. Ib

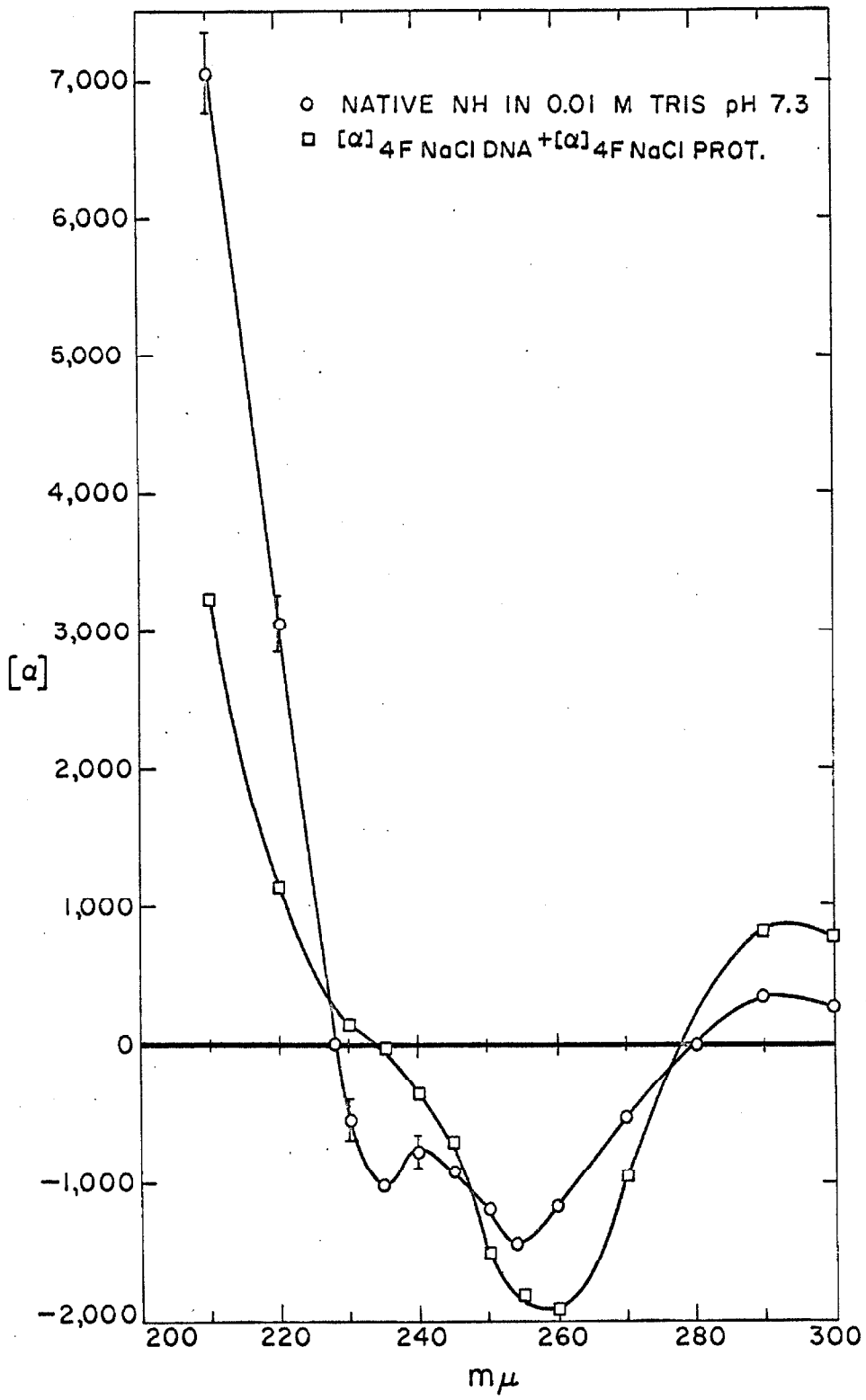

NATIVE NH IN 0.01 M TRIS pH 7.3
[α]4F NaCl DNA +[α]4F NaCl PROT.
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
−1,000
−2,000
[α]
200
220
240
260
280
300
mμ

Fig. IIa

Fig II_b presents another addition spectrum constructed by taking the weighted sum of the ORD spectra (in 0.01 F tris) of DNA-bound whole histones (from reconstituted NH) and that of 4 F NaCl extracted DNA. The shape of the spectrum resembles that of the addition spectrum in Fig II_a with similar magnitudes of rotation at the extrema which occur at the same wavelengths. The cross-over point are, however, at 278 mu and 241 mu. The specific rotations at shorter wavelengths (below 240 mu) are greater than those of the addition curve of Fig II_a.

The ORD of NH was also recorded in 4 F NaCl as is shown in Fig II_c. The shape of the spectrum is different from that of NH in 0.01 F tris in that the weak maximum and minimum at 240 and 235 mu are replaced by a suggestion of a plateau. Specific rotations at the extrema are: $[\alpha]_{290\ mu}$=+680°, $[\alpha]_{255\ mu}$=-2000°. The cross-over points are at 280 mu and 238 mu.

A weighted sum of the ORD spectra of 4 F NaCl extracted DNA in 4 F NaCl and of 4 F NaCl extracted protein in 4 F NaCl is shown in Fig II_c in addition to the NH spectrum recorded in 4 F NaCl. Specific rotations at extrema are: $[\alpha]_{290\ mu}$=+750°, $[\alpha]_{255\ mu}$=-1800°; the cross-over points are at 280 mu and 245 mu. The shape and magnitudes of rotations of both curves of Fig II_c are similar.

THE ORD SPECTRA OF DNA IN NATIVE NH AND IN NH'S TREATED WITH VARIOUS CONCENTRATIONS OF NACL.

The ORD spectrum of DNA in native NH calculated as described previously is compared to that of 4 F NaCl extracted DNA in Fig III_a. 4 F extracted DNA rather than Sevag DNA is chosen so that the ORD contribution of nonhistone protein need not be considered. The spectrum

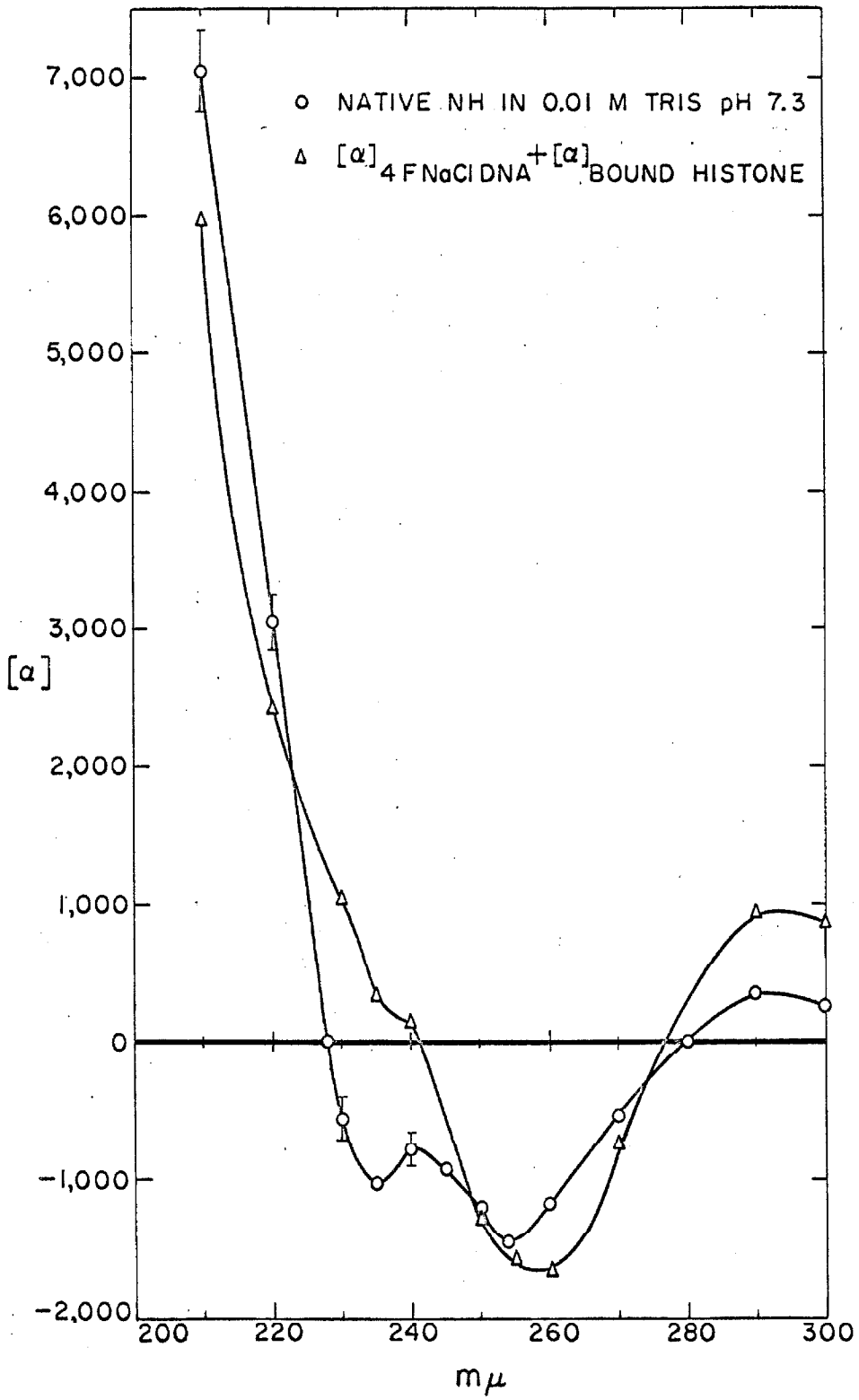

7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
-1,000
-2,000
[α]
200
220
240
260
280
300
mμ
o NATIVE NH IN 0.01 M TRIS pH 7.3
Δ [α]4 F NaCl DNA + [α]BOUND HISTONE

Fig. IIb

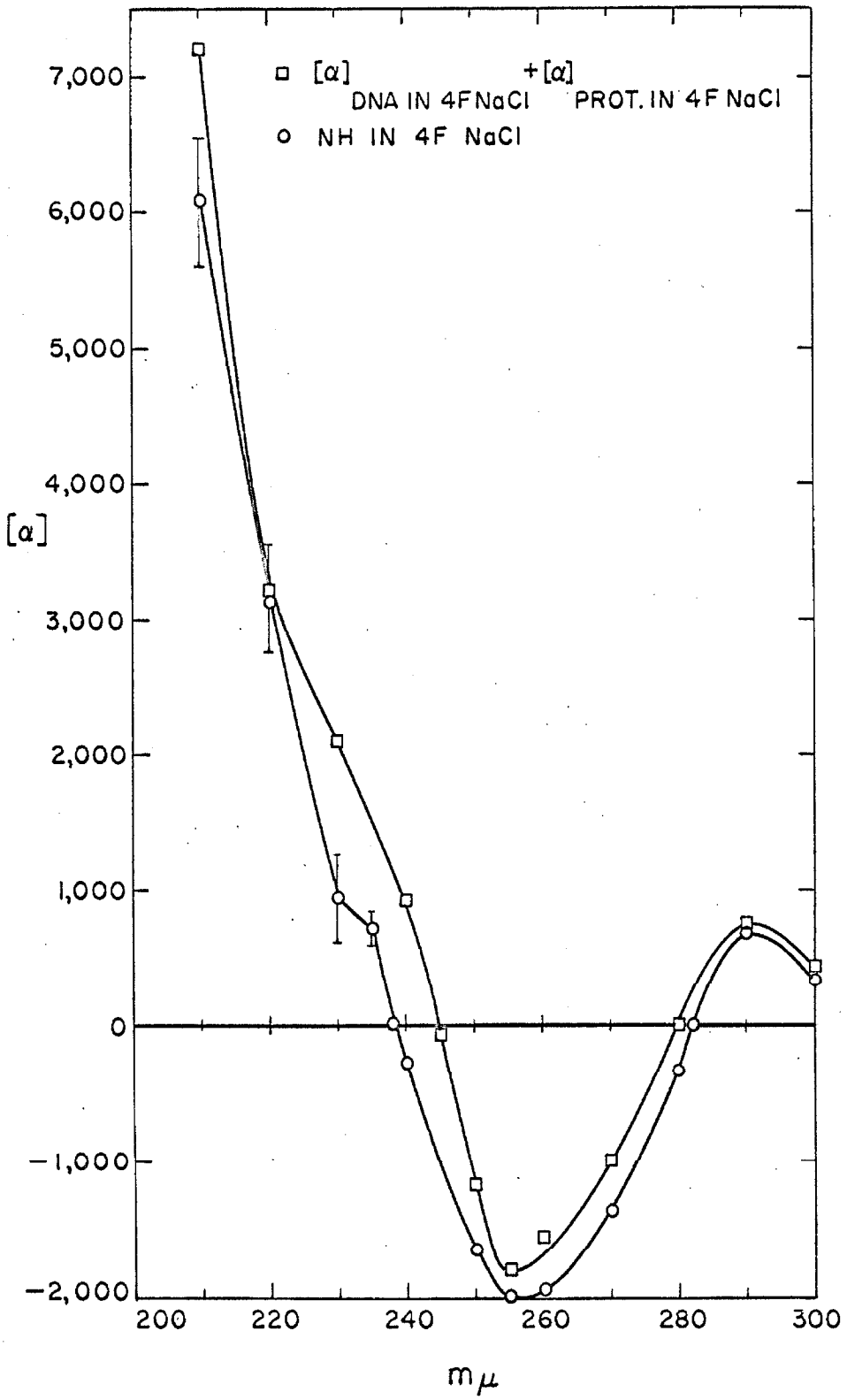

□ [α] DNA IN 4F NaCl
+[α] PROT. IN 4F NaCl
○ NH IN 4F NaCl
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
−1,000
−2,000
[α]
200
220
240
260
280
300
mμ

Fig. IIc

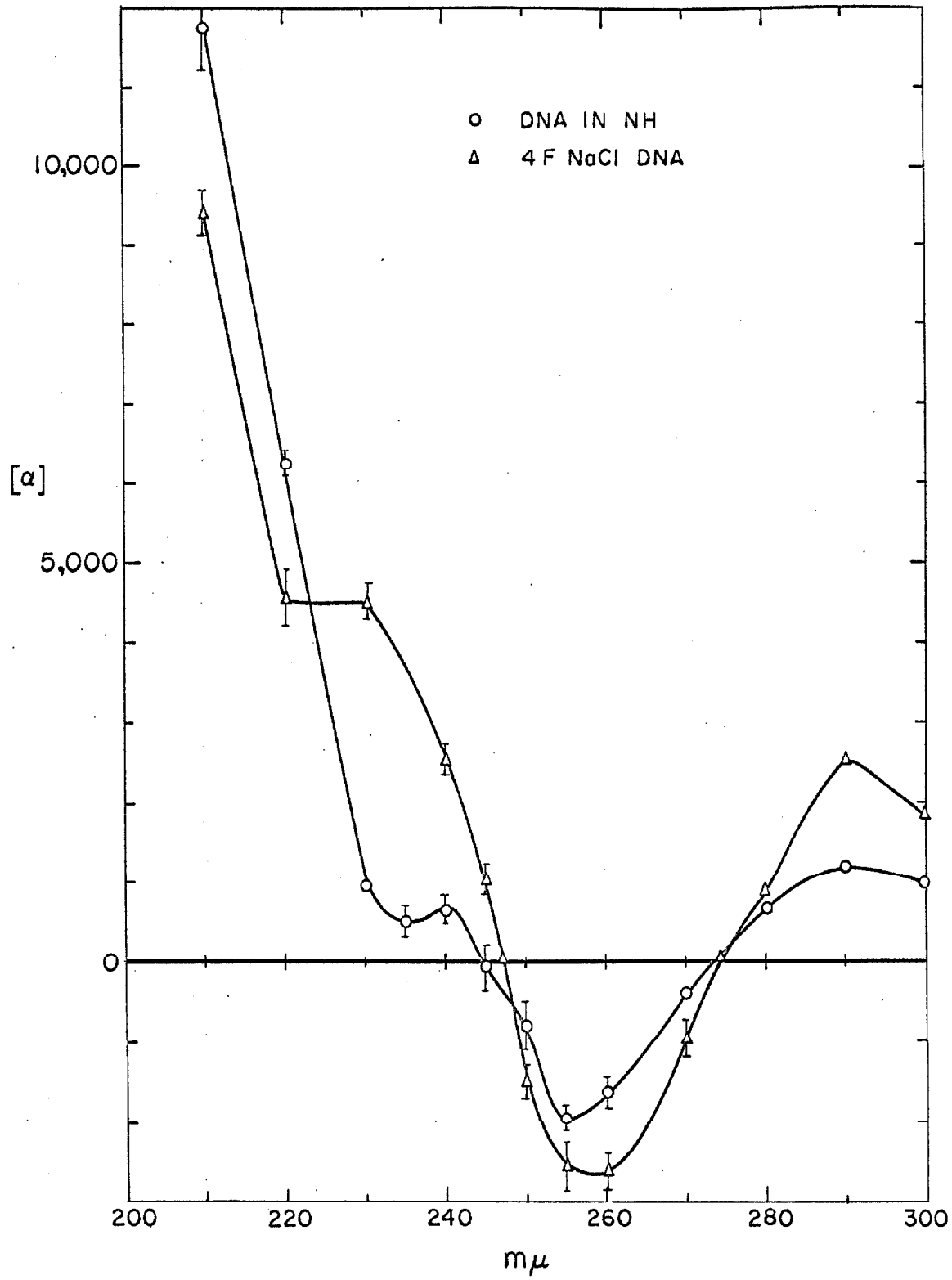


Fig. IIIa

of DNA in NH has two peaks (290 mu, 240 mu) and two troughs (255 mu, 235 mu), which is to be expected from the shape of the ORD spectrum of native NH. The specific rotations at the extrema are: $[\alpha]_{290\ mu}$=+1200°, $[\alpha]_{255\ mu}$=-1970°, $[\alpha]_{240\ mu}$=+670°, $[\alpha]_{235\ mu}$=+520°, $[\alpha]_{210\ mu}$ is +11750°. The cross-over points are at 274 mu and 245 mu.

The ORD spectra of DNA in 0.6 F 0.8 F, and 1.6 F NaCl treated NH's are shown in Fig III_b. The spectrum of DNA in 0.6 F NaCl treated NH is similar both in shape and in magnitude of rotation to that of DNA in native NH. The spectrum of DNA in 1.6 F NaCl treated NH exhibits, however, more resemblance to that of 4 F NaCl extracted DNA. The specific rotations at the extrema for DNA in 0.6 F NaCl treated NH are: $[\alpha]_{290\ mu}$=+1210°, $[\alpha]_{255\ mu}$=-1870°, $[\alpha]_{240\ mu}$=+640°, $[\alpha]_{235\ mu}$=+470°, $[\alpha]_{210\ mu}$=+10930° and cross-over points are at 276 mu, 247 mu; for DNA in 1.6 F NaCl treated NH, specific rotations at the extrema are: $[\alpha]_{290\ mu}$=+1810°, $[\alpha]_{255\ mu}$=-2500°, $[\alpha]_{210\ mu}$=+9500°, and the cross-over points are at 275 mu, 245 mu.

The ORD spectrum of DNA in 2 F NaCl extracted NH as well as that of 4 F NaCl extracted DNA is shown in Fig III_c. They exhibit close similarity both in shape and magnitude of rotation. The specific rotations at the extrema for DNA in 2 F NaCl extracted NH are: $[\alpha]_{290\ mu}$=+2300°, $[\alpha]_{255\ mu}$=-2470°, $[\alpha]_{210\ mu}$=+9250°. There is a suggestion of a plateau between 230 mu and 220 mu ($[\alpha]_{230\ mu}$=+4000°). The cross-over points are at 274 mu and 247 mu.

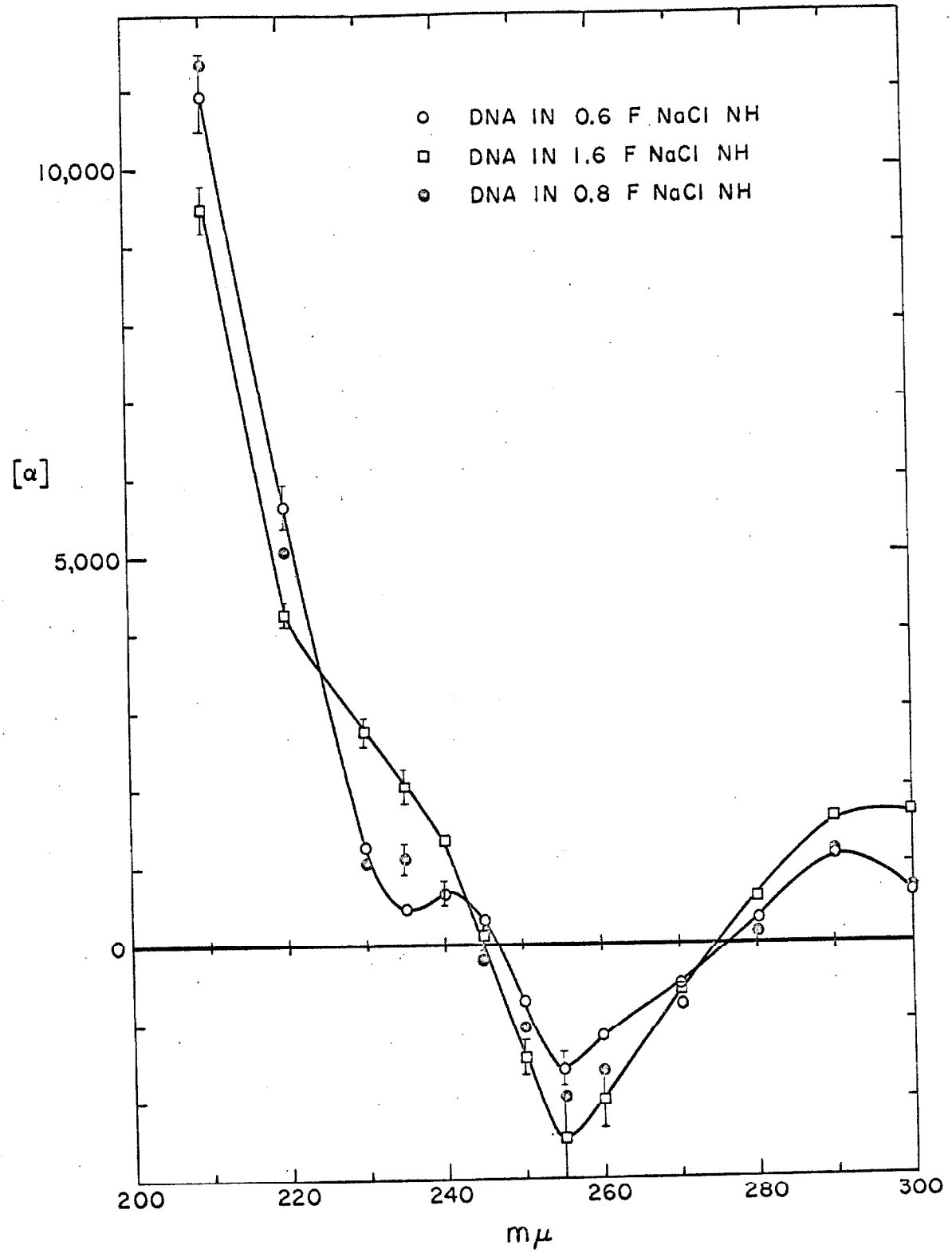

DNA IN 0.6 F NaCl NH
DNA IN 1.6 F NaCl NH
DNA IN 0.8 F NaCl NH
10,000
5,000
0
[α]
200
220
240
260
280
300
mμ

Fig. IIIb

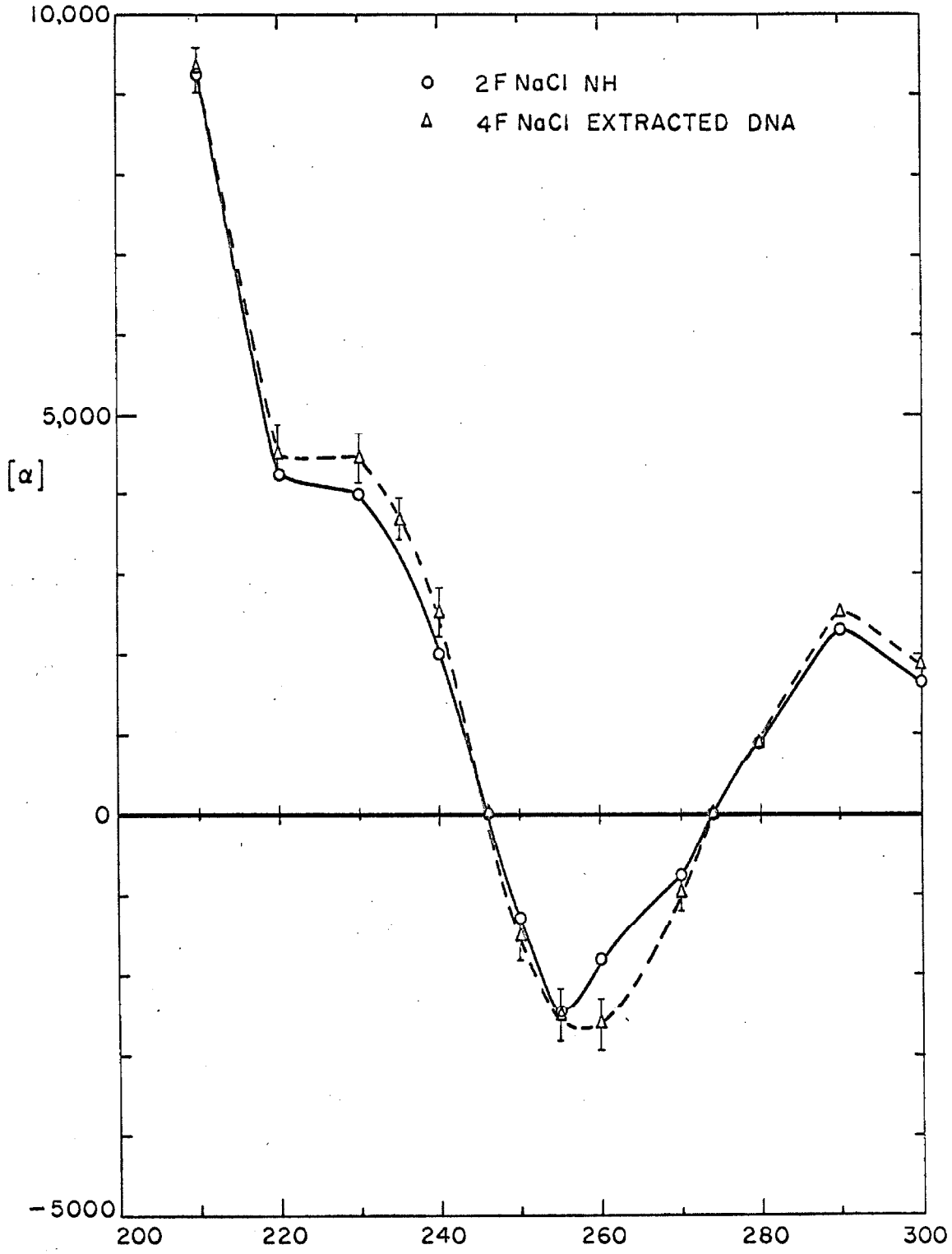

10,000
5,000
0
−5000
[α]
o 2F NaCl NH
Δ 4F NaCl EXTRACTED DNA
200
220
240
260
280
300
mμ

Fig. IIIc

IV. DISCUSSION:

The objective of the present study concerns:

A) Is there a conformational change of DNA in Native NH as compared to free DNA?

B) We shall present evidence that the answer to A) is: the conformation of DNA in NH is altered as a result of the DNA-histone interaction in the NH, the next question is then: which class or classes of histone cause the conformational change of DNA? (there are three main classes of histones: I,II,III+IV).

C) What kind of DNA conformational change does the association of histone induce ? Is the DNA supercoiled in NH?

The experimental approach consists of measurement of extinction coefficients and ORD spectra of DNA: free, in native NH, and in various dehistonized NH's. In the following the experimental evidence bearing on each of the above questions will be discussed:

A) CONFORMATIONAL CHANGE OF DNA IN NATIVE NH AS COMPARED TO FREE DNA

Experimental evidence for this is:

(1) Extinction coefficients Table I presents the extinction coefficients of DNA: free, heat denatured and in native NH. There is 11-12% hyperchromicity for DNA in native NH as compared to free DNA. Of the 11-12% observed hyperchromicity, 2-3% can be attributed to the A_{260mu} of the associated proteins (chapter 1), the remaining 8-10% is then an effect of the interaction between DNA and protein in NH.

The E(P) for DNA in NH is intermediate between those for native and denatured DNA. This would seem to indicate that the direction of DNA structural change in NH is toward that of melted DNA. When histones

are completely dissociated from DNA and the DNA-histone interaction abolished, the E's of DNA in the NH's return to the value of free DNA.

It is well known that the optical absorption of DNA at 260 mu is conformation dependent (7) and that, at constant pH, is independent of ionic environment up to at least 1 M NaCl. It follows that changes in extinction coefficients at 260 mu most probably indicate a conformational change in the DNA. One very attractive hypothesis is that this conformational change is associated with a distortion of the base stacking of DNA. Thus, it is proposed that the DNA bases in NH, because of binding to the histones, are differently oriented in such a way as to produce 8 to 10% hyperchromicity. When histones are completely dissociated from DNA and the DNA-histone interaction abolished, the E's of DNA in the NH's return to the value for free DNA.

2) ORD measurements: The ORD spectrum of DNA has been shown to be conformation sensitive (8). An alteration of the ORD spectrum of DNA in NH from that of free DNA may thus reflect a conformational change of the DNA in NH. The ORD spectrum of DNA in native NH is however not experimentally measurable: only the ORD spectrum of NH can be directly recorded from the spectropolarimeter. The native NH being mainly composed of DNA and histones, the difference spectrum between the native NH and that of the histones in NH, if available, should then be the ORD spectrum of DNA in NH. Unfortunately, the ORD spectrum of histones in NH is unavailable, so we resort to its analog, the ORD spectrum of whole DNA-bound histones which can be computed from the

spectrum of the reconstituted NH's (chapter two, Method section). The ORD spectrum of DNA in NH (Fig III_a) is then calculated from the difference spectrum of the native NH and DNA-bound histones.

There is as yet no direct experimental support for this substitution. It is possible that the histone in native NH may have an as yet unknown conformation of anomalous ORD pattern and different from that of histone in reconstituted NH; This is however improbable. The ORD study of ribosomes (9), in which the RNA is reported to possess the same conformation as free ribosomal RNA (10), demonstrates that the ribosomally bound basic protein does not exibit any peculiar ORD pattern except a deepening of the 233 mu trough which is a result of the increase in the α-helical content (as with histones bound to DNA). If the situation is the same with the ORD pattern of histones in NH, then the above substitution will yield at least a qualitatively correct spectrum of DNA in NH. With the assumption inherent in the substitution in mind, we go on with the analysis of ORD data.

It should also be pointed out that, in the process of computing the DNA spectrum in NH, the possible contribution of nonhistone protein is not incorporated into the ORD spectrum of the protein in NH. However, nonhistone protein constitutes 10% or less of the 4 F NaCl extractable protein and this 10% of nonhistone protein does not change the ORD of histone protein (Fig III of preceding chapter). It will also be shown later that its

presence in NH does not alter the ORD of DNA in NH either.

Fig. III_a is an ORD spectrum of DNA in NH obtained as described. It is significantly different from that of free DNA. The shallower peak at 290 mu and trough at 255 mu, the additional peak at 240 mu, and trough at 255 mu, and the higher degree of rotation at 210 mu of ORD spectrum of DNA in NH are indications of conformational change.

B) HISTONES II, III+IV ARE THE HISTONES RESPONSIBLE FOR THE CONFORMATIONAL CHANGE OF DNA IN NH

Before discussing the question of which class of histone causes the observed conformational change of DNA in NH, it is necessary to show first that the DNA conformation change is due not to the mere presence of protein and DNA in the same solution but indeed to the interaction of protein and DNA in NH.

The ORD spectrum of native NH differs from the addition spectrum (Fig. II_a) obtained by adding the ORD spectra of DNA and protein according to the composition of native NH. The ORD spectrum of native NH exibits a shallower peak at 290 mu and a shallower trough at 255 mu, an additional peak at 240 mu, a trough at 235 mu and a higher degree of rotation at 210 mu. The above differences may be caused as stated above by i) the mere presence (without interaction) of protein and DNA in the same solution; or ii) the interaction between DNA and protein.

The following experiments were designed to differentiate between the two possibilities. ORD spectra of NH, 4F NaCl extracted DNA, and of protein, were recorded in 4 F NaCl, in which native NH dissociates into protein and DNA coexisting in the same solution yet without interacting with each other. If there is an ORD effect due to i), the ORD spectrum of native NH recorded in 4 F NaCl should be different from the addition spectrum which is a weighted sum of the ORD spectra of DNA and protein in 4 F NaCl. If ii) is the correct explanation, then the NH spectrum in 4 F NaCl and the addition spectrum of DNA and protein in 4 F NaCl should be the same. It can be seen from Fig. II_c that there is a close similarity in both the shape and magnitude of rotation between the ORD spectrum of NH and the weighted addition spectrum. The high ionic strength has abolished the DNA-protein interaction and concomitantly removed the difference in the spectra. Therefore, the conformational change of DNA in NH is indeed due to the DNA-protein interaction.

It has been shown in chapter 1 that different concentrations of NaCl solutions dissociate histone and nonhistone proteins from NH in a rather selective manner. Thus, histone I is totally dissociated at 0.6 F NaCl; histone II in the range of 0.8 F to 1.6 F NaCl, although most of it is dissociated below 1.2 F NaCl; histone III+IV over a wide range, 0.9 F up to 2 F NaCl. For the case of nonhistone protein, half of it is dissociated below 0.6 F, most of the

rest stays with DNA even after 4F NaCl extraction. It is thus possible to tackle the problem of which class of histone causes the observed conformational change of DNA in NH by making measurements on the dehistonized NH's obtained by pelleting native NH through various concentrations of NaCl.

1) ORD measurements: The composition of associated protein of NH after various concentrations of NaCl extractions can be estimated from Lowry and gel electrophoresis analyses. The ORD spectra of DNA in the various preparation of NH's can therefore be computed from the ORD spectra of DNA-bound histones and of the NaCl extracted NH.

The ORD spectrum of DNA in 0.6 F NaCl treated NH (Fig. III_b) is very much the same as that of DNA in native NH. The shallower peak at 290 mu and trough at 255 mu, the peak at 240 mu and trough 235 mu all persist; the degree of rotation at 210 mu is however lower than that of DNA in native NH. The 50% of nonhistone protein and the histone I which have been dissociated by 0.6 F NaCl extraction are thus not the protein components which induce the ORD spectral change of DNA in NH.

Between 0.6 F and 0.8 F. NaCl, roughly one sixth of total histone II and only histone II is dissociated, with an accompanying change in ORD spectrum of DNA. The ORD spectrum of 0.8 F NaCl treated NH has a plateau between 235 mu to 230 mu in place of the 240 mu peak and

235 mu trough. Hence, histone II is responsible, at least in part, for the ORD spectral change of DNA in NH.

The bulk of histone II and histone III+IV are dissociated in the range of 0.8 to 1.6 F NaCl; the majority of the less than 10% of histones still associated at 1.6 F NaCl belongs to the class of histone III+IV. In Fig. III_b is shown the ORD spectrum of DNA in 1.6 F NaCl treated NH, which resembles the spectrum of 4 F NaCl extracted DNA except that it exhibits a smaller degree of rotation at 290 mu, lacks the 230 to 220 mu plateau which is characteristic of 4 F NaCl extracted DNA. Since the dissociation of histone III+IV is almost concurrent with that of histone II, it is difficult to differentiate the ORD spectral change associated with removal of histone II from that associated with removal of histone III+IV, or indeed to determine if there is any such change associated with histone III+IV removal.

The ORD spectral change of DNA at 1.6 F NaCl is not due to the dissociation of nonhistone protein, because very little of it (4% is dissociated between 0.8 and 1.6 F NaCl.

The ORD spectrum of DNA in 2 F NaCl extracted NH (which does not contain any histone protein) bears a remarkable resemblance to that of 4 F NaCl extracted DNA (Fig. III_c). This is a natural consequence of the close similarity in their chemical composition. From the resemblance in ORD spectra above 230 mu of Sevag DNA (Fig. I_a)

which does not contain any nonhistone protein, and 2 F and 4 F NaCl extracted DNA which contain a significant amount of nonhistone protein (Fig. III_c), it seems that nonhistone protein does not contribute in any significant manner, to the ORD spectral change of DNA in NH.

2) Extinction coefficients measurements: There is an initial slight increase of extinction coefficient of NH treated with NaCl at concentrations between 0. and 0.6 F.NaCl This coincides with the dissociation of histone I. Treatment with concentrations greater than 0.6 F NaCl causes the extinction coefficients to drop continuously. Or, considering the variations in the measurements, one may say that the difference in extinction coefficients between NH and 0.6 F NaCl extracted NH is not significant; therefore, so far as the optical absorption of DNA is concerned, histone I binding has little influence. Both of the above facts suggest that the mode of histone I binding to DNA is different from that of the other histones. It is possible to argue, however, that the mode of histone I binding to DNA is not different from that of the other histones but that the removal of 30% of histones, which is the amount of histone being dissociated at 0.6 F NaCl, is not sufficient to cause a change in extinction. This possibility, in view of the lack of experimental evidence, is not discussed here. The drastic drop in extinction coefficient, paralleled by the dissociation of one sixth of histone II which is observed between 0.6 to 0.8 F NaCl treat-

ment implies that association of histone II to DNA is highly effective in inducing the inferred disorientation of DNA bases. Thus removal of a small amount of histone II causes a large drop in extinction coefficient. The trend of decrease in extinction coefficient is followed by 1.2 F and 1.6 F NaCl treatment, in which range almost all of the histone II and histone III+IV are dissociated from DNA in NH. After 1.6 F NaCl extraction, up to 4 F NaCl, the value of extinction coefficient is virtually that of free DNA.

The trends of changes of DNA properties in NaCl-treated NH's indicated by extinction coefficient as well as by optical rotations at 4 wavelengths are depicted in Fig, IV.

C) SUPERCOILING OF DNA IN NH:

The hypothesis that the length of NH (or chromatin fibre) is much shorter than the DNA it contains is supported by the following observations:

1) Calculation of the total length of DNA per nucleus (11) and the total length of chromatin fibre per nucleus (12) shows that the chromatin fibre is approximately one fourth the length of the DNA it contains (1).

2) Measurements of rotatory diffusinn constants of the various NaCl dehistonized NH preparation (13) indicates that the NH molecule elongates as a result of histone dissociation. The length after

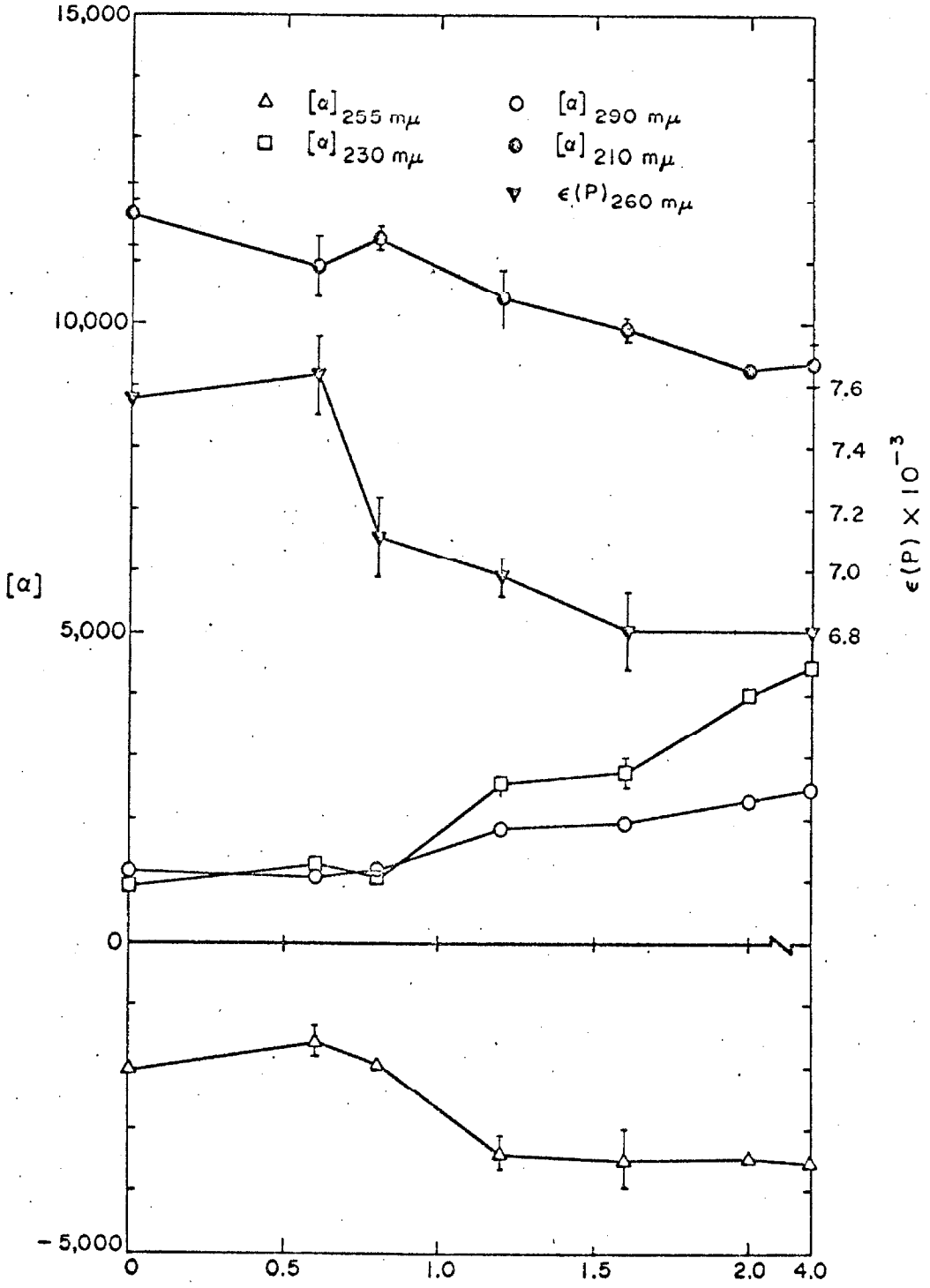

15,000
10,000
5,000
0
-5,000
[α]
△ [α]255 mμ
□ [α]230 mμ
○ [α]290 mμ
◎ [α]210 mμ
▽ ε(P)260 mμ
7.6
7.4
7.2
7.0
6.8
ε(P) X 10⁻³
0
0.5
1.0
1.5
2.0
4.0
F NaCl EXTRACTED NH

Fig. IV

dehistonization is about 1.5 times that of the original NH.

3) Hydrodynamic measurements (14) on NH and DNA indicates that the NH molecule is less asymmetric than DNA.

The above observations suggest that DNA is probably supercoiled in some way to yield the shorter NH molecule. The X-ray diffraction pattern (15) of NH exhibits a set of reflections absent from DNA patterns; some of which disappear when the fibre is stretched and indicate a model of supercoiled DNA with a pitch of 110 Å (16). The presence of supercoiled DNA in chromatin fibres has also been inferred from analysis of the effects of polarized U-V light on DNA induced birefringence (17).

As a result of DNA supercoiling, the planes of the DNA bases are no longer perpendicular to the molecular axis and indeed this is what flow birefringence and flow dichroism studies of NH and DNA demonstrate. Flow birefringence and flow dichroism studies (13) on DNA and NH, having the same rotatory diffusion constant, demonstrate that average orientation of base pairs perpendicular to the molecular axis of NH appears to be approximately 40% of that of DNA. When histones are removed by NaCl treatment, both flow birefringence and flow dichroism of the treated NH increase toward that of DNA, the concentrations of NaCl which are effective

are exactly those which are effective in alteration of absorption and of ORD spectra of NH. This suggests that as more and more histone II and III+IV are removed from NH supercoiling of DNA in NH is gradually diminished. When all histones are removed the DNA of NH is restored to the state of free DNA. The previously described hyperchromicity of NH and the ORD measurements can also find their explanation in the base pair disorientation of DNA in NH.

The exact mechanism of how the binding of histone II and III+IV can cause supercoiling of DNA and the question of why there are four kinds of histones having different chemical and physical properties and different effects on binding to DNA are a challenge for future investigation.

REFERENCES:

(1) Dupraw, E. J. (1965) Nature 206 338.

(2) Littau, V. C. Allfrey, V, G. Frenster, J. H. and Mirsky, A. E. (1964) PNAS 52 93.

(3) Zubay, G, Doty, P. (1959) J. Mol. Biol. 1 1.

(4) Marmur, J. (1961) J. Mol. Biol. 3 208.

(5) Chen, P.S. Jr. Toribara T. Y. and Warner, H. (1956) Anal. Chem. 28 1756.

(6) Chargaff, E. Davidson J. N. The Nucleic acids, Vol 1.

(7) Doty, P. Marmur, J, Eigner, J. and Schildkraut, C (1960) Proc. U. S. Nat. Acad. Sci. 46 460.

(8) Samejima, T. and Yang, J. T. (1964) Biochem. 3 613.

(9) McPhie, P. and Gratzer, W. B. (1966) Biochem. 5 1310.

(10) Schlessinger, D. (1960) J. Mol. Biol. 2 92.

(11) a) Leuchtenberger, C., Leuchtenberger, R. and Davis, A. M. (1954) Amer. J. Path. 30 65.

b) Rudkin, G. T., Hungerford, D. A. and Nowell, P. C. (1964) Science 144 1229.

(12) Osgood, E. E., Jenkins, D. P., Brooks, R. and Lawson, R. K. (1964) Ann. N. Y. Acad. Sci. 113 717.

(13) Ohba, Y. (1966) Bioch. Biophy. Acta 123 76.

(14) Gianonni, R. and Peacocke, A. (1963) Biochim. Biophy. Acta. 68 157.

(15) a) Wilkins, M. H. F., Zubay, G. and Wilson, H. R. (1959) J. Mol. Biol. 1 179.

b) Pardon J. and Wilkins M. H. F. (1967) In preparation.

(16) Pardon, J. and Richards, B. (1967) In preparation.

(17) Inuoe, S., and Sato, H., (1962) science 136 1122.

PART II

DORMANCY ASSOCIATED WITH REPRESSION OF GENETIC ACTIVITY

Chapter 1

Dormancy Associated with Repression of Genetic Activity [1, 2]

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Dormancy of buds and seeds is a well known and common phenomenon in the plant world. It is of interest because it has not yet become understandable. The dormant tissue has available to it by definition all of the environmental conditions of temperature, water supply, nutrition, etc., required for growth. The dormant tissue is alive, it respires, and in some cases grows very slowly in size (10). Yet a dormant tissue lies idle; it does not grow normally. There is within the dormant tissue some factor or mechanism which restricts growth and cell multiplication.

Many physical and chemical factors are known which possess the ability to end dormancy in one or another tissue or organ. These factors include, for example, proper photoperiod, treatment with the appropriate temperature, application of gibberellic acid, application of ethylene chlorohydrin, application of potassium thiocyanate, or thiourea, and in some cases, the mere passage of time. It is not immediately obvious that these dormancy-breaking factors possess any characteristic in common. Nonetheless, it is probable that there must be some point at which the mechanisms by which the several factors act upon dormancy merge, and play upon one or a few common facets of cell function.

If we view the problem of dormancy within the framework of molecular biology, a hypothesis immediately suggests itself, namely, that in the dormant cell the genetic material is completely, or nearly completely, repressed. According to this hypothesis, the genetic material would be unable to express itself in the form of production of the messenger RNA which is essential to enzyme synthesis and hence to growth and metabolism. The present paper constitutes a first approximation analysis of whether the hypothesis of gene repression as the cause of dormancy is a tenable one. The method consists firstly in comparison of the rates of RNA synthesis by dormant and awakening buds, and secondly in comparison of the abilities of the chromatin of such buds to support DNA-dependent RNA synthesis.

[1] Received Jan. 31, 1964.

[2] Report of work supported in part by the Herman Frasch Foundation, the National Science Foundation, Grant G-25150, and the United States Public Health Service, Grant RG-5143.

Materials and Methods

The dormant buds of potato tubers formed the experimental material for the present investigation. Potato tubers exhibit a rest period of 2 or more months after harvest. During this period the buds of the tuber will not grow, even if they are placed in a physically favorable environment. The dormancy of potato tuber buds may, however, be broken at any time by application of ethylene chlorohydrin (4, 5). Bud growth commences 10 to 17 days after the beginning of a 3-day treatment with ethylene chlorohydrin (8).

Freshly harvested potato tubers were immediately stored at 4°. Under these conditions, complete dormancy is maintained for a period of several months. Tuber samples sufficient to supply 30 to 200 buds per treatment were removed from cold storage at the beginning of each experiment on in vivo incorporation of precursor into RNA, and tuber samples sufficient to supply approximately 5 g fresh weight of bud tissue removed from cold storage at the beginning of each experiment on chromatin isolation.

Treatment for the breaking of dormancy was carried out in closed containers containing ethylene chlorohydrin (The Matheson Co., Inc.) in a concentration of 2 ml per kg tubers (8). The ethylene chlorohydrin was contained on a piece of cotton which in turn was contained in a petri dish in the bottom of the container. After an ethylene chlorohydrin treatment period of 3 days, the tubers were removed to the open air for the remainder of the period before final harvest. In the present experiments, visible growth of potato buds commenced approximately 4 days after the end of ethylene chlorohydrin treatment, or 7 days after its commencement. Control buds were similarly harvested from nontreated tubers. The buds of nontreated tubers remained dormant over the 2-month period of cold storage during which the several lots of tubers used were observed.

Incubation of Excised Potato Buds in Uridine-2-C^{14}. Rate of RNA synthesis by potato buds was determined with excised buds incubated with the metabolite under investigation. Buds were excised from the tuber with minimal amount of contaminating adjacent nonbud tissue. Such contamination as did occur did not contribute importantly to the results since the nonbud (tuber) tissue is very inactive in

RNA synthesis (see below). Each sample was incubated in a petri dish containing 5 ml of H_2O and 3 μg of penicillin as well as 0.2 μmole of uridine-2-C^{14}, specific activity 24 μc per μmole. Incubation was for 2.5 hours at 24°. In certain incubations, as outlined below, a pretreatment of the buds for 2 hours with 6 mg actinomycin D per ml preceded the incubation in uridine-2-C^{14}.

Extraction and Determination of RNA and DNA. At the end of the incubation period the buds were first washed to free them of uridine-2-C^{14} containing solution, and next ground in a glass homogenizer. The samples were depigmented by washing twice in cold methanol. They were then washed one time in cold acetic acid methanol, 3 times in cold 5% trichloroacetic acid, and twice in ethanol-ether (1 : 1 mixture). This washing procedure freed the ground tissue of small-molecule labeled metabolites. The tissue was next treated for hydrolysis of RNA and DNA by the general methods of Schmidt and Tannhauser as outlined by Ts'o and Sato (11). The washed homogenate was incubated for 17 hours in 0.3 N KOH at 37°. This treatment hydrolyzed the RNA to 2',3' ribonucleotides. The hydrolyzed solution was then made 5% in perchloric acid and the precipitate of potassium perchlorate and DNA centrifuged off. On the supernatant fraction the content of ribonucleotides was determined by optical density at 260 mμ and by colorimetric orcinol reaction. Radioactivity of the ribonucleotide solution was determined on aliquots plated on planchets and counted in a Nuclear Chicago D-47 gas flow counting system. DNA was determined in the potassium perchlorate precipitate by hydrolysis at 100° for 10 minutes in 0.5 N perchloric acid. The resulting hydrolysate was then neutralized with KOH, the potassium perchlorate centrifuged off, and the deoxyribonucleotide content of the supernatant fraction determined by optical density at 260 mμ and by the diphenylamine reaction of Burton (2). Radioactivity was determined as in the case of the RNA ribonucleotides.

Isolation of Chromatin. Chromatin was isolated from dormant and awakened potato buds by the general methods of Huang and Bonner (6). The excised buds were ground with sand in a mortar at 4° in a grinding medium consisting of sucrose, 0.25 M, tris pH 8 0.05 M, $MgCl_2$ 0.001 M, and β-mercaptoethanol, 0.001 M. The ground material was filtered successively through cheese cloth and miracloth to remove cell debris, and centrifuged at 10,000 × g for 30 minutes to pellet the chromatin. The resulting pellet was successively washed by resuspension and repelleting in grinding medium, and twice with Tris, pH 8, 0.05 M. The recovery of DNA in the so purified chromatin amounted to 35 to 40% of that present in the tissue.

Chromosomally Supported RNA Synthesis. The effectiveness of the isolated chromatin in support of DNA-dependent RNA synthesis was determined according to the general methods of Bonner, Huang, and Gilden (1). For this purpose use was made of a standard reaction mixture (1) for conduct of DNA-dependent RNA synthesis, namely per 0.34 ml: 0.1 μmole each of GTP, CTP, and UTP, 10 μmole Tris buffer, pH 8.0, 0.1 μmole C^{14} ATP (1 μc/μmole), 1 μmole $MgCl_2$, 0.25 μmole $MnCl_2$, 3 μmole β-mercaptoethanol. This reaction mixture was fortified with 10 to 20 μg of *E. coli* RNA polymerase purified by method of Chamberlin and Berg (3) to the stage of their fraction 3. To the polymerase-containing reaction mixture was added 50 μg DNA, either as pure DNA or as chromatin of the desired variety and the whole was then incubated at 37° for 10 minutes. The reaction mixture was stopped by rapid filtration on a Schleicher and Schull Type B-6 filter followed by 4 washings with cold 5% trichloroacetic acid. The dried filters were then counted in a Nuclear Chicago D-47 gas flow counting system.

Results

The data of figure 1 concern a typical experiment in which buds pretreated for 3 days with ethylene

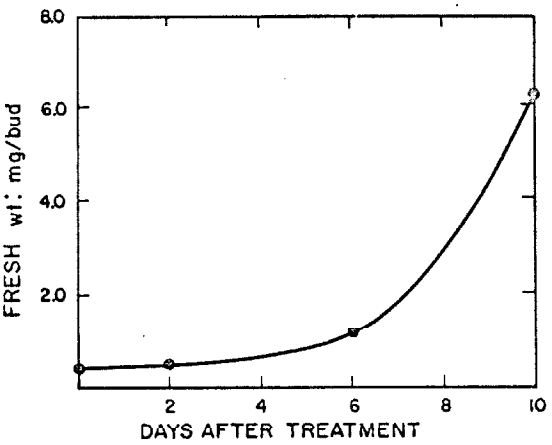


FIG. 1A. Fresh weight of the buds of potato tubers at varying times after a 3-day pretreatment with ethylene chlorohydrin.

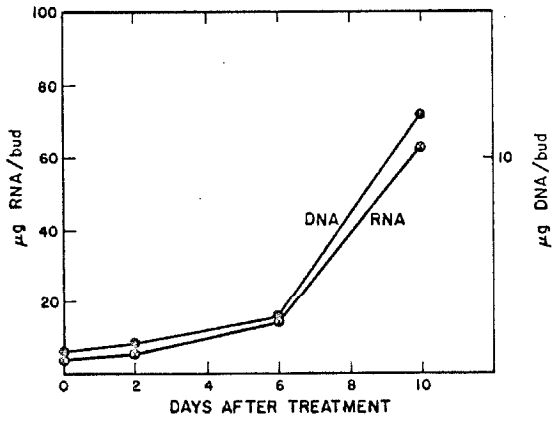


FIG. 1B. RNA and DNA content of buds of potato tubers at varying times after 3-day pretreatment with ethylene chlorohydrin.

chlorohydrin were harvested at various periods after the end of such treatment. Growth of buds as followed by increase in fresh weight increased by 2.5-fold above the initial weight by the sixth day after treatment, and rapid growth occurred by the tenth day after treatment. Changes in RNA content parallel, although they are slightly more dramatic than those in fresh weight, and start at an earlier time, namely are detectable within 2 days after treatment. DNA content, a measure in this instance of cell number, parallels growth in fresh weight. We may conclude then, that as a result of ethylene chlorohydrin treatment, the buds of previously dormant potato tubers acquire the ability to increase not only in volume but also in cell number as measured by DNA content, and in RNA content.

The data of figure 2 concern the ability of buds harvested at different times after ethylene chlorohydrin treatment to synthesize RNA and DNA. Synthesis is in this instance measured by incorporation of uridine-2-C^{14} into the 2 different kinds of nucleic acid. It is apparent from the data of figure 2 that the buds of dormant potato tubers possess an exceedingly limited ability to incorporate uridine into RNA or DNA, and are in fact almost totally devoid of this ability. Rate of RNA synthesis doubles within 2 days after the end of ethylene chlorohydrin treatment and is 130-fold the dormant level by 10 days after treatment. Alterations in rate of DNA synthesis are equally evident. This rate increases markedly over the first 2 days after ethylene chlorohydrin treatment, increases by 20-fold over a 6-day period and by 130-fold over a 10-day period, as is also shown in figure 2. The amount of RNA synthesis as measured by uridine-2-C^{14} incorporation into RNA, per unit of bud DNA, provides a measure of RNA

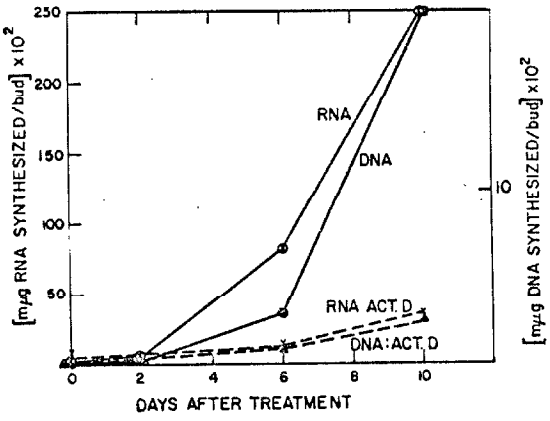


FIG. 2. Rate of incorporation of the carbon of uridine-2-C^{14} into RNA and DNA by buds of potato tubers at varying times after 3-day pretreatment with ethylene chlorohydrin. In the 2 lower curves of figure 2, the buds were incubated in solution containing not only uridine-2-C^{14}, but also actinomycin D.

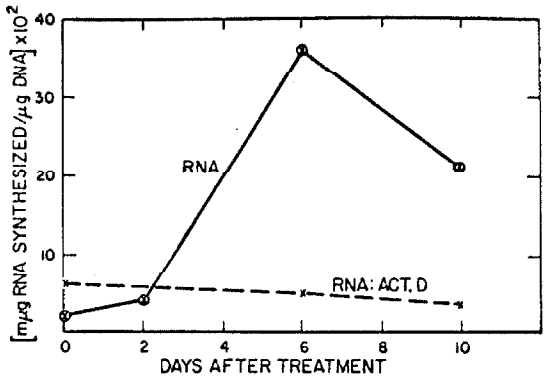


FIG. 3. Rate of RNA synthesis in buds of potato tubers at varying times after a 3-day pretreatment with ethylene chlorohydrin, and on the basis of RNA synthesized per unit DNA per unit time. In the lower curve the buds were subjected to treatment with actinomycin D.

production per cell. This also increases as a result of ethylene chlorohydrin treatment (fig 3).

The production of RNA by nondormant potato buds is inhibited by actinomycin D pretreatment (fig 2). It is clear therefore that the RNA production by such buds is of the DNA-dependent variety (7, 9).

The experiments outlined above establish that dormant potato buds synthesize RNA at a rate which is exceedingly small compared to that in growing, nondormant buds. They do not, of course, establish the basis of this difference. The experiments outlined below establish that the block to RNA synthesis in dormant buds lies at the level of the repression of chromosomal activity.

It is already established that chromatin may be isolated from the tissues of plant material with the genetic control machinery intact (1). It has further been established that the DNA of the derepressed genes of chromatin is available for transcription by added exogenous RNA polymerase (1). The rationale of the present experiments is then that of isolation of chromatin, and determination of the extent of derepression of such chromatin by determination of the ability of chromatin to support DNA-dependent RNA synthesis in the presence of added exogenous RNA polymerase. The data of table I concern a typical experiment. For this experiment one set of tubers was first treated for 3 days with ethylene chlorohydrin, and then left to grow at 25° for 10 days. Three days before the expiration of the 10-day period, 2 further sets of tubers were removed from cold storage, and one treated for 3 days with ethylene chlorohydrin. The third set remained at 25° in a container similar to that used for the ethylene chlorohydrin treatment, but without ethylene chlorohydrin. This third set then serves as the dormant, untreated control. From each set 5 to 10 g fresh weight of buds were removed and chromatin prepared

Table I. *Effectiveness of Chromatin of Dormant and of Non-dormant Potato Buds in the Support of DNA-dependent RNA Synthesis by Exogenous RNA Polymerase*

For composition of reaction mixture see Materials and Methods.

50 μg of DNA supplied to system as:	RNA synthesized $\mu\mu$mole AMP incorp per 10 min
Potato DNA (deproteinized)	3370*
Chromatin of potato tuber	0
Chromatin of dormant buds	122
Chromatin of buds from tubers at end of 3-day treatment with ethylene chlorohydrin	1412
Chromatin of buds from tubers 10 days after 3-day treatment with ethylene chlorohydrin	1538

* Incorporation due to polymerase alone (150 $\mu\mu$mole) subtracted.

as outlined under Materials and Methods. It may be remarked that roughly 25 kg of potatoes yield about 5 g fresh weight of dormant potato buds. The ability of each kind of chromatin thus obtained to support DNA-dependent RNA synthesis in the presence of added exogenous *E. coli* RNA polymerase was then determined. The data of table I show that potato DNA is highly effective in the support of DNA-dependent RNA synthesis, as would be expected. Chromatin from the potato tuber itself is totally ineffective in this function. Chromatin from dormant potato buds is almost, but not quite completely, inactive in the support of DNA-dependent RNA synthesis, less than one-thirtieth as effective as deproteinized potato DNA. The effect of ethylene chlorohydrin treatment upon the chromatin of potato buds is dramatic. Chromatin isolated from buds harvested at the end of the 3-day treatment with ethylene chlorohydrin is more than 10-fold more effective in the support of DNA-dependent RNA synthesis than is the chromatin of dormant buds. Only a further small increase in ability of bud chromatin to support DNA-dependent RNA synthesis takes place during the succeeding 10 days after the 3-day ethylene chlorohydrin treatment.

Discussion

Dormant potato buds possess a very limited capability for the conduct of both DNA-dependent RNA synthesis and of DNA replication. Treatment with ethylene chlorohydrin, a classical compound for the breaking of dormancy, removes the strictures to both RNA and DNA synthesis. The RNA synthesized by potato buds after ethylene chlorohydrin treatment is made by DNA-dependent RNA synthesis since it is actinomycin D-inhibitable in vivo. The RNA synthesized by the potato buds represents therefore transcription of the genetic material of the potato genome. Why do dormant potato buds not make RNA in quantity? The block to RNA synthesis could in principle be attributable to any one of a great number of defects, as for example, lack of RNA polymerase, limited availability of one or all of the requisite riboside triphosphates, etc. Among the possible causes of lack of RNA synthesis in dormant potato buds is the possibility that the genetic material of dormant potato buds is repressed. This is shown to be in fact the case. Chromatin isolated from dormant potato buds and incubated in a complete reaction mixture containing all of the requisites for the conduct of DNA-dependent RNA synthesis, namely riboside triphosphates, RNA polymerase, etc., is little effective in the conduct of RNA synthesis. Chromatin isolated from potato buds which have been caused to become nondormant by ethylene chlorohydrin treatment is highly active in the conduct of DNA-dependent RNA synthesis. We may say, therefore, that the genome of dormant potato buds is very largely repressed. Whether such repression is the sole cause of dormancy remains to be established.

Summary

The buds of dormant potato tubers incorporate uridine-2-C^{14} into RNA and DNA at a highly limited rate. Rate of such synthesis is markedly increased after pretreatment of the tubers with ethylene chlorohydrin, a treatment which also breaks dormancy.

RNA synthesis by the buds of nondormant potato tubers is inhibited by actinomycin D, and is hence of the DNA-dependent type.

Chromatin of the buds of dormant potato tubers is almost totally incapable of the support of DNA-dependent RNA synthesis by added exogenous RNA polymerase. The chromatin of nondormant buds of potato tubers (in which dormancy has been broken by treatment with ethylene chlorohydrin) is highly effective in the support of DNA-dependent RNA synthesis by added exogenous RNA polymerase.

It is concluded that the genetic material of the buds of dormant potato tubers is largely in a repressed state, and that the breaking of dormancy is accompanied by derepression of the genetic material.

Acknowledgments

We are indebted to Dr. Herman Timm of the University of California, Davis, and Dr. Bishop of the University of California, Shafter, for constant supply of freshly harvested dormant potatoes. We acknowledge, too, the fine technical assistance of Mrs. Cornell Phillips and Mrs. Stella Tan, and in particular we are indebted, for continuing counsel and assistance, to Dr. Ru-chih C. Huang.

Literature Cited

1. Bonner, J., R. C. Huang and R. Gilden. 1963. Chromosomally directed protein synthesis. Proc. Natl. Acad. Sci. 50: 893–900.
2. Burton, K. 1956. A study of the condition and mechanism of the diphenylamine reaction for the colorimetric estimation of DNA. Biochem. J. 62: 315–23.

3. CHAMBERLIN, M AND P. BERG. 1962. DNA-directed synthesis of RNA by an enzyme from *Escherichia coli*. Proc. Natl. Acad. Sci. 48: 81–94.
4. DENNY, F. E. 1926. Hastening the sprouting of dormant potato tubers. Am. J. Botany 13: 118–25.
5. GUTHRIE, J. D. 1939. Role of glutathione in the breaking of the rest period of buds by ethylene chlorohydrin. Contrib. Boyce Thompson Inst. 11: 261–70.
6. HUANG, R. C. AND J. BONNER. 1962. Histone, a suppressor of chromosomal RNA synthesis. Proc. Natl. Acad. Sci. 48: 1216–22.
7. KAWAMATA, J. AND M. IMANISHI. 1961. Mechanism of action of actinomycin D, with special reference to its interaction with DNA. Biken's J. 4: 13–24.
8. MICHENER, D. 1942. Dormancy and apical dominance in potato tubers. Am. J. Botany 29: 558–68.
9. REICH, E. AND E. L. TATUM. 1962. Action of actinomycin D on animal cells and viruses. Proc. Natl. Acad. Sci. 48: 1238–45.
10. ROSA, J. T. 1928. Relation of tuber maturity and of storage factors to potato dormancy. Hilgardia 3: 99–124.
11. TS'O, P. O. P. AND C. SATO. 1959. Synthesis of RNA by plants. Exptl. Cell Res. 17: 227–36.

Chapter 2

THE DORMANCY OF ONION BULBS

I. INTRODUCTION:

It has long been known that mature onion bulbs do not sprout if planted in favorable conditions soon after harvest, and are said to be dormant. After a dormant period of approximately two months, shoots and roots gradually begin to emerge. The bud does however continue to grow in size during dormancy. The dormant onion bud is mitotically inactive. The scales which surround and protect the bud grow and are, mitotically active during the dormant period (1). If one adopts visible growth and/or mitotic division as a yardstick for measuring dormancy, it would thus seem that during dormant period of the bulb, the bud is not completely dormant, and the scales are not either.

The main difficulty in isolating potato chromatin lies in the contamination of starchy material and the minute bud size. In addition to the sizable bud, the onion bulb possesses another merit, namely, the small content of starch granules, which promises a less contaminated chromatin.

II. MATERIALS AND METHOD:

Isolation of onion chromatin: Freshly harvested onion bulbs from the Imperial Valley was first disected into bud and scale tissues. 350g of scales was blended with 200g of precooled glass beads in 300 ml of grinding medium (0.5 M sucrose, 0.05 F tris pH 8, 0.024 M EDTA) in a waring blendor for one minute at 50 volts followed by 2 minutes at 40 volts.

For bud tissue, 60g of starting material was used and the amount of glass beads and grinding medium proportionally decreased. The suspension was filtered through 2 layers of cheese cloth, then through one layer of miracloth. The filtrate was centrifuged for 40 minutes at 2000xg (4000 rpm). The pellet was washed twice with grinding medium and then dispersed in 0.05 F tris PH 8. The crude chromatin in portions of 7 ml was layered on top of 20 ml of 1.7 M sucrose and centrifuged in SW 25 rotor for 2 hours at 22,000 rpm. The transparent pellet after dispersion in DSC (0.015 F NaCl, 0.0015 F Nacitrate) was dialyzed against 50 x volumes of DSC for 12 hours with one change of outside solution. The dialyzate was sheared in the Virtis container at 30 volts for 90 seconds, centrifuged at 2500 rpm for 20 minutes. The supernatant is soluble nucleohistone.

PREPARATION OF ONION DNA:

DNA is isolated from onion chromatin according to the Sevag procedure.

CHEMICAL COMPOSITION:

This is done by spectral analysis of 320 mu to 230 mu as is described in chapter 1, part 1.

HEAT DENATURATION AND DETERMINATION OF MELTING TEMPERATURE (Tm):

The melting profile is recorded in DSC on a Gilford spectrophotometer. Tm is defined as the arithmatic average of starting and final temperature of melting.

ASSAY OF TEMPLATE ACTIVITY:

The ingredients and condition for in vitro DNA dependent RNA synthesis are the same as for potato chromatin.

III. RESULTS AND DISCUSSION:

The protein to DNA ratio for both the onion bud and scale nucleohistone is the same (protein/DNA=2). The melting profiles of the two are similar with Tm's of 81-82°C and 33-34% hyperchromicity (Fig. I). Both bud and scale possess significant template activity for RNA synthesis, and this is reduced as compared to DNA (Fig. II). While the template activity of dormant potato buds is only 5% that of potato DNA, the template activity of the onion bud is 40% that of onion DNA. It seems unlikely from Fig. III that this enhanced template activity is created during the isolation process. The onion bud, which is not dormant according to the criterion of no visible growth, as well as the scale tissue, both yield chromatin of high template activity. Therefore, the chromatin of the slowly growing and therefore not truly dormant buds of onion bulbs, resembles more the chromatin of growing potato buds than it does the chromatin of truly dormant potato buds.

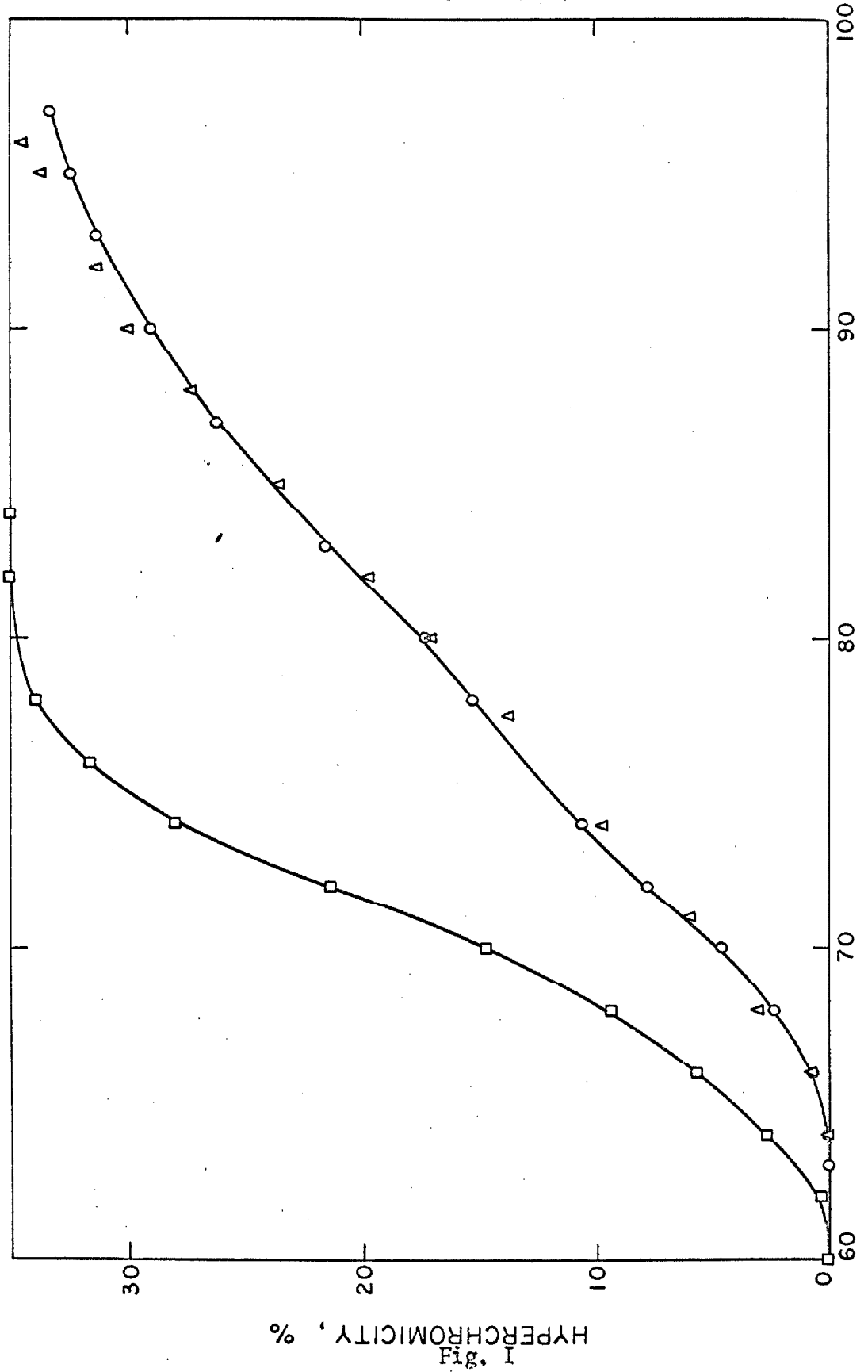


Fig. I

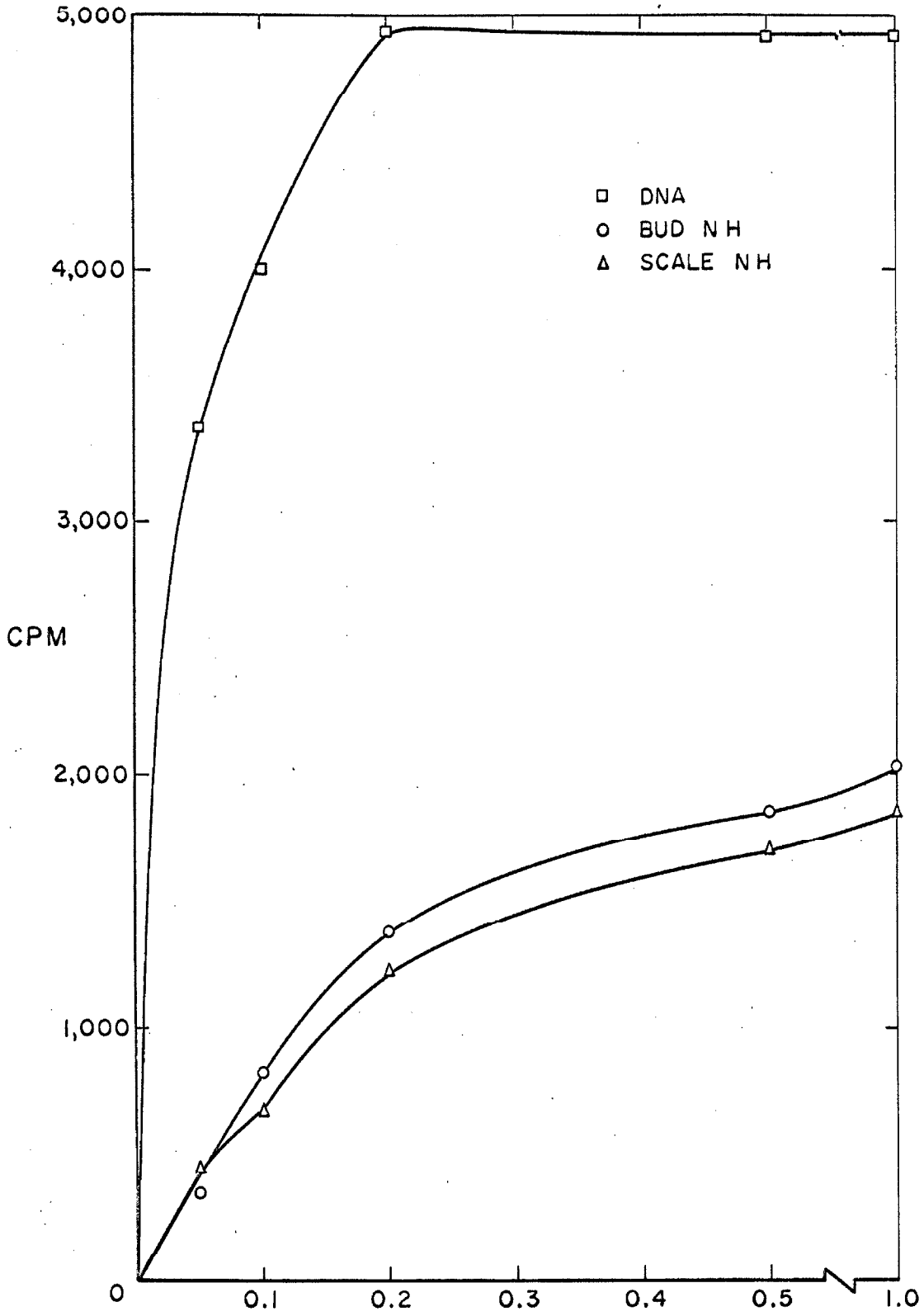
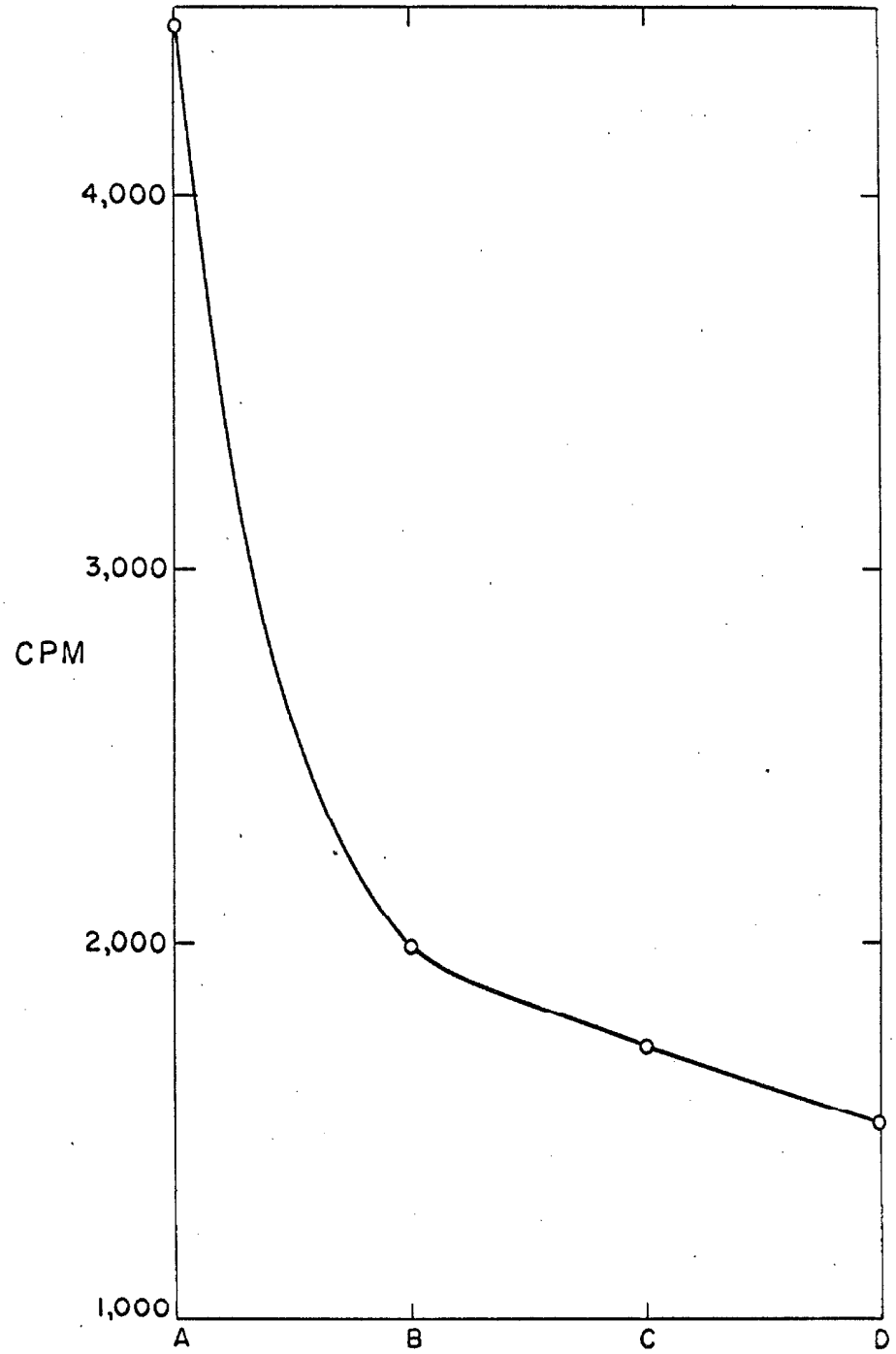

5,000
4,000
3,000
2,000
1,000
0
CPM
DNA
BUD N H
SCALE N H
0.1
0.2
0.3
0.4
0.5
1.0
O. D. 260 mμ OF TEMPLATE

Fig. II



A: crude chromatin
B: purified chromatin
C: purified chromatin dialyzed against DSC
D: NH

Fig. III

REFERENCE:

(1). Abdalla, A. A., and L. K. Mann, Hilgardia 35, 85 1963

Chapter 3

ISOLATION OF GLADIOLUS CHROMATIN

I. INTRODUCTION:

It is demonstrated in the proceding chapters that chromatin of the dormant tissue is almost totally incapable of the support of DNA-dependent RNA synthesis by added exogenous RNA polymerase. The chromatin of nondormant tissues in which dormancy has been broken by either treatment with ethylene chlorohydrin or the mere passage of time is highly effective in the support of DNA-dependent RNA synthesis. The question of what makes the two chromatin behave so differently in directing the DNA-dependent RNA synthesis can not be sought from the potato because of the difficulty in obtaining workable amount of purified potato chromatin which is a result of the minute bud size and exuberant starch content of the potato tuber. Neither can the answer be sought from onion bulbs which do not possess defined dormancy.

The Gladiolus corm would seem to be an ideal substitute. The bud is considerably bigger and easily accessible. Dry storage at room temperature after harvest allows a dormant period of six to eight months. Treatment with ethylene chlorohydrin breaks the dormancy (1). One to two months after the treatment, a full and healthy plant is developed. As soon as six days after the administration of ethylene chlorohydrin, chemical changes within the corm can be detected. The activity of some enzymes is increased ten folds (2).

A working scheme was therefore set up. Isolation of purified chromatin from Gladiolus dormant and nondormant organs would first be tried. If purified chromatin could not be prepared, discard Gladiolus

and look for other suitable plant material. If chromatin could be prepared, template activity of both dormant and nondormant chromatin would be tested. If a significant difference could be observed, chemical analysis would then follow; if the result turns out otherwise, drop this project.

II. MATERIALS AND METHODS:

Isolation of chromatin: 200g of Gladiolus bulb or 10g of bud were used. Several isolation procedures were employed in an attempt to isolate Gladiolus chromatin in good yield and with acceptably small contamination. The various procedures were in essence modifications in homogenization and speed of centrifugation of the procedure of Huang and Bonner (3).

Optical absorption: The pellet of the 1.7 M sucrose centrifugation was diluted with DSC (0.015 M NaCl, 0.0015 M Nacitrate) and optical absorption at 320 mu, 260 mu, and 230 mu recorded on a Hitachi-Perkin-Elmer U.V. spectrophotometer.

III. RESULTS AND DISCUSSION:

Both the Gladiolus bulb and bud gave very low yield of chromatin. From 200g of bulb merely 3 $O.D._{260\ mu}$ of chromatin was recovered; while buds(after correction for scattering) yielded virtually no chromatin at all. The recovered bulb chromatin, did not exibit the 260 mu absorption peak and 240 mu trough, which are characteristic of the purified chromatin from other sources.

The reasons for the poor yield can be either or both of the following:

i) the Gladiolus bulb and bud have very little chromatin material to start with,

ii) the chromatin was lost during the process of isolation.

The poor yield of chromatin is not the most severe drawback because one can always start with more bulbs and buds. It is the contamination of chromatin with scattering material which made us desert Gladiolus and quit this project.

REFERENCES:

(1). Denny, F. E., Boyce Thomson Series, Vol. 5 P. 435

(2). Boyce Thomson Series, Vol 4 131

(3). Huang, R. C., and J. Bonner, Proc. Nat. Acad. Sci. 48, 1216 1962