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MODEL PROTEINS AND DEOXYRIBONUCLEIC ACIDS

by

Margaret F. Pinkston

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Executive Officer

Supervisory Committee

The City University of New York

Abstract

INTERACTIONS OF MODEL PROTEINS AND DEOXYRIBONUCLEIC ACIDS

by

Margaret F. Pinkston

Advisor: Professor Hsueh Jei Li

Random copolypeptides containing lysine and one other amino acid, either hydrophobic or hydrophilic, were complexed with deoxyribonucleic acids by direct mixing and their interactions studied by the methods of thermal denaturation and circular dichroism (CD). Such copolypeptides serve as model proteins with which to probe the contribution made by individual amino acids to the binding and conformation of protein-DNA complexes in solution, with special reference to the basic histones complexed to DNA in native chromatin.

In the free state, basic/hydrophobic model proteins with varied proportions of lysine and alanine possess differing amounts of alpha-helix and/or random coil, depending on the relative lysine/alanine content. When bound to DNA, a substantial increase in the alpha-helical content is observed for model proteins with 40-60% alanine; when the percent alanine is either higher or lower than this intermediate value, there is no significant change in alpha-helicity with binding.

In the presence of intermediate ionic strength (0.1 to 0.5M NaCl), complexes in the 40-60% alanine range undergo a drastic transition from B- to A-type CD. Poly($Lys^{19}Ala^{81}$), on the other hand, shows only a slight transition to A-type CD at 0.4M NaCl, despite full alpha-helix in the free state. This indicates that additional factors other than

alpha-helix and the presence of alanine must be responsible for the B to A transition. At the other extreme of lysine/alanine ratio, with high lysine content, the presence of NaCl induces a B to ψ transition. These differences in conformational response to binding and the presence of salt may be related to the location of binding, whether in major or minor groove of DNA.

The A-type CD response was studied further by complexing of poly ($Lys^{40}Ala^{60}$) to poly(A)·poly(U) (an RNA model) and to DNA's of different (G + C) content (Cl. perfringens, 30%; E. coli, 53%; M. luteus, 70%). Physical studies on such complexes were done in both absence and presence of NaCl. Effects of temperature on A-type complexes were investigated by running CD spectra at different equilibrated temperatures from 25^{o} to 100^{o}C. Irreversibility of the A-type conformation was established by dialysis from 0.2M NaCl back to 0.0M NaCl; qualitatively, the A-type CD remained, despite a pronounced reduction in amplitude.

When complexed with a basic/hydrophilic model, poly($Lys^{52}Ser^{48}$), the DNA B-type conformation remained unaltered until just before complete charge neutralization, at which time partial C-form was effected. This was true in both absence and presence of NaCl. Therefore, water molecules must contribute to maintenance of the status quo (B-form), whereas tight binding by lysine residues usually excludes water and promotes transition to C-form DNA.

Poly($Lys^{66}Ile^{34}$), which incorporates a hydrophobic residue with some capacity for steric hindrance, is completely insoluble in all common solvents. However, with copolypeptide in suspension, binding occurred, and the DNA moved from B- toward C-type conformation; little

additional change transpired with dialysis to 0.2M NaCl.

The biological relevance of such model protein systems is maintained through continual reference to their natural counterparts, here, the basic histones. Observations in this system are also valid for protein-DNA interactions in general. Thermal denaturation and circular dichroism have been used to probe and characterize the physical properties of all the histones, individually, and in combination with each other and with DNA. Through correlation of results from model protein-DNA complexes with those of histone studies, many features of the more complicated biological system are seen to be both reproducible and amplified in the model system. This makes possible the study of relationships between a controlled number of components, whereas in the native system such relationships are obscured by an overwhelming number of complexities. The random copolypeptides, therefore, can simplify the study of protein-DNA interactions and throw light on the important questions being addressed to their biological counterparts.

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Chapter I

INTRODUCTION

Interactions between histones and DNA and between the histone molecules themselves are felt to hold the key to the structural organization of chromatin, and perhaps to the complex system of genetic control in eukaryotic organisms. The five histones common to most eukaryotes are very basic proteins: two are arginine-rich (H3 and H4), one lysine-rich (H1), and two slightly lysine-rich (H2A and H2B). Of these, the two arginine-rich and two slightly lysine-rich histones appear to be arranged in clusters along the DNA, approximately one each per hundred base pairs, while the very lysine-rich histone binds independently of the others along intervening stretches of DNA which are otherwise free of histones[1].

Each histone has been studied separately, and its individual characteristics determined, through extraction of histones from chromatin, separation into histone fractions, and reconstitution with homologous DNA[2,3]. However, the methods required to accomplish extraction and separation, and to prevent aggregation before renewed binding, require the use of strong acids, salts and denaturants, so that it is difficult to distinguish in the subsequent physical studies which of the observed effects can be considered to be natural and which artifactual.

A model system which begins with simple ingredients, representative

of known histone components and selected to illuminate specific characteristics, has several advantages: stringent conditions are not required for handling of model proteins prior to complexing; attention can be focussed on a relatively small number of complexities without the overwhelming presence of unrelated factors; and results obtained can be fitted back, piece by piece, into the biological puzzle, solidifying its outlines and confirming details of the larger and more complicated system.

To be able to explore questions thoroughly, the model system must be narrowly selective, and, within the model system, further restrictions must prevail.

For these studies, the biological reference system was restricted to the lysine-rich and slightly lysine-rich histones. For the model system, only two types of amino acids were chosen as subjects for copolymerization: lysine, as representative of all the basic histone residues, and alanine, for all those of a hydrophobic nature. Then, so that the system might be representative of more than one kind of histone, the proportions of lysine and alanine within the copolymer were varied. Subsequently, the initial restriction on the lysine partner was adjusted to permit the exchange of a representative hydrophilic residue, serine, for the hydrophobic alanine, or to permit the introduction of an alternate hydrophobic residue in its place. In this way, the contribution of each amino acid in the copolypeptide to the interactions of the model proteins under study could be assessed realistically and related to reactions observed in their original histone counterparts, as well as, ultimately, to reactions observed in proteins other than histones.

Whereas the reconstitution or complexing of individual histones with DNA is usually accomplished by gradient dialysis from high to low salt in the presence of urea or guanidinium chloride[4], complexing of copolypeptide was by the slow, direct mixing of model proteins with rapidly stirring nucleic acids, performed initially in the absence of salt, so that this effect, too, might be studied.

The major physical methods chosen for investigation of protein-DNA interactions were also restricted to two: circular dichroism (CD), and thermal denaturation. Each of these methods complements the other in the information it imparts. Circular dichroism reflects changes in the conformation of macromolecules, in response both to binding and to environmental changes in the solution medium. Thermal denaturation reports on the amount of DNA bound by protein, and on various characteristics of the binding event: the tightness of binding, the protection it affords the bound nucleotide, modifications of binding in the presence of salt. When explored in the model system, both these parameters can be compared with results obtained from use of the same physical methods in studies of the biological reference system.

Chapter II

MATERIALS AND METHODS

Synthesis of Copolymers

Two methods were used to synthesize copolymers of L-lysine and L-alanine: thermal copolymerization at very high temperature, and copolymerization through reaction of N-carboxyanhydrides of these amino acids in the presence of an initiator. The first method was chosen as an inexpensive preliminary experiment to determine the feasibility of binding model copolymers to DNA and of performing meaningful physical studies on complexes so formed. Low yields and inability to control possible branching reactions with the ε-amine of the lysine stimulated a change of method to one promising more quantitative yields and guaranteeing linearity of the copolymer through protection of the reactive amine.

Method I. Preliminary Thermal Copolymerization.

L-lysine and L-alanine from Sigma Chemical Company were copolymerized using different proportions of amino acids through adaptation of procedures for thermal synthesis published by Fox and Waehneldt[5]. Since successful thermal copolymerization requires dry heating of otherwise thermo-labile neutral amino acids, together with sufficient proportions of those which are non-neutral, different ratios of Lys/Ala (Lys always greater than 50%) were heated together in side-

armed test tubes under a slow stream of nitrogen.

Prior to introduction of the reactants, the test tubes were individually isolated from the rest of the system by means of clamps, and flamed, with nitrogen flowing, to insure absence of both moisture and oxygen during polymerization. The tubes were then submerged up to the side arms in an oil bath containing a thin-wire heating coil attached to a Variac rheostat, by means of which it was possible to control the heat precisely.

The oil used for the bath was mineral oil, but it became black and viscous at the high temperatures required (200°C). Silicone fluid, which remains clear at such temperatures, would have permitted observation of the reactants during the heating process. A sleeve of insulation around the oil bath beaker was required in order to achieve temperatures above 175°C. By use of the sleeve and a large magnetic stirring bar, and with constant adjustment of the Variac, the recorded temperature varied no more than 2°C during the required six-hour heating period, counted from the time 200°C was actually reached. Arrival at this temperature required an additional one to two hours, depending on the efficiency of the insulation.

After the six-hour period of polymerization at high heat, the Variac was turned off and the products were allowed to return to room temperature slowly, with nitrogen still flowing. The cooling process required 5 1/2 to 6 hours.

Before heating, the amino acids were ground together to a fine powder which liquified at high temperature and solidified with cooling. When treated with 5-6 parts water, the resulting Lys-Ala copolymers could be brought completely into solution. Those of Lys-Leu (also

synthesized with L-leucine from Sigma) were solubilized to a large extent, but some undissolved portions remained. These were retained in the solution medium and dialyzed, as was the Lys-Ala copolymer, in the cold against water for three days, with four changes per day. The remaining non-diffusate was filtered and lyophilized.

Method II. Copolymerization of N-carboxyanhydrides.

Linear random copolymers containing varying percentages of lysine and alanine were synthesized from N-carboxyanhydrides of ε-carbobenzyloxy-L-lysine and L-alanine (purchased from Miles Laboratories) according to the methods of Morita, Simons, and Blout[6] and Fasman, et al[7]. The anhydrides, in proper amounts for the desired mole ratios, were weighed under one atmosphere of argon in a dry box (Vacuum/Atmospheres Corporation Dri-Train HE-193-1, Los Angeles, Calif.) to insure absolute exclusion of moisture during the weighing procedure. Before removal from the dry box, the anhydrides were placed in separate jars and stoppered with rubber serum caps. After removal, the solvent could then be introduced by means of a hypodermic needle, using a second hypodermic syringe filled with silica gel, to permit equalizing of pressure without introduction of moisture.

The solvent was a 1:4 (v/v) mixture of dry dioxane and benzene, freshly distilled over lithium aluminum hydride and calcium hydride as the respective drying agents. For mixing, the distillates were removed from the receiving flask through serum caps, and introduced into serum-capped bottles using two syringes, one for filling with liquid, one containing silica gel to allow for expulsion or introduction of dry air to equalize pressure.

The weighed, individually bottled anhydrides of lysine and alanine were first solubilized, using the same syringe technique of solvent transfer, before mixing for polymerization. Once in solution, the contents of one bottle was quickly poured into the other, with simultaneous addition of the weighed initiator, sodium methoxide (Sigma Chemical Co.), and the total contents swirled to mix. The anhydride/initiator ratio was either 20 or 200; the total concentration of anhydride, 1-2%.

Polymerization was allowed to proceed undisturbed for two days at 25°C, after which anhydrous hydrogen bromide was bubbled through the polymerization solution for 45 minutes, to promote precipitation and removal of the benzyl group. Nitrogen was then bubbled through the solution, with stirring, for ninety minutes, to remove excess HBr, and the insoluble product allowed to stand overnight in solution. After washing in the dioxane-benzene mixture, the product was extracted for three hours with acetone and dried in a vacuum. The amino acid composition of the resulting copolymers was analyzed, after dialysis and lyophilization, by Mr. Carl Homnick at Merck Institute.

Other Copolymers and Materials

Miles Laboratories provided random copolymers of L-lysine-L-serine and L-lysine-L-isoleucine. Orders were placed for Lys-Ser in a 50/50 ratio and for Lys-Ile at ratios of 35/65 and 65/35. The analyzed 50/50 product was received as poly(Lys^{52} Ser^{48}), molecular weight, 37,375. The poly(Lys,Ile) products could not be analyzed for composition due to complete lack of solubility in any common solvents. Poly L-lysine hydrobromide, polylysine, was also a product of Miles

Laboratories.

Calf thymus DNA was purchased either from Sigma Chemical Company or from Miles Laboratories and purified by repeated phenol extraction. DNA's of Escherichia coli, Clostridium perfringens, and Micrococcus luteus came from Worthington Biochemicals, and the double-stranded sodium salt of Poly(A)·Poly(U) from P-L Biochemicals, Inc.; these were used without further purification. Trypsin and soybean inhibitor were purchased from Sigma Chemical Company.

Buffer Solutions

All experiments were carried out in ethylenediamine tetraacetate (EDTA) buffer, 2.5 x 10^{-4}M, at pH 8.0. For those experiments designed to determine the effects of salt, varying amounts of sodium chloride were added to the same EDTA buffer, either for purposes of dialysis or for complexing directly in salt.

Preparation of DNA and Copolypeptide Solutions Prior to Complexing

DNA solutions: Calf thymus DNA, stored after phenol purification in a buffer of 0.01M Tris, 0.001M NaCl, was dialyzed overnight with four changes of EDTA buffer before being adjusted spectrophotometrically to a concentration of 10^{-4}M, using an extinction coefficient of 6500 at 260nm[8]. All other DNA's and polynucleotides were dissolved directly into EDTA buffer, without dialysis, and adjusted to the same concentration, using their respective extinction coefficients at 260nm: E. coli, 6700; Cl. perfringens, 7400; M. luteus, 7000[8]; Poly(A) Poly(U) 5525 (calculated from information provided by P-L Biochemicals, Inc.).

Copolypeptide stock solutions: For stock solutions, the dry copolypeptide was dissolved in EDTA buffer at concentrations from two to four times that used for complexing and dialyzed against several changes of the same buffer. Concentrations of solutions used for complexing of each copolypeptide were calculated on the basis of the quantity required for approximate charge neutralization of phosphates with addition of about one milliliter, the required dilution being determined on the basis of an empirical precipitation curve.

Concentration Determinations of Copolypeptide Stock Solutions

Copolypeptide stock solution concentrations required for calculations were determined by means of the Ninhydrin Method of Spies[9].

Preparation of Ninhydrin Solution: Methyl cellosolve (Sequinal grade, Pierce Chemical Co.) was tested for peroxide with a 10% KI solution. Under the hood, 2gm ninhydrin (Pierce reagent grade) were introduced quickly into 50ml of peroxide-free methyl cellosolve already stirring under nitrogen, and stirred until completely dissolved. To this solution was added an equal quantity of freshly made citrate buffer (2gm citric acid monohydrate dissolved in 20ml 1N NaOH, diluted to 50ml) in which stannous chloride (80mg Pierce Reagent Grade $SnCl_2$/ 50ml citrate buffer) had been dissolved. The light-sensitive ninhydrin solution was immediately transferred to a brown bottle which had been previously flushed out with nitrogen to avoid oxygen contamination.

Preparation of Stock Solutions for Standard Curves: Amino acids of high purity (Pierce AMAC Standard Kit 22) were solubilized in water at 5mM concentrations. From these amino acid stock solutions, copolypeptide standard stock solutions were prepared in 4ml quantities, using

the appropriate proportion of each amino acid according to the composition determined by amino acid analysis. A standard curve for each copolypeptide was prepared by serial dilution of the copolymer standard stock solution, using five different concentrations, in a range of 0.5-2.0mM, since Beer's law is obeyed through an O.D. of 0.50 with a deviation of 4% at O.D. = 1.0.

Preparation of Samples: 0.5ml concentrated hydrochloric acid was added to 0.5ml of the solution whose concentration was to be determined, and the solution was allowed to hydrolyze at 110°C for 22-24 hours, after which it was neutralized with 1.0ml of 6.0N NaOH.

Determination of Concentration: 0.1ml each of sample or standard was added to 1ml ninhydrin solution in tubes large enough to contain 10ml, mixed, the tubes capped, and the solution boiled for 20 minutes, after which 5ml of diluent (1:1 H_2O:propanol) was added and mixed. Spectrophotometric readings were taken at 570 nm, the standard readings graphed, and sample concentrations determined from the graph.

Method of Complexing

All complexing of copolypeptide with nucleotides was accomplished at room temperature by direct mixing, through the drop-by-drop addition of copolypeptide to rapidly stirring nucleic acid. Reproducible results required the use of standard size test tubes (18 x 150mm) and a uniform quantity of nucleic acid (5ml). Stirring was by means of a Lab-line Super Mixer (Arthur H. Thomas, Inc.) set at highest speed and operated by the Touch-Plate. Complexes were allowed to stand approximately fifteen minutes before physical studies were begun.

Digestion of Complexes by Trypsin

Trypsin solution (0.01mg/ml) was added to the complex in the amount required to give a final concentration of 1, 2, 5, or 10μg/ml of complex. The reaction was carried out at room temperature for 1 1/2 hours and terminated by the addition of 0.2ml of soybean trypsin inhibitor (1mg/ml) to the total complex solution.

Methods Used for Physical Studies of Complexes

Thermal Denaturation: Measurements of thermal denaturation were made at 260nm, or 320nm, on a Gilford recording spectrophotometer, Model 2400-S. Temperature increase was controlled at 2/3°C per minute by a NESLAB Programmer (TP-2) connected to a Haake circulating bath (Type FE) using ethylene glycol to permit elevation of temperature above 100°C. Three samples and a reference cell were run simultaneously and the percent increase in absorbance, the hyperchromicity (h), recorded as a function of temperature. The resulting curves were analyzed in terms of the derivative of the hyperchromicity (dh/dT) to allow a better visualization of small changes in the hyperchromic curve.

Calculation for derivative melting curves employed the equation

$$dh/dT = \frac{h(T + 1) - h(T - 1)}{2}$$

where T is the measured temperature. To determine the actual hyperchromicity from the recorded graph, the change in absorbance (ΔA), which equals the difference between the location (X) on the recorded graph and the baseline (ΔX) multiplied by a scale factor, is divided by the initial absorbance at 260nm (A_0):

$$h = \frac{\Delta A}{A_o} = \frac{\Delta X \text{ (scale factor)}}{A_{260}}$$

The Gilford Model 2400-S is accurate to about 0.001. Under the experimental conditions used, a change equal to the instrumental error corresponds to a change of about 0.1 in a dh/dT plot. Therefore, plotted changes of this magnitude are evaluated on the basis of reproducibility between samples of different preparations and different concentrations, and curves between points are drawn or averaged according to such evaluation[10].

Circular Dichroism (CD): All circular dichroism spectra were recorded by a Jasco Spectropolarimeter, Model J-20, at room temperature, with the exception of those experiments in which the A-type CD spectrum was studied as a function of temperature. For the latter experiments, a Lauda K-2/R circulating bath (Brinkman Instruments) with both heater and compressor was used. Temperatures were allowed to stabilize before taking spectral readings between 340nm and 200nm; the temperature of the ethylene glycol bath was then adjusted to the next level, equilibrated for five minutes, and the spectrum run again.

Spectral data are directly recorded in terms of ellipticity, θ, measured in degrees and related to the chart paper by means of a scale factor. At a given wavelength, the CD signal represented on the chart corresponds to the distance across the chart between the baseline and the sample recording at that wavelength.

The CD spectra are reported as $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ in $M^{-1}cm^{-1}$, where ε_L and ε_R are, respectively, the molar extinction coefficients for left and right circularly polarized light. $\Delta\varepsilon$ is related to the measured

ellipticity by the equation

$$\Delta\varepsilon = \frac{\theta}{33.03\ C\ \ell}$$

where C is the concentration in moles per liter and ℓ is the path length of the solution in centimeters.

Chapter III

POLY($LYS^{m}ALA^{n}$): COPOLYPEPTIDES AND THEIR COMPLEXES

The emphasis of these investigations is upon interactions between model proteins and DNA rather than upon the methods of synthesis and characterization of the model proteins themselves. Synthesis was necessary because variation in composition was essential to the binding studies and copolymers of the desired compositional range were unavailable commercially. However, since synthesis was a means to an end, rather than an end in itself, discussion of the synthetic procedure and product determination will be brief and restricted to characterizations related to the legitimate concerns of this research.

Following synthesis of copolymers which contained varying proportions of lysine and alanine, the initial binding studies first were focussed on one copolymer, poly($Lys^{40}Ala^{60}$), in order to define its characteristic parameters as a basis for comparison. Then the field of scrutiny was enlarged to include the other copolymers of greater and lesser lysine/alanine proportions, so that the process of evaluating the relative importance of hydrophobic and electrostatic interactions in the observed results might be refined and controlled.

In reporting the results of these studies, the same procedure will be followed: the characteristic parameters observed and determined with poly($Lys^{40}Ala^{60}$) will be described and evaluated, then considered in the light of similar experiments performed on its sister compounds.

In this way a comprehensive view of the contribution of each amino acid to the interaction between DNA and its binding proteins can be assessed.

Copolypeptides Synthesized by the Method of Thermal Copolymerization

The method of thermal copolymerization was developed at the Institute of Molecular Evolution, where the major interest is in determining how the first proteins could have evolved in the origins of life[11]. Although, in the procedures described, as many as eleven amino acids were polymerized simultaneously, mention was made in the referenced article[5] that lysine and alanine had been sucessfully copolymerized.

Several runs were made with this method of copolymerization, one of lysine and leucine, two of lysine and alanine. Each run, comprised of four test tubes heated simultaneously, was made at a slightly different percent composition. Product yield was determined by the Lowry method of protein determination. Quantitative results suggested that different temperatures favor different percent composition: at higher temperatures ratios of 1:1 and 2:1 of Lys:Ala showed better yield; lowering the temperature only five degrees favored a 3:2 proportion over both of the others.

The yield from 2-3gms of a lysine-leucine mixture was only 1-2mg of fluffy, whitish material; the lysine-alanine mixture yielded no solid whatsoever. However, for the latter, staining was observed on the sides of the lyophilizing tubes. By washing each tube with 2ml H_2O, a solution was obtained which gave positive protein results by the Lowry method.

Having determined the protein content of the copolymers, it was

possible to make appropriate dilutions so as to complex them with DNA in the desired ratios. Thermal denaturation of the Lys/Leu-DNA complexes showed no evidence of stabilization by the copolymer, and this combination was abandoned without further work. The Lys/Ala-DNA complex, however, showed 27% stabilization to a temperature of 91°C in the 67:33 input ratio Lys:Ala sample. The 60:40 sample showed 16% stabilization to the same temperature. With the 50:50 sample there was little evidence of stabilization by the copolymer, almost all (89%) of the absorbance change occurring around 50°C, the temperature at which the calf thymus DNA melts.

Results from these preliminary experiments served to demonstrate that a random copolymer of lysine and alanine would in fact bind to the DNA and provide stability, which would vary according to its compositional make-up. The stage was then set for the use of a more precise synthetic method which would yield linear copolymers in sufficient quantity to provide for sustained and detailed studies of their interaction with deoxyribonucleic acids.

Copolymerization of N-Carboxyanhydrides of L-Lysine and L-Alanine

Random copolymers of L-lysine and L-alanine were produced from their N-carboxyanhydrides following the method described by Morita, et al[6], and Fasman, et al[7]. The ε-amine of the lysine was protected by a carbobenzyloxy group which was removed by hydrogen bromide after polymerization was complete. Three runs were made, each run yielding four different copolymers of various percent composition. To permit more detailed studies, work was restricted to the use of six different input ratios of Lys:Ala, drawn from the second and third runs: 19:81, 40:60,

50:50, 60:40, 70:30 and 90:10. Amino acid analysis, performed by Mr. Carl Homnick of Merck Institute, showed the following products, respectively: 19:81, 41:59, 48:52, 57:43, 67:33 and 81:19. Since the only requirement as to size of the copolymer for binding was that it be comparable to the histones on which it was modeled, accurate molecular weight determinations were not performed. However, gel electrophoresis of copolymers, run concurrently with whole histones by Mr. I. Michael Leffak of our laboratory, showed considerable variation in size, but all fell within the histone range, i.e. between 10,000 and 25,000[12]. Reliability of the polymerization method was assumed, and further characterization of products was not deemed necessary for the success of the projected studies.

Copolypeptide-DNA Complexes

A titration precipitation curve for each copolymer of different percent composition was drawn (Figure III-1). With each copolymer the complex remains completely soluble until a certain input ratio, r, is reached, at which point precipitation occurs sharply, more sharply the higher the lysine content. The midpoint, r_{mid}, of the curve is determined from the plot. Table III-1 shows this value expressed in two ways: in terms of amino acid/nucleotide, as is customary for histones, and in terms of lysine/nucleotide, which emphasizes the electrostatic positive/negative (+/-) relationship. The midpoint using the latter term varies irregularly between 0.84 (for 81% alanine) and 1.00 (for 81% lysine); using the former term, there is progressive increase in the number of amino acid/nucleotide with increase in alanine content, ranging from 1.2 to 4.4 when percent alanine reaches 81.

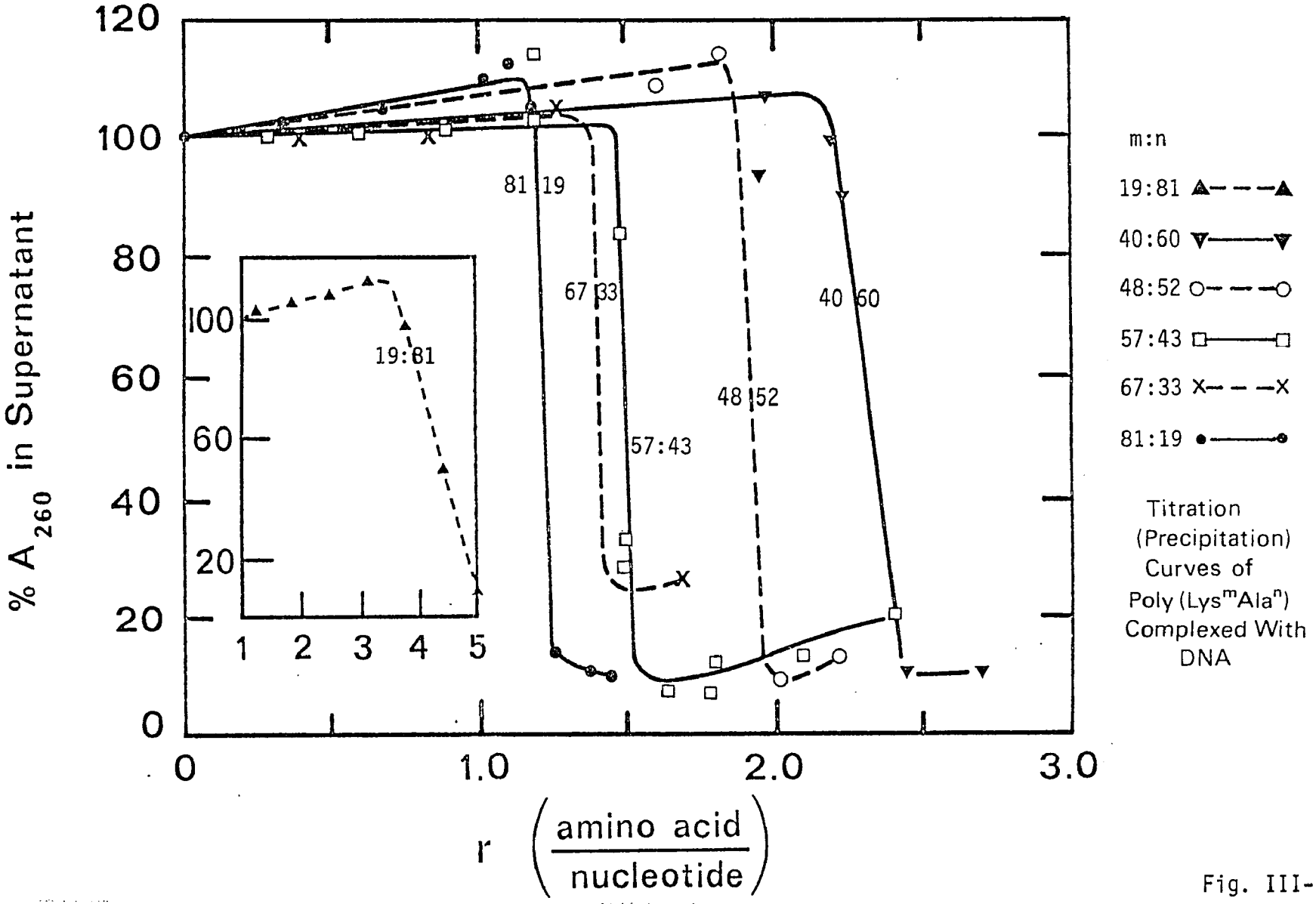

120
100
80
60
40
20
0
% A_{260} in Supernatant
0
1.0
2.0
3.0
r $\left(\frac{\text{amino acid}}{\text{nucleotide}}\right)$
81 19
67 33
40 60
48 52
57:43
19:81
100
60
20
1 2 3 4 5
m:n
19:81
40:60
48:52
57:43
67:33
81:19
Titration (Precipitation) Curves of Poly (Lys^mAla^n) Complexed With DNA

Fig. III-1

TABLE III-1

Midpoints of Precipitation Curves

of Poly($Lys^{m}Ala^{n}$)·DNA Complexes

Copolypeptide m/n	19/81	40/60	48/52	57/43	67/33	81/19
r_{mid} (amino acid/ nucleotide)	4.40	2.33	1.93	1.50	1.42	1.23
r_{mid} (lysine/ nucleotide)	0.84	0.96	0.93	0.86	0.95	1.00

Thermal Denaturation of Poly($Lys^{m}Ala^{n}$) Complexes

Figure III-2 shows derivative melting curves from complexes of different r value, using the copolymer poly($Lys^{40}Ala^{60}$) for binding on calf thymus DNA. Readings were taken at both 260nm and 320nm. At 260nm, as more copolypeptide is bound to DNA, the melting area of free base pairs at about $50^{o}C$ (Tm) is reduced, while that of bound base pairs at about $93^{o}C$ (Tm') is proportionately increased. Compared with melting curves of polylysine-DNA complexes prepared by the same method in the same buffer[14], there are three major differences. In polylysine-DNA complexes, the melting occurs exclusively in Tm and Tm' regions, and the hyperchromicity change between them is negligibly small, whereas in poly($Lys^{40}Ala^{60}$)-DNA complexes the residual melting between these two melting bands is significant, as shown in Figure III-2. Secondly, for copolypeptide-DNA complexes of higher r values, there is a rather large upward shift of about 7^{o} in the Tm melting band, compared with a shift of approximately 3^{o} in polylysine-DNA complexes. A third significant difference is the fact that, while in polylysine-DNA complexes the maximum hyperchromicity, h_{max}, is reduced when r of the complex is increased, in copolypeptide-DNA complexes h_{max} is increased by as much as 6% above that of pure DNA.

These properties, found in poly($Lys^{40}Ala^{60}$)-DNA but not in polylysine-DNA complexes, could result from the presence of alanine in the complex which might favor some hydrophobic contact among bound regions, inter- or intramolecularly, during melting. If such is the case, some excess hyperchromicity at 260nm could be contributed by an increase of light scattering. The melting curves monitored at 320nm, shown in Figure III-2, support this explanation, as the hyperchromic change at

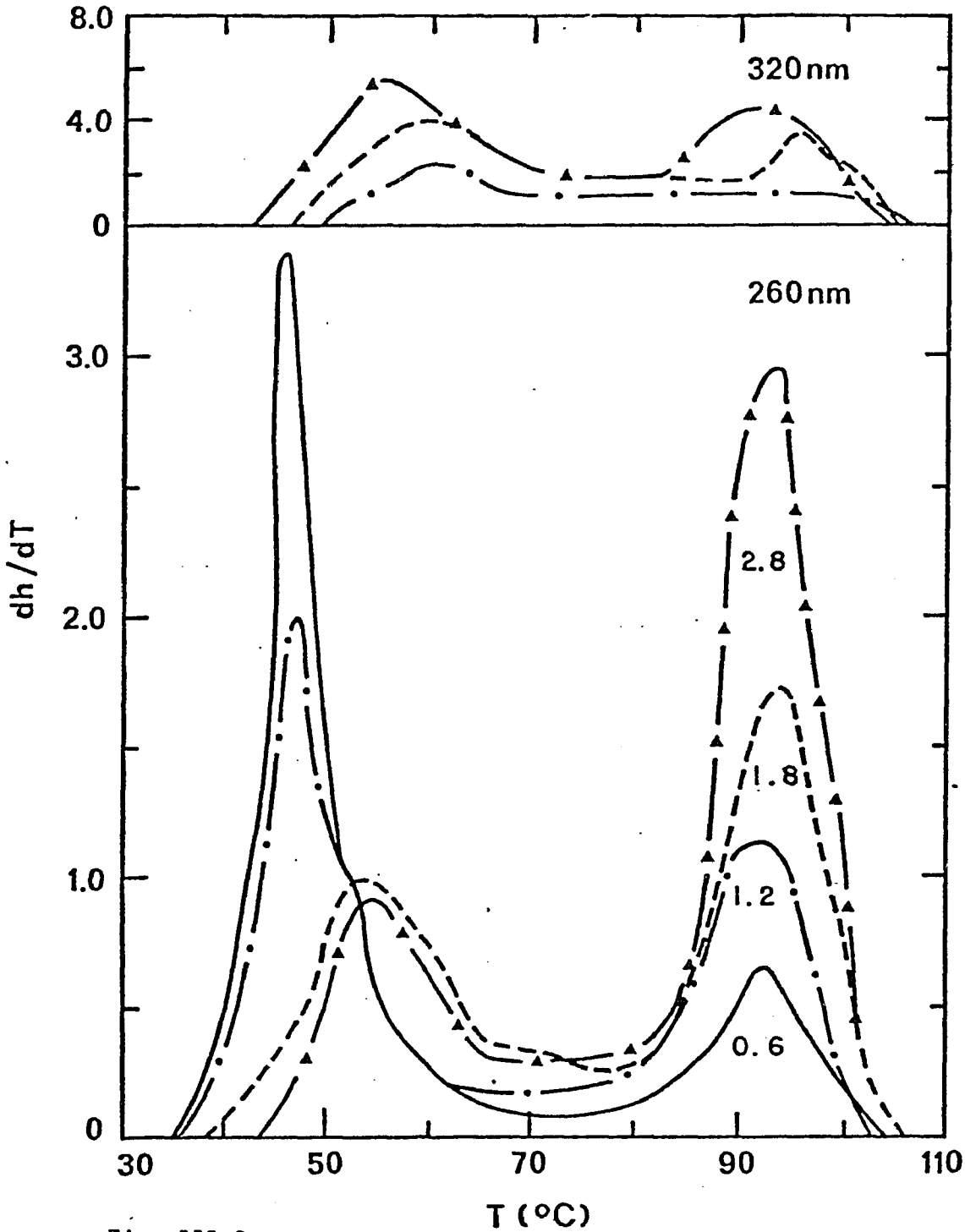


Fig. III-2

DERIVATIVE MELTING PROFILES OF POLY($Lys^{40}Ala^{60}$)·DNA COMPLEXES AT DIFFERENT r-VALUES (amino acid/nucleotide)

320nm becomes more significant for complexes of higher coverages. Similar phenomena were reported before[15] for reconstituted protamine-DNA complexes.

Derivative melting curves from three additional copolymers are shown in Figure III-3. In all cases, biphasic melting curves are induced by the binding of copolypeptide to DNA: one phase of melting occurs about 48°C (Tm) and another phase between 90°and 100°C (Tm'). Although the patterns of melting are approximately the same among various copolypeptides, two distinctions can be observed. First, the Tm' is 91-93°C for complexes with poly($Lys^{19}Ala^{81}$), 93-94°C with poly ($Lys^{48}Ala^{52}$), and 95-98°C with poly($Lys^{67}Ala^{33}$). In the case of polylysine, the Tm' is 99-101°C[14]. The Tm' therefore gradually increases as the lysine content of the model protein is increased. Secondly, the Tm' melting band is broader for a model protein of higher alanine content. The width of the Tm' melting band at its half-height is about 15°C for complexes with poly($Lys^{19}Ala^{81}$), 10°C with poly($Lys^{48}Ala^{52}$), 7°C with poly($Lys^{67}Ala^{33}$) (Figure III-3), and 5°C with polylysine[14], which seems to indicate that the melting of protein-bound DNA is less cooperative when the α-helical content of the protein is increased.

As is true for poly($Lys^{40}Ala^{60}$), there is residual melting between the two major melting bands of Tm and Tm' which is more pronounced for those complexes having a higher alanine content in the model protein.

Thermal denaturation results have been used to determine the fraction of protein-bound base pairs in a nucleoprotein; this fraction, in turn, was used to calculate the CD of protein-bound base pairs, for polylysine-DNA[16], protamine-DNA[17], and polyarginine-DNA[18]. The same method can be used here for poly($Lys^{40}Ala^{60}$)-DNA, though light scatter-

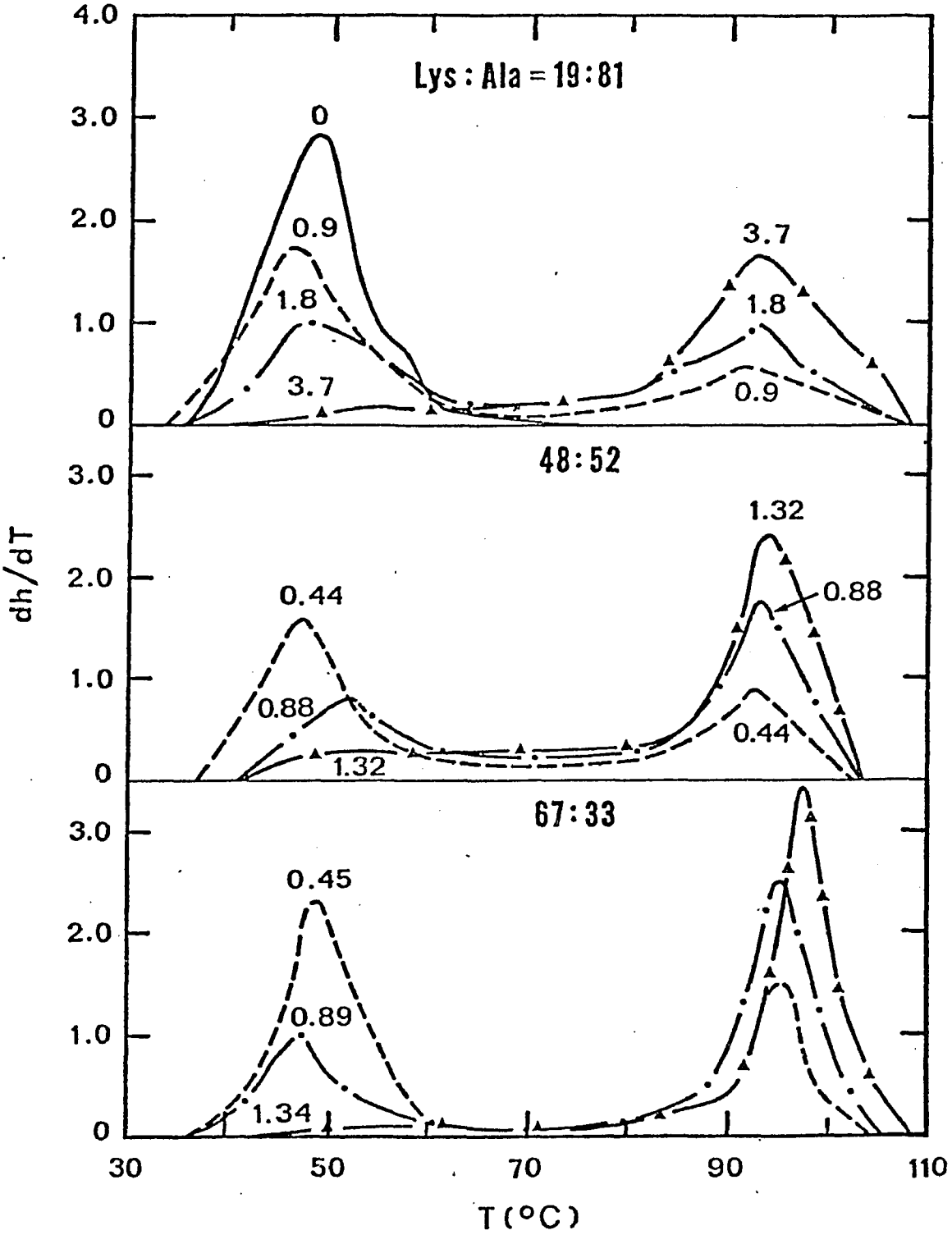


Fig. III-3

DERIVATIVE MELTING PROFILES OF POLY(Lys^m Ala^n)·DNA COMPLEXES

(m:n and r-values are indicated)

ing during melting makes some contribution to the hyperchromicity change in this system. Because of the residual hyperchromicity between the two melting bands at Tm and Tm', both equation 1 and 2 below are used, where equation 2, but not equation 1, includes the residual melting between the two melting bands as part of the melting of copolypeptide-bound base pairs:

$$r = \beta A_{Tm'}/A_T \qquad (1)$$

and

$$r = \beta\ 1 - (A_{Tm}/A_T) \qquad (2)$$

In these equations, β represents the amino acid residues per nucleotide in copolypeptide-bound regions, A_{Tm} and $A_{Tm'}$ the areas under melting bands of Tm and Tm', respectively, and A_T the total area under the curve which is equal to h_{max}.

Linear plots of these two equations are shown in Figure III-4, for poly($Lys^{40}Ala^{60}$), and in Figure III-5, for three other copolypeptides representing a wide compositional range. For the same complex, β values obtained by equation 1 are always greater than those by equation 2. From Figure III-4, the number of amino acids/nucleotide in copolypeptide-bound regions is 3.2 by equation 1 and 2.7 by equation 2, giving an average of 3.0 residues/nucleotide for poly($Lys^{40}Ala^{60}$). Since lysine comprises only 40% of the residues in this copolypeptide, this means there should be approximately 1.2 lysine/nucleotide in the bound regions. Averaging the β values derived from both equations as plotted in Figure III-5, there are 3.6 amino acid/nucleotide for poly($Lys^{19}Ala^{81}$), 1.7 for poly($Lys^{48}Ala^{52}$), and 1.1 for poly($Lys^{81}Ala^{19}$).

Figure III-6 shows the dependence of both the average β value and the Tm' of the complexes on the alanine content in the model protein.

Fig. III-4

DETERMINATION OF β VALUES FOR POLY($Lys^{40}Ala^{60}$)·DNA

LINEAR PLOTS OF EQUATIONS (1) AND (2)

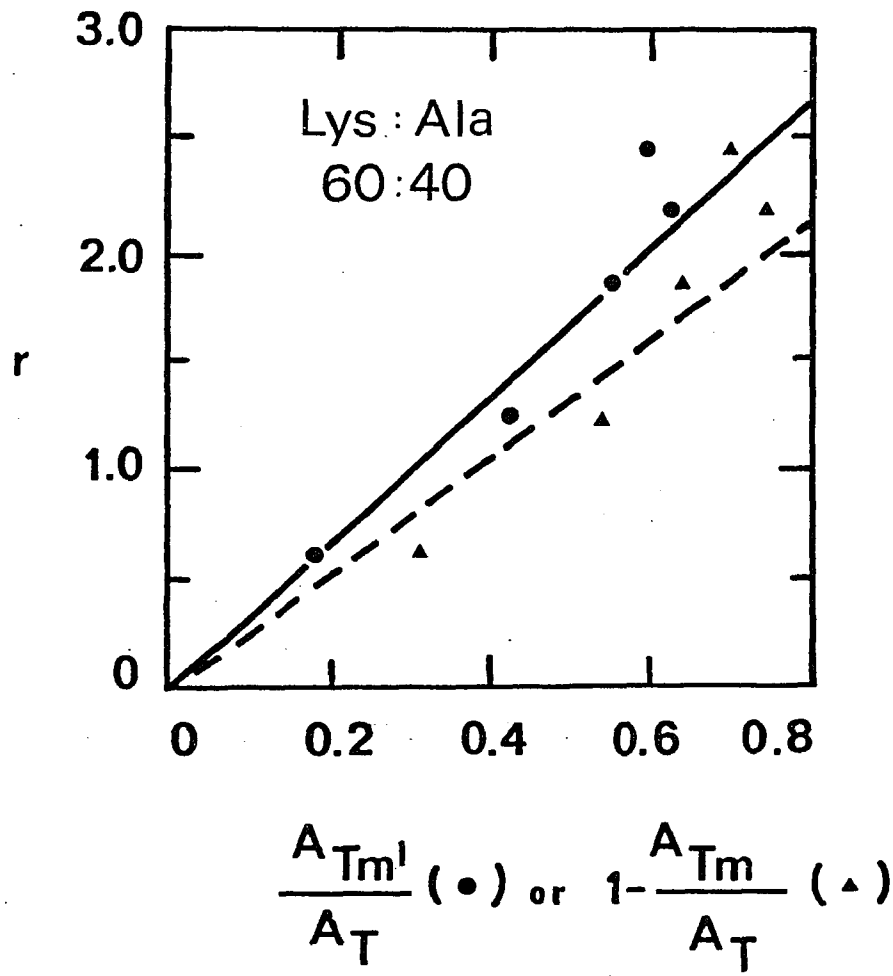
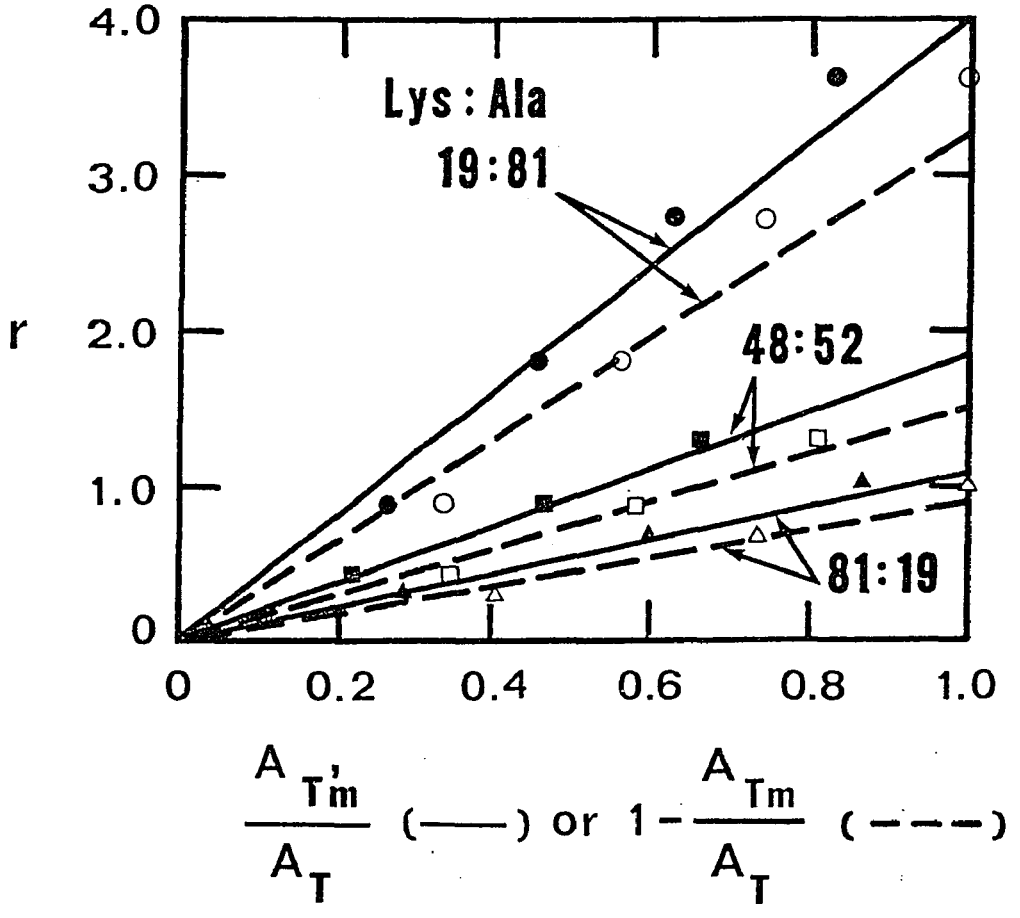


Fig. III-5

DETERMINATION OF β VALUES FOR POLY(Lys^m Ala^n)·DNA COMPLEXES

LINEAR PLOTS OF EQUATIONS 1 AND 2

(m:n is indicated)



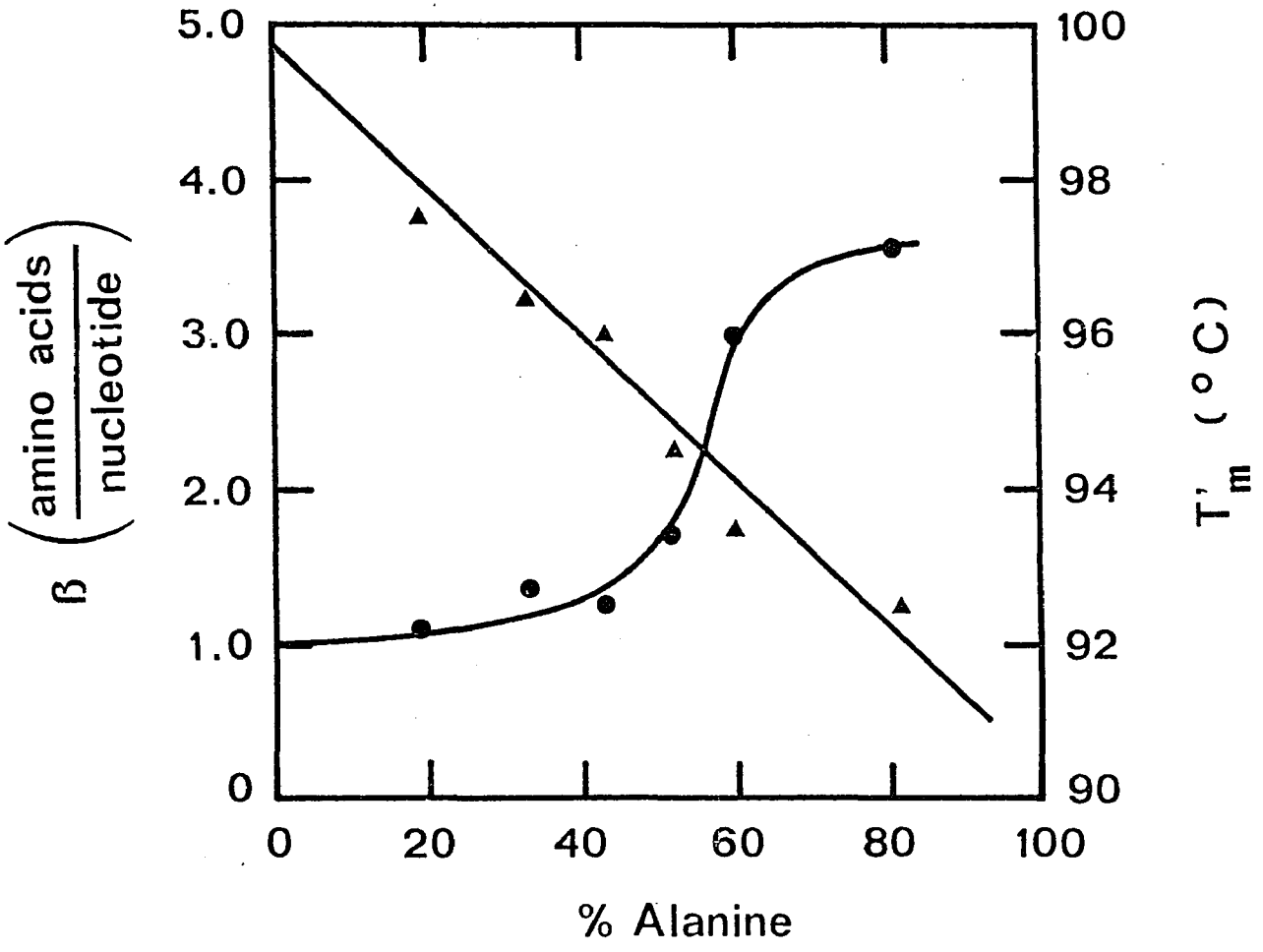


Fig. III-6 DEPENDENCE OF MELTING TEMPERATURES AND β VALUE ON ALANINE CONTENT OF MODEL PROTEIN

The Tm' of each model protein represents the average value obtained from complexes of various r values. In protein-bound regions the Tm' decreases linearly as the alanine content of the protein is increased. On the other hand, the β value in the protein-bound regions increases sharply when the alanine content reaches 50%. This seems to correlate with a sharp increase in α-helical content of these copolypeptides when they are bound to DNA, as is demonstrated later in Figure III-13.

Circular Dichroism (CD) Studies on Copolypeptide-DNA Complexes

The CD effect of varying the r value of a complex is demonstrated in Figure III-7 with spectra from poly($Lys^{40}Ala^{60}$)-DNA complexes. As more copolypeptide is bound to DNA, two significant changes in CD spectra can be seen. The CD band near 220 nm becomes more negative, and the CD band of DNA near 275nm is slightly reduced and red-shifted. The latter effect is similar to that found with polylysine binding but is of much lesser magnitude.

Figure III-8 shows variation in r values for copolypeptide-DNA complexes of different Lys/Ala composition. Complexes of poly(Lys^{19}-Ala^{81}) (Figure III-8a) produce a large negative CD band at 220nm and a shoulder at 210nm, with the amplitude proportional to the r value of the complex. Such CD in this wavelength region indicates a great deal of α-helix in the protein moiety of the complex. The effect on the DNA CD in the 275nm region produced by the binding of poly($Lys^{19}Ala^{81}$) is, however, quite small, both in amplitude and in red shift. As the alanine content of each copolypeptide chosen for complexing is reduced (Figure III-8, a-d), there is a great reduction in the protein CD near 220nm, accompanied by an increasing effect on the DNA CD near 275nm.

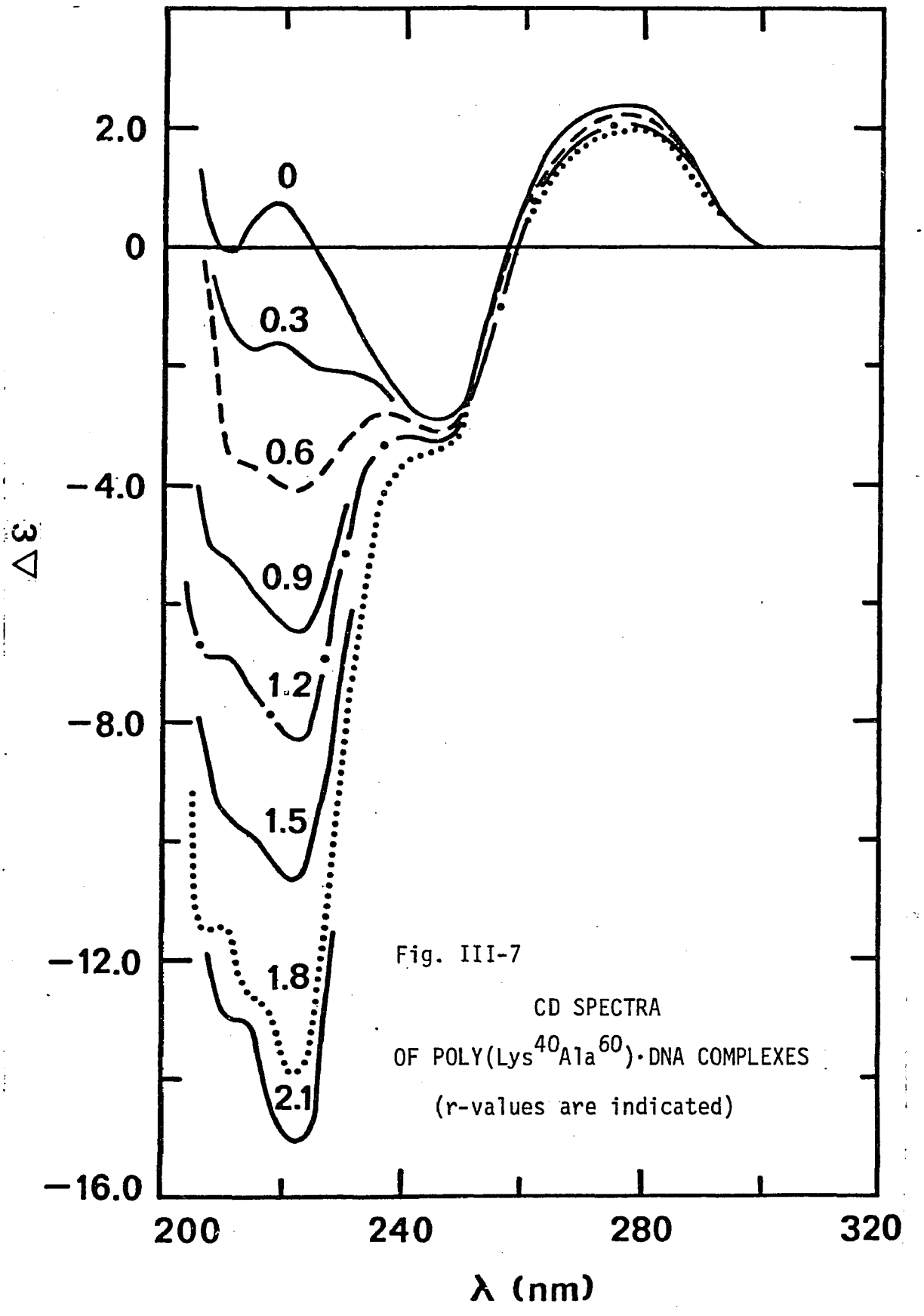


Fig. III-7

CD SPECTRA

OF POLY($Lys^{40}Ala^{60}$)·DNA COMPLEXES

(r-values are indicated)

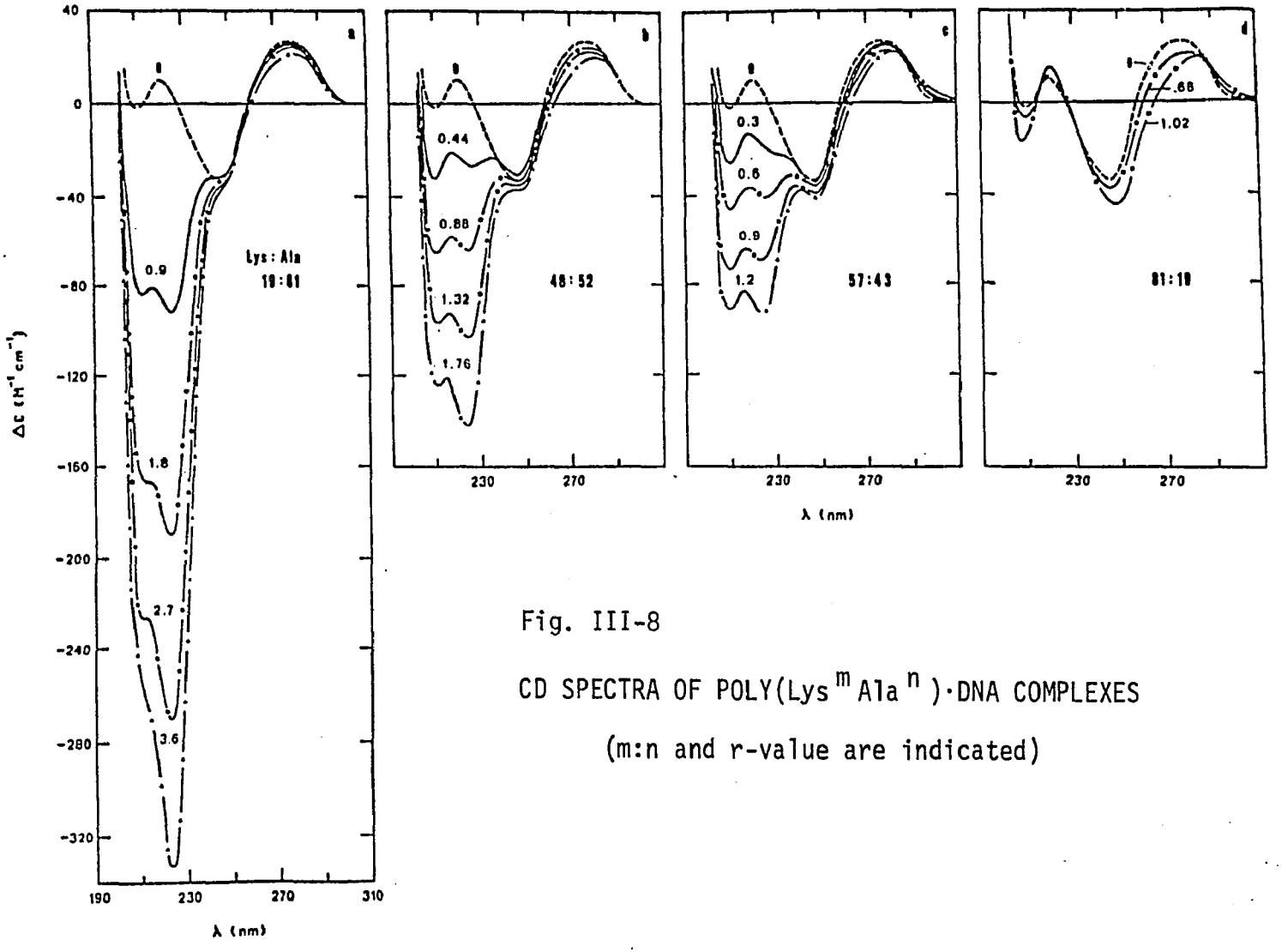


Fig. III-8

CD SPECTRA OF POLY(Lys^{m}Ala^{n})·DNA COMPLEXES

(m:n and r-value are indicated)

Above 250nm there is no CD contribution from copolypeptide. The CD of a complex in this region, therefore, is the sum of the CD from free and copolypeptide-bound base pairs. If F is the fraction of base pairs bound by copolypeptide in the complex, and $\Delta\varepsilon_m$, $\Delta\varepsilon_f{}^D$, and $\Delta\varepsilon_b{}^D$ the measured CD of the complex and the CD of free and bound base pairs, respectively, equation 3 can be used:

$$\Delta\varepsilon_m = (1 - F)\Delta\varepsilon_f{}^D + F\Delta\varepsilon_b{}^D \qquad (3)$$

This equation has been used before for polylysine-DNA[16], protamine-DNA[17], polyarginine-DNA[18], and poly(Lys^{58}Phe^{42})[19]. F for the complex can be calculated from the equation

$$F = r/\beta \qquad (4)$$

where β, as defined earlier, is the ratio of amino acid residues to nucleotide in copolypeptide-bound regions. With the aid of $\beta = 3.0$, as determined by thermal denaturation (Figure III-4) for poly(Lys^{40}-Ala^{60}), $\Delta\varepsilon_b{}^D$ of copolypeptide-bound base pairs from three complexes of different r values were calculated from equation 3. As shown in Figure III-9a, they do agree with one another. The characteristics of the CD are λ_{max} 279nm for the peak, λ_c 259nm for the crossover, and $\Delta\varepsilon_{279} = 1.8$. Also included in Figure III-9a is $\Delta\varepsilon_b{}^D$ of polylysine-bound base pairs in direct-mixed polylysine-DNA complexes. It may be seen that the conformational change in bound DNA induced by copolypeptide, as judged by CD, is similar to that induced by polylysine, but is significantly smaller in amplitude. The shift in λ_c from 256.5nm in $\Delta\varepsilon_f{}^D$ to 259nm in $\Delta\varepsilon_b{}^D$ in the case of copolypeptide is much smaller than its shift from 256.5nm in $\Delta\varepsilon_f{}^D$ to 272nm in $\Delta\varepsilon_b{}^D$ for polylysine. It should be noted that, in chromatin, histone binding on DNA also causes a great reduction in the amplitude of λ_{max} but only a small shift for λ_c.

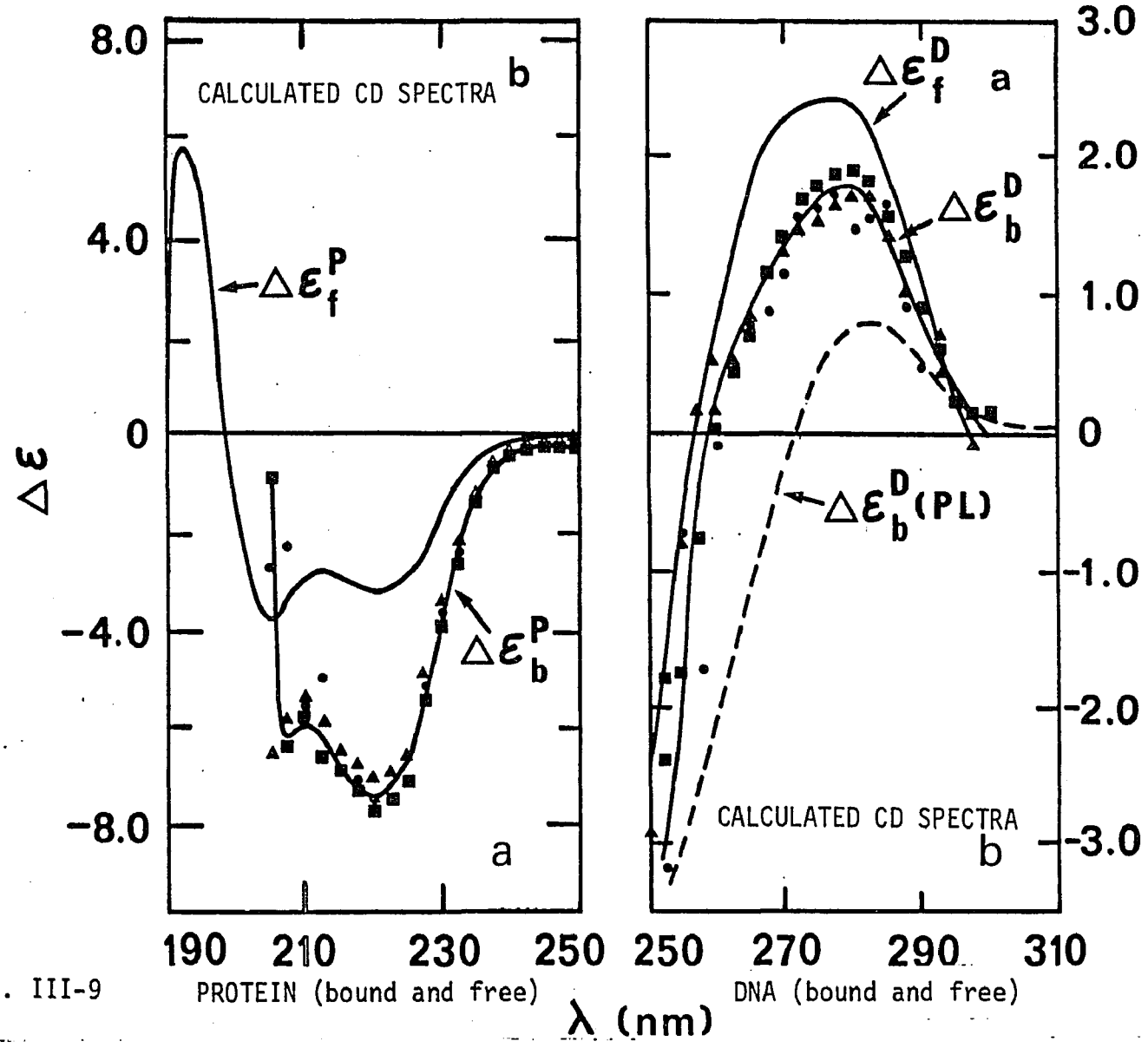

8.0
4.0
0
-4.0
-8.0
Δε
CALCULATED CD SPECTRA
b
$\Delta\varepsilon_f^P$
$\Delta\varepsilon_b^P$
a
190
210
230
250
PROTEIN (bound and free)
3.0
2.0
1.0
0
-1.0
-2.0
-3.0
$\Delta\varepsilon_f^D$
a
$\Delta\varepsilon_b^D$
$\Delta\varepsilon_b^D$(PL)
CALCULATED CD SPECTRA
b
250
270
290
310
DNA (bound and free)
λ (nm)

Fig. III-9

Perhaps this similarity in effect is due to the presence of α-helix in bound proteins, in poly($Lys^{40}Ala^{60}$) in the present system, and in histones in chromatin.

Figure III-10 shows the calculated CD in the same spectral region for three additional model protein complexes. In each case, the calculated DNA CD spectrum, $\Delta\varepsilon_b^D$, is independent of the r value of the complex. With poly($Lys^{19}Ala^{81}$), $\Delta\varepsilon_b^D$ is very close to that of free DNA, $\Delta\varepsilon_f^D$, having a reduction of about 10% in amplitude but no appreciable red shift in the spectrum. For poly($Lys^{57}Ala^{43}$), there is a substantial red shift in both peak and crossover, as well as a reduction of 20% in amplitude at 275nm. When the lysine content is increased to 100%, both the red shift and the reduction in amplitude are considerably greater.

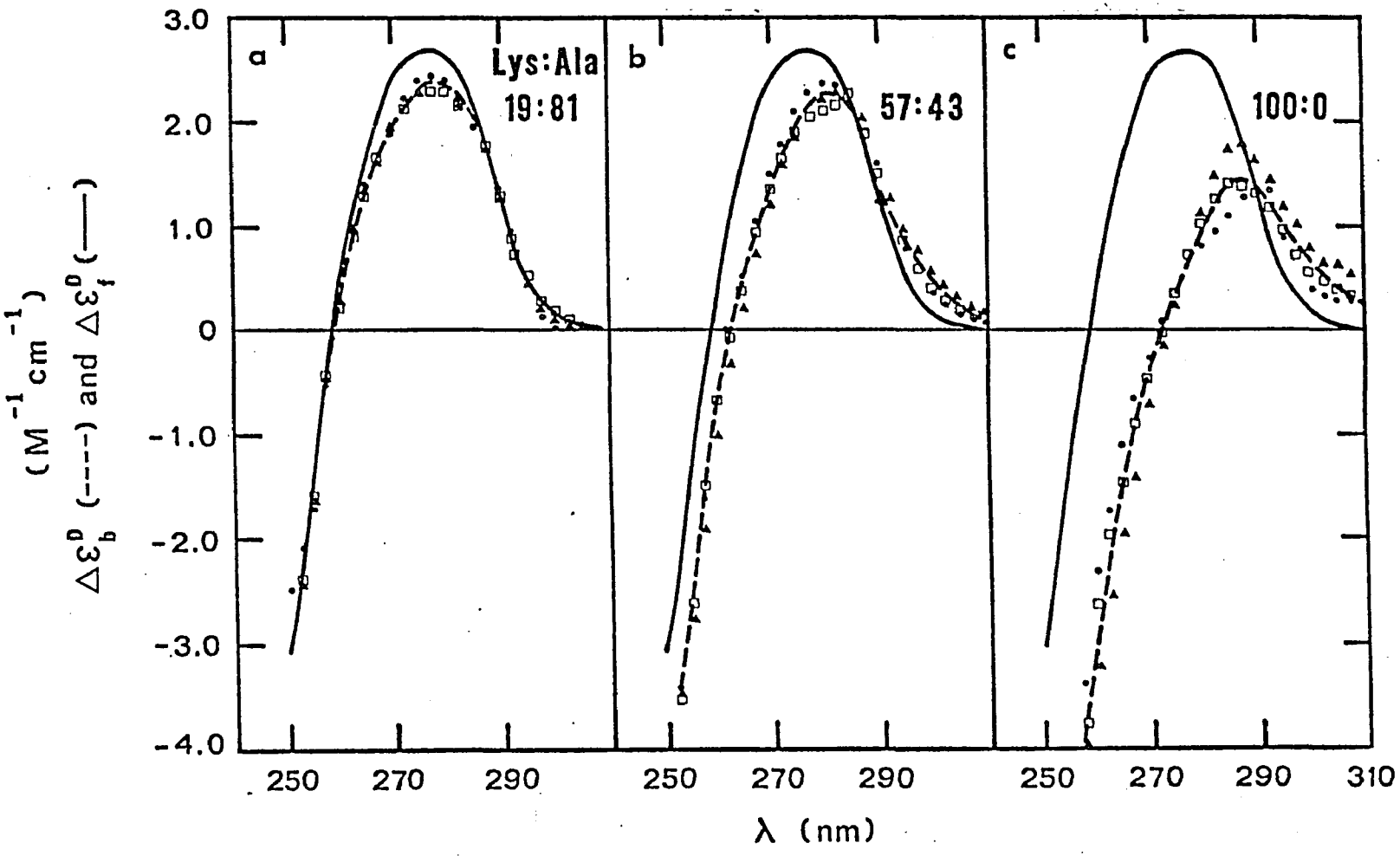
Below 250nm, both DNA and copolypeptide contribute to the measured CD. There is a possibility that the CD of DNA in this region might be affected by the binding of copolypeptide. However, this change is probably small, as is the case for DNA in the presence of salt[20,21,22], or for DNA in polylysine-DNA complexes[16]. Further, the CD of DNA in this region is relatively small compared to that of copolypeptide, as shown in Figure III-7. Therefore, as an approximation, it is assumed that the CD change in DNA below 250nm caused by the binding of copolypeptide is negligibly small compared to that of the copolypeptide alone. It is then possible to use the following equation to estimate the CD of bound copolypeptide in the complex:

$$\Delta\varepsilon_m = \Delta\varepsilon_f^D + r\Delta\varepsilon_b^P \qquad (5)$$

where $\Delta\varepsilon_m$ and $\Delta\varepsilon_f^D$ are as defined earlier in equation 3 and $\Delta\varepsilon_b^P$ is the CD of bound protein in the complex. The calculated $\Delta\varepsilon_b^P$ from three

Fig. III-10 CALCULATED CD SPECTRA OF DNA, FREE AND BOUND BY POLY($Lys^{m}Ala^{n}$)

(m:n is indicated)



complexes of different r value are shown in Figure III-9b to agree with one another. Also included in this figure is the CD spectrum of free copolypeptide, $\Delta\varepsilon_f^P$. This CD has double negative peaks at 222 and 205nm and a positive peak at 193nm, which indicates the existence of α-helix [23,24]. When copolypeptide is bound, the amplitude of its CD is nearly doubled, and the shape is changed to a major negative peak at 220nm with only a shoulder at 207nm. As the helical copolypeptide is bound to DNA, the enhancement in the amplitude can be interpreted as possibly resulting either from a distorted α-helix or from a greater proportion of α-helix when the copolypeptide is bound to DNA, or, more likely, from both. An increase in α-helical content is not impossible, because charge neutralization of lysine by phosphate could possibly stabilize more α-helix in bound copolypeptide.

Figure III-11 shows the measured CD spectra of free ($\Delta\varepsilon_f^P$) and the calculated spectra of bound ($\Delta\varepsilon_b^P$) model proteins. In Figure III-11a both spectra are shown for poly($Lys^{19}Ala^{81}$). With free copolypeptide, there are two negative peaks, at 222 and 208nm, and a positive peak at 191nm. Both shape and amplitude of these peaks suggest that, when free, this model protein is in completely α-helical conformation[23,24,25]. After complex formation, the CD of bound protein ($\Delta\varepsilon_b^P$) above 205nm is approximately identical to that of the free protein ($\Delta\varepsilon_b^P$); below this wavelength no reliable signal could be obtained under the experimental conditions employed.

With poly($Lys^{48}Ala^{52}$), the free model protein ($\Delta\varepsilon_f^P$) has one major negative peak at 202nm and a minor one at 222nm. Both shape and amplitude of peaks suggest that this model protein possesses a mixture of both α-helix and random coil. When complexed with DNA, the CD of bound

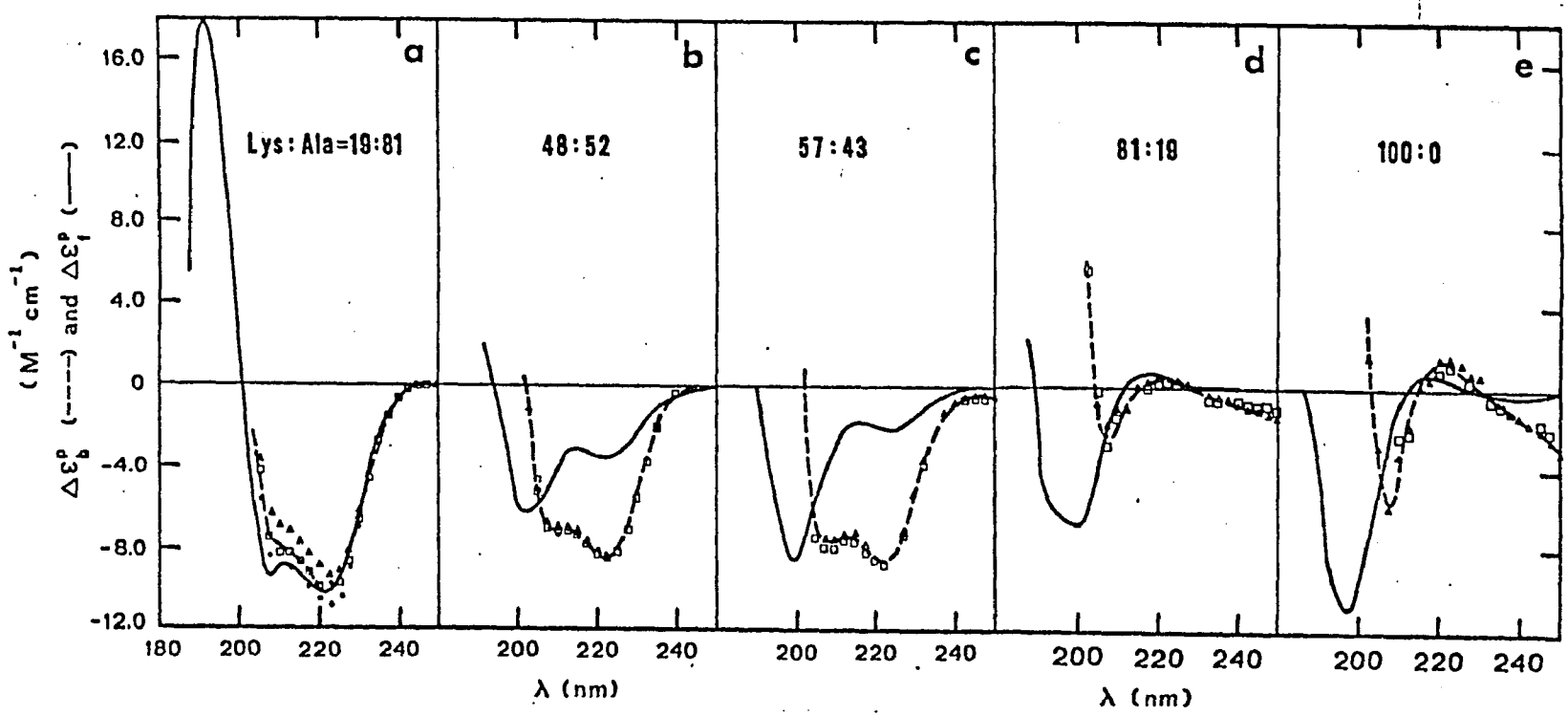


Fig. III-11 CALCULATED CD SPECTRA OF POLY(Lys^mAla^n), FREE AND BOUND

(m:n is indicated)

protein ($\Delta\varepsilon_b^P$) differs greatly from that of free protein in that a minor negative peak becomes a major peak at 222nm, with a minor one at 210nm. These CD changes induced by binding are similar to those with poly($Lys^{40}Ala^{60}$).

The results with poly($Lys^{57}Ala^{43}$), both free and complexed, are similar to those of poly ($Lys^{48}Ala^{52}$), except that, in the free state ($\Delta\varepsilon_f^P$), the former has more random coil and less α-helix than the latter (a smaller CD at 220nm and a bigger CD at 200nm). This is expected from its higher content of positively charged lysine residues.

It is interesting to compare the CD results in Figure III-11a, b, and c with Figure III-9a. Although the CD of free model protein ($\Delta\varepsilon_f^P$) is changed substantially when the lysine content in the protein is increased from 19 to 57%, the CD of bound proteins ($\Delta\varepsilon_b^P$) is not significantly changed. In other words, although the secondary structures of these model proteins in free state are very different, due to their difference in lysine or in alanine content, they do have similar secondary structure after being bound to DNA. Perhaps charge neutralization of lysine residues, effected by their binding to phosphates in DNA, stabilizes α-helical structures.

When the lysine content in the copolypeptide is increased to 81% Figure III-11d), or 100% (Figure III-11e), $\Delta\varepsilon_f^P$ shows a negative peak at 198-200nm and a small positive peak at 218nm, suggesting that these model proteins are mainly in random coil conformation[23,24,25]. When they are complexed with DNA, the calculated CD's can be greatly influenced both by the accuracy of the assumption basic to equation 4 namely, no change in the CD of DNA in this wavelength region after complexing with model protein, and by the lower ratio of signal to

noise. Nevertheless, the results clearly indicate that no α-helical structures have been induced in these complexes. In addition, the consistent upturn of the CD of these bound model proteins below 208nm, no matter which protein is used, indicates the great effect on the CD of random coil in the complex.

Figure III-12 shows the linear dependence on r value of the CD of poly($Lys^{40}Ala^{60}$)-DNA complexes both at 220nm, for bound copolypeptide, and at 278nm, for bound DNA. This linear relationship validates the assumptions on which the calculated CD spectra are based.

Figure III-13 summarizes the calculated CD results as a function of the alanine content in the copolypeptide: $\Delta\varepsilon_b^D$ at 278nm for DNA, and $\Delta\varepsilon_b^P$ and $\Delta\varepsilon_f^P$ at 222 and 210nm for protein. As the alanine content is increased, the protein-bound DNA $\Delta\varepsilon_b^D$ at 278nm gradually increases toward the B-type CD observed in free DNA. With free model protein, the main decrease in $\Delta\varepsilon_f^P$ at 222nm occurs when the alanine content is greater than 40%, whereas such transition occurs at lower alanine content when the protein is bound.

Figure III-14 shows the difference CD between the free and bound state of DNA or protein. When the model protein contains more alanine, the difference in DNA CD becomes smaller, as seen before in Figure III-10. The difference in protein CD, measured either at 222nm or at 210nm, shows a maximum at 40-50% alanine. In other words, the greatest CD change induced in protein by binding to DNA occurs for those model proteins containing 40-50% alanine.

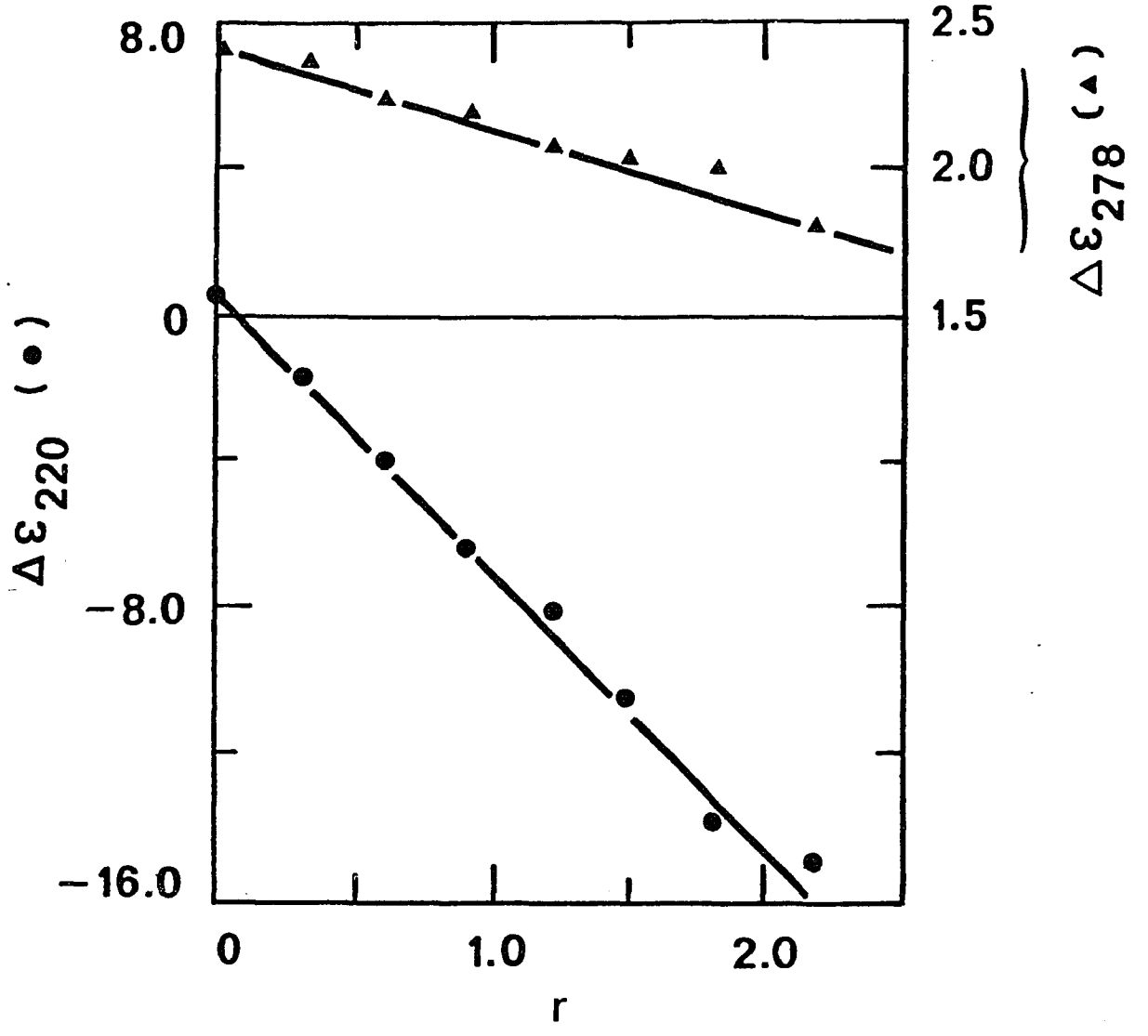


Fig. III-12 LINEAR DEPENDENCE OF CD ON r-VALUE OF POLY($Lys^{40}Ala^{60}$)·DNA COMPLEX

FIG. III-13

DEPENDENCE OF CALCULATED SPECTRA ON ALANINE CONTENT

ON MODEL PROTEIN

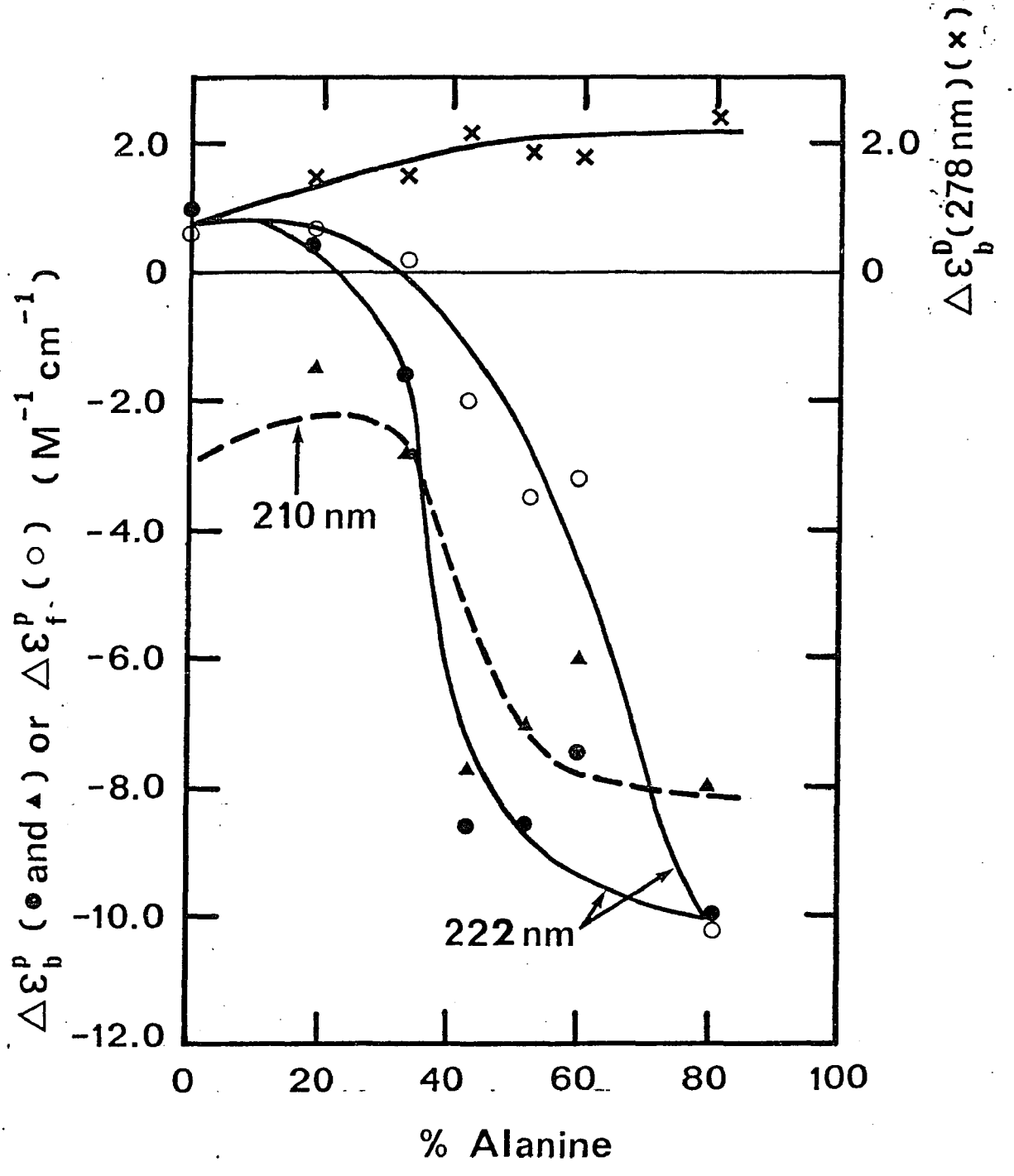
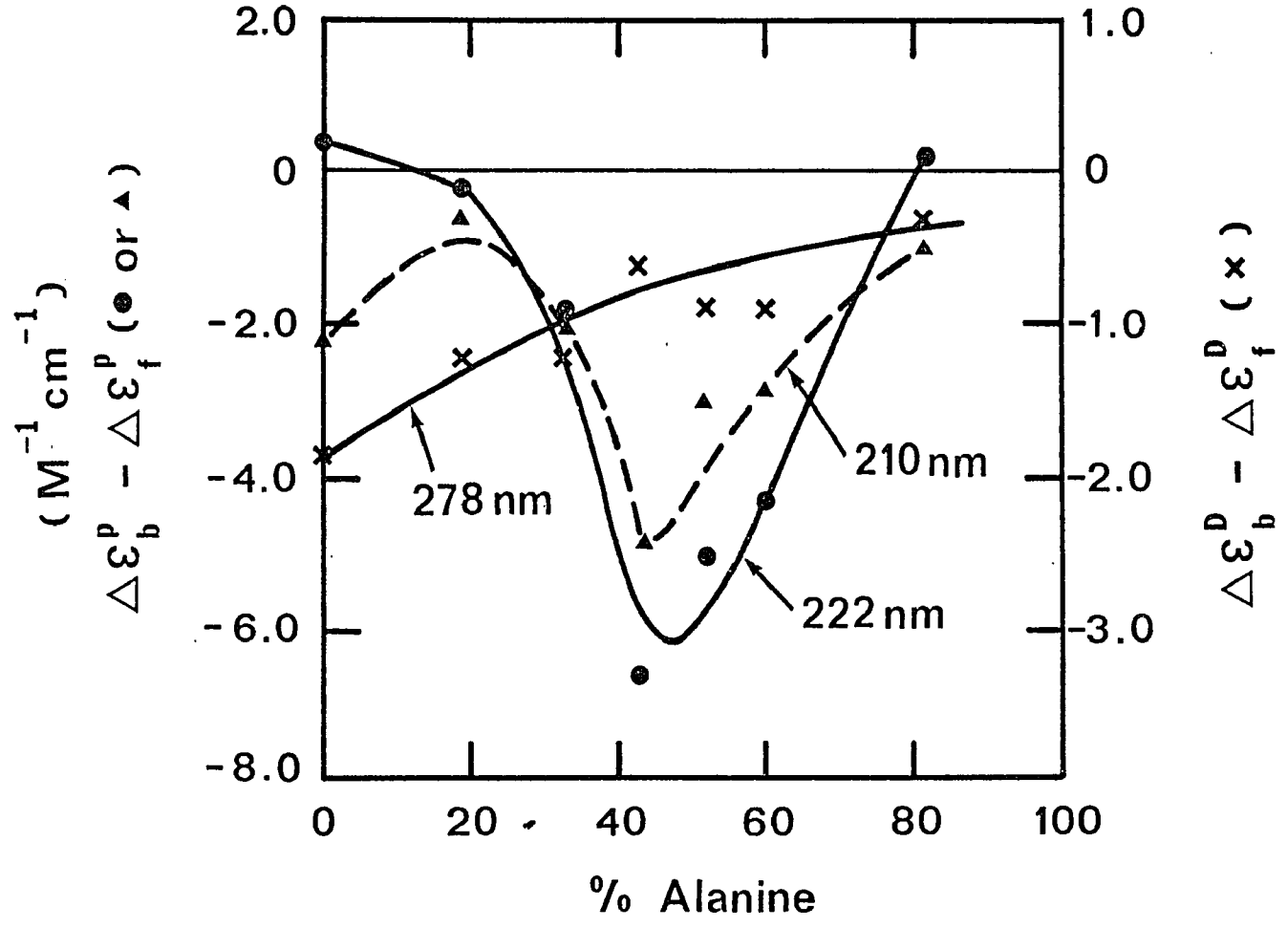


Fig. III-14 DEPENDENCE OF DIFFERENCE CD BETWEEN BOUND AND FREE STATE FOR DNA(278nm) AND FOR PROTEIN (222nm and 210nm)



Discussion

Studies of protein-DNA interaction are much more complicated than those of pure DNA or proteins alone, because the individual properties of both DNA and proteins have to be considered simultaneously. Three major aspects of interaction are commonly considered when a protein is bound to DNA: selectivity of a DNA sequence for binding[26,27,28,29], thermal stabilization of bound base pairs[30,31,32,33,34,35,36,37], and structural effects, both on protein[21,38] and on DNA[39,40,41,42,38,16]. The last two aspects are dealt with extensively in the work on model proteins.

In nucleohistones, the melting temperatures of histone-bound regions are generally lower than those found in basic homopolymers such as polylysine and polyarginine. The most obvious explanations for the lowering of these melting temperatures are either (a) that the presence of non-basic amino acid residues destabilizes DNA base pairs or (b) that phosphates on the DNA are not fully neutralized by direct interaction with cations on the proteins.

One of the major concerns in the study of model protein-DNA complexes is to determine whether or not any particular non-basic amino acid residues have the potential to destabilize the DNA helix. One approach has been to examine the melting temperature of protein-bound DNA (Tm') using as a reference the Tm' of polylysine-bound DNA. It was observed that neither aromatic tyrosine residues in poly(Lys^{50}-Tyr^{50})[43] nor alanine residues in poly($Lys^{40}Ala^{60}$)[44] tend to destabilize the DNA helix. On the other hand, the presence of aromatic phenylalanine in poly($Lys^{58}Phe^{42}$) does destabilize the DNA helix in the course of thermal denaturation[19]. A gradual decrease in Tm' as the alanine

content in the model protein is increased (Figures II-3 and III-6) could be either an indication of destabilization of the DNA helix by hydrophobic alanine residues, or a lack of full charge neutralization on the phosphate lattice resulting from the presence of rigid α-helical structures in the model proteins having a higher alanine content. The gradual shift of Tm' to higher temperatures in the presence of a small amount of NaCl (shown later in Figure IV-1) favors the latter possibility. In other words, the hydrophobicity of alanine residues does not in itself destabilize the DNA helix to a significant degree. Instead, alanine in the free copolypeptide simply causes it to form a rigid α-helix, which, when bound to DNA, prohibits full ionic interaction between lysine and phosphate, and this leads to the lower Tm'. The fact that melting temperatures for histone-bound base pairs in nucleohistones are lower than those of polylysine-DNA, despite roughly equal proportions of basic amino acid residues and phosphates in histone-bound regions of chromatin[37,14], may be explained in a similar way.

Although it has been reported previously that the CD of histone IV (f2a1) is distorted when it is complexed with DNA in reconstituted nucleohistone[21,38], with reconstitution, the cause of the change is more complicated than with direct mixing, because during salt gradient dialysis it is not known under what specific condition the histone is complexed to DNA. In addition, in the free state, histone IV has not only α-helix, but β sheet and random coil as well[45]. The simpler copolypeptide-DNA system under study has a great advantage in that copolymers of higher alanine content have an α-helical structure in the free state, and, by using the method of direct mixing, the condition of

interaction can be controlled.

The enhanced but distorted CD spectrum of the bound copolymer shown in Figure III-9b could be explained in two ways: perhaps there is a strong interaction between the amide of the copolypeptide and the DNA; or there may be a distortion of the regular α-helix of the copolymer when it is bound. Because of these dual possibilities, it is necessary to be cautious in analyzing the protein CD spectrum of a protein-DNA complex. Simply using as reference the spectra of free proteins in α-helix, β-sheet, or random coil conformations, without considering the possible distortion of its secondary structure by binding, could be misleading.

The effect on CD resulting from complex formation can be divided into two parts, namely, the effect on DNA CD and the effect on protein CD.

As far as the DNA CD is concerned, at low ionic strength the directly mixed protein-DNA complexes always show some degree of red shift and a reduction of the positive CD band near 275nm, a phenomenon characterized as a distorted B or a B $\longrightarrow$ C transition[48,20]. The extent of this transition depends upon the model protein used. The B $\longrightarrow$ C transition in DNA has been considered to be a result of dehydration, because this transition as measured by CD, can be generated by lowering humidity[20,21,22,46], or by the binding of such model proteins as polylysine[16] and other polypeptides, copolypeptides, and histones. Dehydration of DNA can occur either on the phosphate lattice or in the major or minor groove, if the water molecules, bound or in the vicinity of DNA, are replaced by salts or by protein molecules. If dehydration is the main cause for this B $\longrightarrow$ C transition observed in CD, the results

(Figures III-10 and III-13) suggest that less dehydration of DNA has been effected by the binding of model proteins having higher alanine content than apparently occurs as a result of binding by polylysine. This seems surprising, since the model proteins with more alanine possess more α-helical structure (Figure III-11) and, therefore, are expected to be more hydrophobic, However, it is possible that the rigid α-helical structure of these model proteins cannot fit so tightly into the grooves of DNA as does polylysine, with the result that water molecules are not replaced sufficiently by α-helical proteins to effect dehydration. It is also possible that, due to this same α-helical rigidity, the phosphate residues in protein-bound regions are not fully neutralized and therefore can attract more water molecules in the vicinity of DNA. If either suggestion is correct, the results reported here seem quite compatible with the concept of a relationship between dehydration of DNA and a B $\longrightarrow$ C transition in the CD spectrum.

Above 210nm, the major CD characteristics of the α-helix do not seem to change appreciably when a model protein in this conformation is complexed with DNA, although some minor distortion does occur (Figure III-11a-c). The change in amplitude of major and minor peaks in these spectra as the protein moves from a free to a complexed state can be regarded as due to an increase in α-helical content after complex formation. Below 210nm, as a free model protein in random coil is complexed with DNA, its characteristic CD is substantially changed (Figure III-11 d-e), which is not altogether unexpected because of the obvious loss of flexibility of the polypeptide chain after it is tightly bound to DNA, whether it was initially in a free or in a coiled state. The main effect of binding occurs below 210nm, in the range of

π,π^* transition of the amide groups[47,49,50,51,52,23]. Apparently the π,π^* transition is much more sensitive to protein binding on DNA than is the n,π^* transition around 220nm, as was observed previously in a report on histone H4-DNA complexes based upon results of both vacuum uv absorption and CD[21].

In summary, these results indicate that:

1. The binding of poly(Lys^mAla^n) can induce a structural transition in DNA from B to C conformation; this effect diminishes as the alanine content of the model protein increases.

2. Model proteins which contain 40-50% alanine show the greatest change in the protein CD upon binding to DNA (Figure III-14). In the free state these proteins contain a mixture of α-helix and random coil (Figure II-11); binding forces them to form more α-helix at the expense of unordered coil. When α-helix is present, there appears to be a certain optimum amount which permits maximum interaction of lysine residues with phosphates of the DNA; this is demonstrated by the similarity between bound spectra of all model proteins in the 40-60% alanine range (Figures III-9b and III-11), despite marked differences in the CD of their unbound states.

3. The presence of α-helix in itself reduces the efficiency of direct charge neutralization of DNA phosphates by lysine, yielding a Tm' which is always lower than the corresponding melting temperature of polylysine-DNA complexes.

Chapter IV

EFFECTS OF IONIC STRENGTH ON POLY($LYS^{m}ALA^{n}$)-DNA COMPLEXES

With changes in ionic strength there are attendant changes in all parameters defined under low salt conditions by both thermal denaturation and circular dichroism. The low salt conditions employed for studies described in the preceding chapter are not physiological, and therefore not realistic. However, by using them as a reference against which to observe change with increasing ionic strength, it is possible to separate the effects of binding by copolymer from those of binding by ions and approach a better understanding of the contribution of each type of binding to the functional aspects of the biological system.

The following results stem from experiments utilizing the same copolypeptides and the same methods of study used for the low salt studies. Evaluations should then prove relevant and valid as a base for future experiments.

Thermal Denaturation

The derivative melting profiles of several poly($Lys^{m}Ala^{n}$)-DNA complexes at various ionic strengths (0.00, 0.01, 0.03M NaCl) are shown in Figure IV-1 (a,b,c,d). In all cases, a higher ionic strength in the medium raises the melting band of free DNA (Tm) to a much greater extent than it does that of bound DNA (Tm'), which is affected only slightly. The fact that it is affected at all contrasts with results

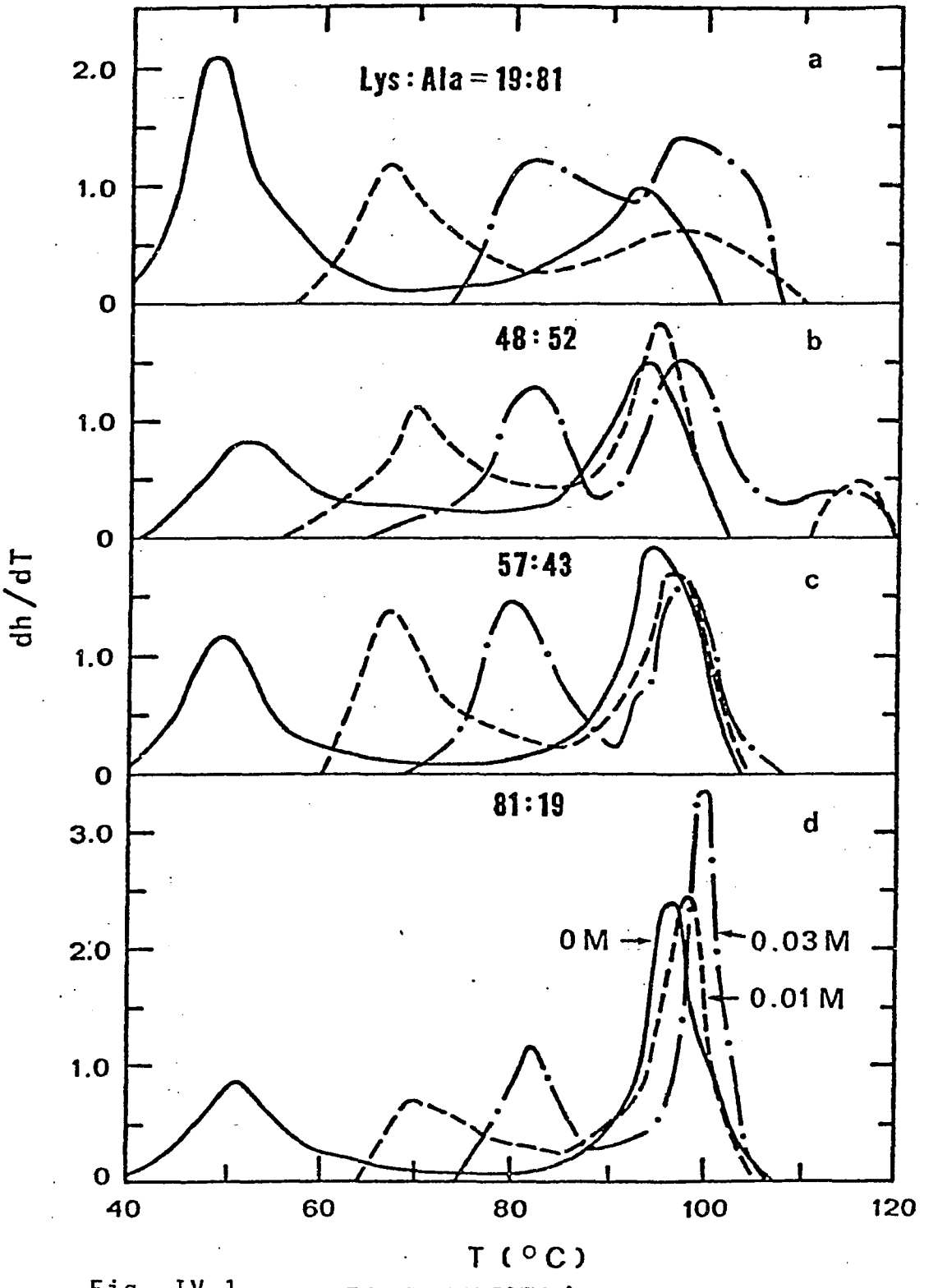


Fig. IV-1
DERIVATIVE MELTING PROFILES OF POLY($Lys^m Ala^n$)·DNA COMPLEXES AT VARIOUS IONIC STRENGTHS

(m:n and NaCl concentration are indicated)

obtained with direct mixed polylysine-DNA[53], protamine-DNA[17], and polyarginine-DNA[54] complexes, where Tm' is independent of ionic strength. The small shift in Tm' can be interpreted as due to a lack of full charge neutralization of phosphates in protein-bound regions by lysine residues in the proteins. The complex with poly($Lys^{48}Ala^{52}$) produces an additional melting band at 110°C in higher ionic strength, which was also observed with poly($Lys^{40}Ala^{60}$) complexes (not shown). This 110°C band was not observed for complexes with alanine content either higher or lower than 40-60%. It should be noted that model proteins containing alanine in the 40-60% range also exhibit the greatest CD changes when bound (see Figures III-9a, III-11, III-13, III-14). At 110°C, light scattering is probably increased, and this, in turn, contributes to the hyperchromicity at 260nm. Therefore, the analysis of ionic strength dependence of Tm and Tm' is limited to NaCl $\leq$ 0.06M.

Equations 1 and 2 have been used previously for describing the effect of ionic strength on Tm and Tm'[17]:

$$Tm = Tm^{o} + a \log Na^{+} \quad (1)$$

$$Tm' = (Tm')^{o} + a' \log Na^{+} \quad (2)$$

These equations are essentially the same as those used with pure DNA[55,56]. Though the theory behind equation 2 for protein-bound base pairs has not yet been developed, the concept of electrostatic shielding by NaCl and its effect on the melting temperature of pure DNA[55,56,57,58] are used here to gain insight into the electrostatic shielding of DNA by bound proteins in a nucleoprotein[35,53]. With poly($Lys^{40}Ala^{60}$), the plots of both equations (Figure IV-2) yield $Tm^{o} = 102^{o}$, $(Tm')^{o} = 100^{o}$, $a = 17^{o}$, and $a' = 2.7^{o}$. These results show $a' << a$, which implies that copolypeptide-bound base pairs are much

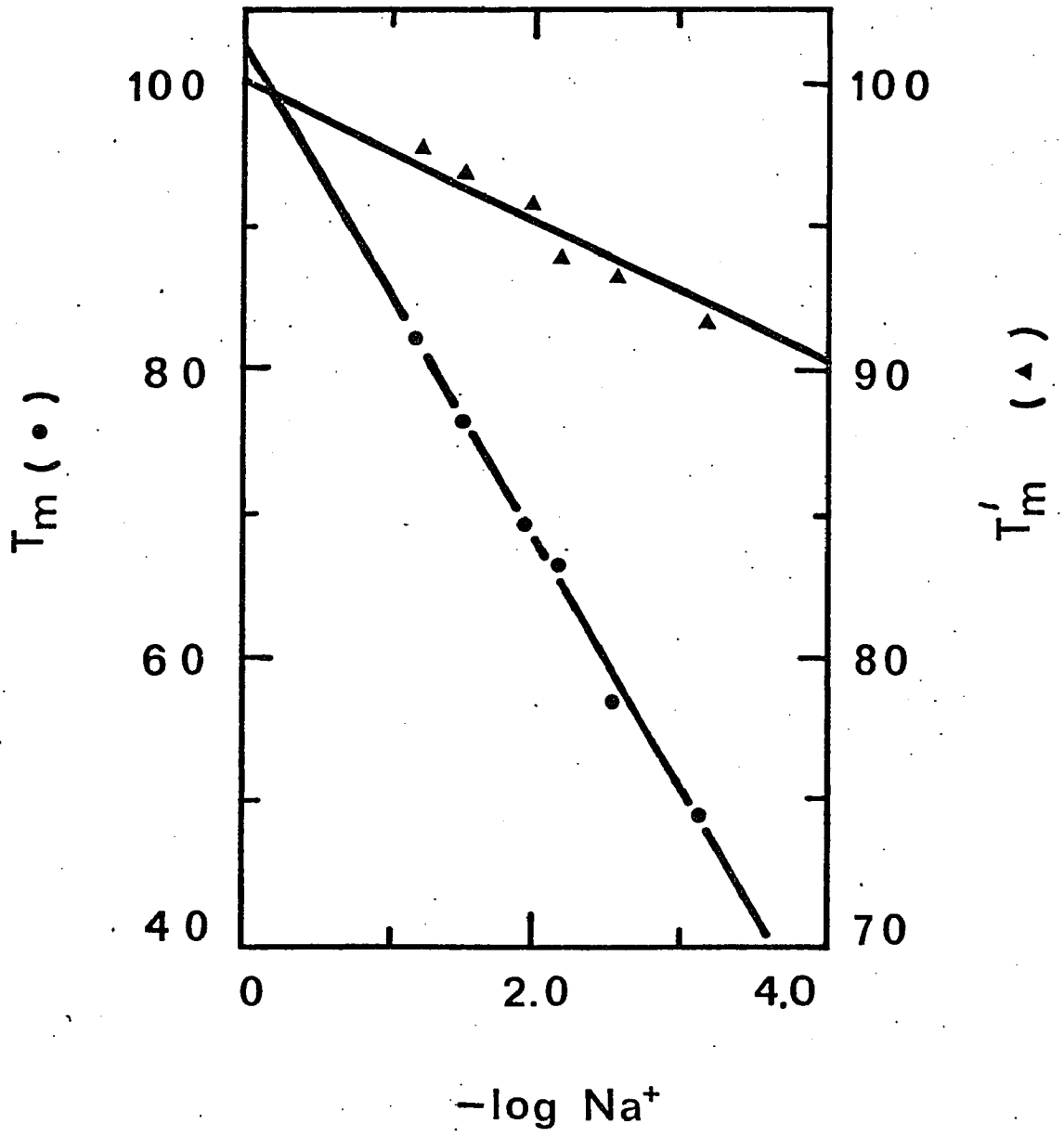


Fig. IV-2

LINEAR PLOTS OF EQUATIONS (1) AND (2)

more electrostatically shielded than are free base pairs, but this shielding is not as great as in protamine-DNA[17] or polylysine-DNA complexes[53] where a' is zero. In addition, $(Tm')^o$ is approximately equal to Tm^o, which may imply further that the presence of alanine in the protein does not adversely affect the thermal stability of DNA. It should be noted that the tyrosine residues in poly($Lys^{50}Tyr^{50}$) also do not stabilize DNA in thermal denaturation[43]. In EDTA buffer, the Tm of the latter complex is slightly lower than the $(Tm')^o$, and also lower than the Tm' of polylysine-DNA, which again may be due to a lack of full charge neutralization on the phosphate lattice caused by the α-helical structure of the protein.

Circular Dichroism

The effect of ionic strength on the CD spectra of poly(Lys^mAla^n)-DNA complexes is still more striking. While the binding of poly ($Lys^{40}Ala^{60}$) to DNA in EDTA buffer without salt causes a slight red shift for the positive CD band of DNA near 275nm, the transfer of this complex to another environment of higher ionic strength causes an opposite and much more pronounced CD effect (Figure IV-3). In addition to marked enhancement of the positive band and a blue shift of its peak from 278 tc 260nm, a new negative band at 295nm appears. The positive peak at 260nm is very similar to that of DNA dissolved in a solvent mixture of water-ethanol or water-dioxane at low water content[22], as well as to the theoretically calculated CD spectrum of A-form DNA[48,59]; it is also very similar to that of the A-form double-stranded RNA[60,61]. The negative peak of the copolypeptide-DNA complex at 295nm, however, is much more pronounced than in other systems with A-type CD spectra.

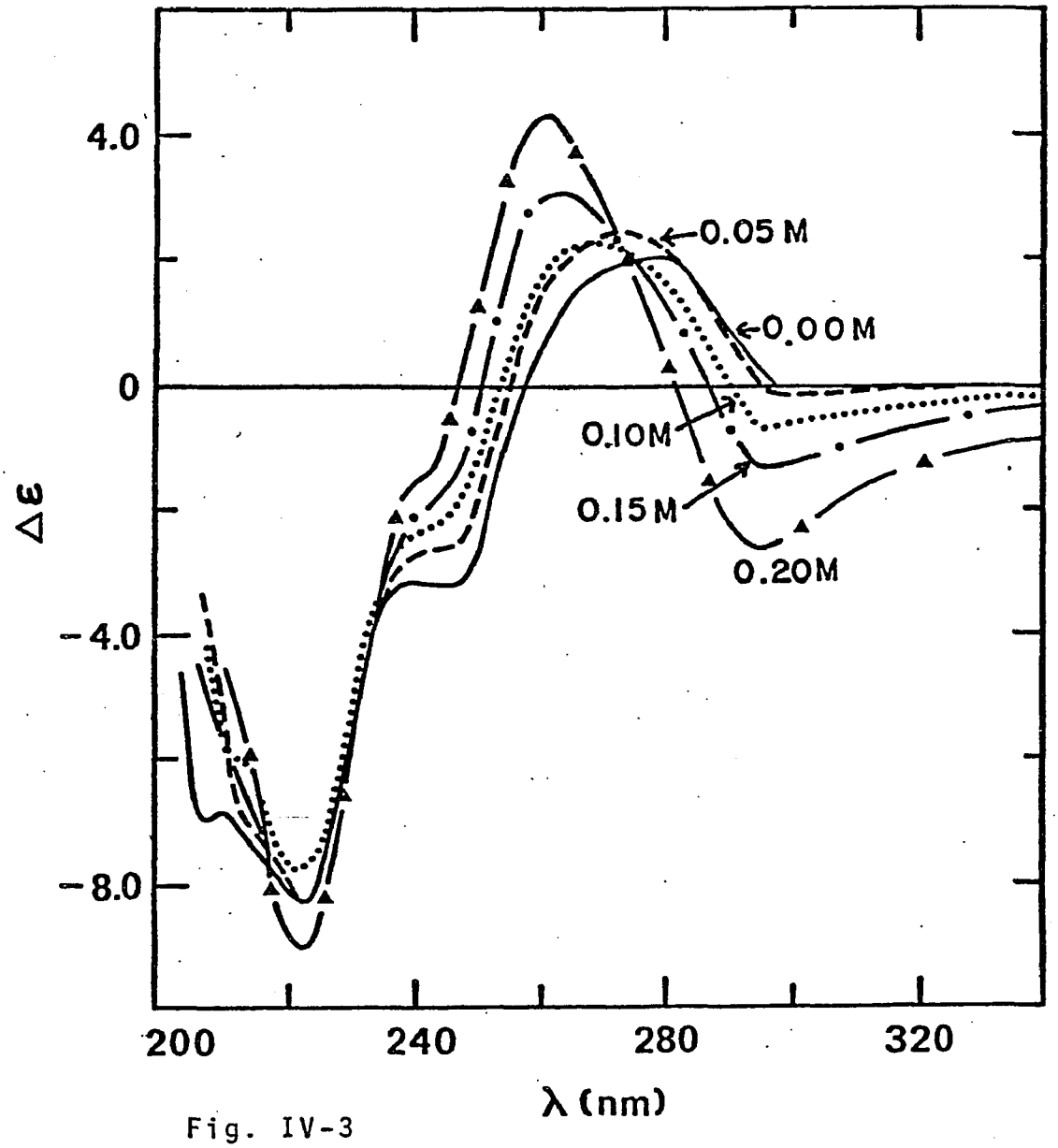


Fig. IV-3
EFFECTS OF CHANGES IN IONIC STRENGTH ON CD SPECTRA OF POLY(Lys$_{40}$Ala$_{60}$)·DNA COMPLEX, r-value = 1.2
(NaCl concentration indicated)

For the poly($Lys^{19}Ala^{81}$)-DNA complex (Figure IV-4a), raising the concentrations of NaCl to 0.2M results in a slight decrease in the positive CD band near 275nm. A further increase of NaCl in the medium results in a slight blue shift and enhancement of the positive CD band near 275 nm, together with the appearance of a new negative band at 295nm. The usual negative CD band at 222nm is slightly reduced and the shoulder at 210nm disappears altogether. Above 250nm, the effect of ionic strength on the DNA CD is qualitatively similar to that of poly($Lys^{40}Ala^{60}$), producing a transition toward A conformation, but quantitatively the CD effect is very much smaller in poly($Lys^{19}Ala^{81}$) than in poly($Lys^{40}Ala^{60}$).

Figure IV-4b shows the results from poly($Lys^{48}Ala^{52}$)-DNA complexes. Here the induced changes in DNA CD above 250nm are much greater than with poly($Lys^{19}Ala^{81}$), with a more pronounced CD pattern of positive peak at 260nm and negative peak at 295nm resembling that of poly ($Lys^{40}Ala^{60}$) in salt, A-form DNA in ethanol[62,22,63] and in DNA film[64], calculated DNA CD in A conformation[48], or A-form double-stranded RNA[60,61].

With the poly($Lys^{81}Ala^{19}$)-DNA complex (Figure IV-4c), where the alanine content is small, the salt-induced CD changes are completely different. The positive DNA CD band above 250nm first red shifts with a reduction in amplitude; with increasing NaCl this reduction progressively develops into a negative CD band near 270nm. In addition, the original 245nm negative peak is lost and a deeper one develops near 230nm. Increase in salt concentration also induces a gradual red shift in the positive CD of the polylysine-DNA complex and development of two big negative CD bands, one near 260nm and another near 215nm

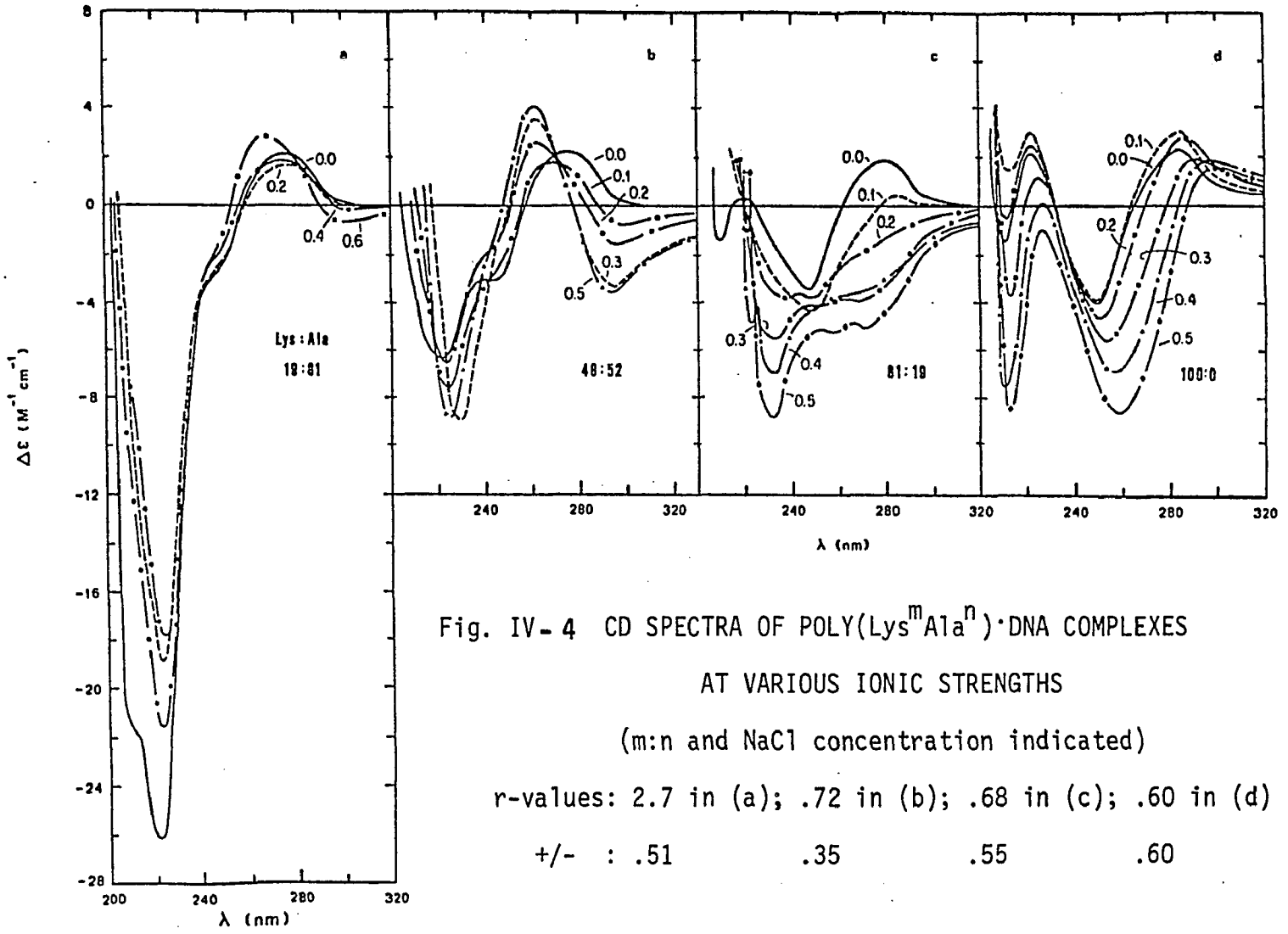


Fig. IV-4 CD SPECTRA OF POLY($Lys^{m}Ala^{n}$)·DNA COMPLEXES AT VARIOUS IONIC STRENGTHS

(m:n and NaCl concentration indicated)

r-values: 2.7 in (a); .72 in (b); .68 in (c); .60 in (d)

+/- : .51 .35 .55 .60

(Figure IV-4d). The salt induced CD spectra in the latter two cases resemble those involved in the B $\longrightarrow$ ψ transition reported before for polylysine-DNA in 1.0M NaCl[65], for reconstituted polylysine-DNA complexes[66,67], reconstituted histone H1-DNA complexes in 0.14M NaF or 0.15M NaCl[39,68], for DNA in poly(ethylene oxide) plus NaCl[69], and for lithium films of DNA and poly(dAT) at 92% or lower relative humidity[70].

The transition to A structure depends not only upon ionic strength but also upon the coverage of DNA by copolypeptide, as shown in Figure IV-5 for poly($Lys^{40}Ala^{60}$) complexes in 0.2MNaCl. The A-type CD spectrum above 250nm becomes more pronounced as more copolypeptide is bound to DNA.

Figure IV-6 shows the CD spectra of poly($Lys^{m}Ala^{n}$)-DNA complexes as a function of r value, when complexes were made in EDTA buffer and then dialyzed to 0.2M NaCl. The positive CD near 275nm of poly ($Lys^{19}Ala^{81}$)-DNA complexes (Figure IV-6a) is not noticeably changed when r is first increased from 0 to 0.9; with further increase it then is reduced significantly in amplitude but only slightly red shifted. This CD change is similar to that of the B $\longrightarrow$ C transition observed for DNA in film[20], in salts[20,21,22,71], and in ethylene glycol[72]; it is also similar to that of directly mixed polylysine-DNA[16], protamine-DNA[17], and polyarginine-DNA complexes in EDTA[18]. The protein CD near 220nm is proportional to its r value, except at r = 3.6 where the complex is close to a full charge neutralization and resultant precipitation (Table III-1). The results in Figures IV-4a and IV-6a show that the slight CD change in the poly($Lys^{19}Ala^{81}$)-DNA complex whether toward A- or C-type spectrum, depends upon both the ionic strength and the r value of its complex.

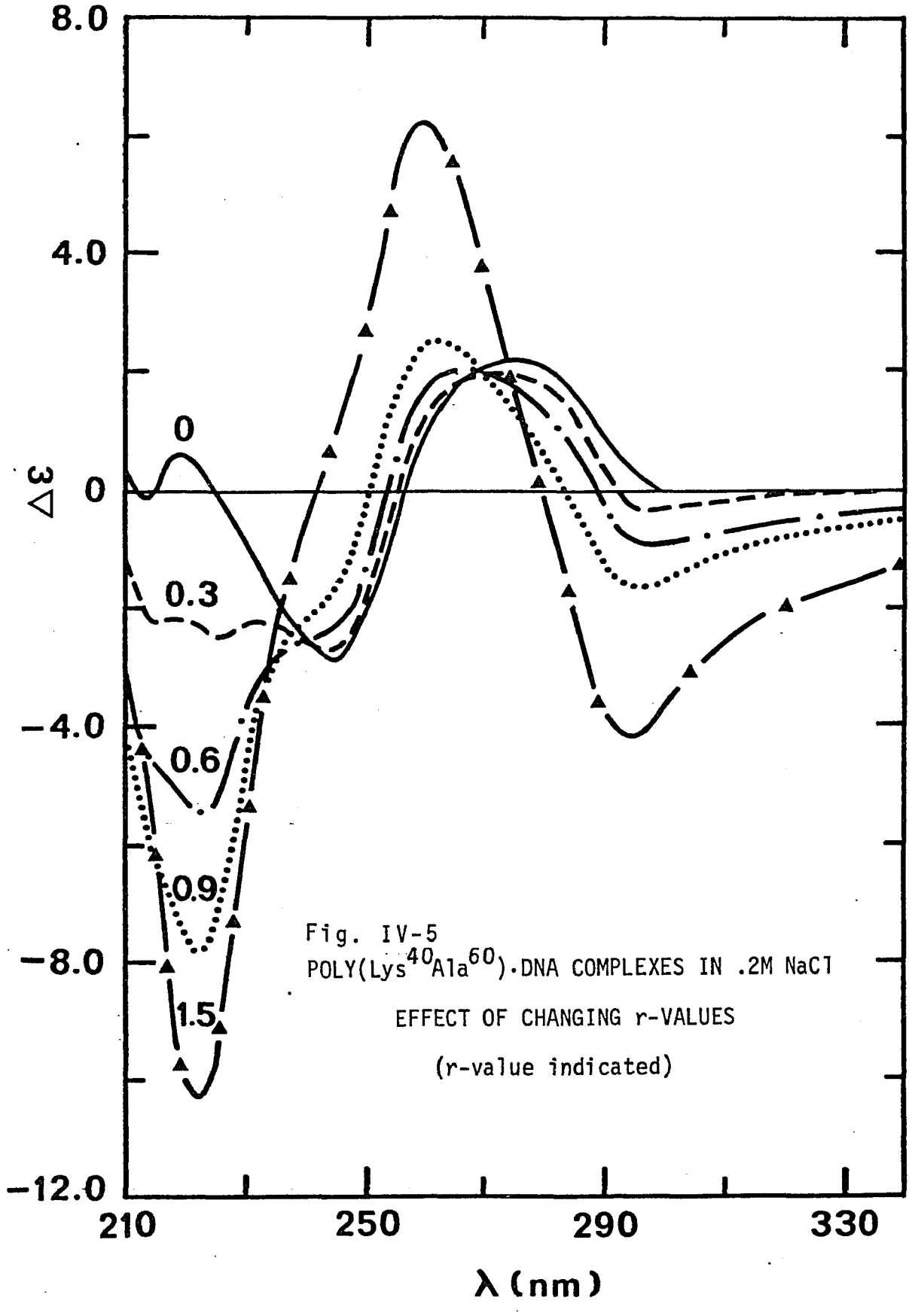


Fig. IV-5
POLY($Lys^{40}Ala^{60}$)·DNA COMPLEXES IN .2M NaCl
EFFECT OF CHANGING r-VALUES
(r-value indicated)

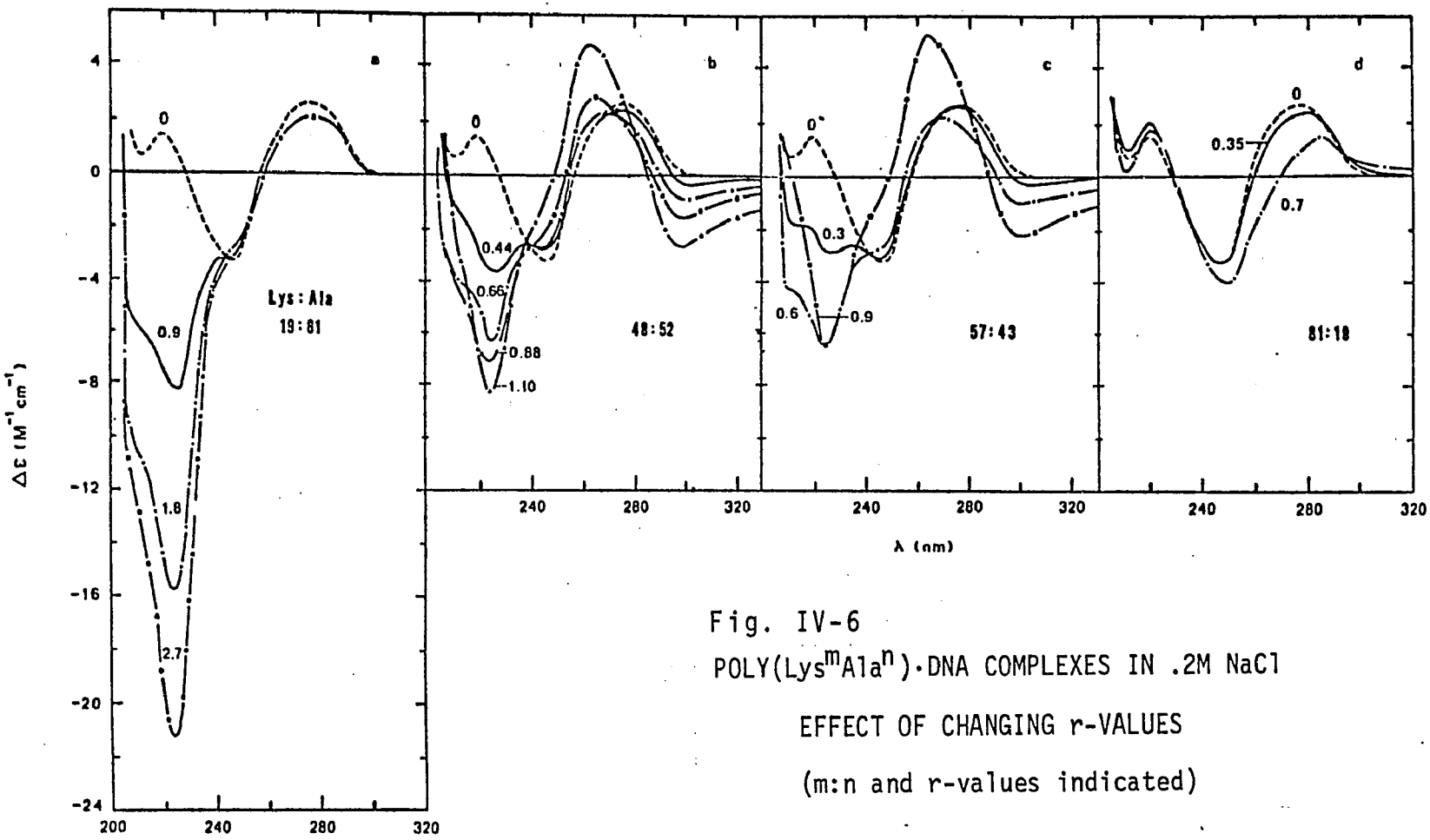


Fig. IV-6

POLY($Lys^{m}Ala^{n}$)·DNA COMPLEXES IN .2M NaCl

EFFECT OF CHANGING r-VALUES

(m:n and r-values indicated)

CD spectra resembling the distorted B $\longrightarrow$ A transition are observed in all the complexes in the 40-60% alanine range: in poly ($Lys^{40}Ala^{60}$) (Figure IV-5), in poly($Lys^{48}Ala^{52}$) and poly($Lys^{57}Ala^{43}$) (Figure IV-6 b-c). If the CD changes at 260nm or 295nm are plotted against r^2, rather than r, a linear dependence can be demonstrated. Such dependence on r of an order higher than one implies either an intermolecular interaction among complex molecules or a cooperative intramolecular structural distortion within the complex molecule. Dependence of the CD change in DNA on complex concentration in 0.2M NaCl was tested on poly($Lys^{40}Ala^{60}$) by measuring the CD spectra of a complex at various dilutions up to tenfold for periods of time extending up to 20 hours. In no case did dilution change the shape of the CD spectrum, and the amplitude at 260 or 295nm was simply proportional to the concentration. This experiment may exclude the possibility that the transition to an A-type CD in the presence of salt is due to a reversible intermolecular interaction, although an irreversible interaction among the complex molecules as a cause of this transition still cannot be ruled out. An alternative suggestion for the dependence of CD change above 250nm upon r^2 rather than r is a cooperative structural distortion of DNA molecules bound by this copolymer. Such a possibility is not unreasonable, since, with respect to base rotation, tilting, and stacking, the change from B to A form DNA is of much greater magnitude than is the transition to C structure[73,59]. The B $\longrightarrow$ A transition may be less favorable energetically than a transition from B to C form, so that maintenance of a copolypeptide-bound region in A conformation with the neighboring free regions in B structure would be unlikely. However, the presence of another copolypeptide in the

neighborhood of this bound region might provide additional energy to permit cooperative action within the DNA molecule and cause a conformational change toward A structure.

With very little alanine content, as in the case of poly(Lys^{81}-Ala^{19})-DNA complexes in 0.2M NaCl, the positive CD spectrum near 275nm is red shifted and reduced as the r value increases, a phenomenon similar to the B $\longrightarrow$ C transition. If the CD spectra in Figure IV-6d and in Figure IV-4c are compared, it becomes apparent that the B $\longrightarrow$ C transition in Figure IV-6d may well be an intermediate of the B $\longrightarrow$ ψ transition, a suggestion made earlier, following the investigation of difference CD spectra for B $\longrightarrow$ C and B $\longrightarrow$ ψ transitions[67]. Figure IV-6d further shows that the induced CD change in this type of transition depends upon r of an order higher than one, again indicating either cooperative structural alteration of DNA by protein binding or an intermolecular interaction among the complex molecules[44].

The long tails in the CD above 320nm in Figures IV-4 and IV-6 could be attributable to light scattering in the complex solution at intermediate ionic strength, as there is a significant increase in the ratio of absorbance at 320nm to that at 260nm. However, although such a contribution might affect the amplitude of the CD at lower wavelengths, it is not expected that the characteristic shape of the CD spectra in Figures IV-4 and IV-6 would be changed appreciably. Therefore, interpretations of these spectra are not invalidated by the presence or absence of light scattering.

Discussion

Theoretically, the optical properties of DNA have been studied

extensively by Tinoco and his collaborators [74,48]; experimentally they have been explored by many laboratories. In general, the three different types of CD correlate with the three conformations of DNA described as A, B, and C forms[73,59]. Depending upon the proteins and the manner of making the complexes, when a protein is bound to B form DNA, the conformation of the DNA, as judged by CD, may be unchanged[75,76,21,38], distorted toward C form[16,17,18,77], distorted toward A form[38,78], or toward another structure exhibiting a huge negative CD near 270nm[65,79,39,68,66,69].

Transitions from B conformation, B $\longrightarrow$ C and B $\longrightarrow$ A belong to different categories with respect to base rotation, tilting, and stacking[59]. Earlier reports on the CD of nucleoproteins dealt almost exclusively with transitions from B conformation: B toward C, B toward A, or B toward other structures. The evidence in Figure IV-3 demonstrates a transition of B toward C followed by a further transition toward A. The B $\longrightarrow$ C transition occurs when DNA is complexed with the copolypeptide in EDTA buffer; the further transition toward A is then induced as the initially distorted complex is transferred from EDTA buffer containing NaCl.

Since the CD of the bound copolypeptide is not significantly changed in salt, its secondary structure presumably is not changed either. However, charge distribution along the complex molecule changes markedly from free to copolypeptide-bound DNA regions. The presence of significant concentrations of Na^+ and Cl^- ions along the complex molecule could apply different forces to bound and free regions of DNA, and cause base tilting. The base tilting of A form produces a 20^o angle between the base pairs and the plane perpendicular to the

helical axis of DNA, which represents the greatest tilt among the three common structures[59]. In the presence of salt, it is also possible that the tertiary structure of bound copolypeptide is changed, and that hydrophobic interaction between DNA base pairs and alanine residues, or between the alanine residues themselves, would become important. Since the bases in A conformation are more exposed to the outside than in B or C structure, hydrophobic interaction between these bases and alanine residues might be favored in the A structure.

The results in Figures IV-4 and IV-6 show that neither the alanine residue itself nor the α-helix is the principal source of this kind of transition in the presence of NaCl, because poly($Lys^{19}Ala^{81}$), containing the maximum percentage of both alanine and α-helix of all the model proteins thus far studied, induces only a slightly detectable transition to A-type CD at high salt (Figure IV-4a), whereas model proteins of intermediate alanine content show a very pronounced transition for A-type CD under the same conditions (Figures IV-4b, IV-6b and c). Possibly the additional α-helical structures forcefully formed in the process of binding (Figures III-11 and III-14) might have some effect on the transition of DNA structure.

Another possible explanation for the failure of poly($Lys^{19}Ala^{81}$) to produce significant A-type CD, despite its full α-helix in the free state, is that it may bind DNA in one particular groove (the major groove, for example), while the other model proteins, with less α-helix in the free state, may bind DNA in another groove (such as the minor groove). This suggestion is based upon the finding that poly($Lys^{19}Ala^{81}$) complexed with DNA is well protected from trypsin digestion while other model proteins, poly($Lys^{40}Ala^{60}$), poly($Lys^{67}Ala^{33}$) and polylysine,

complexed with DNA are not. It was therefore suggested that perhaps poly(Lys^{19}Ala^{81}) binds DNA in the major groove, and that the amide groups next to lysine residues are buried in this groove, protected from trypsin approach, while such protection is not available for other model proteins binding DNA in the minor groove[80].

The results of these studies support the following conclusions: (a) of all the model proteins complexed with DNA, those having 40-60% alanine undergo the largest transition in transfer from low to intermediate ionic strength; (b) the complexes formed with poly(Lys^{19}Ala^{81}) undergo either a slight transition to A at higher salt or simply retain the distorted B or B $\longrightarrow$ C transition at an intermediate salt, while the complexes formed with model proteins poly(Lys^{81}Ala^{19}) and polylysine, both of which have unordered structures, undergo a transition toward ψ structure.

Chapter V

STUDIES ON A-TYPE COMPLEXES

Since complexes of calf thymus DNA with poly($Lys^{40}Ala^{60}$) had been shown to exhibit the greatest A-type response to the presence of various concentrations of salt, it was decided to use this copolymer to study such response in greater detail. Accordingly, poly($Lys^{40}Ala^{60}$) was complexed with different types of nucleic acids to determine whether or not its binding could induce in them transitions of a similar nature. There is considerable controversy over the source of the A-type conformation, whether it is due to changes in secondary or tertiary structure, and what part is played by aggregation of molecules or condensation of the DNA[81]. To determine the contribution of light-scattering to the apparent CD spectra, absorbance measurements were taken at different wavelengths and the A_{320}/A_{260} ratio calculated. (Measurements less than 0.04 indicate negligible light-scattering.)

Since double-stranded RNA's are normally in A-form[22], Poly(A)·Poly (U) (poly-adenine, poly-uridine) in its double-stranded form was used as an A-form model and complexed with copolypeptide, to determine whether binding and subsequent dialysis into salt, or binding directly in salt, would enhance its A-type spectrum. Since there have been suggestions that the percentage of guanine-cytosine (G·C) base pairs in a DNA might be responsible for different abilities to assume the A-type conformation[81], complexes were also made with DNA's of different G+C content to permit comparisons in response.

Different methods of producing A-type complex were also explored, using either dialysis into salt after binding, or complexing directly with reactant already in salt. Finally, effects of temperature increase upon the A-like CD spectrum were studied by heating complexes already in this form until denatured, then looking for evidences of renaturing with cooling. Reversibility of the B $\longrightarrow$ A transition induced by salt was investigated through dialysis back to EDTA buffer after the change in conformation had occurred. It seemed likely that study of results from experiments examining many different parameters might provide information which ultimately would answer questions concerning the structural origin of A-form DNA.

Complexes with Poly(A)·Poly(U)

Double-stranded Poly(A)·Poly(U), a model for double-stranded RNA, maintains its double helix only in salt concentrations ranging from 0.01 to 0.1M NaCl[82]. For this reason, no experiments could be performed in zero salt for control purposes. However, two sets of experiments were carried out, one in which the dry nucleic acid was both dissolved and complexed in EDTA buffer with 0.01M NaCl, and another wherein dissolution and complexing were effected in EDTA buffer plus 0.1M NaCl. In both cases, the complex being studied was dialyzed to higher salt after initial readings, it being assumed that binding would protect the Poly (A)·Poly(U) from loss of double-strandedness. For both sets of experiments, the copolypeptide was maintained in 0.01M NaCl for complexing.

The precipitation curve for Poly(A)·Poly(U) (Figure V-1) was plotted only for complexes made at the lowest possible concentration of salt (0.01M NaCl). In contrast to the DNA precipitation curves with

FIGURES

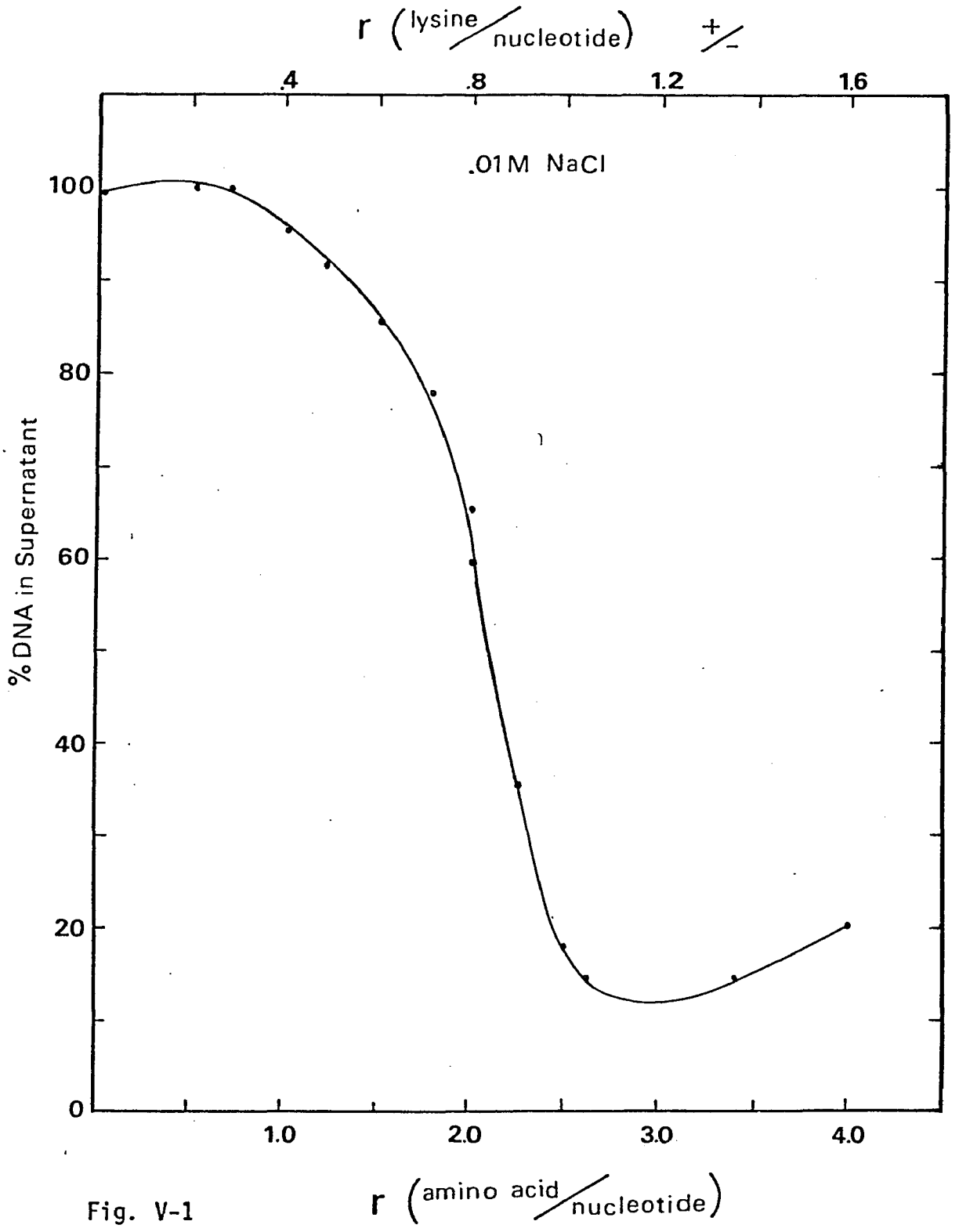
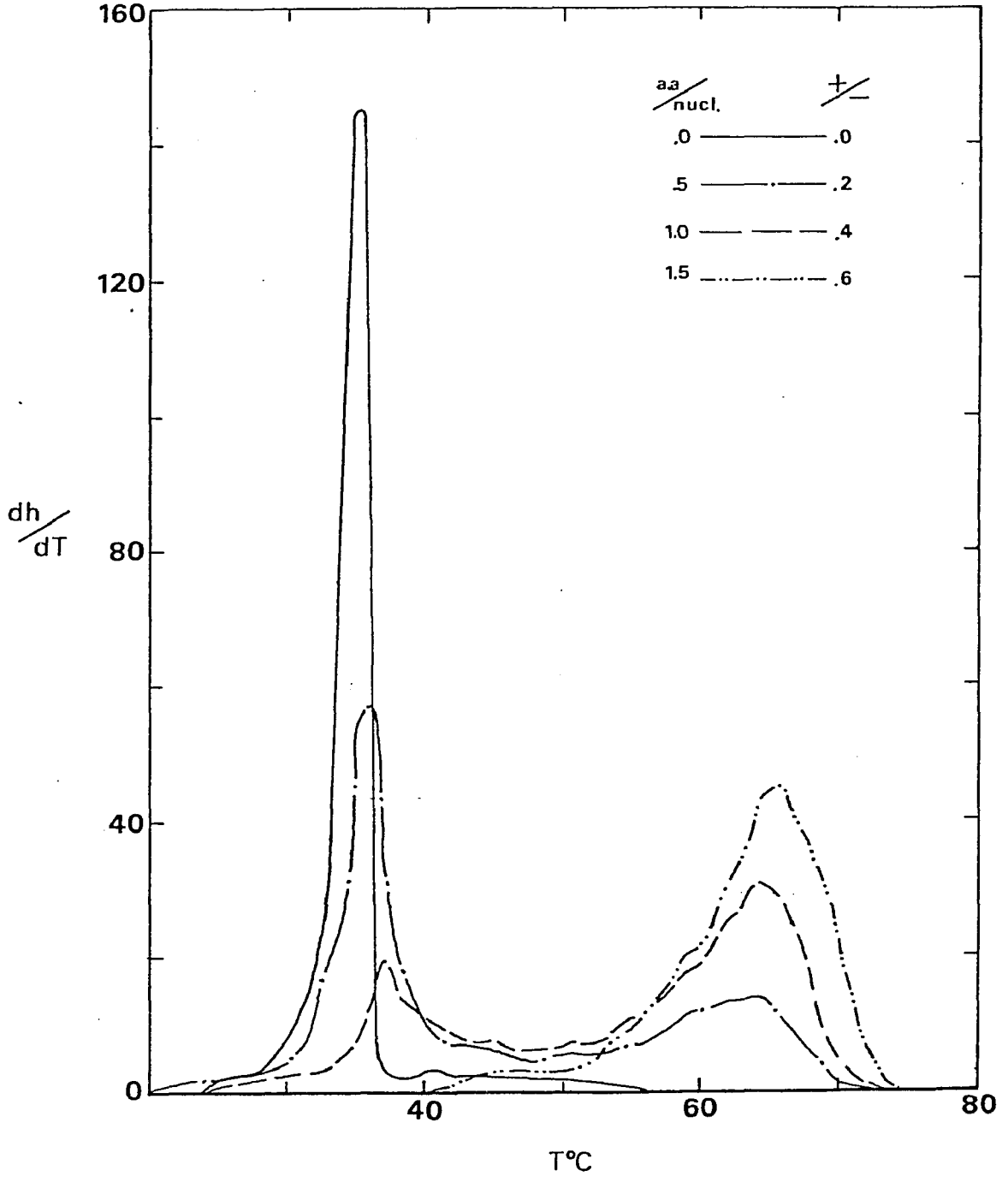


Fig. V-1

Lys^{40}-Ala^{60}/Poly(A)·Poly(U) Complexes



Lys^{40}–Ala^{60} / Poly(A)·Poly(U) Complexes

Fig. V-2 .01 M NaCl

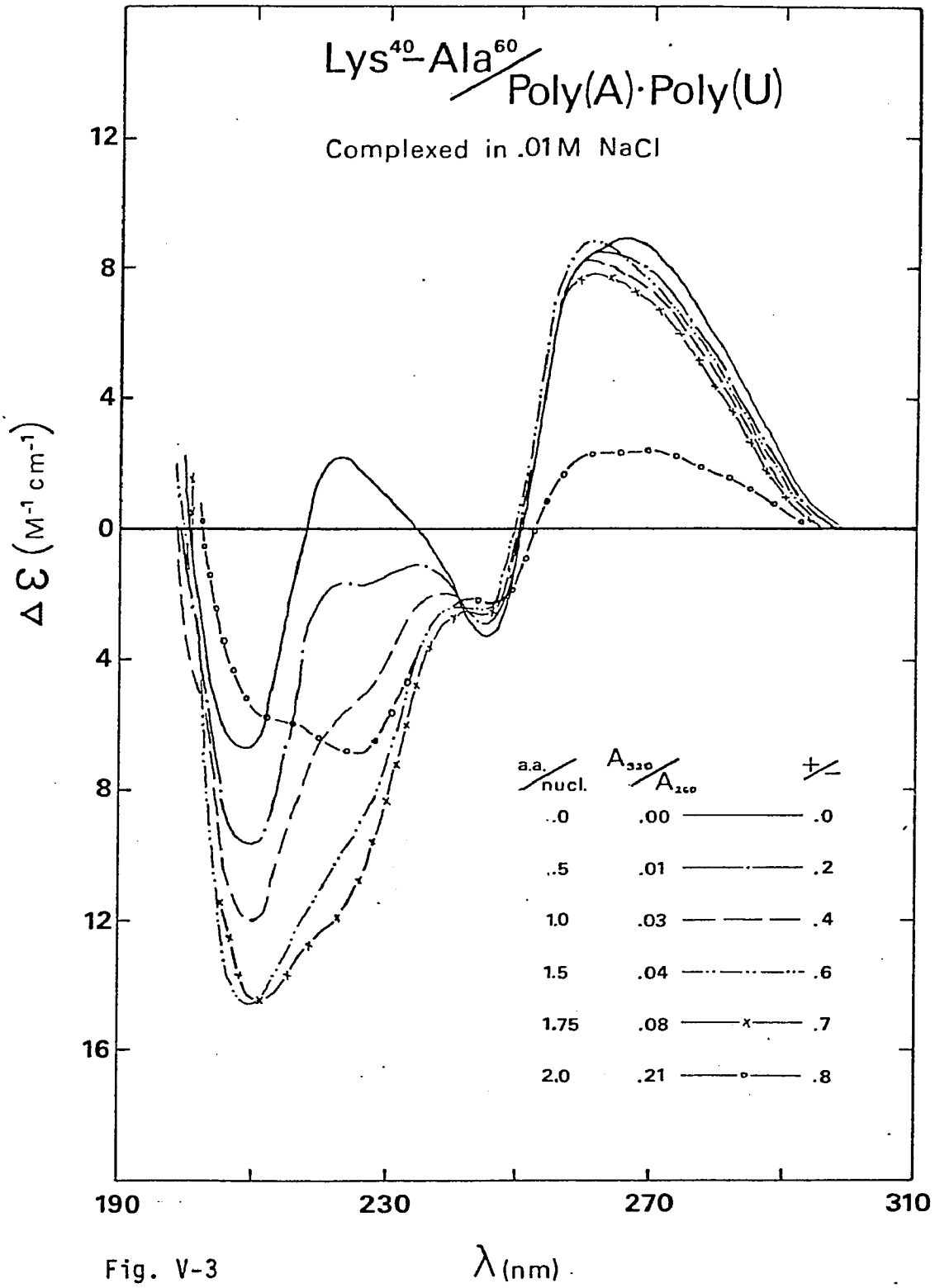

Lys40–Ala60
Poly(A)·Poly(U)
Complexed in .01 M NaCl
Δε (M⁻¹ cm⁻¹)
λ (nm)
12
8
4
0
4
8
12
16
190
230
270
310
a.a./nucl.
A320/A260
+/−
.0 .00 .0
.5 .01 .2
1.0 .03 .4
1.5 .04 .6
1.75 .08 .7
2.0 .21 .8

Fig. V-3

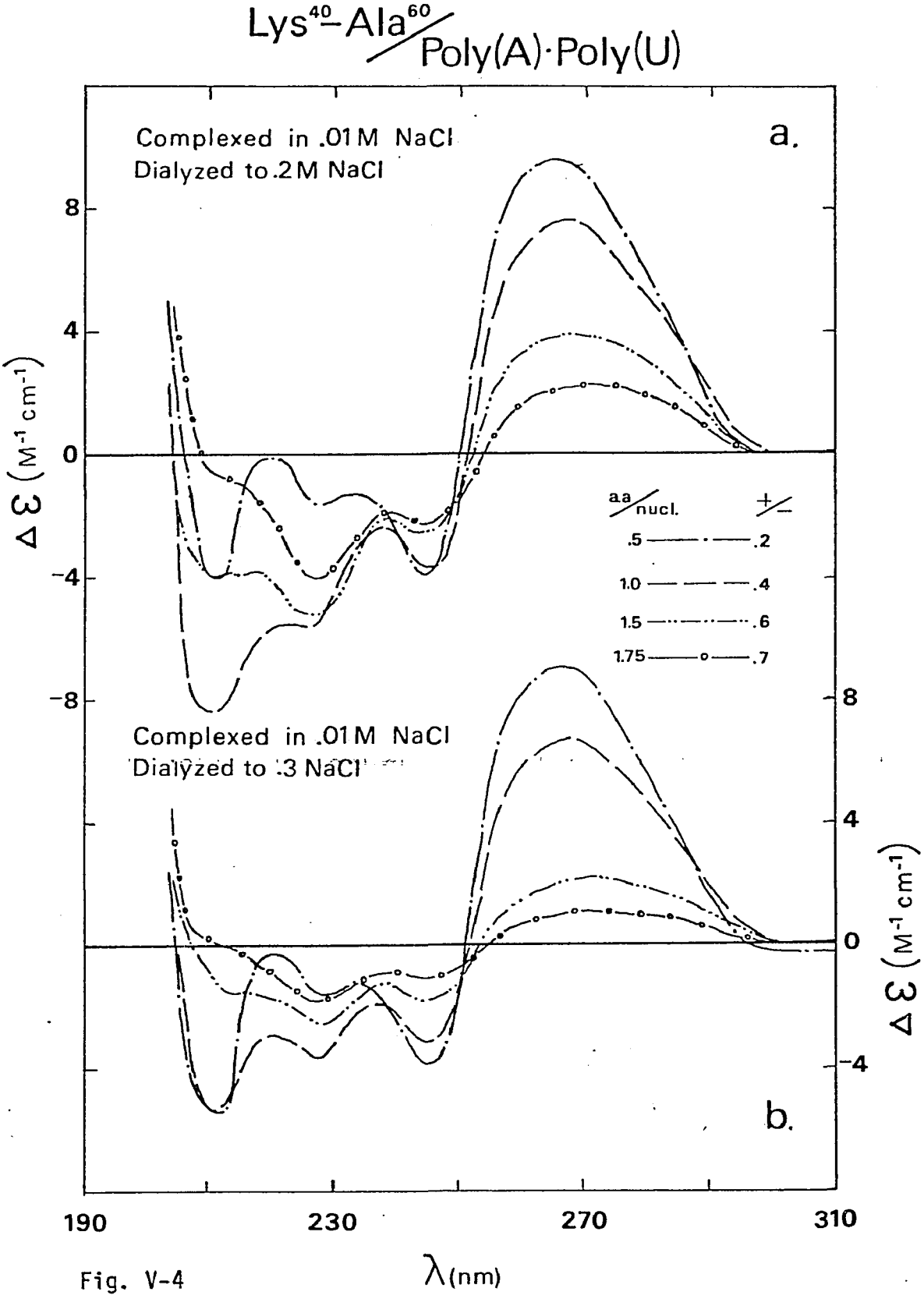

Lys40–Ala60
Poly(A)·Poly(U)
Complexed in .01 M NaCl
Dialyzed to .2 M NaCl
a.
Complexed in .01 M NaCl
Dialyzed to .3 NaCl
b.
aa/nucl.
+/−
.5 — .2
1.0 — .4
1.5 — .6
1.75 — .7
Δε (M⁻¹ cm⁻¹)
8
4
0
−4
−8
190
230
270
310
λ(nm)

Fig. V-4

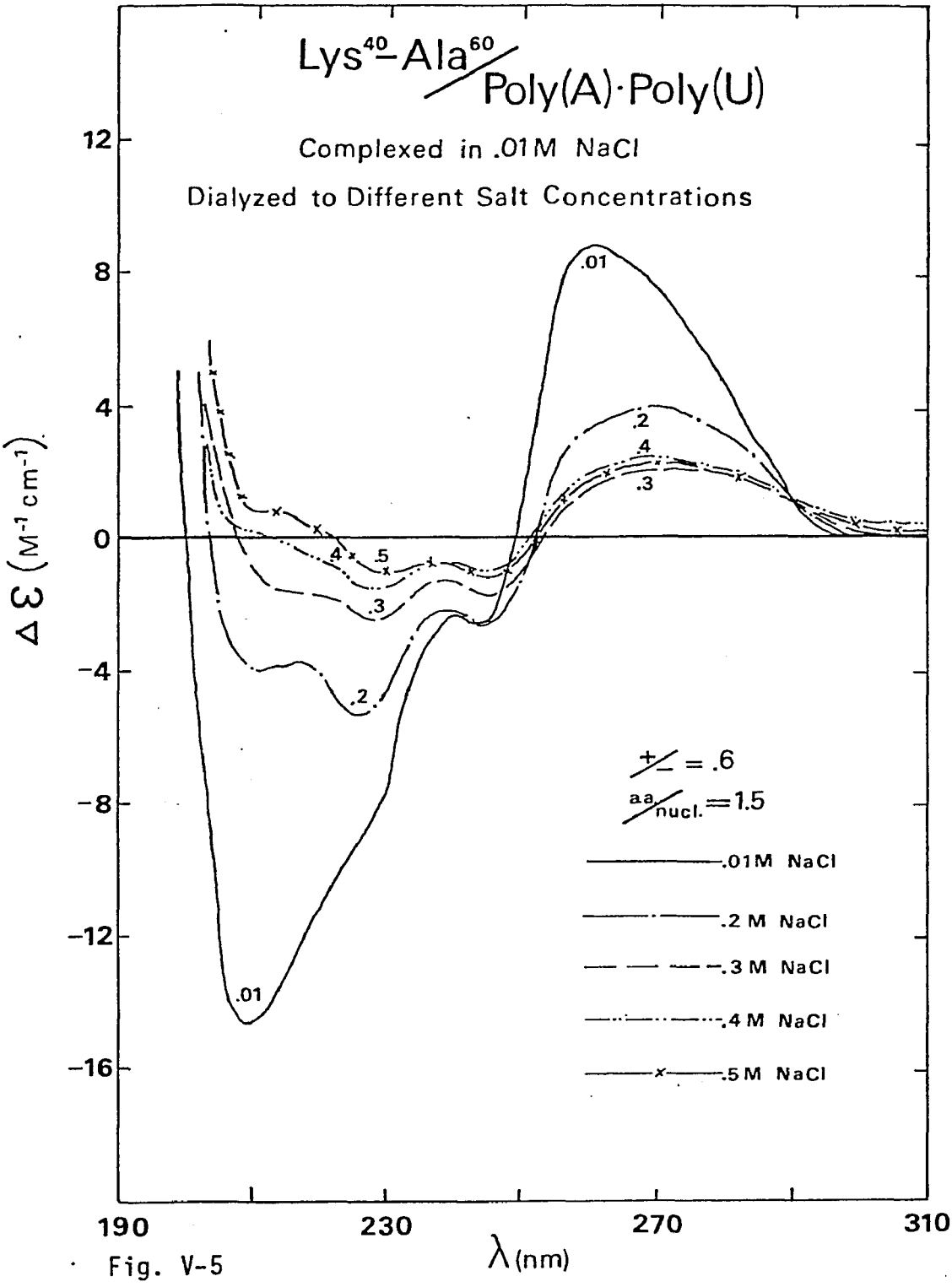
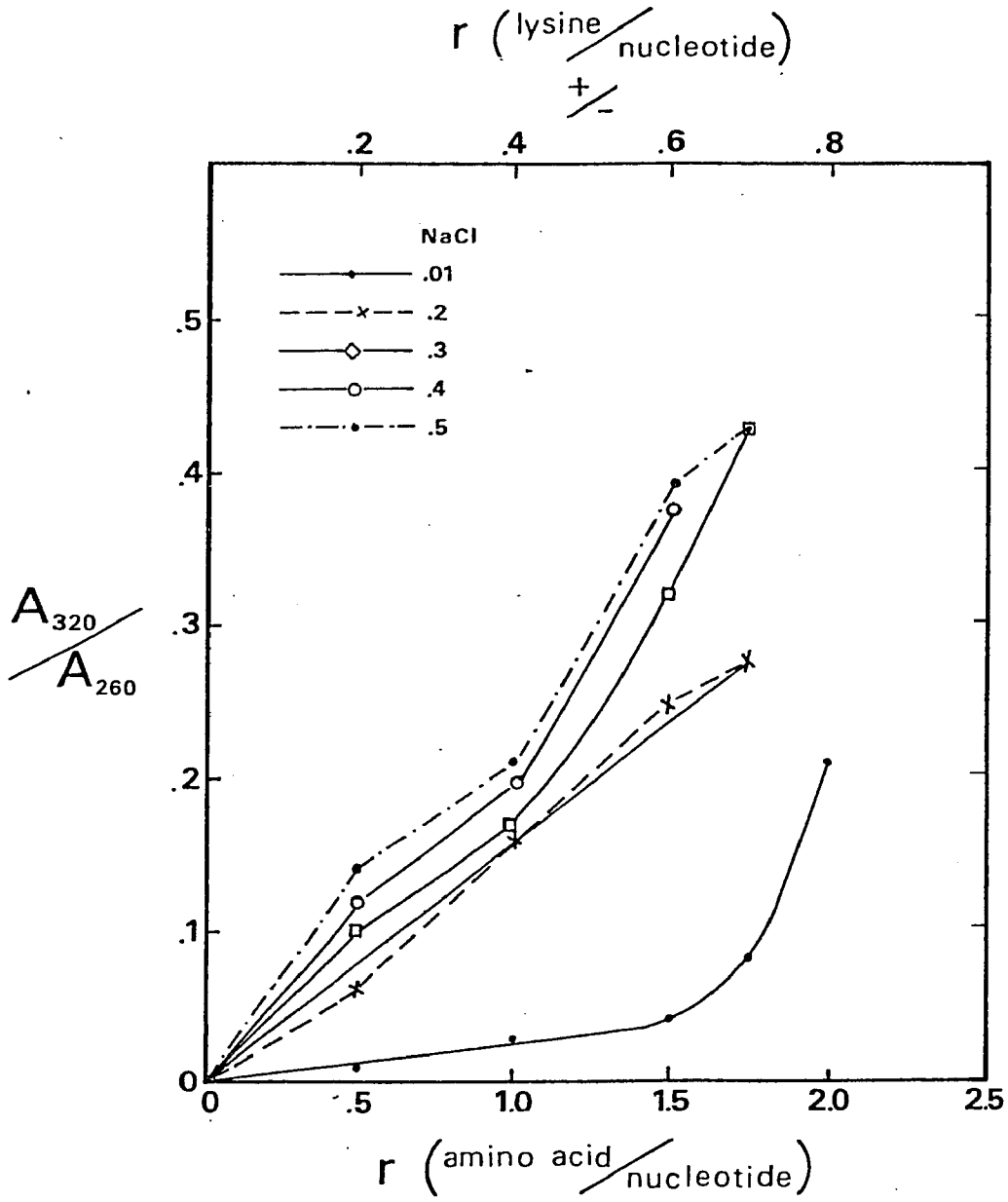

Lys40-Ala60 / Poly(A)·Poly(U)
Complexed in .01M NaCl
Dialyzed to Different Salt Concentrations
12
8
4
0
-4
-8
-12
-16
Δε (M-1 cm-1)
190
230
270
310
λ (nm)
.01
.2
.4
.3
.5
+/- = .6
aa/nucl. = 1.5
.01M NaCl
.2 M NaCl
.3 M NaCl
.4 M NaCl
.5 M NaCl

Fig. V-5



Contribution of Light Scattering at Different Degrees of Coverage

Lys^{40}–Ala^{60} / Poly(A)·Poly(U)

Complexed in .01 M NaCl
Dialyzed to Different Salt Concentrations

Fig. V-6

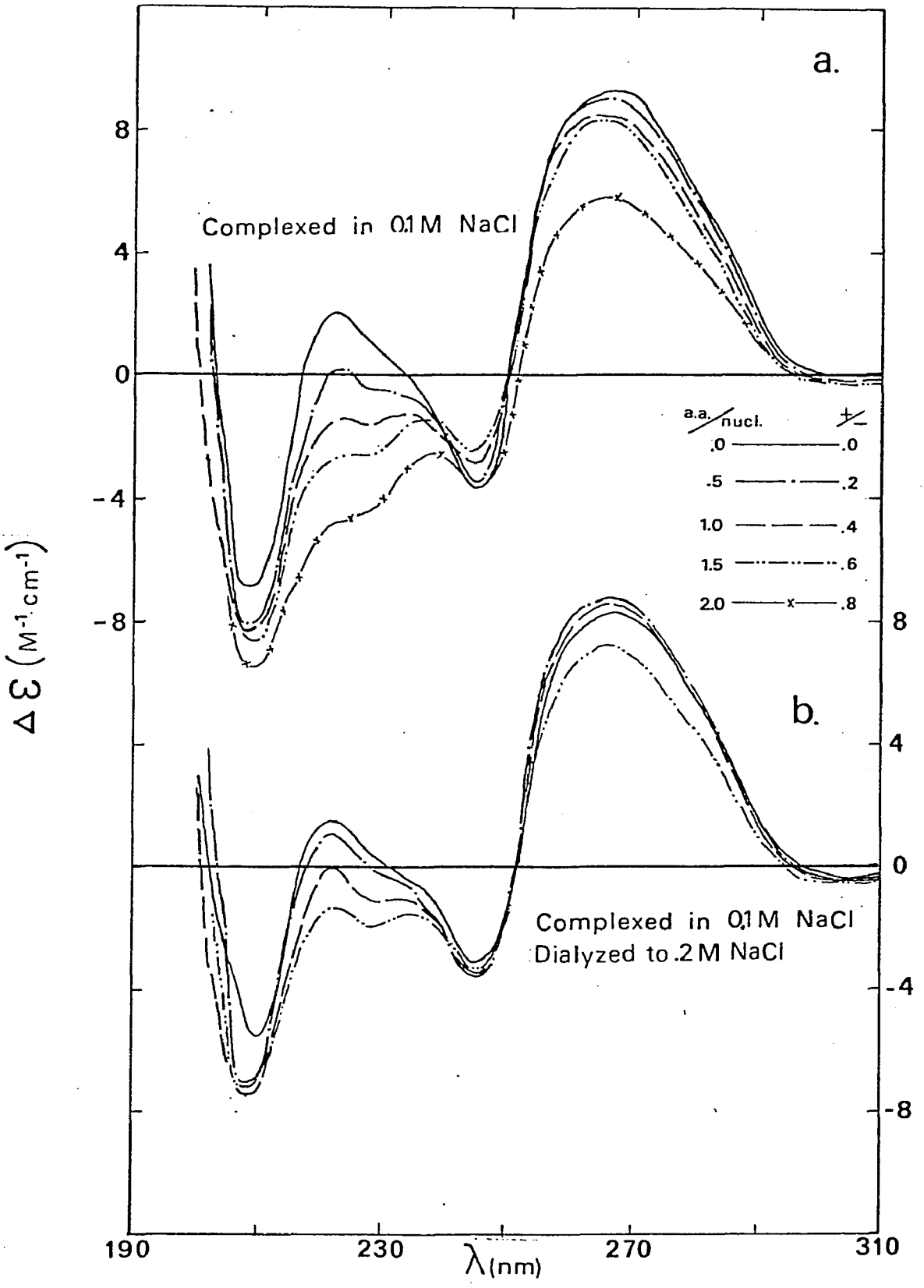


Fig. V-7 $\frac{Lys^{40}-Ala^{60}}{Poly(A)\cdot Poly(U)}$

this copolymer, the Poly(A)•Poly(U) curve shows detectable cooperativity of binding, with some precipitation of complexes occurring long before complete charge neutralization is effected. This was apparent in the reduced magnitude of the Tm' bands upon thermal denaturation (Figure V-2) and probably contributed to the unusually low average β value of 1.38 amino acid/nucleotide (+/- = .55).

The initial melt of free Poly(A)•Poly(U) (in 0.01M NaCl) is highly cooperative, demonstrated by the narrow half-width of the Tm band in Figure V-2. When bound by poly($Lys^{40}Ala^{60}$) the character of the melt changes, yielding Tm' bands which are broad by comparison, thus indicating less cooperativity of melting in the bound regions. The temperatures of the melts are low, despite the presence of salt. Also, there is a noticeable increase in Tm' with increasing coverage, the peaks moving from 64°C, at a +/- ratio of 0.2 (r = .5), to 66°C at +/- = 0.6 (r = 1.5). Above this ratio precipitation occurs with heating. The average β value as determined from the plots of $r = \beta \frac{A_{Tm'}}{A_T}$ and $r = \beta\left(1 - \frac{A_{Tm}}{A_T}\right)$ was 1.38 amino acid/nucleotide or 0.55 lysine/nucleotide which is unusually low for this copolypeptide. Poly($Lys^{40}Ala^{60}$) complexed with calf thymus DNA has an average β value of 3.0 amino acid/nucleotide or 1.2 lysine/nucleotide. See Figure III-4.

The CD spectrum of free Poly(A)•Poly(U) (Figure V-3) shows the positive nucleic acid peak to be blue shifted from the customary calf thymus DNA position of about 278nm (in B-form) to below 270nm, a shift which is characteristic of the B ⟶ A transition. Complimentary RNA is unable to assume the B-like conformation at all; double-stranded RNA's have been observed only in the A-type structure and the conformation is thought to be attributable to the necessity of fitting the

sugar oxygen into the ribonucleic acid backbone, a condition not encountered by the deoxyribonucleic acid[22].

With the first degree of binding there is a sudden further blue shift of the peak to a position at 265nm, where it remains throughout further coverage. The amplitude of the free peak is greatly enhanced over that expected for DNA, being increased from a $\Delta\varepsilon$ of around 2.5 to one of greater than 8.0. This effect is also characteristic of A-form. However, whereas increasing coverage is required for the production of large amplitudes in A-form DNA complexes, increased binding of copolypeptide to Poly(A)·Poly(U) up to a +/- ratio of about .7 (r = 1.75), tends to decrease the nucleic acid peak slightly. Beyond this point, in the .8 (r = 2.0) complex, there is a sudden elevation of the A_{320}/A_{260} ratio from .08 (at +/- = .7, r = 1.75) to .21 and a sharp decrease in amplitude of the signal, indicating aggregation.

Below 250nm, in the protein region, the free Poly(A)·Poly(U) spectrum contains the positive and negative extrema expected of nucleic acids, but with the 220nm peak skewed and the magnitude of the 210nm trough markedly increased. Binding by copolymer further enhances this trough indicating that the bound protein makes considerable contribution to the apparent CD. The reduction in amplitude observable in the complex of r = 2.0 (+/- = .8) possibly results from aggregation and precipitation, judging from the titration (precipitation) curve of Figure V-1 and the absorbance ratio, A_{320}/A_{260} = 0.21.

In Figure V-4a, the effect of dialyzing into higher salt is shown. The 0.8 (+/-) sample (r = 2.0) has been eliminated through precipitation. In the nucleic acid peak, where initially (in 0.01M NaCl) an increase in coverage affected the amplitude only slightly, up to a +/-

ratio of .7 (r = 1.75), in 0.2M NaCl, an increase in amplitude observable at the first degree of coverage (+/- = .2, r = .5) was followed by a marked decrease with each of the following complexes and a progressive shift of the peak to higher wavelengths. Such decrease in amplitude might be due to precipitation and loss of soluble material. On the other hand, in the protein region, compared to Figure V-3, the amplitude of the prominent negative trough is cut in half by the .2 (+/-, r = .5) binding and progressively minimized for the other complexes, while at the same time the relative importance of the 222nm negative peaks are further enhanced.

At 0.2M NaCl, double-stranded Poly(A)•Poly(U) separates to form one single-stranded Poly(A) and a triple-stranded Poly(A)•2Poly(U)[83,84]. No attempt was made to ascertain whether binding of model protein to nucleic acids did indeed protect against loss of double-strandedness as assumed. The slight increase in the nucleotide peak at a +/- ratio of .2, r value of .5, when only twenty percent of the phosphates were protected by binding, may well have been caused by partial denaturation or triple-strandedness.

With dialysis into 0.3M NaCl, complexes with the same degree of coverage remain in solution, as shown in Figure V-4b, and the effects observed in 0.2M NaCl are further amplified. Such effects may well be due to aggregation. Figure V-5 shows the results from one complex at higher coverage (+/- = .6, r = 1.5) when dialyzed into progressively higher concentrations of salt. At this degree of coverage the fraction of bound nucleotide pairs should be sufficient to prevent a change in strandedness, making it possible to evaluate the effect of salt on conformation without attention to this consideration. The increased

sodium ion concentration yields a drastic reduction in amplitude of both major and minor peaks, in moving from 0.01 to 0.2M NaCl. Additional increase is associated with still further reduction in amplitude; the overall change in the trough position encompasses elevation from a negative $\Delta\varepsilon$ equal to about -15 ($M^{-1}cm^{-1}$) to a positive value, while the relative prominence of the 222nm peak shows continual growth despite decreases in the amplitude of the total spectrum.

The contribution of aggregation to the effects observed with increasing salt concentration is summarized in Figure V-6, which shows the A_{320}/A_{260} ratios plotted against input ratios, the latter being expressed in terms of both +/- ratios and r values: the +/- ratio emphasizes the degree of charge neutralization effected by binding; the amino acid/nucleotide ratio (r value) emphasizes the presence of uncharged amino acids and their contribution to the overall effect of binding, through inter- and intramolecular hydrophobic interactions.

When Poly(A)•Poly(U) was dissolved and complexed in 0.1M NaCl (with the copolymer remaining in 0.01M prior to complexing), the composite shape of the complex spectra (all degrees of coverage considered as a unit) changes markedly (compare Figure V-3 and V-7); the total spectrum of the uncomplexed Poly(A) Poly(U) changes very little. In 0.1M NaCl, the amplitude of the positive CD peak near 270nm changes little until a +/- ratio of .8 (r = 2.0) and even at that degree of coverage the A_{320}/A_{260} ratio is increased to only 0.086 (from 0.005) and the peak amplitude at 265nm is reduced only slightly, from a $\Delta\varepsilon$ of 8.0 to about 6.0, rather than to little more than 2.0. In addition, the peak position of Poly(A)•Poly(U) is shifted very little as a result of binding

in 0.1M NaCl. Below 250nm, the differences in the effects of binding at this salt concentration, compared to binding in 0.01M, are considerably greater. Whereas, by +/- = .7 (r = 1.75) in 0.01M NaCl, the major negative peak has been approximately doubled in magnitude, descending to over -14 ($M^{-1}cm^{-1}$), in 0.1M NaCl, the $\Delta\varepsilon_{210}$ is changed only from -7.0 to -9.6. Nevertheless, the changes aroung 220nm still follow a fairly regular pattern with binding, presumably due to the increasing increments of protein at higher r values.

When the above complexes are dialyzed into 0.2M NaCl, the uncomplexed Poly(A)·Poly(U) spectrum changes, the 210nm negative peak being reduced to $\Delta\varepsilon$ = -5.6 (see Figure V-7). A change is to be expected, since at this salt concentration double-stranded Poly(A)·Poly(U) is transformed into two different entities: triple-stranded Poly(A)·2Poly(U) and single-stranded Poly(A)[83,84]. Here, too, the complex with +/- = .8, r = 2.0, precipitates out of solution, as does the 0.01M NaCl complex at the same degree of coverage when dialyzed into 0.1M NaCl. The spectra of the other complexes, however, change relatively little with the increase in salt, as shown in Figures V-7 a-b; those of Figures V-3 and V-4a, however, differ considerably.

Complexes with DNA's of Different G·C Content

Calf thymus DNA has a guanine-cytosine (G+C) content of 43%[81]. To determine whether or not the presence of these base pairs contributes to the tendency of a DNA molecule to assume the A-type conformation when bound to poly($Lys^{40}Ala^{60}$) in the presence of salt, three other DNA's containing different percentages of G+C were used for complexing: Clostridium perfringens DNA, 30%; Escherichia coli, 53%; Micrococcus

FIGURES

Lys^{40}-Ala^{60} Complexed with Different DNA's

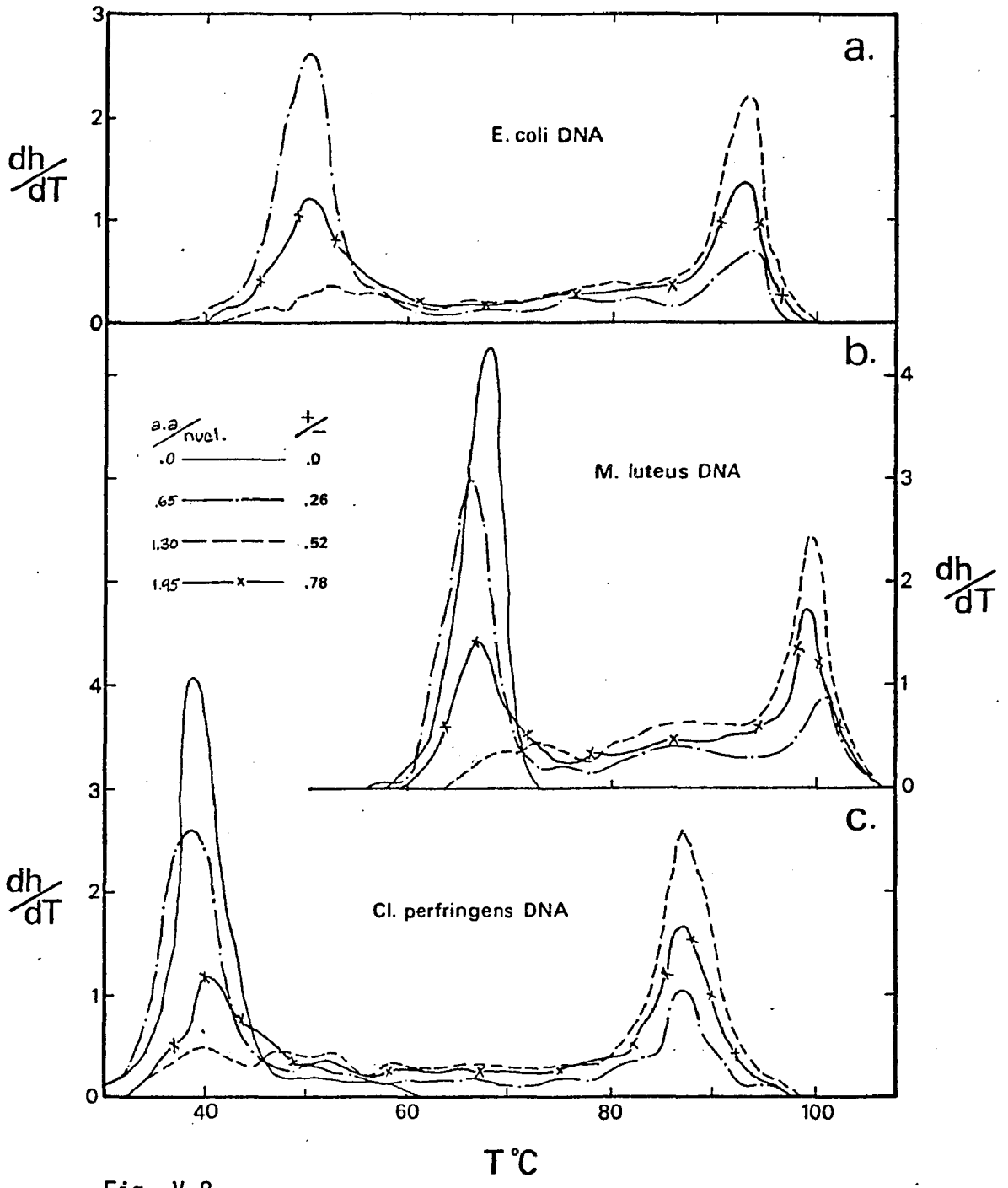


Fig. V-8

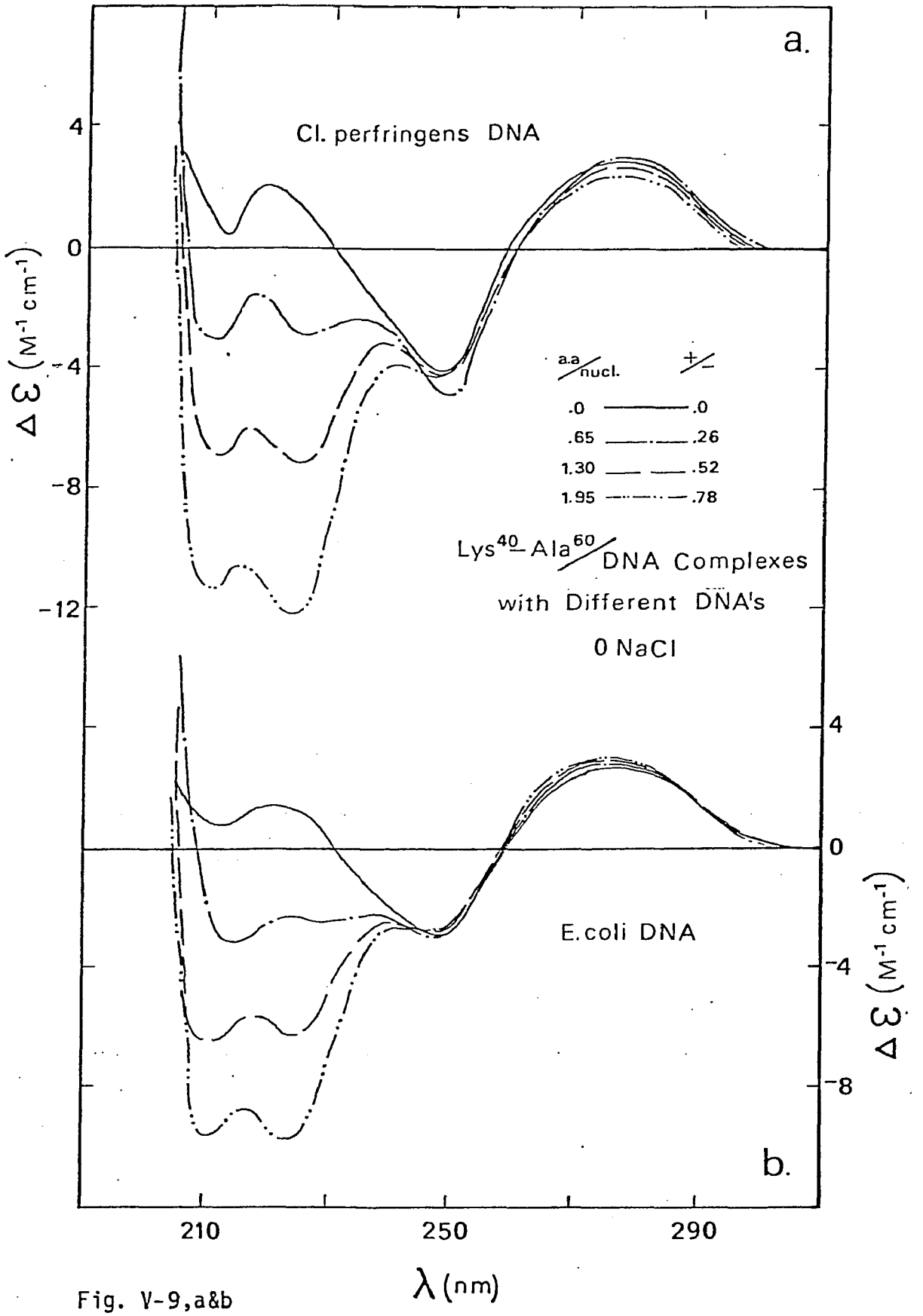

a.
Cl. perfringens DNA
4
0
-4
-8
-12
Δε (M⁻¹ cm⁻¹)
a.a/nucl.
+/−
.0 ——— .0
.65 —·—·— .26
1.30 — — — .52
1.95 —··—··— .78
Lys40-Ala60/DNA Complexes
with Different DNA's
0 NaCl
E.coli DNA
b.
210
250
290
λ (nm)

Fig. V-9,a&b

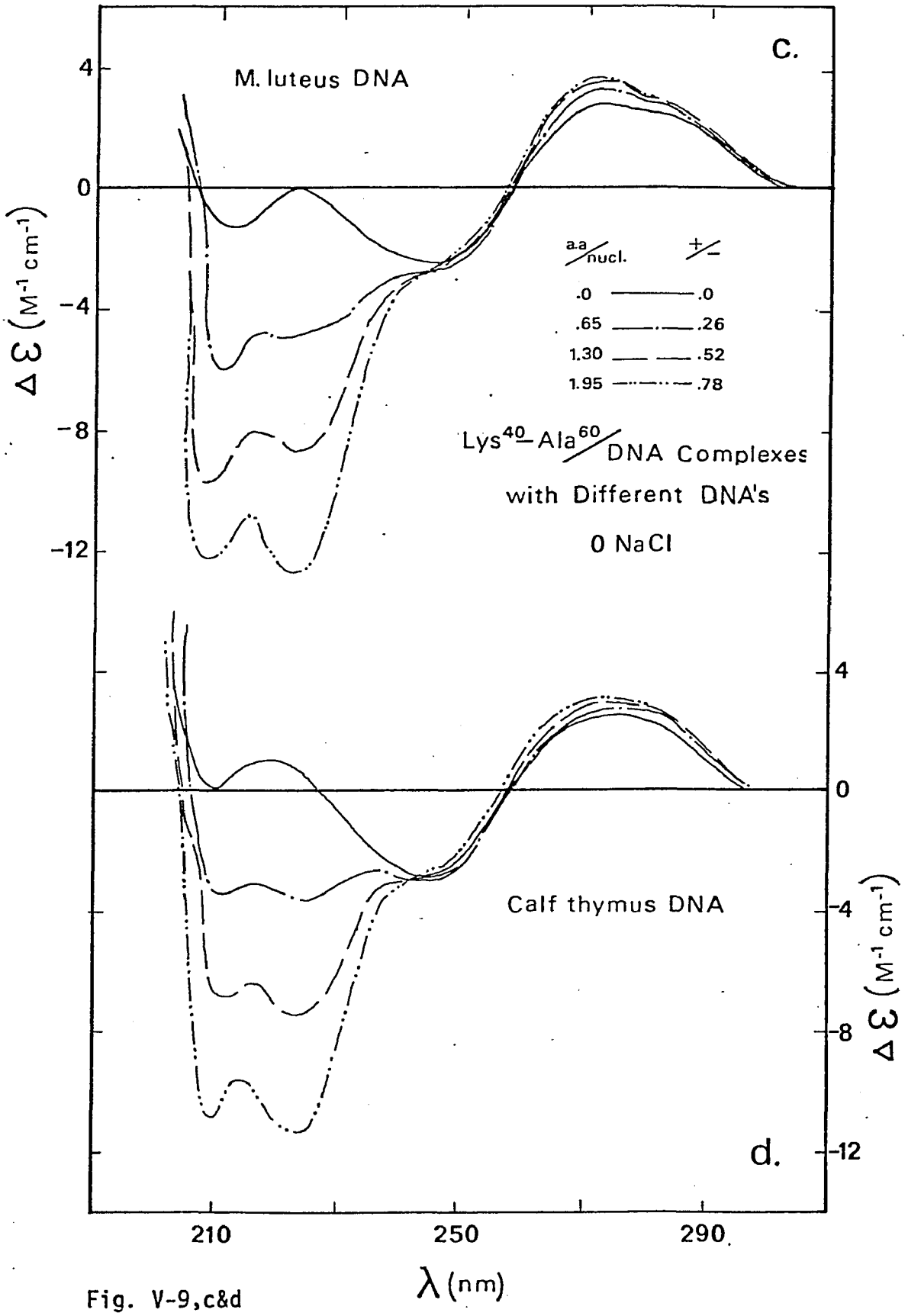

c.
M. luteus DNA
a.a/nucl.
+/−
.0 .0
.65 .26
1.30 .52
1.95 .78
Lys40–Ala60/DNA Complexes
with Different DNA's
0 NaCl
Calf thymus DNA
d.
Δε (M-1 cm-1)
λ (nm)
210
250
290
4
0
-4
-8
-12

Fig. V-9,c&d

luteus, 70% [81].

The derivative melting curves of complexes made with these DNA's are shown in Figure V-8 (a-c). Differences in their G+C content are evident from locations of the melting bands, since the stability afforded by three hydrogen bonds, as opposed to the two present in A•T (adenine-thymine) pairs, is reflected in higher or lower melting temperatures of both Tm and Tm'. The character of the melting bands, at comparable r values with different DNA's, is generally similar in shape, with the heights and breadths of bands being much the same. M. luteus has more residual melt between the bands; Cl. perfringens shows least variation in the temperature of Tm'; with all three types, by an r value of 1.95 (+/- = .78), the Tm peak is largely eliminated. At +/- ratios greater than .78, increasing temperature tends to reduce the amplitude of the Tm' band due to loss of material by precipitation. The following average β values were determined for each DNA bound by poly(Lys^{40}-Ala^{60}): Cl. perfringens, 3.2 amino acid/nucleotide (+/- = 1.3); E. coli, 2.8 amino acid/nucleotide (+/- = 1.1); M. luteus, 3.1 amino acid/nucleotide (+/- = 1.2).

The CD spectra of complexes between poly($Lys^{40}Ala^{60}$) and each type of DNA, including calf thymus, show great similarity in the absence of salt, as seen by comparing graphs a, b, c and d of Figure V-9. Binding does not affect the shape, and has little effect upon the amplitude, of the DNA positive peak. The negative protein peaks below 250nm, for all DNA's, show increasing α-helical content with increasing coverage, the relative shapes all being much the same as those characteristic of the copolymer when bound to calf thymus DNA.

With the advent of sodium ion into the medium, the picture changes. Figure V-10 a-c shows the effect upon each uncomplexed DNA of increasing the NaCl concentration from 0.0 to 0.5M. Although in general no substantial changes occur in the DNA CD, it is worth noting that beyond 0.4M NaCl, Cl. perfringens tends to percipitate out of solution, which could be related to the observable change in the spectrum below 230nm.

For complexes formed in the absence of salt and dialyzed into solutions of increased ionic strength, the next set of graphs (Figures V-11,-12,-13) show, for each DNA, the effect at different degrees of coverage of increasing the NaCl concentration. At low coverage (+/- = .26, r = .65) there are wide differences in the salt responses of the different DNA's, in both DNA and protein regions of the spectra. Effects of salt on the Cl. perfringens spectra are about equally apparent in both regions (Figure V-11a): the DNA peak shifts and changes amplitude, but retains its rounded shape; the 222nm protein negative peaks are changed in amplitude though not in shape, but, as expected from the salt effect on the uncomplexed DNA, the negative 210nm peak, apparent in the absence of salt, is eliminated altogether. With E. coli DNA (Figure V-11b), response is minimal in the DNA portion, and, although there is noticeable change below 230nm, the effect here, too, is comparatively small.

The .26 (+/-, r = .65) complex with M. luteus (Figure V-11c) begins to show changes in the DNA region recognizable as the beginnings of A-form, and the whole protein region is more affected in shape than is that of the Cl. perfringens spectrum. It is the composite calf thymus spectrum, however, which shows the greatest change.

FIGURES

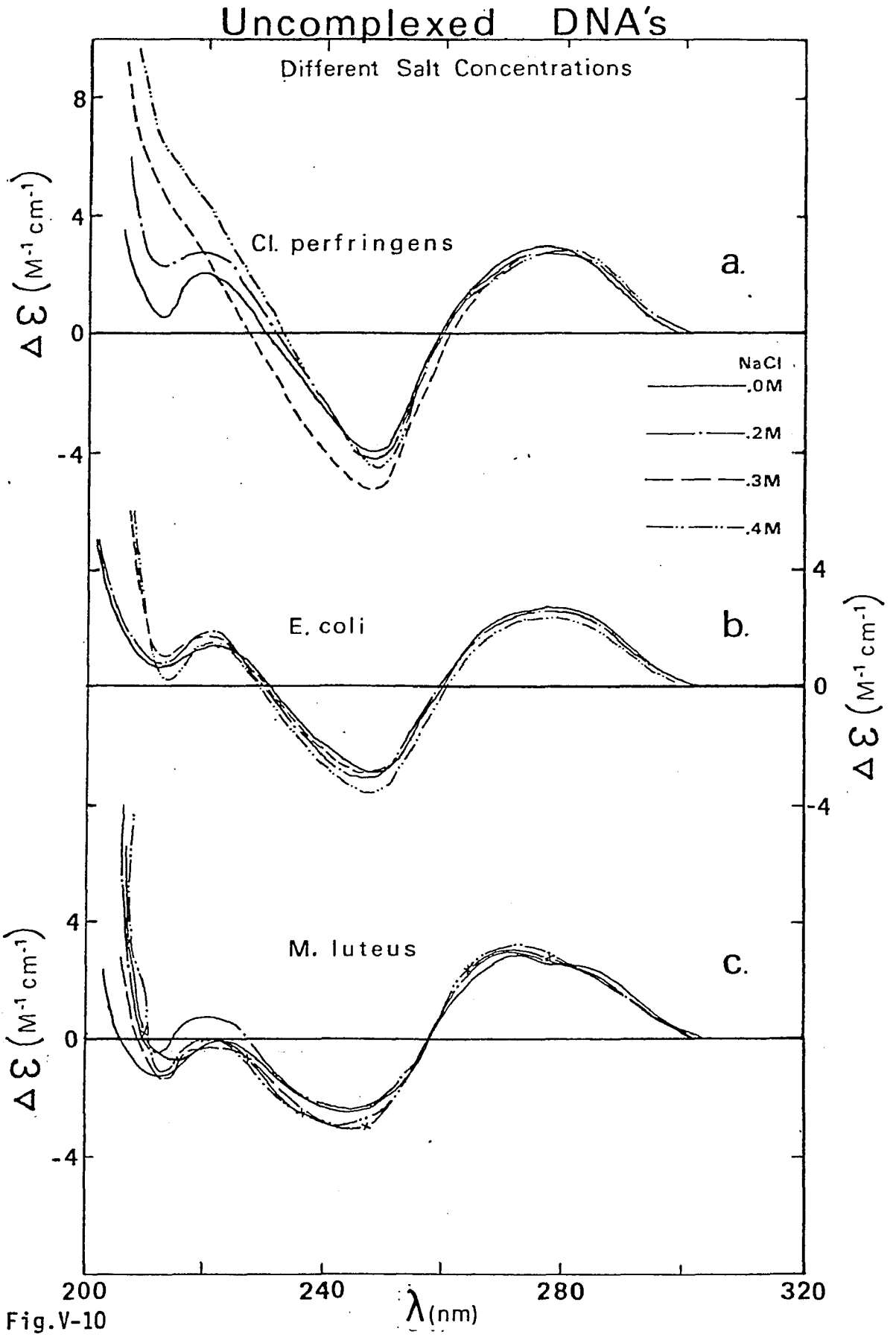

Uncomplexed DNA's
Different Salt Concentrations
Cl. perfringens
a.
E. coli
b.
M. luteus
c.
NaCl
.0M
.2M
.3M
.4M
Δε (M⁻¹cm⁻¹)
8
4
0
-4
200
240
280
320
λ(nm)

Fig.V-10

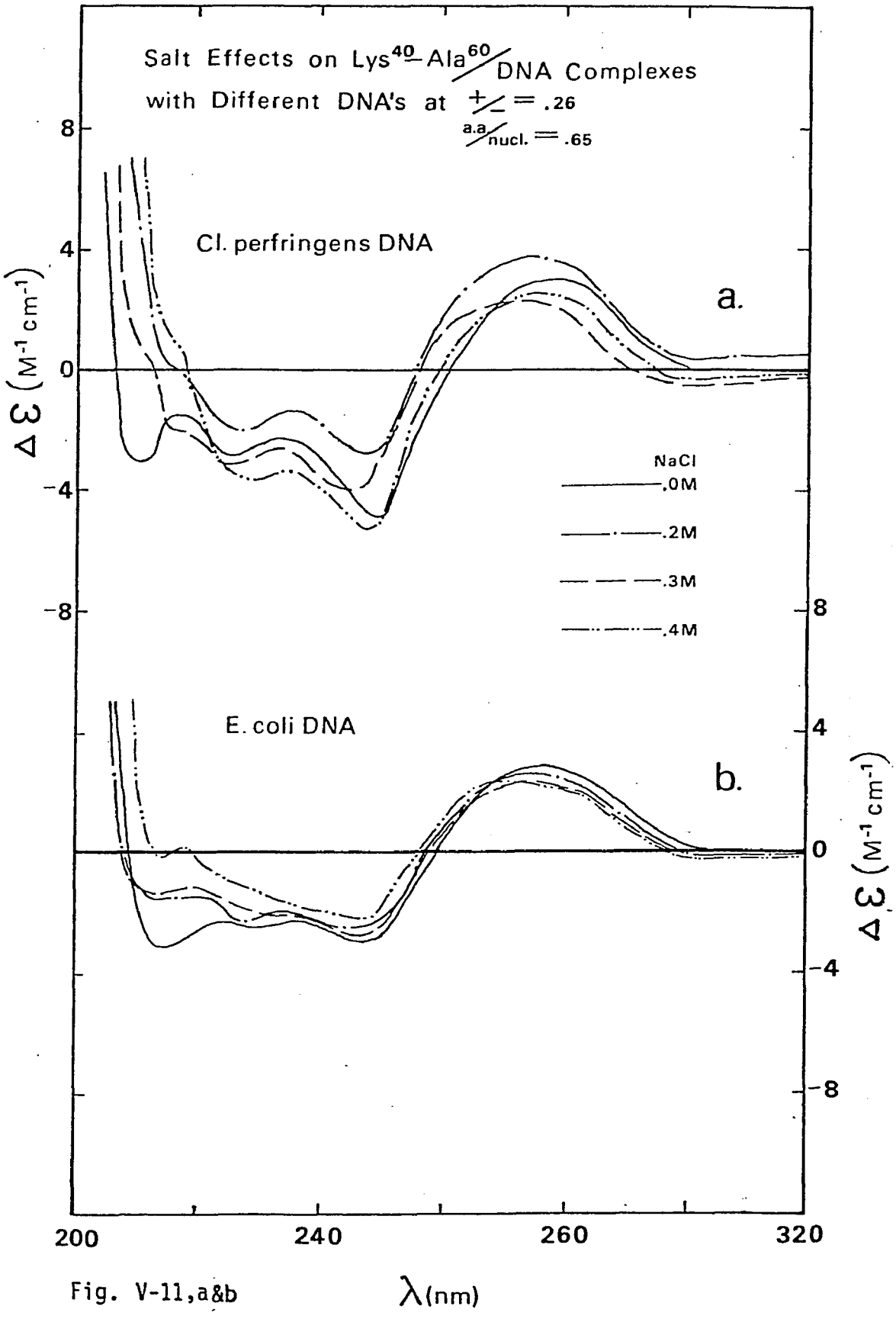

Salt Effects on Lys40-Ala60/DNA Complexes
with Different DNA's at +/- = .26
a.a./nucl. = .65
Cl. perfringens DNA
a.
E. coli DNA
b.
NaCl
.0M
.2M
.3M
.4M
Δε (M-1 cm-1)
Δε (M-1 cm-1)
8
4
0
-4
-8
200
240
260
320
λ(nm)

Fig. V-11,a&b

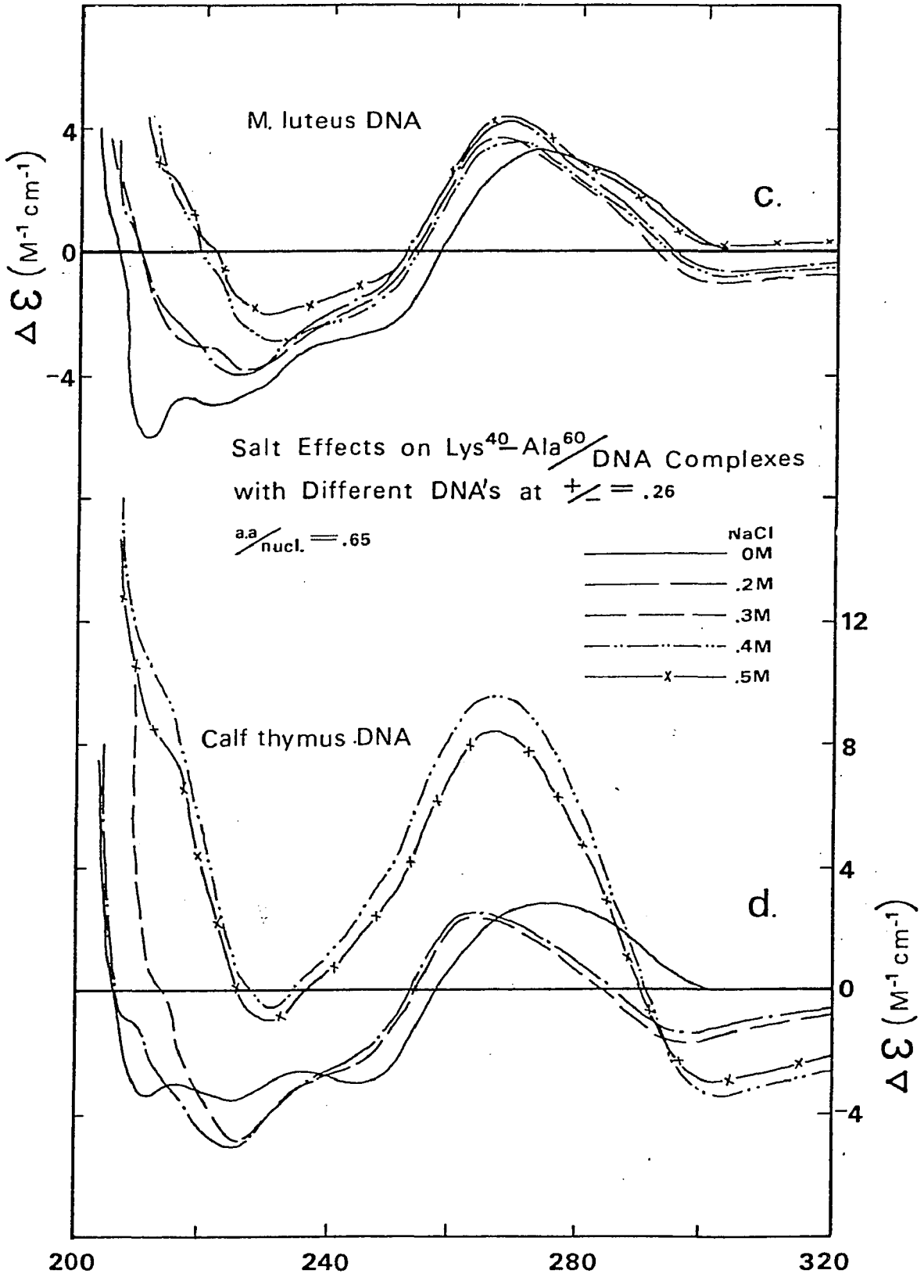

M. luteus DNA
c.
Salt Effects on Lys40–Ala60/DNA Complexes
with Different DNA's at +/− = .26
a.a/nucl. = .65
NaCl
0M
.2M
.3M
.4M
.5M
Calf thymus DNA
d.
Δε (M⁻¹ cm⁻¹)
Δε (M⁻¹ cm⁻¹)
4
0
−4
12
8
4
0
−4
200
240
280
320
λ(nm)

Fig. V-11,c&d

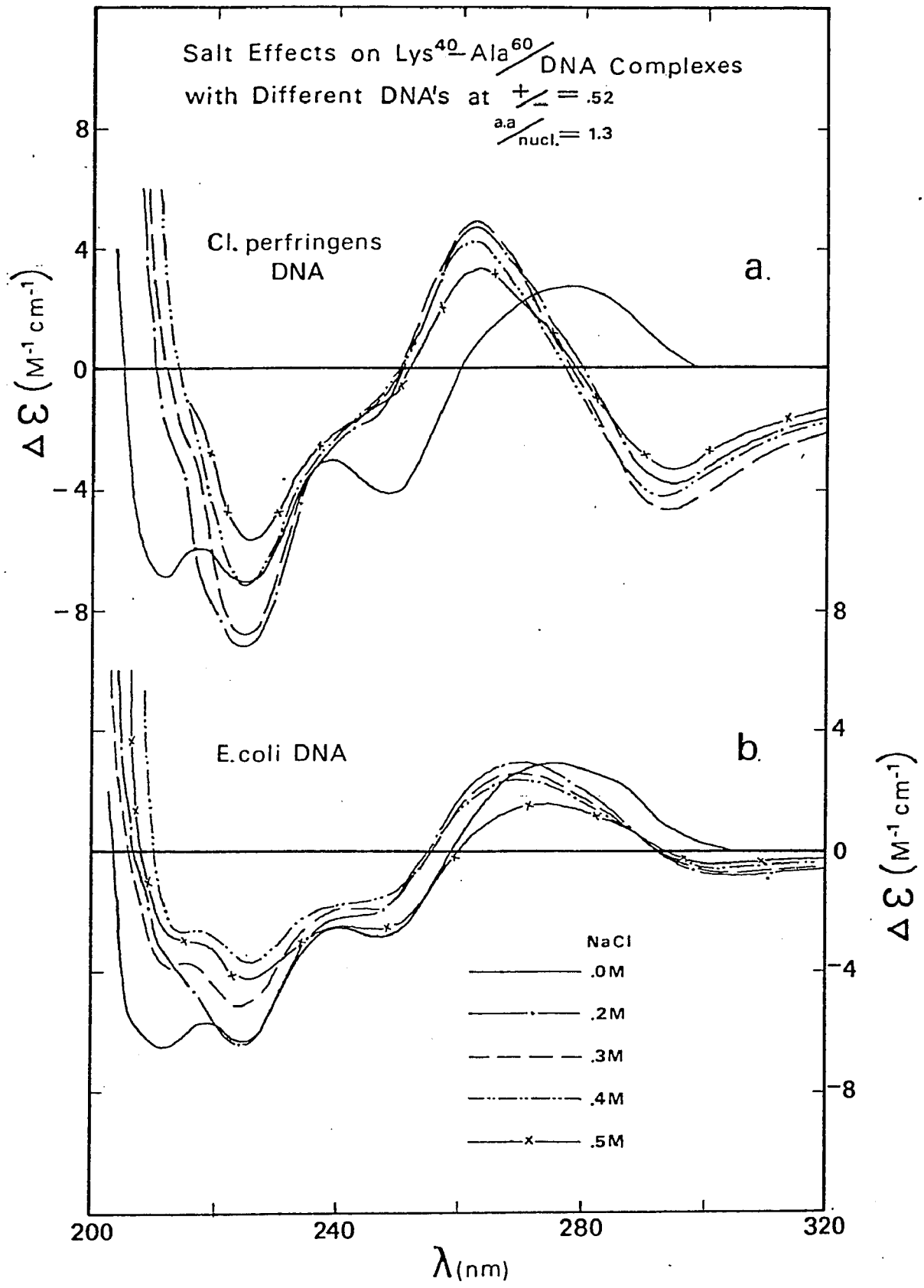

Salt Effects on Lys40-Ala60/DNA Complexes
with Different DNA's at +/- = .52
a.a/nucl. = 1.3
Cl. perfringens
DNA
a.
E.coli DNA
b.
NaCl
.0M
.2M
.3M
.4M
.5M
Δε (M-1 cm-1)
λ(nm)
200
240
280
320
8
4
0
-4
-8

Fig. V-12,a&b

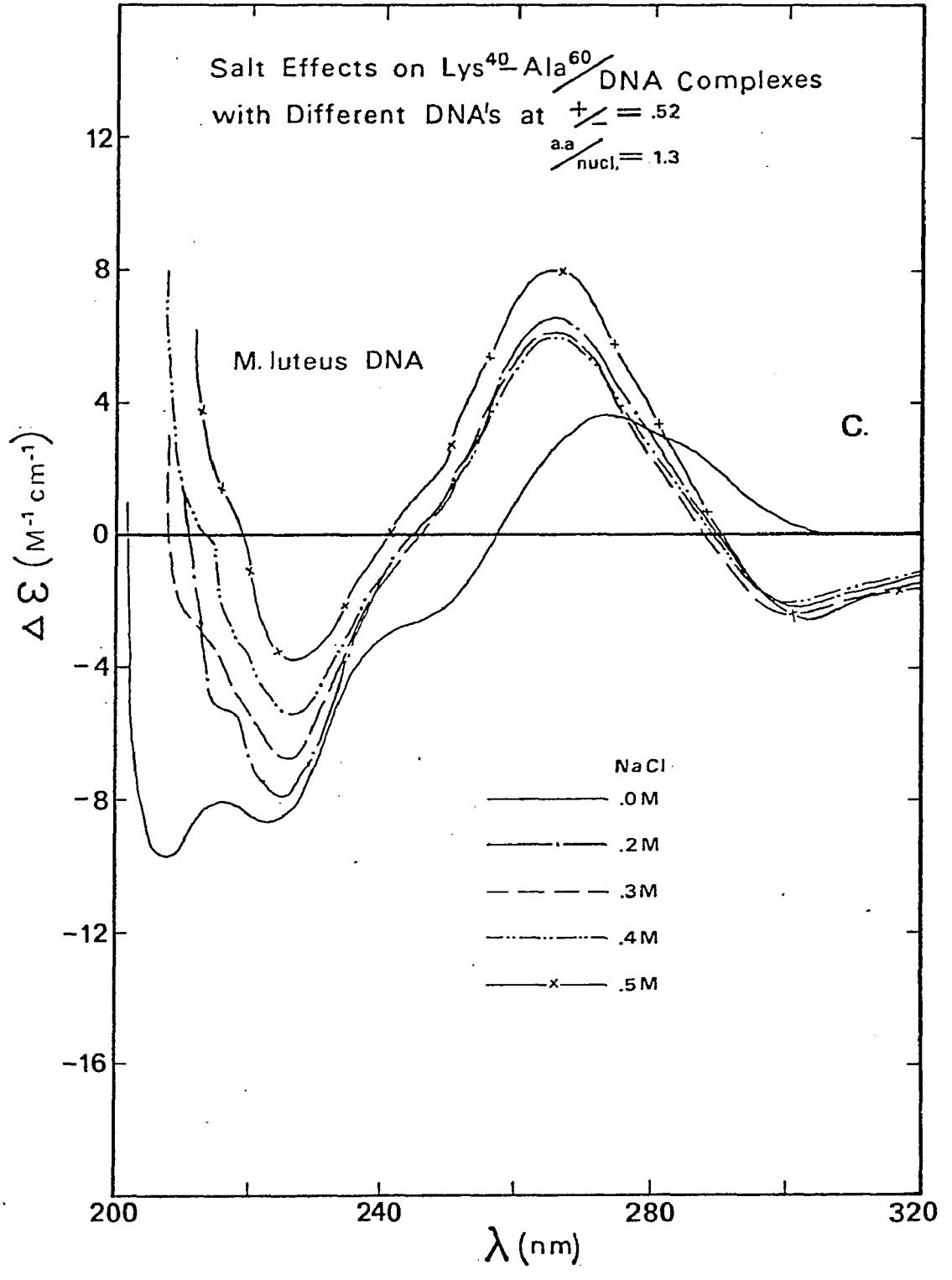

Salt Effects on Lys40-Ala60/DNA Complexes
with Different DNA's at +/- = .52
a.a/nucl. = 1.3
M. luteus DNA
C.
NaCl
.0M
.2M
.3M
.4M
.5M
Δε (M-1 cm-1)
λ (nm)
12
8
4
0
-4
-8
-12
-16
200
240
280
320

Fig. V-12,c

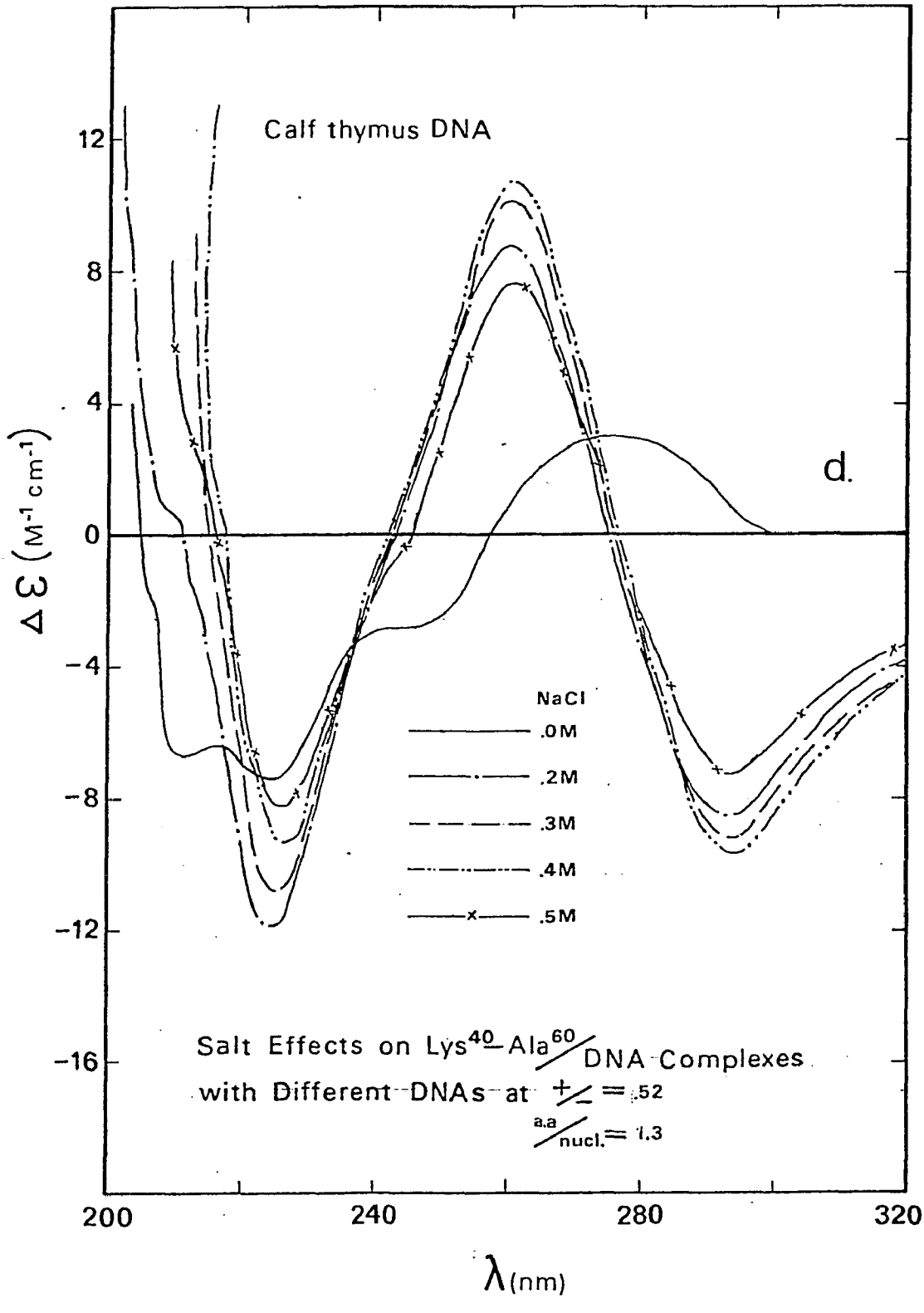

Calf thymus DNA
d.
12
8
4
0
-4
-8
-12
-16
Δε (M⁻¹cm⁻¹)
NaCl
.0M
.2M
.3M
.4M
.5M
Salt Effects on Lys40-Ala60/DNA Complexes
with Different DNAs at +/- = .52
a.a/nucl. = 1.3
200
240
280
320
λ(nm)

Fig. V-12,d

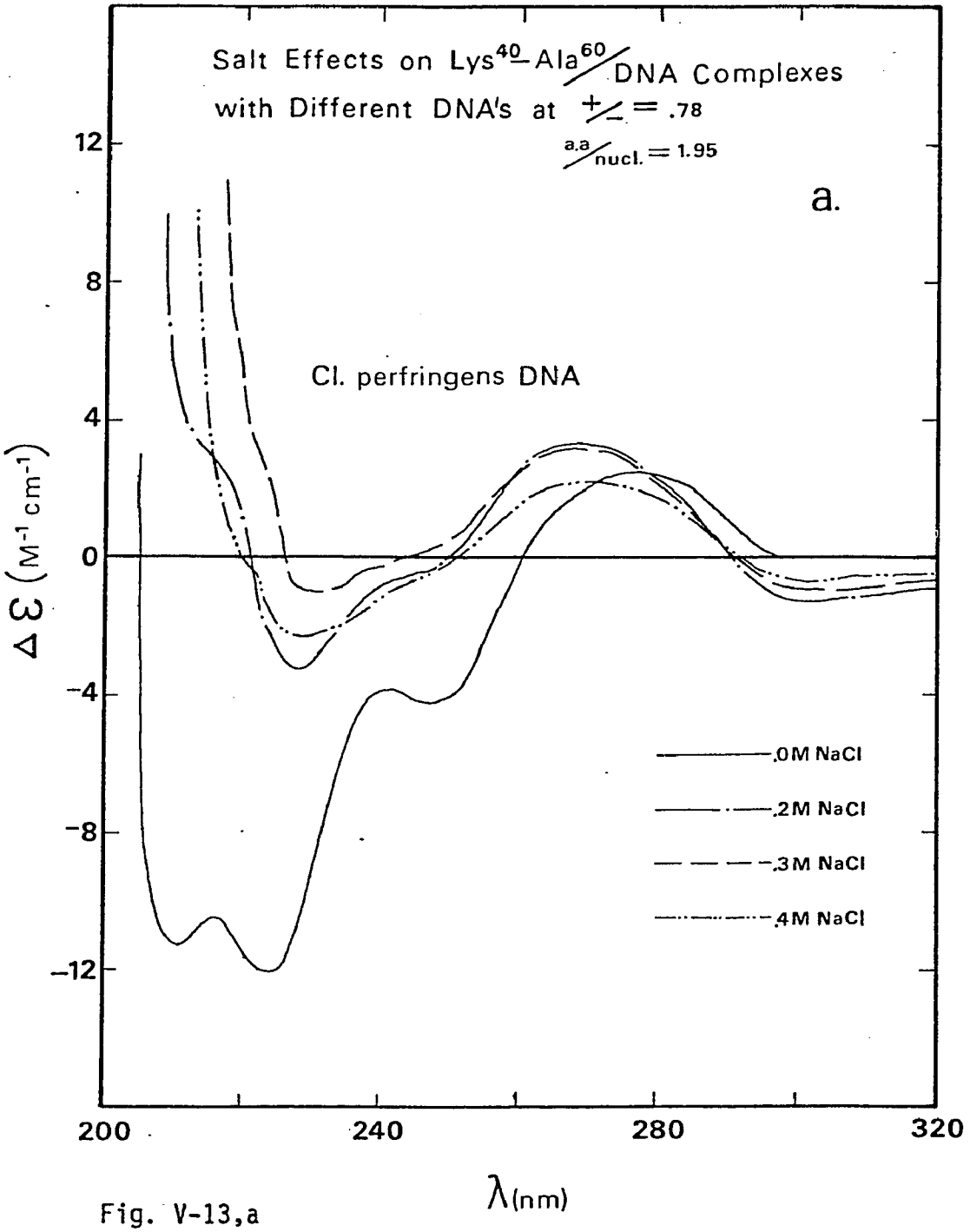

Salt Effects on Lys40-Ala60/DNA Complexes
with Different DNA's at +/− = .78
a.a/nucl. = 1.95
a.
Cl. perfringens DNA
12
8
4
0
−4
−8
−12
Δε (M⁻¹ cm⁻¹)
200
240
280
320
λ(nm)
.0M NaCl
.2M NaCl
.3M NaCl
.4M NaCl

Fig. V-13,a

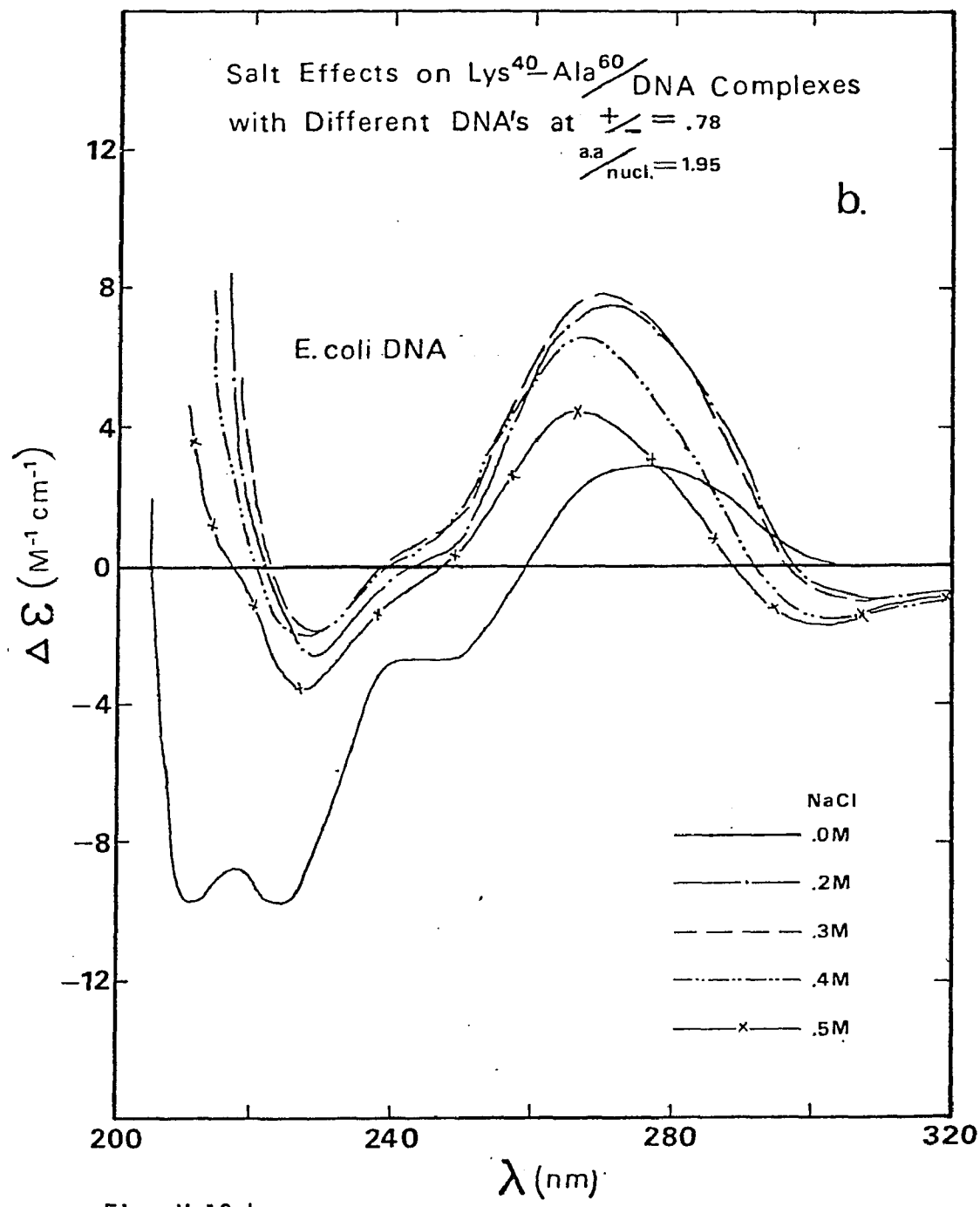
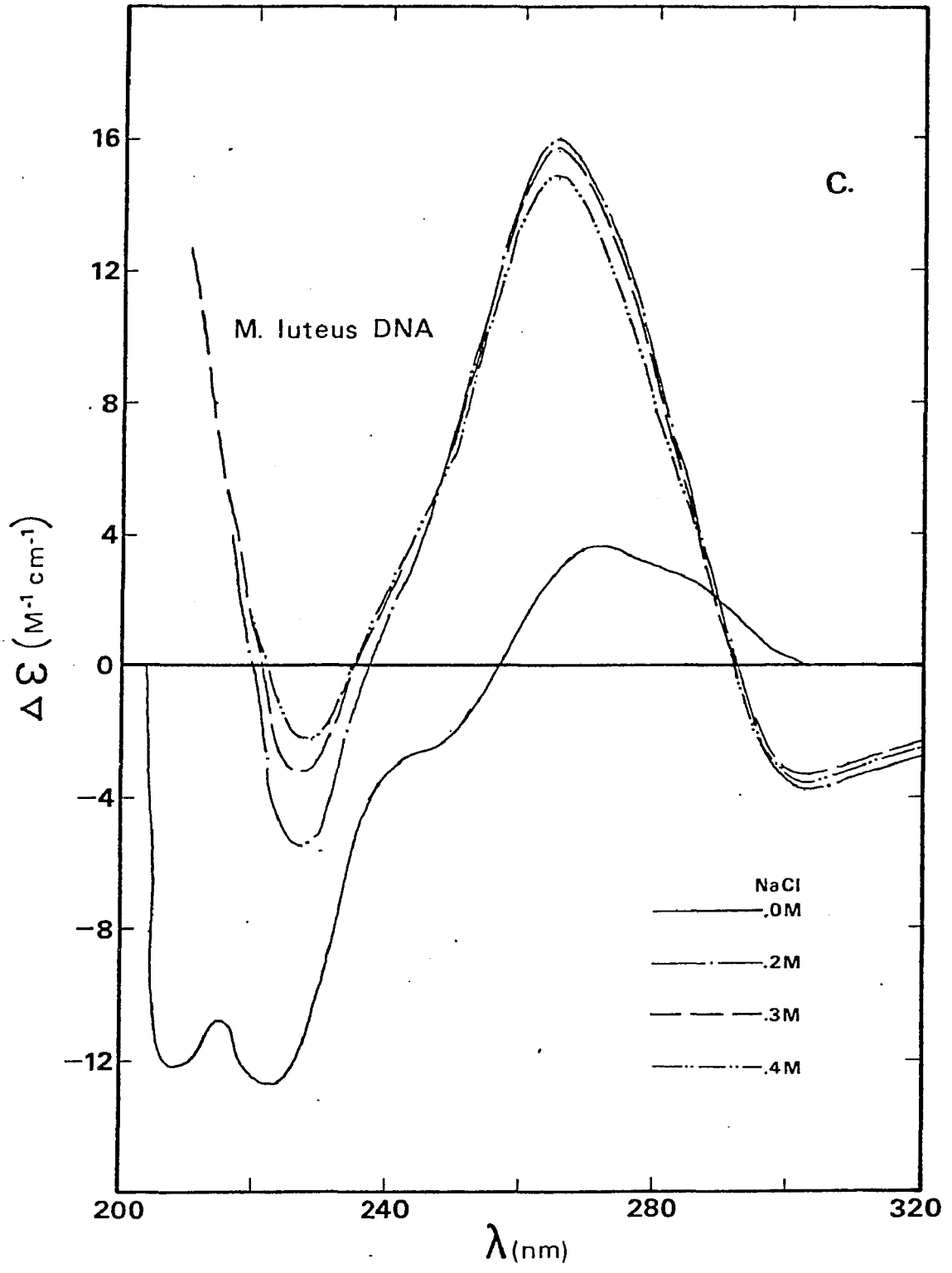

Salt Effects on Lys40-Ala60/DNA Complexes
with Different DNA's at +/- = .78
a.a/nucl. = 1.95
b.
E. coli DNA
12
8
4
0
-4
-8
-12
Δε (M-1 cm-1)
200
240
280
320
λ (nm)
NaCl
.0M
.2M
.3M
.4M
.5M

Fig. V-13,b



Salt Effects on Lys^{40}—Ala^{60}/DNA Complexes with Different DNA's at $+/- = .78$; $a.a./nucl = 1.95$

Fig. V-13,c

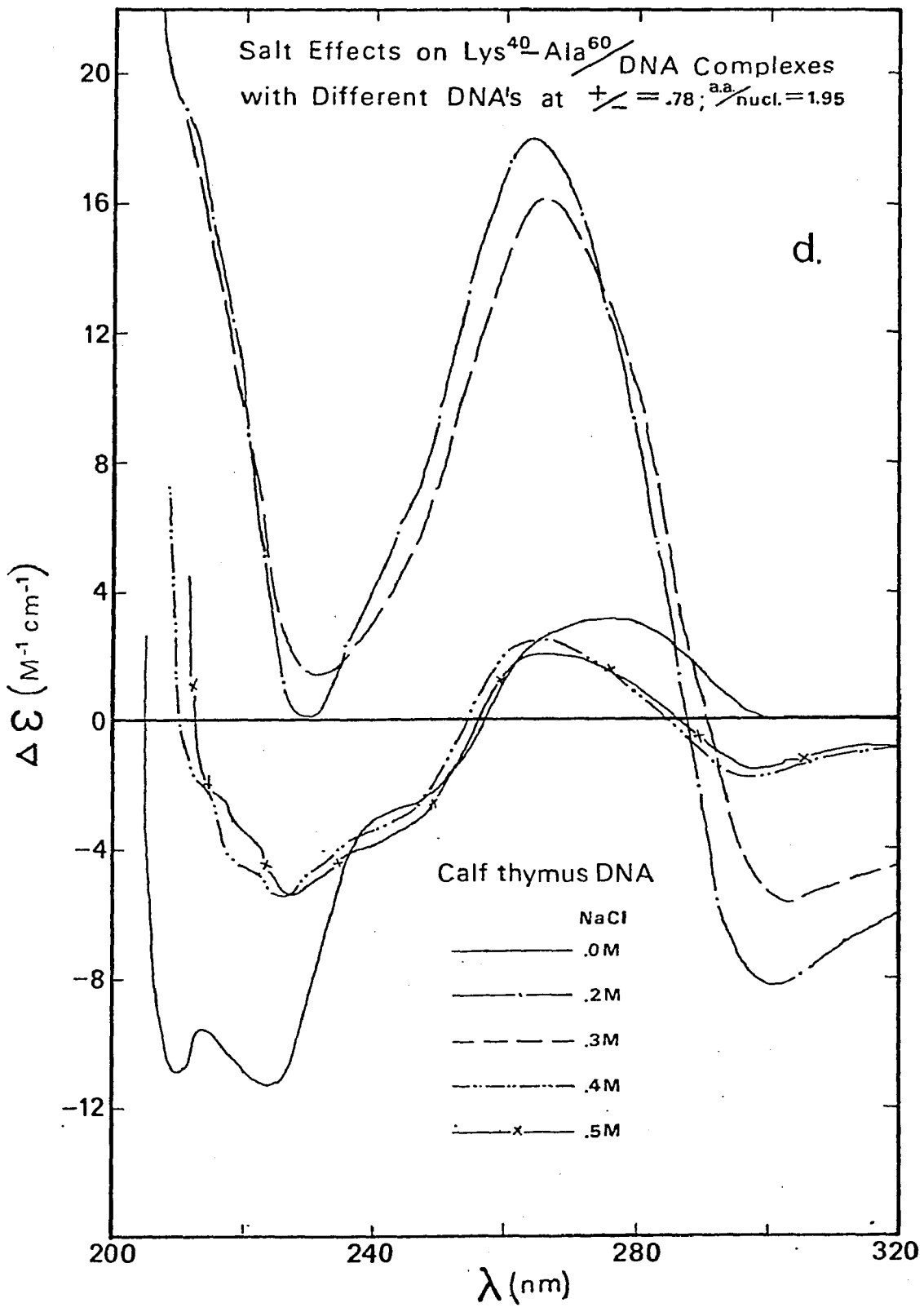

Salt Effects on Lys40-Ala60/DNA Complexes
with Different DNA's at +/− = .78; a.a./nucl. = 1.95
d.
Calf thymus DNA
NaCl
.0M
.2M
.3M
.4M
.5M
Δε (M⁻¹ cm⁻¹)
λ (nm)
20
16
12
8
4
0
−4
−8
−12
200
240
280
320

Fig. V-13,d

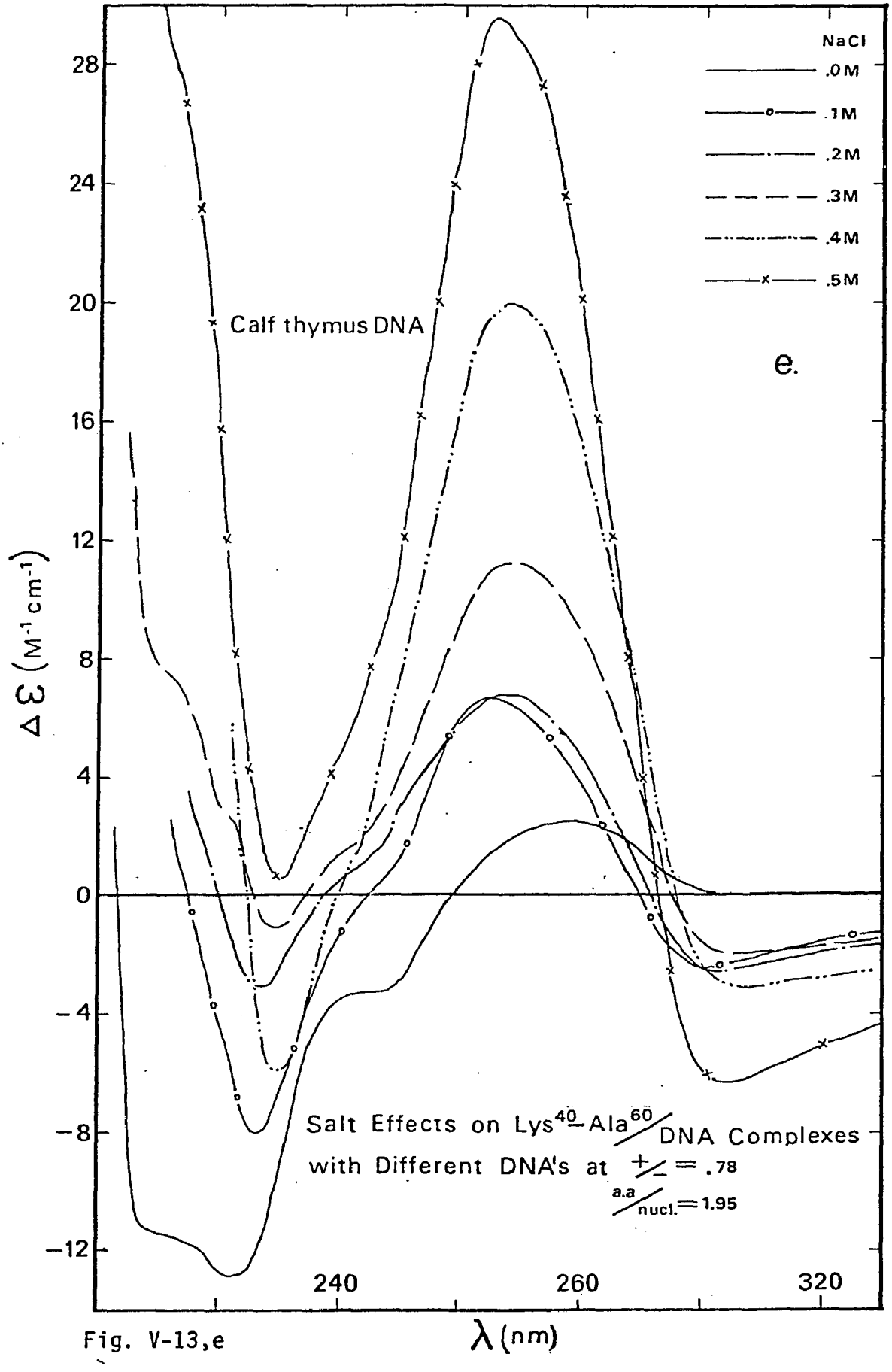

NaCl
.0M
.1M
.2M
.3M
.4M
.5M
Calf thymus DNA
e.
28
24
20
16
12
8
4
0
−4
−8
−12
Δε (M⁻¹cm⁻¹)
240
260
320
λ (nm)
Salt Effects on Lys40-Ala60/DNA Complexes
with Different DNA's at +/− = .78
a.a/nucl. = 1.95

Fig. V-13,e

Even at this low coverage (+/- = .26, r = .65) the calf thymus DNA has assumed the A-form characteristics: the pronounced negative peak at 300nm; the markedly enhanced, blue shifted, more pointed positive DNA peak; also, the negative protein peaks of the α-helix have disappeared, while the whole spectrum in this region has been elevated until only a small portion of the spectrum below 250nm remains negative; in addition, the crossover point is lowered to about 235nm from its normal position of 275nm.

At this degree of coverage, the relative effects of salt on the DNA's do not follow the course expected if percent G+C content is a dominant feature in production of the A-type conformation. Among these DNA, the order of G+C ranks as follows: M. luteus > E. coli > calf thymus > Cl. perfringens. Yet, in order of manifestation of the A-form, as shown at this degree of coverage, they rank: calf thymus >> M. luteus > Cl. perfringens > E. coli. It is interesting to observe, then, whether this same ordering holds for complexes of higher coverage.

At r = 1.3 (+/- = 0.52) the set of spectra depicted in Figure V-12 (a-d) show the same overall ordering in terms both of magnitude of effects and of characteristic evidence of A-type conformation, although the difference in magnitude between calf thymus and M. luteus is no longer so great. E. coli begins to assume the characteristic A-form shape, but has not yet evidenced an increase in magnitude. The other three DNA's all have pronounced negative peaks at 300nm, positive DNA peaks which are pointed, markedly enhanced, and blue shifted by from 10 to 15nm, and crossover points which are blue shifted by an equal amount. The negative peak or shoulder at 245nm, prominent in all DNA complexes at zero salt, has been largely or totally eliminated with

these three DNA's whereas it is still a contributing feature of the E. coli curve. The most noticeable characteristic of bound DNA's which are committed to A-form by salt at this degree of coverage is the differentiation, with each type of DNA, into just two basic types of spectra, a salt-form and a no salt-form. The amplitudes change with changing salt concentrations, but the basic salt-form at this degree of coverage is unchanged, in marked contrast to the variation in curve shape observed with change in salt at r = .65 (+/- = .26) (compare with Figure V-11 a-d).

At r = 1.95 (+/- = .78) there is a sudden shift in the ranking of DNA's with respect to A-form manifestation in the presence of salt. The order now becomes: calf thymus > M. luteus > E. coli > C. perfringens. In Figure V-13a, complexes of Cl. perfringens are shown to have lost their major A-form characteristics to a startling degree, whereas, in Figure V-13b, E. coli spectra have begun to acquire those same qualities, amplified in the DNA positive peak over 3 1/2 times. This DNA, now, has developed a "salt-form", "no salt-form" pair of spectra, though its salt-form shows greater variation than those of the r=1.3 (+/- = .52) complexes. The M. luteus composite spectrum (Figure V-13c), on the other hand, shows an intensification of the salt, no salt-forms, with little variation in its greatly increased positive peak at all concentrations of salt.

Different samples of complexes at r = 1.95 (+/- = .78) coverage of calf thymus DNA by the same copolymer showed such wide variation in composite spectra at different salt concentrations that two examples are shown. In one instance (Figure V-13d), the amplitude of the positive peak in 0.2 and 0.3M NaCl is very little greater than that of

M. luteus, while at higher concentrations the structure appears to have collapsed, in somewhat the same way as was evidenced by all the complexes of Cl. perfringens with r = 1.95 (+/- = .78). In the other case (Figure V-13e), with increasing salt, the positive peak expands progressively upward to a $\Delta\varepsilon$ of almost 30 ($M^{-1}cm^{-1}$), or somewhat less than twice that of the maximum M. luteus peak. Perhaps these conflicting results can be explained in terms of competing forces responsible for the conformation, or in terms of differences in the average composition of the random copolymer sample, or perhaps both. In both calf thymus examples, the initial response to salt is characteristic and greater in amplitude than with any other type of DNA, regardless of its G+C content.

Studies in 0.2M NaCl

In the above studies with different types of DNA, the salt concentration which most closely approximates physiological conditions is 0.2M NaCl. Therefore, complexes at this molarity of NaCl were analyzed in greater detail and approached from several different angles. Comparisons were made of results with complexes made in zero salt and dialyzed to 0.2M NaCl against those complexed directly in salt, with both reactants in solutions of 0.2M NaCl prior to complexing. Spectra were taken immediately after complexing, after several hours had elapsed, and again after 24 hours, to determine whether both fast and slow reactions were involved in development and maintenance of the A-type conformation.

After recording their spectra in salt, complexes were dialyzed back to EDTA buffer and checked by thermal denaturation to make certain that all the salt ions had been removed. (The Tm is highly sensitive to the

FIGURES

Different DNA's Complexed with Poly($Lys^{40}Ala^{60}$) (cont.)

Dialysis into 0.2M NaCl

V-14 CD Spectra of Complexes at Same r-value with DNA's of Different (G+C) Content
- a. r = 0.65, +/- = .26
- b. r = 1.3, +/- = .52
- c. r = 1.95, +/- = .78
- d. uncomplexed DNA's

V-15 Effect of Changing r-values on CD of Complexes with Different DNA's
- a. Cl. perfringens
- b. E. coli
- c. M. luteus
- d. Calf thymus

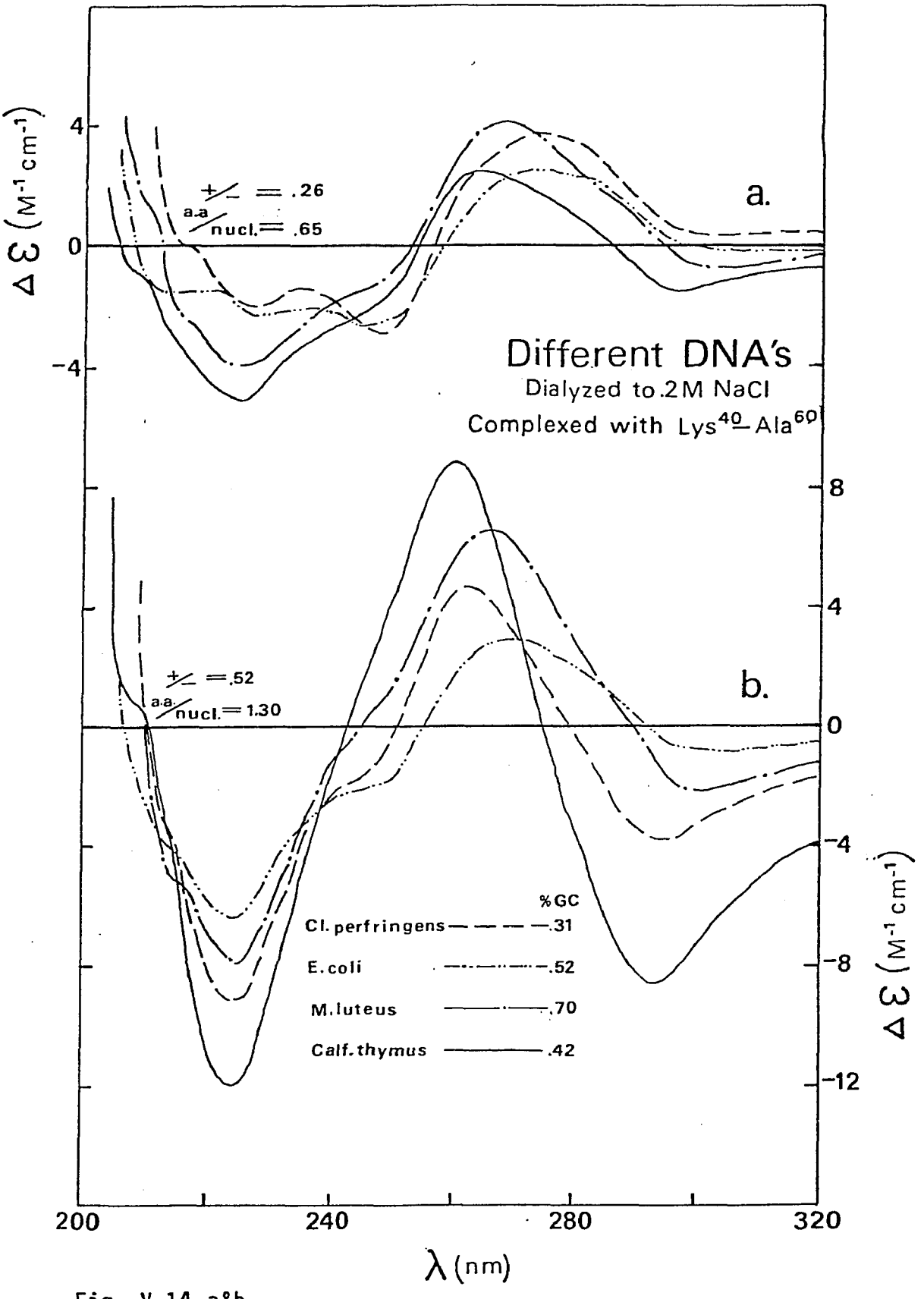

Δε (M⁻¹cm⁻¹)
4
0
-4
+/- = .26
a.a/nucl. = .65
a.
Different DNA's
Dialyzed to .2M NaCl
Complexed with Lys⁴⁰-Ala⁶⁰
8
4
0
-4
-8
-12
+/- = .52
a.a/nucl. = 1.30
b.
%GC
Cl. perfringens — — — .31
E. coli .52
M. luteus .70
Calf. thymus .42
200
240
280
320
λ (nm)
Δε (M⁻¹cm⁻¹)

Fig. V-14, a&b

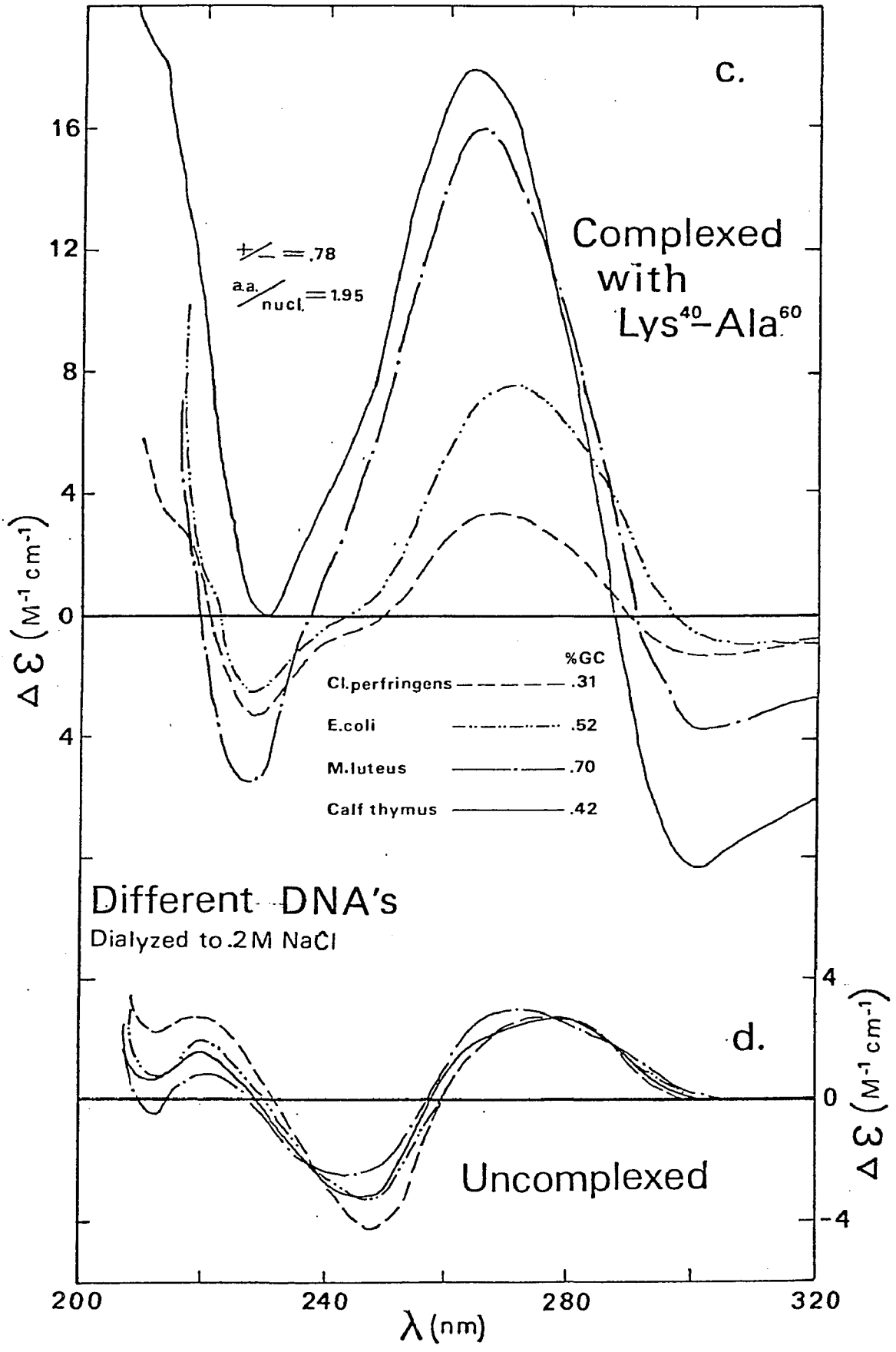
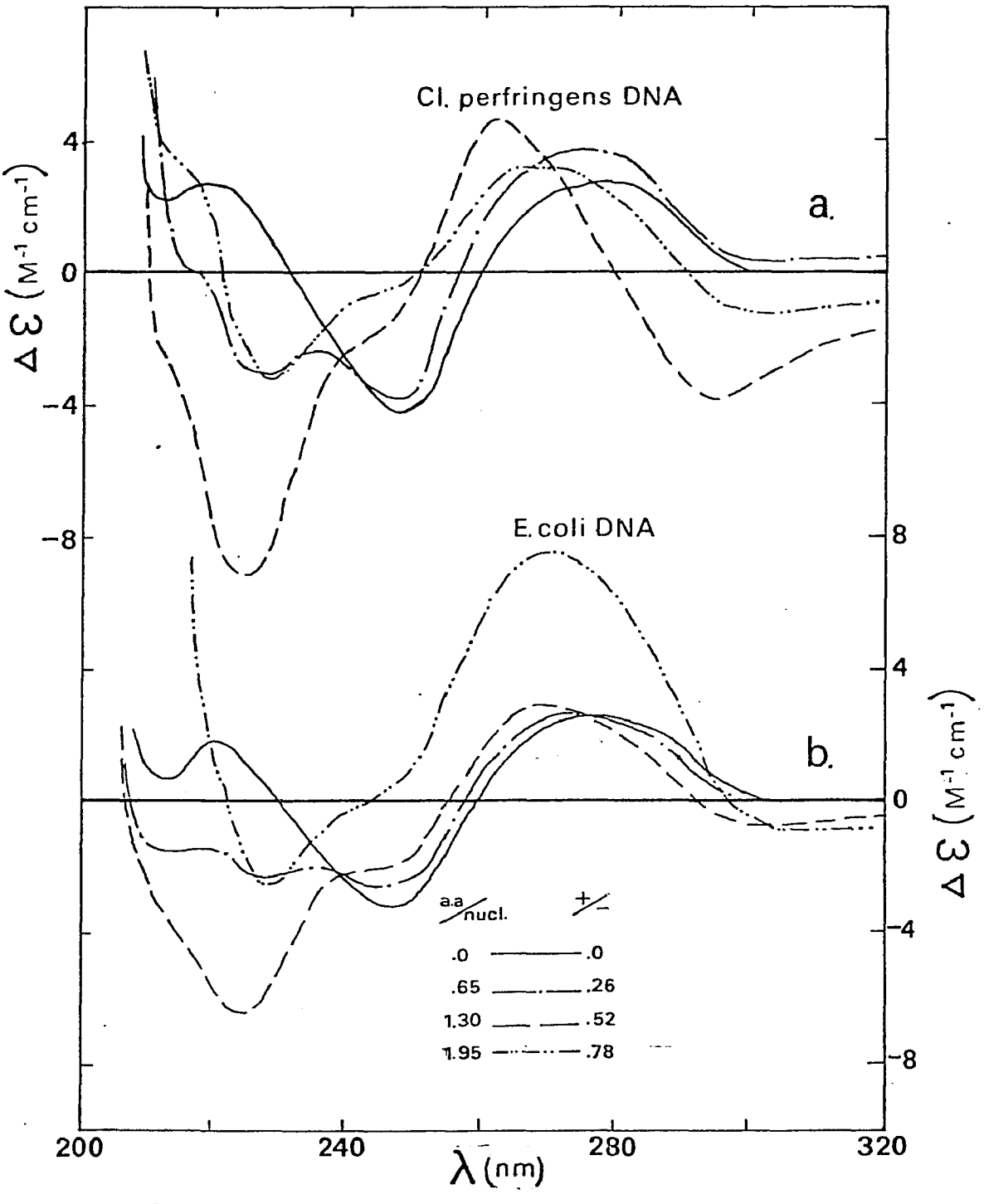

c.
Complexed with Lys40–Ala60
+/− = .78
a.a./nucl. = 1.95
%GC
Cl.perfringens .31
E.coli .52
M.luteus .70
Calf thymus .42
Different DNA's
Dialyzed to .2M NaCl
d.
Uncomplexed
Δε (M-1 cm-1)
λ (nm)
200
240
280
320
16
12
8
4
0
4
4
0
-4

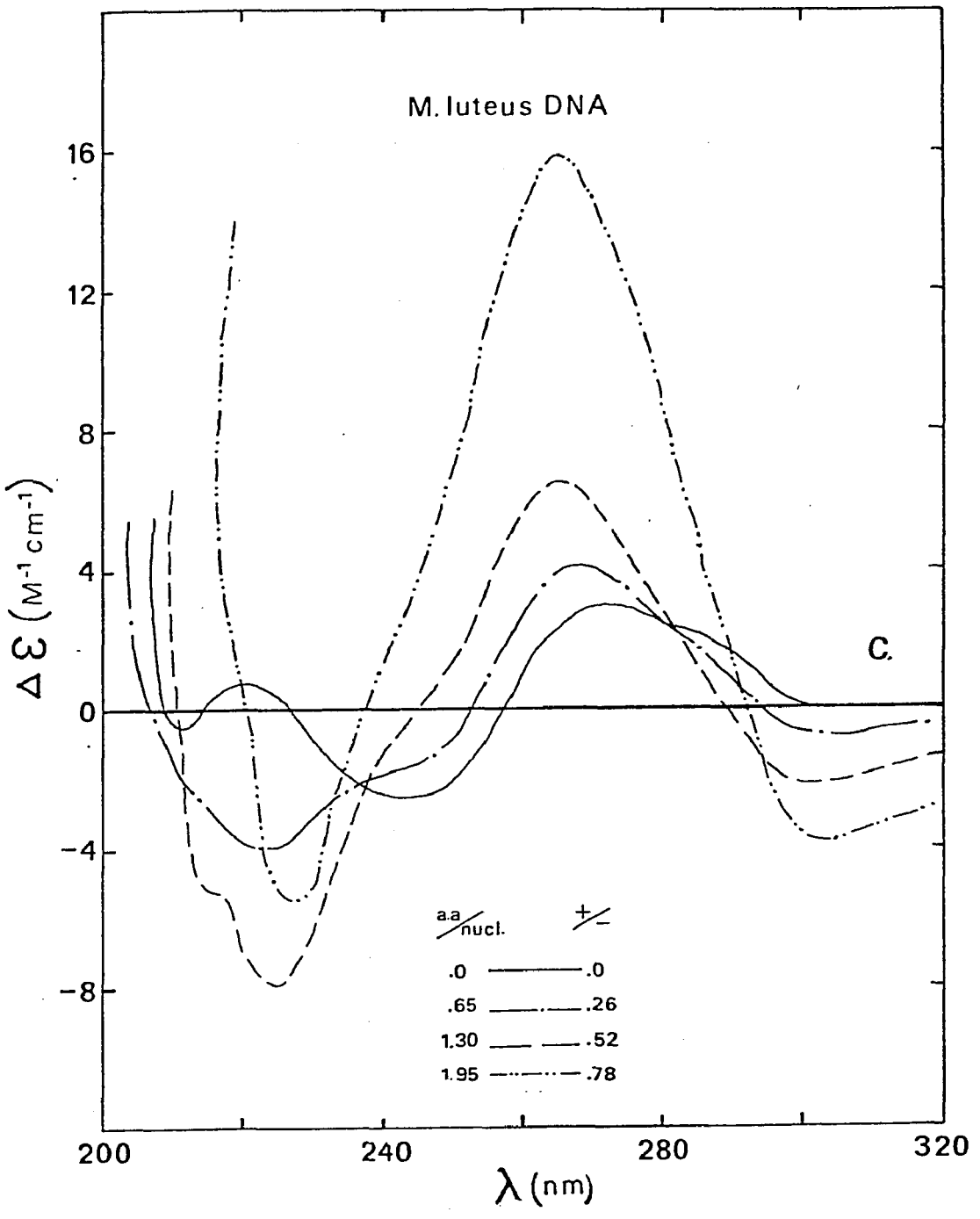
Fig. V-14,c&d



Lys^{40}–Ala^{60}/DNA Complexes with Different r-values

Dialyzed to .2M NaCl

Fig. V-15,a&b



Lys^{40}–Ala^{60}/DNA Complexes with Different r-values

Dialyzed to .2M NaCl

Fig. V-15,c

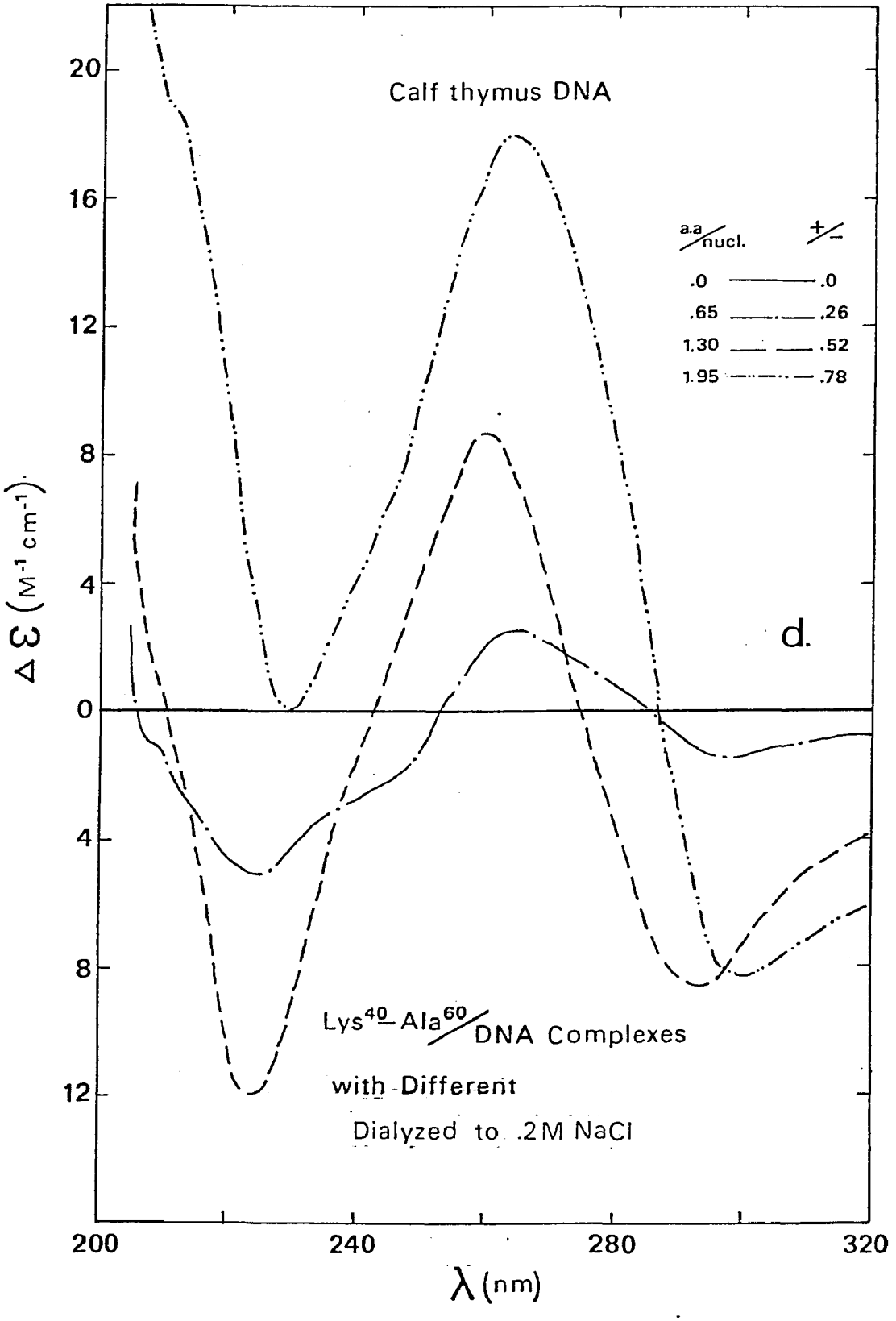

Calf thymus DNA
a.a/nucl.
+/−
.0 —— .0
.65 —·— .26
1.30 — — .52
1.95 —··—·— .78
d.
Lys40–Ala60/DNA Complexes
with Different
Dialyzed to .2M NaCl
Δε (M⁻¹ cm⁻¹)
λ (nm)
20
16
12
8
4
0
4
8
12
200
240
280
320

Fig. V-15,d

presence of ions, and shows marked elevation if salt removal has not been complete.) The CD spectra were then run again, to see if the complexes would retain their A-form in the absence of salt or would revert to their no-salt conformation.

The effect of temperature increase on the CD of A-form samples complexed in 0.2M NaCl was also studied, through use of a jacketed cell in the spectropolarimeter with circulation of ethylene glycol and recording of the CD spectrum at different stabilized temperatures.

For complexes dialyzed into 0.2M NaCl, the set of graphs in Figure V-14 a-d compares the salt effect at different degrees of coverage as a function of DNA type. Figure V-14 a-c show differences in the type of response elicited from each type of DNA when the same amount of copolypeptide is added; Figure 14 d shows the uncomplexed DNA's in 0.2M NaCl. Comparison of the graphs with each other facilitates evaluation of the quantitative response to salt by each DNA as a function of degree of binding; comparisons within the set of Figure V-15 a-d illuminate the same question, by holding steady with each graph the particular DNA and varying the r value.

When complexing was to be carried out only after conformations of both copolypeptide and DNA had been equilibrated in salt solution, the CD spectrum of each reactant was first recorded in both the absence and presence of 0.2M NaCl (Figure V-1). Usually, the spectrum of calf thymus DNA in salt of this concentration changes only slightly from that of zero salt but it may change up to the amount shown.

Both Complex I, in Figure V-17 a-c and Complex II, in Figure V-18, are sets of calf thymus DNA complexes made in 0.2M NaCl. Both sets of

FIGURES

Poly($Lys^{40}Ala^{60}$)·Calf Thymus DNA

Complexed in 0.2M NaCl

V-16 CD Spectra of Uncomplexed Copolypeptide and DNA in 0.0M and 0.2M NaCl

V-17 Complex I. Change in CD with Changing r-value at Different Times After Complexing
- a. at time of complexing
- b. 4 hours later
- c. after 24 hours

V-18 Complex II. Change in CD with Changing r-value

V-19 After Dialysis Back to EDTA Buffer
- a. CD Spectra
- b. Derivative Melting Profiles 4 days after Dialysis

V-20 Contribution of Light Scattering at Different Degrees of Coverage

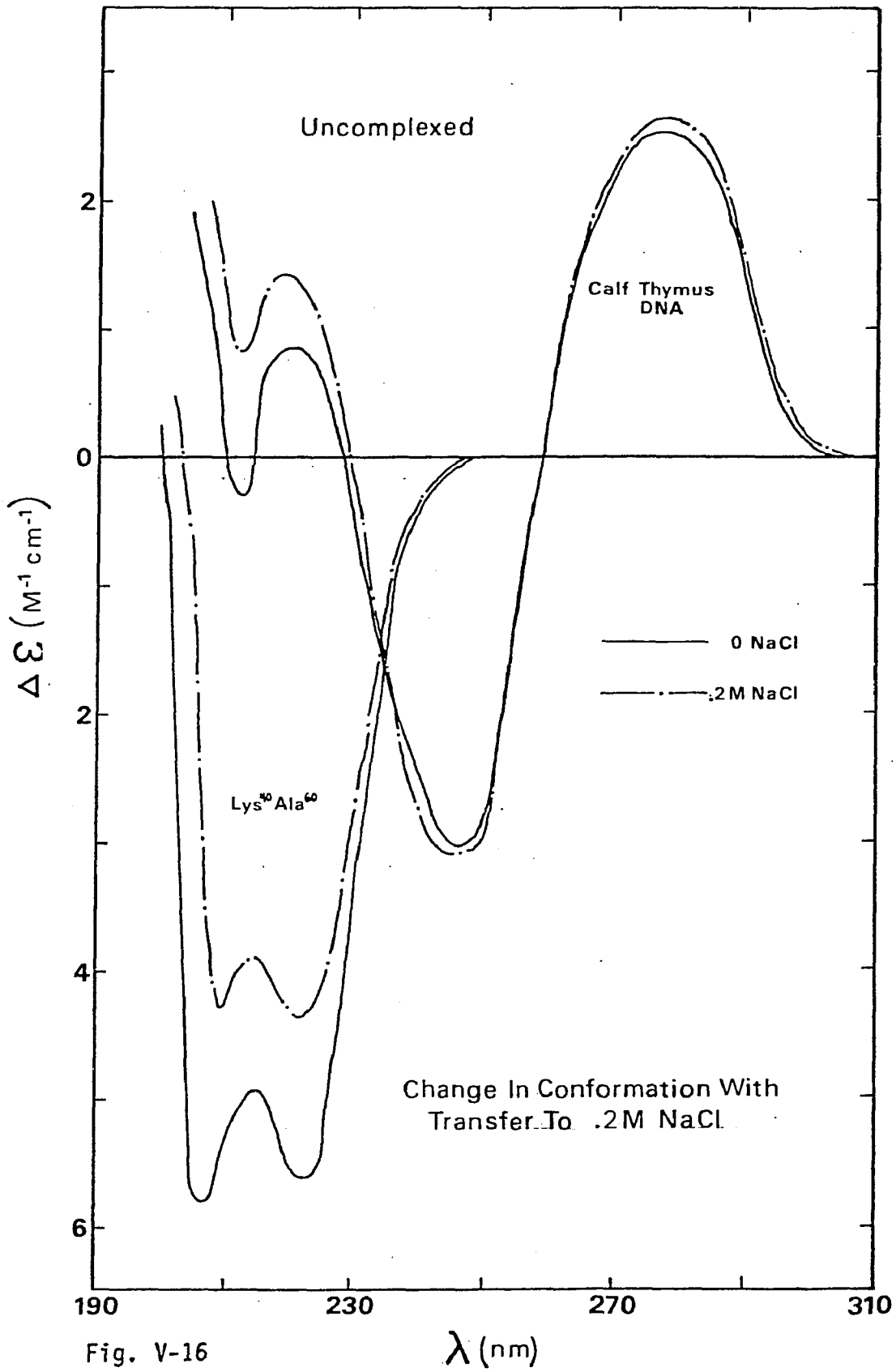

Uncomplexed
Calf Thymus DNA
0 NaCl
.2M NaCl
Lys^{40}Ala^{60}
Change In Conformation With Transfer To .2M NaCl
2
0
2
4
6
190
230
270
310
$\Delta\varepsilon$ ($M^{-1}cm^{-1}$)
λ (nm)

Fig. V-16

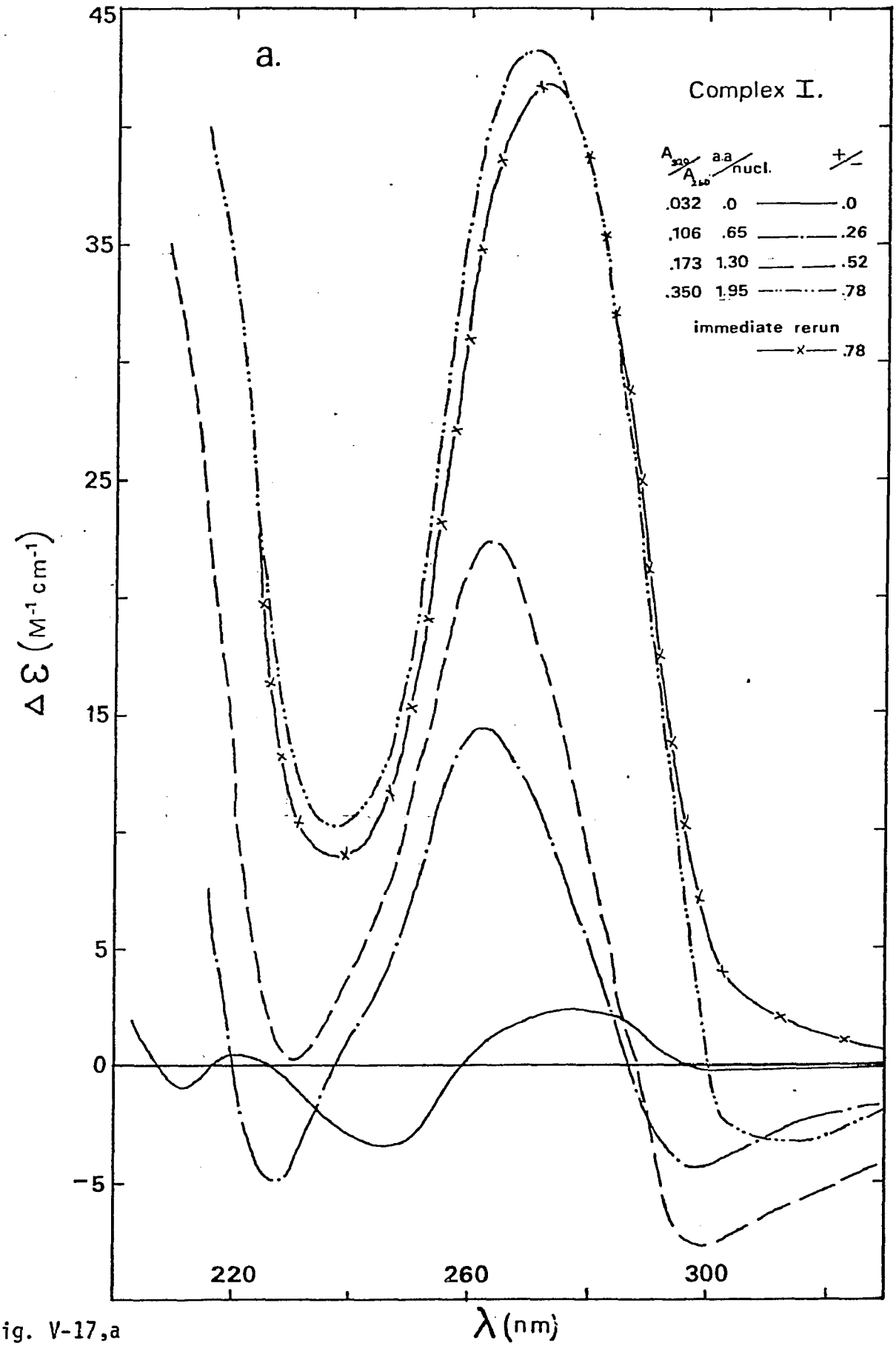

a.
Complex I.
A330/A260 a.a/nucl. +/−
.032 .0 .0
.106 .65 .26
.173 1.30 .52
.350 1.95 .78
immediate rerun
.78
45
35
25
15
5
0
−5
Δε (M⁻¹ cm⁻¹)
220
260
300
λ (nm)

Fig. V-17,a

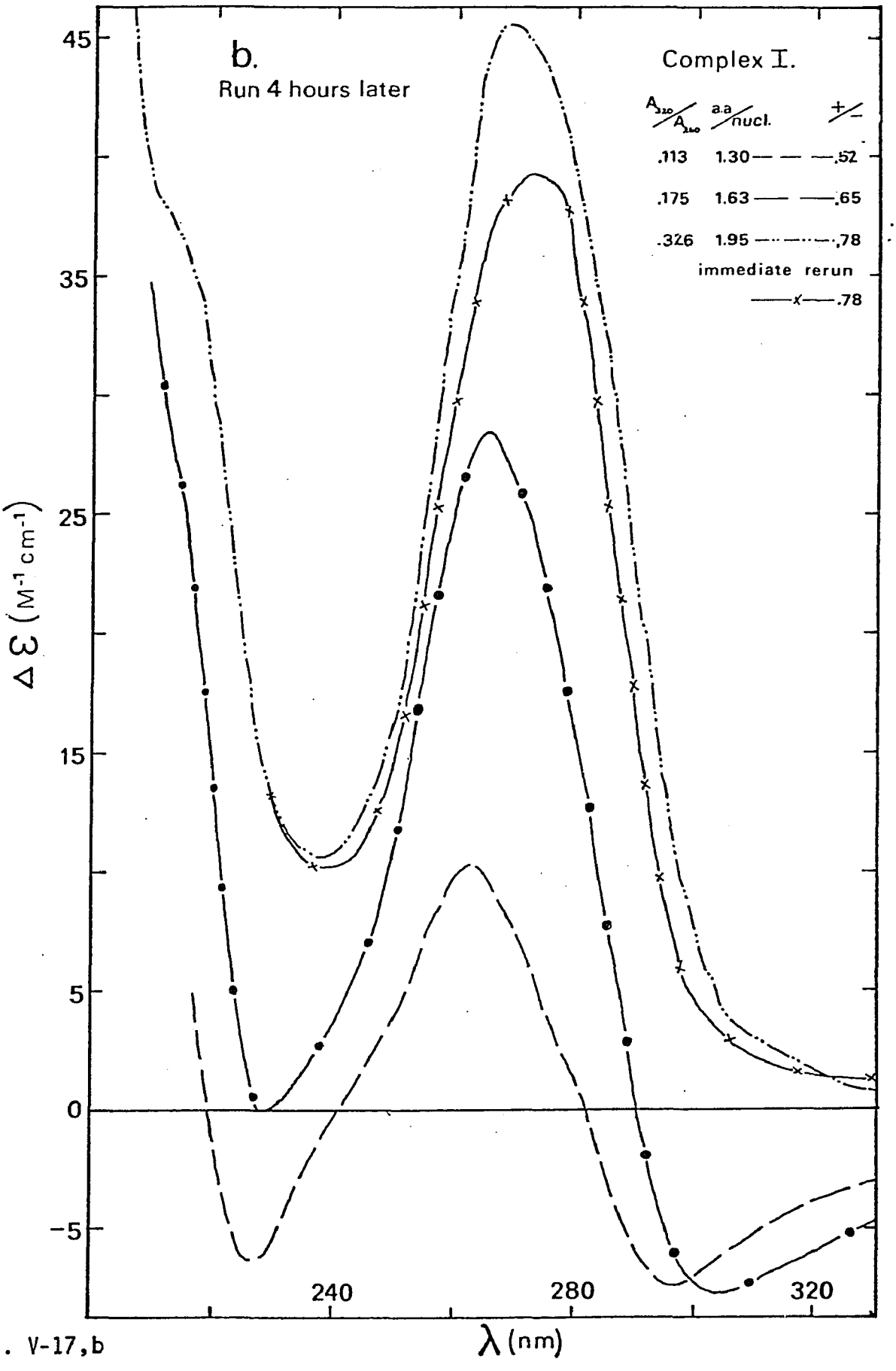

b.
Run 4 hours later
Complex I.
A320/A260 a.a/nucl. +/−
.113 1.30 — — — .52
.175 1.63 —— —— .65
.326 1.95 —·—·—·— .78
immediate rerun
——x—— .78
45
35
25
15
5
0
−5
Δε (M⁻¹ cm⁻¹)
240
280
320
λ (nm)

Fig. V-17,b

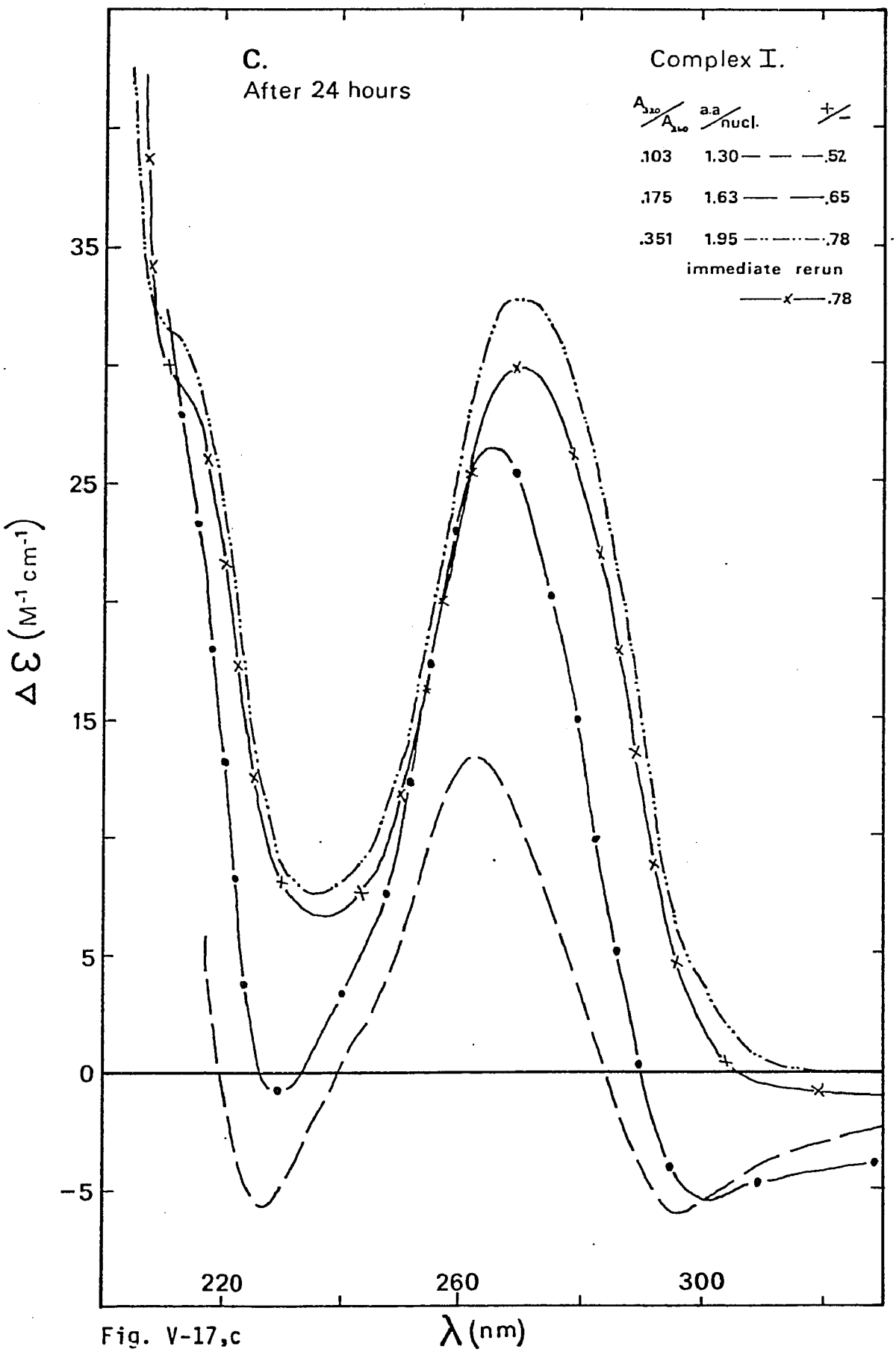

C.
After 24 hours
Complex I.
A320/A260 a.a/nucl. +/−
.103 1.30 —— —— —— .52
.175 1.63 —— —— .65
.351 1.95 —— ·—·—· .78
immediate rerun
—— x —— .78
35
25
15
5
0
−5
Δε (M⁻¹ cm⁻¹)
220
260
300
λ (nm)

Fig. V-17,c

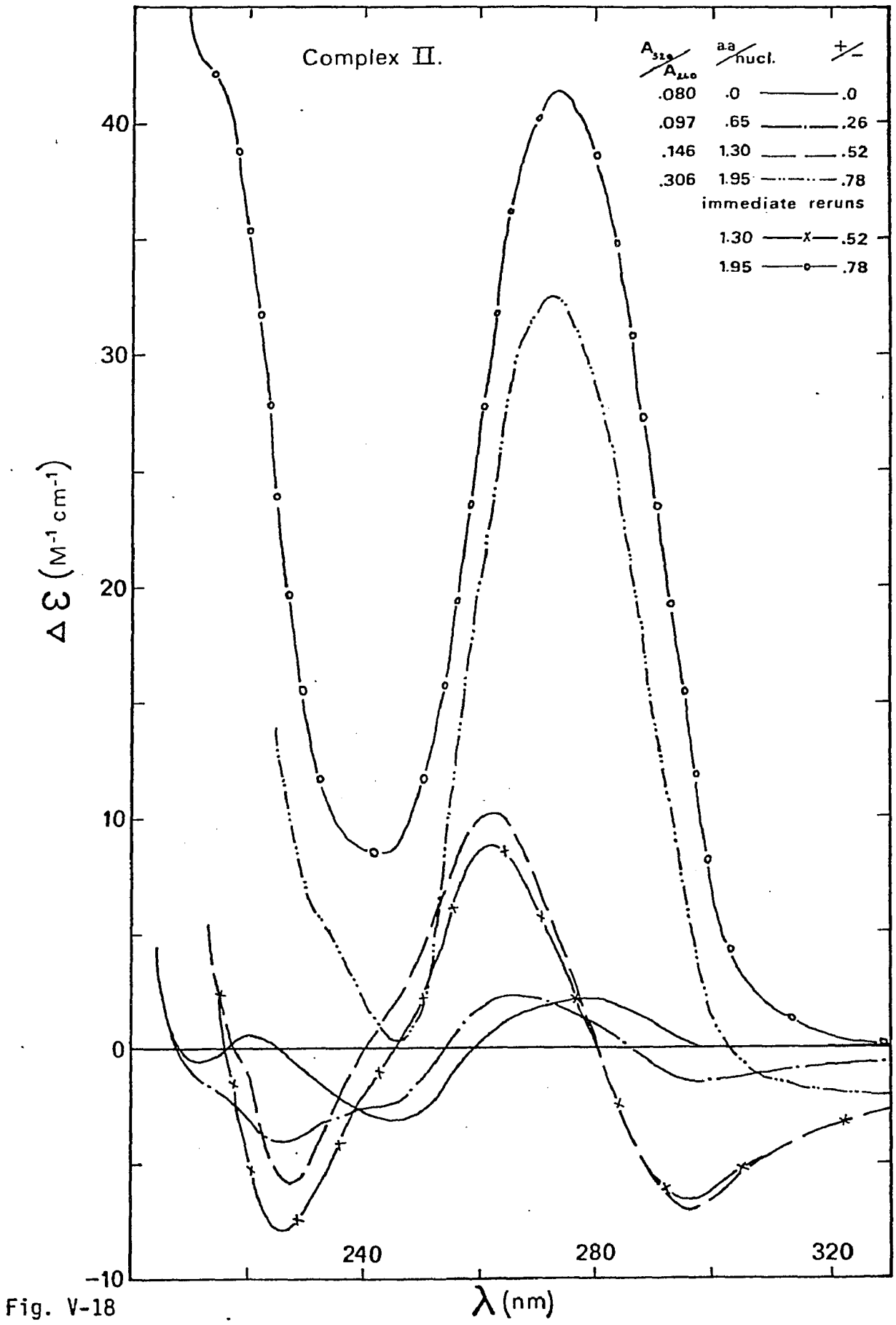

Complex II.
A 325 / A 440
a.a / nucl.
+/-
.080 .0 .0
.097 .65 .26
.146 1.30 .52
.306 1.95 .78
immediate reruns
1.30 .52
1.95 .78
40
30
20
10
0
-10
Δε (M⁻¹ cm⁻¹)
240
280
320
λ (nm)

Fig. V-18

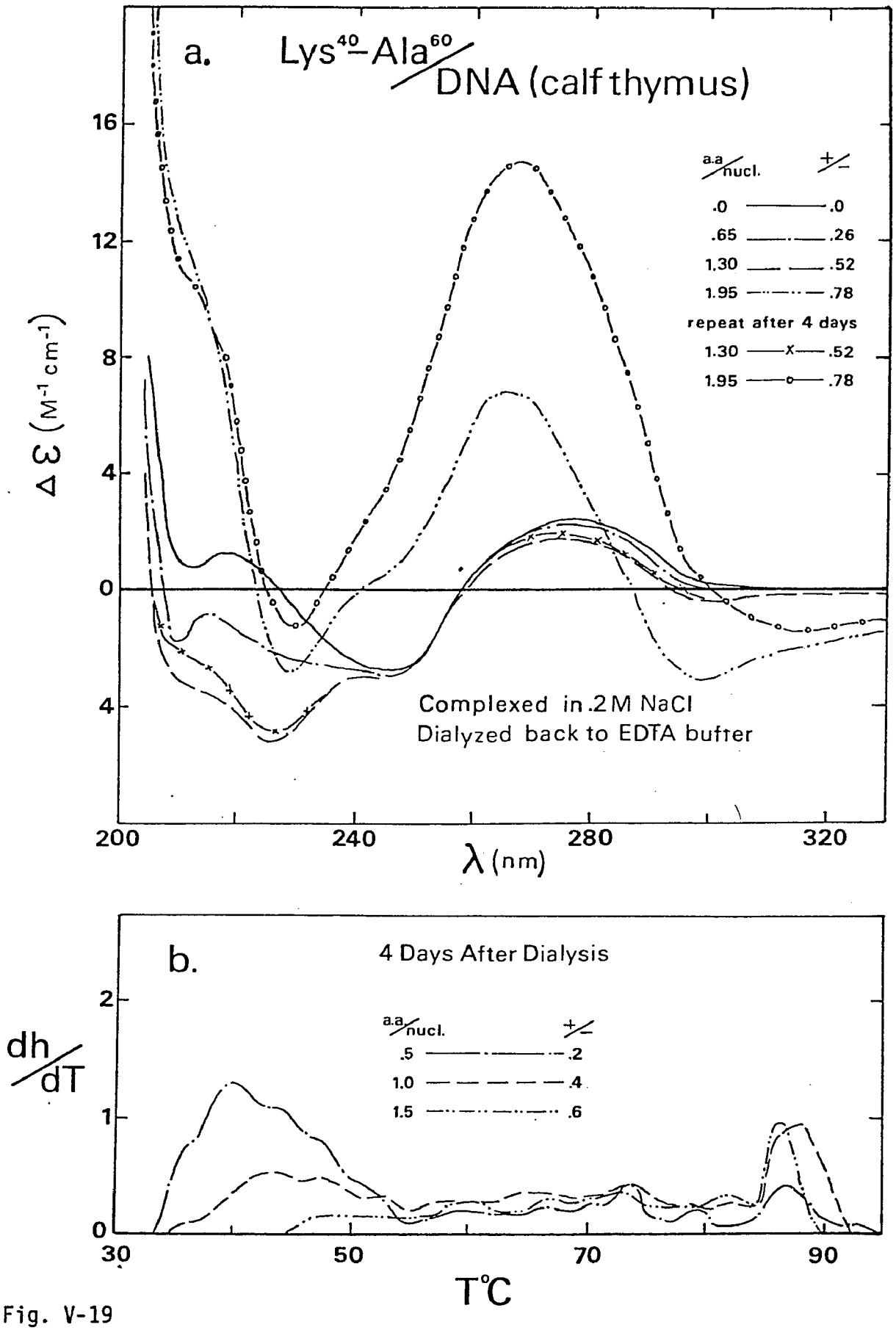

a.
Lys40–Ala60
DNA (calf thymus)
a.a/nucl.
+/−
.0 .0
.65 .26
1.30 .52
1.95 .78
repeat after 4 days
1.30 .52
1.95 .78
Complexed in .2M NaCl
Dialyzed back to EDTA buffer
Δε (M-1 cm-1)
λ (nm)
b.
4 Days After Dialysis
a.a/nucl.
+/−
.5 .2
1.0 .4
1.5 .6
dh/dT
T°C

Fig. V-19

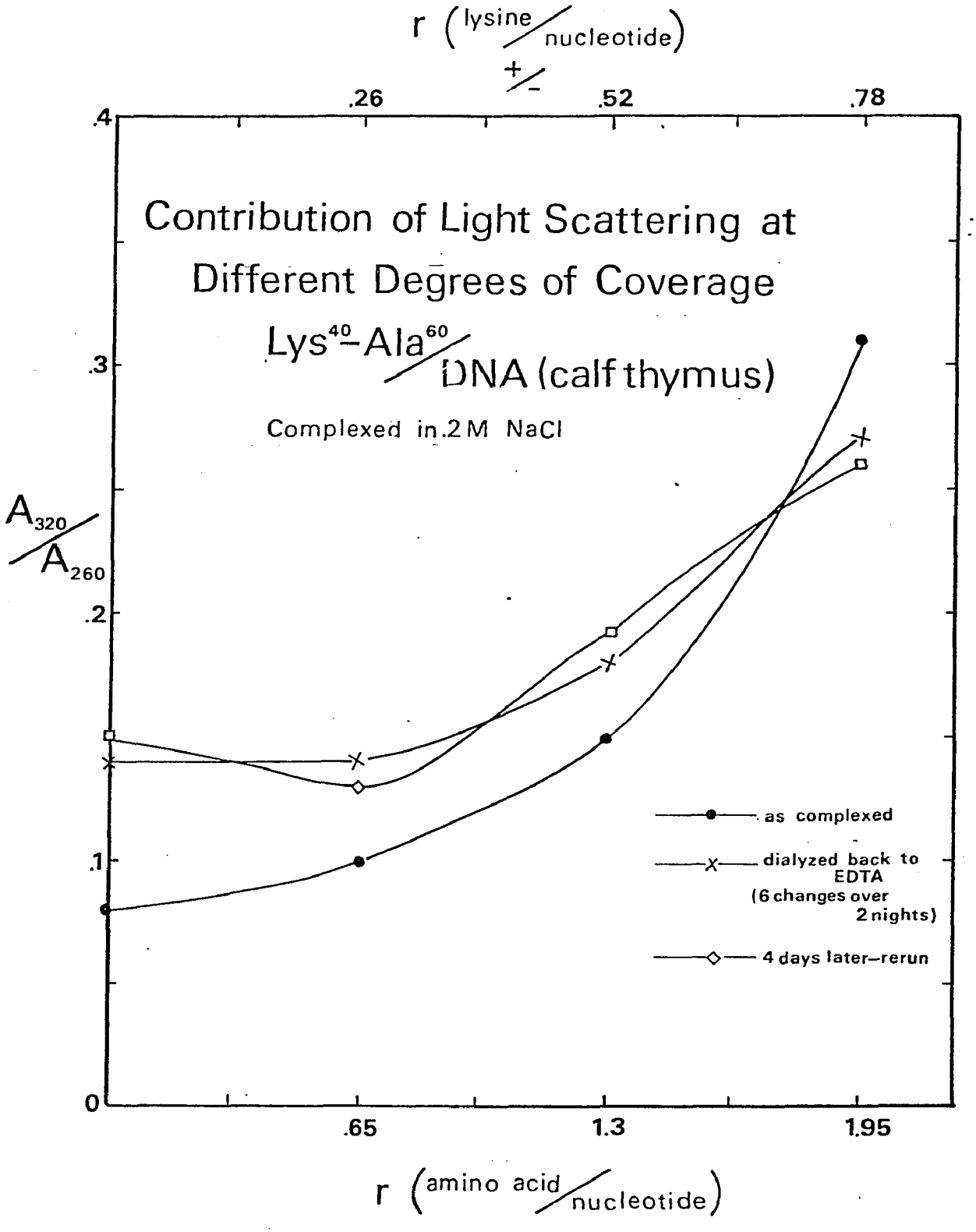

r (lysine/nucleotide)
+/−
.26
.52
.78
.4
.3
.2
.1
0
Contribution of Light Scattering at
Different Degrees of Coverage
Lys40–Ala60/DNA (calf thymus)
Complexed in .2 M NaCl
A320/A260
as complexed
dialyzed back to EDTA (6 changes over 2 nights)
4 days later—rerun
.65
1.3
1.95
r (amino acid/nucleotide)

Fig. V-20

complexes develop huge positive CD spectra of characteristic A-form at higher coverage; both were made with copolymer from the same stock solution and DNA from the same phenol extraction. The spectra of each were recorded shortly after complexing, with immediate re-runs for complexes of higher r value. As shown in both figures, the response upon complexing directly in salt is an immediate conformational change to the same general form expected with dialysis into 0.2M NaCl. The amplitudes of response, however, are very different. Neither DNA sample in the uncomplexed state shows much change in spectrum from that observed in zero salt, but there is a vast difference between sets of complexes in the magnitude of response at the highest degree of coverage. This was also true for the calf thymus complexes with r values of 1.95 (+/- = .78) at different salt concentrations (Figure V-13 d-e). There is one notable difference in the recorded spectra of these samples which were complexed in, rather than dialyzed into, 0.2M NaCl: the apparent kinetic effect. This is shown by changes in the spectral levels of the immediate re-runs, observable at all three extrema. There is not only a quantitative difference in the change observed with complexes of the same r value, there is also a directional change. In Complex I, the positive peak moves down slightly and shifts to the red; in Complex II, the wavelength of the peak remains constant, but the measured $\Delta\varepsilon$ moves upward by almost nine units during the period of thirty minutes between spectral runs. The net adjustment in both directions leaves the complexes finally adjusted to approximately the same level after the re-run.

The course of change was followed further with the set called Complex I, by recording spectra after four hours (Figure V-17b), and again after 24 hours (Figure V-17c). After fours hours, change in amplitude

of the positive peak continues; this time the re-run after half-an-hour interval shows a much greater decrease than before, although the initial run of the second round showed a slight increase over the initial run of the fresh complex. After 24 hours, the peak level of the complex with r = 1.95 (+/- = .78) has settled considerably from the level achieved when fresh (from a high of about 43 when fresh to a $\Delta\varepsilon$ of about 33), yet the intermediate complex with r = 1.63 (+/- = .65) has moved up during the same time scale. Similar unpredictable fluctuations possibly due to aggregation can be observed at 300nm, were, within certain boundaries, the negative peak changes shape and becomes positive. Characteristically, as this peak deepens, the positive peak tends to blue shift; as it moves upward into the positive realm, the position of the major positive peak may move back toward the red. With very large amplitudes, the crossover point below 250nm is completely eliminated, and what was once a negative peak rises on a grandiose scale to display a minimum far above the maximum level of the ordinary DNA spectrum.

Figure V-19 shows that after dialysis back to EDTA, over a period of two nights with six changes of dialysis buffer, the complex with highest coverage in the Complex II set was still able to maintain the A-form in the absence of salt. The amplitude of the spectrum was considerably diminished, but the irreversibility of the conformational change when the DNA is largely bound is undeniable. This proved to be equally true for complexes dialyzed into 0.2M NaCl and back again to EDTA. In both cases, the spectra after return to zero salt look essentially the same. That zero NaCl was actually achieved by dialysis is demonstrated in Figure V-19b, where both Tm and Tm' of the complexes are at temperatures lower than normal rather than elevated.

In the spectrum of the complex with r = 1.95 (+/- = .78) which was recorded a second time four days after removal from dialysis, again there is marked enhancement. This may indicate a partial increase in stability with cessation of dialysis, stability gained through a strengthening of the forces governing maintenance of the A conformation. It is interesting to note in the plot of A_{320}/A_{260} against r value, or input ratio (Figure V-20), that in the complex with r = 1.95 (+/- = .78) these values are not significantly changed four days after removal from dialysis, yet the amplitude of the positive CD peak is almost double. This means that aggregation and light scattering cannot be completely responsible for the enhancement. The curves, under all conditions of this experiment, show marked increase in the A_{320}/A_{260} ratio with increasing r value.

To determine the response of the A-form complexes to changes in temperature, samples complexed in 0.2M NaCl either with calf thymus or with E. coli DNA were studied by recording their CD spectra at different equilibrated temperatures and correlated with continuous thermal denaturation runs in which absorbance was recorded.

The circular dichroism results shown in Figure V-2a are numbered sequentially at increasing and decreasing temperature levels in a series of runs on one complex, so as to show progress in enhancement, or decrease, of each extremum. The positive peak at 270 and the minimum in the 230-240nm range both reach their highest point with the curve at 56.7°C (curve #4); the initial minimum at 300nm moves from negative to positive and continues to rise until 76.4°C (curve #6). Above these temperatures the intensities diminish, and by 89°C the spectrum closely

FIGURES

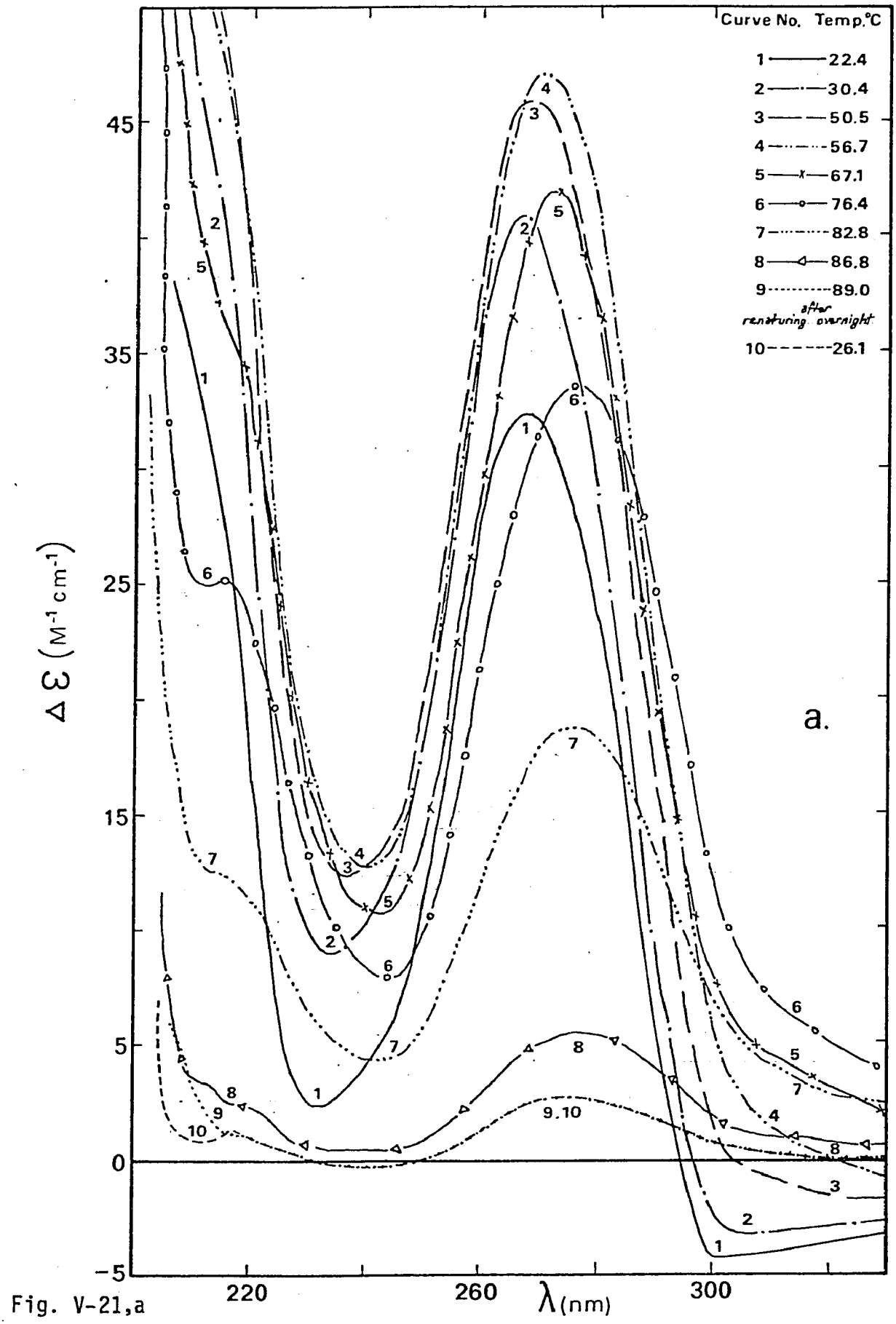

Curve No. Temp.°C
1 22.4
2 30.4
3 50.5
4 56.7
5 67.1
6 76.4
7 82.8
8 86.8
9 89.0
after overnight renaturing
10 26.1
a.
Δε (M⁻¹ cm⁻¹)
45
35
25
15
5
0
-5
220
260
300
λ(nm)

Fig. V-21,a

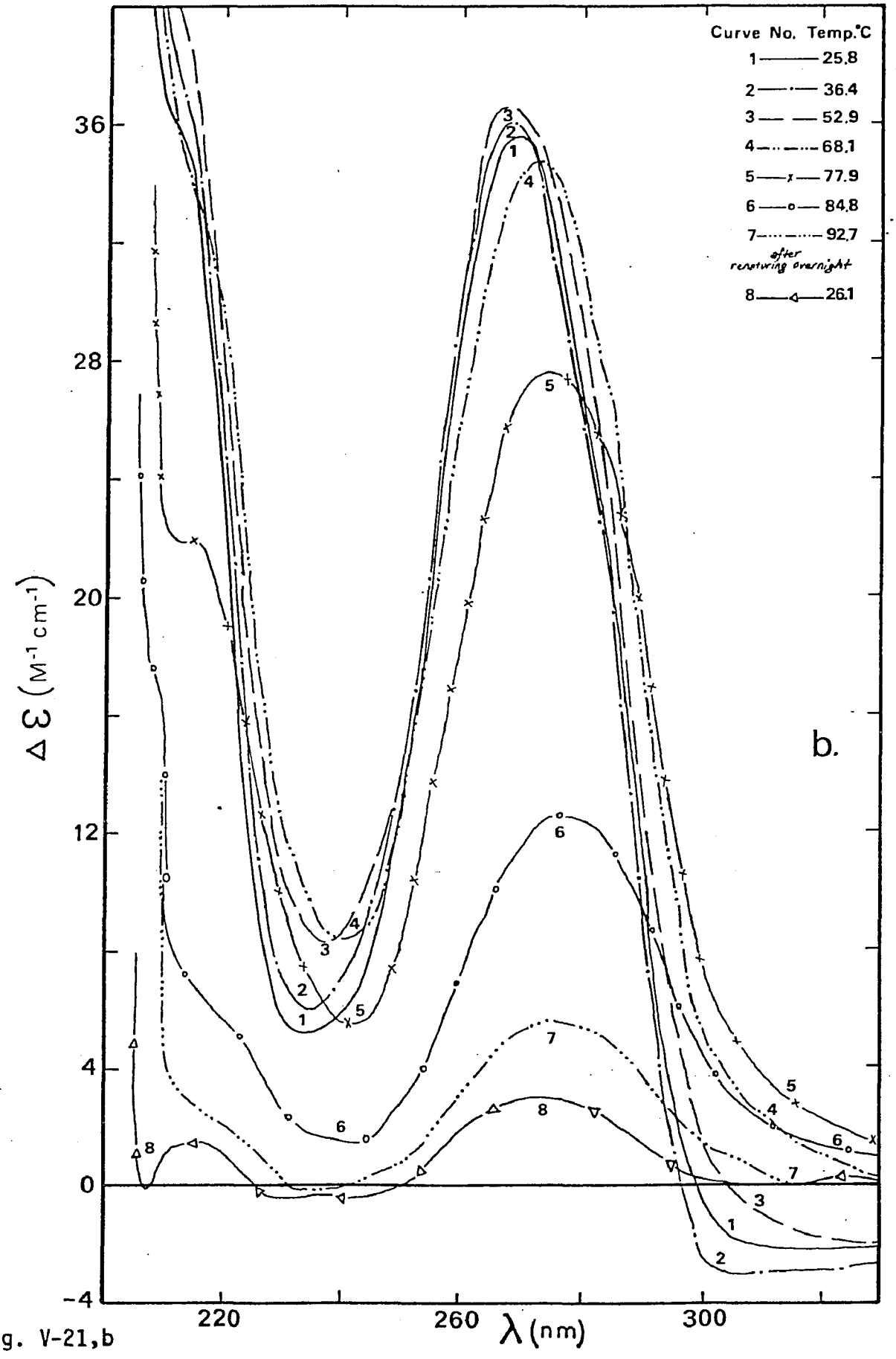

Curve No. Temp.°C
1 25.8
2 36.4
3 52.9
4 68.1
5 77.9
6 84.8
7 92.7
after renaturing overnight
8 26.1
b.
Δε (M⁻¹cm⁻¹)
36
28
20
12
4
0
-4
220
260
300
λ (nm)

Fig. V-21,b

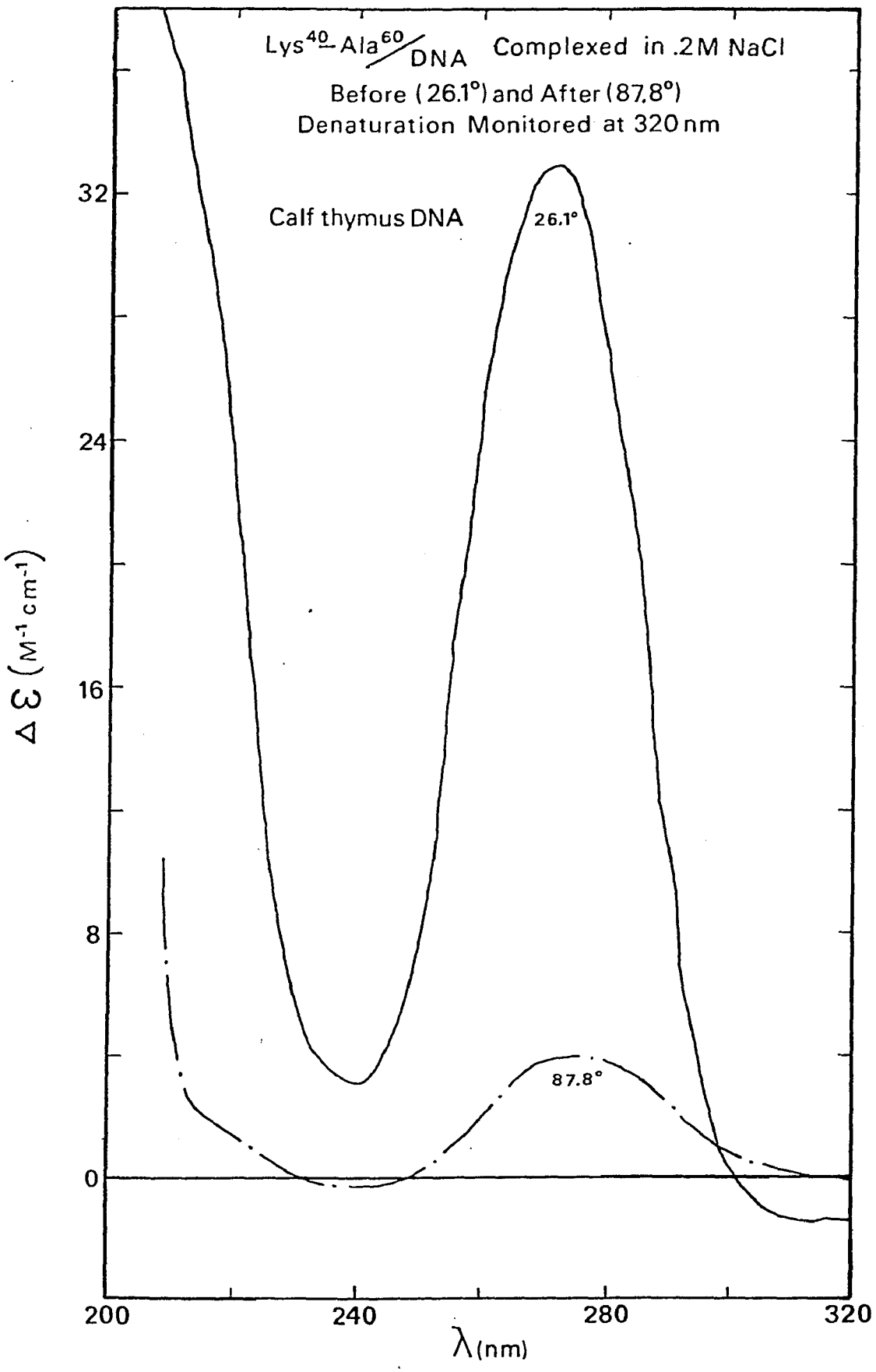

Lys40-Ala60/DNA Complexed in .2M NaCl
Before (26.1°) and After (87.8°)
Denaturation Monitored at 320 nm
Calf thymus DNA
26.1°
87.8°
32
24
16
8
0
200
240
280
320
Δε (M⁻¹ cm⁻¹)
λ(nm)

Fig. V-22

Thermal Denaturation of $Lys^{40}-Ala^{60}$/DNA (Calf thymus)
Complexed in .2M NaCl; Monitored at 320 nm

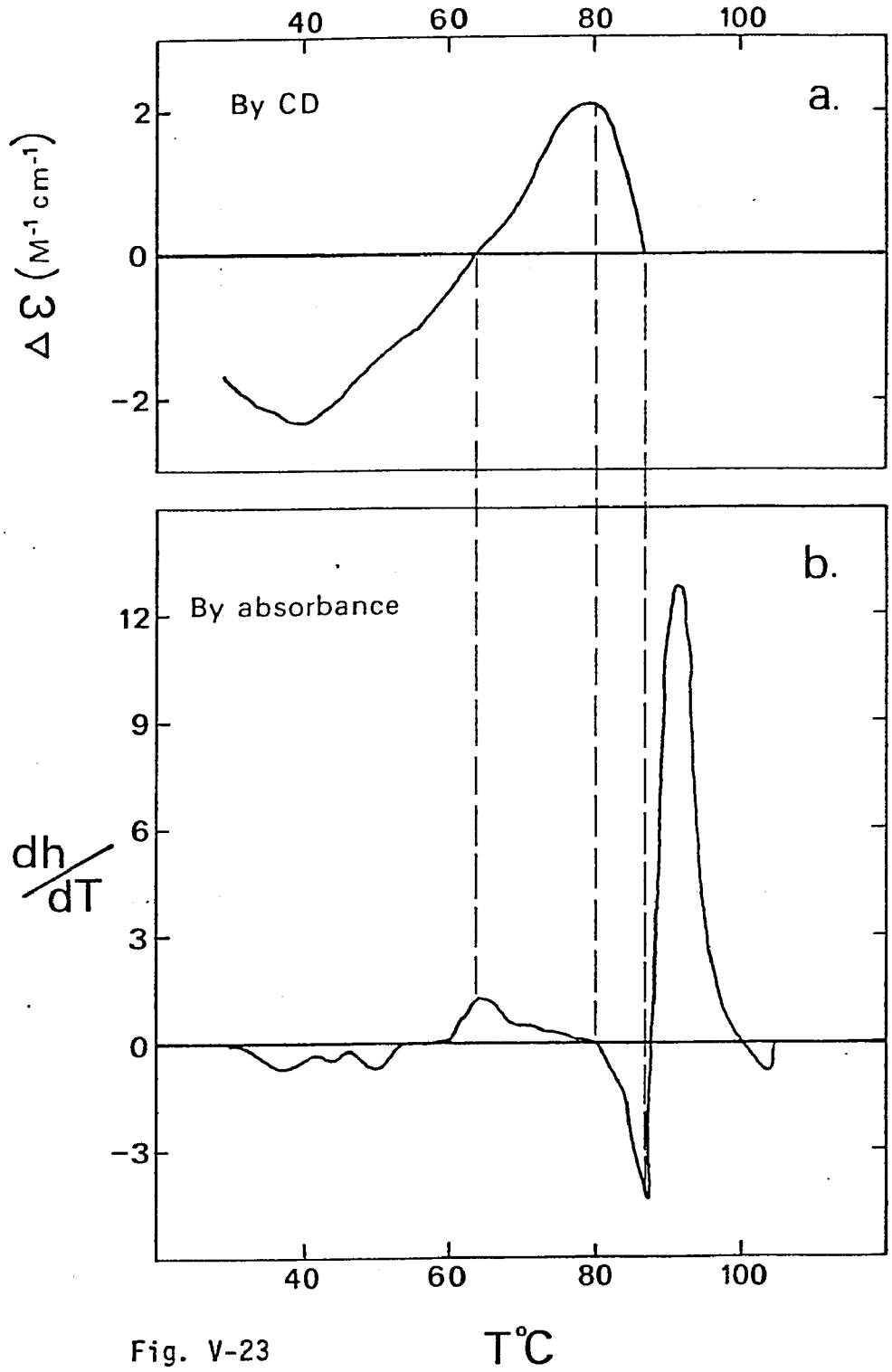


Fig. V-23

resembles that of uncomplexed DNA in salt. After cooling to 26.1°C the complex was allowed to renature overnight and the spectrum was taken again. No A-form was regenerated, and the spectrum remained unchanged.

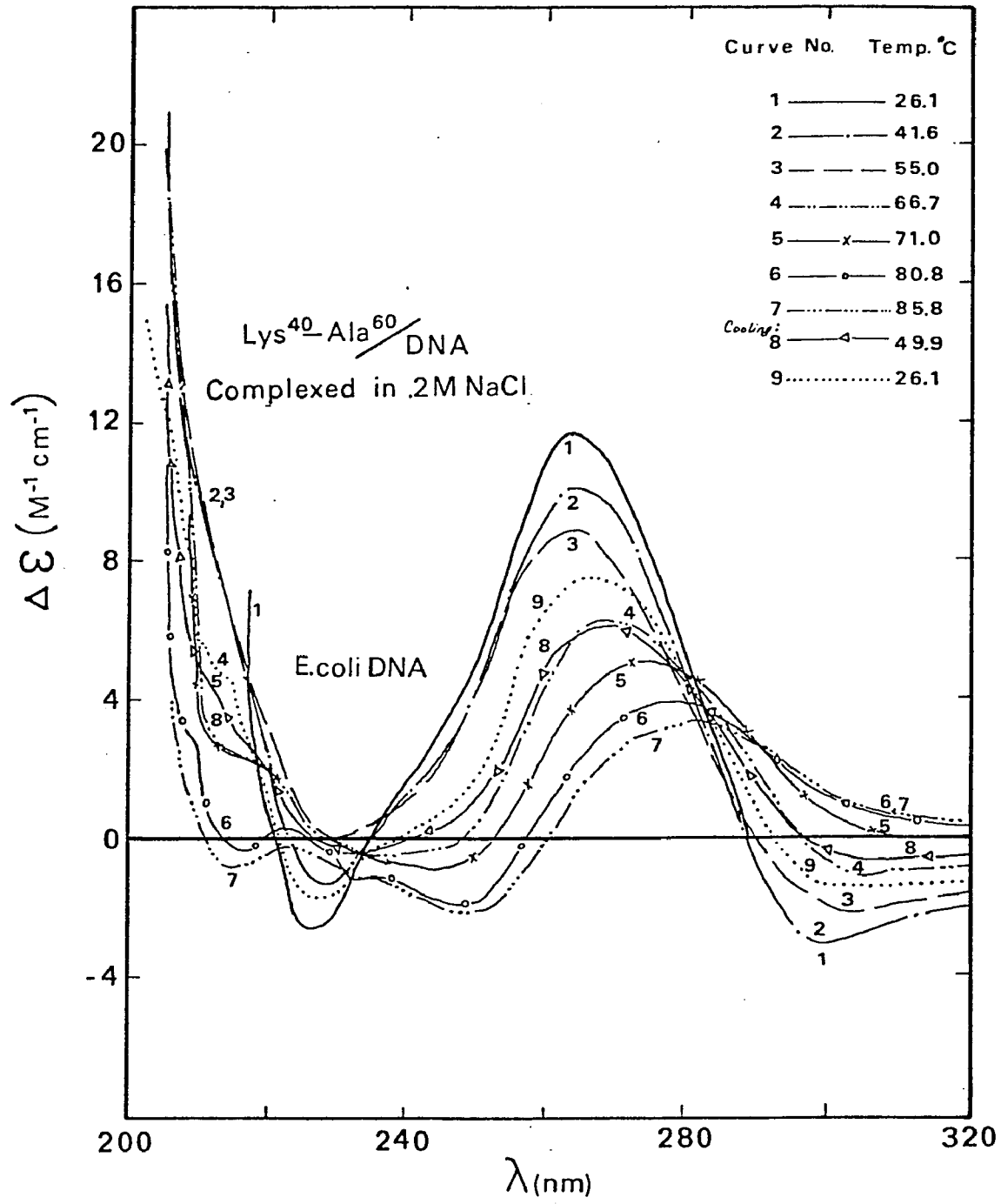
A fresh sample of the same complex was run again 24 hours later, with somewhat different results. Variation in the positive 270nm peak between room temperature and 60°C was reduced from a difference in $\Delta\varepsilon$ of 15 units increase (curves 1 and 4 in Figure C-2a) to that of one unit (curves 1 and 3 in Figure V-22). The highest point reached with the fresh was $\Delta\varepsilon = 47$; after 24 hours, it was only 37. In the same temperature range, variation in the 230-240nm minimum shows a difference in $\Delta\varepsilon$ of 11 units; after 24 hours, the difference is reduced to 3. Overall variation in the 300nm region shows a general decrease in intensity in going from the fresh to the 24 hour sample, but it follows much the same progression.

Two days after the initial complexing, fresh samples of the same complex were thermally denatured on the Gilford spectrophotometer, recording at 320nm the changes in absorbance with increasing temperature, and on the Jasco spectropolarimeter, recording the changes in the CD spectrum at the same wavelength. Since DNA does not absorb at 320nm, any changes reflect differences due to aggregation and light scattering. To make certain that the two-day-old complex still was representative of properties defined by the samples run earlier, the full CD spectrum was run before and after thermal denaturation on the 320nm run, during which the temperature was increased at a steady rate of 2/3°C per minute with the 320nm spectrum recorded continuously. Since the Jasco spectropolarimeter does not record temperature automatically, bath

temperature readings were taken periodically and corrected for heat loss en route to the cuvette by a previously determined calibration curve. Figure V-23a shows the CD curve defined by these readings, Figure V-23b, the recorded absorbance spectrum during denaturation. The two graphs are shown to be related by their extrema and crossover points. The most striking feature of the lower graph is the sharp dip at 87°C, corresponding to the CD zero point, followed by a precipitous rise and steep descent to zero at 100°C. Whether or not this feature represents the sudden aggregation and precipitation of protein molecules is uncertain. There is little evidence of protein structure in the CD spectrum after denaturation, but it is also apparent that there is DNA present in the solution. Further study should include centrifugation of the solution after denaturation and analysis of both pellet and supernatant to determine the composition of each.

The change in the circular dichroism spectra of E. coli complexes at various stages of thermal denaturation follows a different sequence. Results at various temperature levels are shown in Figure V-24, where sequential curves again are numbered. The A-form is defined most intensely by the very first curve, recorded at 26.1°C. After this, the positive peak decreases progressively as temperatures rise to 85.8°C (curve #7). At this temperature, the point of the peak has become rounded and its center red shifted from 262 to 278nm, the peak location of B-form DNA. The magnitude, however, is still somewhat enhanced. Cooling the complex to 49.9°C, after thermal denaturation up to 86°C, showed a marked increase in amplitude (curve #8); there was still further increase on return to room temperature (curve #9). Determination of Tm and Tm' (Figure V-25) of E. coli DNA in 0.2M NaCl, both

FIGURES



Progressive Change in Conformation with Change in Temperature

Fig. V-24

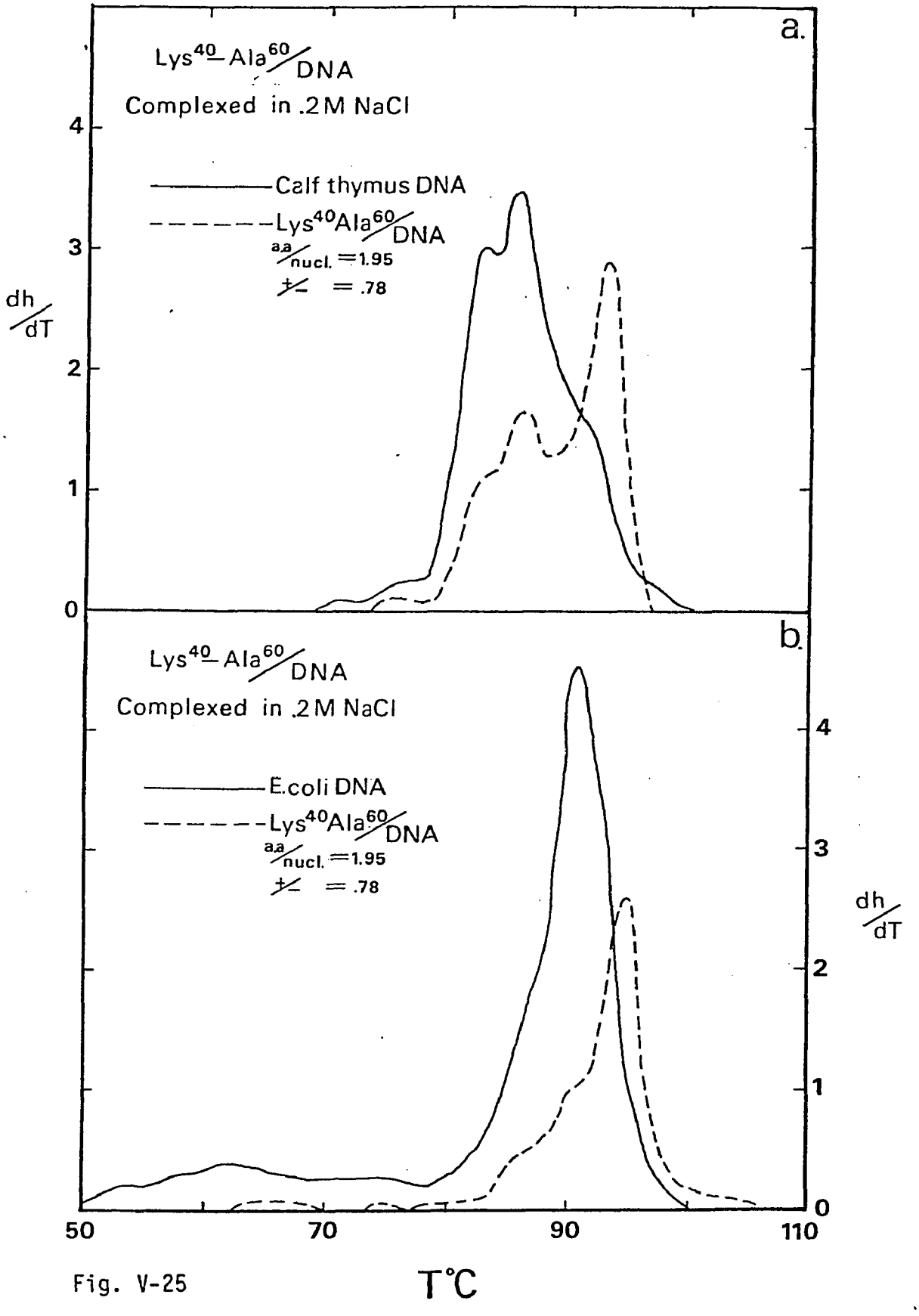

a.
Lys40-Ala60/DNA
Complexed in .2M NaCl
Calf thymus DNA
Lys40Ala60/DNA
aa/nucl. =1.95
+/- = .78
dh/dT
b.
Lys40-Ala60/DNA
Complexed in .2M NaCl
E.coli DNA
Lys40Ala60/DNA
aa/nucl. =1.95
+/- = .78
dh/dT
0
1
2
3
4
50
70
90
110
T°C

Fig. V-25

free and complexed with poly($Lys^{40}Ala^{60}$), yielded a Tm of 91°C and a Tm' of 95°C, indicating that neither point of denaturation was reached in the CD study. Therefore, the apparent partial renaturation of the E. coli complex after cooling does not necessarily denote a greater capacity for renaturation than that demonstrated by the calf thymus complex, wherein full denaturation was achieved before cooling. The progressive loss of intensity with heating, however, does indicate a difference in physical characteristics of the two types of DNA, as does the lower potential of E. coli for production of A-form CD spectra of huge dimension.

Discussion

These studies were designed to define and illuminate the various factors which contribute to the production and maintenance of the DNA conformation known as A-form. The circular dichroism spectra of A-, B-, and C-forms were first defined by Tunis-Schneider and Maestre[20] in studies relating the CD spectrum of DNA films to forms originally identified by X-ray, by reproducing as nearly as possible the conditions of humidity and ionic strength which produced those forms. Brahms and Mommaerts[62] reported observations of A-type DNA in 80% ethanol solutions of low ionic strength, and Ivanov et al[22], studied DNA and tRNA conformations, by varying the type of both solvent and salt ions.

From the studies by Ivanov and co-workers[22], it was reported that an A-like CD spectrum in 78% ethanol appeared to have only a slight dependence (if any) on the G+C content of the DNA, that it was not dependent on temperature in the range of stability of the double helix, that it remained stable and unaltered throughout several days, and that

it was fully reversible, returning to B-form with administration of a small amount of water. The last point was substantiated by showing a family of curves from a reverse titration of the 78% ethanol solution with water. In these curves, the positive peak had a maximum height in $\Delta\varepsilon$ of less than 10 units ($M^{-1}cm^{-1}$), although the DNA used for these results was calf thymus. Similar transitions to A-form were reported by Ivanov for DNA in water-isopropanol and water-dioxane solutions.

Ivanov and co-workers also observed that, with increase in the NaCl concentration of 80% dioxane, the negative band at 295nm such as is seen with copolypeptide-DNA complexes in salt, appeared. Its presence had also been reported in the earlier studies of Tunis-Schneider and Maestre on DNA films[20], and in RNA solutions at increasing ionic strength[85]. This band was attributed by Ivanov, et al, to an aggregation phenomenon induced by the increase in cation concentration.

According to Ivanov, in the less-polar water-ethanol solution, as opposed to dioxane, increase in NaCl did not produce the negative band, but instead red shifted the A-form maximum and reduced the depth of the minimum at 210nm while retaining the other A-form characteristics. With tRNA, at low ionic strength in the absence of divalent cations, the spectrum was reported to closely approximate the CD of DNA in water-ethanol, but, with increase in ionic strength the effect on tRNA was said to be the same as with decrease in ionic strength for DNA in 78% ethanol (i.e., a blue shift of the maximum and a deepening of the 210nm band). The effect upon copolymer-DNA complexes, then, with increase in salt, resembles that of tRNA under the above conditions and DNA in 80% dioxane rather than in water-ethanol solutions. With tRNA, a non-cooperative transition is reported, with an abrupt change in the CD

spectrum at a critical electrolyte concentration comparable to that of tRNA at high temperatures when the structure of the molecule collapses with denaturation[22]. This is suggestive of the Cl. perfringens DNA collapse in salt at higher coverage (Figure V-13a) and of the first example of the calf thymus DNA complex at r = 1.95 (+/- = .78) (Figure V-13d).

Elements of all the above observations reported by Ivanov, et al, are present in the results of copolypeptide-DNA studies in salt. However, in experiments at higher ionic strength where A-form is produced by copolypeptide binding rather than by solvent dessication, it is to be expected that not all results would necessarily agree.

In substantial agreement are the findings with respect to the influence on A-form of the G+C content of the DNA. Calf thymus ranks in G+C content behind both E. coli and M. luteus DNA's, yet, under all circumstances studied, its manifestations of A-form were by far the most spectacular. Undoubtedly the fact that, of the DNA's investigated, calf thymus alone is eukaryotic, contributes to this difference, yet, even with the elimination of calf thymus from consideration, the rank in ability to produce A-form is not consistently related to G+C content.

Conflicting results were noted, in comparing other observations of copolypeptide-DNA complexes with those of studies on uncomplexed nucleic acids, particularly with respect to both stability and reversibility of the A-type conformation. Major changes in spectra of complexes at higher coverage in 0.2M NaCl continued to be apparent for hours and even days after complexing (Figure V-17 and V-18), yet, despite the magnitude of these changes, the spectra remained within the broad outlines defining the A-form. Reversibility was never completely achieved. Drastic de-

creases in amplitude were indeed observed after prolonged dialysis into EDTA buffer containing no added NaCl but, with complexes of higher coverage, the outlines of A-form were never eliminated but rather increased after removal from dialysis (Figure V-19a).

As predicted from Ivanov's opposing results with tRNA and DNA, contrasting effects were observed between the model RNA complexes of Poly(A)·Poly(U) and those of DNA with increase in ionic strength. However, with A-form induced by copolypeptide rather than solvent, the effects with each were opposite to those reported with uncomplexed nucleic acids. Figure V-5 with Poly(A)·Poly(U) curves shows a sudden decrease in both positive and negative extrema, rather than an intensification, and the positive peak is shifted to longer rather than to shorter wavelengths. Figure V-13e, on the other hand, shows a set of calf thymus curves, complexed at the same degree of coverage, exhibiting marked enhancement and blue shift of the positive peak with increasing NaCl concentration. The latter observations correlate well with observations of DNA in 80% dioxane rather than ethanol; the response of the Poly(A)·Poly(U) complex may well be related to the "critical electrolyte concentration" at which the structure of the tRNA molecule collapses with denaturation.

There were no data in the Ivanov paper to support the statement that the A-form was not dependent upon temperature in the range of stability of the double helix. Unpublished results in our laboratory[86] had shown earlier that, in the absence of salt, a certain degree of A-form could be produced in poly($Lys^{40}Ala^{60}$)-DNA and other copolypeptide-DNA complexes (when the relative amino acid content of the copolypeptide was in the range of 40-60% lysine/alanine) by increasing the

temperature, the greatest effect being observed in the temperature range between 30 and 60°C. The lack-of-dependence statement is also challenged by studies of Girod, et al[63], who reported changes in the CD spectrum of A-form DNA in 80% ethanol as a function of temperature. These studies were directed toward determining whether transition to this form was the result of intrinsic changes in the secondary structure of the DNA molecule or a reflection of aggregation or condensation of the molecule just prior to precipitation, as indicated by light scattering. They reasoned that, if the DNA were forming a compact tertiary structure, it should be possible to watch such structure melt prior to denaturation of the secondary structure into random coil. Nothing definitive could be demonstrated with the calf thymus DNA, but with the T_2 phage DNA, which has equally pronounced positive and negative extrema, two phases of melting were shown, the first affecting only the positive peak at temperatures between 35 and 49°C, the second affecting the negative peak between 49 and 60°C. This evidence prompted the conclusion that A-form resulted from aggregation or condensation of the DNA.

Since a marked increase in the A_{320}/A_{260} ratio always accompanies the appearance of huge A-form CD spectra, it seems justifiable to conclude that aggregation does play an important part in the magnitude of the response. Whether or not it is justifiable to attribute the generation of the A-type conformation itself to aggregation is another question altogether. The data from reversibility studies upon removal of salt do not seem to support such a conclusion.

Since, in the presence of salt, the transition of DNA to its A form is effected by the binding of copolypeptide, yet, with removal of salt,

the transition is not altogether reversible (though the amplitude is decreased), it seems likely that forces other than, or in addition to, those of aggregation are at work. The fact that sixty percent of the amino acid residues in that copolypeptide which is most effective in producing the A-type conformation, namely, poly($Lys^{40}Ala^{60}$), are hydrophobic, suggests that interactions of a hydrophobic nature might make a significant contribution to intra- as well as inter-molecular structures. This is borne out by the relationship between the degree of coverage and the extent of the transition, demonstrated in the studies in 0.2M NaCl (Figure V-14 a-d). With fewer hydrophobic residues present at lower coverage, equally effective interactions cannot occur. It seems probable that these hydrophobic interactions are responsible, not only for the irreversibility of the transition (intra-molecular interaction), but also for enhancing the tendency of DNA molecules to aggregate in the presence of salt (inter-molecular interaction). Hydrophobic interaction between amino acid residues might explain the amplitudes of A-form spectra exhibited by copolypeptide-DNA complexes, amplitudes which are three times the height of those seen with pure DNA in 80% ethanol or dioxane solutions. They might also explain why, after complete thermal denaturation of a complex exhibiting A-form, the presence of salt in the medium on cooling does not stimulate a renewed transition (Figure V-22 a-b); yet, when denaturation is only partial and the basic secondary structure of DNA is able to regenerate, the A-form becomes progressively enhanced on cooling (Figure V-24). This suggests that the initial stimulus for the generation of A-form comes from intra-molecular events requiring an intact helix; inter-molecular interactions then build upon the basic form so generated. The strength

of hydrophobic interactions would be likely to increase with time and lack of disturbance in the medium, thus solidifying conformations, which would explain the greater stability with heating observed in the peak height of the 24 hour complex vs the fresh (compare a and b of Figure V-21).

Obviously, from the fact that copolypeptides containing more than 60% alanine are unable to produce impressive A-form structures, hydrophobic interactions and the presence of α-helices are not sufficient in themselves. Apparently, tight binding by the lysine residues is also required to make possible initial distortion of the normal DNA conformation and permit maintenance of the new form. An overwhelming percentage of α-helix in a copolypeptide might interfere with such tight binding, whereas a certain optimum proportion, when tightly bound, might, through hydrophobic interactions, hold the DNA to the more rigid conformation imposed on RNA structures by the presence of the additional oxygens of the ribonucleotides. In copolypeptide-RNA complexes, these same oxygens might prevent the tight binding which would be required to further enhance the A-form of RNA, with the oxygen, in this case, providing the interference, rather than a superfluity of α-helix.

In summary, the results of these studies appear to justify the following conclusions with respect to generation and maintenance of the A-type conformation in copolypeptide-DNA complexes:

1. If the G+C content of a DNA influences the transition to A-form, it is certainly not the only determining factor.

2. DNA and RNA complexes give opposite responses to increases in ionic strength.

3. Light scattering due to aggregation undoubtedly contributes to the magnitude of the spectral response, but it is not responsible for the qualitative aspects of the characteristic A-form CD spectrum.

4. The irreversibility of the A-type conformation of DNA, when it has been generated jointly by the binding of poly($Lys^{40}Ala^{60}$) and the presence of salt, is attributable to strong hydrophobic interactions which are not eliminated by the removal of salt.

5. Changes in temperature and/or concentrations of sodium chloride contribute to the strengthening or weakening of those hydrophobic interactions (both inter- and intra-molecular) which are responsible for the generation and maintenance of A-form DNA complexes.

Chapter VI

COMPLEXES WITH OTHER ALIPHATIC COPOLYPEPTIDES

The binding of DNA by histone molecules is influenced not only by basic and hydrophobic amino acids, but by hydrophilic as well. Whereas alanine was chosen to be the model for hydrophobic residues in the initial investigations, serine was selected to represent the hydrophilic group in a new copolypeptide combining lysine and serine. In still another combination, isoleucine was exchanged for the alanine to probe the additional effect of steric hindrance on the hydrophobic characteristics. With the exchange of only one type of residue, any new effects would be attributable solely to its influence. By altering one parameter at a time, more subtle shades of difference could by high-lighted. Polylysine, which was employed throughout all model protein experiments as a fundamental reference by which to evaluate new approaches, was used in these studies to assist in the interpretation of results from trypsin digestion of the lysine-serine copolymer.

Binding Characteristics of poly($Lys^{52}Ser^{48}$)

The precipitation curve of the random copolymer poly($Lys^{52}Ser^{48}$), seen in Figure VI-1, is notable in two ways: first, for its complete lack of cooperativity in binding, signified by the almost vertical drop from 100% DNA remaining in the supernatant after centrifugation to about 6%; second, for its retention in solution until just beyond, rather than just before, the complete neutralization of the DNA phos-

phates as judged on the basis of input ratio (r_{mid} = 1.04). Both observations appear to be due to the same factor, namely, the presence of the hydrophilic hydroxide which has been substituted for the hydrogen of alanine. The -OH group apparently is able to hold the complex completely in solution by its interaction with the surrounding water molecules rather than by participation in any other types of interaction. This is supported by the total absence in the precipitation curve of evidence suggesting light-scattering and aggregation: the level horizontal line maintained at 100% DNA in the supernatant should be compared with the increasingly elevated lines of the poly($Lys^{m}Ala^{n}$) complexes (Figure III-1) containing higher percentages of alanine. The centrifuged A_{320}/A_{260} remains at less than 0.1 throughout the precipitation curve measurements.

The β-value, on the other hand, indicates that not every lysine introduced into solution is bound to phosphate. The equation, $r = \beta \frac{A_{Tm'}}{A_T}$, gives a straight line through zero (as shown in Figure VI-2) intersecting the 100% bound axis at a β-value of .91 lysine/nucleotide. The other method of calculating β, using $r = \beta(1 - \frac{A_{Tm}}{A_T})$, also gives a straight line intersecting the baseline, not at zero, but at 0.12, indicating a residual melt of 12%. The β-value calculated by the first method appears to represent the number of tightly bound lysines/nucleotide, while the second method indicates the qualitative effect of serine on the lysine binding.

Characteristics of binding are best judged by consideration of the melting curves. Figure VI-3 shows this qualitative effect by the tailing of each band, above the Tm and below the Tm', at each degree of coverage. The tailing to some extent affects the peak locations of

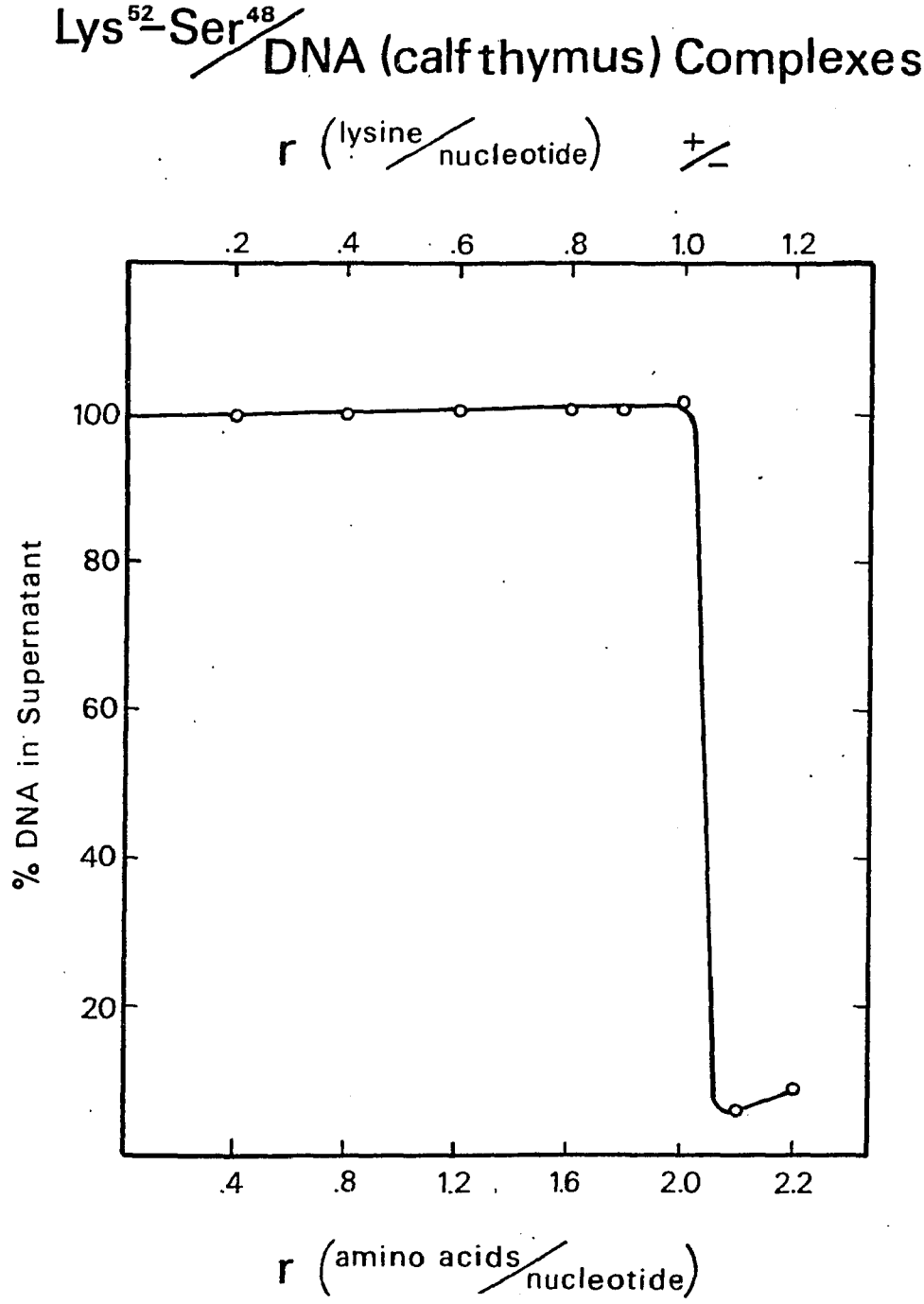
both bands: the Tm peaks lean toward higher temperatures at higher coverage, the Tm' peaks bend backward slightly, but the major portion of the Tm' band remains consistently at about 96°C, indicating that the bound portion of the DNA is tightly bound.

Effects of Trypsin Digestion on Thermal Denaturation Curves

Figure VI-3b is placed in apposition to VI-3a to permit comparison and demonstrate the effective destruction of the Tm' band resulting from digestion by 5μg trypsin for a period of 1 1/2 hours. Since 52% of the amino acids in the copolypeptide are lysines, upon which the enzyme is maximally active, the linear random polymer of 35,375 molecular weight could be expected to be chopped by enzymatic action into fairly small pieces of differing lengths during this time. It is hardly surprising that these small pieces were unable to provide any degree of cooperative protection to a high Tm'. One might expect, however, that the free DNA band would be more apparent in the complexes of lower coverage. Instead, in the .4 (+/-, r = .8) complex the height of the Tm band is cut in half, and in all complexes there is an extremely broad, low melt, centered, for the complexes of higher coverage, at around 62 C. Perhaps uneven distribution of the small, bound pieces along the DNA would afford a limited, localized protection against thermal instability; the pieces might be too small and separated to influence other portions of the molecule, yet large enough to stabilize a few nucleotides.

The above explanations being purely speculative, polylysine, the fundamental reference polymer, was subjected to action by different

FIGURES

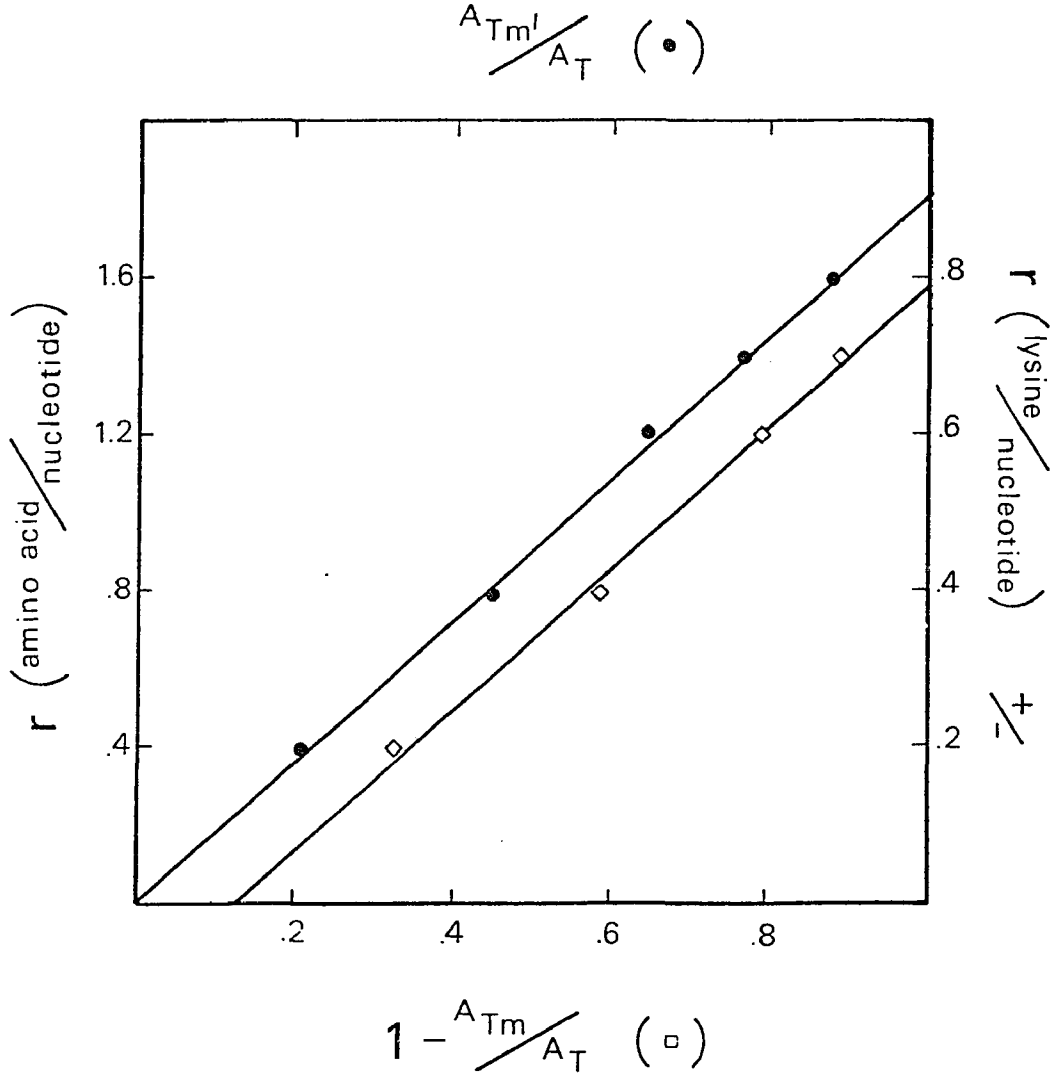


Titration (Precipitation) Curve

Fig. VI-1

Lys^{52}–Ser^{48}/DNA Complex

Determination of β values



$$r = \beta\left(A_{Tm'}/A_T\right) \quad \text{or} \quad r = \beta\left(1 - A_{Tm}/A_T\right)$$

Fig. VI-2

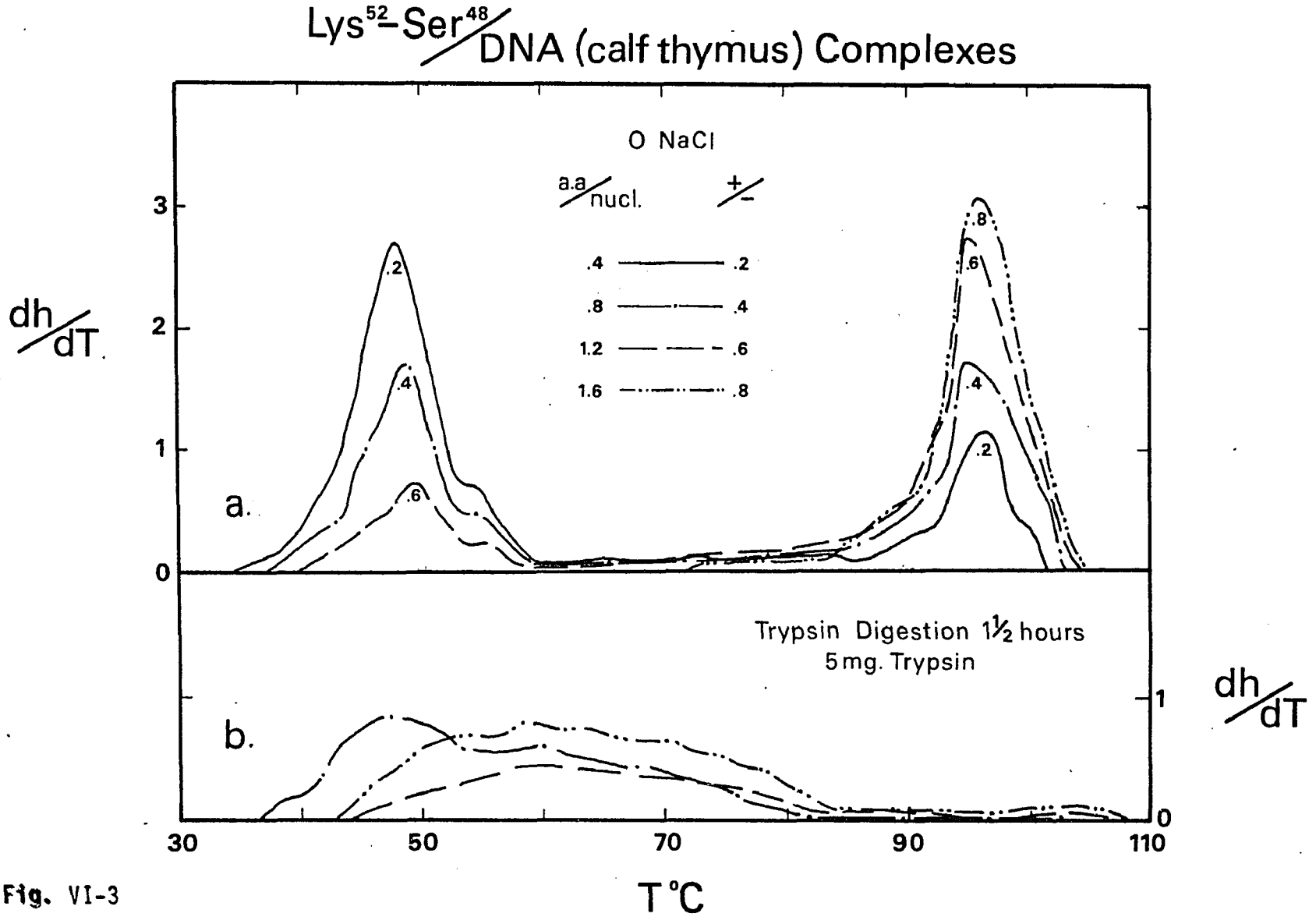

Lys52-Ser48/DNA (calf thymus) Complexes
O NaCl
a.a/nucl.
+/−
.4 .2
.8 .4
1.2 .6
1.6 .8
dh/dT
a.
b.
Trypsin Digestion 1½ hours
5 mg. Trypsin
T °C
30
50
70
90
110

Fig. VI-3

quantities of trypsin for the same 1 1/2 hour period of digestion. Figure VI-4a shows, plotted on a larger scale, the polylysine melts before and after the introduction of 5μg or 10μg trypsin. At zero trypsin, polylysine, at a 0.6 ratio of lysine/nucleotide, gives its characteristic melting bands with calf thymus DNA: with little free DNA available, the Tm is comparatively short and located at its normal temperature of 47°C; the Tm' of DNA bound by polylysine is tall and sharply defined at 97°C, manifesting strong protection and a cooperative melt once denaturation is initiated. Samples of the same complex after digestion by 5μg (the same as used for the lysine-serine complexes) and 10μg of trypsin have lost both Tm and Tm' bands and have replaced them with a single melting mound between. The 5μg mound begins to rise, essentially, at about 45°C and terminates at 85°C, with a thin tail to higher temperatures; the 10μg mound starts at 50°C, rises a little higher than the first, then settles back in the same manner. Compared to the copolymer melts, where the lysine binding was qualified by the presence of serine, the bound mounds of polylysine are somewhat higher and not quite so broad, but the same reasoning as to the source of the melting phenomena would seem to apply, in attempting to explain the replacement of two melts with one central mound.

It was reasoned further that, if the pieces resulting from enzymatic action were small enough, they might be dialyzed away, provided the tight binding was first loosened by salt. Accordingly, the 5μg and 10μg samples, together with unbound DNA for control, were dialyzed to 0.5M NaCl with three changes, then dialyzed back to EDTA buffer, with five changes to ensure complete removal of salt. Figure VI-4b shows the results of thermal denaturation after dialysis. The central mound

FIGURES

Derivative Melting Profiles of Polylysine-DNA Complexes
(+/- = .6)

VI-4 Trypsin Digestion (0, 5, 10μg; 1 1/2 hrs)
a. no dialysis
b. after dialysis to 0.5M NaCl and back to EDTA buffer

VI-5 Trypsin Digestion (0, 1, 2μg; 1 1/2 hrs)
a. no dialysis
b. after dialysis to 0.5M NaCl and back to EDTA buffer

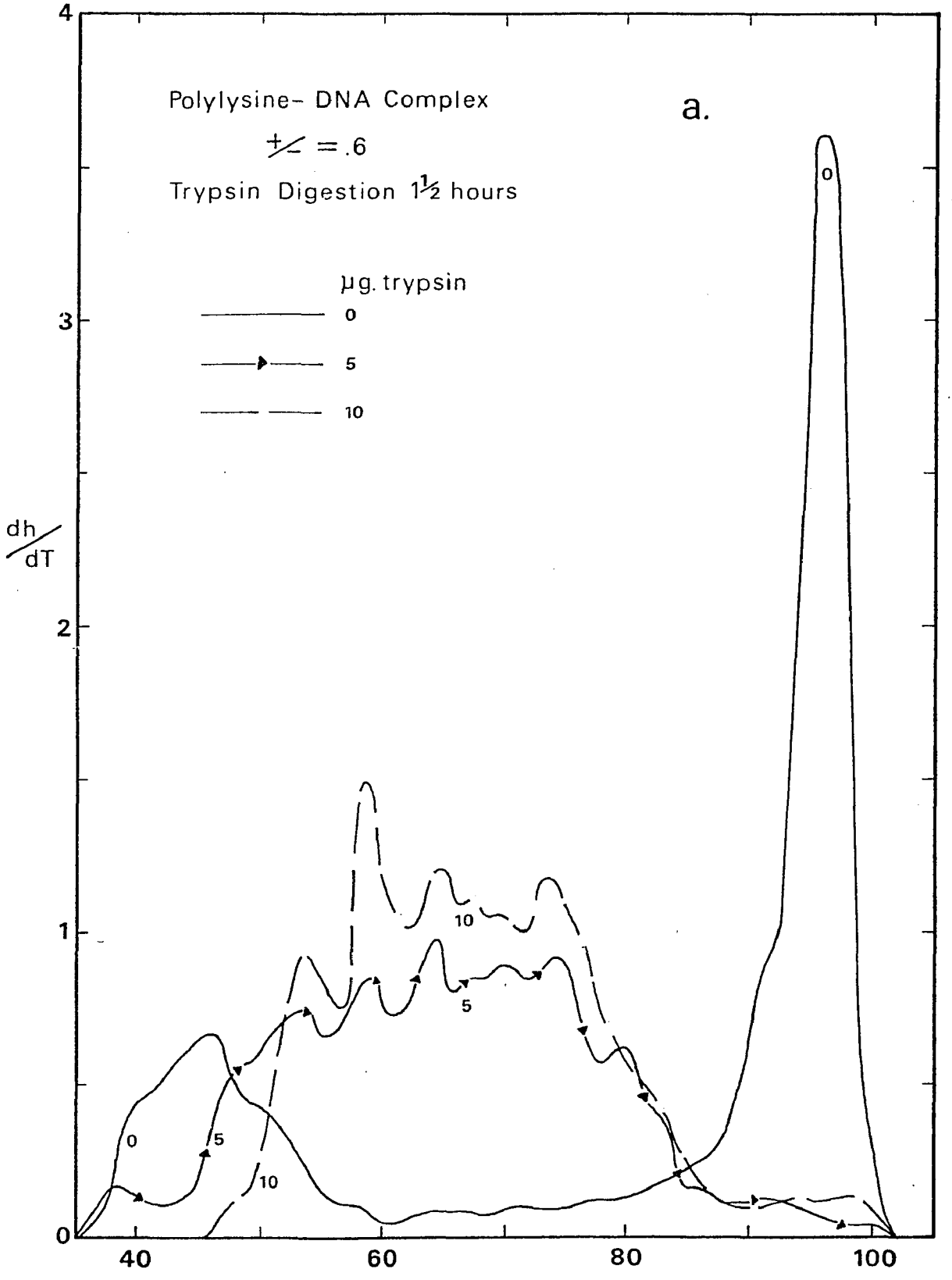

Polylysine- DNA Complex
+/- = .6
Trypsin Digestion 1½ hours
a.
µg. trypsin
0
5
10
dh/dT
T°C
4
3
2
1
0
40
60
80
100

Fig. VI-4,a

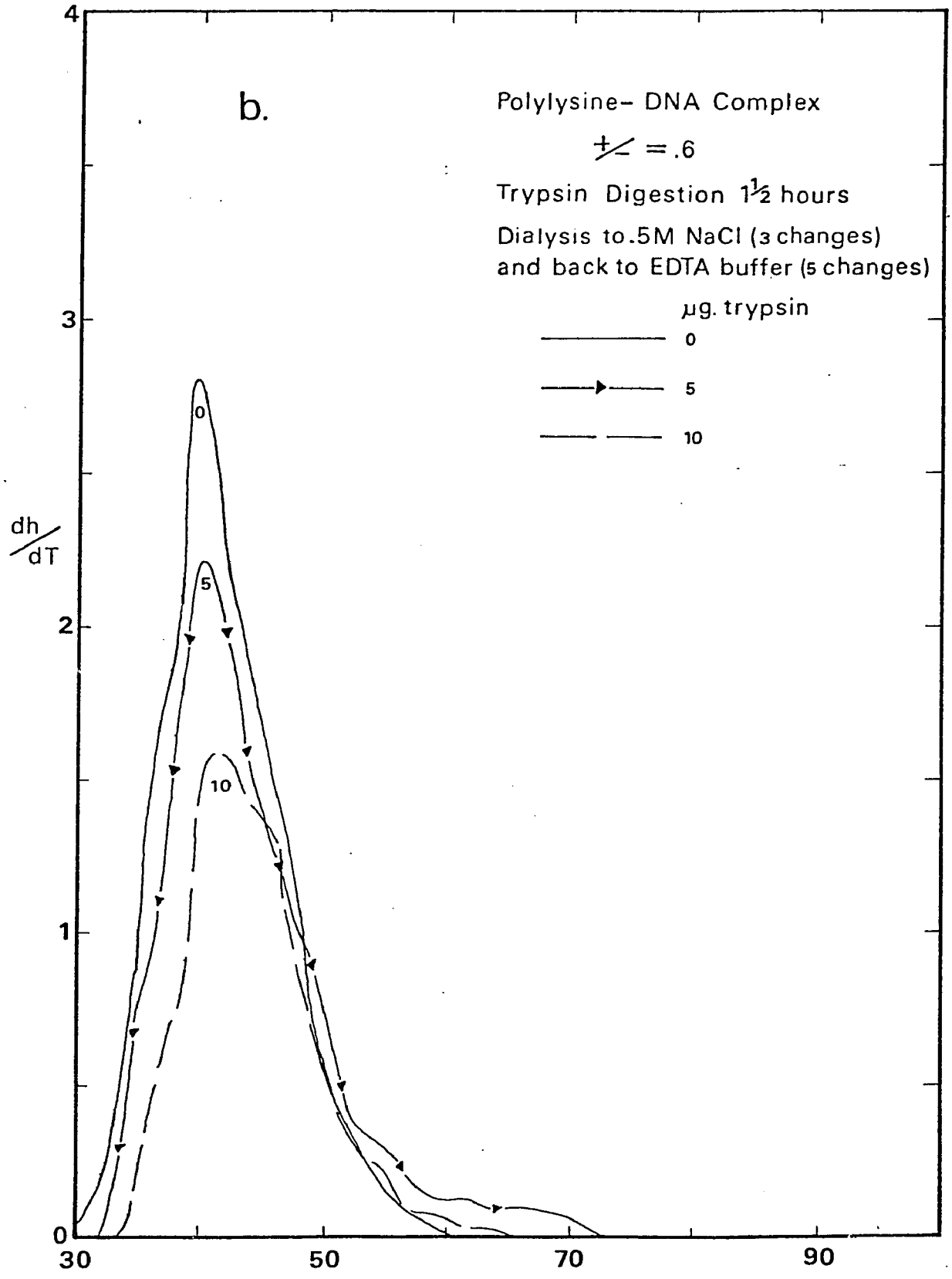

b.
Polylysine- DNA Complex
+/- = .6
Trypsin Digestion 1½ hours
Dialysis to .5M NaCl (3 changes)
and back to EDTA buffer (5 changes)
µg. trypsin
0
5
10
dh/dT
T°C
4
3
2
1
0
30
50
70
90

Fig. VI-4,b

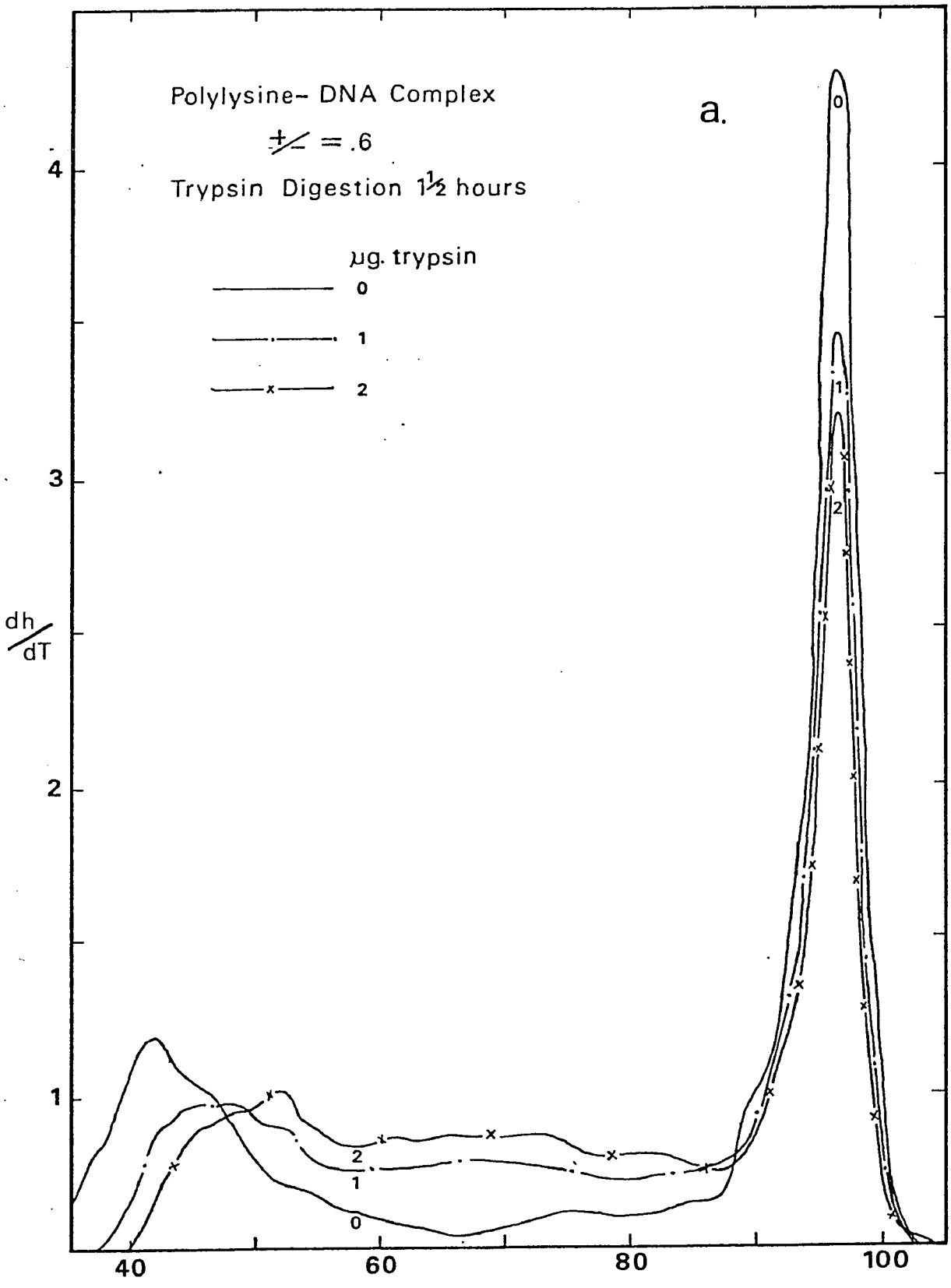

Polylysine- DNA Complex
+/- = .6
Trypsin Digestion 1½ hours
µg. trypsin
0
1
2
a.
dh/dT
4
3
2
1
40
60
80
100
T°C

Fig. VI-5,a

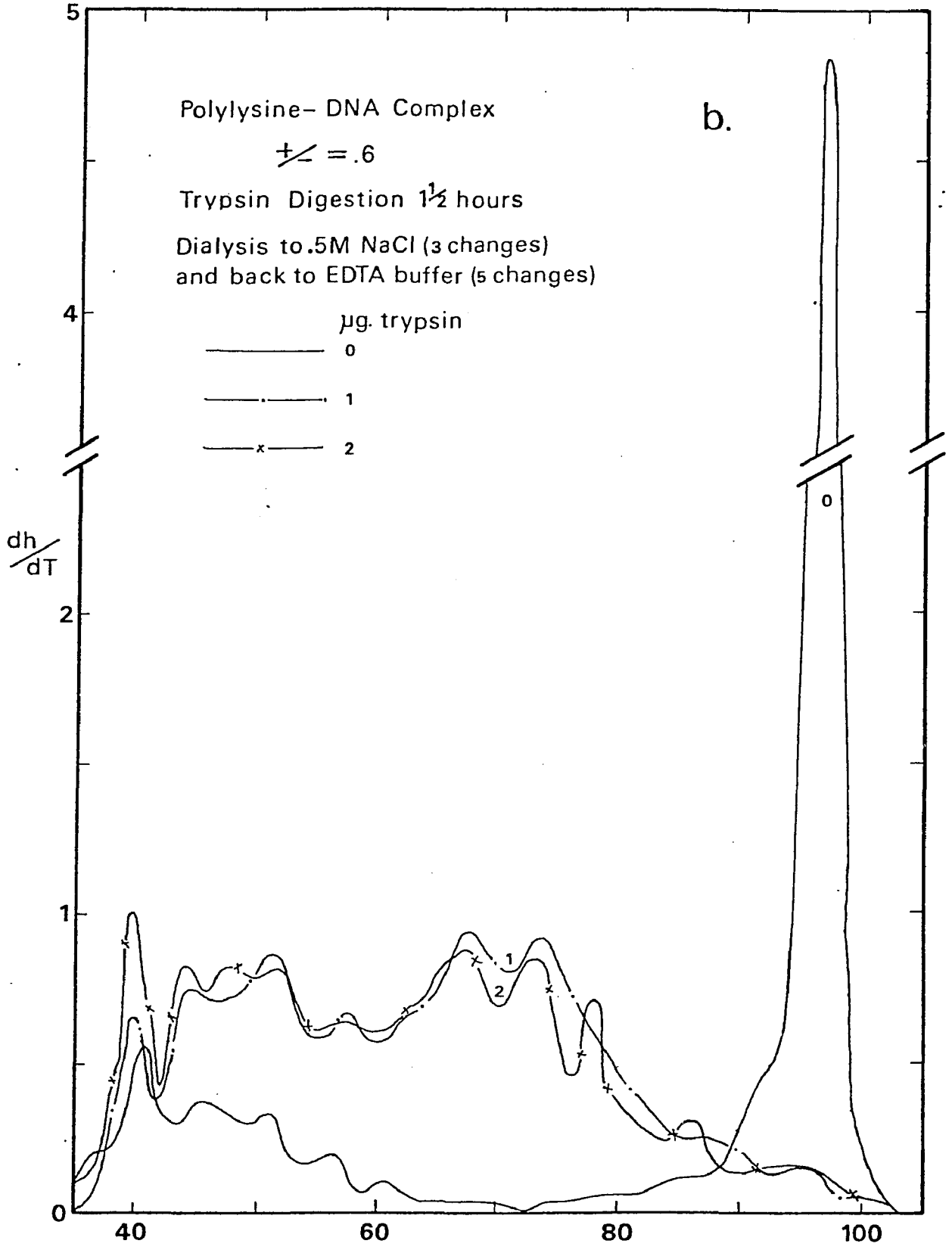

Polylysine– DNA Complex
+/− = .6
Trypsin Digestion 1½ hours
Dialysis to .5M NaCl (3 changes)
and back to EDTA buffer (5 changes)
µg. trypsin
0
1
2
b.
dh/dT
T°C
0
1
2
5
4
2
1
0
40
60
80
100

Fig. VI-5,b

is seen to have disappeared entirely, in both 5μg and 10μg samples, and to have been replaced by free DNA bands. The longer trailing edge extending from the somewhat shorter 5μg band suggested that, with this quantity (5μg) of trypsin, there still remained a few polymer pieces long enough to be prevented from passing through the dialysis bag.

Therefore, another experiment was performed using 1μg and 2μg trypsin to digest the same polylysine-DNA complex (lysine/nucleotide = 0.6) for the same period of time. The melting results are shown in Figure VI-5a. With a restricted amount of enzyme, the cooperative Tm' band is still well and characteristically defined in both treated complexes. The free Tm band is, perhaps, still recognizable as such, but it has leveled into a central plateau, the lower edge and central level of which are higher for 2μg than for 1μg.

Dialysis of these samples into salt, then, should indicate what part is played by length of copolymer in the maintenance of characteristic melting patterns. Following the same dialysis procedure, Figure VI-5b shows what the restricted digestion procedure actually accomplished. The pieces of digested polymer still remained long enough that, even though dislodged into the dialysis medium by salt, they were unable to pass through the bag; on further dialysis into EDTA buffer, with removal of salt they became rebound to the DNA, randomly and uncooperatively. Neither of the treated samples is able to cooperate in stabilizing DNA to a high temperature, because of spaces intervening between the pieces; whereas, after the cuts had been inflicted by the enzyme, but before dialysis, the pieces remained bound side by side on the DNA backbone, and collectively, in close proximity, they still were able to cooperate in producing the sharp, high melting Tm' of bound DNA

(Figure VI-5a). When separated by salt dialysis, different length pieces were able to stabilize the DNA to different temperatures, depending upon their length. On the other hand, after digestion with 5μg and 10μg of trypsin, even without salt dialysis, there were no pieces long enough to act cooperatively despite the possibility that they may have remained sufficiently close together.

Circular Dichroism of Poly($Lys^{52}Ser^{48}$)

Qualitatively the circular dichroic pattern of unbound poly(Lys^{52}-Ser^{48}) is shown, in Figure VI-6, to be largely in random coil, but quantitatively, the spectrum is extremely small. For purposes of comparison, a sample of unbound poly($Lys^{48}Ala^{52}$) containing approximately the same proportion of lysine is shown beneath. Both copolymers had an input ratio of 50:50 at the time of polymerization, and the difference in analyzed content is undoubtedly reflective of differences in the rates of polymerization between the alanine and serine N-carboxyanhydrides in the presence of ε-carbobenzyloxy-L-lysine N-carboxyanhydride. The source of the difference in magnitude of the CD spectra is less readily apparent.

When bound, the lysine-serine copolymer appears to have relatively little effect on the DNA conformation (Figure VI-7) until the complex approaches the point of neutralization, when there is some slight movement toward the C-type conformation. The protein area of the spectrum is dominated by DNA, rather than by copolymer, which makes a small contribution to the minimum at 210nm, but the amplitude is little affected by increasing r-value.

Predictably, from the thermal denaturation results, after digestion

FIGURES

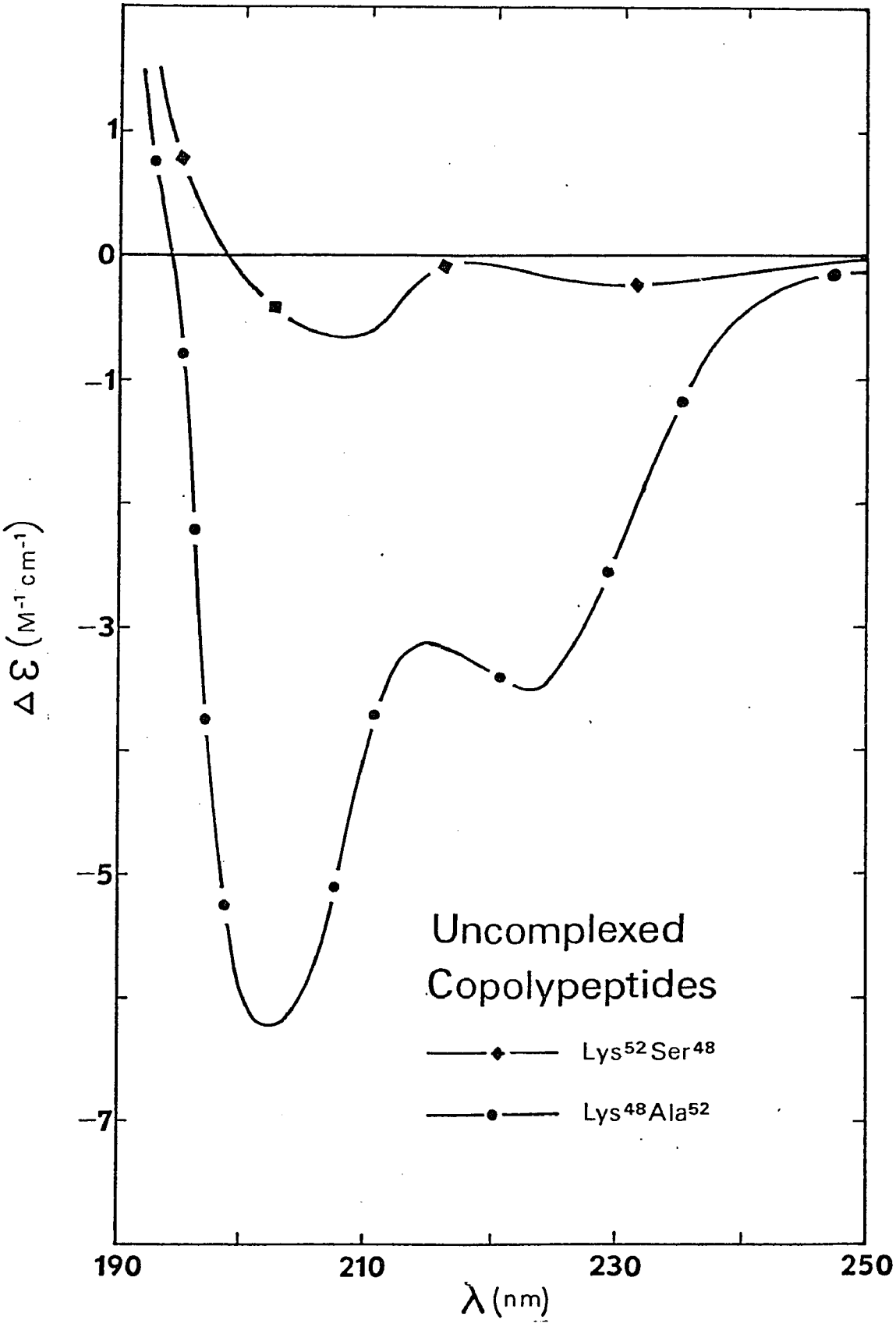

1
0
−1
−3
−5
−7
Δε (M⁻¹cm⁻¹)
190
210
230
250
λ (nm)
Uncomplexed
Copolypeptides
Lys52Ser48
Lys48Ala52

Fig. VI-6

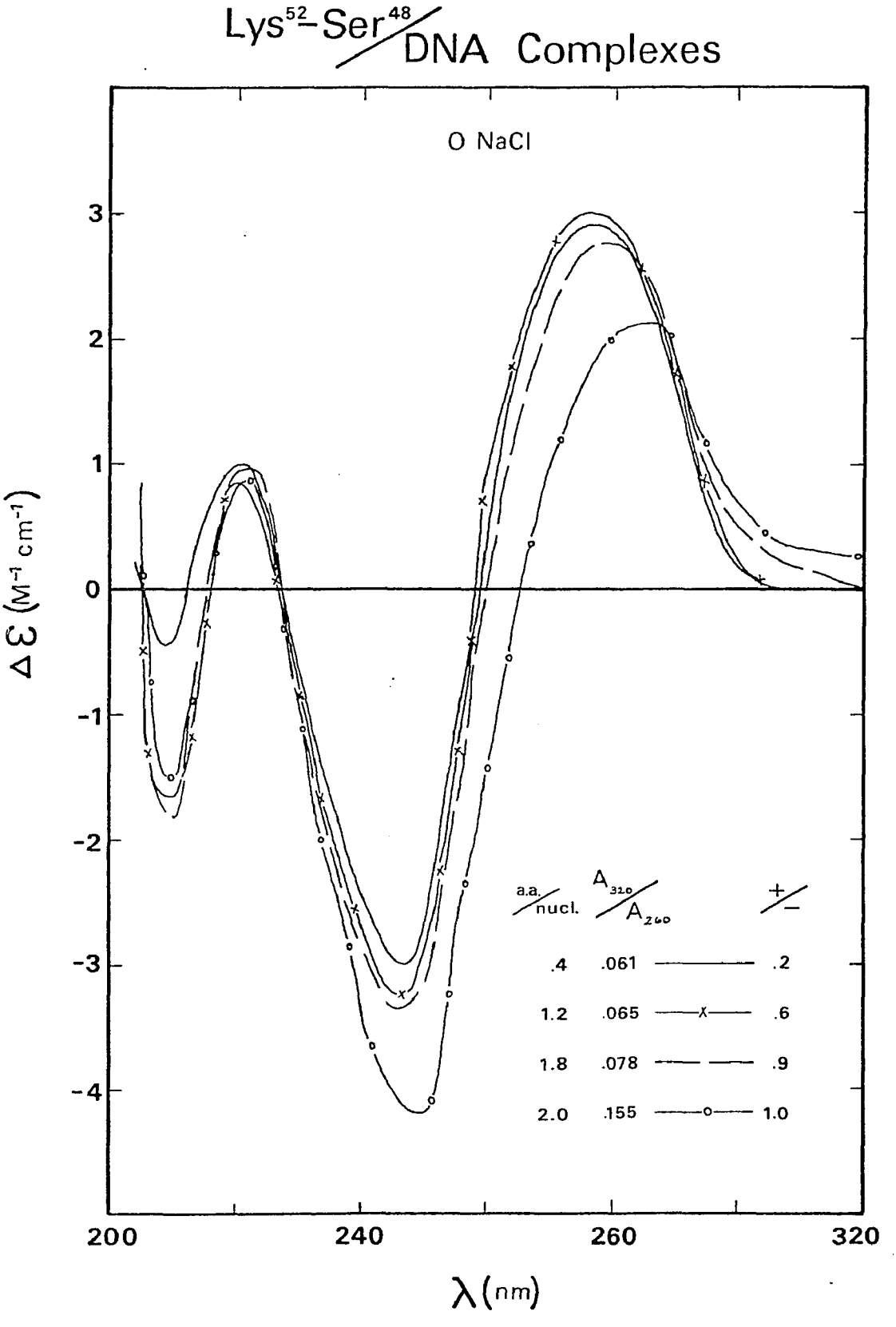

Lys52-Ser48/DNA Complexes
O NaCl
Δε (M-1 cm-1)
λ(nm)
200
240
260
320
3
2
1
0
-1
-2
-3
-4
a.a./nucl.
A310/A260
+/−
.4 .061 .2
1.2 .065 .6
1.8 .078 .9
2.0 .155 1.0

Fig. VI-7

Lys^{52}–Ser^{48}/DNA Complexes

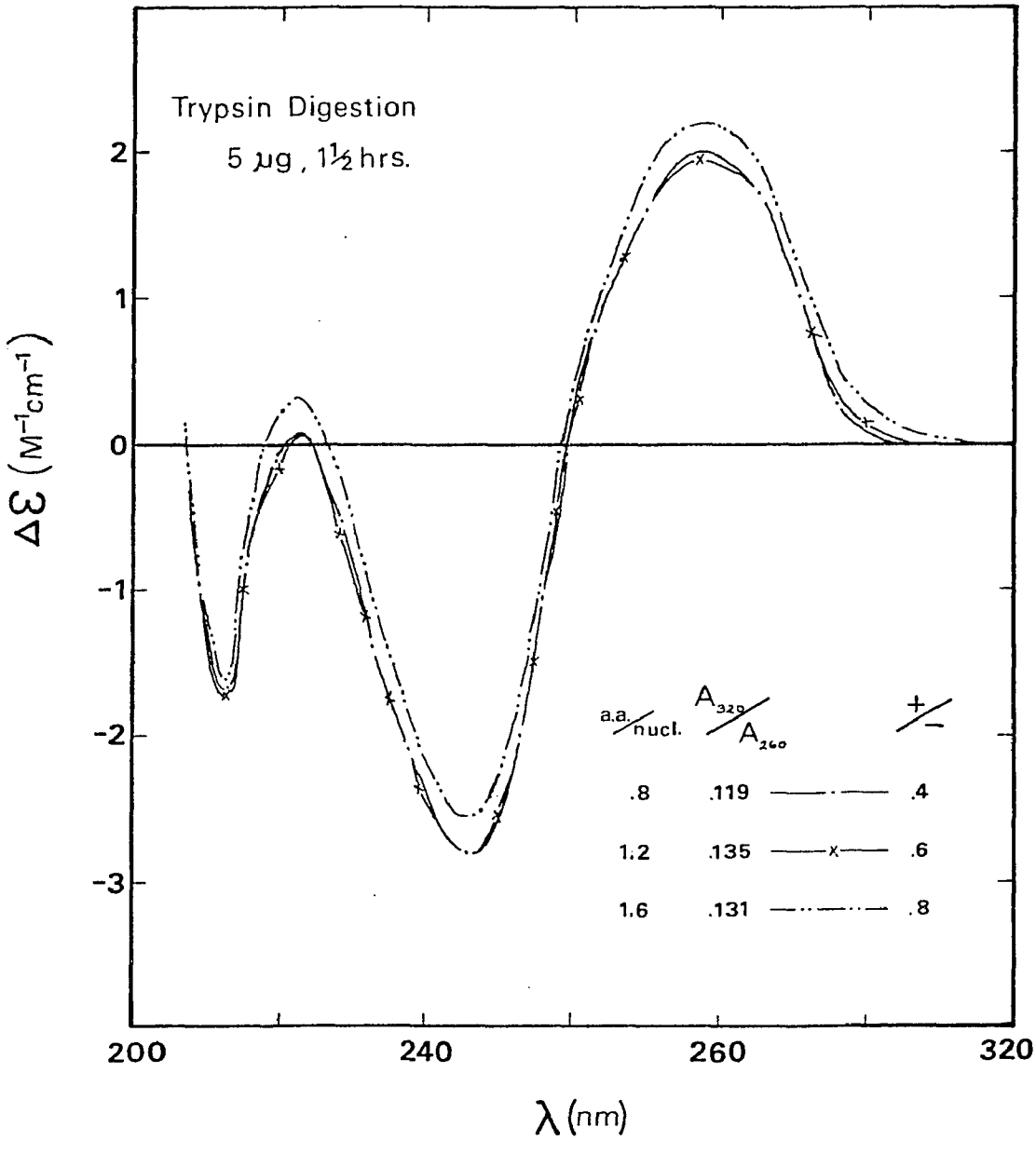


Fig. VI-8

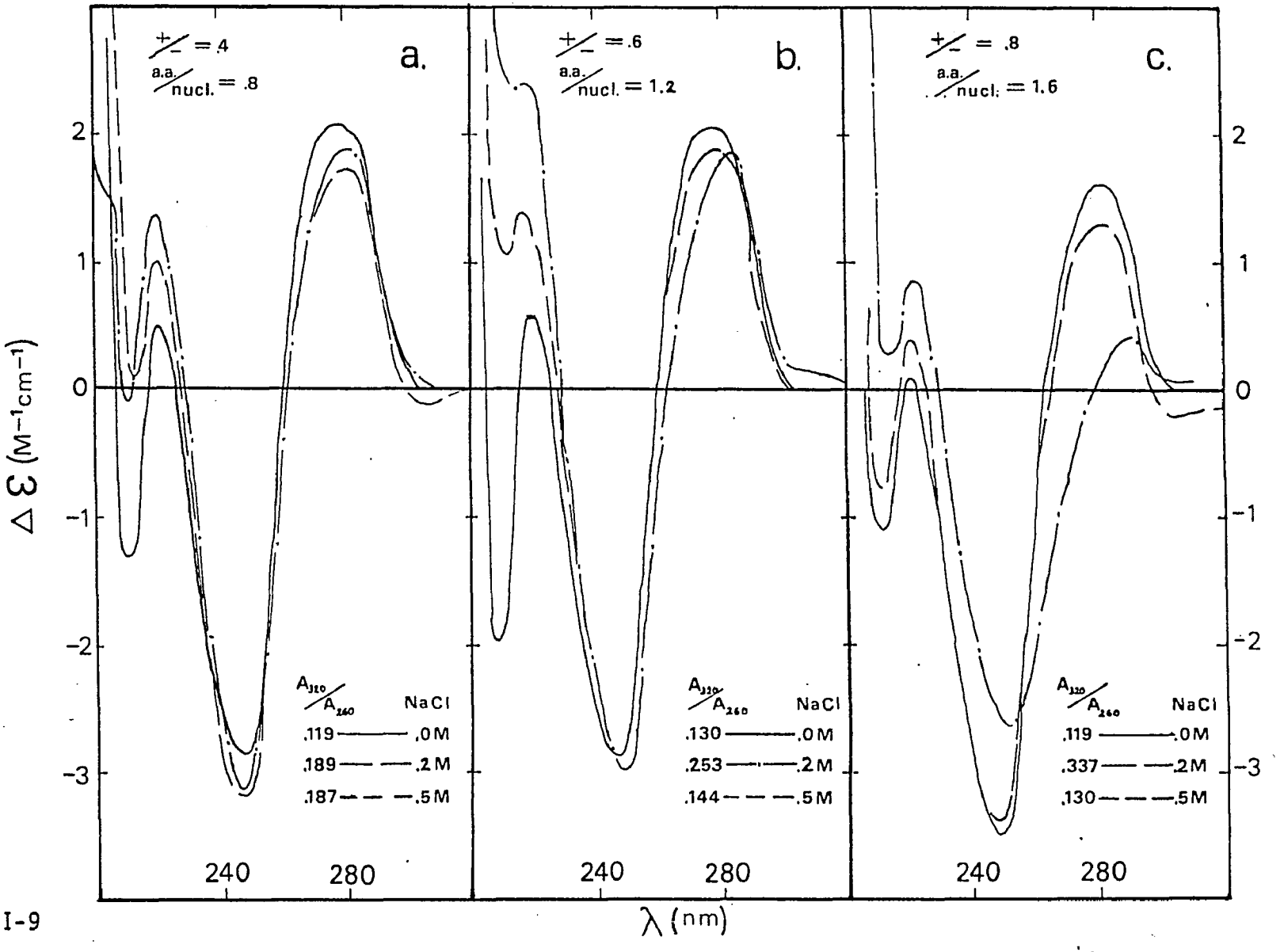

+/− = .4
a.a./nucl. = .8
a.
+/− = .6
a.a./nucl. = 1.2
b.
+/− = .8
a.a./nucl. = 1.6
c.
A320/A260 NaCl
.119 .0M
.189 .2M
.187 .5M
A320/A260 NaCl
.130 .0M
.253 .2M
.144 .5M
A320/A260 NaCl
.119 .0M
.337 .2M
.130 .5M
Δε (M⁻¹cm⁻¹)
λ (nm)
240
280
2
1
0
−1
−2
−3

Fig. VI-9

with 5μg trypsin, the already minimal effects of binding are further decreased, despite an increase in the A_{320}/A_{260} ratio of all complexes to approximately .12-.13 with change in coverage (Figure VI-8). These ratios would suggest some contribution from aggregation and lead one to expect an increase rather than decrease in amplitude.

When treated with salt of various concentrations (Figure VI-9 a-c), the effect in the DNA region is greatest in the .8 (+/-, r = 1.6) complex (c), but the 0.6 (r = 1.2) complex (b) appears to be most responsive in the protein region, reacting with a sharp elevation in the 210nm minimum. What is most surprising is the relatively much greater effect on each of the complexes of 0.2M NaCl as opposed to 0.5M. At the latter concentration the conformation of the complex in both regions of the spectrum assumes an intermediate position, somewhere between that characteristic of zero and 0.2M NaCl. In each complex, the A_{320}/A_{260} is highest at 0.2M and drops back at 0.5M; therefore, aggregation must play some part in the amplitude of the response. The lower concentration may permit certain conformational changes which are proscribed at higher levels. The structure is by no means collapsing in higher salt, but this salt (0.5M) may serve to relax the tighter winding initiated by the lower salt concentration, or possibly promote partial dissociation of the copolypeptide from DNA. Another surprising feature of the salt spectra is the faint suggestion of a negative peak at 300nm, such as might be expected with A-form DNA, but would hardly be looked for in combination with the decreased and red shifted peak of incipient C-form. This would support the suggestion of relaxation in winding, which is a prerequisite for production of A-type conformation.

Complexes with Poly($Lys^{66}Ile^{34}$)

Although copolymers at two different ratios of lysine/isoleucine were ordered (35/65 and 65/35) from Miles Laboratories, and two products were ultimately received, meaningful studies were almost totally precluded by the complete lack of solubility of these copolymers in any common solvents (listed by Miles as "Acetic Acid, D.M.F., D.C.A., Dioxane, H_2O, 6N HCl, HBr in Acetic Acid"). Specifications stated molecular weight to be "indeterminable"; in the copolymer for which input of isoleucine was 65%, acid hydrolysis remained "incomplete after 150 hours at 120 C in either 6N HCl or concentrated HCl:Acetic acid 1:1". Total acid hydrolysis did prove possible when the isoleucine input dropped to 35%, and the product was determined by automatic amino acid analysis to contain 66:34 Lys/Ile.

It seemed possible that, being insoluble in HBr in Acetic acid, the protective group on the Lysine residue, which is customarily removed after polymerization by bubbling HBr through the polymerization medium, might have been retained, and that this group might be interfering with solubilization of the product. Numerous IR spectra were run, looking for evidence of its presence, but no definitive results were obtainable.

Attempts were made on the copolymer of higher lysine content to bring into solution even an extremely small amount, so as to determine if binding was possible in the usual EDTA buffer. A cloudy, semi-gelatinous substance resulted, which resisted table-top centrifugation. Finally it was possible to take absorbance readings on the second supernatant of a 1 ml pellet to which 5 ml EDTA buffer had been added. This proved to be a suspension rather than a solution, since it was ultimately spun out completely, but it was possible to use this diluted

FIGURES

CD Spectra of Poly($Lys^{66}Ile^{34}$)·DNA Complexes

VI-10 in 0.0M NaCl

VI-11 after Dialysis into 0.2M NaCl

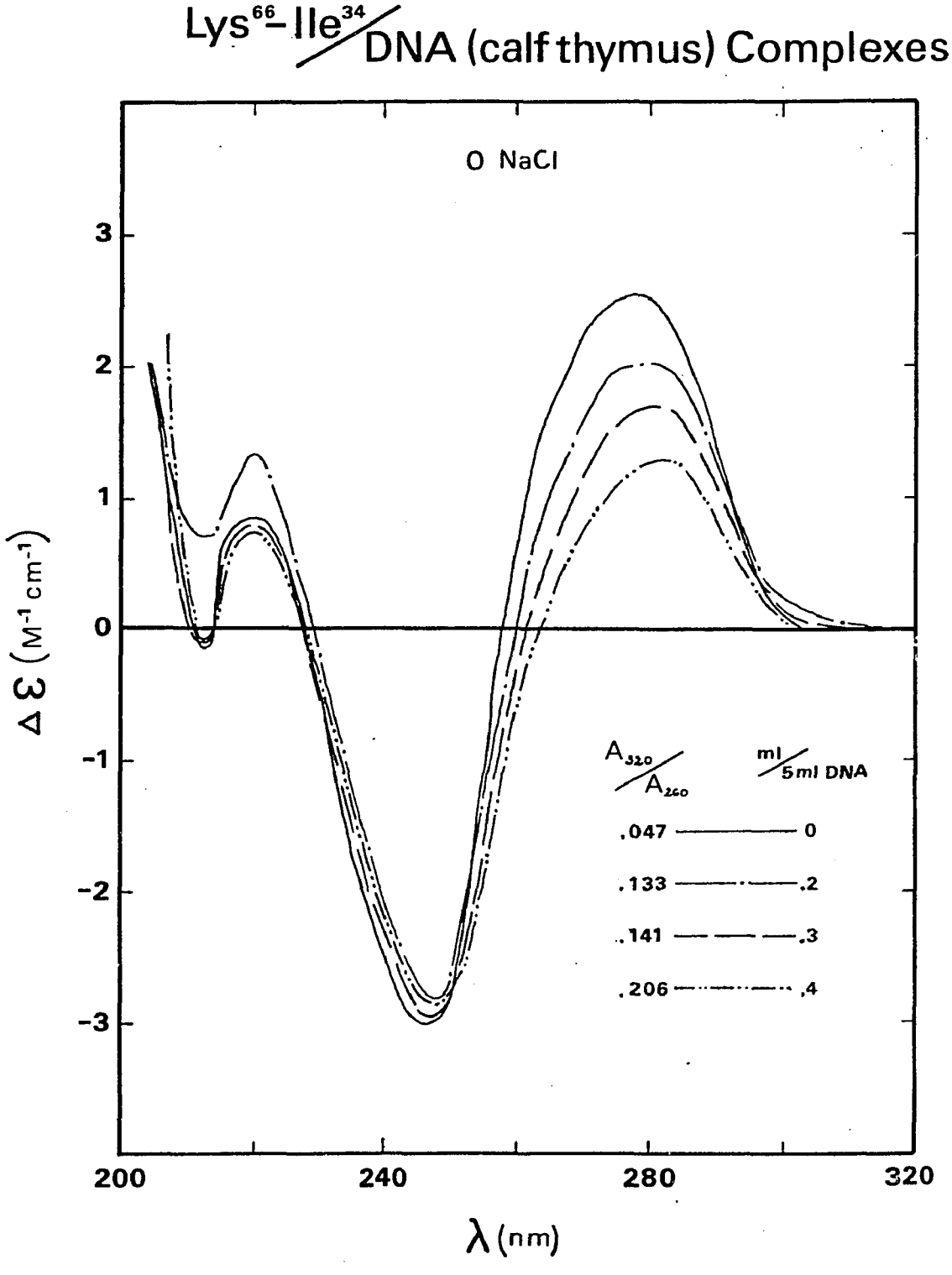

Lys66-Ile34/DNA (calf thymus) Complexes
0 NaCl
3
2
1
0
-1
-2
-3
Δε (M-1 cm-1)
200
240
280
320
λ (nm)
A320/A260
ml/5ml DNA
.047 0
.133 .2
.141 .3
.206 .4

Fig. VI-10

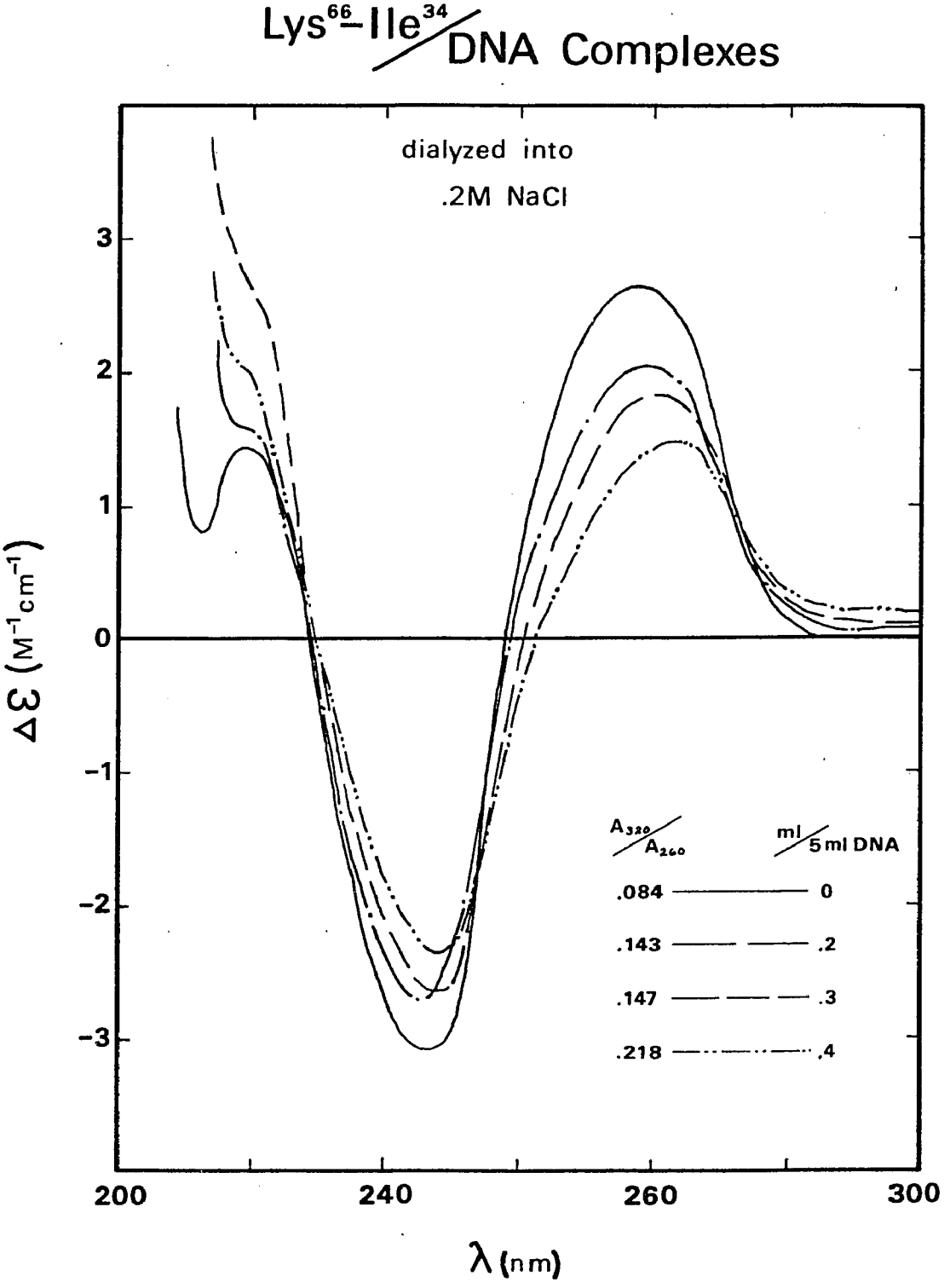

Lys66-Ile34/DNA Complexes
dialyzed into
.2M NaCl
3
2
1
0
-1
-2
-3
Δε (M-1cm-1)
200
240
260
300
λ (nm)
A320/A260
ml/5ml DNA
.084 0
.143 .2
.147 .3
.218 .4

Fig. VI-11

second supernatant for limited complexing. As expected the A_{320}/A_{260} ratios were very high, increasing from 0.133 (with 0.2ml "solution" added to 5ml DNA) to 0.206 with addition of 0.4ml "solution". Despite these problems, the lysine residues present in the semi-solution were not prevented from binding to the DNA in the reaction medium, and the spectra pictured in Figure VI-10 resulted. Binding by the copolymer produced a clear red shift and reduction of the positive peak, both of which are characteristic of complexes moving toward C-form DNA; the effects were linear with increasing r-value. In the protein region, structure in the bound copolymer is not apparent, and the DNA spectrum there is largely unaltered.

The same complexes were then dialyzed into 0.2M NaCl. With increased ionic strength, the DNA peaks show almost no change, although light scattering becomes more significant above 300nm. Below the cross-over point there is considerably more variation in the minimum at 245-250nm, and the upward extension of the 210nm minimum is reminiscent of the Cl. perfringens DNA spectrum in increasing salt (Figure V-11a).

Thermal denaturation of complexes was attempted, but precipitation in both Tm and Tm' areas made the results meaningless. Since there was no true solution, no concentration determinations, precipitation curves, or quantitative evaluations of any sort, could be made. Fortunately, the CD spectra are calculated in terms of the known DNA concentration, so these could be reported. From the character of the CD spectrum of DNA bound by poly($Lys^{66}Ile^{34}$), it seems likely that the lysine portion of the copolymer was partially solubilized, so that it was able to approach the DNA and bind.

Discussion

Studies on these other copolymers were not extensive. Each one points to new possibilities for future investigations and is seen in its present context as a logical extension of the more detailed studies on lysine-alanine complexes.

To evaluate and interpret the differing effects of lysine-alanine, -serine, and -isoleucine copolymers on DNA conformation, it is necessary to measure their effects against established criteria.

According to Ivanov, et al[22], the magnitude of the positive CD band is determined by the helical rotation angle, which in turn controls the width of the narrow groove. The different DNA forms are recognized by different degrees of winding, B-form being intermediate, with C-form more, and A-form less tightly wound. The tighter the winging the narrower the groove, and, as reflected in the CD, the smaller the amplitude of the CD positive band. These particular forms of the double helix (i.e., A-, B-, and C-form) are controlled by three competing forces:

1) H-bonding between complementary bases;

2) Vertical stacking interactions;

3) Electrostatic repulsion of the negative phosphate groups.

At low ionic strength, there is greater repulsion of the phosphates, and the narrow groove widens; with increase in ionic strength, there is greater concentration of cations surrounding the phosphates, the repulsion is diminished, and the groove narrows in response to solvophobic reactions, which attempt to exclude the solvent. Tighter binding of the helix provides for greater base pair overlap, and stacking interactions are thereby increased; the resulting angle prevents effective H-bonding between the complementary bases, and the

solvophobic interactions are intensified.

A decrease in polarity of the medium, on the other hand, weakens the stacking interactions and strengthens the intrachain H-bonds, thus effectively defusing the solvophobic interactions so that they can no longer compensate for the constraint imposed upon the DNA backbone by winding. A sudden reversal to a more relaxed condition of winding occurs: the transition from B- to A-form. The less polar medium intensifies electrostatic interactions, thus producing counteractive effects (increased repulsion of phosphates, but more effective ion binding); however, this intensification allows spectral changes to be seen at lower concentrations of salt.

By itself, salt gives a non-cooperative change, from B- to C-form, a linear increase in winding of the helix with increasing ionic strength. Combining the effects of salt with those of an apolar medium gives a cooperative reverse transition to the conformation, recognized as A-form.

One other set of observations by Ivanov appears relevant: with DNA in water, there is no apparent difference in the ability of different ions to effect the C-form; all the metal ions, ranging from Li^+ to Cs^+, acted quantitatively in the same way. However, in solutions of high (90%) methanol concentration, where the ions bind to, rather than shield, the phosphates, there was clear specificity in action: of the metal ions, Li^+ stabilizes DNA at the greatest width of the narrow groove, Cs^+ at the narrowest (in other words, Li^+ gives B-form, Cs^+ gives C-form DNA). Related to these facts is the information that Li^+ is the most hydrated of the metal ions, Cs^+ the least, and the size of the Li^+ ion, when surrounded by a double layer of hydrate water, is said to correspond well to the size of the less-twisted narrow groove.

Against this background, the hydrophilic serines appear to be serving the same function as the highly hydrated lithium ions in methanol-water solutions. By embracing the surrounding water molecules, they may prevent the DNA from twisting as the phosphates are bound by lysine, thus maintaining the B-type conformation (Figure VI-7) until close to charge neutralization. Differences in ionic strength appear to affect the balance of forces in different ways at different degrees of coverage (Figure VI-9 a-c). The presence of the water molecules does not provide the positive counteractive power contributed by a less polar medium; therefore, the effect of binding by lysine-serine, essentially, seems to be maintenance of the status quo.

The extreme hydrophobicity of isoleucine apparently does not prevent the lysine from binding. The resulting charge neutralization of the phosphates permits the DNA to wind more tightly in an attempt to exclude the solvent, and the CD positive peak, accordingly, is decreased (Figure VI-10) and red shifted. It seems surprising that the bulky substituents of the isoleucine residues did not interfere sterically with the tight winding, unless, perhaps, the binding of lysine was effective only on the external phosphates. However, if this were true one might expect that increased ionic strength would further neutralize the phosphates, and there would be nothing to prevent the tighter winding in response. Figure VI-11 shows that this did not occur.

If, with increasing r-value in the lysine-serine complexes, the serine hydroxyl group continues to protect DNA from major conformational change, even in the presence of increasing salt concentrations, through

preventing dehydration of the DNA helix, what are the unique qualities contained in the lysine-alanine complexes that make it capable of transition to A-form?

In the presence of NaCl, the effect on DNA of binding by poly ($Lys^{40}Ala^{60}$) mimics that of unbound DNA in 80% dioxane-water medium. It, therefore, must act to:

1) weaken stacking interactions;

2) strengthen intrachain H-bonds;

3) decrease the effects of solvophobic interactions;

4) intensify electrostatic interactions.

How could these effects be accomplished through binding?

Remembering that the swing to A-form is actually a backlash from a delicate balancing of opposing forces, and that it occurs only in the presence of elevated salt concentrations or temperature, it is possible to envision an interplay of forces which permits the sudden change of conformation.

The hydrophobic alanine residues form strong alpha-helices in EDTA buffer, as shown by the CD spectra of complexes in zero salt (Figure V-10 a-d), and such structures tend to be rigid, being stabilized by H-bonds. The constant A-form of RNA in the absence of salt has been explained as due to the rigidity imposed on the backbone by its inability to accommodate the 2' hydroxyl[22]; perhaps, in the case of the copolypeptide, the alpha-helix would impose similar restrictions in the presence of salt, thereby weakening the stacking interactions; hydrophobic interactions of the alanine, increased by the salt, would combat the tendency toward twisting, and this in turn would strengthen the intrachain H-bonds; these effects coupled with the strong electro-

static binding of lysine should prove sufficient to overbalance the solvophobic interactions, and, quite suddenly, produce the A-form.

Together, then, alanine and lysine are able to accomplish in DNA the same end as that effected by the less polar medium in the absence of binding; serine and lysine unite to stabilize the DNA through binding with inclusion of water; isoleucine, in its isolation, allows the lysine to act independently, but in so doing makes a proper evaluation of its potential impossible. In future studies, perhaps the inclusion of a very small proportion of isoleucine in a copolymer containing two other amino acids, whose joint properties already had been studied extensively, would not present solubility problems of the same dimensions and would permit its contribution to protein interactions to be realistically assessed.

Chapter VII

BIOLOGICAL RELEVANCE OF THE MODEL PROTEIN SYSTEM

In order to maintain the biological relevance of a model system, the problems to which it may address itself must be defined in the context of its larger and more complicated native counterpart. A system related to histones must be defined by studies on histones themselves and on the organizational level immediately above them. These studies in turn are defined by all relevant studies at the next higher level, so that an understanding of the whole may be based upon an understanding of the parts which make up that whole. Component parts at every level of dissection are made up of parts of parts of still other parts.

The descent from functioning organism to chromatin within the nucleus of its cells contains a myriad of different levels; a further descent from chromatin to the operational level of individual histones is required in order to arrive at the level appropriate for model protein studies.

The questions addressed to chromatin relate to its structure and function, to the packaging of enormous lengths of DNA into a tiny nucleus, to the mechanisms for activation and control of transcription during the cell cycle[88,89]. Various models of chromatin structure, based on physical studies, have been devised: initially, from X-ray studies, a regular supercoiled structure[90]; then, from electron microscopy, a string of beads, which, after nuclease digestion of chromatin,

appeared as particles, called "ν-bodies"[91]; more recently, the concept of chromatin subunits composed of histones and DNA, visualized in a number of different ways[92,93,94,95], through combining results from kinetic and equilibrium studies which utilized circular dichroism, fluorescence, nuclear magnetic resonance, and other techniques.

By extraction of nucleohistones from the chromatin, the field is narrowed to interrelationships between the five histones and DNA, and to histone-histone interactions which might contribute to the subunit structure. It is further narrowed, then, to the fundamental protein-protein and protein-DNA interactions with which the model protein system is qualified to deal.

The model proteins used in these studies, lysine-alanine, lysine-serine, and lysine-isoleucine, have as their natural counterparts those histones having a higher proportion of lysines among their basic residues, histones f1 (H1), f2a2 (H2A), and f2b (H2B). However, observations made on these models can be related to general characteristics of all histones, including the arginine-rich histones f2a1 (H4) and f3 (H3), as well as to proteins in general, since all natural proteins share the common ingredients of basic, hydrophobic, hydrophilic, and acidic amino acid residues. Results which relate directly to these general properties have the more universal application.

With respect to histones, the varying proportions of these ingredients in the histones of calf thymus are presented in Table VII-1, adapted from tables in Hnilica's Structure and Biological Function of Histones[95]. The figures in parentheses show the percentage of the chosen copolymer partner which represents each type of ingredient in the

TABLE VII-1

Amino Acid Composition of the Main Histone Fractions

Histone	%Basic	(Lys) %	%Hydro-phobic	(Ala) %	%Hydro-philic	(Ser) %	%Acidic
f1 (H1)	30.4	(28.7)	43.7	(25.1)	19.5	(6.7)	5.4
f2a2(H2A)	25.3	(12.5)	38.9	(13.2)	21.2	(5.0)	14.2
f2b (H2B)	25.4	(16.7)	35.1	(10.2)	26.5	(10.9)	12.5
f3 (H3)	26.7	(10.1)	39.8	(13.5)	18.5	(3.8)	14.6
f2a1(H4)	26.7	(9.8)	33.4	(7.5)	21.5	(2.5)	11.2

Adapted from Table 2, Structure and Biological Function of Histones, by L. S. Hnilica, (Chemical Rubber Company, 1972).

TABLE VII-2

Relation of Input to Product Composition

Input Lys:Ala	Analyzed Product Lys:Ala	Percent Lys:Ala	Deviation %
19:81	1.0:4.4	19:81	0
40:60	40:58.5	41:59	1
50:50	1.0:1.1	48:52	2
60:40	53.3:40	57:43	3
70:30	61.5:30	67:33	3
90:10	42:10	81:19	9

model protein. Isoleucine, not included in the table, is about 5% in all histones except f1, where it is only 0.8%. Such figures do not address themselves to the distribution of these types within the various histone molecules. However, it has been demonstrated that all histones are characterized by a distinct asymmetry in distribution of the basic residues, the majority being located in the N-terminal end of four of the histones (H2A, H2B, H3, and H4)[89] and in the C-terminal end of the very lysine-rich histone (H1). In the other half of the molecules the composition is similar to that found in globular proteins[88].

The use of random copolymers have advantages and disadvantages. The statistical average of random distribution endows the copolymer with certain general properties inherent in the percentage composition. It is quite likely, however, that the actual distribution of different residues within the molecules is not, in fact, random, due to apparent differences in rates of polymerization of the individual N-carboxyanhydrides in the polymerization mixture. These differences became evident upon comparison of input ratios with the product compositions reported from amino acid analysis. Table VII-2 shows a progressive deviation, from 0 to 9%, with increasing proportions of lysine, which suggests that, in the 1:4 dioxane-benzene solvent mixture, the polymerization of alanine proceeded at a faster rate than that of lysine. In the lysine-alanine 50:50 copolymer (analyzed product, poly($Lys^{48}Ala^{52}$), the deviation favors alanine; in the lysine-serine 50:50 copolymer, on the other hand, the deviation favors lysine over serine analyzed product, poly($Lys^{52}Ser^{48}$) . In the solvent provided for the latter polymerization, the protected epsilon-amino group of the lysine apparently permitted a faster rate than did the OH group of the serine.

Differences in rates imply that there may well be randomly placed blocks of basic residues interspersed in what are probably larger blocks of hydrophobic residues. This may actually have proved to be an advantage in the mimicking of molecules which are asymmetric by nature. Also it might explain some of the difficulties experienced in reproducing results with samples from different stock solutions. Histone sequences remain fixed and results from complexing are, therefore, reproducible; random copolymer complexes show greater variation in average quantitative results, but qualitatively they remain much the same upon repetition.

The techniques of thermal denaturation and circular dichroism have been used to probe and characterize the physical properties of each of the histones; when individually complexed with DNA, each shows characteristics in melting and CD patterns which differ from those of the others. Reports from many laboratories, including our own, have provided an impressive array of data, from which to draw material for study at the model protein level and against which to measure relevant observations within that system.

Thermal Denaturation

Thermal stabilization or destabilization of DNA by proteins or ions is the result of differences in binding strengths and preferences of the ligands with respect to native versus denatured DNA[36]. Redistribution of the ligands between native and denatured segments over the melting range leads to broadening of the melting profiles. Histones bind double-stranded native DNA twice as readily as single-stranded,

whereas the protein RNase shows a preference eight times stronger for denatured than for native DNA's[36]. The former protein stabilizes the DNA and raises the melting temperature; the latter destabilizes and lowers it. These capabilities serve to increase the efficiency of the biological function of each.

Binding by salt, which neutralizes charges on the phosphates and excludes water molecules from the phosphate groups, is not localized along the DNA chain, so that melting of NaCl-DNA complexes yields single-phase helix-coil transitions; binding by nucleohistones and basic polypeptides, on the other hand, is localized, and, as a result, gives multiple-phase transitions, with half-widths and temperatures which are insensitive to the fraction of bound sites[16]. In chromatin and in nucleohistone the DNA base pairs bound by histones melt at two temperatures, which are associated, respectively, with the more-basic and the less-basic halves of all the histones, rather than with individual histone types[14].

The G+C content of natural DNA's also affects the character of their melts, showing a linear relationship of the Tm to the percent G+C[46]. Studies on pure calf thymus DNA, which is 42% G+C[96], consistently showed a non-symmetrical melting profile, with major band at 47°C and shoulder at 54°C, giving evidence of heterogeneity with respect to base composition and seeming to imply a minor fraction with higher G+C content. The theory was tested through exploiting the cooperative, preferential binding and precipitation by polylysine of (A+T)-rich DNA under conditions of salt gradient dialysis. The supernatant following centrifugation contained only free (G+C)-DNA, the pellet, fully-covered (A+T)-DNA. From this it could be demonstrated that, although the

average composition of calf thymus DNA is 42% G+C, a minor fraction (13% of the total) contains 55% G+C base pairs.

Under other conditions of low ionic strength with direct mixing, binding by polylysine is non-cooperative and irreversible (at high salt it is reversible)[98]. Polyarginine at high salt binds and precipitates (G+C)-rich DNA, in the same manner as polylysine with its preference for A•T pairs. Such demonstrated differences are the background materials against which results from copolymer complexes can be evaluated. The biological importance of investigating factors related to the G+C content of DNA's is underscored by the suggestion of Szybalski et al[99], that these sequences may be promoter regions associated with initiation of transcription by RNA polymerase.

Circular Dichroism

Whereas the thermal denaturation studies of histone-DNA complexes emphasize their common asymmetry, circular dichroism has been widely used to probe their individual conformational differences. However, these CD studies have also utilized the characteristic asymmetry through the recording of spectra following cleavage of each histone into its N-terminal and C-terminal halves.

After cleavage of f1 (H1), only the C-fragment changes the CD spectrum with binding, markedly depressing the positive DNA band; the N-fragment binds, but without specificity. When in the intact molecule, it does modify the binding of the C-fragment, however; in so doing, it alters the conformation of the DNA[4]. These experiments, reported by Fasman, et al, were done on complexes prepared by stepwise salt gradient dialysis, from 2M NaCl 0.002M Tris to a final 0.14M NaF 0.002M Tris,

yet, despite the differences in buffer medium and method of preparation, the CD spectrum shown for f1-DNA (in figure 2 of the article) is remarkably similar, qualitatively, to that of the 81:19 Lys/Ala complex in 0.5M NaCl made by direct mixing (shown in Figure IV-4c). The similarities undoubtedly are related to the very high lysine content of both f1 and the 81:19 complex. The spectrum of DNA bound by the C-fragment alone gave a 270nm trough, double that of intact f1-DNA, as does the 100:0 polylysine 270nm trough (Figure IV-4d) compared to that of the copolymer-DNA complex.

The spectra of histone f2a2 (H2A)[100] and f2b (H2B)[78] are entirely different from that of f1, causing blue shifts and greatly increased positive CD bands in the DNA region. Similar changes are also effected by f2a1 (H4), although this is one of the arginine-rich histones [101]. Such changes give evidence of the familiar A-form observed in the Lys/Ala complexes when copolymer composition was in the range of 40-60% alanine. The lysine and alanine content of these histones is around half that of f1 (see Table VII-1), while their acidic content is two to three times as great. Also, the molecular weight of f1 is almost twice that of any of the other histones, so it is not difficult to accept the assigned biological role of the very lysine-rich histone as distinctly different from the rest. With respect to the A-form spectrum observed in complexes with the less basic histones, it was stated by the authors of the above experiments that alterations in the DNA CD spectrum were not artifacts attributable to the effects of light-scattering, based on calculations of $O.D._{400}/O.D._{258}$ (only slightly different from the measure of light-scattering used for the copolymer complexes). A linear increase in this value was observed with increasing histone/DNA ratio

for each histone, all showing the same amount of light-scattering, while the CD spectrum of each remained essentially unchanged. They did suggest, however, that the chiral pattern might be due to specific folding of the DNA and not necessarily to change in the geometry of the double helix[101].

The slightly lysine-rich histone f2b (H2B), when cleaved into two fragments, has an N-fragment richer in basic residues than its C-fragment. The N-fragment has the same effect on the CD as the intact histone, when bound, only smaller; the C-fragment has no effect at all. Yet the C-fragment is required in the intact molecule to give the maximum CD response. This is in contrast to the f1 molecule, where the maximum change was produced by the more basic C-fragment alone and attenuated by the intact molecule. Histone f2b shows an increase in secondary structure and an increase in aggregation with increase in ionic strength. Compared with the arginine-rich f2a1 (H4), which also exhibits the A-type conformation when complexed with DNA in the presence of salt, f2b shows a greater enhancement of the positive DNA band, and the distortion is not reversed at high histone/DNA ratio, as is characteristic of f2a1 at ratios above 1.5[78].

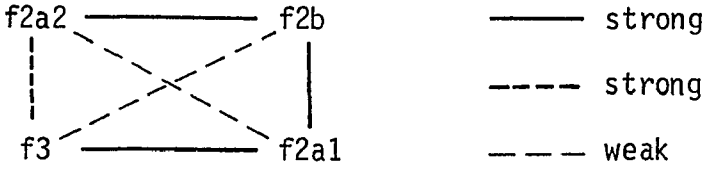
Adler, et al[101], also reported that studies of complexes made with each histone separately and then summed gave results far different from those observed with native nucleohistones, suggesting that among the histones themselves interaction occurred which qualified their binding in complexes which comprised all the histones and DNA. When complexed to DNA each of the histones f1 and f2a1 inhibit the CD distortion caused by the other. Histone f2b was observed to combine with itself, with f2a1, and with f2a2, suggesting that its role might be to organize

clusters for specific binding with DNA. It is interesting to note that all three of these strongly interacting histones, individually, are generators of A-form in DNA[78]. In all three (f2b, f2a2, f2a1), the helical content is essentially the same; therefore they must have similar secondary and tertiary structures which facilitate interactions[100].

Nuclear magnetic resonance experiments showed that, under proper ionic conditions, the central, uncharged portion of histone f2b self-interacts, while the basic ends combine with DNA[102]. It is possible that the rate differential in lysine-alanine copolymerization might furnish just such a self-interacting central portion; since the tempering effects of acidic and hydrophilic residues present in histones are lacking in the copolymer, this would explain the exaggerated A-form spectra displayed by poly($Lys^{40}Ala^{60}$) when complexed in 0.2M NaCl. Their further exaggeration with increasing temperature is explained by the fact that hydrophobic interactions are strengthened at high temperatures[103].

Protein-Protein Interactions Related to DNA

Studies by D'Anna and Isenberg[104] on histone-histone complexes showed that complexing increases the alpha-helical content while it inhibits aggregation and formation of β-sheet. They suggested that the change in secondary structure implied an induced fit, so that the partners could bind to form a compact linear structure composed of four histones held together by interactions of various strengths:



With four different histones, the unit then has direction, which could be associated with one of the DNA strands. Another unit might then exist in the other direction, with weaker crosslinks holding the units together, thus increasing the stability of a DNA duplex.

Demonstrated interactions among histones which make possible this sort of model, on which the larger models of chromatin structure are built, also serve as subjects for dissection into their component parts, from which they may be integrated into the model protein systems. There through constant reference to the natural phenomena, the observations in the simpler system become relevant and provide a means for answering the questions of why and how the circular dichroism spectrum of DNA is altered by the binding of histone molecules.

Chapter VIII

CONCLUSION

The model proteins used in these studies have been shown to be related to their histone counterparts, not only in amino acid content, but in similarities of physical properties, and in their effect upon DNA with binding. Complexes have responded in recognizable patterns to investigation by both thermal denaturation and circular cichroism. Effects due to hydrophobic interactions have been confirmed in their origin by the generation of exaggerated spectra. The dampening effect of hydration on the magnitude of the DNA CD peak has been demonstrated through the presence of hydrophilic serine in the complex. Salt effects have been separated and compared to physical properties observed under no-salt conditions, and effects of temperature on both conformation and binding have been examined.

Physical studies of model protein systems are fascinating in and of themselves, but their primary value lies in simplifying the complex relationships inherent in their biological counterparts. The foregoing research has been dedicated to that end.

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