

AN ABSTRACT OF THE THESIS OF

RICHARD RANDALL WICKETT for the DOCTOR OF PHILOSOPHY
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Title: CONFORMATIONAL CHANGES IN HISTONE IV AS INDUCED
BY SALTS AND NUCLEOSIDE TRIPHOSPHATES

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Irvin Isenburg

The addition of salts or nucleoside triphosphates to histone IV solutions induces conformational changes in the histone molecule. These conformational changes are investigated by measurement of the fluorescence anisotropy of the histone tyrosine emission, the circular dichroism (CD) of the peptide bond and of the tyrosine CD. Changes induced by Na_2HPO_4 , NaH_2PO_4 , Na_2SO_4 , NaClO_4 , NaCl , MgCl_2 , NaF , ADP, ATP, GTP, CTP and TTP are investigated.

Salt induced conformational changes are separated into two steps which occur on vastly different time scales. There is a change which is very fast and recorded simply as a rise. At sufficiently high salt concentration the fast change is followed by a slower one which takes minutes to hours depending on solution conditions.

CD results demonstrate that α -helix formation accompanies the fast step. Fluorescence anisotropy measurements indicate that other

conformational folding, to which the CD is insensitive, and histone dimerization also occur in the fast step. Equations are developed for a quantitative comparison of the salts studied.

The slow step is found to be highly co-operative having a sharp onset at a given salt concentration followed by a steep rise to a maximum. CD spectra and kinetic CD measurements demonstrate that the slow step involves intermolecular β -sheet formation. Both static and kinetic measurements indicate that there is more than one process occurring in the slow step.

Nucleoside triphosphates induce conformational changes at nucleotide concentrations which are of the same order of magnitude as the histone concentration. ATP and GTP interact with histone IV so strongly that plots of fractional conformational change versus nucleotide/histone are indistinguishable over a four fold range of histone concentrations. CTP and TTP are less effective but are at least two orders of magnitude more effective than any of the inorganic salts studied.

Conformational Changes in Histone IV as Induced by
Salts and Nucleoside Triphosphates

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Richard Randall Wickett

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Professor of Biophysics

in charge of major

Redacted for privacy

Acting Chairman of Department of Biochemistry
and Biophysics

Redacted for privacy

Dean of Graduate School

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Typed by Clover Redfern for

Richard Randall Wickett

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CONFORMATIONAL CHANGES IN HISTONE IV AS INDUCED BY SALTS AND NUCLEOSIDE TRIPHOSPHATES

I. INTRODUCTION

Histones: Characterization and Nomenclature

Histones are basic proteins that are found in association with DNA (Murray, 1964). They are rich in lysine and arginine and contain no tryptophan. Histones have been found in all multicellular organisms thus far examined (Johns, 1971). It is probable that they exist in all differentiated organisms with the possible exception of some fungi (Leighton et al., 1971).

Histones were discovered nearly ninety years ago (Kossel, 1884). Stedman and Stedman (1950) first suggested that histones were gene repressors more than two decades ago. Since 1963 when Bonner and Huang (1963) discovered that histones repress DNA dependent RNA synthesis, they have been intensely studied. Such studies were long handicapped because methods for obtaining pure histone fractions have only become available in the past few years.

Histones may be separated from homogenized cell components or nuclei in a number of ways including acid extraction (Luck et al., 1958) or perchloric acid and ethanol (Barr and Butler, 1963). Alternatively, chromatin, which is a complex of DNA, histones, non-histone acidic proteins and perhaps a small amount of RNA

(Bonner et al., 1968), may be isolated in relatively pure form and the histones then extracted. Chromatin is insoluble in 0.14 M NaCl and is usually isolated by treatment of homogenized nuclei with this solvent (Bonner et al., 1969) followed by centrifugation and repeated washing of the precipitate. The histone may then be dissociated from the DNA by treatment with 0.20-0.25 M HCl or H_2SO_4 or 2.0 M NaCl. Following this treatment the DNA, which is still complexed to the non-histone proteins, may be removed by either ultracentrifugation or precipitation with ethanol to yield whole histone protein.

Two major systems of histone nomenclature have been developed. Each reflects the differing techniques used to separate histone fractions. Luck et al. (1958) used column chromatography on IRC-50 with a guanidinium chloride gradient at pH 6.8 to separate whole histone into four fractions which were called I, II, III and IV. Johns et al. (1960) separated three main fractions on carboxymethylcellulose using pH 4.2 buffer followed by dilute HCl. These fractions were labeled F1, F2 and F3. Although it has subsequently become clear that neither of these methods yield homogenous proteins the two types of nomenclature have been applied to the histone fractions which have been purified from these mixtures. This, combined with the practice of labeling histones as lysine rich, slightly lysine rich, arginine rich, etc. has led to some confusion in the past. It is now clear that there are five major histone fractions; accordingly, the nomenclature has

been standardized.

Histone I, or F1, is a very lysine rich histone (lys/arg = 15.0) and is also distinguished from other histones by its high content of proline. Histone I (F1) is the largest of the major histones with a molecular weight of approximately 21,000 (Teller, Kincade and Cole, 1965; Haydon and Peacocke, 1968; Rall and Cole, 1971). This protein exhibits microheterogeneity, that is, there are a number of components of approximately equal molecular weight with nearly identical properties occurring in tissue (Kincade and Cole, 1966; Stellwagen and Cole, 1968; Bustin and Cole, 1969; Kincade, 1969).

Histone Iib2 (F2B) is a lysine rich histone (lys/arg = 2.5). Iib2 (F2B) has been sequenced (Iwai et al., 1970; Hnilica, Kappler and Jordan, 1970). This histone has a molecular weight of 13,770 from the complete amino acid sequence and is the only histone fraction with N-terminal proline.

Histone Iib1 (F2A2) is an intermediate or slightly lysine rich histone (lys/arg = 1.1) and has a high content of leucine. The complete sequence is not yet known but fragments accounting for most of the amino acids have been determined (Phillips, 1971; Hayashi and Iwai, 1971). Comparative gel exclusion chromatography and the mobility in gel electrophoresis suggest a molecular weight of about 15,000 (Phillips and Clarke, 1970) which is consistent with a value of 14,700 found for Iib1 from sedimentation studies (Hnilica, 1967) on

fraction F2A (a mixture of IIb1 and IV) and $16,800 \pm 400$ (Diggle and Peacocke, 1971) on fraction F2A2 (IIb1) in guanidium chloride.

Histone IV (F2A1) is an arginine rich histone with a high content of glycine and is sometimes referred to as glycine-arginine rich or GAR histone. The complete amino acid sequence has been determined (Delange et al., 1969a; Ogawa et al., 1969). The protein has a molecular weight of 11,300. The amino-terminal residue is acetyl-serine and the carboxy-terminal residue is glycine.

Histone III (F3) is arginine-rich ($\text{lys/arg} = 0.8$) and is the only histone containing cysteine. The complete amino acid sequence of calf thymus histone III (F3) has recently been determined (Delange, Hooper and Smith, 1972) and the molecular weight is 15,324. The 1/2 cystine residues are at residues 96 and 110. There is also a minor component of F3 (III) in calf thymus consisting of about 20% of the F3 (III) of calf thymus and containing only 1/2 cystine residue (Marzluff et al., 1972).

As the histones have been partially or completely sequenced a striking feature of the primary structure has emerged. The amino acid distributions are very irregular with a high density of basic residues in one portion of each molecule. In histone IV (F2A1) the amino terminal half of the molecule (residues 1-50) contains 9 arginines, 6 lysines, and only 1 glutamic acid for a net total of 14 positive charges (Delange et al., 1969a; Ogawa et al., 1969). This

half of the molecule also has 12 glycine residues and relatively few hydrophobic residues. In the carboxy-terminal half molecule (residues 50-102), the picture is quite different. There are 4 lysine and 4 arginine residues but 3 glutamic and 2 aspartic acid residues totaling only 2 net positive charges. This portion of the molecule also contains a much higher proportion of residues with hydrophobic side chains: leucine, isoleucine, valine, threonine, methionine, alanine, phenylalanine, and tyrosine.

A similarly uneven distribution of basic residues is seen in histone Iib2 (F2B) (Iwai et al., 1970), histone III (F3) (Delange et al., 1972) and histone I (F1) (Bustin et al., 1969; Rall and Cole, 1971). In histone I (F1), however, the preponderance of basic residues is in the carboxy-terminal half of the molecule. While the complete amino acid sequence of histone Iib1 (F2A2) has not yet been determined, it is apparent from the analysis of tryptic peptides that this histone also has a very uneven distribution of amino acids (Phillips, 1971; Hayashi and Iwai, 1971).

Thus a general picture of histones emerges. They are relatively small basic proteins with molecular weights on the order of eleven to twenty thousand. Approximately one-half of the molecule carries a high density of positive charges while the other half has an amino acid composition similar to that of enzymes or other globular proteins.

Biological Functions of Histones

The biological functions of the histones have not yet been determined. It has been suggested that histones are responsible for the repression of genes in differentiated organisms (Stedman and Stedman, 1950; Ts'o and Bonner, 1964). It has also been suggested that they are structural proteins (Wilkins, Zubay and Wilson, 1959; Littua et al., 1965; Stellwagen and Cole, 1969; Shih and Fasman, 1971; Bradbury and Crane-Robinson, 1971) which provide for the packaging of the DNA in the chromosome and the morphological changes observed in chromosomes during cell division. These proposed functions are not mutually exclusive.

In differentiated cells only a small and very specific portion of the DNA is available for transcription. The discovery that histones inhibit in vitro DNA dependent RNA synthesis (Bonner and Huang, 1963; Allfrey and Mirsky, 1964) lends support to the hypothesis that histones act as gene repressors. Whole chromatin is very inefficient in supporting in vitro DNA dependent RNA synthesis but removal of the histones under conditions which leave most of the non-histone protein intact yields material with a template activity almost as high as that of free DNA (Allfrey et al., 1964; Marushige and Bonner, 1966). However, not all of the repression found in chromatin is due to histones (Spelsberg, Hnilica and Ansevin, 1971).

The different histone fractions have been found to vary somewhat in their ability to inhibit in vitro DNA dependent RNA synthesis (Barr and Butler, 1963; Allfrey and Mirsky, 1964; Spelsberg and Hnilica, 1971a). The transcription of RNA from reconstituted histone-DNA complexes is inhibited most by fraction F3 (Barr and Butler, 1963) and least by fraction F1 (Allfrey and Mirsky, 1964). However, when excess histone is present, all fractions yield at least 80% inhibition (Barr and Butler, 1963). While the selective extraction of histone F1 by $(\text{NH}_4)_2\text{SO}_4$ was found to produce no change in RNA synthesis from isolated chromatin, the removal of the other histone fractions produces a dramatic increase in in vitro RNA synthesis (Spelsberg and Hnilica, 1971a).

There are clear examples of changes in the histone content of cells during differentiation and development. During sea urchin embryogenesis histones do not appear until the blastula stage at which time they are synthesized rapidly (Nemer and Lindsay, 1969). As avian erythrocytes mature, histone I (F1) is replaced by a unique histone fraction F2C (V) with a high serine content (Vidaldi and Neelin, 1968). Fambrough, Fujimaru and Bonner (1968), found a large variation in the quantitative distribution of fractions, I (F1), IIa (F2B with some others) and IV (F2A1) in the different organs of peas but no variation in fractions IIb (F2A2 with some F2B) and III (F3). In spite of these differences, the limited number of different histone

molecules, even with the micro-heterogeneity of histone I (F1) (Kincade and Cole, 1966), as well as their presence in essentially constant amounts and proportions in nuclei of a wide variety of cell types (Hnilica, Johns and Butler, 1962; Hnilica, 1966; Laurence et al., 1963, 1966; Johns, 1971), argues against histones as highly specific repressors of transcription. This has led to a search for additional factors which might be involved in mediating the histone-DNA interaction to produce more specificity.

The RNA transcribed from isolated chromatin is tissue specific and competitive RNA-DNA hybridization experiments give evidence that this specificity is the same for in vivo and in vitro transcription (Paul and Gilmore, 1968; Gilmore and Paul, 1969; Bonner et al., 1968; Tan and Miyagi, 1970; Spelsberg and Hnilica, 1971b).

Chromatin components can be dissociated by salt and reassociated by dialysis without the loss of this specificity if 5 M urea is present during reassociation (Paul and Gilmore, 1968; Bonner et al., 1969; Huang and Huang, 1969).

Huang and Bonner (1965) reported the presence, with histones, of a class of RNAase resistant RNA molecules of 40-60 nucleotides in length, with a high content of dihydrouridine. These molecules, known as chromosomal RNA, were reported to be co-valently bound to histone molecules in a complex that contained one RNA and several histone molecules (Bonner et al., 1968). Bekor et al. (1969)

presented evidence from RNA-DNA hybridization experiments with native and reconstituted chromatin that this RNA is essential for the specific translation of chromatin. Artman and Roth (1971), however, have suggested that this RNA is an artifact of histone isolation. Thus the reality of these results is still in doubt.

Chromatin also contains a complex mixture of acidic proteins (Stellwagen and Cole, 1969) and there is increasing evidence that these are involved in genetic control mechanisms, possibly as specific derepressors. Non-histone chromosomal proteins stimulate in vitro DNA dependent RNA synthesis from chromatin (Wang, 1970) and may also stimulate RNA synthesis in vivo (Allfrey, 1971). Gilmore and Paul (1969) concluded that the presence of such proteins is necessary for the specific reassociation of chromatin. The most direct evidence for the importance of non-histone proteins comes from the work of Spelsberg, Hnilica and Ansevin (1971) who found that the specific behavior of RNA transcribed from reconstituted liver or thymus chromatin, in competitive RNA-DNA hybridization experiments, is dependent on the presence of the respective liver and thymus non-histone proteins. When liver non-histone protein was replaced by thymus non-histone protein before reassociation, the RNA transcribed from the product chromatin behaved as if it had been transcribed from native thymus chromatin. Replacing thymus non-histone protein with that of the liver yielded RNA similar to that transcribed from liver

chromatin. As the other chromatin constituents were presumably left intact, these results were taken as strong evidence that the non-histone proteins play a vital role in the specific control of transcription. However, Bonner et al. (1968) used similar RNA-DNA hybridization techniques to conclude that non-histone proteins are not important for specific transcription. Difficulties in interpreting RNA-DNA hybridization results arise from the many repetitious or very similar nucleotide sequences found in the DNA of higher organisms (Britten and Kohne, 1968). This leads to cross-reaction between RNA molecules and very similar but not identical DNA sequences (Britten and Kohne, 1968).

The chemical modification of histones which occurs in vivo is a possible means of either introducing more specificity into the histone-DNA interaction or of allowing transcription from specific regions of a chromosome which has been generally repressed by histones. The phosphorylation of serine (Ord and Stockton, 1968; Stockton and Stevely, 1968; Lagan, 1969) and the methylation (Murray, 1964, Delange et al., 1969a) and acetylation (Phillips, 1971; Delange et al., 1969a) of lysine are well established. All of these changes reduce the net positive charge on the histone molecules and could weaken their interaction with DNA. Even small amounts of acetylation significantly reduce the ability of histones to inhibit in vitro DNA dependent RNA synthesis (Allfrey et al., 1964) as does the phosphorylation of F1 (I)

(Stockton and Stevely, 1968; Ord and Stockton, 1968).

Both phosphorylation of F1 (Lagan, 1969) and acetylation of arginine rich histones (Allfrey, 1964) have been observed during hormone induced stimulation of RNA synthesis in vivo. These chemical modifications have also been observed during growth and development. The phosphorylation of F1 is extensive in regenerating rat liver or dividing rat heptoma cells but is absent from non-dividing cells (Ord and Stockton, 1968; Gutierrez-Cernosak and Hnilica, 1971; Balhorn, Chalkly and Granner, 1972) and acetylation of histones F3 (III) and F2A1 (IV) has been correlated with metabolic changes in differentiating chick embryos (Boffa and Vidaldi, 1970).

Other types of chemical modifications may be significant. Thiol/disulfide conversions in histone III (F3) have been reported to occur (Ord and Stockton, 1968) during growth and development. The methylation of histidine (Gershey et al., 1969) and arginine (Paik and Kim, 1970) may also occur in vivo in small amounts but the evidence for their occurrence is not clear cut (Allfrey, 1971).

Histone IV (F2A1) molecules from pea seedling and calf thymus have only two conservative differences in amino acid sequence, a lysine replaced by arginine and an isoleucine by a leucine (Delange et al., 1969b). This amazing sequence preservation after two billion years of divergent evolution is unique (Dickerson and Geiss, 1969) and indicates that the function of histone IV involves the entire

primary structure of the molecule. This sequence homogeneity and the apparently universal occurrence of the five major histone fractions (Johns, 1971) suggests that histones may play an integral role in the structure of chromatin. They may be involved in the folding required to pack DNA with a chain length of several meters into a chromosome with dimensions of a few microns, and in the morphological changes which chromatin undergoes during cell division, differentiation and dedifferentiation.

Such functions should be reflected in the physical properties of histones and histone DNA-complexes which will be reviewed in the next two sections.

Studies on the Conformation of Histones

The structure of chromatin has not yielded to X-ray diffraction analysis in the way that the structures of DNA and many globular proteins have. Chromatin is an extremely complex substance and forms gels instead of crystals. Although some interesting results, which will be subsequently discussed, have been obtained with X-ray techniques, the three dimensional structure of chromatin is still largely a mystery. One approach toward solving the problem has been to study the conformational properties of histones and histone mixtures. Such studies are undertaken with the hope of obtaining information about the structure and interactions of these less complex constituents which

will eventually be useful in helping to elucidate both the structure of chromatin and the biological functioning of histones.

Some of the earliest physical studies of histone conformation were made on films cast from solutions of whole histone preparations. Infrared spectra of histone films (Bradbury et al., 1962) showed a strong amide I band at 1660 cm^{-1} and an amide II band at 1550 cm^{-1} which were tentatively attributed to α -helix. It is known that random coil absorbs near these frequencies (Elliot et al., 1958) but a fraction (~22%) of the amide protons were found to exchange very slowly. This fraction was completely deuterated only after three weeks exposure to an atmosphere of 94% relative humidity of D_2O . Since no infrared absorbance bands corresponding to β -sheet structure were found in freshly prepared films, this shielding of amide protons was interpreted as further evidence for the presence of α -helical structure. Optical rotatory dispersion (ORD) measurements (Elliot, Bradbury and Zubay, 1962) also gave evidence for the presence of α -helix in histone films cast from water. The helix content was estimated from the Moffitt constant, b_0 (Moffitt and Yang, 1956), as 25% ($b_0 = -155$). The X-ray patterns of such films depend upon the conditions of preparation (Zubay and Wilkens, 1962). Films cast after isolation of histone by the addition of 0.50 M HCl to nucleohistone and sedimentation of the DNA by centrifugation gave rise to X-ray diffraction patterns with diffuse rings at 4.5 and 10 \AA which were interpreted as the

result of α -helix. The diffraction patterns from histone films cast after isolation of histone from nucleohistone by the addition of 4 M NaCl and precipitation of the DNA with ethanol showed sharp reflections at 4.7 \AA which are characteristic of β -sheet structure.

ORD studies on histones in solution (Elliot, Bradbury and Zubay, 1962; Jirgensons and Hnilica, 1965; Bradbury et al., 1965, 1967; Tuan and Bonner, 1969) have consistently found very little α -helix or β -sheet in water and that their secondary structure is quite dependent on solution conditions.

Elliot, Bradbury and Zubay (1962) found that b_0 was equal to -20 for whole histone solutions both in water and 8 M urea, while Jirgensons and Hnilica (1965) found $b_0 = 0$ for whole histone in water. A b_0 value close to 0 (compared to -620 for fully helical protein (Bradbury et al., 1967)) and the same in water and 8 M urea is strong evidence that the histones have very little secondary structure in water. In 0.10 M NaCl b_0 was found to be -97 and in 1.0 M NaCl b_0 had decreased to -146 (Jirgensons and Hnilica, 1965) demonstrating that histones have considerable secondary structure in salt solutions. Elliot et al. (1962) found a b_0 value of -412 in the helix promoting solvent ethylene chlorhydrine.

ORD studies on histone fractions (Jirgensons and Hnilica, 1965; Bradbury et al., 1965, 1967; Tuan and Bonner, 1969) have found that there are important differences among the histone fractions in salt

solutions but that all fractions have little secondary structure in water. Jirgensons and Hnilica (1965) studied histone fractions in water and 0.10 M NaCl. For fraction F1 (I), b_0 was +13 in water and -26 in 0.10 M NaCl, indicating that F1 (I) has little helical content at this ionic strength. However, for fraction F2A (a mixture of F2A1 (IV) and F2A2 (IIB1)) b_0 was -123 in 0.10 M NaCl and for F2b (IIB2) and F3 (III) in 0.10 M NaCl, b_0 was -147 and -87 respectively. These authors found little difference in the salt effect when Na^+ was replaced by K^+ , Mg^{++} , or Al^{+++} but phosphate was found to be much more effective than chloride in inducing ORD changes.

Bradbury et al. (1965, 1967) and Tuan and Bonner (1969) have also found that the additions of salts to histone fractions causes an increase in their secondary structure and that the ORD of histone I (F1) is the least affected by salts. However Taun and Bonner (1969) did find that in 3.4 M NaCl histone I has an ORD spectrum consistent with either 25% α -helix and 75% random coil or 35% β -sheet and 65% random coil.

Bradbury et al. (1967) pointed out that ORD changes can result from either α -helix or β -sheet formation. These same authors presented data from infrared absorbance studies in D_2O solutions. There was little absorbance characteristic of β -sheet in fractions F2A, F2B or F3 in 1.0 M NaCl. Fraction F1, however, has an infrared peak at 1630 cm^{-1} in 1.0 M NaCl which corresponds to the absorbance of

amide vibrations in β -sheet structure.

The dye 8-anilino-naphthalene-1-sulfonic acid (ANS) is essentially non-fluorescent in water (quantum yield 0.004) but has a high quantum yield in non-polar solvents, or when absorbed to hydrophobic regions of proteins (Weber and Young, 1964). Laurence (1966) used ANS as a probe to investigate the effect of salts on histone solutions. The fluorescence intensity of F2A or F3 with excess ANS was very sensitive to salt and increased by an order of magnitude when the NaCl concentration was raised from 0.0 to 0.50 M (in 17 mM acetate pH 4.7) and leveled off as the NaCl concentration was further increased. The fluorescence intensity of ANS with F2B or F1 showed little dependence on NaCl and was much lower than with F2A or F3. The increase in fluorescence intensity in NaCl was interpreted as the result of ANS binding to conformationally altered histone molecules. The fluorescence intensity of ANS-F2A showed a further increase with time after NaCl addition but was not dependent on the time of ANS addition. This time dependent increase took one to several hours to reach completion. The change was faster at 8 mg/ml protein than at 0.26 mg/ml and was interpreted as due to slow histone aggregation. Histone aggregation has been observed in the ultracentrifuge (Cruft, Mauritzen and Stedman, 1958; Haydon and Peacocke, 1968; Edwards and Shooter, 1969; Diggle and Peacocke, 1971) for all fractions and fraction F2A is particularly prone to aggregate (Edwards and Shooter,

1969). Cruft et al. (1958) observed time dependent aggregation in " β -histone," which has an amino acid composition approximately corresponding to a mixture of F2A and F3. In agreement with the ORD results of Jirgensons and Hnilica (1965), Laurence (1966) found that divalent anions were much more active than chloride in inducing changes in fraction F2A. Phosphate and sulphate induced a maximum increase in fluorescence intensity at a concentration 30 times less than that for chloride.

It should be pointed out that the histone fractions used in these early studies were generally not pure histone proteins. This is especially true of fraction F2A which is now known to be a mixture of two histones, F2A1 (IV) and F2A2 (IIB2). This may be an important consideration as Laurence (1966) reported that fraction F2B inhibited the slow salt induced changes in F2A-ANS mixtures, indicating that there may be cross reactions among histone species.

As mentioned previously, techniques for separating histones into homogenous fractions have recently become available. Furthermore, the complete amino acid sequences of F3 (III) (Delange et al., 1972), F2B (IIB2) (Iwai et al., 1970; Hnilica et al., 1970) and F2A1 (IV) (Delange et al., 1969a; Ogawa et al., 1969) give reliable criteria of purity for these proteins. Thus the most recent physical studies, which will now be discussed separately, have been done on well defined histone proteins. Of course, fraction F1 (I) as currently

isolated, is still a mixture of a number of proteins with slight differences in their primary structure (Bustine and Cole, 1969; Kincade and Cole, 1966).

The nuclear magnetic resonance (NMR) spectrum of whole histones in D_2O has many sharp peaks which can be assigned to protons of the various amino acid residues (Boublik et al., 1970a, 1970b; Bradbury and Crane-Robinson, 1971). The sharpness of these peaks results from rapid rotational averaging. As histones undergo conformational changes, residues which are incorporated into more rigid structures will have much broader resonance peaks with an apparent loss of peak area. Such studies are particularly interesting because of the very non-uniform distribution of amino acid residues in the primary structure of histones.

The effect of salt on the NMR spectra of histones F2A (IV) and F2B (IIb2) is to broaden the resonance peaks from protons of the hydrophobic amino acid residues (Boublik et al., 1970a, 1970b) which are located predominantly in the C-terminal halves of both molecules (Delange et al., 1969a; Iwai et al., 1970). A particularly noticeable effect was the complete disappearance of the tyrosine resonance peaks of F2A1 (IV) in 0.10 M NaCl. This is significant because all four of the tyrosine residues of F2A1 (IV) are in the C-terminal half of the molecule (Delange et al., 1969a; Ogawa et al., 1969). For both proteins, peaks assigned to leucine, isoleucine and

phenylalanine were considerably broadened in 0.10 M NaCl, while those from glycine, histidine, lysine and arginine were much less affected.

An NMR and ORD study on the half-molecules of F2B (IIb2), cleaved between residues 58 and 63, confirmed that the salt induced conformational changes occur primarily in the C-terminal half of the molecule (Bradbury et al., 1972). ORD measurements showed that the half-molecule containing residues 63-125 undergoes large conformational changes as the salt is raised from 0 to 0.5 M NaCl. NMR measurements further localized this change to residues 66 to 102. The N-terminal half-molecule (residues 1-58) showed no significant ORD changes even at 2.5 M NaCl but NMR line broadening indicated that some interchain interactions occur in 2.5 M NaCl. These interactions were localized to a segment between residues 30 and 50.

With histone I (F1) considerably more salt is needed to produce significant line broadening (Boublik et al., 1970a). In 1.0 M NaCl the resonance peaks from phenylalanine, tyrosine, leucine, isoleucine, and valine are broadened while the peaks from protons of the lysine side chain and alanine are broadened very little, if at all. The observed changes were attributed to restriction of the mobility of the residues between 52 and 100. This region contains the highest proportions of leucine, isoleucine and valine as well as the only tyrosine

and phenylalanine residues in F1 (I) (Rall and Cole, 1971).

Boublik et al. (1970a, 1970b) applied the "helical wheel" analysis of Schiffer and Edmundson (1967) to the primary structure of both F2A1 (IV) (Boublik et al., 1970a) and F2B (IIb2) (Boublik et al., 1970b). In the case of F2A1 the wheels predict that the portion of the sequence with the highest potential for α -helix formation lies between residues 55 and 72. Some potential for α -helix formation is found between residues 37-54 and 73-90. Helical wheels for F2B (IIb2) predict that helix formation will be between residues 37-125.

Bradbury and Crane-Robinson (1971) combined the NMR studies with the helical wheel analysis and the previously discussed ORD, IR and sedimentation results to propose models for the salt induced conformation changes in F1 (I), F2B (IIb2) and F2A1 (IV).

The proposed model for F1 (I) is for either inter- or intrachain β -sheet formation involving residues 52-100. This β -sheet formation was proposed to begin in the range of 0.20-0.40 M NaCl and lead to aggregation, possibly by interchain β -sheet formation, at NaCl concentrations above 1.0 M. The authors noted that the presence of some α -helical structure at higher salt cannot be ruled out.

For both F2A1 (IV) and F2B (IIb2) the proposed model was α -helix formation in the C-terminal half of the molecule at NaCl concentrations below 0.10 M followed by histone-histone interactions at higher NaCl concentrations. Both F2B (IIb2) and F2A1 (IV) have been

found to aggregate in 0.10 M NaCl (Diggle and Peacock, 1971; Barkley and Eason, 1972). The circular dichroism (CD) spectrum of histone IV (F2A1) in 0.14 M NaF (Shih and Fasman, 1971) also gives partial support to the proposed model. The spectrum is considerably changed from that in water and indicates the presence of considerable secondary structure. However, the CD spectrum appears to result from a combination of α -helix and β -sheet and may be fit by a combination of polypeptide CD spectra (Greenfield and Fasman, 1969) having 24% α -helix and 36% β -sheet.

The salt induced changes discussed above depend not only on the ionic strength but on the type of salt used (Jirgensons and Hnilica, 1965; Laurence 1966). Indeed Li et al. (1971) reported that 0.005 M phosphate buffer at pH 7.4 induces a drastic change in the CD spectrum of histone IV (F2A1) altering the spectrum from that in water to that of a protein with considerable α -helix and β -sheet.

The Conformation of Nucleohistone

Investigations of nucleohistone structure have centered around several key questions. While X-ray techniques have partially illuminated the conformation of DNA, the conformation of the histone molecules and their distribution along the DNA chain is still largely unknown. Other important questions concern the superstructure of nucleohistone. What is the tertiary structure of the DNA-histone

complex? What are the roles of individual histone molecules in maintaining the structural integrity of the complex?

To answer these questions, three approaches have been employed. The first and most obvious method is to study isolated whole nucleohistone. Many workers have also studied nucleohistone which has been selectively depleted of part of the histone by various techniques. Lastly, much work has been done on complexes of purified histone fractions with DNA.

There is considerable evidence that the major portion of the DNA in nucleohistone is in the B form. The infrared dichroisms of the in-plane vibrations of DNA bases and the phosphate ester chain are almost as high in stretched oriented nucleohistone films as in free DNA (Bradbury et al., 1962). The X-ray diffraction patterns of nucleohistone fibers (Zubay, Wilkens and Blout, 1962; Zubay and Wilkens, 1962; Wilkens, Zubay and Wilson, 1959), while more diffuse than those from DNA fibers, exhibit both the 3.4 Å reflection on the meridian, arising from basestacking, and the layer line pattern from the helical sugar phosphate chain. Wide angle X-ray scattering patterns from histone gels and solutions have been found to be consistent with B-form DNA (Wilkens et al., 1959; Bram, 1971; Bram and Ris, 1971) but not with A or C forms. Bram (1971) concluded that in both nucleohistone and 2.0 M NaCl the DNA is in a slightly distorted B form with a pitch of 32 Å. This interpretation is supported by the

close similarity of the near ultraviolet CD of DNA in nucleohistone and 2.0 M NaCl (Permogorov et al., 1970; Henson and Walker, 1970).

Circular dichroism results indicate that the conformation of DNA in reconstituted histone-DNA complexes depends upon the method of preparation. When histone I (F1)-DNA (Olins, 1969; Fasman et al., 1970; Adler and Fasman, 1971) or histone IV (F2A1)-DNA (Shih and Fasman, 1971; Li et al., 1971) complexes are prepared by gradient dialysis from 2.0 M NaCl (and 5M urea in the case of histone IV) to an ionic strength of 0.01 or less, the CD from 240-300 nm, where there is no contribution from histone, is unchanged from that of native DNA. When the dialysis is stopped at 0.14 M NaF the results are quite different. Under these conditions histone I (F1) causes the positive ellipticity band at 275 nm to disappear while the negative band at 245 nm more than doubles in size and is red shifted to 250 nm. Histone IV (F2A1) causes the positive band at 275 nm to increase in size while the negative band at 245 nm decreases somewhat and a new, smaller, negative band appears at 305 nm. The two histones act antagonistically in mixed complexes in 0.14 M NaF (Shih and Fasman, 1972). Histone I blocks the changes induced by histone IV at a ratio of I/IV of 1/4 but a ratio of IV/I of 1/2 is necessary for histone IV to block the changes induced by histone I. Phosphorylation at serine 37 significantly reduces the effectiveness of histone F1 (I) in inducing the observed conformational changes (Adler et al., 1971).

In addition to those reflections characteristic of B-form DNA, X-ray patterns of nucleohistone exhibit features which are absent from the patterns of either isolated DNA or histones. Wilkens et al. (1959) observed a semi-meridionally oriented ring at 37 \AA and attributed this spacing to histone bridges between roughly parallel DNA molecules. Subsequently, a series of low angle reflections at 110 , 55 , 37 and 27 \AA have been observed in nucleohistone gels (Luzatti and Nicolaieff, 1963) and fibers (Pardon et al., 1967; Richards and Pardon, 1971). These reflections disappear when fibers are mechanically stretched (Pardon, Wilkens and Richards, 1967; Richards and Pardon, 1971). This observation led Pardon et al. (1967) to postulate a super-helical structure for nucleohistone. The proposed super-helix has a pitch of about 120 \AA and a diameter of approximately 100 \AA . Recent electron micrographs of nucleohistone have shown fibers with a diameter of 80 to 100 \AA (Bram and Ris, 1971) and when these fibers were stretched prior to fixation the diameter of some decreased to a minimum of 25 \AA .

The characteristic "supercoil" X-ray pattern was also found in reconstituted whole histone DNA complexes prepared either by dialysis from 2.0 M NaCl or direct mixing at 0.14 M NaCl (Richards and Pardon, 1971). The selective removal of histone F1 (I) by 0.70 M NaCl or treatment with 1.0 M NaCl which removes all of F1 and some of the other histone fractions (Ohlenbush et al., 1967) did

not alter the X-ray patterns but fibers of nucleohistone which had been treated with 1.1 M NaCl showed only reflections characteristic of B form DNA. A partial nucleohistone prepared from isolated DNA and a mixture of histones F3 (III), F2A2 (IIb1) and a trace of F2A1 (IV) also exhibited the series of low angle rings attributed to a nucleohistone superhelix.

Flow dichroism (Ohba, 1966) and birefringence measurements indicate that the length of nucleohistone increases by about 50% when the histones are removed. This change was effected primarily by removal of the arginine rich histones. The specific dichroism was found to be lower than that of native DNA as is the specific electric birefringence (Houssier and Fredericq, 1966). This implies that the bases are tilted with respect to the axis of orientation. Both base tilting and a decrease in overall length are consistent with the presence of super-helical structure.

Histones stabilize DNA against thermal denaturation and the melting of histone-DNA complexes has been extensively studied by following the increase in DNA absorbance that accompanies melting. DNA isolated from pea embryos has a T_m of 70° in 0.16 M sodium citrate while whole pea embryo chromatin has a two step melting profile with T_m 's at 69.5° and 84° (Bonner and Huang, 1963). This was interpreted as melting of uncomplexed DNA and 69.5° and fully complexed nucleohistone at 84° . The effect of histones is similar in

magnitude to that obtained by raising the ionic strength from 0.007 to 0.5 M (Lee, Walker and Peacocke, 1963). Ohlenbush et al. (1967) observed a broad melting profile for calf thymus nucleohistone with a T_m of 76° . As histones were removed by salt extraction the curve progressively shifted to lower temperatures and DNA extracted with 2.0 M NaCl melted at 49° . The biggest shift in T_m was caused by the removal of histone I (F1). Studies on reconstituted histone-DNA complexes (Huang, Bonner and Murray, 1964) indicate that histone I is the most effective of the histones in stabilizing DNA. A histone I-DNA complex with a histone/DNA weight ratio of 1.4 had a T_m of 81° compared to $70-75^\circ$ T_m for similar complexes with other histone fractions.

Ohba (1966) applied the analysis of hypochromic dispersion (Felsenfeld and Sandeen, 1962) to nucleohistone. In nucleohistone, as distinct from DNA, the G-C denaturation curve was found to precede the A-T denaturation curve as the temperature was raised. Nucleohistone with 68% of the histones removed still showed the reversed order of denaturation. DNA-polylysine complexes also showed a reversed order of denaturation producing the conclusion that there was some preferential interaction between lysine residues and A-T base pairs.

In whole chromatin the number of basic amino acids is roughly equal to the number of negatively charged phosphate groups (Vendrely

et al., 1960). This approximate charge neutrality does not necessarily mean that every positively charged residue is in contact with a negatively charged phosphate group. Indeed, electrometric titrations indicate that about one-third of the basic amino acids are not in tight contact with phosphate groups (Walker, 1965) and as many as 48% of the phosphate groups are free to bind cationic dyes (Itzahaki, 1970, 1971b). Clark and Felsenfeld (1971) reported that only 50% of the DNA in chromatin was released by nuclease treatment and no histones were found with the digested DNA. It was concluded that the 50% of the DNA which was digested was not covered by histones. This conclusion was supported by polylysine binding data. Polylysine was found to bind to only 50% of the DNA in chromatin, on a nucleotide phosphate to lysine basis, while it binds to 100% of free DNA. The addition of polylysine further protected the DNA against nuclease digestion. The polylysine used was of an average degree of polymerization of 670 which was presented as evidence that free DNA is present in fairly long stretches.

Iztahaki (1971a) reported that nuclease digestion of chromatin is very sensitive to conditions and that 75% digestion can be achieved. It was also reported (Iztahaki, 1971a) that histones were released during the digestion but were precipitated with the undigested chromatin when the techniques of Clark and Felsenfeld (1971) were used. Li, Chang and Weiskopf (1972) reported on changes in the thermal

denaturation profile which occur when polylysine binds to chromatin. These changes were reported to be consistent with polylysine binding to regions of chromatin which are covered by either non-histone protein or the less basic halves of histone molecules. Both Itzahaki (1971a) and Li et al. (1972a) concluded that much less than 50% of the DNA was completely free of protein. Thus the amount of DNA in chromatin which is not covered by histones is currently the subject of controversy.

The conformation of the histone molecules in nucleohistone has proven to be difficult to determine. ORD and CD studies are complicated by the presence of overlapping DNA bands. ORD estimates of the α -helix content of nucleohistone have varied from 18% (Oriel, 1966) to 41% (Taun and Bonner, 1969). Frič and Šponar (1971) concluded from a CD study that histone had more secondary structure in nucleohistone than in a 2.0 M NaCl solution. However, Taun and Bonner (1969) concluded that the histone had less secondary structure in nucleohistone than in 3.4 M NaCl.

Both X-ray diffraction (Zubay and Wilkens, 1962) and infrared dichroism measurements (Bradbury, 1962) indicate that at least some α -helical structure is present in nucleohistone and CD changes resulting from the addition of acridine orange to nucleohistone are more characteristic of the interaction of the dye with α -helical polypeptides than with DNA (Simpson and Sober, 1970).

Bradbury and Crane-Robinson (1971) have estimated the amount of α -helix as between 37% and 45% based on the infrared absorption of hard to exchange amide protons. This estimation is based on the assumption that all of the shielded protons are incorporated into α -helical structure. Incorporation into other types of rigid structure, for example by hydrophobic interactions in the C-terminal halves of F2A1 (IV) or F2B (IIb2), could also produce hard to exchange amide protons making the estimate of α -helix content too high.

X-ray diffraction patterns of nucleohistone do not show a sharp reflection at 4.7 Å (Zubay and Wilkens, 1962; Wilkens et al., 1959). The absence of this reflection is good evidence against the presence of substantial amounts of extended β -sheet conformation in nucleohistone.

The melting profile of reconstituted histone-DNA complexes is biphasic (Huang et al., 1964; Shih and Bonner, 1970) with a low temperature T_M corresponding to the melting of free DNA regions. From an extrapolation of the percent of hyperchromicity in the low temperature T_M versus histone/DNA to zero hyperchromicity Shih and Bonner (1970) concluded that a single molecule of histone IV (F2A1) occupies a DNA segment of nine base pairs while a single molecule of histone I (F1) occupies 41 base pairs. Since histone I (F1) is only about twice as large as histone IV (MW 21,000 vs 11,300) it would appear that histone I is more extended along the DNA chain than is histone IV. This conclusion is consistent with circular dichroism results which indicate

that histone I is in an extended conformation in the histone DNA complex (Adler and Fasman, 1971) while the histone IV contribution to the CD of the histone IV-DNA complex indicates the presence of secondary structure (Shih and Fasman, 1971; Li et al., 1971) in the histone molecule. This spectrum could not, however, be fit to a combination of random coil, β -sheet and α -helix using polypeptide CD spectra as standards (Li et al., 1971).

II. MATERIALS AND METHODS

Preparation of Samples

Histone IV from calf thymus was prepared and purified by the method of Ogawa et al. (1969). Our samples were electrophoretically homogeneous and their amino acid composition agreed with the published sequence (Delange et al., 1969a; Ogawa et al., 1969). The histone IV concentration in water was determined spectrophotometrically by taking $\epsilon_{230} = 4.7 \times 10^{-2} \text{ cm}^{-1}$ per mole of residue per liter (Ohlenbusch et al., 1967; Shih and Bonner, 1970). H_0 will denote the total histone concentration in moles of protein per liter.

Salt stock solutions were added to histone IV in water with constant mixing. The time of salt addition is called time zero in this work. The temperature was controlled to $22 \pm 1^\circ \text{C}$. For a given stock salt solution the variation in pH was less than 0.05 pH units. Phosphate was used as its own buffer. When other salts were used, $5 \times 10^{-3} \text{ M}$ cacodylate buffer was used at the pH's noted. Cacodylate, by itself, induced no detectible changes over the times tested. ATP, GTP, TTP and CTP were also added as concentrated stock solutions. Stock solutions were refrigerated or frozen until used. ATP stock solutions were enzymatically assayed after use by an assay kit purchased from the Sigma Chemical Co. No hydrolysis of ATP was found. ADP stock solutions were prepared immediately before use.

Fluorescence Anisotropy Measurements

Fluorescence anisotropy was measured on an instrument built in our laboratory (Evetts and Isenberg, 1969; Evetts et al., 1970) modified in two ways. Two Jarrel Ash 1/4 meter monochromators were used in series in the excitation beam. Single monochromators and Cs-054 Corning filters were used in the two emission beams. The light source was a Hanovia 977-B 1000 Watt Hg-Xe lamp. Samples were excited with plane polarized light at 280 nm. The intensity of emitted light, polarized parallel to the excitation beam will be called I_E ; that perpendicular to the polarization of the excitation beam will be called I_B . Emission was measured at 325 nm.

Histone IV contains four tyrosines and two phenylalanines, but no tryptophan. The fluorescence is essentially due to the tyrosines because phenylalanine has a much lower absorbance and quantum yield than tyrosine (Teale and Weber, 1957; Teal, 1960). The anisotropy of the emission is defined (Jablonski, 1960) as $r = (I_E - I_B) / (I_E + 2I_B)$ where $I_E + 2I_B$ is proportional to the intensity emitted by the sample (Weber, 1952). The experimental error in anisotropy measurements is estimated to be less than 0.003.

With histone IV samples, it is important to test for, and eliminate, possible scattering artifacts (Weber, 1956). Scattering artifacts were tested for by using additional filters, and different path

length cuvettes, as described by Weber (1956). None of the samples described here showed scattering artifacts.

We denote the anisotropy in water by r_w and let $r(t)$ be the anisotropy at time t in the presence of salt. $r(t)$ was measured by averaging $(E-B)$ for 30 secs., and then $E + 2B$ for 30 secs. Since $(E-B)$ changes much more rapidly than $(E+2B)$, t denotes the mid-point of the $(E-B)$ measurement. The changes reported here were slow enough so that any error due to this procedure was less than one-third of the experimental error due to other causes.

Circular Dichroism

CD spectra, and CD changes as a function of time, were recorded on a Model CD-SP Durrum-Jasco CD recorder. $\Delta\epsilon_w(\lambda)$ will denote the CD of histone IV in water at a wavelength λ . $\Delta\epsilon(t, \lambda)$ will be the CD in salt, at time t , and wavelength λ . CD results are reported as $\Delta\epsilon = \epsilon_l - \epsilon_r$ where ϵ_l and ϵ_r are extinction coefficients for left and right handed circularly polarized light. The units of $\Delta\epsilon$ will be cm^{-1} liter per mole of residue. The techniques used to obtain and analyze CD difference spectra will be explained in the results section. CD spectra in the vacuum ultraviolet were recorded on an instrument in the laboratory of Dr. W. C. Johnson (1971).

III. RESULTS

Part A: Histone IV Salt Interactions

Fast and Slow Changes Resulting from Salt Addition

All of the salts studied induce both fast and slow conformational changes. At sufficiently low salt concentrations only the fast change occurs but at higher concentrations this change is followed by the slower one, which takes minutes to hours depending on solution conditions. Two techniques--circular dichroism (CD) in the region of 190-250 nm, and the polarization of fluorescence of the sample when excited at 280 nm, which is within the absorbance band of the tyrosines of histone IV, have been used to study these changes.

In the region of 190-250 nm, the CD is principally that of the peptide linkage (Holzworth and Doty, 1965). The fluorescence anisotropy, on the other hand, depends on the rotational mobility of the transition moments of the tyrosine residues. This, in turn, is a function of the local structural rigidity and the size and shape of the molecule. It also is a function of dimerization or the formation of higher order units or aggregates.

Figure 1 shows $\Delta\epsilon(t, 220)$ of histone IV as a function of time, at a number of NaCl concentrations. Figure 2 is a similar plot of fluorescence anisotropy, $r(t)$. As can be seen, both quantities

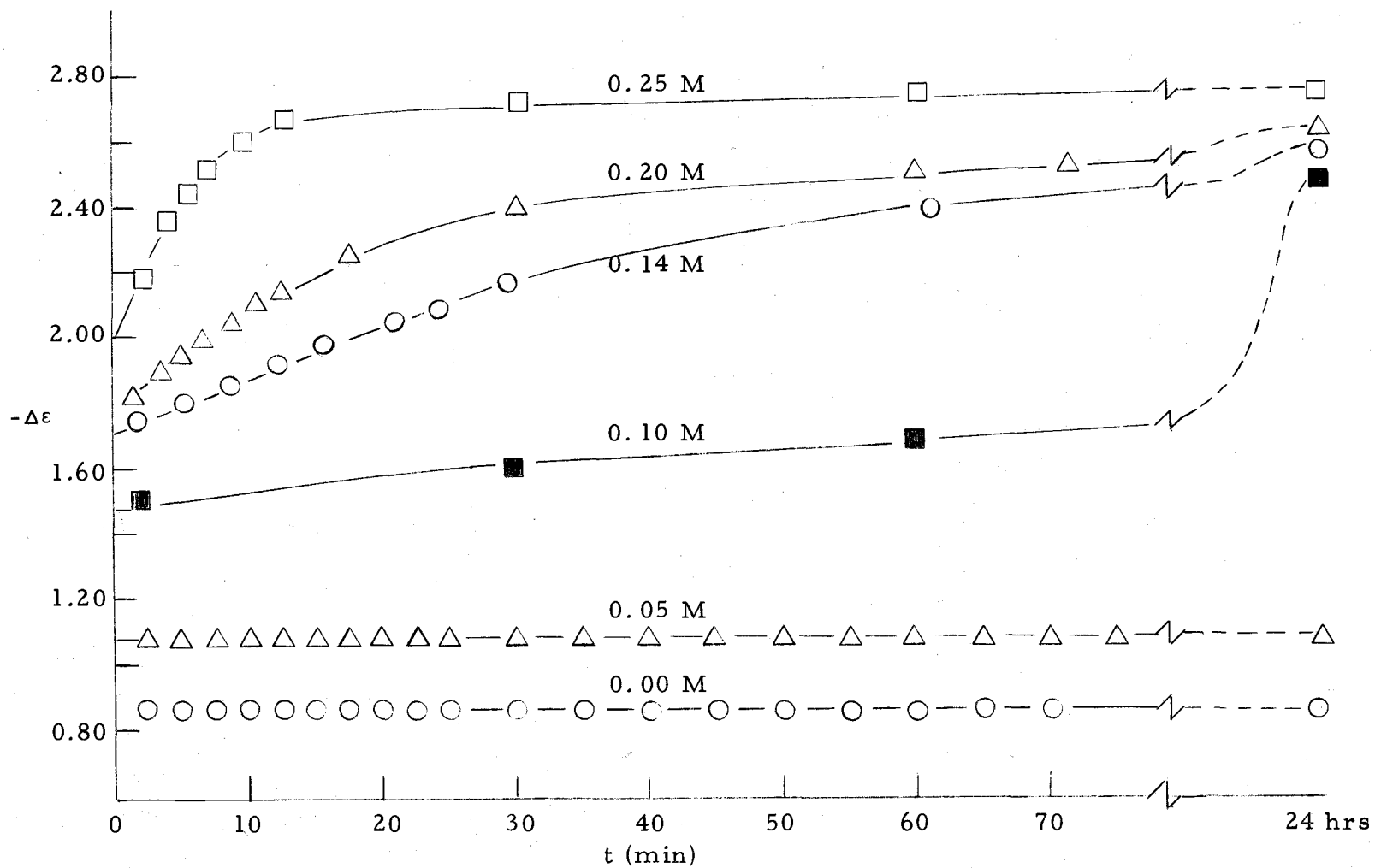


Figure 1. Time dependence of NaCl effects on the CD of histone IV, salt concentrations as shown.

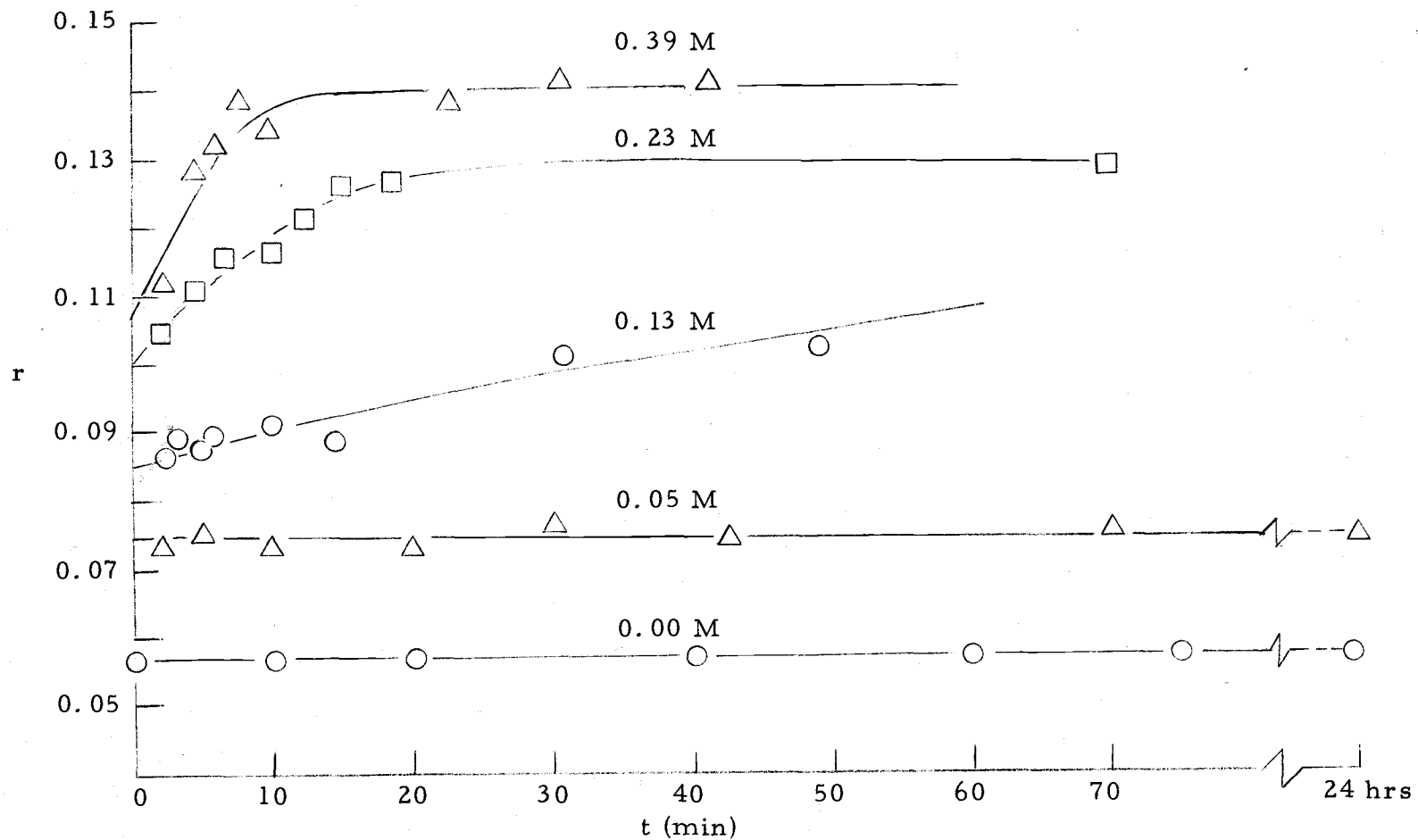


Figure 2. Time dependence of NaCl effects on the fluorescence anisotropy of histone IV, salt concentration as shown.

increase immediately upon salt addition, that is $r(0)$ varies with salt concentration. At sufficiently high salt concentrations, a slow change also occurs. This change becomes more rapid as the salt concentration is raised. However, at sufficiently low concentrations, there is only a fast, but no slow, change. For example, at 1.7×10^{-3} M phosphate, pH 7.4 the initial, rapid, increase in anisotropy is from $r_w = 0.050$ in water to $r(0) = 0.067$ but there is then no further rise for more than 24 hours. At higher phosphate concentrations, following the initial rise, the anisotropy and CD increase further over a period of time and eventually reach a plateau. This plateau value is denoted $r(\infty)$ or $\Delta\epsilon(\infty, \lambda)$.

The rate of the slow change has been found to fit reasonably well to a single exponential decay for all salts studied.

For anisotropy:

$$\frac{r(\infty) - r(t)}{r(\infty) - r(0)} = e^{-t/\tau_F} \quad (1)$$

and for CD:

$$\frac{\Delta\epsilon(\infty, 220) - \Delta\epsilon(t, 220)}{\Delta\epsilon(\infty, 220) - \Delta\epsilon(0, 220)} = e^{-t/\tau_{CD}} \quad (2)$$

τ_F and τ_{CD} are used to distinguish between the time constants for fluorescence anisotropy and CD changes. Figures 3 and 4 show semilog plots of Equations 1 and 2 for phosphate at several concentrations. The plots are linear and extrapolation to zero time allows

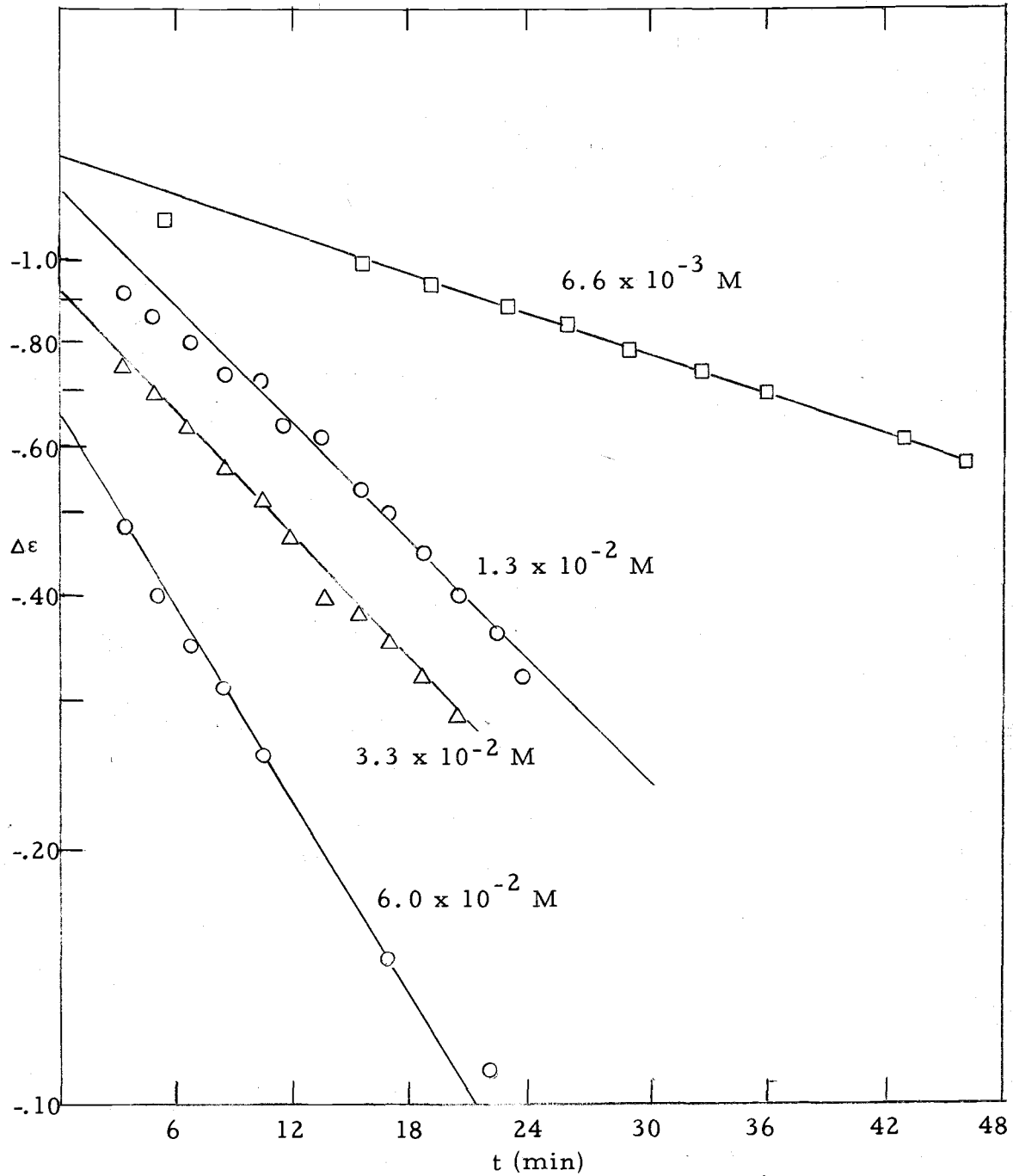


Figure 3. Semi-logarithmic plots of $\Delta\epsilon(\infty, 220) - \Delta\epsilon(t, 220)$ in phosphate as a function of time, phosphate concentrations as shown.

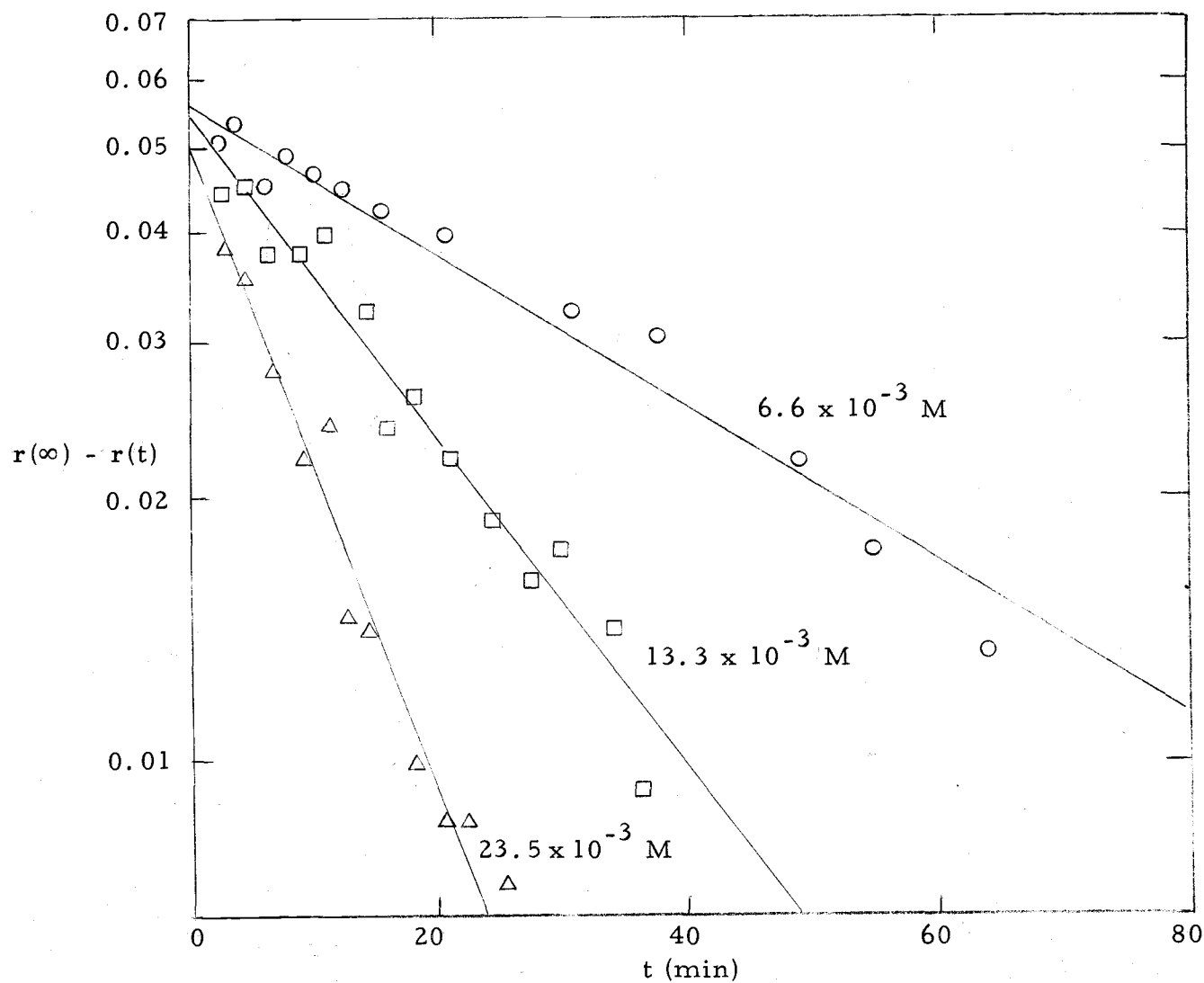


Figure 4. Semi-logarithmic plots of $r(\infty) - r(t)$ in phosphate as a function of time, phosphate concentrations as shown.

the determination of $r(0)$ or $\Delta\epsilon(0, 220)$. These quantities represent the value of the anisotropy or CD after the fast conformational change but before any slow change. τ_F and τ_{CD} are also determined from such plots.

$r(0) - r_w$ measures the anisotropy change of the fast process, while $r(\infty) - r(0)$ measures the total anisotropy change for the slow process. All of these values, $r(\infty)$, $r(0) - r_w$, and $r(\infty) - r(0)$ depend upon the phosphate concentration as shown in Figure 5.

Figure 6 shows the dependence on the phosphate concentration of the final CD, $\Delta\epsilon(\infty, 220)$, the CD change of the fast step, $\Delta\epsilon(0, 220) - \Delta\epsilon_w(220)$, and the CD change of the slow process, $\Delta\epsilon(\infty, 220) - \Delta\epsilon(0, 220)$. The results in Figure 6 are similar to the fluorescence measurements of Figure 5. Both the total CD and the CD change of the fast step increase monotonically with phosphate concentration. On the other hand, the CD change of the slow process reaches a maximum at around 7×10^{-3} M phosphate and then decreases.

Figure 7 shows CD and anisotropy data for the total change in the slow step for all of the salts studied. Three regions of these curves may be distinguished. At low salt there is no slow change. Then, at higher concentrations, there is a steep rise to a maximum. At still higher concentrations there is a gradual decline.

Whether or not the anisotropy and CD data coincide in any portion

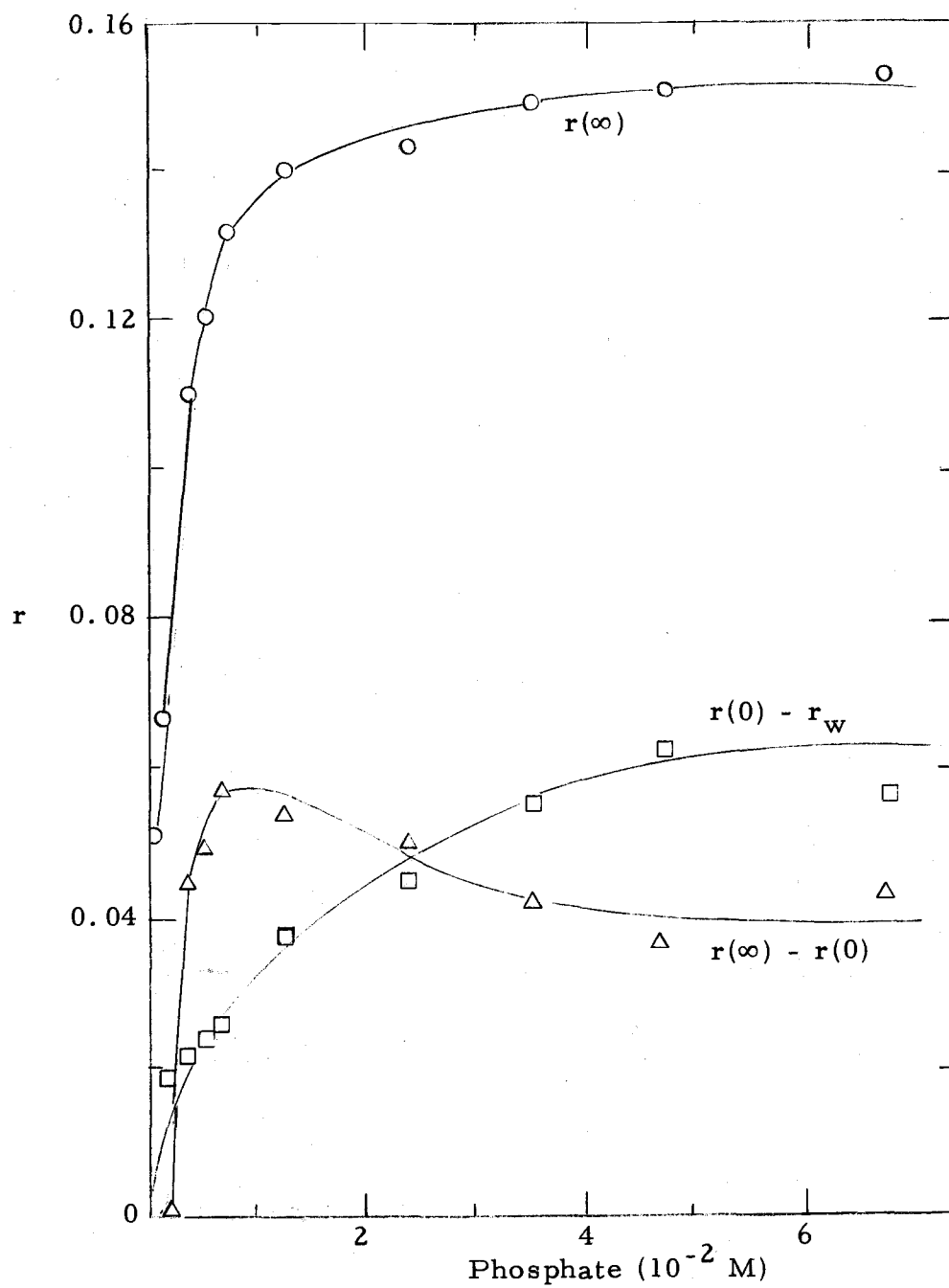


Figure 5. Phosphate concentration dependence of the final anisotropy and anisotropy changes of the fast and slow steps.

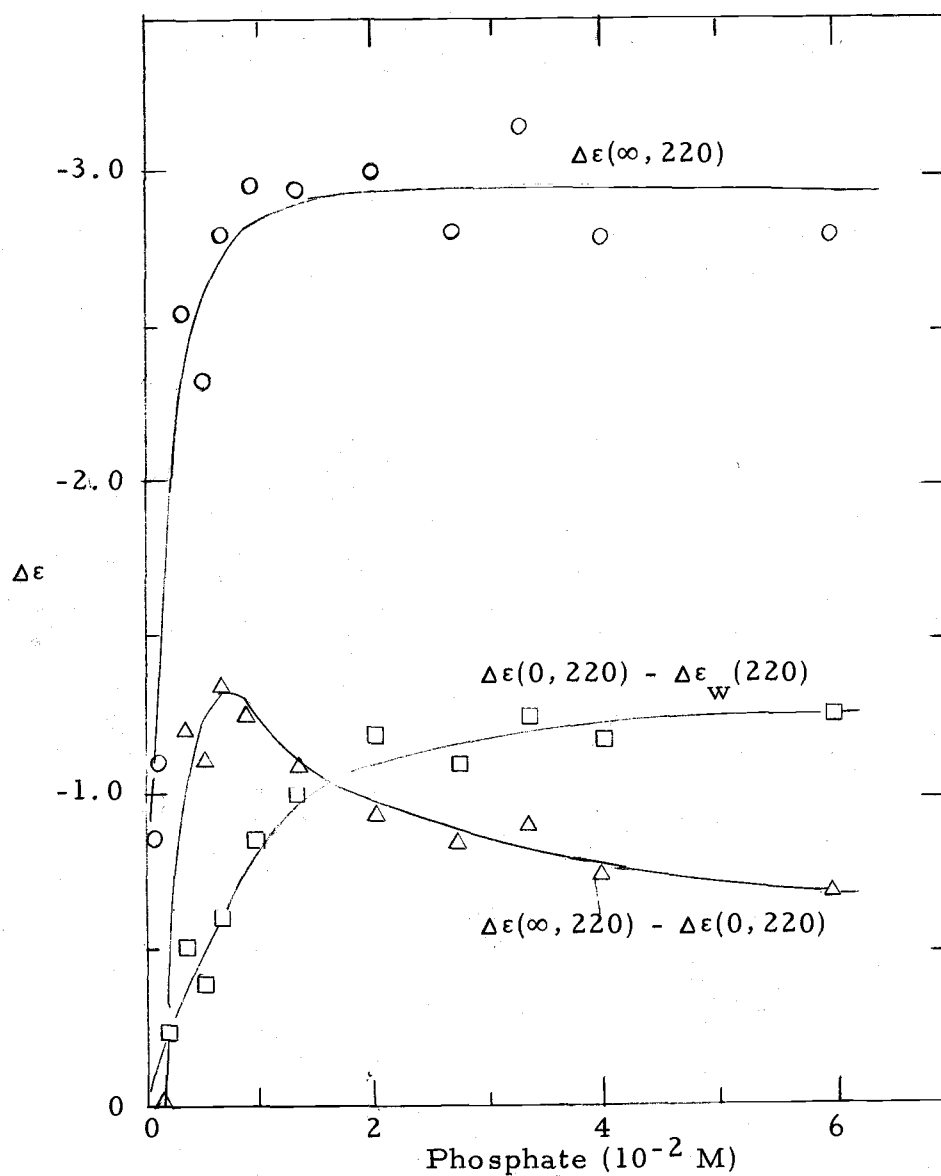


Figure 6. Phosphate concentration dependence of the final CD and CD changes of the fast and slow steps.

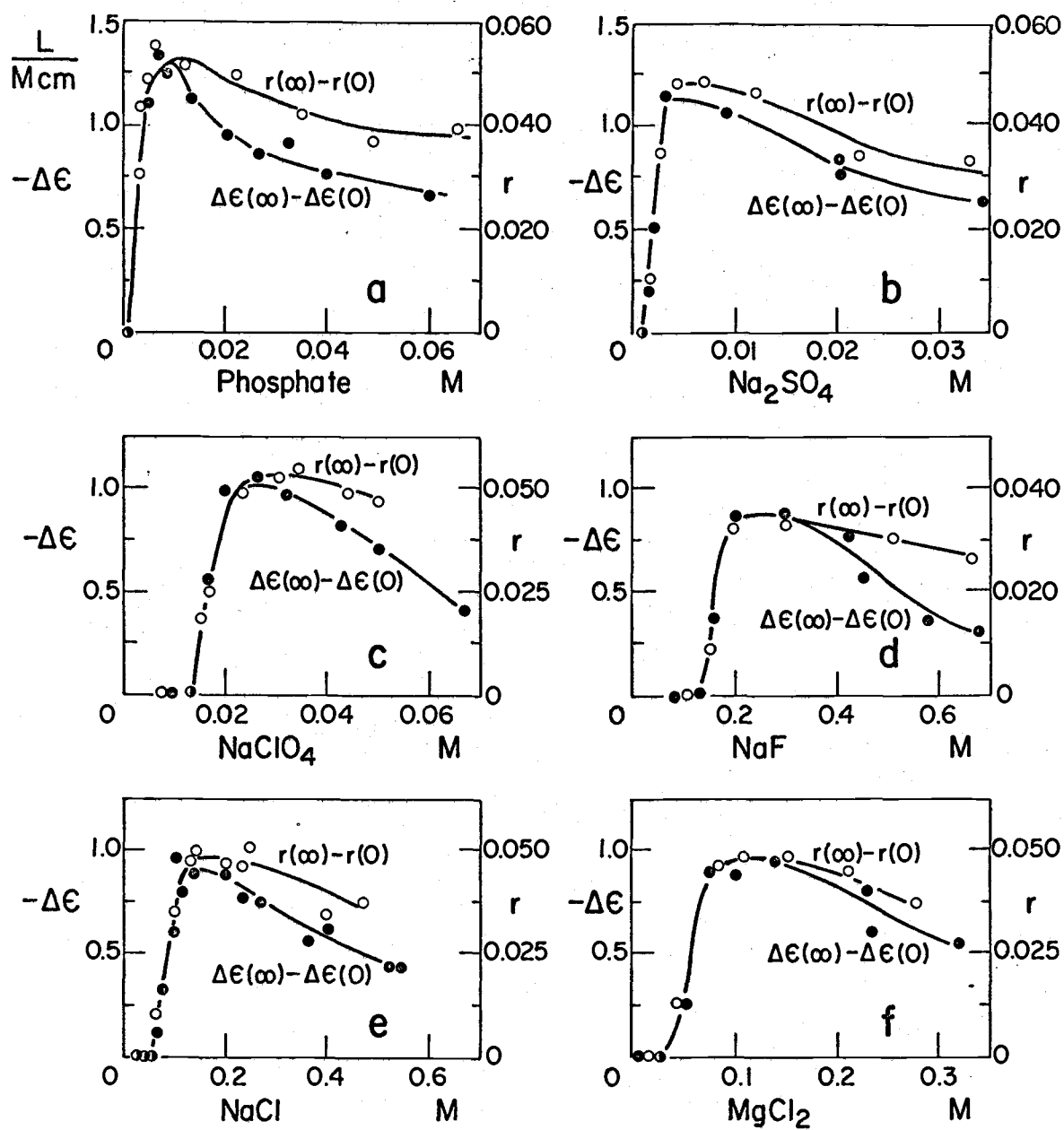


Figure 7. The magnitude of the slow change by CD and fluorescence anisotropy.

of a curve shown in Figure 7 depends, of course, on the scales used to plot $\Delta\epsilon$ and r . The coincidence that appears in the region of sharp rise is due therefore only to the scales chosen. However, the fact that both parameters rise sharply in the same range of salt concentrations is physically meaningful.

The conformational change in the slow step is highly cooperative. We see that there is a break point in the salt concentration below which there is no slow change. Above the breakpoint, however, there is appreciable change. This breakpoint varies markedly with the salt used, and ranges from 1.0×10^{-3} M for Na_2SO_4 to 0.13 M for NaF.

It should not be concluded that the existence of a break point means that the salt binding is in itself necessarily cooperative although this may, of course, be true. However, the cooperativity may be entirely in the structural change of the protein, i.e., when solution conditions favor a new structure the molecule undergoes a co-operative conformational change.

Figure 8 shows that the rate constants, as measured by both CD and polarization of fluorescence, are the same for low salt concentrations, but diverge at high concentrations. The divergence begins slightly after the point where the net change is a maximum.

It should be noted that the rate constants plotted in Figure 8 may be directly compared to each other, although the CD and polarization

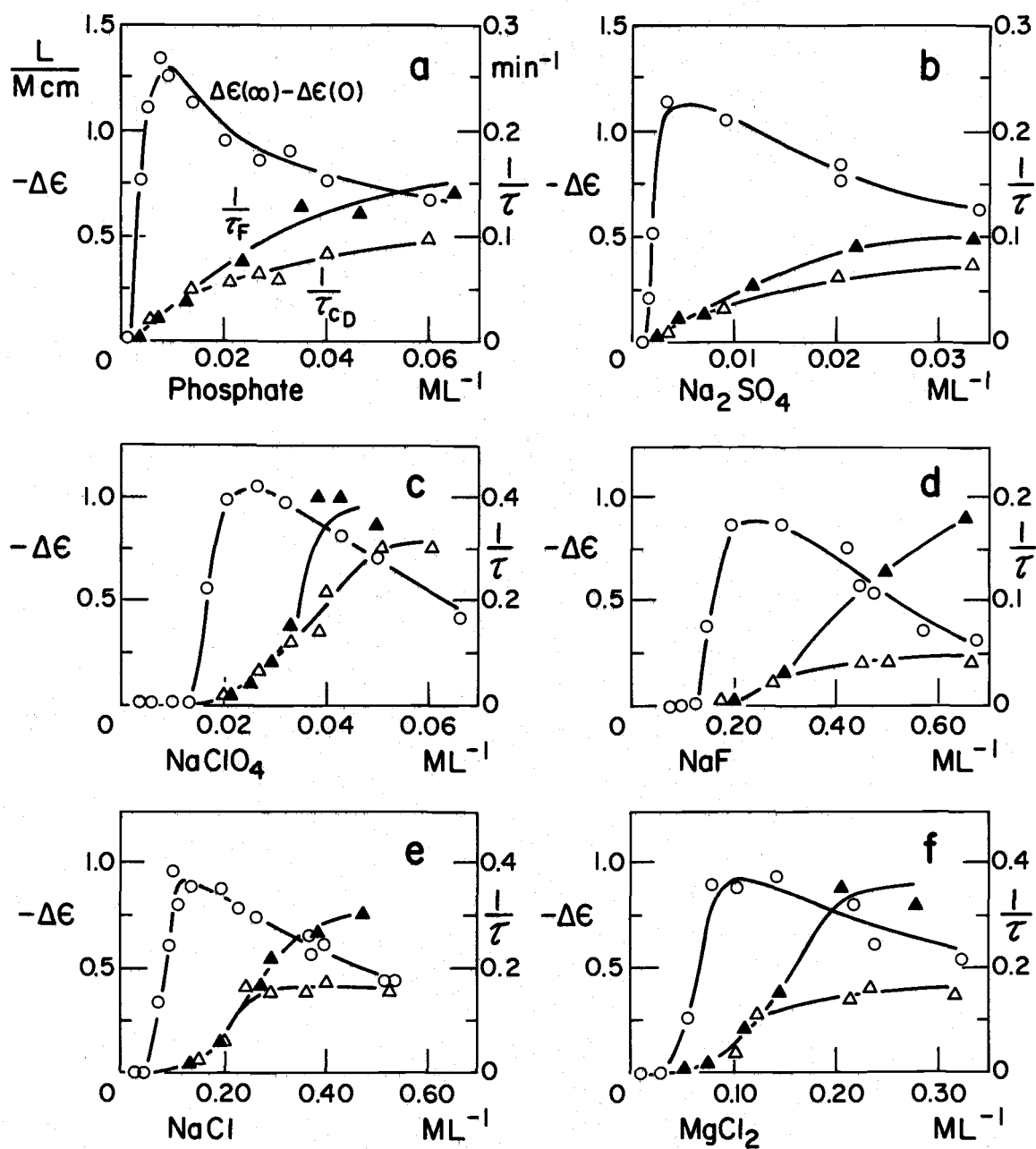


Figure 8. Magnitude of the slow change by CD and the experimental rate constants $1/\tau_{CD}$ and $1/\tau_F$.

comparisons of Figure 7 depend, as discussed, on the scales used to draw the figures.

These data suggest that there are at least two different processes occurring in the slow change, one that dominates at concentrations just above the break point, and another that occurs at higher concentrations.

Circular Dichroism of the Amide Group

The CD of a protein in solution can be divided, approximately, into three categories, that of a random coil, of an α -helix and of a β -sheet (Holzwarth and Doty, 1965; Greenfield and Fasman, 1969). The classification, of course, is not rigorous as the CD depends on side chain interactions, and the details of the tertiary structure of the protein (Morita, Simons and Blout, 1968; Vournakis, Yan and Scheraga, 1968; Fasman, Hoving and Timasheff, 1970). The division, though approximate, is useful and helps elucidate conformational properties.

The continuous slow change creates difficulties in measuring CD as a function of wavelength. About 10 minutes is needed to scan from 240 nm to 200 nm on the Durrum-Jasco spectrometer, and around 30 minutes to scan from 240 nm to 180 nm using the vacuum UV CD spectrometer. It is necessary, therefore, to use an appropriate value for the added salt--one that will induce changes slow enough for

adequate recording, but large enough to allow accurate measurements of the CD change. At 3.3×10^{-3} M phosphate, pH 7.4, there are significant CD changes due to both the fast and the slow processes. Nevertheless, the relaxation time, $\tau_F = 290$ min, is still long enough for a good spectral scan. These solution conditions consequently become useful for spectral studies.

The CD spectra of histone IV in water and 3.3×10^{-3} M phosphate, pH 7.4, down to 180 nm, are shown in Figure 9. There is an immediate change of the CD spectrum when phosphate is added. We note again, that since each scan takes around 30 minutes, there is a small change of the CD during the scan, and the CD spectral change in phosphate (spectra B and C in Figure 9) is slightly larger at lower wavelengths than it should be to correspond to the time at which the spectral scan begins. Nevertheless, under the conditions shown, the error is small and the spectra are accurate to a good approximation.

The difference CD spectrum (Figure 10) between histone in water and that in phosphate, taken in the first hour after the addition of phosphate, measures mainly the CD change of the fast process, although, of course, a small change due to a slow process will also be present. This difference CD spectrum shows a large positive CD band at 193 ± 2 nm and negative CD bands at 212 ± 2 nm and 222 ± 1 nm.

The difference CD spectrum due to the slow process, i. e., the

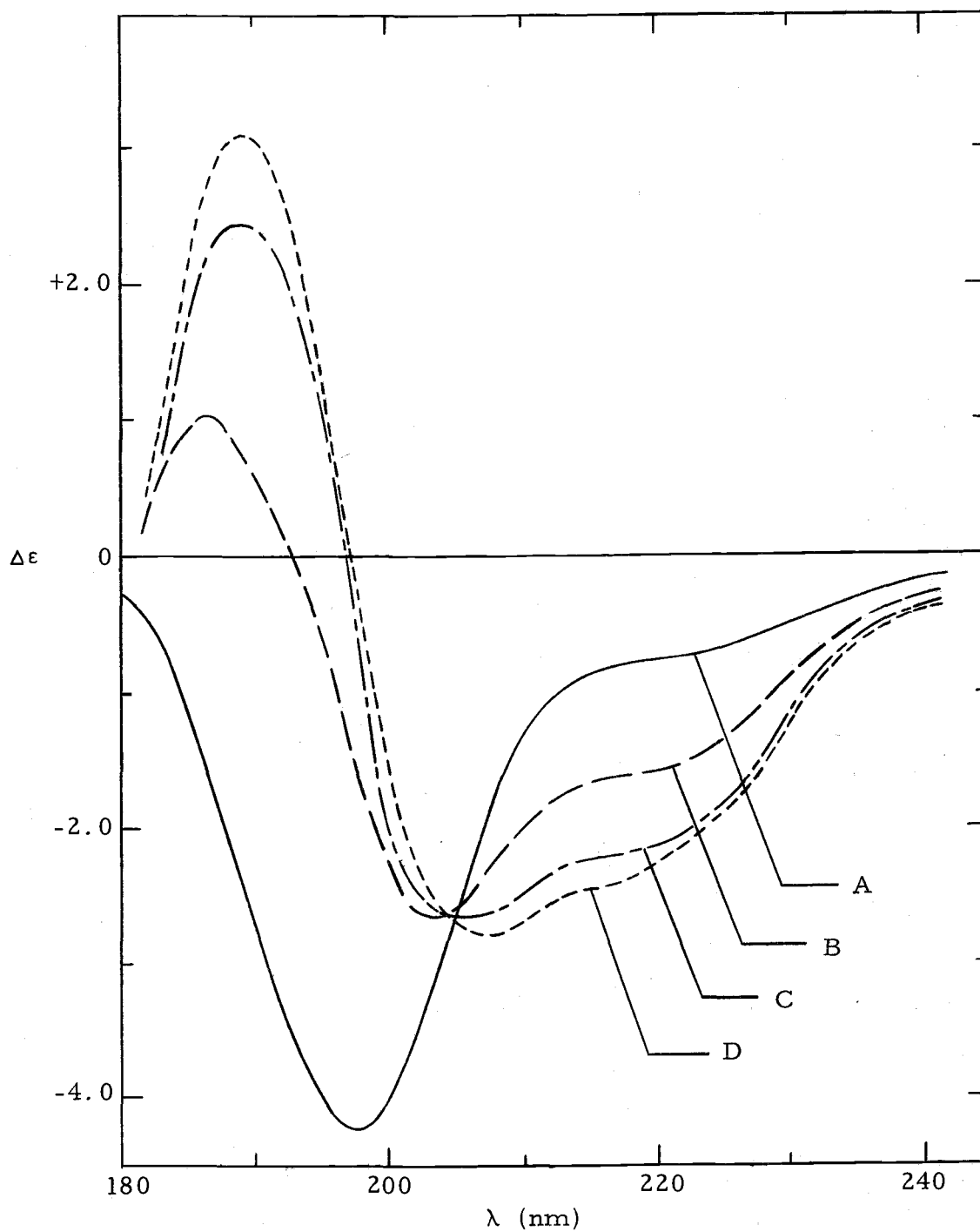


Figure 9. CD spectra of histone IV in water (curve A), in 3.3×10^{-3} M phosphate, pH 7.4 recorded from $t = 25$ to 55 min (curve B), from $t = 290$ to 320 min (curve C) and from $t = 960$ to 990 min (curve D).

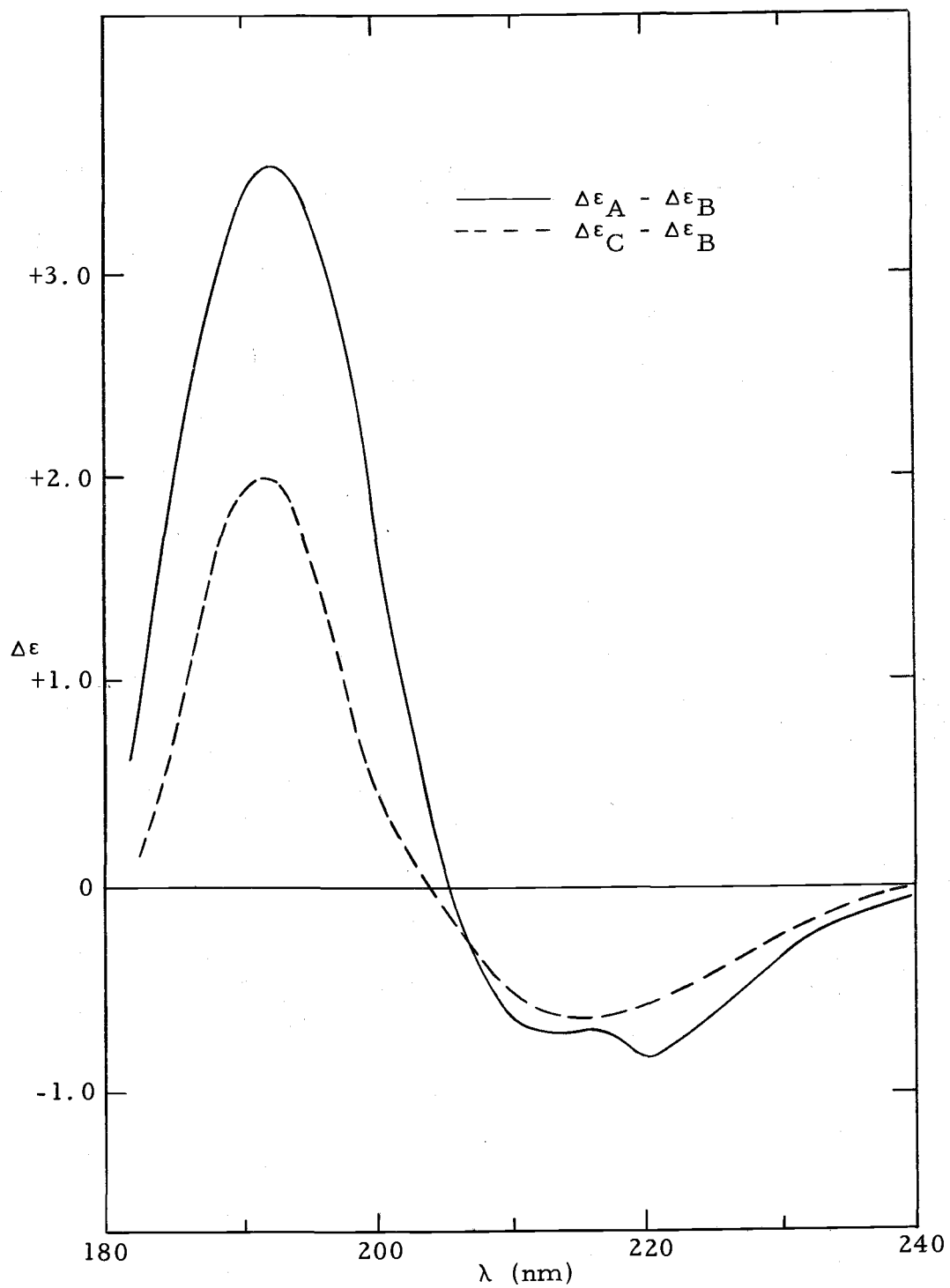


Figure 10. Difference CD spectra computed from Figure 9.

difference between the one taken in the first hour and the one taken in the 5th hour, shows a large positive CD band 192 ± 2 nm and a single negative band at 216 ± 1 nm.

The difference CD spectra of the fast and the slow process, above 200 nm, were also measured using the Durrum-Jasco CD recorder (Figure 11) with closely similar results. This similarity is significant since a scan on the Durrum-Jasco CD recorder over this wavelength range takes only ten minutes. The close agreement between the two spectra, one taking 30 minutes for a scan, and the other taking 10 minutes, demonstrates that the changes that occur during a scan time are negligibly small.

To interpret our data we relied on a comparison with the CD of α -helix and β -sheet for polylysine (Holzwarth and Doty, 1965; Greenfield and Fasman, 1969).

Figure 12 shows two theoretical difference CD spectra. The one with two negative bands is obtained by assuming that 100% of the histone residues change from the structure in water to a conformation having the CD spectrum of polylysine α -helix. The other was computed in a similar way using the CD spectrum of polylysine β -sheet.

There is a striking agreement in shape between the theoretical curves of Figure 12 and the experimental difference spectra of Figures 10 and 11.

Experimental difference spectra were obtained for the other

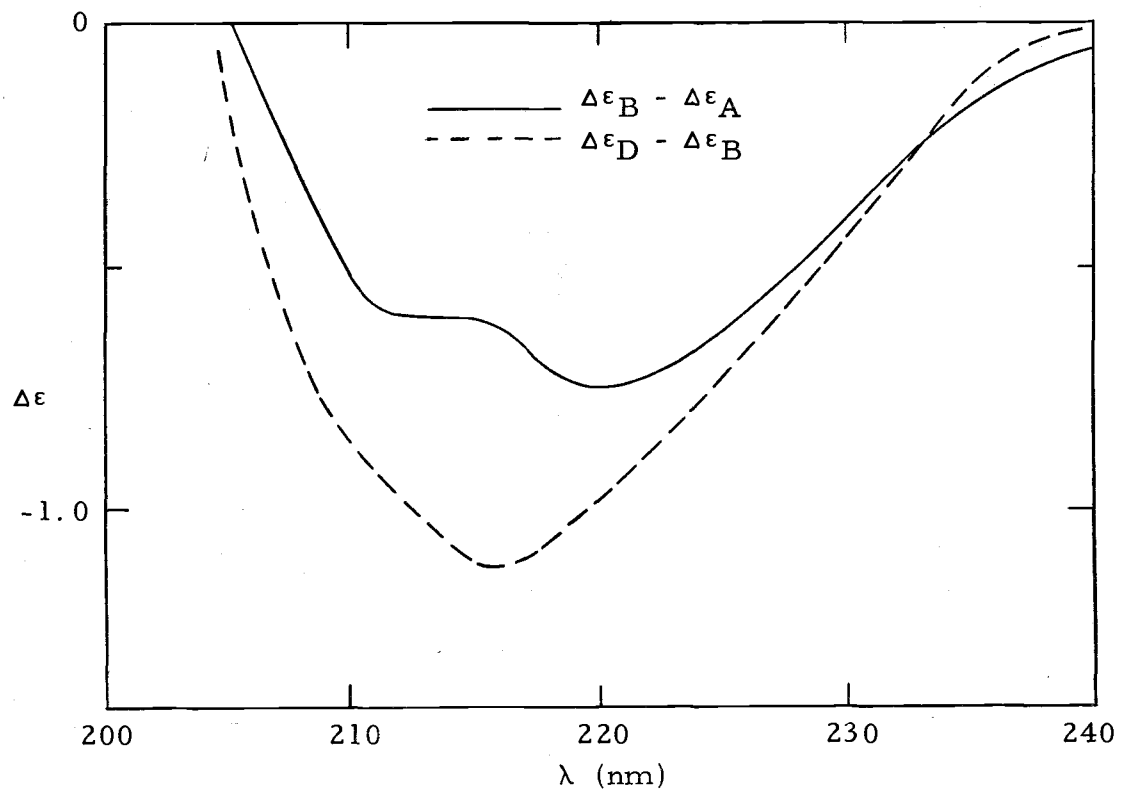


Figure 11. Difference CD spectra of histone IV computed from the CD spectra recorded on the Durrum-Jasco CD recorder for the fast (—) and slow (---) steps.

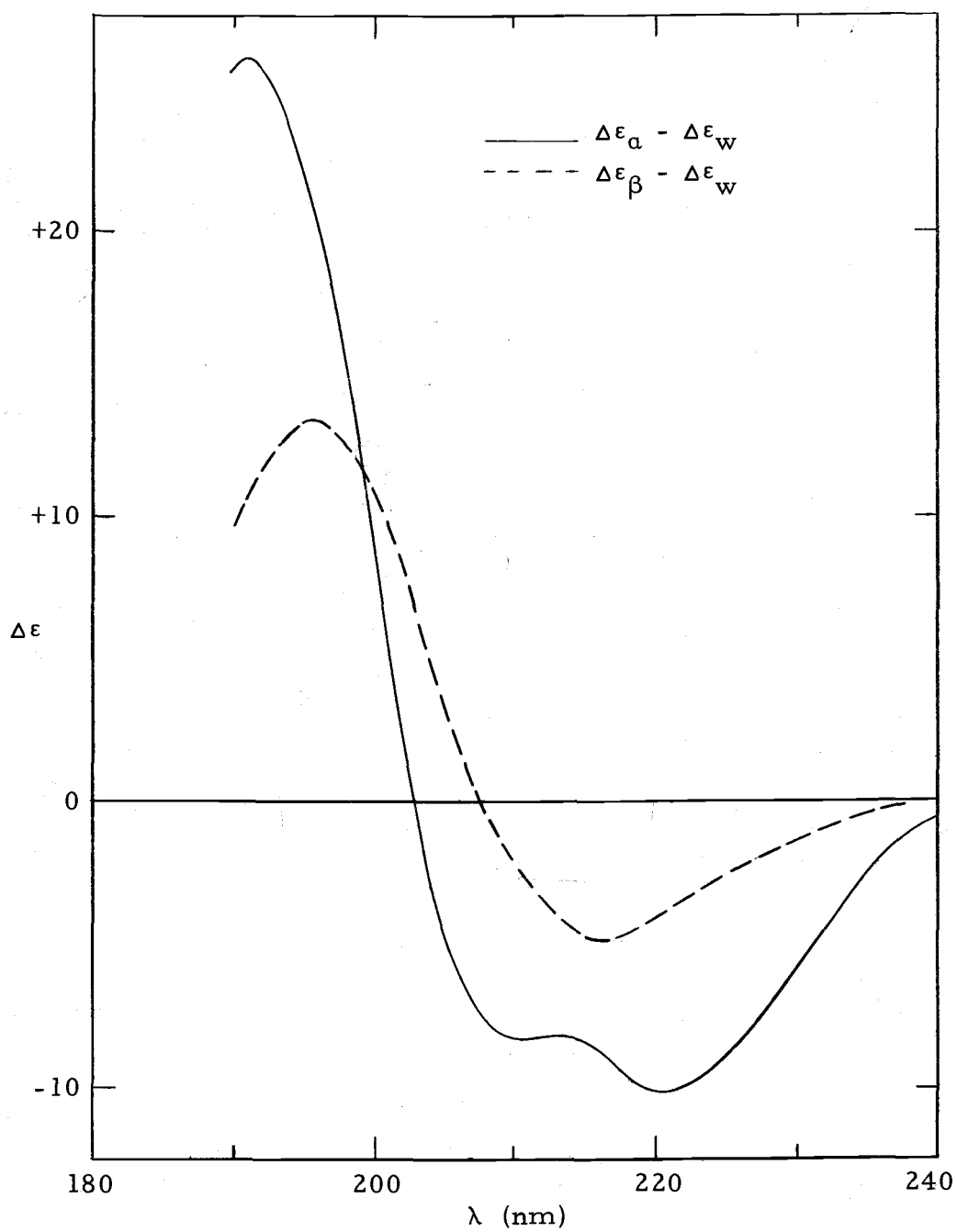


Figure 12. Difference CD spectra of histone IV from that in water to α -helix (—) and to β -sheet (---) typical of polylysine.

salts studied and are shown in Figure 13. Because of cacodylate absorbance the spectra only extend to ~204 nm. In every case there is remarkable agreement between the experimental spectrum for the fast step and the theoretical spectrum for α -helix formation. All of the difference spectra for the slow step have almost exactly the same shape as the theoretical spectrum for β -sheet formation.

The amplitude of an experimental difference spectrum differs, of course, from that for the theoretical, and, by a comparison, we obtain an estimate for the α -helical or β -sheet content of the protein. It will be shown that only a fraction of the residues participate in α -helical or β -sheet formation. The other residues probably undergo some accompanying conformational changes, but if they do, such changes do not contribute significantly to the circular dichroism.

At higher phosphate concentrations the slow change is not slow enough to permit accurate spectral scans of the CD. One may still ask, however, if the processes at these higher phosphate concentrations may still be characterized as involving α -helix formation in a fast step, following by slower β -sheet formation. We approached the question indirectly by measuring the changes at two fixed wavelengths, 222 nm and 216 nm, as a function of time. These experiments were run at 0.91×10^{-5} M histone and 4.0×10^{-2} M phosphate, pH 7.4. For the initial rapid change we obtained

$$\Delta\epsilon(0, 216) - \Delta\epsilon_w(222) / \Delta\epsilon(0, 216) - \Delta\epsilon_w(216) = 1.0. \text{ For the slow}$$

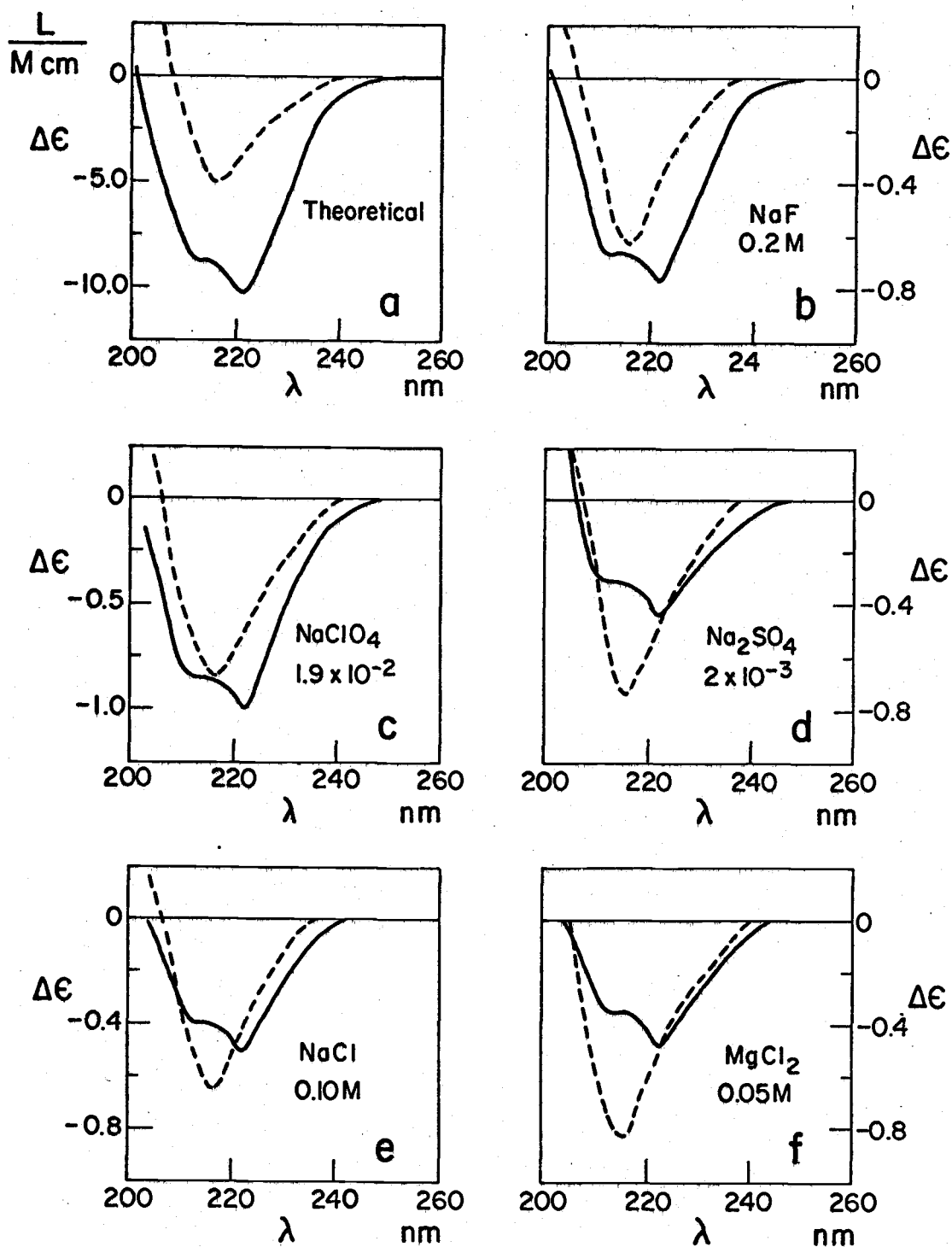


Figure 13. Theoretical and experimental difference spectra from the fast and slow steps of histone IV in the other salts tested.

process we obtained $\Delta\epsilon(\infty, 222) - \Delta\epsilon(0, 222) / \Delta\epsilon(\infty, 216) - \Delta\epsilon(0, 216) = 0.67$.

From Figure 12 corresponding values of 1.1 and 0.77 are obtained.

The close agreement suggests that the same types of changes occur, at least to a good approximation, at low and high phosphate values.

As expected, the time constant for the slow process was the same when measured at either of the two wavelengths.

Histone Salt Equilibrium Constants for the Fast Step

In order to obtain a measure of the effectiveness of various ions in inducing the changes that we have described, it is necessary to assume something about the nature of the ion interaction. For simplicity we assume that there are a set of independent ion binding sites on histone IV. We, of course, have no way of judging the validity of this assumption at present, but it has the virtue of permitting us to obtain effective binding constants and therefore to compare the salts quantitatively.

We describe the interaction between anions and histone IV by the following equation.



[B] is the concentration of free binding sites, [A] is the

concentration of bound sites. Let us further assume that there is a linear relationship between the changes measured by CD or fluorescence anisotropy in the fast step, and the amount of phosphate bound. K is then the effective equilibrium constant for the overall process. It is then possible to calculate the fraction of bound sites, f from,

$$f_{\text{CD}} = \frac{[\text{BA}]}{[\text{B}_0]} = \frac{\Delta\epsilon_{A_0}^{(0)} - \Delta\epsilon_w}{\Delta\epsilon_{A_0=\infty}^{(0)} - \Delta\epsilon_w} \quad (4)$$

for CD and

$$f_r = \frac{[\text{BA}]}{B_0} = \frac{I_{A_0}^{(0)}}{I_{A_0=\infty}^{(0)}} \times \frac{r_{A_0}^{(0)} - r_w}{r_{A_0=\infty}^{(0)} - r_w} \quad (5)$$

for fluorescence anisotropy, where $I_{A_0}^{(0)}$ is the fluorescence intensity at zero time at anion concentration A_0 (Evet and Isenberg, 1969) and B_0 is the total concentration of binding sites.

At salt concentrations in great excess of the histone concentration, $A \approx A_0$ where A_0 is the total anion concentration. Then

$$\frac{1}{f} = 1 + \frac{1}{K[A_0]} \quad (6)$$

There is no a priori reason why f_{CD} must equal f_r , because Equations (4) and (5) are derived under the assumption that the measured effect is, in each case, proportional to the number of bound sites. If, for example, ions specifically bound in one position

affected the tyrosine rigidity without altering the α -helical content, or vice-versa, then f_{CD} would not equal f_r . Therefore, experimental observations on the equality, or lack of equality, of the equilibrium constants yield valuable information on the actual conformational change.

Combining Equations 4 and 6, or 5 and 6, we obtain

$$\frac{1}{(\Delta\epsilon_{A_0}(0) - \Delta\epsilon_w)} = \frac{1}{(\Delta\epsilon_{A_0=\infty}(0) - \Delta\epsilon_w)} \left(1 + \frac{1}{K[A_0]}\right) \quad (7)$$

and

$$\frac{1}{I_{A_0}(0)(r_A(0) - r_w)} = \frac{1}{I_{A_0}(0)(r_{A_0=\infty}(0) - r_w)} \left(1 + \frac{1}{K[A_0]}\right) \quad (8)$$

Figure 14 shows data plotted according to Equations (7) and (8) for phosphate. In the case of phosphate I_A is constant and only $1/(r_0 - r_w)$ need be plotted.

Figure 15 shows data plotted according to Equation (7) for the other salts used. Figure 16 shows similar plots according to Equation (8).

It may be seen that all plots are good straight lines.

Table 1 summarizes the pertinent parameters obtained from the plots of Figure 14, 15 and 16. Several features may be noted.

The equilibrium constants for NaClO_4 and NaH_2PO_4 are an order of magnitude larger than those of the halides. The constants for

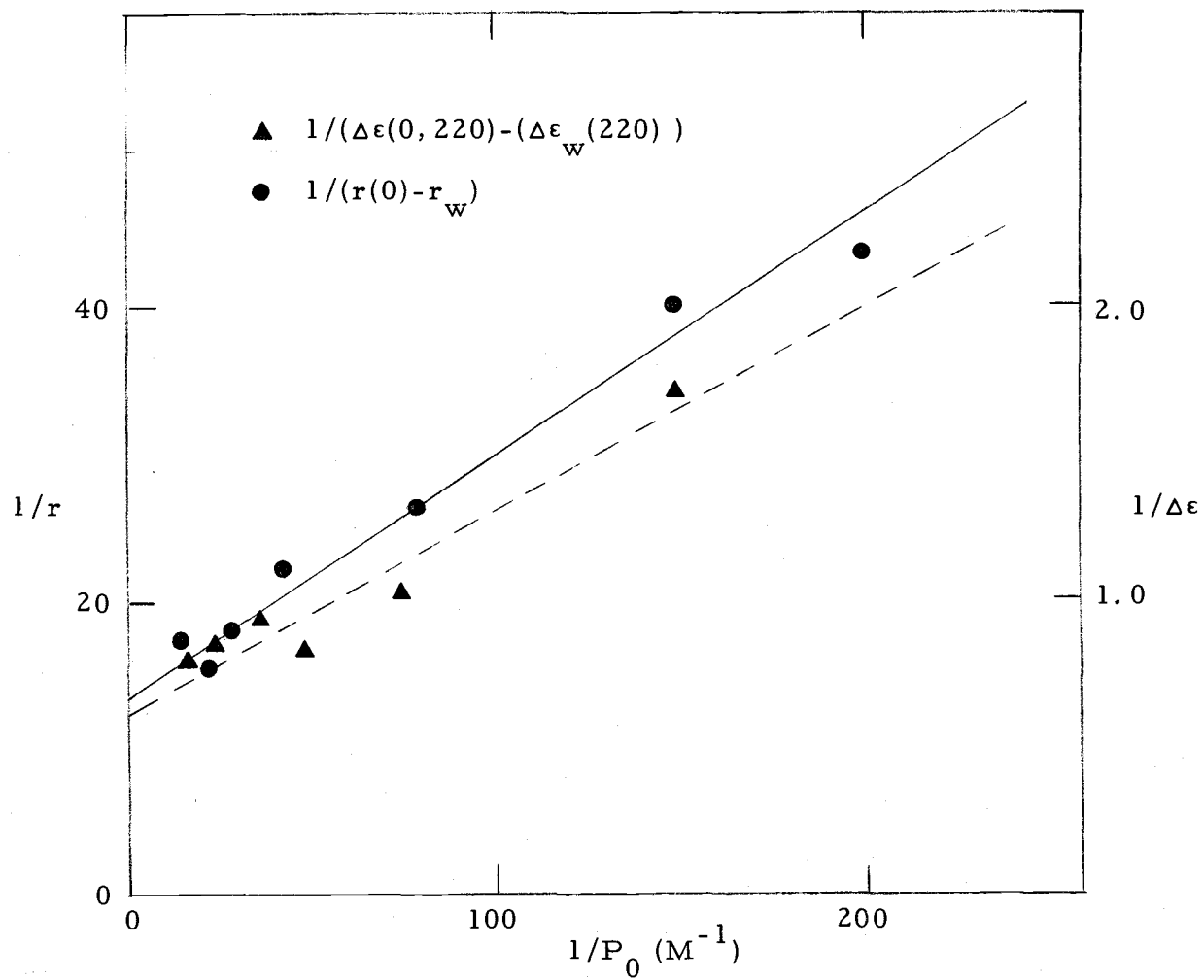


Figure 14. Plots of Equations 7 and 8 for the fast step in phosphate.

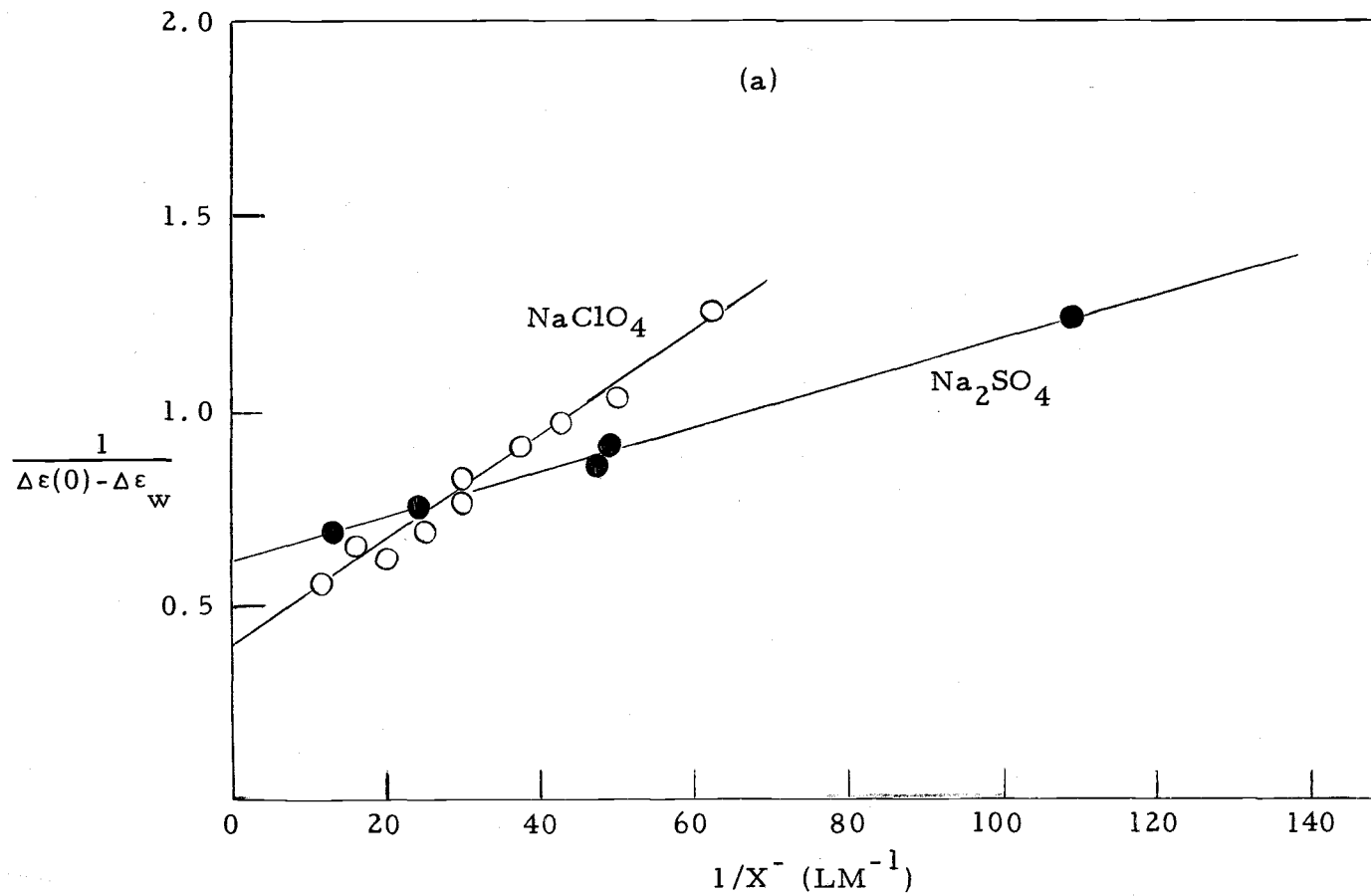


Figure 15. Plots of Equation 7 for the fast CD change for the other salts.

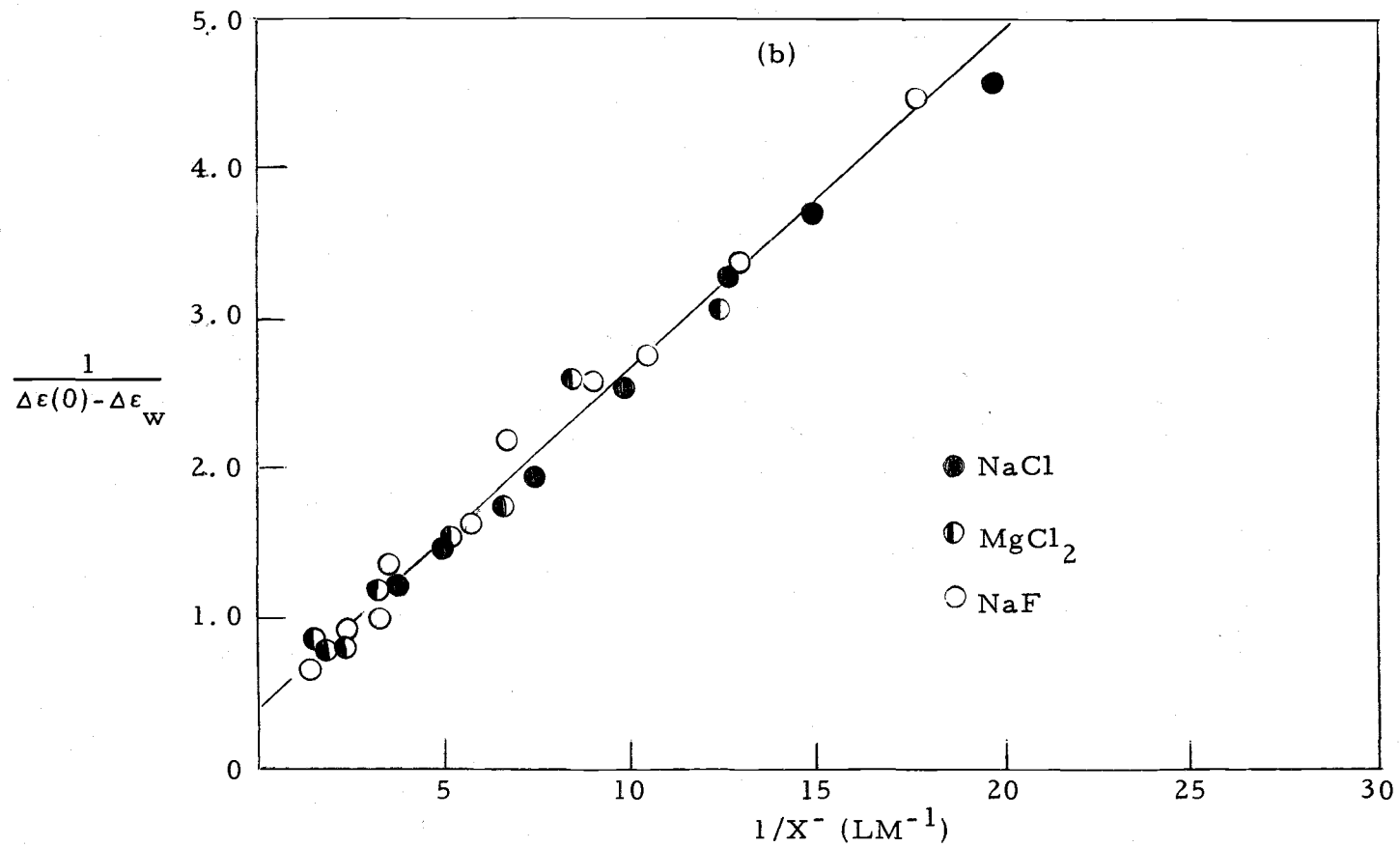


Figure 15. Continued.

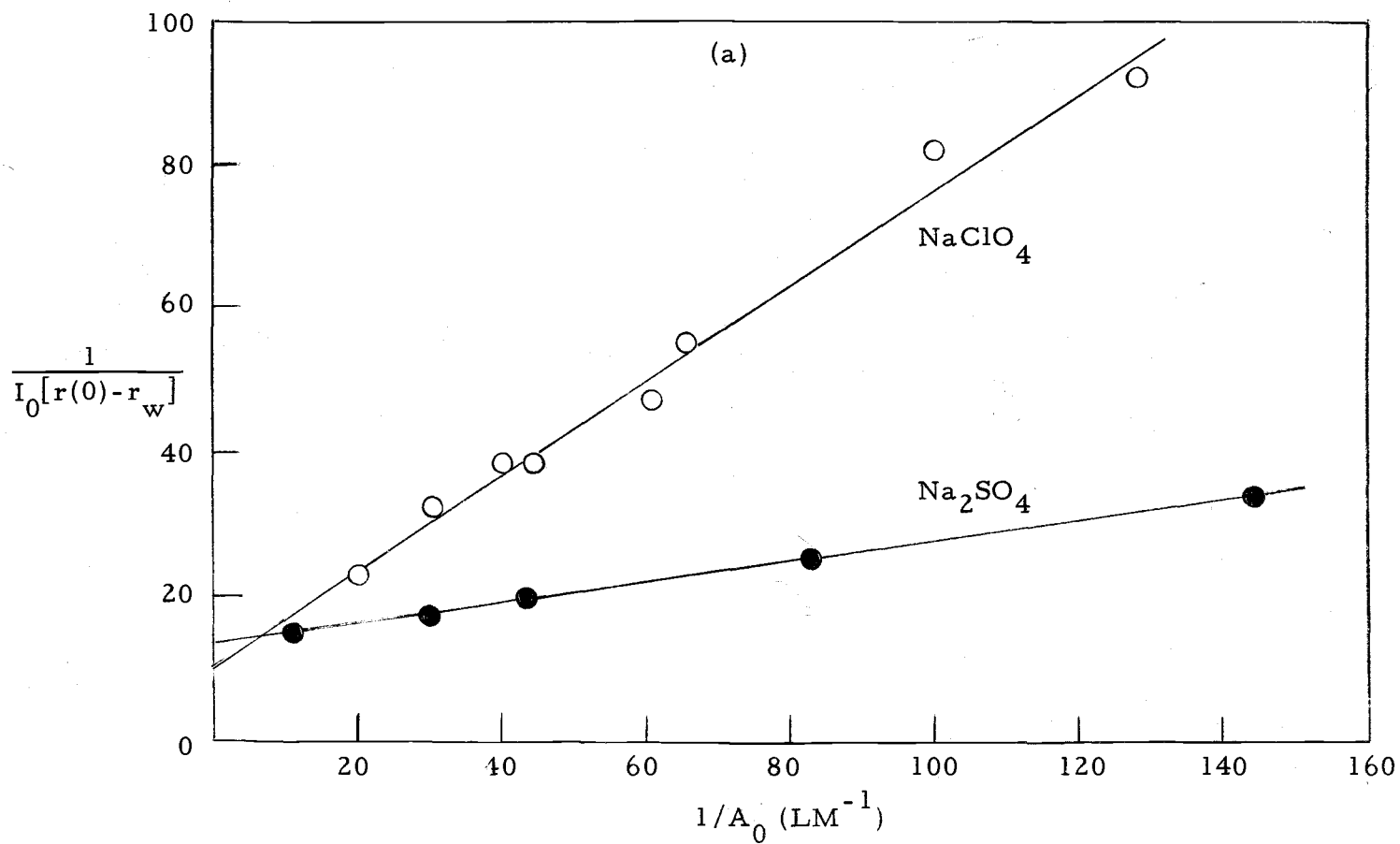


Figure 16. Plots of Equation 8 for the fast anisotropy change for the other salts.

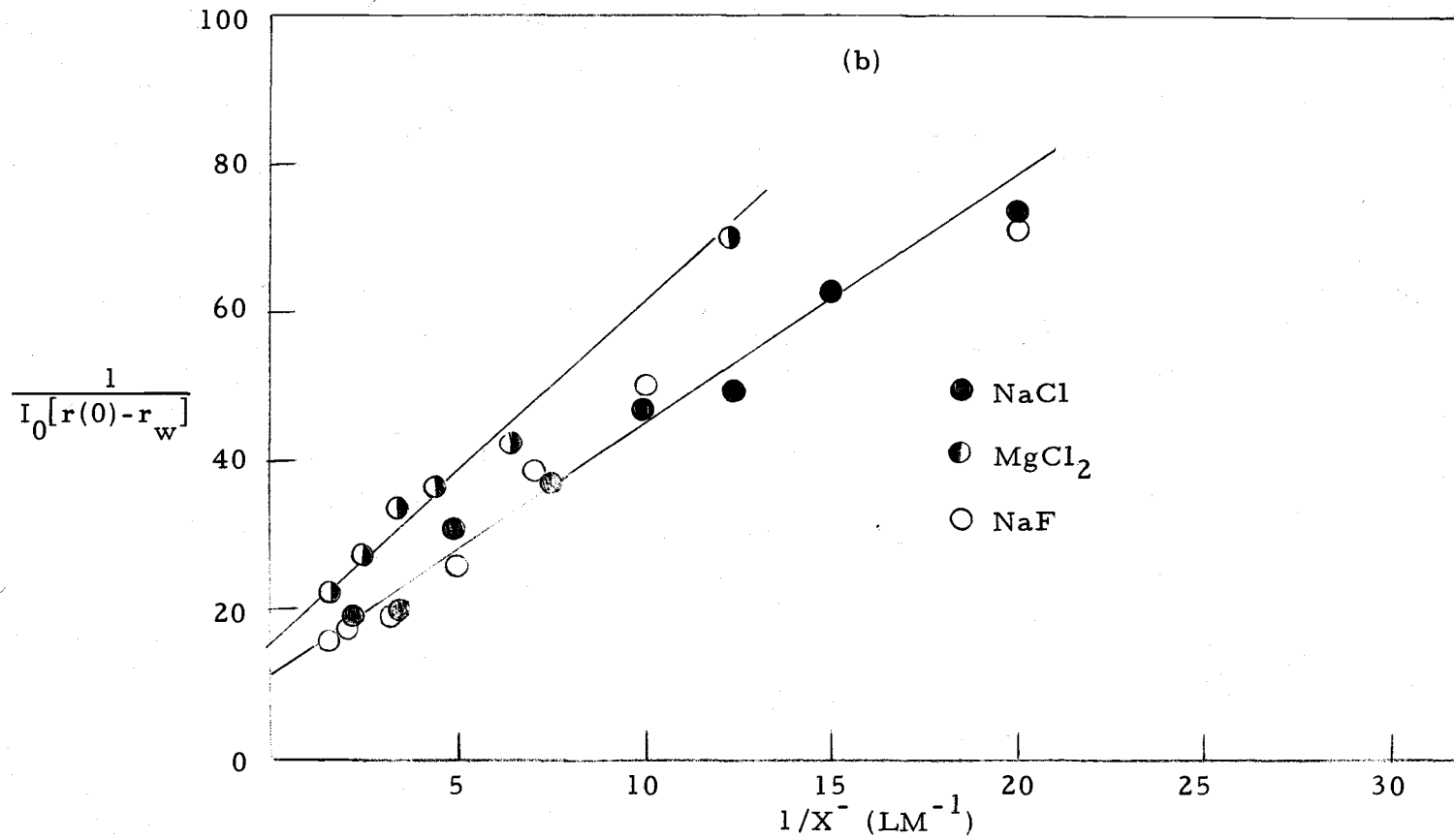


Figure 16. Continued.

the divalent anions are still another order of magnitude larger.

Table 1.

Salt	(1) McmL ⁻¹	K _{CD} LM ⁻¹	(2)	K _F LM ⁻¹
Na ₂ SO ₄	0.61	105	13	95
NaClO ₄	0.40	32	10	16
NaCl	0.45	2.2	11	2.6
MgCl ₂	0.45	2.5	15	3.3
NaF	0.45	2.0	11	2.2
NaH ₂ PO ₄ ⁽³⁾	----	10	--	14
Na ₂ HPO ₄ ⁽³⁾	----	120	--	120
Sodium phosphate pH 7.4	0.60	85	14	80

$$(1) \frac{1}{(\Delta\epsilon_A(0, 220) - \Delta\epsilon_w(220))_{A_0=\infty}}$$

$$(2) \frac{1}{I(0)[r(0) - r_w]_{A_0=\infty}}$$

(3) Calculated as discussed in Li et al. (1972b).

As discussed above, the CD change in the rapid process is essentially due to α -helix formation. The values of the CD change extrapolated to infinite salt concentration were also obtained from Figures 14 and 15 and are shown in Table 1. If the CD of α -helical polylysine and that of histone IV in water are used as standards, as described previously, $\Delta\epsilon_{220} = -10$ for 100% α -helix formation (Figure 8). α -helix formation is cooperative. At a particular, finite,

salt concentration, the fast transition to a new state will not be maximal. If this means that some molecules have undergone a transition and the rest have not, and, furthermore, if all of the altered molecules are in the same state, then the extrapolation to infinite salt concentration will permit the calculation of the number of α -helical residues per molecule at any salt concentration. It is, of course, not necessary to assume that there is only one stretch of α -helix per molecule and there may be more than one. From the extrapolation to infinite phosphate concentration (Table 1) we estimate that 17 to 22 residues, of histone IV are converted to α -helix in the fast process. If it is not true that all altered molecules are in the same state, such a procedure will lead only to an average value for the α -helical residues per molecule.

Histone Concentration Dependence

In this section the dependencies of the fast and slow processes on the histone concentration are examined. Figure 17 shows the fast step histone concentration dependence of the anisotropy and the CD.

As can be seen the magnitude of the CD change in the fast step has at most a small dependence on histone concentration. In contrast the magnitude of anisotropy changes considerably with histone concentration. This dependency may be fit by a simple model for dimer equilibrium.

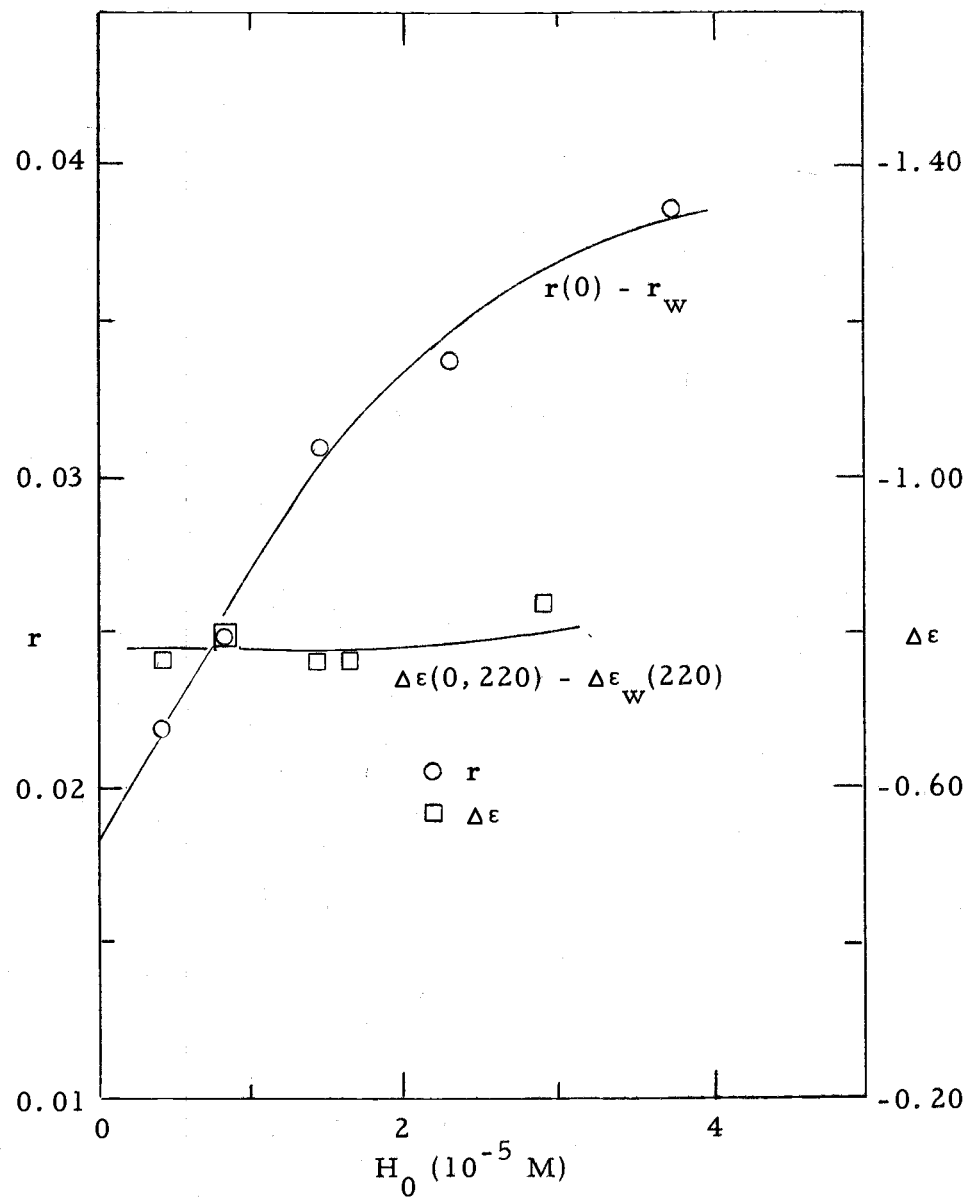


Figure 17. Histone concentration dependence of the fast step by anisotropy and CD in 6.6×10^{-3} M phosphate pH 7.4.

For a dimerization, we have



and

$$K_D = \frac{[H_2]}{[H]^2}$$

We have observed that the ratio of intensity of histone in water to that immediately after the fast step is independent of histone concentration, at least in the range studied. We may write, therefore (Evelt and Isenberg, 1969),

$$f_d = \frac{r_{H_0}^{(0)} - r_{H_0=0}^{(0)}}{r_{H_0=\infty}^{(0)} - r_{H_0=0}^{(0)}} \quad (10)$$

where $r_{H_0}^{(0)}$ is $r(0) - r_w$ at histone concentration H_0 .

H_0 is the total histone concentration and $f_d = 2H_2/H_0$ is the fraction of histone monomers in the associated state. It is necessary to find $r_{H_0=\infty}^{(0)}$, but this is easily obtained by the extrapolation of a plot of $(r_{H_0}^{(0)} - r_{H_0=0}^{(0)})^{-1}$ vs. H_0^{-1} to the intercept at $H_0^{-1} = 0$. The extrapolation yields $r_{H_0=\infty}^{(0)} - r_{H_0=0}^{(0)} = 0.05$ and we then are able to obtain f_d from Equation 10.

The dimer test, namely a plot of $[H_2]$ vs. $[H]^2$, is now possible, and is shown in Figure 18. The plot is a straight line, and we

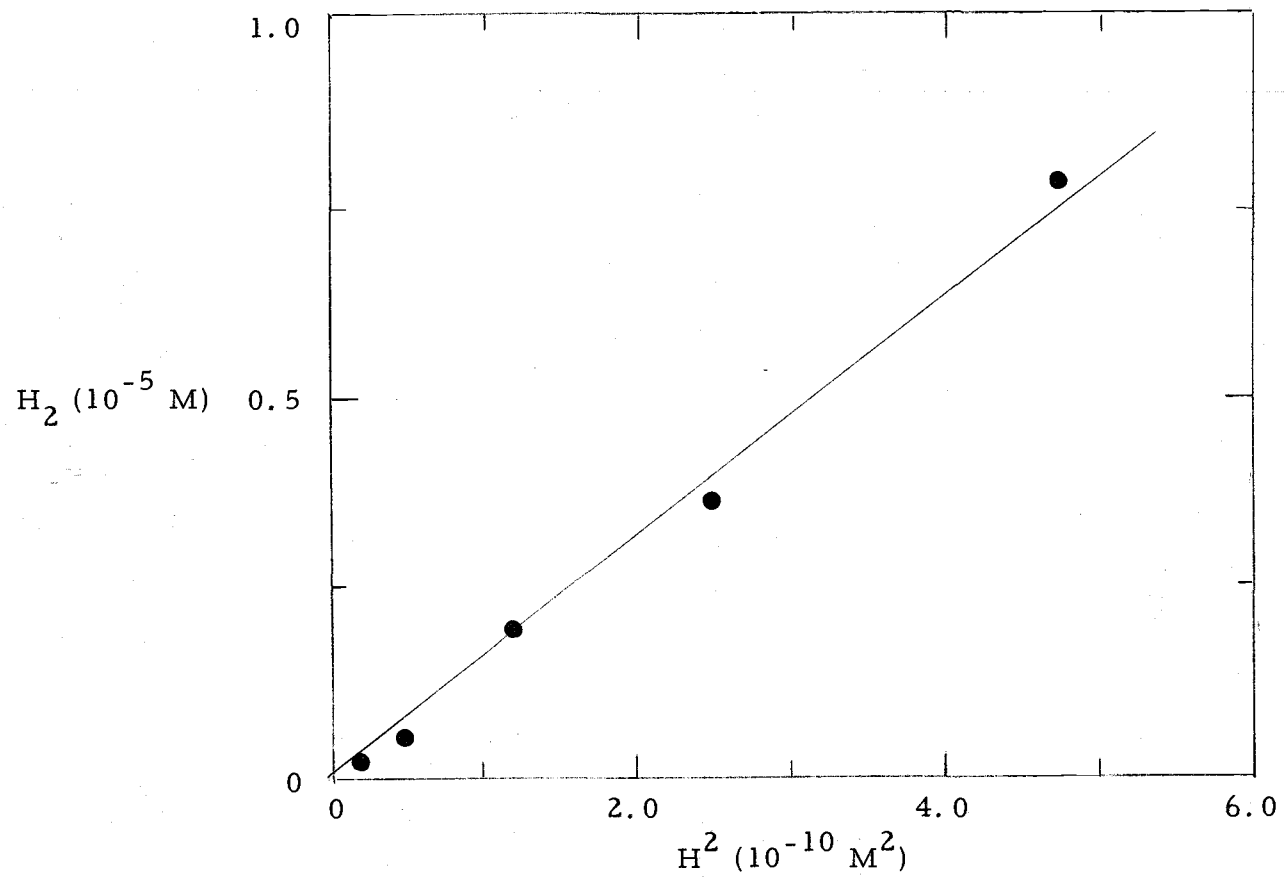


Figure 18. Plot of $[H_2]$ versus $[H]^2$ for dimerization test.

conclude that our data is consistent with a rapid histone dimerization. The equilibrium constant is 1.5×10^4 (Moles)⁻¹ liter in 6.6×10^{-3} M phosphate, pH 7.4.

Figure 19 is a plot of the rate constants for the anisotropy and CD measured during the slow process. The rate increases with histone concentration. This dependency implies that the slow process occurs intermolecularly. Thus two different processes are intermolecular; the initial dimerization, which is rapid, and the slow process which involved β -sheet formation.

Although the anisotropy change in the fast step depends on the histone concentration, the final value of the anisotropy does not. The same final value, $r(\infty) \simeq 0.145$, is reached regardless of the histone concentration, at least over the range of histone values that have been tested, i. e., from $H_0 = 4 \times 10^{-6}$ to 4×10^{-5} M.

We have also found that $\Delta\varepsilon(\infty)$ is independent, or nearly independent, of histone concentration.

This section may be summarized by saying that the initial salt induced conformational change is fast and intramolecular, as might have been expected. A fast intermolecular interaction also occurs. This interaction satisfies dimer equilibrium equations. These rapid changes are followed by a slow conformational change. The final anisotropy and CD are independent, or nearly independent, of histone concentration.

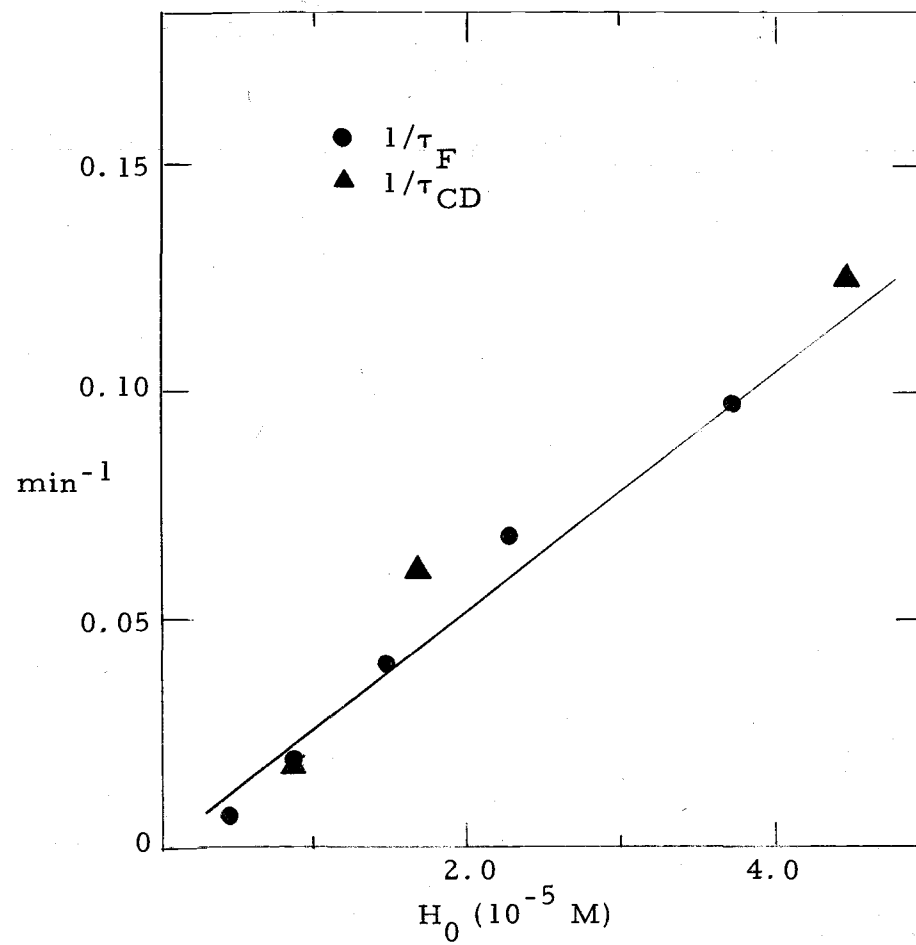


Figure 19. The histone concentration dependence of the rate constants by anisotropy and CD.

In the original intramolecular change a fraction of the residues go into an α -helix, while in the slow change a fraction of the residues go into a β -sheet. As a result of the slow conformational change the fraction of molecules in the monomer form decreases. It is possible that the number of dimers also decreases because the slow process may involve a higher order of aggregation.

Circular Dichroism of the Tyrosine Residues

CD spectra of histone IV in water, and in phosphate, were taken from 300 to 254 nm using 10 cm pathlength cells and twice the usual histone concentration ($H_0 = 1.8 \times 10^{-5}$ M). The results are shown in Figure 20. In water the CD is very weak (A). A significant non-conservative and negative CD in this region is induced immediately when phosphate is added (B), and this is followed by a continuous slow change until a final steady state is reached (C). The three CD spectra in Figure 20 are similar in shape to one another, with a minimum at 277 nm. Histone IV has four tyrosines, two phenylalanines and no tryptophan. The absorbance spectrum of histone IV shows a maximum at 278 nm (Li et al., 1971). The absorbance is due to the tyrosine and hence it is reasonable to ascribe the CD to the tyrosine residues as well. The units for $\Delta\epsilon$ presented in this section are therefore, cm^{-1} per mole of tyrosine per liter.

Analogously to the fluorescence anisotropy and the CD changes

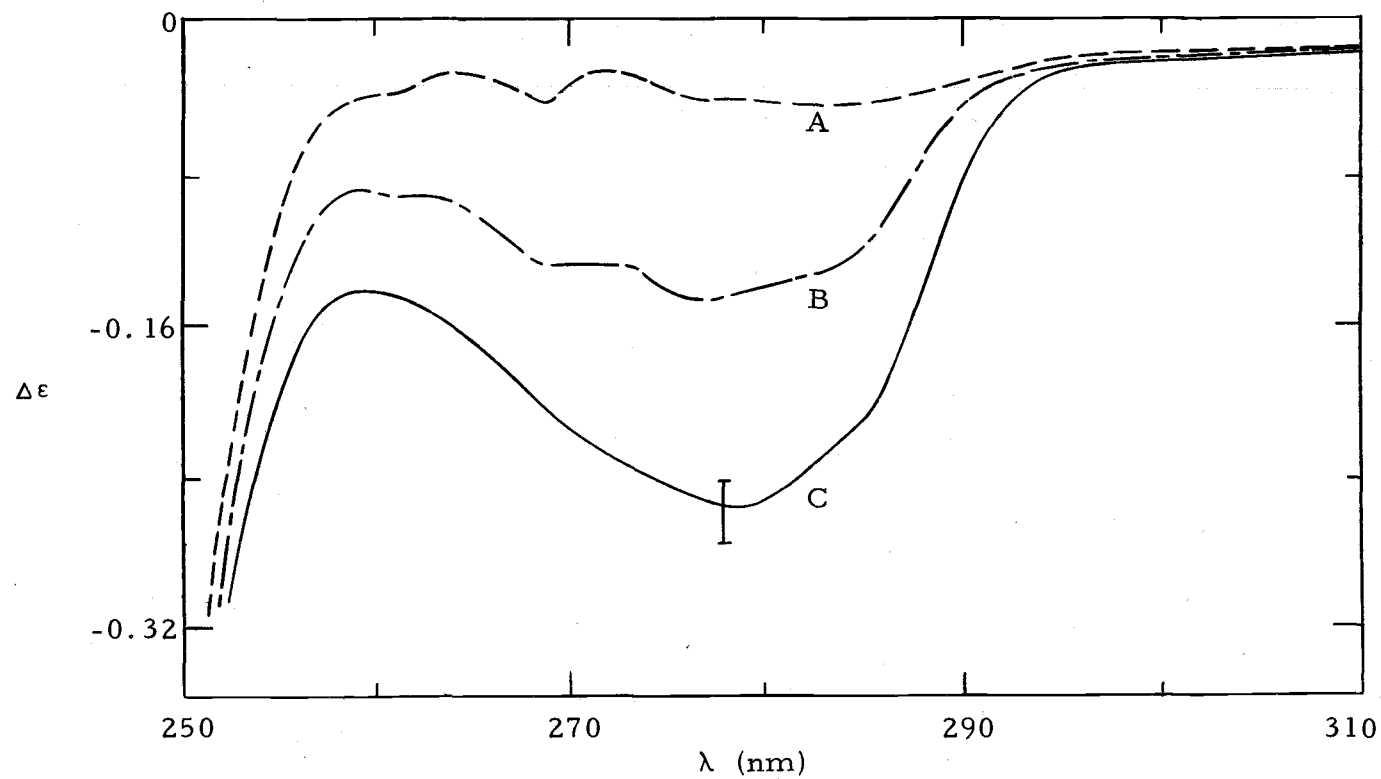


Figure 20. Long wavelength CD spectra of histone IV: A) in water, B) in 6.0×10^{-3} M phosphate pH 7.0 scanned 3-10 min after mixing, C) scanned from 120-127 min after mixing.

discussed previously, the CD change at 277 nm can be separated into two processes. The fast step is finished before the measurement starts, and the slow step fits exponential kinetics although the signal to noise ratio is much lower for this weak CD. Figure 21 shows the magnitude of the slow and fast steps and the final value as function of phosphate concentration.

It should be noted here that the CD of tyrosine in proteins can be either positive or negative and depends greatly on the specific protein conformation (Chen and Woody, 1971; Yang *et al.*, 1968; Schiffer and Edmundson, 1967). The parallel results between the increase of fluorescence anisotropy and of the CD of tyrosine on histone IV after adding phosphate indicate that a more rigid structure is formed surrounding the tyrosines.

Reversibility

To test the reversibility we chose a phosphate concentration such that divalent phosphate would induce a large change, but monovalent would not. Then by lowering the pH after the change occurred we could check to see if there was reversibility. The histone IV was initially at pH 7.4 in 6.6×10^{-3} M phosphate. After 12 hours, when changes had gone to completion, titrations were made to lower pH values. We noted that there was some time dependence of the back titration, and changes occurred for a number of minutes. In Figure

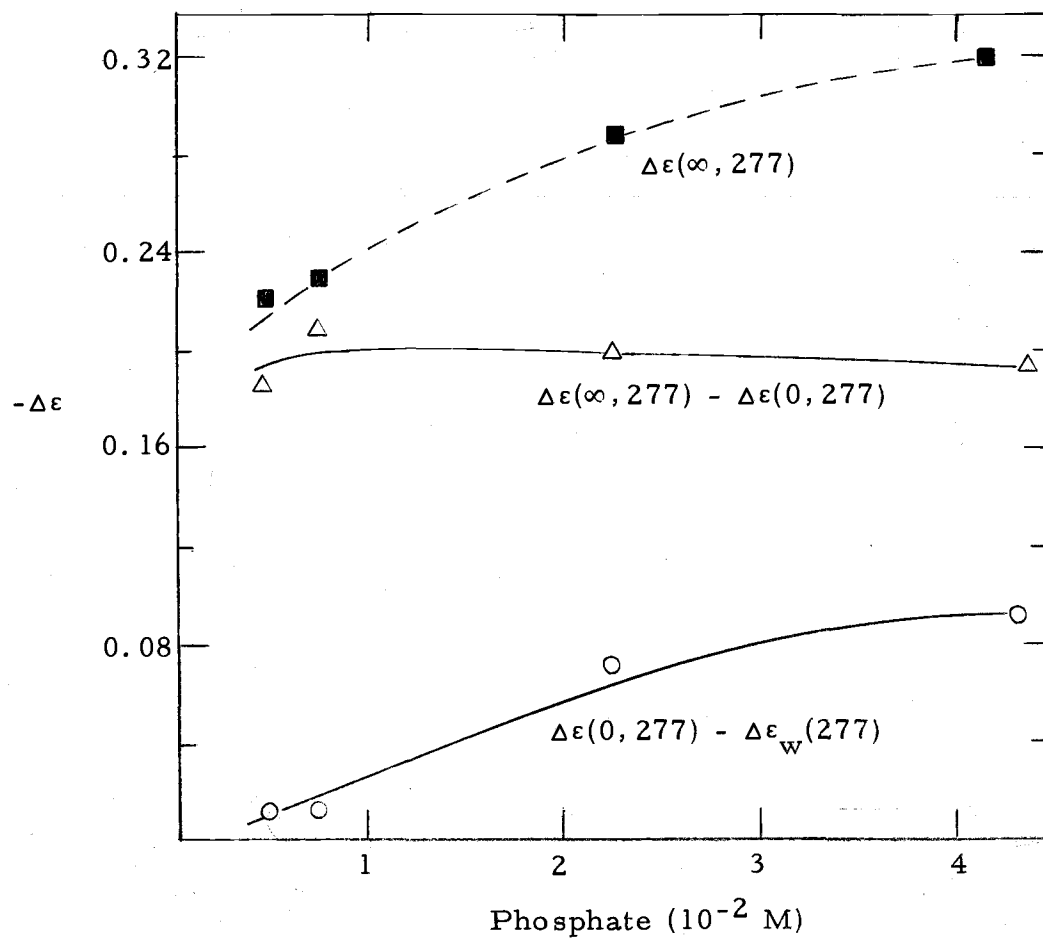


Figure 21. Phosphate dependence of the final CD and of the fast and slow changes at 277 nm.

22, the final values for the anisotropy and the CD at 220 nm are shown. Figure 23 shows the CD spectrum at several of the pH values studied. It is worth noting that at low pH values both the CD and the anisotropy have essentially reverted to the values for histone in water with no added salt. The CD spectrum at pH 1.8 is identical with that of histone IV in water at pH 7.0 (Li, Isenberg and Johnson, 1971).

Experiments with sulfate showed no difference in either the magnitude of fast and slow steps or the rate of the slow step when the pH was changed from 6.7 to 5.5, a range which covers the steepest part of the back titration. These experiments showed that the reversibility described above does indeed result from a di-mono basic phosphate shift, rather than a direct proton action on the protein.

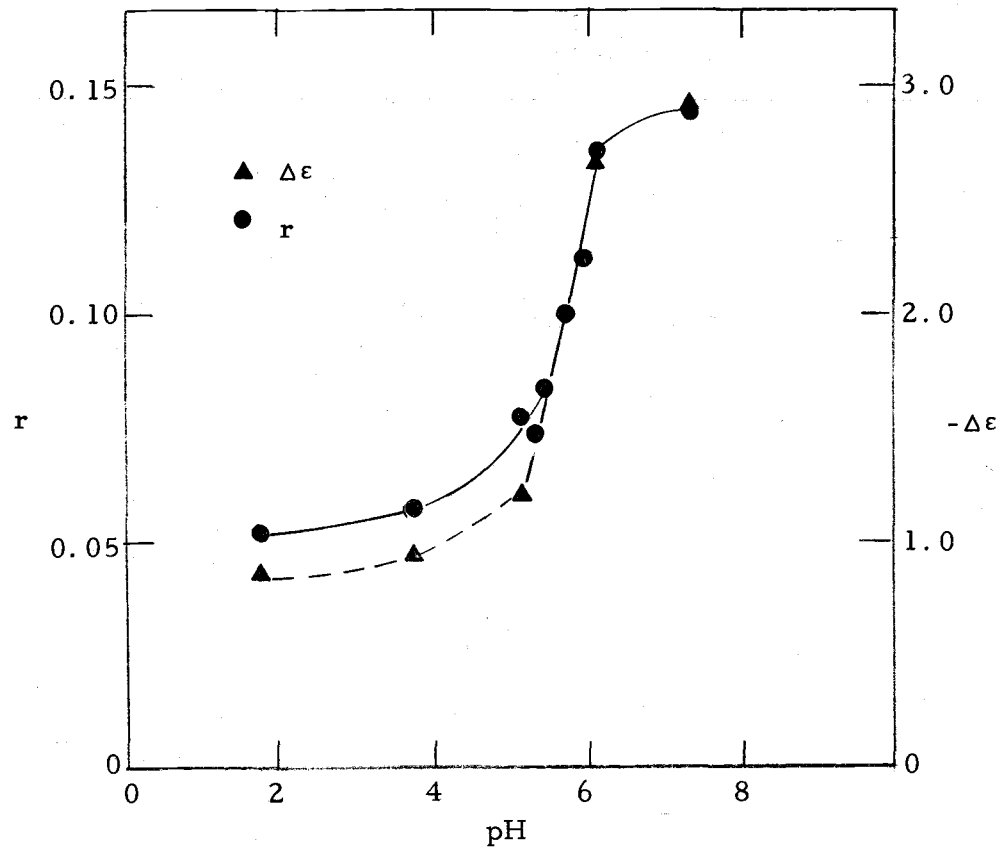


Figure 22. Back titration curve in phosphate as measured by anisotropy and CD.

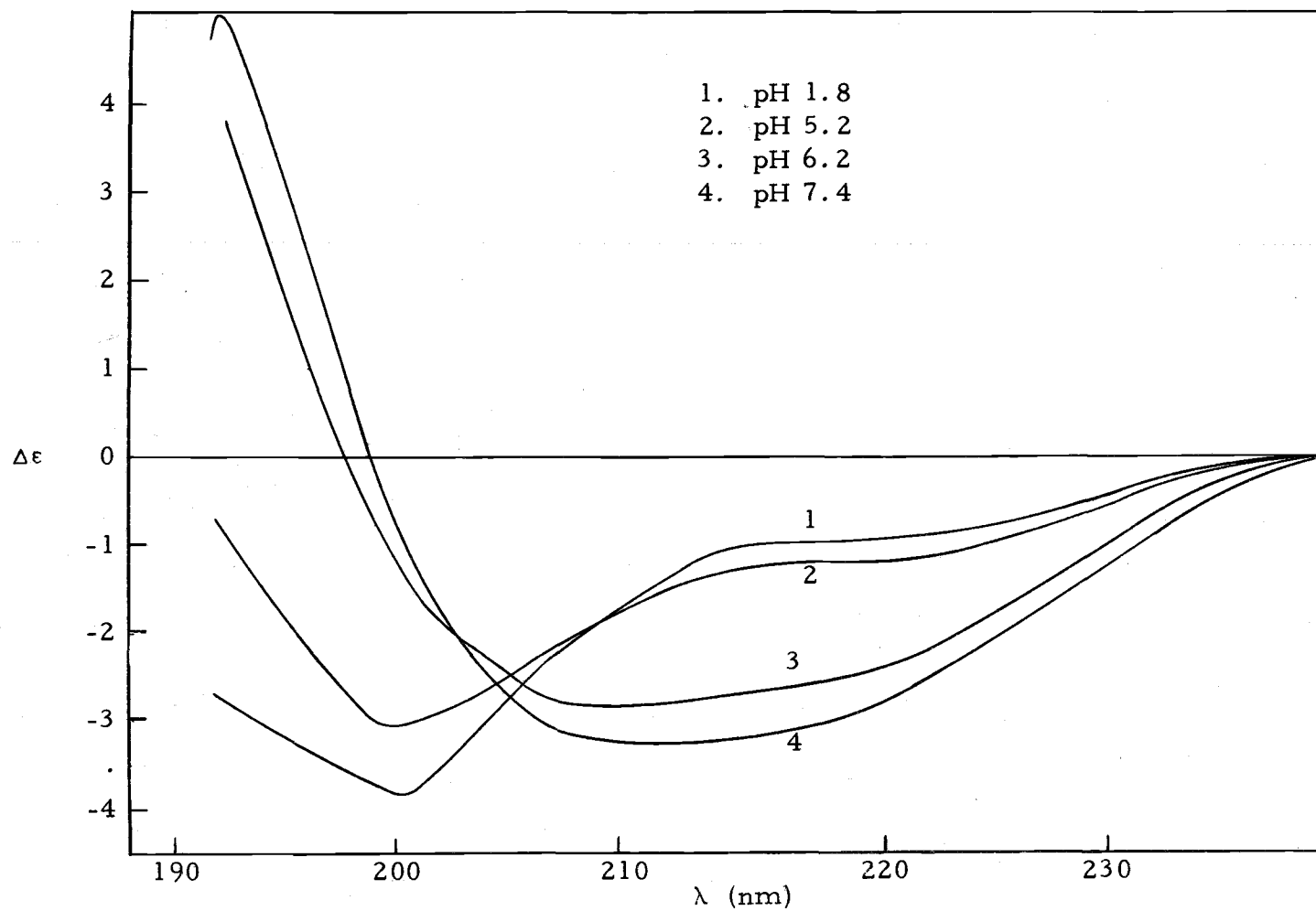


Figure 23. CD spectra of histone IV in 6.6×10^{-3} M phosphate as a function of pH.

Part B: The Interaction of Histone IV with
Nucleoside Triphosphates

Fluorescence Anisotropy Changes Induced by ATP and GTP

Figure 24 shows the effects of ATP, ADP and GTP on the anisotropy of the tyrosine emission at $H_0 = 4.4 \times 10^{-6}$ M. Two features of the curves are of note. First, ATP and GTP are effective in inducing fluorescence anisotropy changes at low concentrations where the ratio of ATP or GTP to histone is a small number. Second, the curves have a sigmoidal shape.

A small amount of time dependent change was observed above 1.5×10^{-5} M ATP or GTP in the absence of NaCl. In 0.025 M NaCl, or with ADP solutions, no time dependence at all was seen over the range observed. The amount of slow change, when it occurs, is small compared to that induced by small anions. When small changes were observed the anisotropy was extrapolated to zero time, as described in Part A, to obtain the data shown in Figure 24.

Reciprocal plots according to Equation 8 are not linear for ATP or GTP and thus, to obtain a more quantitative picture of the interaction, we have calculated f_r (Equation 5), the fraction of histone molecules which have undergone the conformational change. To find

$I_\infty(r_\infty - r_w)$ (Equation 5) it is possible to extrapolate a plot of $\ln \frac{1}{I_A(r - r_w)}$ vs. $\frac{1}{A}$ to $\frac{1}{A} = 0$. These semilog plots are linear for

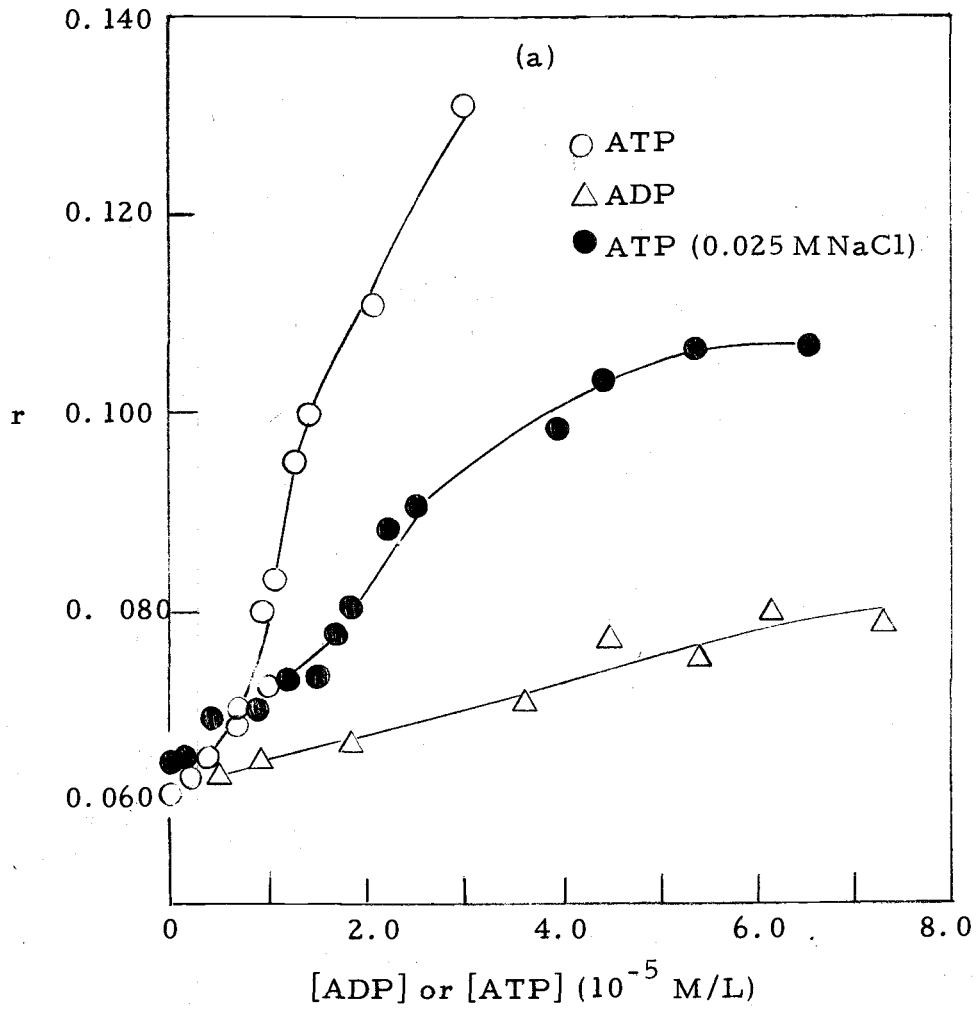


Figure 24. The effects of ATP, ADP and GTP.

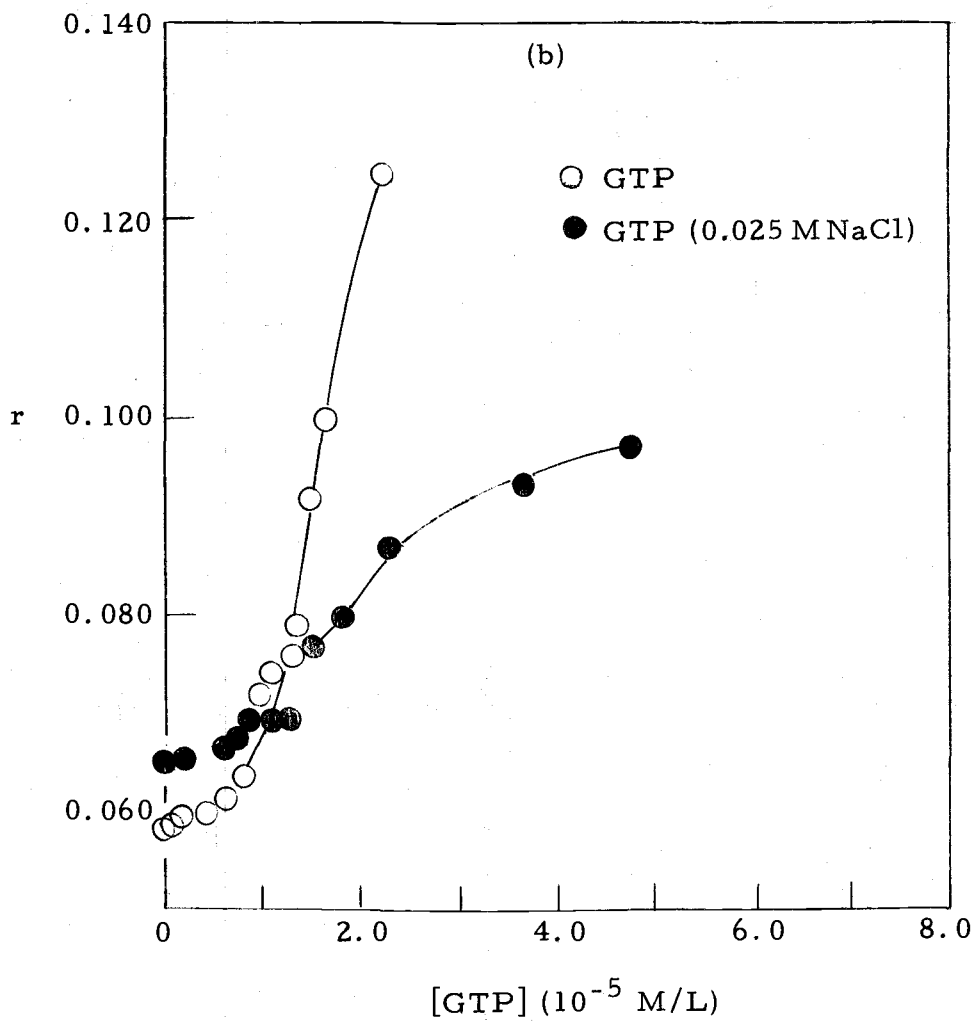


Figure 24. Continued

nucleotide/histone ratios above 1.5 and furthermore extrapolate to the same value for different histone concentrations. The value of $I_{\infty}(r_{\infty} - r_w)$, so obtained, is 0.111 for ATP and 0.125 for GTP. Separate extrapolations of the relative intensity to infinite nucleotide concentration give $I/I_w = 0.77$ for ATP, and 0.70 for GTP, where I_w is the intensity in water. Combining the two extrapolations gives $r_{\infty} - r_w = 0.086$ for ATP and 0.087 for GTP.

Plots of f_r vs ATP at three histone concentrations are shown in Figures 25, while in Figures 26 and 27 we have plotted f_r as a function of the number of ATP or GTP molecules per histone IV molecule. These latter curves do not differ significantly from one another either for ATP or GTP. Furthermore, the curves for ATP and GTP are close to one another.

We note that at nucleotide/histone ratios of about 5.0 the histone begins to precipitate.

CD Changes Induced by Added ATP and GTP

The CD of histone IV is changed markedly by the addition of nucleoside triphosphates. Figure 28 shows $\Delta\epsilon(0, 220)$ as a function of ATP in buffer and 0.025 M NaCl and CD difference spectra are shown in Figure 29. The change is like the fast change seen upon the addition of small anions, but consistent with our anisotropy data, the concentrations of nucleoside triphosphate needed for appreciable

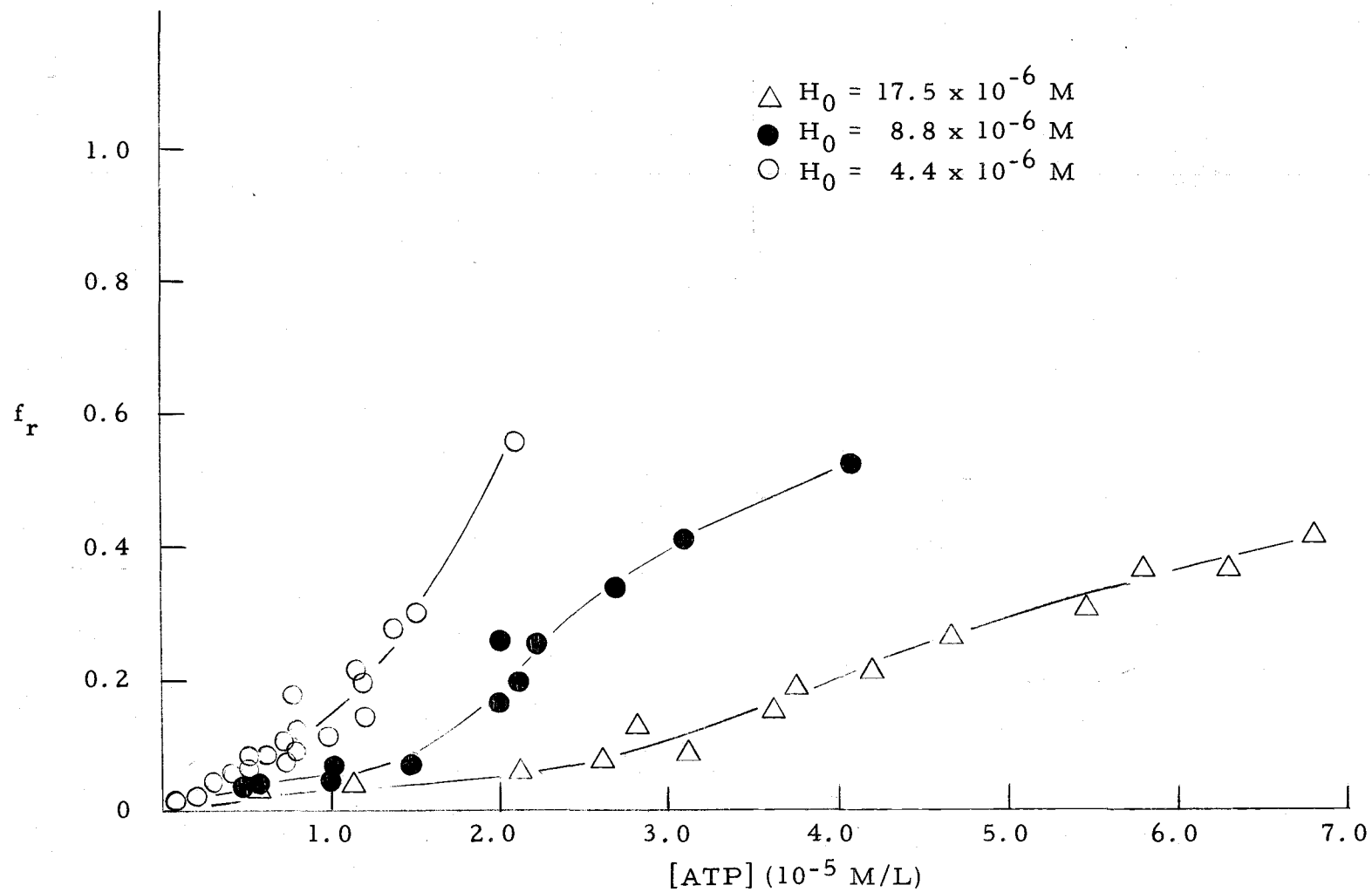


Figure 25. Fraction of conformational change versus ATP at three histone concentrations.

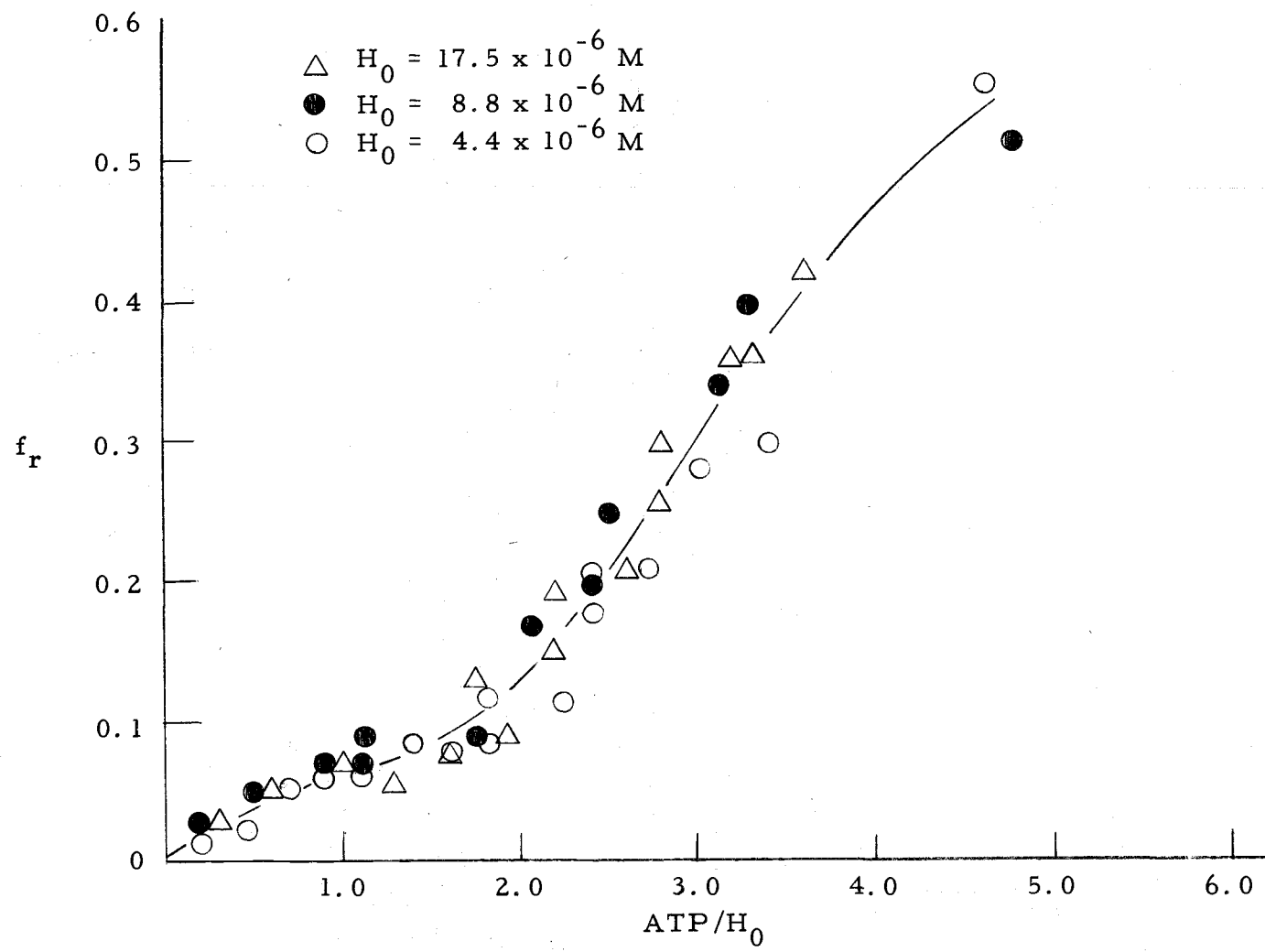


Figure 26. Fraction of conformational change versus ATP/ H_0 at three histone concentrations.

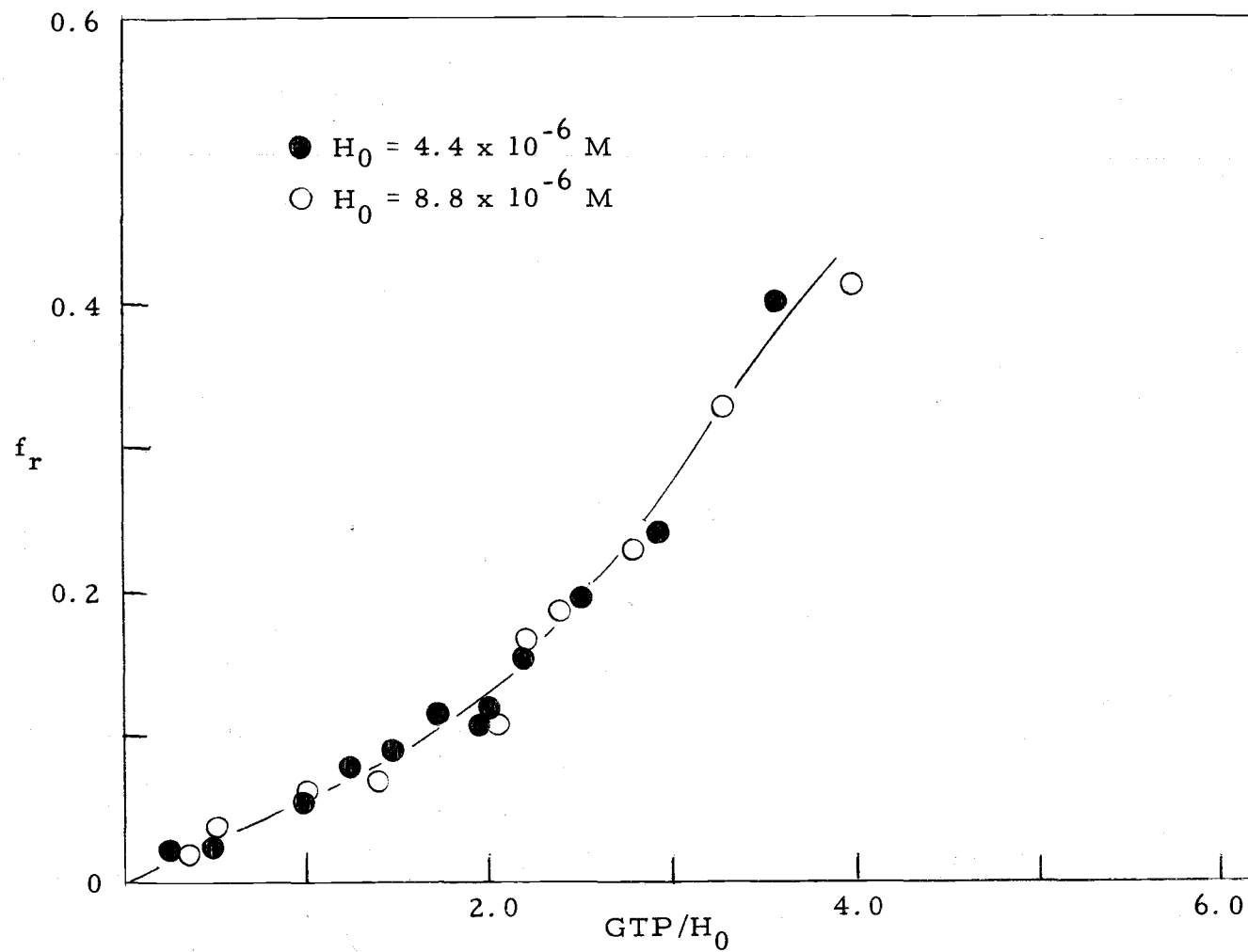


Figure 27. Fraction of conformational change versus GTP/H_0 at two GTP concentrations.

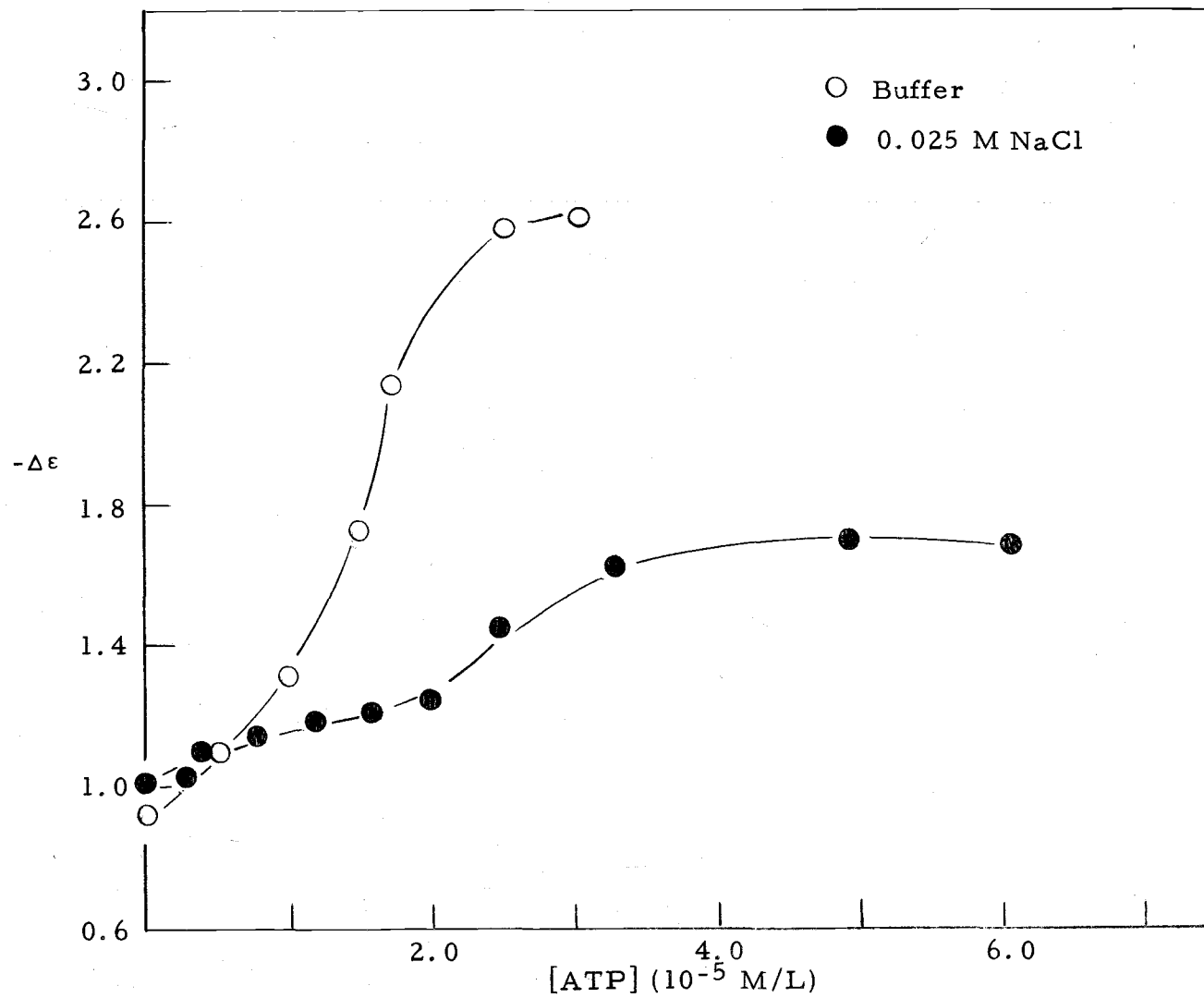


Figure 28. $\Delta\epsilon(0, 220)$ versus ATP in buffer and 0.025 M NaCl.

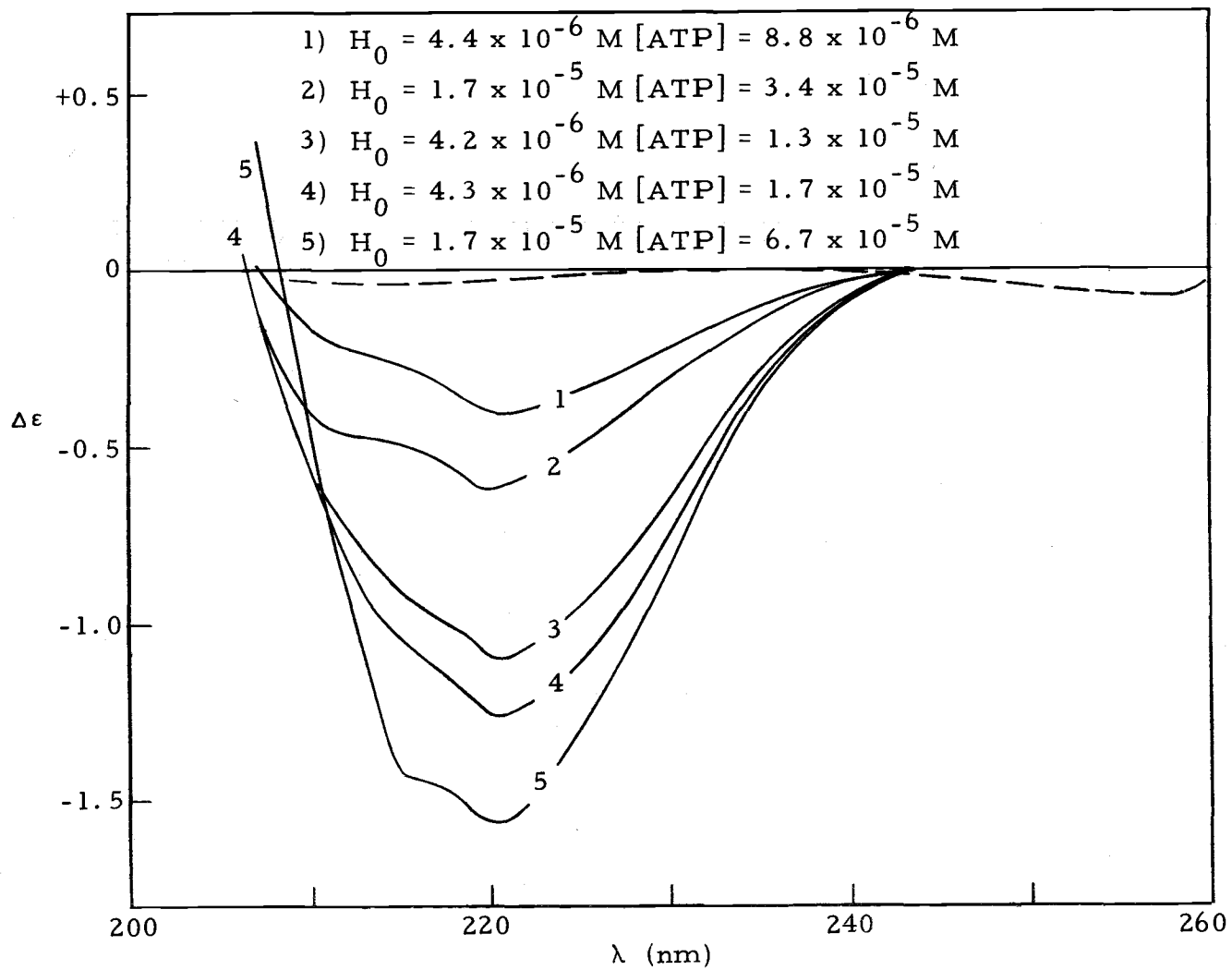


Figure 29. CD difference spectra for ATP relative contribution of ATP at highest ATP is (---).

change is of the same order of magnitude as the histone concentration.

We find appreciably smaller slow changes than occur upon the addition of small anions. At $H_0 = 4.4 \times 10^{-6}$ M we do see small amounts of slow change at nucleotide/histone ratios of 3.0 or higher. However, these slow changes are so much smaller, and faster, than those observed with small anions that we have not been able to obtain CD difference spectra for the slow change. At $H_0 = 1.75 \times 10^{-5}$ M no changes were observed after the start of the measurement (1.5 min after ATP additions) even at the highest ATP concentration studied.

The small CD of the nucleotides above 250 nm was unchanged by the addition of histone and so it seems reasonable to assume that the small CD they possess in the region of protein absorbance is also unchanged by histone. This CD was simply subtracted from the histone CD spectrum. The relative contribution to the CD of ATP, at the highest ATP concentration, is shown as the dashed line on Figure 29.

The CD difference spectra taken at $ATP/H_0 = 2.0$ (1 and 2 of Figure 29) are very similar in shape to those previously obtained for the fast conformational change in histone IV (Figures 9-11, 13). They agree quite well with the theoretical difference spectrum for α -helix formation (Figure 12). At higher ATP concentrations the shape of the difference spectrum changes somewhat. Spectra 3 and 4 have minima at 220 nm but the shoulder at 212 nm is less pronounced. Spectrum 5

($H_0 = 1.70 \times 10^{-5}$ M, ATP = 6.6×10^{-5} M) has a definite shoulder at 216 nm and a crossover point at 208 nm. These perturbations in the α -helical difference spectrum may be the result of β -sheet formation as the β -sheet CD spectrum has a peak at 216 nm. However, as stated, no time dependence was observed in the CD under these conditions. If β -sheet is formed, it is formed in less than 1.5 min.

Figure 30 shows plots of $\Delta\epsilon(0, 220) - \Delta\epsilon_w(220)$ vs. nucleotide/histone for ATP. Figure 31 is a similar plot for GTP. In contrast to the fluorescence results the curves are not superimposable, at least above 1 ATP per histone. Even below 1 ATP per histone, because of experimental error, it is difficult to see if the curves are really the same or simply merge as the ATP concentration is lowered. However, as with the fluorescence data, the slope becomes larger in the higher ATP or GTP range.

Changes Induced by CTP and TTP

The CD as a function of added CTP and TTP per histone molecule is shown in Figure 32. The change per molecule added is not as great as that found with added purine nucleoside triphosphate, but CTP and TTP have effects which are still much stronger than that for phosphate (Figure 6). We note that CTP and TTP have equal effects.

These CD data may be interpreted as resulting from equilibrium

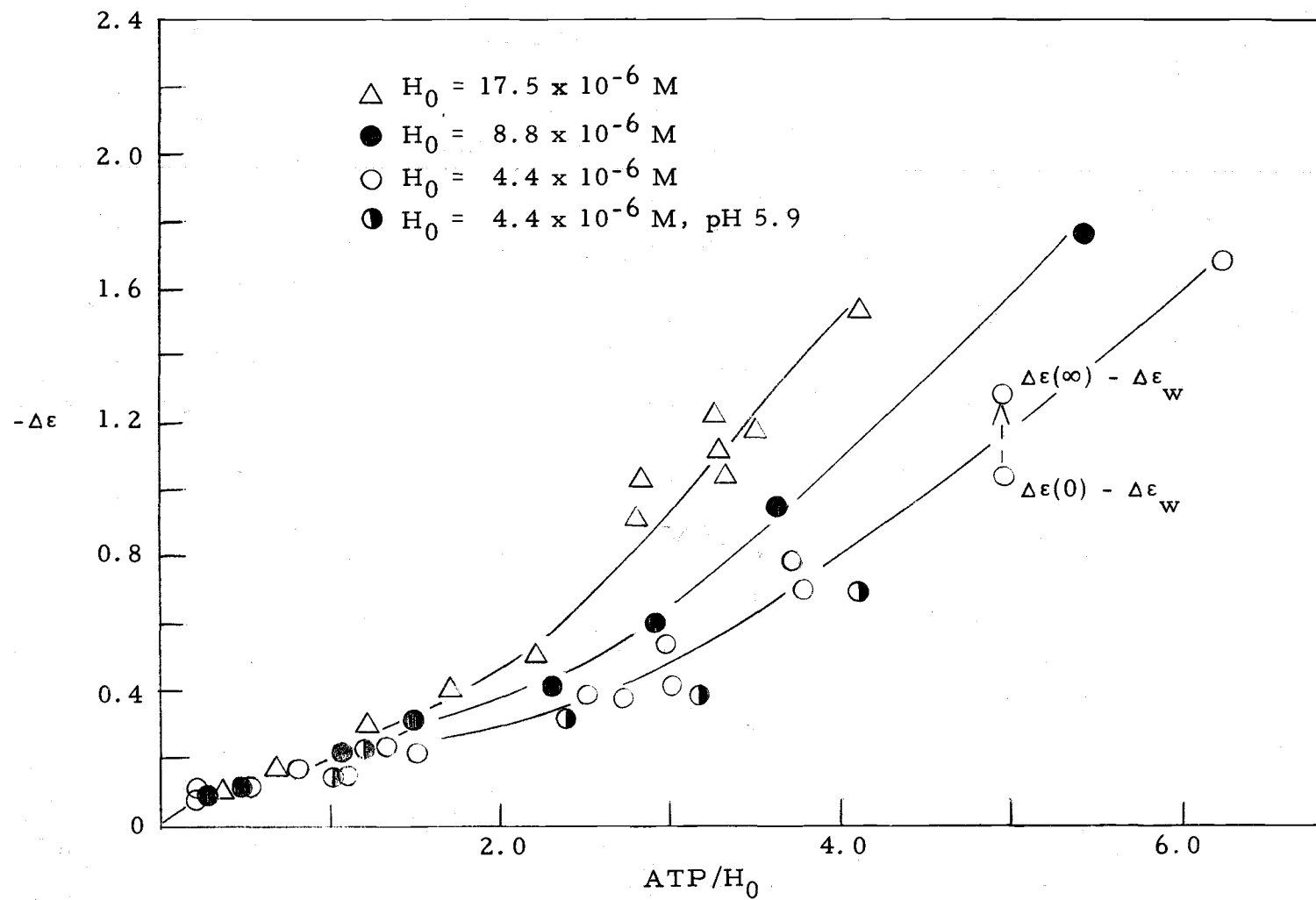


Figure 30. CD change at 220 nm and zero time versus ATP/H_0 .

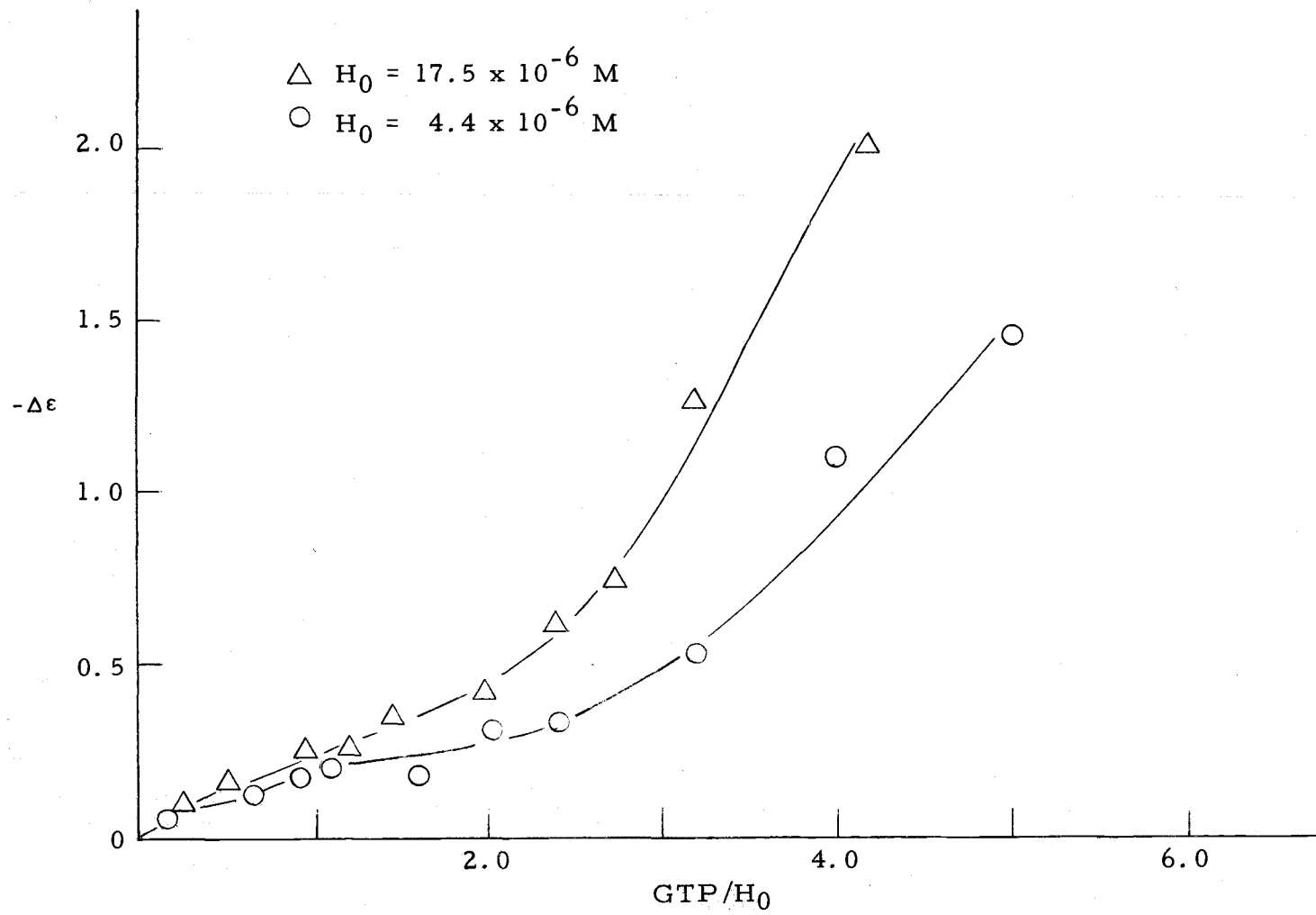


Figure 31. CD change at 220 nm versus GTP/H₀.

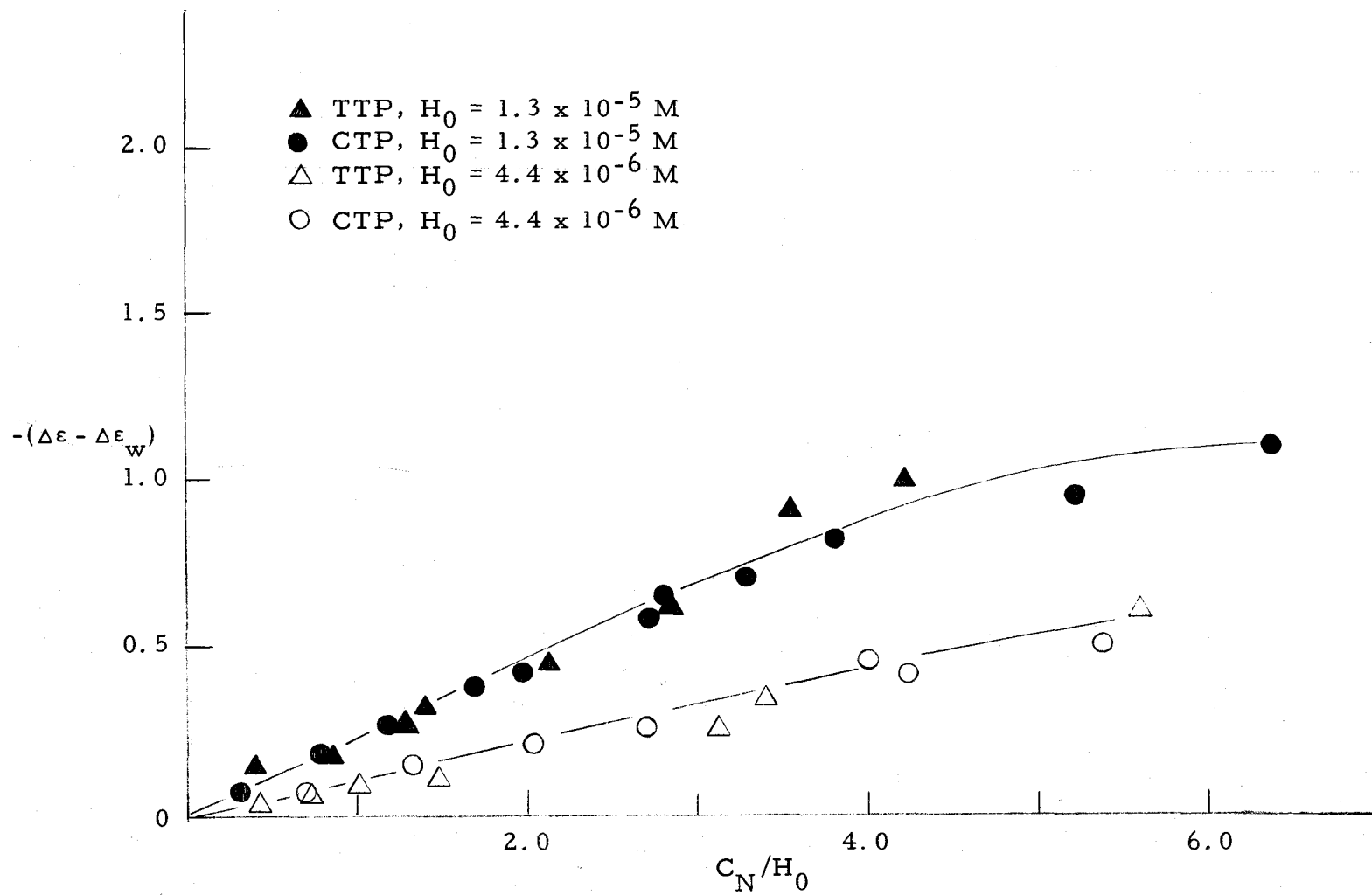


Figure 32. CD change at 220 nm versus C_N/H_0 for CTP and TTP.

binding, plots of $\frac{1}{\Delta\epsilon(0, 220) - \Delta\epsilon_w(220)}$ vs. $\frac{1}{A}$ above nucleotide/histone ratios of 2.0 are linear, as with small anions. Furthermore, the effective equilibrium constant is the same for CTP and TTP at both $H_0 = 4.4 \times 10^{-6}$ M and $H_0 = 1.3 \times 10^{-5}$ M, viz. $1.3 \times 10^4 \text{ M}^{-1}$ which is two orders of magnitude higher than those for divalent anions (Table 1).

The anisotropy change breaks sharply upward at the higher range of nucleotide concentrations (Figure 33). The nucleotide/histone ratio at which this break occurs depends on the histone concentration.

A reciprocal plot analysis of the fluorescence data below the break,

$\frac{ATP}{H_0} = 6.0$, and at the lower histone concentration,

$H_0 = 4.4 \times 10^{-6}$ M, yielded linear plots and gave

$K = 1.2 \times 10^4 \text{ M}^{-1}$ for CTP and $K = 1.4 \times 10^4 \text{ M}^{-1}$ for TTP.

For the higher histone concentration, $H_0 = 1.3 \times 10^{-5}$ M, the sharp break upwards occurs at about a ratio of pyrimidine nucleoside triphosphate to histone of only 2.0. The range from 0.0 to 2.0 is too small to be used for accurate reciprocal plots. Consequently we could not obtain an effective equilibrium constant from these data.

The equilibrium constants obtained from fluorescence data agree with those from CD data.

CD difference spectra are shown in Figure 34. Added TTP and CTP produce the same CD changes. The spectra have a peak at 220 nm but, at the higher histone concentration, where there is more

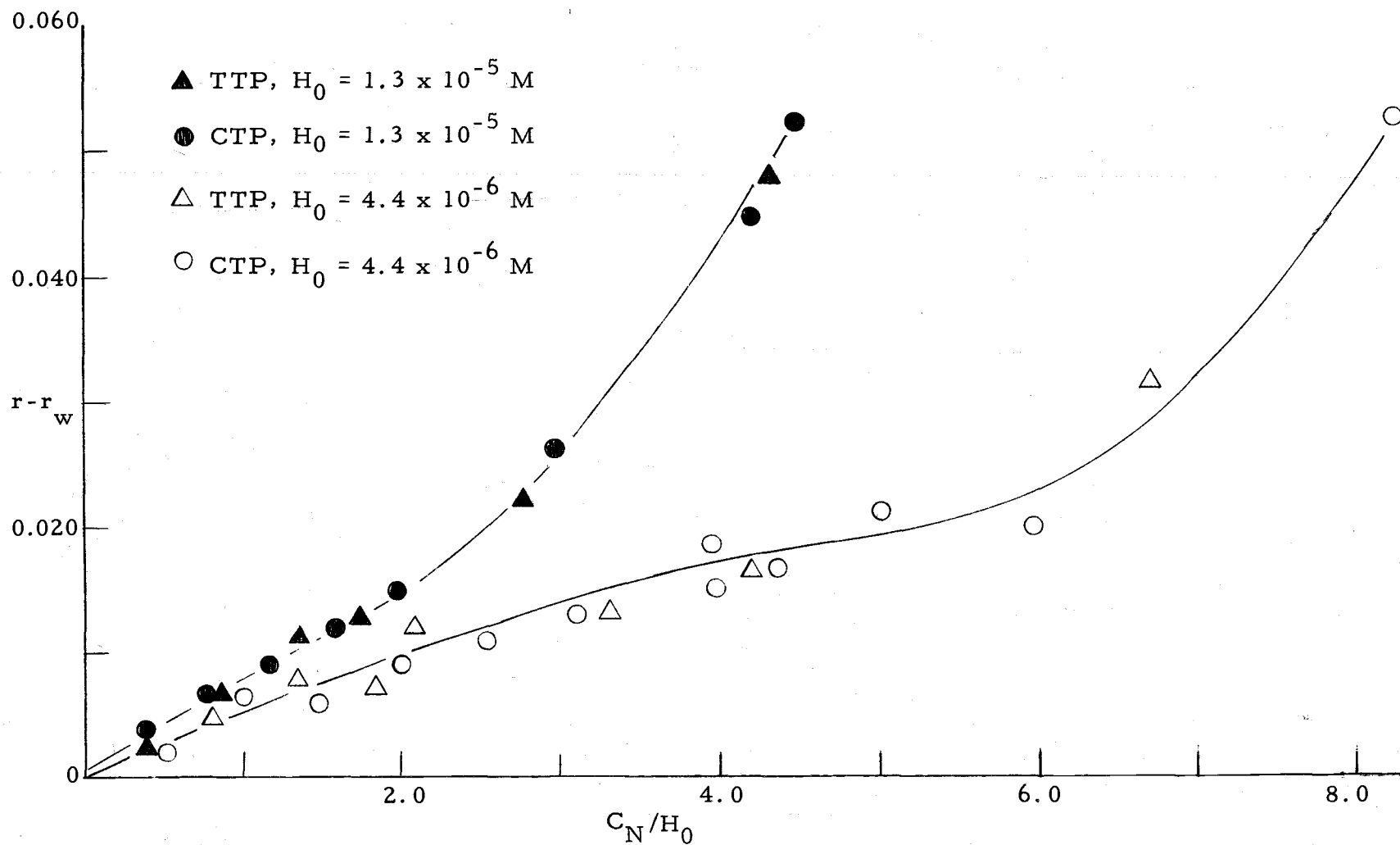


Figure 33. Fluorescence anisotropy change versus C_N/H_0 for CTP and TTP.

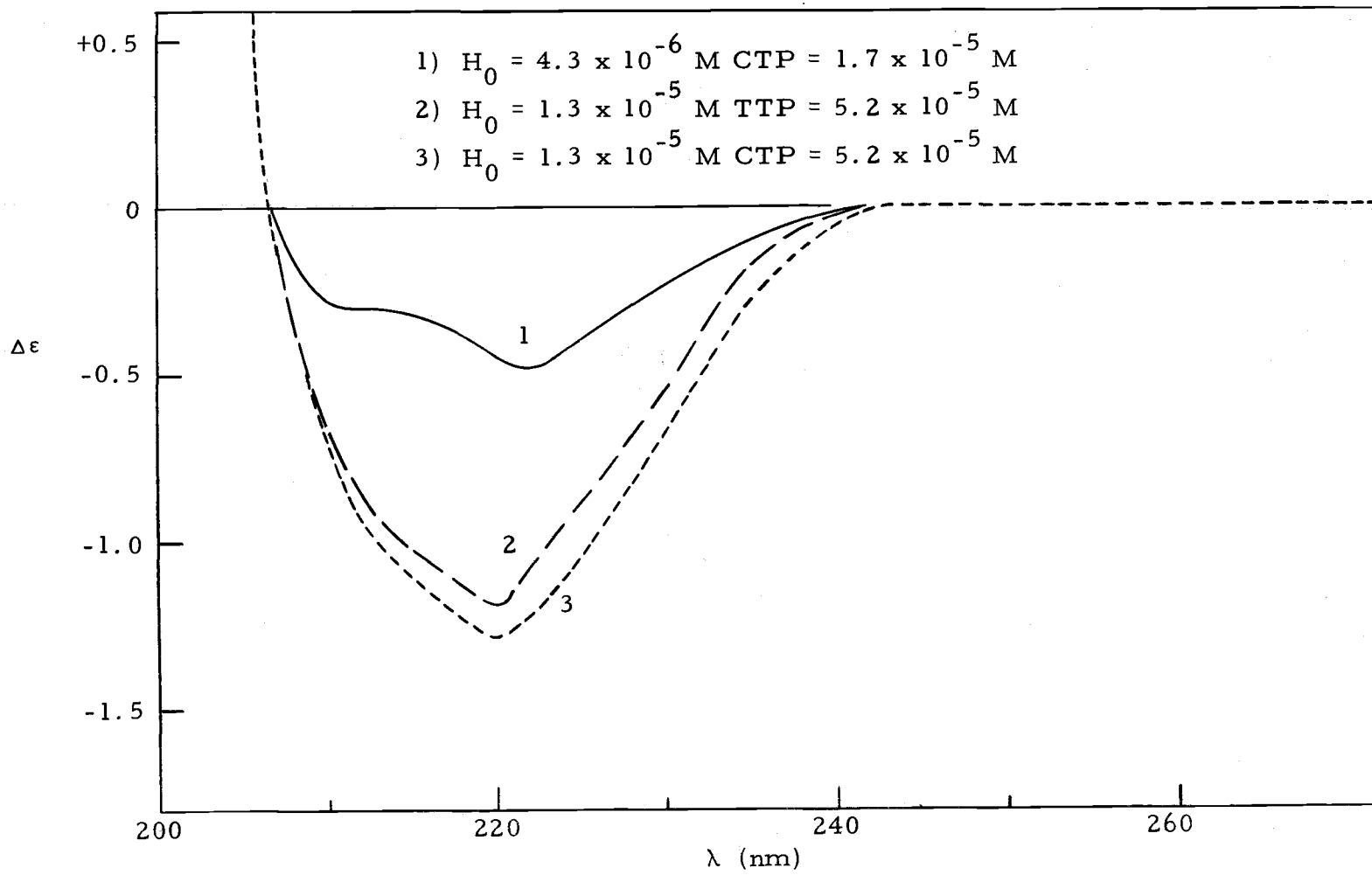


Figure 34. CD difference spectra for CTP and TTP.

total interaction, the shoulder at 210 nm, found in α -helical difference spectra (Figure 12) is less pronounced.

AMP and cAMP

We note that we could detect no change upon the addition of AMP or cAMP in concentrations found effective for di or tri phosphates.

IV. DISCUSSION

We have found that both α -helix and β -sheet are formed when salts are added to histone IV. However they form on two entirely different time scales. Due to the vast difference in the kinetics of the two processes we have found it possible to separate the α -helix and β -sheet formation. In addition, the rapid histone-histone interaction, in the presence of phosphate, satisfies a dimer equilibrium. Thus it is possible to describe an apparently complex process as a superposition of more simple events.

We note that the rapid structural change yields an added CD spectrum close to that of a typical α -helix (Figure 13). If we assume that this spectrum arises solely from α -helix formation then, from an extrapolation to infinite salt concentration, we can deduce that the maximum α -helix formation is 17-22 residues per protein molecule, provided it is legitimate to use the rotatory strength of α -helical polylysine as a standard. This of course neglects any side chain or end effects, and because of the assumption, there is an uncertainty in the value for the number of residues. The values of 17 residues for sulfate and phosphate and 22 residues for the other salts (Table 1) are reproducible to within one or two residues. Although the difference between 17 and 22 is outside of experimental error it is difficult to evaluate its significance. In addition we note that there is no way at

present to tell if the α -helix is in one continuous stretch or two or more shorter ones.

We can also demonstrate that other conformational folding, which does not significantly affect the CD, accompanies α -helix formation in the fast step. When the fast change occurs there is a marked increase in the anisotropy of the tyrosine emission. An interpretation of this increase is aided by a comparison to that found when a random copolymer, try₅glu₉₅, goes from random coil to 100% α -helix (Pesce et al., 1964). For the random copolymer an increase in r of only 0.017 was reported. This small increase for the copolymer is exceeded in the histone IV change even at relatively low salt concentrations (Figures 2 and 5) where only a small fraction of the histone molecules have undergone a conformational change. If, in addition, we estimate the magnitude of the anisotropy increase when all of the molecules undergo the fast change, we find changes of the order of 0.10, an order of magnitude larger than that which is found for the random copolymer.

For the random copolymer studied by Pesce et al. (1964), tyrosine, in the α -helical conformation, projects into the medium. It can then rotate about the side chain saturated bonds, and such Brownian rotation leads to an appreciable depolarization. The larger anisotropy change for histone IV implies that in addition to α -helical formation, there is structure formed to which the CD is insensitive.

This structure further hinders the tyrosine rotation. We are thus led to a picture of salt effects completely analogous to renaturation of denatured enzymes. Upon the addition of salt to histone IV, there is a general conformational change in the fast step. It is highly unlikely that all of the tyrosines are in the region of α -helix formation but, even if they are, the evidence indicates that additional structural changes exist.

It is interesting that for NaClO_4 the equilibrium constant, as measured by CD, is clearly different from that measured by fluorescence polarization, although they are equal for the other salts. We conclude that for most salts one overall conformational change occurs. We simply measure one or another aspect of the change. Once we conclude this, it follows that perchlorate induces a different type of alteration with different effects on the CD and the rigidity of the tyrosines.

The β -sheet formation is quite extensive under optimum conditions but declines somewhat at higher salt concentrations. Application of the theoretical CD change of 100% β -sheet formation (Figures 12 and 13) to the data of Figure 3, yields values for the amount of β -sheet formed under given conditions. The addition of sulphate and phosphate induces a maximum formation of approximately 30 residues β -sheet per molecule (Histone IV has 102 residues). The amount of β -sheet formed gradually falls to a value of less than 15 residues at

higher salt concentrations. With fluoride, perchlorate and chloride the β -sheet formation is slightly less extensive reaching a maximum of 19-21 residues, falling to less than 10 residues at higher salts. As with the values for α -helix the amount of error in these values is difficult to estimate.

Although the amount of β -sheet formed reaches a maximum and then falls off (Figure 3), the amount of the fast step continues to rise with salt. This shows that the high salt effect on histone IV structure is not an example of salt destabilization of the Hofmeister type (Von Hippel and Schleich, 1969). At higher salt, where the amount of slow step begins to decline, the rate constants of the process as measured by CD, τ_{CD} , and fluorescence, τ_F , first begin to differ (Figure 8). Thus both static and kinetic measurements indicate that a new type of structure is formed at high salt.

Since, at higher phosphate levels, there is more α -helix, it is possible that the lower β -sheet formation at high salt is due to competition between α -helix and β -sheet formation. However, we have no evidence to support this conjecture and it is possible that salt addition induces multiple effects. At low salt, by neutralizing some of the histone positive charges, conformational changes are induced, but at higher salt concentrations, some destabilizing interactions might also occur.

Though the effects of the salts are qualitatively similar, they

are very different quantitatively. The break point for the slow step, for example, is markedly different for each anion studied, ranging from 10^{-3} M for sulphate to 0.13 M for fluoride (Figure 3). The order of effectiveness is sulphate > phosphate > perchlorate > chloride > fluoride. It is important to note that this order is not that of the Hofmeister series (von Hippel and Schleich, 1969), nor is the order of magnitude of the salt concentrations typical of that for Hofmeister effects.

The effective rate constant of the slow process, in which β -sheet is found, depends on the histone concentration (Figure 18). This implies that β -sheet formation occurs intermolecularly. Although the rate of the slow step depends on histone concentration, the total overall change, including both fast and slow processes, does not depend on the histone concentration, at least as measured by anisotropy and CD.

If β -sheet formation does indeed form at the interface between molecules in either the dimer, or in higher order associations, such formation will effectively remove the monomer from the original equilibrium governing the rapid dimerization. In this way more molecules will be brought into an associated form. These, in turn, also may form β -sheet and hence the entire process may proceed to the same end point independently of the initial histone concentration. Diggle and Peacocke (1971) observed that histone IV aggregates

extensively in salt; in 0.10 M NaCl histone IV appeared to be monodisperse in the ultracentrifuge with a minimum molecular weight of 2.6×10^6 . The high histone concentration, and relatively long times used in their sedimentation study, virtually insures that the histone had completed the slow conformational change. A molecular weight of 2.6×10^6 indicates a final structure which is an aggregate of 230 histone IV molecules.

As discussed in the introduction, Boublik et al. (1970a) studied salt induced conformational changes in histone IV using NMR techniques. The authors concluded that the conformational changes induced by salt occur primarily in the C-terminal half of the histone IV molecule. The increase in tyrosine anisotropy and the induction of tyrosine CD verify the conclusion that conformational changes occur in the C-terminal half of the molecule. However, we cannot use these data to conclude that such changes occur only in the C-terminal half of the molecule. In fact, it seems unlikely that all of the α -helix and β -sheet formed are in only one-half of the molecule. These two conformations alone may account for nearly half of the residues under optimum conditions (20-30 β -sheet, 17-22 α -helix) and, as discussed above, the anisotropy data indicate that other conformational changes are occurring as well.

The interaction of nucleoside triphosphates with histone IV differs significantly from that of small anions. With phosphate, for

example, there is no measurable effect on the CD or fluorescence anisotropy of histone IV until 10^{-3} M phosphate (Figures 5 and 6). At this concentration there are more than 100 phosphate ions per histone molecule. In contrast the nucleoside triphosphates have measurable effects on both the anisotropy and CD when only 0.20 molecules of nucleoside triphosphate are present per histone IV molecule. A further difference is that extensive slow β -sheet formation is not observed in the interaction of nucleoside triphosphates with histone IV.

The techniques of fluorescence anisotropy and CD, as used here, yield data which are functions of the changes in conformation of the protein. They do not measure the fraction of small molecules bound to histone IV, although the effective binding constants, as we have defined them, are useful in comparing the effectiveness of small molecules in inducing change. However, we emphasize that because of the nature of the data we cannot interpret them as true binding constants except in special cases, such as that in which only one molecule binds per histone. Nevertheless, we can draw some important conclusions about the binding of nucleoside triphosphates to histone IV.

1. The binding of ATP or GTP to histone IV is very strong.

Simple equilibrium calculations for one binding site per molecule show that an equilibrium constant of at least

10^6 M^{-1} is required to produce fractional binding at $H_0 = 4.4 \times 10^{-6} \text{ M}$ and $\text{ATP}/H_0 = 2.0$ which is within 5% of that found at $H_0 = 1.75 \times 10^{-5} \text{ M}$ and $\text{ATP}/H_0 = 2.0$. The value 5% is about the experimental error of the anisotropy measurement in Figures 25 and 26.

2. Either the ATP binding is itself co-operative, or the conformational change is sequential with the binding of an ATP molecule or molecules producing one conformational change, while further binding produces a greater change. Either of these cases requires at least 2 binding sites.
3. There is both phosphate and nucleotide specificity. Triphosphate is stronger than diphosphate which is stronger than monophosphate. ATP and GTP are much more effective than CTP or TTP. AMP and cAMP had no effect over the range tested.

The divergence of the CD curves at differing histone concentrations, Figures 30-31, in contrast to the fluorescence results of Figures 26-27, could arise in a number of ways. The binding which produces the CD change could be at a different site than that which produces the fluorescence change, and there have a lower equilibrium constant. Alternatively, there could be some additional secondary structural formation at the higher histone concentration to which the anisotropy is insensitive. Interchain β -sheet would be a possible

candidate for such a structure. If it occurred in the N-terminal end of the molecule the anisotropy might not be sensitive to it as the CD, since the four tyrosines are all in the C-terminal half of the molecule.

Although CTP and TTP are much less effective than ATP and GTP, the effective equilibrium constants obtained from the data in Figures 32 and 33 are 100 times higher than those found for sulfate or phosphate (Table 1). It should be pointed out that the effective equilibrium constant, $1.3 \times 10^4 \text{ M}^{-1}$, is the true equilibrium constant for the CTP or TTP-histone IV binding if, but only if, there is only one binding site which affects the CD or fluorescence anisotropy. In that case, it is the binding constant for the site.

Histone IV undergoes a series of conformational changes and exhibits a truly remarkable structural polymorphism. The addition of salts to histone IV results in the following sequence of events:

1. Rapid α -helix formation,
2. Rapid dimerization,
3. Slow interchain β -sheet formation.

Furthermore, there are at least two different slow processes -- one at low and one at high salt concentrations, and these processes are reversible. Nucleoside triphosphate induce conformational changes at extremely low nucleotide/histone ratios.

While it is tempting to relate this structural polymorphism to the biological functions of histones, it would be premature because no

simple extrapolation from histone IV to chromatin can exist. Chromatin is a structure involving different histones, as well as DNA and non-histone protein. But in general, the alterations of chromatin in cell division, differentiation, and dedifferentiation must involve histone rearrangement and conformational alterations regardless of the specific role, or roles, the histone is playing. It is possible, therefore, that the polymorphism we are reporting is related to the general polymorphisms displayed by chromatin. The possibility of biologically important molecules such as nucleoside triphosphates exerting controls on histone conformation is especially intriguing.

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