Studies on the structure of Deoxyribonucleohistone

by

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But as for certain truth, no man has known it,
Nor shall he know it.....
For even if by chance he were to utter
The final truth, he would himself not know it:
For all is but a woven web of guesses.

Xenophanes

tr. K.Popper

1

INTRODUCTION

It is a widespread assumption that every somatic cell of an eukaryotic organism contains the information necessary to specify all the characteristics of that organism. Indeed, nuclei from differentiated cells will, on injection into enucleated oocytes, support the development of fertile adults.[75,76] That expression of this information is restricted in any one cell to a well-defined subset of those characteristics is witness to the accuracy of the organisation of the genetic material.

This organisation is of a greater complexity than that in prokaryotes. There exists in eukaryotes a hierarchy of control: not only must there be control over the activation of individual genes but large quantities of the DNA must be kept in a permanently repressed state. This must be achieved while permitting the entire DNA complement to be replicated and equally divided between the daughter cells at mitosis.

The contrast between fine and coarse control elements probably extends to the mechanisms involved. Fine control involves the switching on and off of single genes or groups of genes. This is a situation analogous to that successfully explained for bacteria by the model of Jacob and Monod.[99,134] Hence it is often held to require the intermediacy of tissue and species-specific control substances (the repressors and activators of the model). Coarse control, in contrast, concerns the repression or activation of the entire genome or large portions of it. A less specific mechanism would be suitable in this case and indeed such control is traditionally linked to the condensation of part of the nuclear material known as heterochromatization.

Investigations into the nature of the control mechanisms have concentrated on the effect of various DNA-associated nuclear components on the structure and function of the DNA. A number of systems have been studied which range from whole nuclei to artificial complexes of purified components. Numerous attempts have been made to isolate 'chromatin' from whole nuclei: all such preparations are artefactual to some degree. There is little point in claiming that such extracts are 'isolated chromosomes'.[17] An analytical definition is preferable. Complexes containing little but DNA and histones (deoxyribonucleohistones - DNH) are perhaps the simplest nuclear-derived systems for studying the control of gene expression.

1. The histones

The histones were the first protein constituents of the nucleus to be discovered[107] and they still remain the only well-defined protein fraction. Much is known of their physicochemical properties (for reviews see refs.[159] and[228]) but their role in the structure and function of chromatin has still not been completely elucidated.

There are five main histone fractions, the lysine-rich histone f1, the two slightly lysine-rich histones, f2b and f2a2 and the arginine-rich histones f2a1 and f3. Electrophoretic evidence points to there being at least 12 subfractions making up these main groups.[148] Two of the f1 subfractions are distinct protein species with a molecular weight difference of 1000.[149] Some of the observed heterogeneity is undoubtedly due to chemical modification of various peptide residues - particularly to acetylation and phosphorylation.

There is little evidence for tissue or species-specificity among the histones. Indeed histone f2a1 from calf thymus differs in sequence by only two conservative substitutions from that derived from pea seedlings.[45] If the other fractions do not quite achieve this degree of evolutionary stability, they do display a considerable degree of interspecies homology. Only f1 shows any signs of species specificity. It differs quite considerably both in composition[228] and molecular weight[149] between different species.

Two examples of tissue-specific histones are of outstanding importance. The best documented is the f2c fraction found in the nucleated erythrocytes of a number of species.[141 219 220] Arginine-rich histones are found to replace the normal histones during the maturation of sperm,[15] an extreme example of this being the replacement by protamines during the maturation of fish spermatozoa.[48] Both these cases occur in cells where complete repression of the genome is taking place.

Histones are highly basic proteins which, in nucleohistone, have over 80% of their basic residues bound to DNA.[224] Ionic forces predominate in chromatin but have little potential for endowing specificity. It is becoming increasingly obvious, however, that there is a hydrophobic component to the histone-DNA interaction[78 106] which could provide some specificity. Indeed there have been repeated reports that lysine-rich histones bind preferentially to A-T rich DNA[40 185] while arginine-rich histones are associated with G-C rich DNA.[39 40]

The histones are a well-defined group containing a small

number of basic proteins with little tissue or species-specificity. They interact with DNA mainly through ionic bonds. Of themselves they are not likely candidates for the role of fine controllers of gene expression on a Jacob-Monod-type model.

2. Histones and chromatin structure

Each chromosome in a eukaryotic nucleus behaves as if it were a single double-helix of DNA.[207] The DNA in a human chromosome varies in length between 1.4 and 7.3cm, condensing into a structure less than 10μm long at metaphase.[52] Such an extreme condensation argues that the chromatin is capable of a high degree of organisation.

a. The structure of chromosomes

Most of the information on chromosome structure has been obtained by electron microscopy. Ris was the first to report bumpy nucleoprotein fibres from eukaryotic nuclei with diameters of 20-25nm.[170] Thin-sectioning of nuclei revealed interphase chromosome fibres with diameters of the order of 7.5-15nm whereas preparation of material by surface spreading or critical point drying gave diameters of 23-25nm.[51] Surface spreading of chromosomes on solutions of chelating agents led to the observation of 10nm fibres.[173] The existence of these two major fibres types - the 10nm and 25nm fibres - which can be shown to exist in the same preparation, caused considerable debate. Wolfe and his coworkers[229,230] maintained that the 25nm fibre was a single 10nm fibre coated with protein, while Ris[172] argued for two 10nm fibres held together by metal ion bridges. Ris later withdrew this hypothesis in favour of a model of the 25nm fibre as a coiled 10nm fibre.

Du Praw and Bahr,[53,54] by consideration of the dry masses

of the two types of fibre, deduced that the 10nm fibre was packed into the 25nm by means of a supercoil with a packing ratio of 10:1. The total packing ratio of the DNA double helix into an interphase 25nm fibre was of the order of 56:1.

Lampert[110] has claimed to be able to see the spiral shadow of a supercoil in electron micrographs of 25nm chromatin fibres. X-ray studies of metaphase chromosomes have given no evidence of structure beyond that observed in isolated nucleohistone[152] - such structure would have to be very regular to be observed (J.F. Pardon, private communication).

It is of course possible that the widely held view that 10nm and 25nm fibres are in some way 'typical' of chromatin structure is misleadingly simple. All studies show a considerable range of fibre diameters (typically 5-50nm) and at least one group of investigators has deduced that no one diameter can be held to characterise the structure of chromatin.[232]

Electron microscopy is by no means free from artefacts. Ethanol can cause free DNA to form 10nm structures[111] characterised by a supercoiled configuration.[112] Uranyl acetate - widely used as a stain - is reported to form 23nm fibres under certain conditions.[74] In addition, the treatment undergone in sample preparation appears to destroy all forms of higher order structure present in DNH (A. Pooley, private communication). These results impose some restrictions on the interpretation of data derived solely from electron microscopy.

b. <u>The structure of extracted chromatin</u>

Despite these limitations, a degree of structure -

represented by the 10nm fibre - can be held to be fundamental to the structure of chromatin. Preparations of nucleohistone by the method of Zubay and Doty[233] also show 10nm fibres in the electron microscope.[171]

Investigations into the structure of extracted chromatin have mainly been pursued by X-ray diffraction techniques. The DNA in stretched chromatin is in the B-form; however in unstretched fibres the DNA is poorly oriented.[154] Recently it has been suggested on the basis of solution studies by circular dichroism that there is a considerable amount of C-form DNA in unoriented samples.[79 101] There is as yet no hard evidence to support this hypothesis.

Nucleohistone fibres show a set of X-ray reflections which are wholly typical. These reflections, at 11, 5.5, 3.7 and 2.7nm vary in intensity with the water content of the fibre.[125] Luzzati and Nicolaieff put forward the interpretation that they arose from different packing arrangements of linear DNA molecules into a nucleoprotein matrix.[125] However, the clear meridional orientation of some of these reflections cannot be explained on this theory and Wilkins and his coworkers[153 154 166] have interpreted the reflections as arising from a supercoiled nucleohistone fibre with a pitch of 12nm and a 10nm diameter. Other workers from a consideration both of electron microscopy and of X-ray scattering from dilute solutions, have proposed a second supercoil model.[26] This is a much more tightly wound but irregular coil of 4.5nm pitch and 6nm diameter. None of these models, not even the widely quoted Wilkins supercoil,[154] will explain all the data.

However, these 'supercoil rings' can be used as a qualitative measure of the higher order structure of nucleohistone. Not only fibres but also gels of DNH[152] will give these X-ray reflections. Nucleohistone precipitated by low concentrations of salts also retains the 'supercoil rings'.[67 163]

When histone is removed from the complex in high ionic strength the characteristic diffraction pattern is lost to be regained when the material is returned to low ionic strength and the histone reassociated.[146 234] The specificity of this interaction has been tested by complexing DNA with purified histone fractions. No single fraction will generate the supercoil rings but a mixture of f2a1, f2a2 and f3 was found to reconstitute 'supercoiling' when used in saturating amounts.[166] These results were confirmed by experiments in which the fractions were gradually dissociated in salt.[166]

Other physical parameters can be used as measures of nucleohistone structure. It is generally agreed that those histone fractions which are removed by salt at ionic strengths of 0.7M-1.0M cause the largest change in these parameters.[82 165] Henson & Walker[82 83] have identified the fractions involved as f2b, f2a2 and f3 - if one fraction is involved it is f2a2.[84] However, other workers have identified the major change in the circular dichroism with removal of f2a1.[223] Bonner[198] has insisted that all the changes are related merely to the amount, not the nature, of the histone removed.

3. The histones and gene control

It was Stedman and Stedman[202 203] who first suggested that histones might act to repress the transcription of RNA from the DNA template (its template activity). However, subsequent research has shown that histones have none of the properties necessary for specific repressors - e.g. tissue and species specificity and specificity of interaction with DNA. A good case can still be made out for histones as coarse controllers of DNA transcription.

Coarse control has traditionally been associated with certain structural elements in the nucleus. All the genetic material becomes inactive at metaphase when it is condensed into mitotic chromosomes. Thus the permanently repressed DNA became identified with the heterochromatin - that portion of the chromatin that remained condensed throughout the cell cycle. The active part of the genome belonged to the euchromatin or diffuse chromatin, which became decondensed during interphase.[28] Chromatin condensation also took place in those tissues - e.g. nucleated erythrocytes and spermatozoa - where the DNA became inactivated during differentiation. Indeed, most of the RNA-synthetic activity of a cell is confined to the diffuse chromatin.[117] Heterochromatin is not present in the embryonic nucleus but arises during cytodifferentiation.[28] The extent of heterochromatization depends on the activity of a cell in transcription.[201]

It is generally agreed that chromatin is a less efficient template for RNA polymerases, both homologous and heterologous, than is DNA. Whether this is due to

inhibition of binding or of transcription is still a matter of controversy.[32,104,127,217] The removal of histone restores template activity.[2,90] Artificial DNA-histone complexes also exhibit low template activity.[8,85] Some workers have suggested that these results reflect differences in solubility of the different nucleohistones in the assay mixture.[86,87] However, recent results have shown a similar pattern when measuring the activity of chromatin in the nucleus with endogenous RNA polymerase.[133,192] Evidence here suggests that removal of lysine-rich histones may be more effective in restoring template activity than the removal of arginine-rich. Moreover, it has been known for some time that removal of lysine-rich histones from both interphase and metaphase nuclei causes the condensed chromatin to become diffuse.[118,132] This result is in parallel with the effect that removal of f1 has on the solubility of nucleohistone[68,143] - it will no longer precipitate at physiological salt concentrations. This further exemplifies the correlation between condensation of chromatin and its template activity.

In species where the erythrocyte nucleus is inactivated during development, the tissue-specific histone f2c usurps the structural functions of f1[27,124] without actually replacing it.[14,135] In this situation where very efficient repression is required, a histone fraction better able to bring about condensation is perhaps needed.

The simple hypothesis that it is the binding of histone to DNA itself that causes the decrease in template activity has often been proposed. This is in contrast to the hypothesis, exemplified by the condensation-activity

correlation, that inhibition is due to some structural change brought about by the binding of histone. It has been supposed that a reduction in template activity is due to a physical blocking of binding sites by histone molecules.[20]

Clark and Felsenfeld[38] claimed to show that this was not so and that >50% of DNA in chromatin was free of protein. Their evidence has now been extensively rebutted.[31 98 131] The binding of quantities of histones does not necessarily prevent the binding of other probes, any more than it prevents transcription. A reporter molecule which binds in the minor groove of DNA molecules, binds to the same extent to DNA and to chromatin,[193] nor does its binding prevent transcription.[57] Both histone and RNA polymerase seem to bind in the major groove; other dye binding evidence would seem to confirm this.[3 182] In particular, ethidium bromide binding appears to correlate well with template activity.[182] The greatest increase in ethidium bromide binding occurs when fraction f1 is removed from the complex.[3]

During the process of activation of some types of completely repressed cells, large increases in the dye binding capacity of the chromatin have been reported.[43 105] These results concern acridine orange which binds in a similar fashion to ethidium bromide. Increase in dye binding is accompanied by a decrease in thermal stability of the chromatin.[168 169] These changes, although a necessary precondition for activation, are in themselves not sufficient to ensure RNA synthesis.[5]

Acridine orange binding is localized in the euchromatin of whole nuclei.[62] However, the genetic activity

of euchromatin is not due to any absence of histones. Euchromatin and heterochromatin fractions extracted from nuclei displayed no difference in histone content.[41] However, there are differences in the proportions of RNA and acidic nuclear proteins - both are more abundant in diffuse chromatin.[60]

Modification of histone binding to DNA by binding of polyanions such as these is a possible mechanism for the control of genetic activity. That the non-histone proteins could act in this way was proposed by Paul and Gilmour.[69 156] Non-histone proteins were required for the reconstitution of chromatin with the correct template specificity. Reports of the tissue- and species-specificity of non-histone proteins[9 226] encouraged the hypothesis that they act as specific regulators. However it has now been concluded that present methods are not sensitive enough to detect such proteins if they were to exist.[126]

Huang and Bonner extracted a natural complex of protein and a small RNA molecule from chromatin.[91] This 'chromosomal RNA' is covalently bound to non-histone protein and is essential for the reconstitution of template specificity (for a review see ref.[92]). cRNA hybridises mainly to the repetitive sequences in DNA[195] and is thus not likely to play a role in the restriction of specific DNA sequences.

Other methods of modifying the DNA-histone interaction are enzymatic modification of the peptide side-chains and variations of the ionic strength of the nuclear environment.

Acetylation of histones has been implicated in the activation of lymphocytes after phytohaemagglutinin

treatment[1,160] and in the increase of RNA synthesis observed in the regenerating rat liver.[161] Phosphorylation of histones is a widely reported phenomenon. There is however some conflict of evidence. The peak of phosphorylation of f1 has been identified with metaphase[24,109] and with DNA synthesis.[6,76] Louie and Dixon[122] have proposed that the extent of phosphorylation of this fraction modulates the extent of supercoiling of the chromatin fibril. This is in contrast to other histones where phosphorylation may play a role in the correct binding of the histone to DNA.

Changes in ionic strength can have a great effect on the structure of chromatin. They can cause the condensation and decondensation of chromatin in nuclei[118,132,144] and affect the banding patterns found in salivary gland nuclei.[174] Large increases in ionic strength completely dissociate the histone from chromatin and yield major changes in its structure.[82,83] The concentration of free ions in the cell nucleus is similar to that in the cytoplasm although there are large reserves of bound ions.[191] It is conceivable that nuclear ionic strength could reach a sufficient level to affect the structure of the chromatin. Indeed changes in the ionic concentrations of the nuclei of newt oocytes have been observed at certain stages of development.[138] Variations in the plasma membrane potential of insect salivary gland cells have been reported during ecdysone-induced puffing.[108]

The decondensation of chromatin correlates well with increase in template activity and both can be brought about *in vivo* by removal of histone. Euchromatin and

heterochromatin contain the same amount of histone. However, through the intermediacy of other components the histone could be differently bound to the two fractions such that at the same ionic strength they have different degrees of condensation. Changes in condensation could then be brought about differentially by overall changes in ionic strength.

4. The aims of the thesis

Histones play a vital role in maintaining the integrity of the solution structure of nucleohistone.[82,83] This structure can be removed with the histones in high salt concentrations.

In the first part of this thesis, we are concerned to further elucidate this dissociation of histone from DNH. Thermodynamic investigation of the dissociation and various other studies are undertaken in the hope of obtaining further information on the nature and perhaps mechanism of the dissociation.

The condensation of chromatin and its analogue precipitation are also brought about by changes in the ionic strength of the medium. These processes correlate with the template activity of the DNA.

The second part of the thesis investigates the precipitation of DNH. The physicochemical changes that take place during precipitation are studied. The relationship of these changes to those observed in the electron microscope is discussed. The involvement of histone and metal ions in the mechanism of precipitation is explored.

The thesis attempts an investigation of the interactions between nucleohistone and neutral salts. The observed changes are related to the structure and function of nucleohistone in the cell nucleus.

2

METHODS

1. Preparation of material

a. Deoxyribonucleohistone

Deoxyribonucleohistone (DNH) was prepared substantially by the method of Zubay and Doty.[233] Thymus glands were removed from freshly-killed calves and frozen in solid carbon dioxide. They were stored at -20°C until required.

Routinely 20gm of thymus was suspended in 200ml of buffer (24mM sodium EDTA, 75 mM sodium chloride corrected to pH 8) at 4°C and the precipitate collected. After extensive washing with the same buffer this precipitate was dispersed in a litre of distilled water and then stirred for one hour at room temperature without frothing. It was then dialysed against three changes of 10 volumes each of 0.7mM sodium phosphate buffer pH 6.8, at 4°C for 72h. This gel was then stirred again under similar conditions but overnight, during which period the viscosity was seen to drop. Finally, the solution was clarified by centrifugation at 78000g for 30 min (Spinco Model L, 30 rotor, 30000 rpm). The clear gel-free supernatant was collected. This material is referred to as native DNH.

It was usual to discard, after clarification, no more than a small pellet of particulate matter. However, on some occasions, the stirring was not sufficient to break down the gel and a substantial gel fraction was obtained from the bottom of the centrifuge tube. Such gels could be extracted by extensive stirring with several volumes of buffer. DNH preparations obtained in this way were found not to differ in their physical

properties from the more usually prepared samples.

Modifications in the above procedure were made from time to time. During the investigations into the effect of small concentrations of divalent metal ions on the structure of DNH (Chapter 7), the following variations were made:

1. Preparations were performed with a solution of 0.123M sodium chloride, corrected to pH 8 by the addition of M sodium hydroxide solution, replacing the original buffer. This eliminated the leaching out of divalent metal ions by the EDTA, which was originally included to minimise the action of endogenous nucleases.

2. Occasionally, in preparations of this sort, 5mM sodium bisulphite was included to prevent increased proteolysis.[150] In this case, the washing solution was 0.118M sodium chloride, 5mM sodium bisulphite, corrected to pH 8.

3. One preparation was performed as in variation 1 but with the addition of 20μM magnesium chloride to all solutions. This preparation formed a very heavy gel on centrifugation which could only be dispersed by brief but rigorous treatment in the Waring blendor.

These preparations will be referred to in the following chapters as DNH-Mg 1, 2 and 3 respectively.

DNH preparations were routinely of concentrations in the range 500 - 700μg/ml. They were stored at 4°C where they remained stable for 4 - 6 weeks, eventually being degraded by endogenous proteases. This is a considerably

longer lifetime than that reported by Garrett[66] but ageing samples of our preparations consistently retain their nativity, by all criteria, for this length of time.

All experiments on DNH are performed in 0.7mM sodium phosphate buffer pH 6.8 unless otherwise stated.

b. DNH partially depleted of histone

It has long been known[7] that increasing ionic strength will remove the histones from the DNH complex. This process provides some degree of fractionation by virtue of the fact that the number of salt links involved in binding the histones to the DNA is histone-specific.[137] The original method of producing reproduceably depleted samples for physico-chemical investigation[143] relied on overnight ultracentrifugation to separate the depleted DNH from the dissociated protein. Gel filtration techniques have come increasingly to be preferred.[119,227,82] Such a method using a column of agarose beads was employed in this investigation.

To prepare a depleted sample of DNH, 10ml native DNH was dialysed with stirring, for a period greater than 7h, against 1.5l of an unbuffered solution of sodium chloride of the required ionic strength. 5ml of the dialysate was taken and applied to the top of a 1.5 x 35cm column of Sepharose 4B (Pharmacia) which had been previously equilibrated with the same salt solution. Elution was carried out with this salt solution at a pressure of 50cm of water. 2-3ml fractions were collected and assayed by their adsorption at 260nm and 230nm (for nucleic acid and protein respectively).

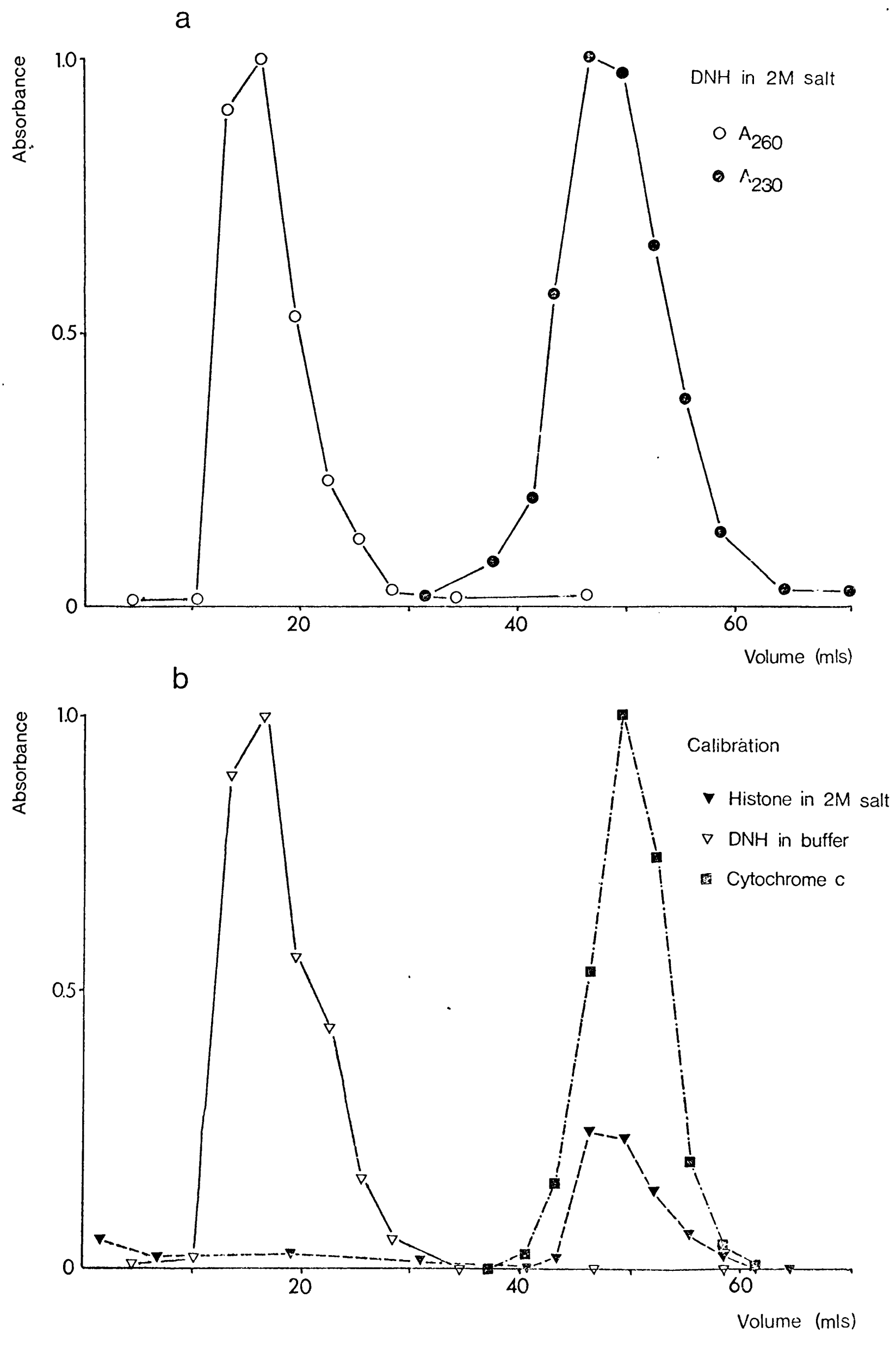
The method was varied only in that when preparing samples depeleted by 2M salt the dialysis step was omitted, 0.584gm of sodium chloride being added to a 5ml aliquot of the native DNH with stirring and this sample applied directly to the column. Columns were washed occasionally with a 0.02% solution of sodium azide to prevent bacterial contamination.

A typical separation of a 2M salt sample is shown in Fig 2.1. The separation is a good one, the depleted DNH being eluted at the exclusion volume and the dissociated protein at the salt peak, with little or no overlap. The depleted DNH is not contaminated by the coelution of any aggregated protein species as has been reported for ultracentrifuge separations[10]. Acid-extracted histone in 2M salt is eluted at the salt peak (Fig.2.1b) suggesting that its molecular weight may not reach those extremities reported under other conditions[46].

Fractions from the exclusion peak were pooled such that >90% of the A_{260} was collected, and this material was dialysed overnight against a large excess of 0.7mM sodium phosphate buffer pH 6.8 prior to analysis. Depleted DNH samples prepared in this manner will be referred to in the text by the molarity of the salt solution used to deplete them; hence $DNH^{0.7}$ is a sample which has been depleted in 0.7M sodium chloride.

The salt-depletion method was used regularly to prepare samples of two well-defined species. $DNH^{0.7}$ is depleted solely of f1 and $DNH^{2.0}$, with an average protein to DNA weight ratio of 0.046, is considered as being pure DNA. Although such a sample contains more

Fig 2.1 Elution profiles from columns of Sepharose 4B



protein than one prepared by organic solvent extraction its CD spectrum is very similar to those published elsewhere[25].

c. Histone protein

Histone was prepared by a modification of the method of Walker.[224]

To two volumes of a solution of DNH was added one volume of N hydrochloric acid; the mixture was vigorously shaken and then kept on ice for one hour, shaking intermittently. The histone was dissociated from the DNA, which precipitated and was removed by centrifugation (International PR-2, 12000 rpm 15 mins). The supernatant was dialysed overnight against a large excess of 1mM hydrochloric acid, then freeze-dried and stored at 4°C.

2. Characterisation of deoxyribonucleohistones

The routine analysis of DNH samples was carried out using methods which investigate three levels of structure of the complex. Chemical and electrophoretic analyses were used to investigate the amount and nature of histone protein in the complex; circular dichroism was used to monitor the state of the secondary structure of both the DNA and the protein and sedimentation velocity measurements gave an indication of the hydrodynamic behaviour of the complex, which is dependent on its shape.

Information was thus obtained on the primary, secondary and tertiary structure of the complex.

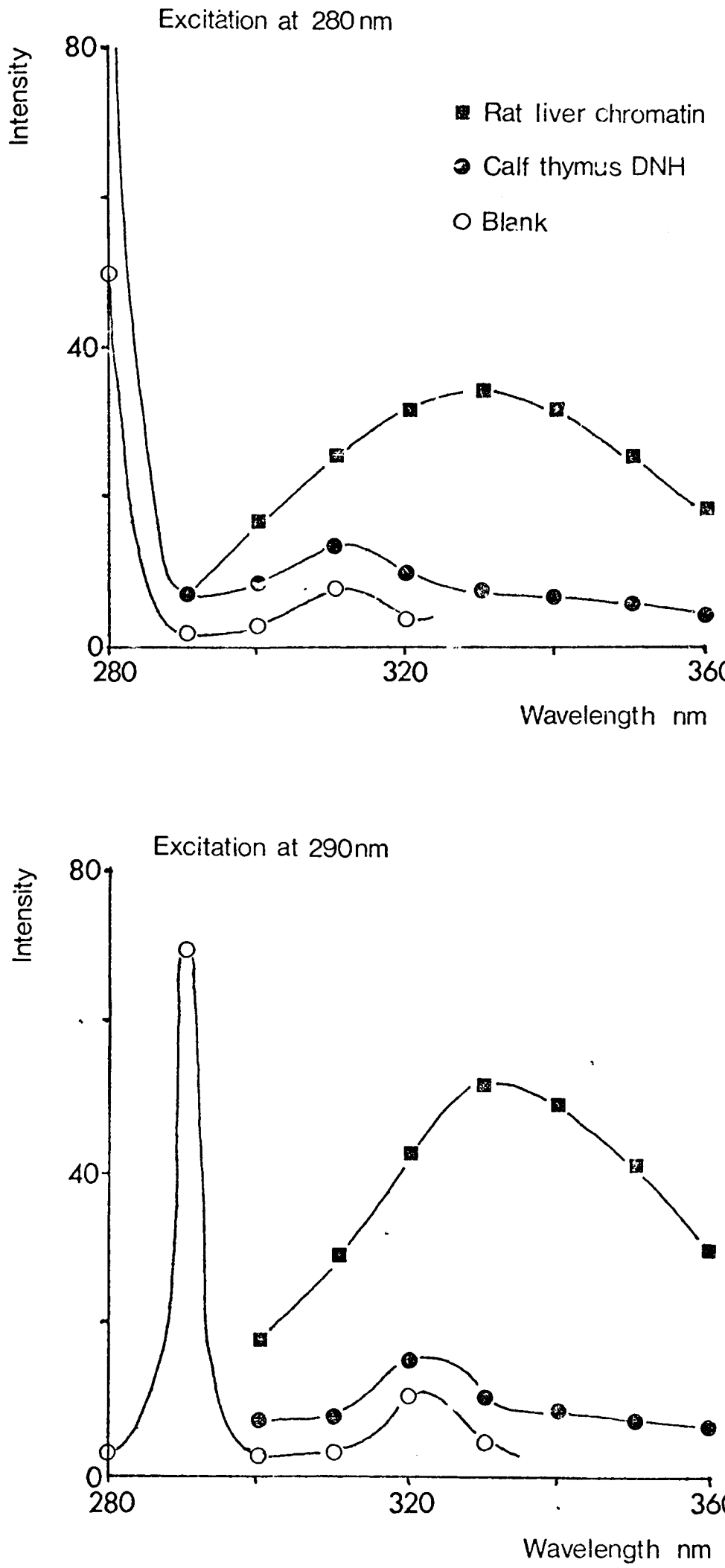
a. Quantitative assays

DNA was assayed in all samples from the absorbance at 260nm using the extinction coefficients 7400 per mole of phosphorus for DNH[115] and all depleted samples with a protein to DNA weight ratio of 0.50 or greater, and 6600 per mole of phosphorus for DNA[158] and all depleted samples with protein to DNA weight ratios of less than 0.50.

Free histone protein in solution was measured from its absorbance at 275nm. Correction was made for scatter by extrapolation of the log-log plot of absorbance against wavelength in the region 340-400nm to lower wavelengths[49]. The extinction coefficient used was $\varepsilon^{1cm}_{0.1\%} = 0.352$[56].

Protein in samples of DNH was measured by a micro-modification of the Lowry technique[123]. The sample, containing 0-30 µg protein, in a volume of 0.5ml was assayed by calibration against a histone standard whose concentration was derived as above. The colour generated was measured

Fig 2.2 Fluorescence emission spectra of chromatin samples



by absorbance at 750nm, a variation of 4% being allowed between duplicates.

Weight ratios of protein to DNA were thus obtained. Estimations on eight separate preparations of native DNH gave an average value of 1.30 ± 0.1.

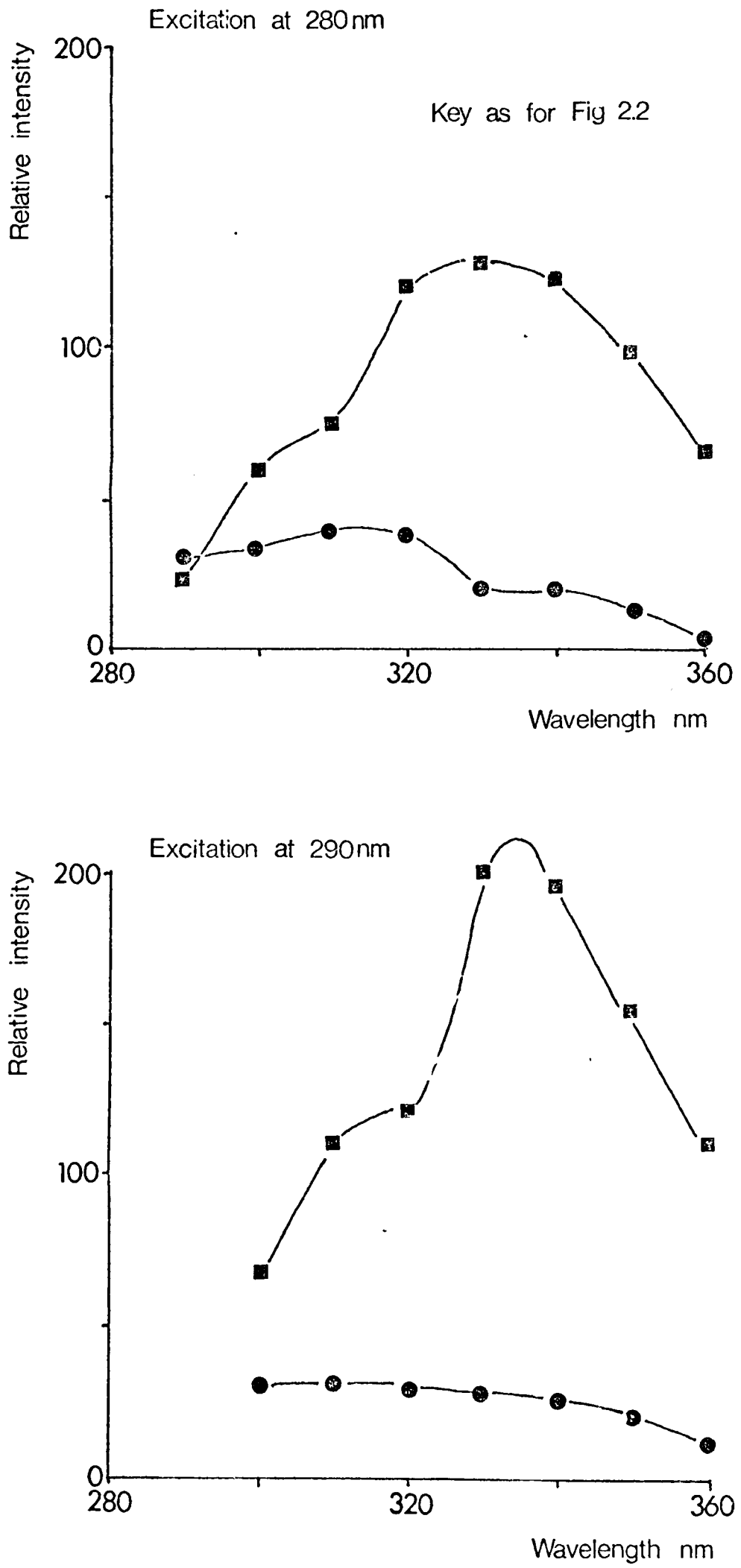
b. Protein fluorescence

The fluorescence spectra of globular proteins derive from the tryptophan and tyrosine chromophores. Tyrosine fluorescence is only visible in the absence of tryptophan since it is severely quenched by the tyrosyl-carboxylate bond. Proteins containing tryptophan have an emission spectrum due to this residue alone, irrespective of the exciting wavelength.[208]

This provides a method of detecting the presence of non-histone protein, which contains tryptophan,[201] in the presence of histone, which does not. Excitation of a sample of histone at 280nm should give an emission spectrum with a peak at about 305nm due to tyrosine.[18] The presence of tryptophan residues will alter this spectrum and will also produce an emission spectrum from an excitation at 290nm, where tyrosine is no longer excited. The presence of nucleic acids should not interfere with such measurements since the bases only fluoresce weakly at these wavelengths.

Fluorescence measurements were obtained on the Perkin-Elmer spectrofluorimeter (by kind permission of Dr. G.K. Radda) using 1cm silica cells. The absorbance of samples at the exciting wavelength was kept at or below 0.1 absorbance units. Emission spectra were recorded for normal DNH, histone protein extracted by

Fig 2.3 Normalised fluorescence emission spectra of chromatin samples



2M salt and rat liver chromatin (kindly supplied by Miss H. Hooper). The spectra, with excitation both at 280nm and 290nm, are shown in Fig 2.2. The rat liver chromatin, which contains non-histone protein (non histone protein to DNA weight ratio approximately one)[89], gives a broad band which peaks at 330-335nm, in response to excitation at both wavelengths. Such a peak is typical of the tryptophan chromophore in proteins.[208] There is no sign of tryptophan fluorescence in the spectrum either of DNH or of the free histone. The DNH spectrum has a very low intensity compared with that of the rat liver chromatin with no peak when excited at 290nm - see the normalised spectra in Fig 2.3.

The small peaks seen even in the blank at 30nm above the exciting wavelength are due to Raman scattering.

The presence of tryptophan - containing proteins in a chromatin complex causes a fluorescence spectrum that is dominated by the tryptophan chromophore. The absence of such an effect in the spectra of free histone or of DNH leads to the conclusion that there can be no measurable level of non-histone protein in DNH.

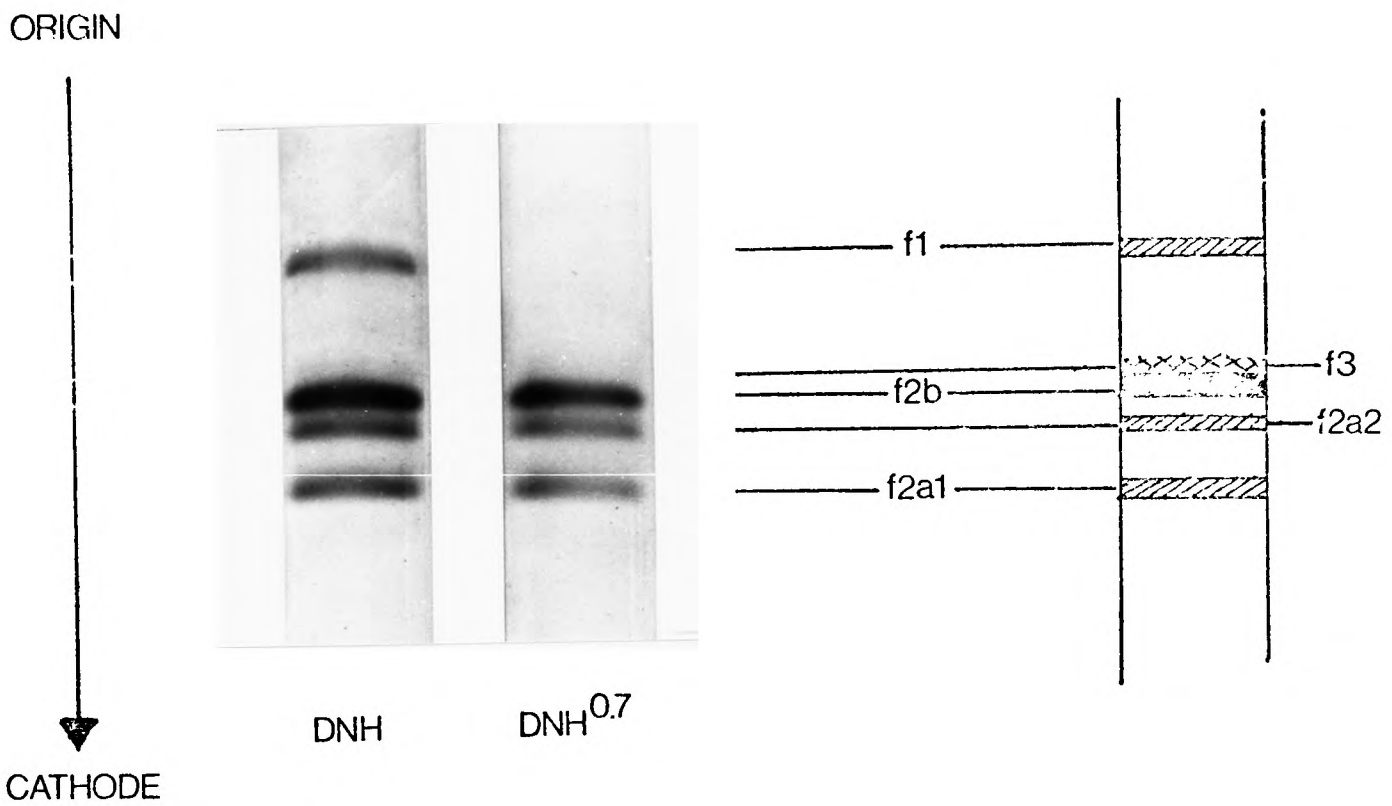
It is of course possible that any tryptophan chromophores in DNH are quenched by interaction inside the complex. However, this would seem unlikely if no such quenching takes place in the rat liver chromatin.

c. Analysis of histone species by polyacrylamide gel electrophoresis.

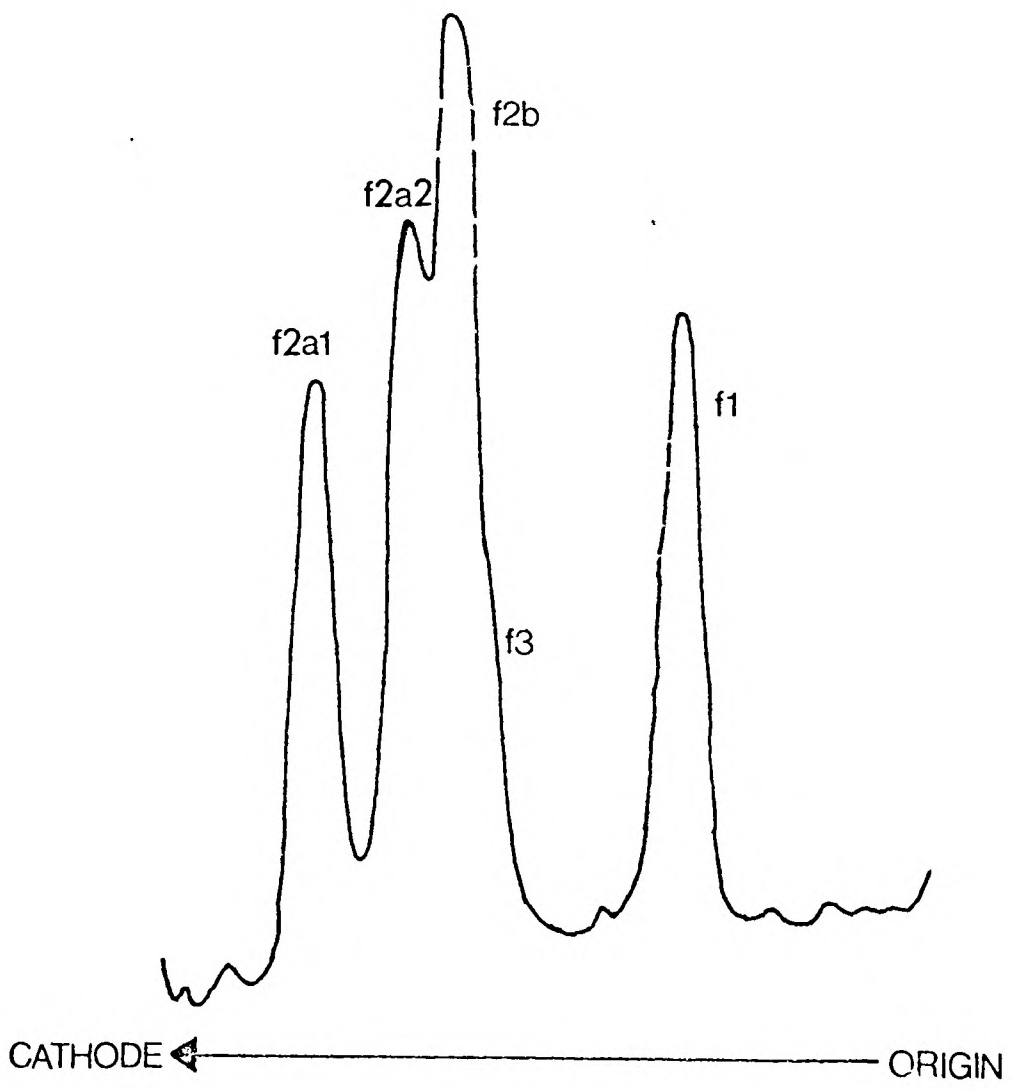
The technique of Panyim and Chalkely was used.[147] This uses a pH 2.7 and 2.5M urea to produce a good separation of the five main histone components.

Fig 2.4 Polyacrylamide gel electrophoresis after Panyim and Chalkley[146]

a



b microdensitometer traces



The gels were 10cm long and 0.6cm in diameter and were polymerised by the use of N,N,N',N'-tetramethylethylene-diamine (TEMED) and ammonium persulphate. Preelectrophoresis removed TEMED $^{2+}$ and $S_2O_8^{2-}$ ions and lowered the ionic strength and pH of the gels. It was continued for about six hours at 2mA/gel, or until a marker of benzene-azo-α-naphthylamine had been electrophoresed through one gel.

Protein samples obtained as reported above were dissolved in 15% sucrose, 0.9M acetic acid and were layered onto the gel surface in a volume not exceeding 100µl. 10µg of protein was sufficient to obtain a good pattern. Electrophoresis, towards the cathode, was routinely carried out at 2mA/gel for 3h. Gels were stained overnight in a 0.1% solution of Coomassie Brilliant Blue R in 7% acetic acid. Destaining was by diffusion into a large volume of 7% acetic acid, with constant stirring.

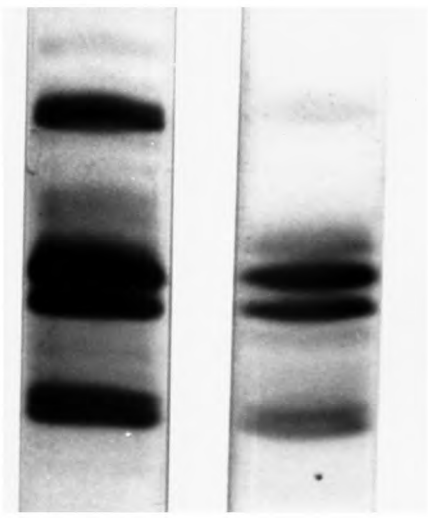
Samples of whole histone run on these gels gave the band pattern shown in Fig 2.4a. The bands have been identified by Panyim and Chalkley[147] and confirmed in this laboratory.

The band patterns on these gels were scanned by a microdensitometer (Joyce-Loebl double beam); a scan of the gel in question can be seen in Fig 2.4b. The bands can be resolved and their relative areas measured electronically by the use of a Dupont curve analyser (by kind permission of Dr. G.D. Meakins). Planimetry has also been used.

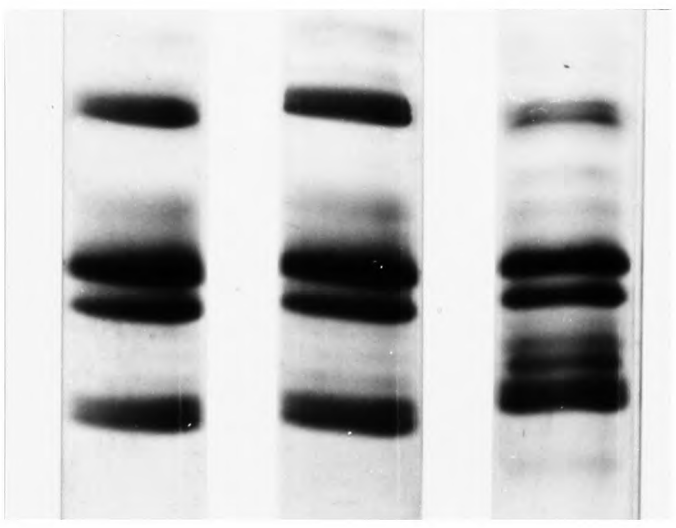
Good optical resolution of all bands except those of f2b and f3 can be obtained. The f3 band is often absent altogether but the large amount of protein in the f2b band

Fig 2.5 Polyacrylamide gel electrophoresis of aged DNH samples

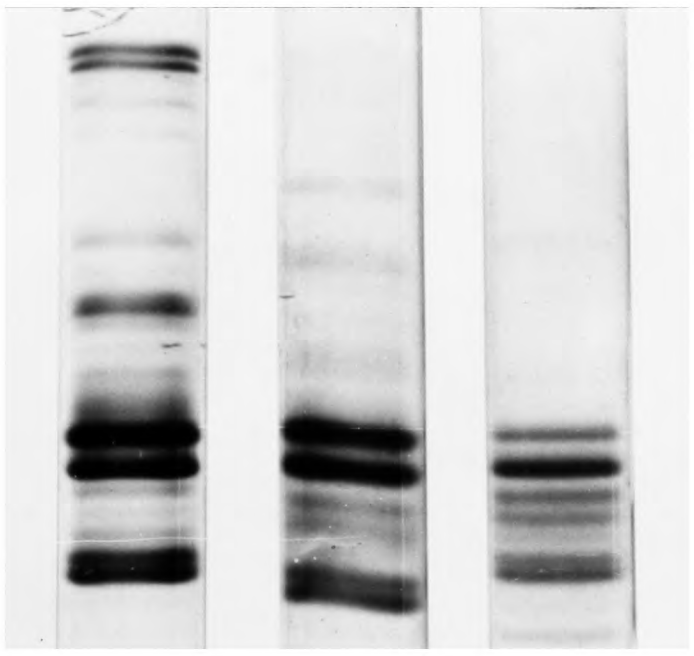
a



b



C



leads one to suspect that these two may well be coelectrophoresing under our conditions. This elusiveness of the f3 histone may also be due to incomplete extraction from the DNH complex or alternatively, to dimer formation through the cysteine residues in f3.[148]

f1 and f2a1 are frequently seen as doublets. Such observations are in keeping with what is known of the structural heterogeneity of these fractions.[148]

Fig 2.4 also contains a gel of a sample of $DNH^{0.7}$ which clearly shows that the only fraction that has been dissociated is f1.

The gel patterns shown in Fig 2.4 are not stable indefinitely. Histone preparations from old samples of DNH show considerable protcolytic degradation of certain histone fractions. The time scale for such degradation varies from preparation to preparation but for normal DNH, the f1 fraction will have been digested after 4-6 weeks.

Fig 2.5 shows the histone electrophoretic patterns given by freshly prepared and old samples of three different preparations. In four weeks the normal DNH preparation (Fig 2.5a) has lost nearly all its f1 protein. Fig 2.5b shows that considerable degradation of a DNH-Mg2 preparation has taken place in 2 weeks. Well defined fragment bands can be seen in the 2 week-old sample.

The DNH-Mg3 preparation (Fig 2.5c) contains an extra slow-moving protein doublet when freshly prepared. This is not an f3-dimer since it is not affected by treatment

with excess β-mercaptoethanol. After the dialysis step of the preparation is complete, this sample is already extensively degraded and only 6 days after preparation has lost all the f1 fraction and considerable amounts of the f3, f2b and f2a1 fractions as well. For this reason, DNH-Mg3 was not used for further experimentation.

It would seem that the omission of sodium EDTA from the washing buffer does indeed increase the activity of endogenous proteases. The addition of magnesium chloride to the preparation buffer even at 20μM level exacerbates this effect.

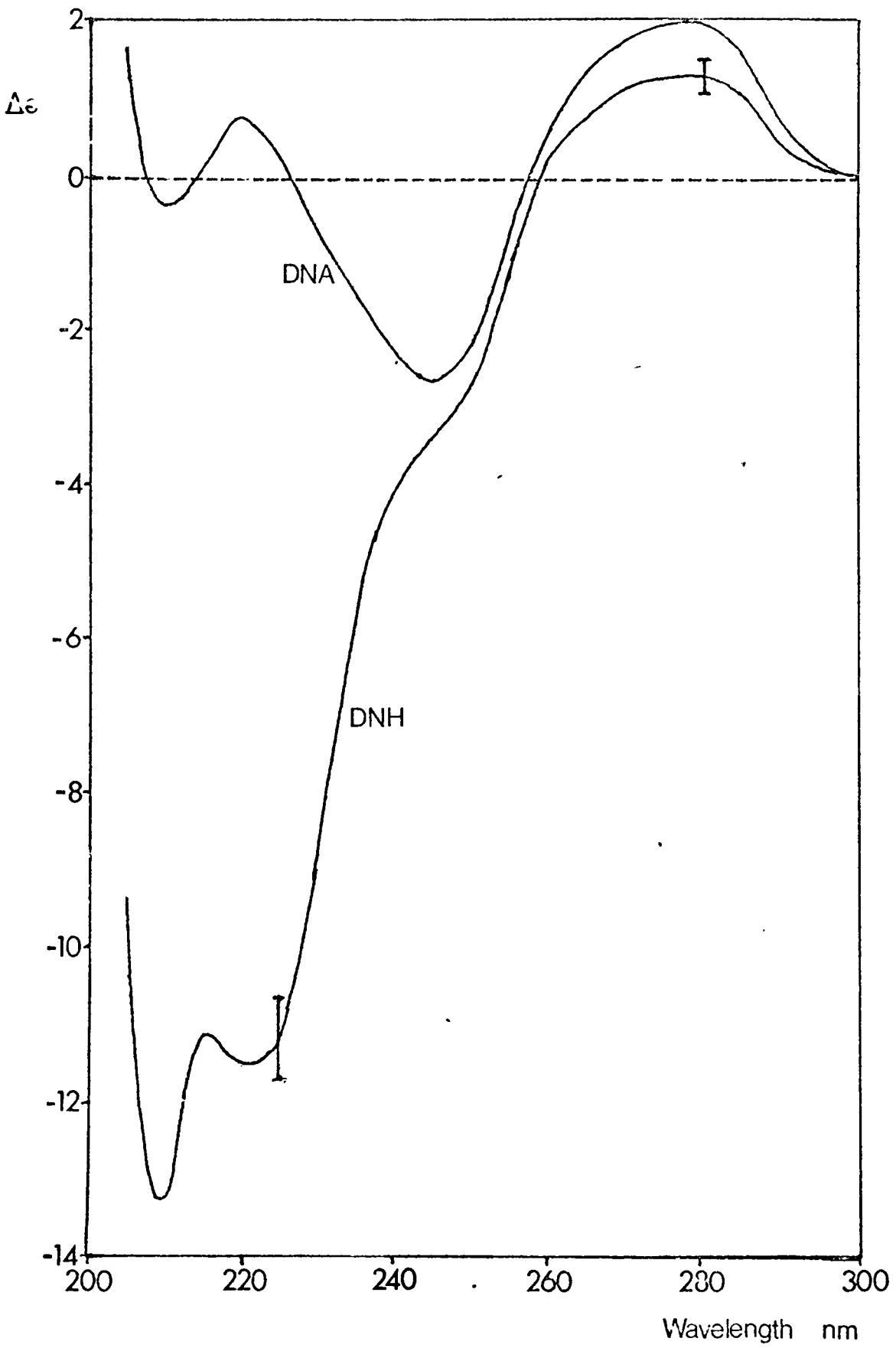
The inclusion of a chelating agent in the buffer by Zubay and Doty is in this respect vindicated.

d. Circular dichroism

When linearly polarised light is passed through an optically-active substance, the right and left circularly polarised components at the wavelengths of the optically active transitions will be absorbed to differing extents. The result is an elliptical polarization of the light at those wavelengths. The circular dichroic bands that arise through this effect are thus a source of information on the optically active transitions. In the case of DNH, this will yield information on those elements of the structure that induce the optical activity.

The asymmetry which in DNA results in optical activity resides in the bases which, although they have a plane of symmetry, are asymmetrically arranged with respect to each other. A spectrum arises which consists of two nearly equal bands of opposite sign with a crossover near the absorption maximum as can be seen in Fig 2.6.

Fig 2.6 Circular dichroic spectra



Tinoco using his theory of optical activity[212], has successfully analysed the CD spectra of nucleic acids in terms of interactions between electronic transitions in the bases.[213] [102] That component of the monomer absorption band which is polarised perpendicularly to the helix axis will give rise to many rotational strengths near the absorption maximum at 260nm. In DNA where the bases are more or less perpendicular to the helix axis[211], interaction of this band in nearest neighbours will cause these contributions to cancel out giving a weak 'conservative' spectrum. Non-conservative effects assume great importance in RNA. Here the tilt of the bases causes interaction between different transitions in the monomers and extensive cancellation does not take place.[102] [214] A much stronger, less symmetrical spectrum results.

Calculations based on this theory give good agreement with the observed spectra.[102] This implies that the CD of nucleic acids is very sensitive to the tilt of the bases. This is not however to rule out the importance of the environment of the bases or of any optical activity that might arise from the n-π^* transition centred about 280nm.[25]

This exciton theory has also been applied to α-helical polypeptides.[210] Other types of protein structure however lack a coherent theoretical basis which would allow similar treatment; and indeed in the case of 'random coil' any coherent structural element at all.[215] In these cases, empirical methods have been developed for analysing spectra using spectra from model compounds[73] or those derived by analysis from the spectra of proteins of known structure.[179] [37] Such attempts may be useful

but can be attacked for ignoring the effect of non-peptide chromophores such as aromatic groups and disulphide bonds.

The CD spectrum of DNH derives essentially from the superposition of peptide elements onto the DNA spectrum. However, as Fig 2.6 shows the maximum around 275nm has a lower magnitude in DNH than in DNA. Detailed analysis of this change is all but impossible. It is undoubtedly derived from the effect of the tertiary structure of DNH on the structure or environment of the DNA bases. It has been variously attributed to the supercoil structure of DNH[81] and to a possible change of the DNA to a mainly C-type structure.[79,101]

All measurements were made on a Roussel-Jouan Dichrographe, Model CD185. This instrument used the Pockels effect to generate circularly polarised light whose angle of polarisation varies continuously through all intermediates between right and left during a half period. After passage through the sample light can be analysed to give $(A_L - A_R)$ directly. Scans were carried out at a speed of 0.125nm/sec with a pen response time of 4 secs. Error in the measurement of a spectrum was taken to be the noise in the signal at any given wavelength.

Ordinary silica spectrophotometer cells of pathlengths 2, 1, 0.5, 0.2 and 0.1cm were used and found to give straight baselines. The optical unit, the lamp housing and the sample chamber were flushed with dry nitrogen for measurements at wavelengths below 220nm. All measurements were recorded at room temperature unless

otherwise stated.

The Dichrographe was calibrated with a standard solution of isoandrosterone in dioxane. All results were calculated in terms of $\Delta\varepsilon=\varepsilon_L-\varepsilon_R$ where ε is the extinction coefficient expressed, for nucleic acids per mole of phosphorus and for a 1cm pathlength.

e. Sedimentation velocity

The structure of chromatin is undoubtedly more compact than that of DNA. Previous hydrodynamic studies[31] have shown DNH to behave as a rigid rod with a compression ratio of about 6 over the DNA contained in it. This agrees very well with the EM data for the A fibril of DuPraw and Bahr[52].

Such a change in shape can be most readily followed by hydrodynamic methods. Sedimentation velocity is the method chosen because of its practical simplicity and speed compared with the other available methods. The sedimentation coefficient, the parameter measured, can be related to the molecular weight, to the partial specific volume and to the frictional coefficient of the molecule by the equation

$$s = \frac{M}{Nf}(1-\bar{v}\rho)$$

However, changes in s are not so easily analysed, especially where, as in histone dissociation, the polyelectrolyte properties of the molecule change as well. For this reason no attempt was made to calculate the $s^o_{20,w}$ since a comparative measure was sufficient.

All experiments were carried out in a Beckmann Spinco Model E analytical ultracentrifuge fitted with UV

absorption optics. The cell used had a standard 4^{o} sector, 12mm pathlength aluminium centrepiece. Runs were performed at $20^{o}C$ and at a speed of 39460 rpm, photographs being taken at 4 min intervals. The photographs were scanned using the Joyce-Loebl microdensitometer.

The sedimentation coefficient was expressed in Svedberg units ($S = 10^{-13}$sec). 0.7mM sodium phosphate was considered to be close enough to standard conditions for a correction to be unnecessary. Nor was a correction made for the concentration dependence of s since at the concentration used for UV optics ($A_{260}<1$ for a 1cm pathlength) this was also considered negligible.

From determination on eleven different preparations, the average sedimentation coefficient was found to be 40.2 ± 9.9.

3. Other methods

a. Viscosity

Another hydrodynamic method which yields considerable information about the dimensions of macromolecules is viscosity. The determination of the variation of relative viscosity with concentration enables the intrinsic viscosity to be determined. The slope of this plot can be related to the ideality of the system under consideration and hence to the extent of macromolecular interaction.

Relative viscosities were measured in a rotating cylinder viscometer similar in design and dimensions to that described by Zimm and Crothers[231]. The apparatus was waterjacketed and kept at a constant temperature of $25^{o}C$.

Measurements were made under conditions where the viscosity was independent of the rate of shear.

The sample to be measured was serially diluted with an appropriate buffer, the relative viscosity and concentration (from A_{260}) being determined at each successive dilution. From this data was derived the intrinsic viscosity of the sample.

The relation of relative viscosity to concentration can be expressed in a number of ways. The most widely used is that due to Huggins[93]:

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c + \ldots$$

where $[\eta]$ is the intrinsic viscosity

η_{sp} is the specific viscosity which is related to η_{rel} the relative viscosity by the formula

$$\eta_{sp} = \eta_{rel} - 1$$

k' is the so-called Huggins constant which, being analogous to a virial coefficient can be held to characterise the ideality of the macromolecule - solvent system. The less ideal the system, the 'worse' the solvent for the polymer the larger will be the value of k'. A large value of k' will thus indicate poor macromolecule-solvent interactions, which indicates a large degree of interactions among the macromolecules themselves.

In situations where tertiary and higher interactions become more important, the Huggins approximation becomes more and more unreliable. For such a case, Mead and Fuoss[129] proposed an alternative form of the relation:

$$\frac{\ln \eta_{rel}}{c} = [\eta] + (k' + \tfrac{1}{2})[\eta]^2 c + \ldots\ldots$$

In this investigation, the Huggins approximation is used routinely since it yields good straight lines in most cases. However, the logarithmic relation has been used in some cases to obtain a meaningful result or to confirm the findings from the Huggins approximation.

In all cases the concentration referred to is not the concentration of DNA in the solution but that of the entire DNH complex. This is contrary to the usage of many workers but would seem to give more relevant results since the intrinsic viscosities obtained are therefore a measure of the volume fraction occupied by the entire macromolecule.

b. Thermal denaturation

The denaturation of the helical structure of DNA by heating is characterised by an increase in A_{260} of approximately 40%, the temperature at which this hyperchromic effect takes place being a measure of the thermodynamic stability of the DNA helix. The binding of histones, and indeed other basic proteins, to DNA increases the thermal stability of the secondary structure. The mechanism of this effect is still not well understood. Loss of protein from the DNH complex causes a gradual displacement of the melting profiles to lower temperature ranges[82]. The absorbance melting profile of DNH is biphasic.

The variation of $\Delta\varepsilon_{275}$ with temperature (circular dichroic melting profile) for DNH is also biphasic[83]. $\Delta\varepsilon_{275}$ increases over the temperature range corresponding to the first step in the absorbance profile and then decreases over a range corresponding to the second

absorbance step. Henson and Walker[83] have interpreted this as being due to the formation of a new complex by migration of histones. $DNH^{1.0}$ no longer shows this biphasic effect but has a circular dichroic melting profile very much like DNA.

Stoppered silica cells containing the sample solutions were placed in an electrically heated and thermally insulated heating block in the light path of the appropriate spectrometer - absorbance was measured in a Pye-Unicam SP500 spectrophotometer at 260nm; circular dichroism in the Roussel-Jouan Dichrographe, $\Delta\varepsilon_{275}$ being interpolated from the trace. The heating block was controlled by a rheostat whose scale had been calibrated against the temperature produced inside the cell.

Readings were taken at approximately 5^{o} intervals and plots of the optical parameter against temperature were constructed. Absorbance melting profiles were characterised by the melting temperature - T_m - which is defined as that temperature at which 50% of the total increase in absorbance has occurred.

c. Controlled proteolysis

The folding of the DNA-histone complex into a compact structure means that the nucleic acid may well be embedded in the middle of a protein matrix[84]. This would cause parts of histone molecules to be removed from the aqueous environment and thus from attack by probes such as enzymes. The proteolytic enzymes, trypsin (EC 3.4.4.4) and chymotrypsin (EC 3.4.4.5) can thus yield information about the gross arrangement of histones in the compact

DNH structure. These enzymes are chosen both for their specificity and for their differential effect on histones. Trypsin cleaves at the basic aminoacid side chains, which have been implicated in binding to DNA whereas chymotrypsin is specific for the aromatic side chains, which are found in the non-basic central or C-terminal portion of most histone primary structures.[55]

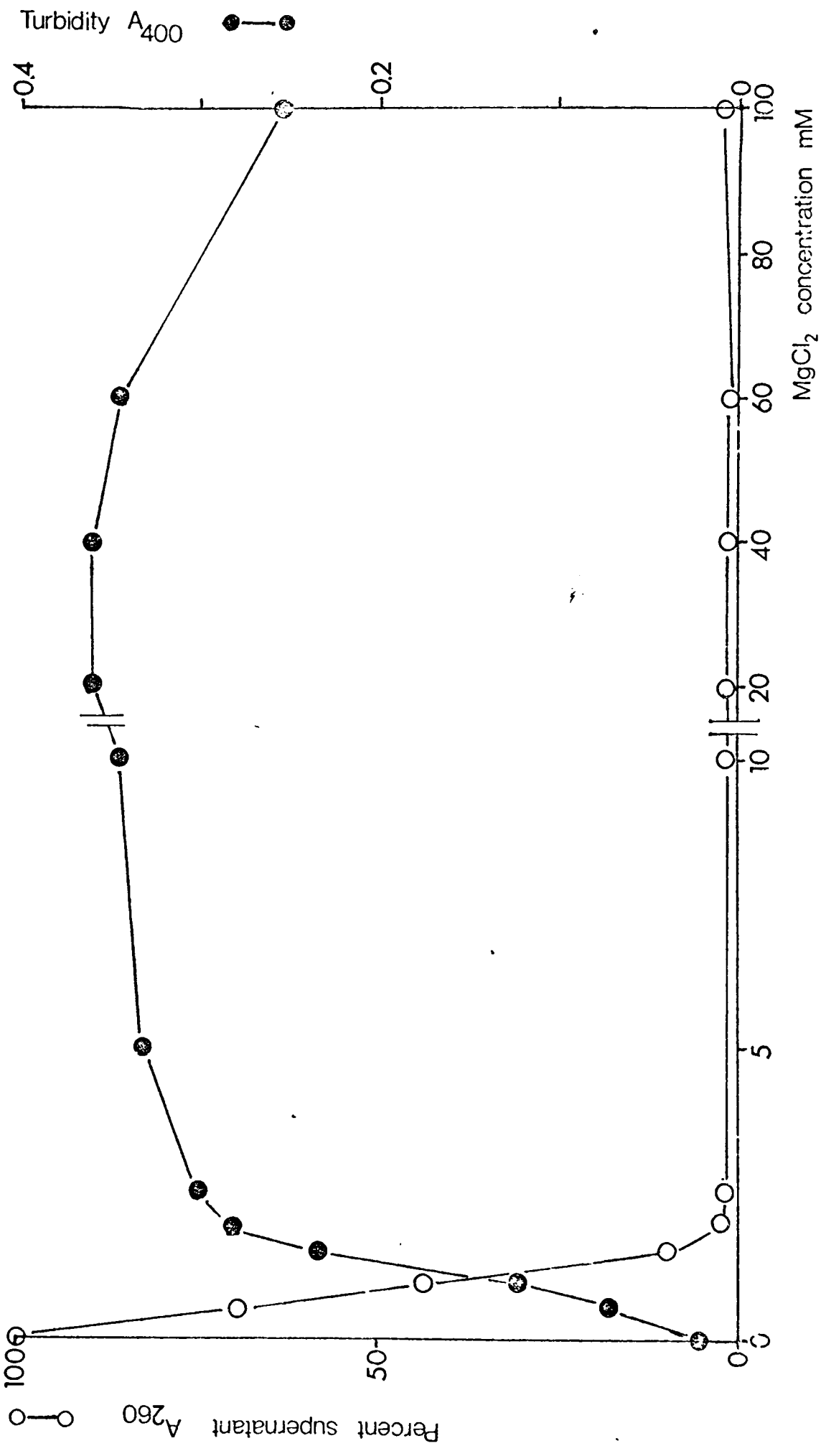
The method used was that of Chatterjee and Walker.[35] Trypsin and chymotrypsin were obtained from Worthington Biochemical Corporation Ltd. and kept at -20°C. Trypsin was treated, prior to use, with L-(1-tosylamino-2-phenylethyl)-chloromethyl ketone to inhibit contaminating chymotryptic activity.[30]

Samples of DNH were incubated at room temperature (18-20°C) with the enzyme at an enzyme:protein ratio of 1:30. Incubations were terminated routinely after three hours, by the addition of a half volume of M HCl. Protein was then prepared from the samples as described above and analysed by polyacrylamide gel electrophoresis. The gel patterns were interpreted with the aid of microdensitometry.

d. The measurement of precipitation curves

The extent of the precipitation of DNH in solutions of low ionic strength was measured in one of two ways. Either the turbidity of the resulting chromatin suspension was measured by absorbance at 400nm or the supernatant A_{260} of the solution after a short low speed centrifugation was used. That these two methods measured the same qualitative effect is shown in Fig 2.7. However, the nephelometric method is not suitable for quantitative measurement of the extent of precipitation. If it is

Fig 2.7 Precipitation of DNH by magnesium ions monitored by two different methods



assumed that the ε_{260} of DNH does not change materially on addition of small amounts of ions, the supernatant A_{260} can be taken as a direct measure of the extent of precipitation.

This assumption is not easily justified. It has long been known that addition of submolar amounts of metal ions to DNA solutions causes a reduction in the ε_{260}.[184 209] A reduction in A_{260} of some 20% is caused by the addition of approximately 100μM Mg^{++} or 100mM Na^{+} to a solution of 100μM in DNA.[184] The effect of magnesium is linked to the tight binding of one magnesium ion to each DNA phosphate. However, Ramm et al.[54] reported that the ε_{260} of DNH remained constant in the range 0-2M sodium chloride. In the absence of definitive data on the effect of magnesium ions on the optical properties of DNH the assumption of a constant ε_{260} over the precipitation range must be considered as an approximation.

Precipitation experiments were routinely carried out by the addition of aliquots of DNH solution to solutions of known ionic composition; after careful mixing the tubes were allowed to stand at room temperature for a few minutes. Addition of DNH to salt solution rather than vice versa ensured that no local areas of high ionic strength arose to cause artefacts. The suspensions were centrifuged at 13500 rpm for 15 minutes in the International PR2 refrigerated centrifuge and the supernatant A_{260} read in a Pye Unicam SP500 spectrophotometer in 1cm silica cells using a buffer blank.

During the course of experimentation, several variations on this procedure were examined. No difference was observed between precipitation curves obtained by

this method and those obtained by consecutive addition of small amounts of precipitant to the same sample of DNH. Neither incubation time greater than 10 minutes nor incubation at 37°C made a difference in precipitation beyond the limits of experimental error.

The reproducibility of the precipitation curves obtained by this technique is ±5%.

3

DISSOCIATION

The study of the physical and biological properties of depleted nucleohistones is a very popular field as reference to recent reviews will show.[55,159,200] Earlier work in this laboratory[81] has attempted to define the dissociation of histone from DNH in terms of physical parameters. This thesis set out initially to complete this definition. Correlation of these parameters with the structure of the system is not possible at a quantitative level. However changes in the parameters allow qualitative statements to be made about changes in the structure.

Much recent work has been published which either duplicates or closely relates to the results reported here. Such findings will be discussed at the appropriate point in the following chapter.

A study of the dissociation of histone from DNH in high salt rapidly leads to the conclusion that a rigidly qualitative definition of the system is not possible. The response of different preparations of DNH to the same concentration of sodium chloride differs. The extent of the difference is shown by the standard deviations marked on the dissociation curve in Fig 3.1. This lack of reproducibility is not due to any great extent to variations in the concentrations of the preparations (see Chapter 4) nor is it due to inherent inaccuracies in the methods used. Several separate treatments of the same preparation of DNH at a given salt concentration yield dissociation results which are identical within the accuracy of the experiments (±5%).

DNH is not a well-defined complex. Variation between preparations is only to be expected when the preparation

Fig 3.1 Dissociation curve of DNH averaged from results on eight different preparations

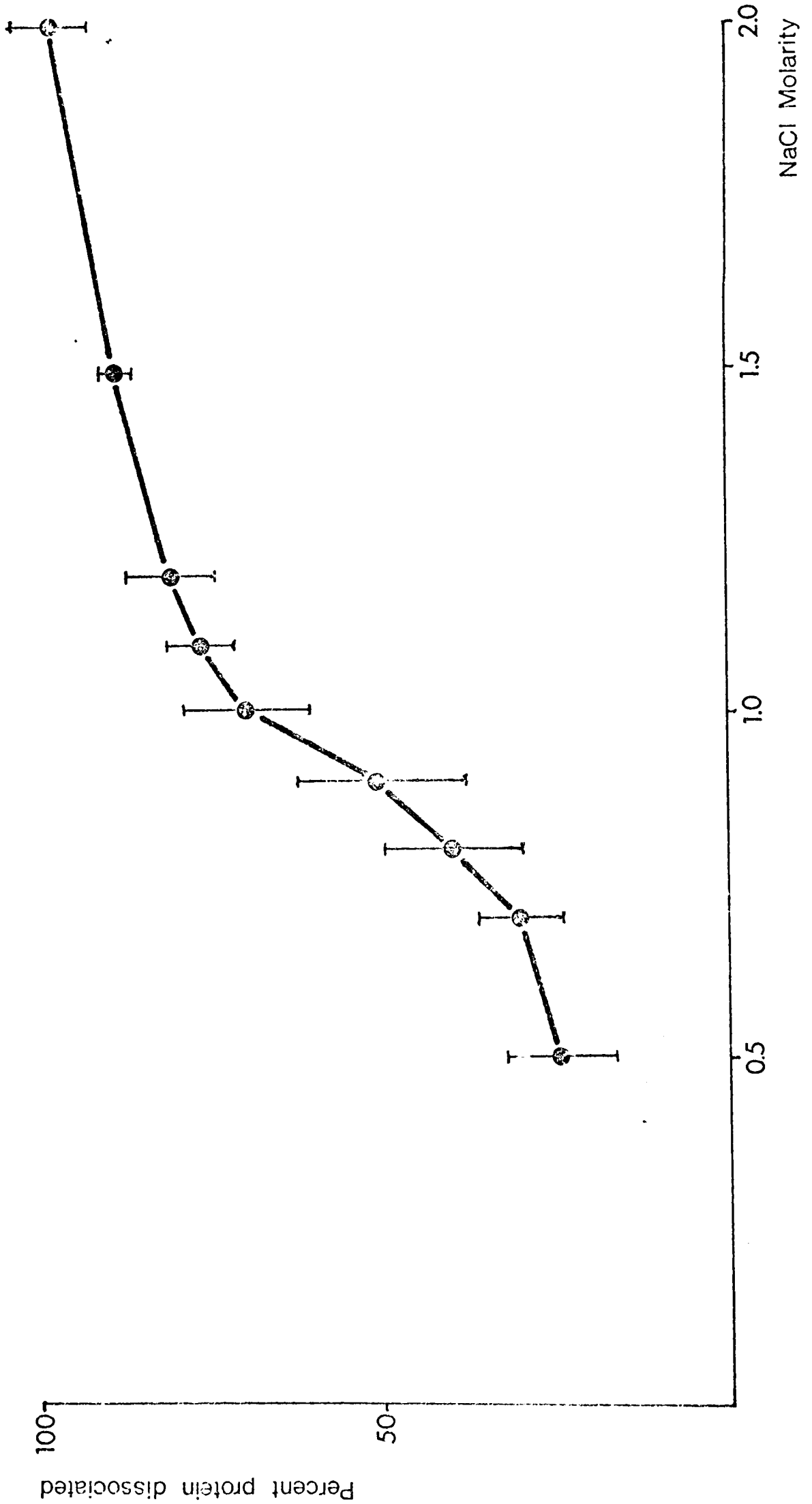
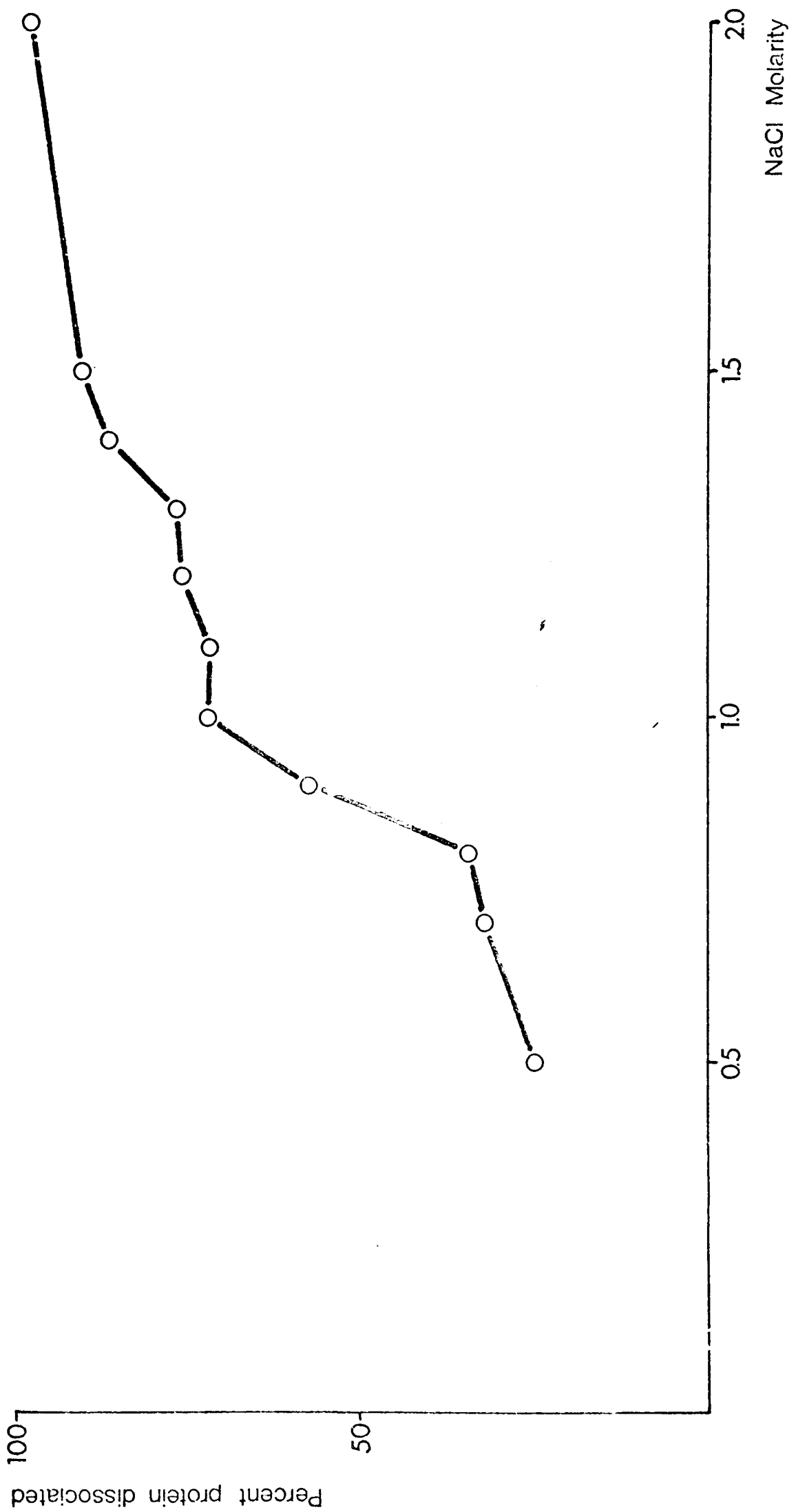


Fig 3.2 Dissociation curve for a 'typical' DNH sample



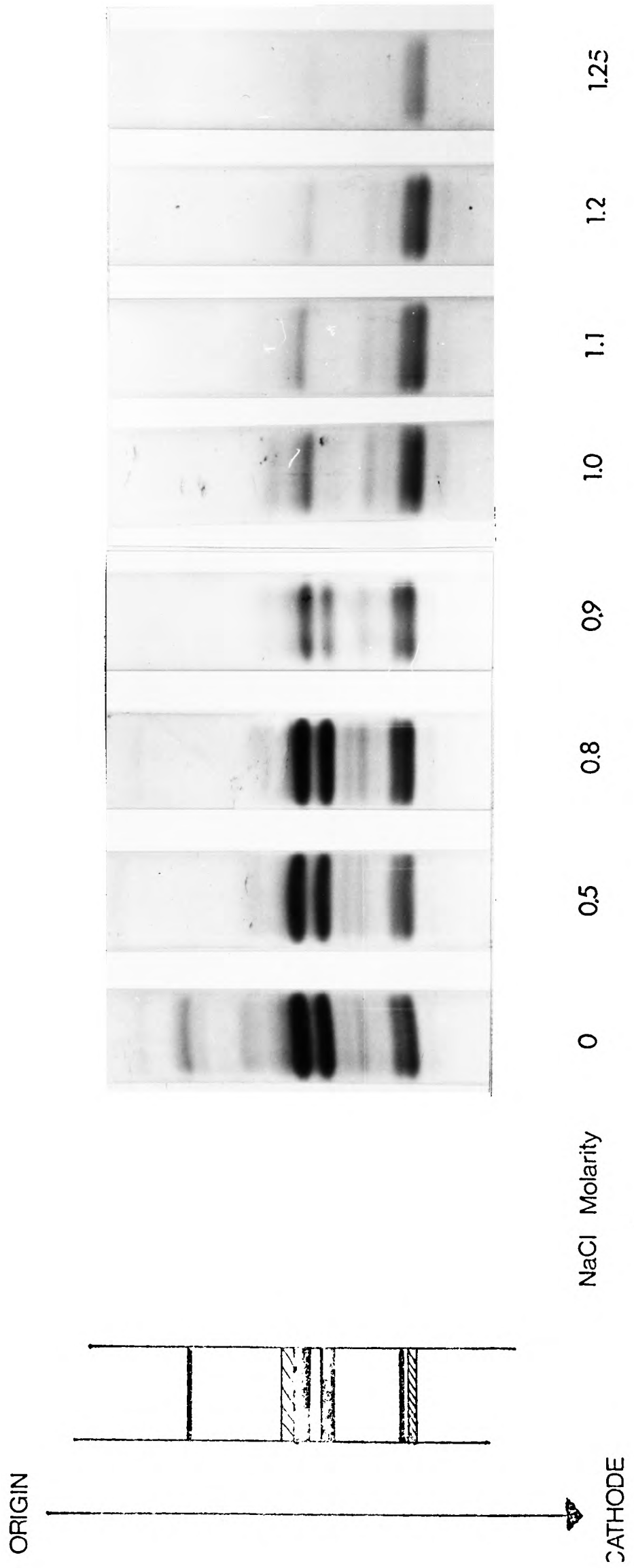
depends on the hardly sophisticated method of precipitation in physiological salt. The exact conditions are those empirically observed to inactivate various endogenous lytic enzymes. That the histone composition is so invariant is strong evidence that the histones are not merely coprecipitants but are bound tightly to DNA in vivo. However, in such a preparation, coprecipitation is bound to occur. The heterogeneity induced by these non-specifically bound components (less than 1-2% by weight) is likely to be the cause of a lack of reproducibility in the behaviour of the complex.

Despite these provisos, many qualitative changes in the physical properties of the complex are highly consistent and it is these which are stressed below. The averaging of data from different preparations often obscures these effects and so the data shown are drawn from single experiments unless otherwise indicated. The features of the data discussed will, however, have been confirmed by the majority of the separate experiments carried out on the eight different preparations involved.

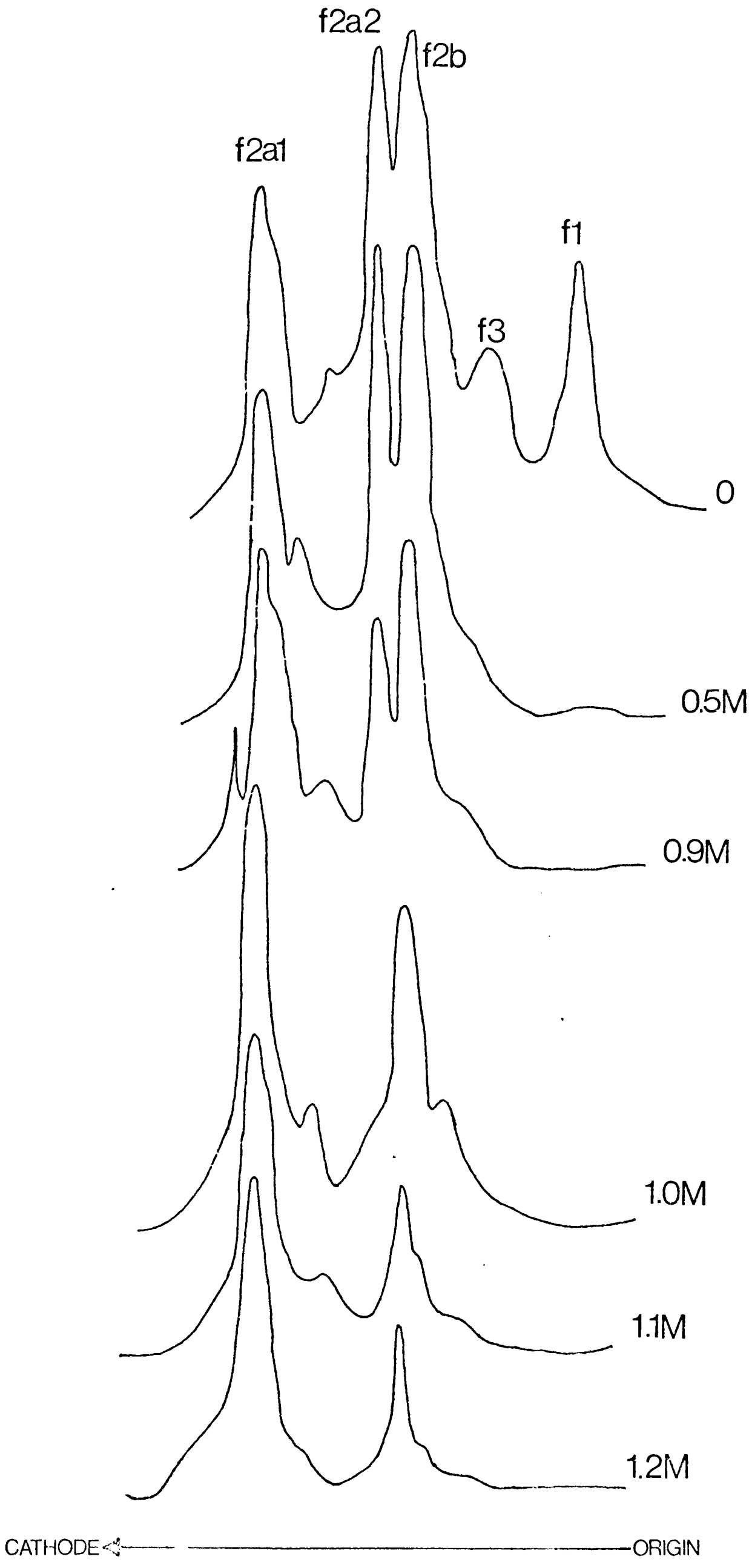
1. Analysis of the protein

Fig 3.1 shows an averaged dissociation curve derived from data from the eight preparations; percentage dissociation was obtained from the protein:DNA ratios. In contrast Fig 3.2 shows the data from a single dissociation experiment which can be considered in some senses as 'typical'. No dissociations were performed at salt concentrations below 0.5M because of the complications introduced in that range by the precipitation of part or all of the nucleohistone.

Fig 3.3 Polyacrylamide gel electrophoresis of depleted nucleohistones



microdensitometer traces



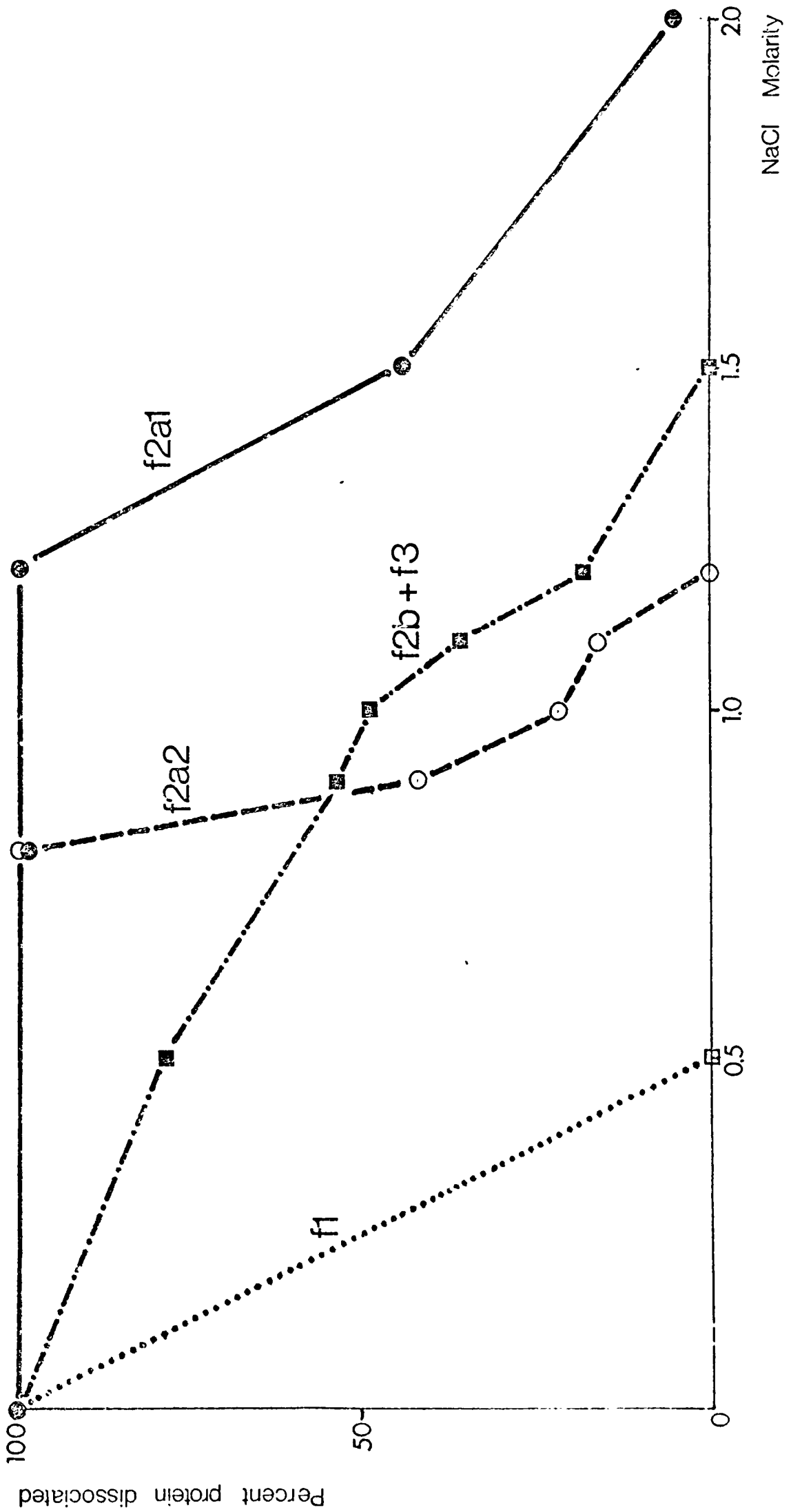
The character of the histone dissociated at various salt concentrations in the experiment of Fig 3.2 is presented in Figs 3.3 and 3.4. Fig 3.3 shows the polyacrylamide gels of several of the depleted nucleohistones together with the microdensitometer traces. Fig 3.4 shows the dissociation curves for the various histone fractions that are derived from these. For the purpose of normalizing the data, fraction f2a1 is assumed to remain bound to DNA at ionic strengths below 1.2M. Analysis of the microdensitometer tracings on this assumption shows that 23.6% of the protein remains bound in 1.2M salt - this agrees well with the value of 24% derived from analysis.

The data for fractions f3 and f2b are not separated because of the inadequate separation of these fractions on the polyacrylamide gel system.

DNH $^{0.5}$ has lost 25% of its protein. Little extra protein is removed on extracting in 0.7M sodium chloride. The protein removed in this range is almost entirely f1 with perhaps some f3. There is certainly no loss of the other fractions and indeed, in some preparations, no f3 is dissociated either. This step is highly repeatable. Any concentration of sodium chloride high enough to dissolve the DNH complex will dissociate the f1 fraction. This effect has been noted by many investigators.[143,227,67] Georgiev and his colleagues[67] maintained that DNH was stable to further dissociation in 0.6M salt. This would seem to be confirmed by the plateau in the dissociation curve observed here.

The majority of the protein is removed from DNH at ionic strengths between 0.7M and 1.1M. In any one preparation this dissociation occurs within a very narrow

Fig 3.4 Dissociation of histone fractions from DNH by salt



salt range. However the position of this range on the ordinate varies considerably between preparations. This variation leads Fig 3.1 to show a very much broader dissociation than takes place. The major histone fraction dissociated in this salt range is f2a2 although a large proportion of f2b+f3 is also removed.

The dissociation curve plateaus again above 1.0M salt. In preparations like that of Fig 3.2 the dissociation curve has a definite plateau followed by a step in which approximately 20% of the protein, identified with f2a1, is removed. This step also varies in position on the ordinate. Other preparations show a gradual loss of protein over the range 1.0M to 2.0M sodium chloride. More recent work[225] on this area of the dissociation has shown the definite step to be a consistent feature.

The dissociation of histones from DNH by increasing ionic strength has always been considered to have little specificity.[143 227 138 139] After the stepwise removal of f1 at relatively low ionic strength, little or no fractionation of the other histones was thought to take place. Early workers did observe that the slightly-lysine rich histones were dissociated before the arginine rich but that the two dissociation ranges overlapped.[143] The validity of these results has been questioned recently since they ignore possible contamination of depleted nucleohistones with histone aggregates.[10]

Such considerations do not affect the results presented above since aggregated histone does not coelute with the depleted complexes on Sepharose chromatography (Fig 2.1).

However an inability to distinguish between f2b and f3 means that a definitive answer cannot be given at this stage. Insofar as a band is seen in the f3 position on polyacrylamide gels as described by Panyim and Chalkley[147] and confirmed in this laboratory[81], this band is removed from depleted nucleohistone by less than 1.0M salt. It is quite possible, though, that this band represents only a subfraction of the f3 molecules.

Table 3.1. Dissociation of histones by salt

Salt range (M sodium chloride)	Fractions dissociated	Characteristics
0.4 - 0.7	f1	discrete
0.7 - 1.2	f2b f2a2 f3(?)	dissociated together over narrow salt range
1.2 - 1.5	f2a1	discrete

Table 3.1 summarises the histone dissociation data. f1 and f2a1 are both removed discretely but at low and high salt respectively. Their dissociations are separated from the major dissociation by two plateau regions. The dissociation of f2a2, f2b and possibly f3 takes place in a narrow salt range at around 0.9M. The exact position of this dissociation on the salt axis varies between preparations.

Recent reports from a Russian group[164 165] support this scheme.

The dissociation of all the protein from a DNH sample in 2M sodium chloride followed by dialysis of the sample back to a lower salt concentration results, after chromatography, in a depleted nucleohistone containing

the same amounts of the different histones as a sample prepared directly by dialysis at the lower salt concentration. Reconstituted nucleohistones prepared in this way have the same intrinsic viscosity as the equivalent depleted DNH.

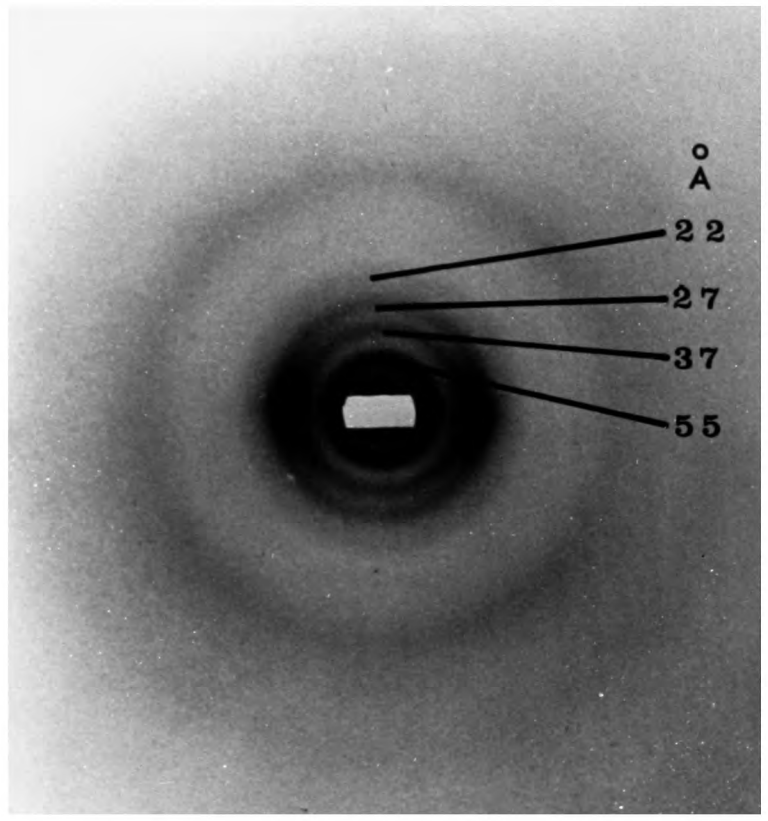
The circular dichroic spectra of reconstituted nucleohistones prepared at 0.6M sodium chloride and above are identical to those of the depleted DNHs. However, reconstitution back to 0.7mM sodium phosphate pH 6.8 does not regularly produce a return to the native DNH CD spectrum. A spectrum much more like $DNH^{0.7}$ is often produced, at least above 260nm.

Reconstitution of the f1 fraction in this salt only system is difficult to bring about. However, the successful reconstitution of all the other fractions provides valuable evidence for the reversibility of the interaction between histone and DNA.

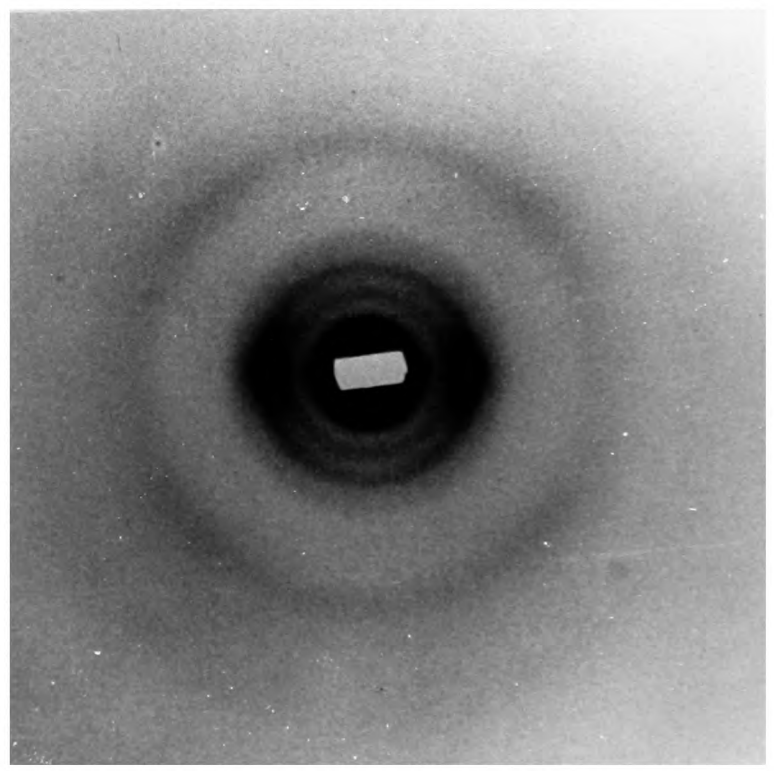
2. X-ray diffraction of depleted DNH

Studies on the X-ray diffraction of depleted DNH were performed in collaboration with Drs. J.F. Pardon and B.M. Richards of Searle Research Laboratories, High Wycombe.

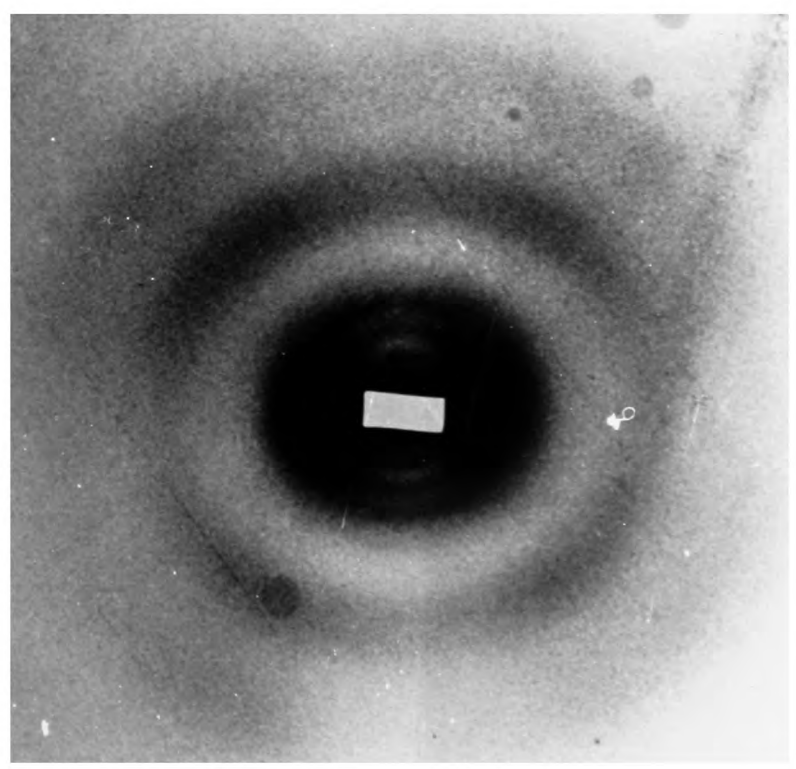
Fig 3.5 X-ray diffraction patterns of depleted nucleohistones



DNH



$DNH^{0.7}$



$DNH^{1.2}$

Measurements were made on two depleted samples of DNH. One was a $DNH^{0.7}$ sample which was lacking only the f1 fraction and the other a $DNH^{1.2}$ sample which contained only f2a1 bound to DNA. The samples were ultracentrifuged overnight and a fibre drawn from the gelled pellet. The fibres were exposed for 20h at 95% relative humidity. The X-ray diffraction patterns obtained are shown in Fig 3.5.

The diffraction pattern for the f1-depleted sample is indistinguishable from that given by native DNH. Both show clearly the 'supercoil rings'. There are two sets of reflections visible in the pattern of $DNH^{1.2}$, the first is similar to that given by native DNH and the second is that expected from DNA in the B-form.

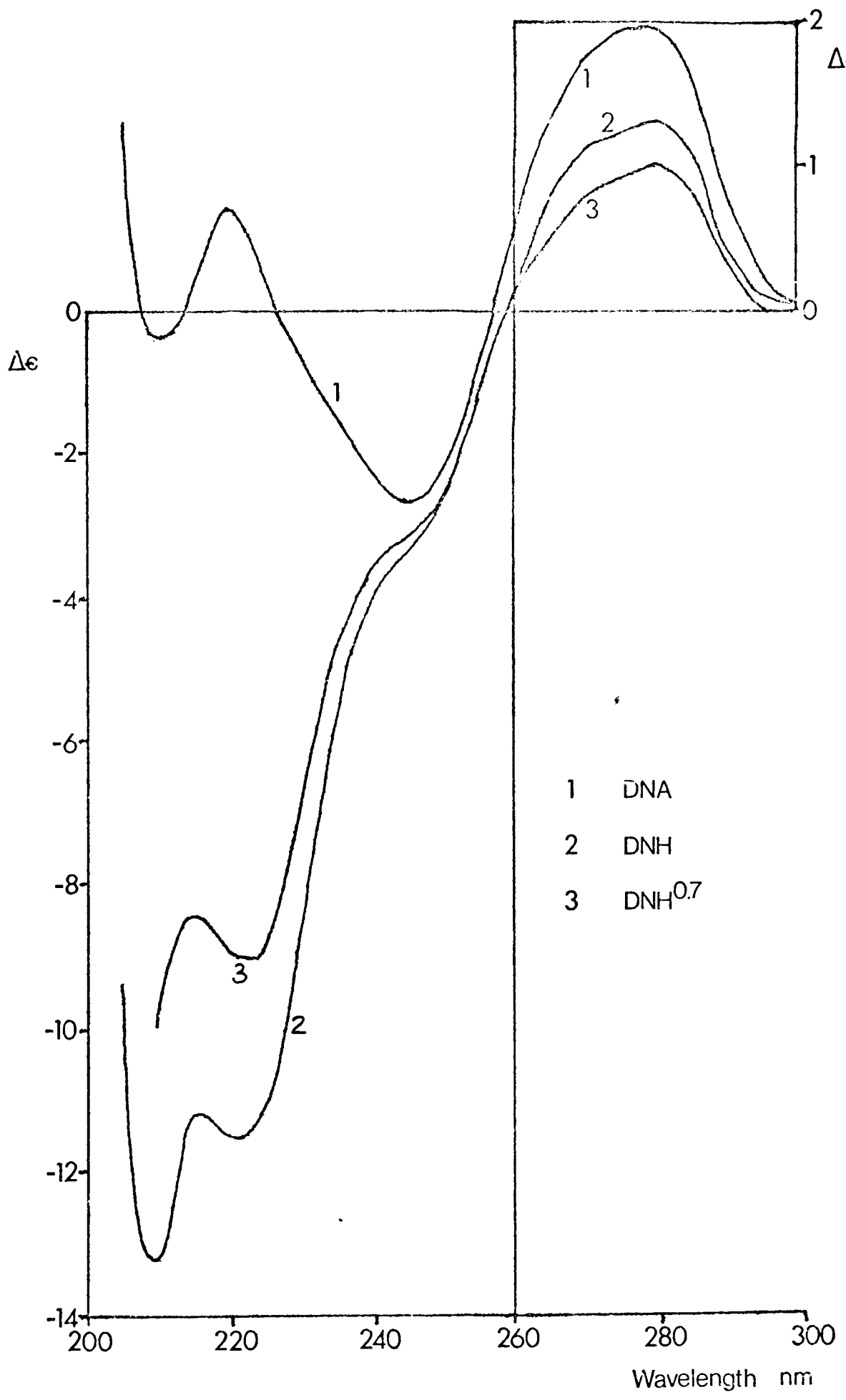
Removal of histone f1 has no effect on the structure of DNH as observed by X-ray diffraction. This result agrees with recently published work from Bradbury's group[22]. Removal of all but histone f2a1 from DNH leaves a mixture of structures - both B-form DNA and 'supercoiled' DNA are present. It can only be inferred that those zones of DNA to which f2a1 molecules are attached retain their DNH-like structure when all the other histone types have been removed. A single histone type can support a DNH-like structure.

3. Circular dichroism of depleted DNH

a. Observed changes in the CD spectrum

The circular dichroic spectrum of whole DNH at low ionic strength (Fig 3.6) is not the sum of the spectra of DNA and whole histone under the same conditions. The positive peak centred at about 277nm is reduced to some 50-60% of its value in the DNA spectrum. There is a

Fig 3.6 Circular dichroic spectra



negative shoulder at 245nm which is also due to DNA and two negative peaks at 222nm and 207nm which are derived from the absorbance bands of the peptide bond. The changes brought about in the CD spectra of DNA and protein on forming DNH are similar to those which take place in high concentrations of salt.[164 165 83]

The CD spectrum of DNA has $\Delta\varepsilon=0$ at 225nm and hence, assuming additivity, the $\Delta\varepsilon_{225}$ value of a DNH spectrum can be used as an index of the protein component. Fig 3.7 shows the variation of this parameter and of $\Delta\varepsilon_{275}$, which is an index of DNA structure, on depletion of DNH with increasing salt concentrations.

The change in the protein dichroism largely follows the change in percentage protein. This is more clearly shown in Fig 3.8 where $\Delta\varepsilon_{275}$ and $\Delta\varepsilon_{225}$ are plotted against the percent protein dissociated for all the depleted samples derived from a single DNH preparation. $\Delta\varepsilon_{225}$ gives a good straight line in this plot thus implying a direct correlation between the two quantities. $\Delta\varepsilon_{225}$ can be used as an empirical measure of the amount of protein in a depleted DNH sample.

$\Delta\varepsilon_{275}$ falls to 75% of its DNH value with the removal of f1 histone at 0-0.5M sodium chloride and thereafter, on further depletion, rises slowly to reach its value in DNA at about $DNH^{1.4}$, where only 10% of the original protein remains on the DNA. The fall in $\Delta\varepsilon_{275}$ on removal of f1 is a highly consistent feature of the data. It has subsequently been reported by Bartley and Chalkley[11] but was not observed in Wagner and Spelsberg's study on rat thymus chromatin.[223]

Fig 3.7 Dissociation curve for DNH as shown by CD parameters

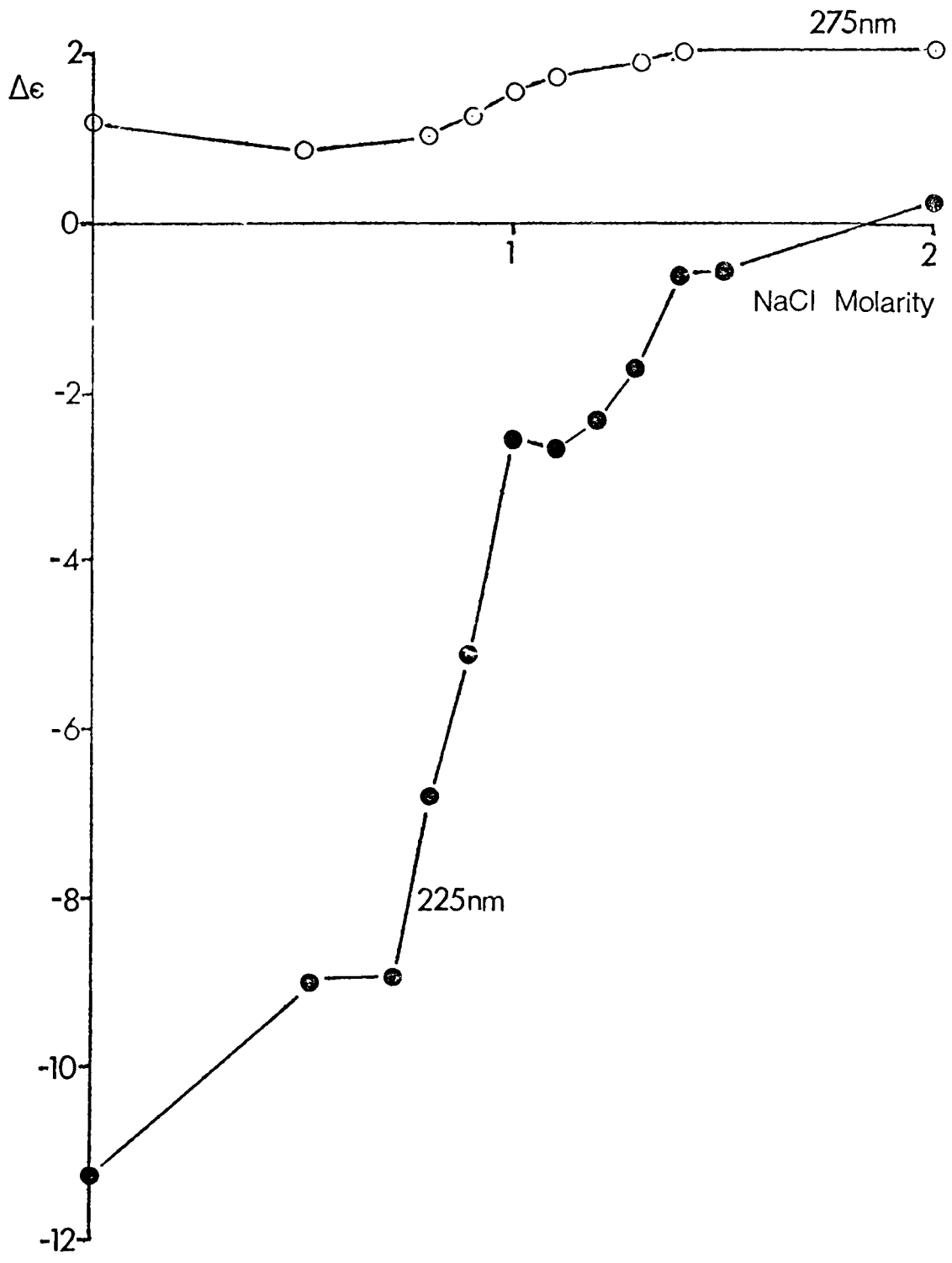
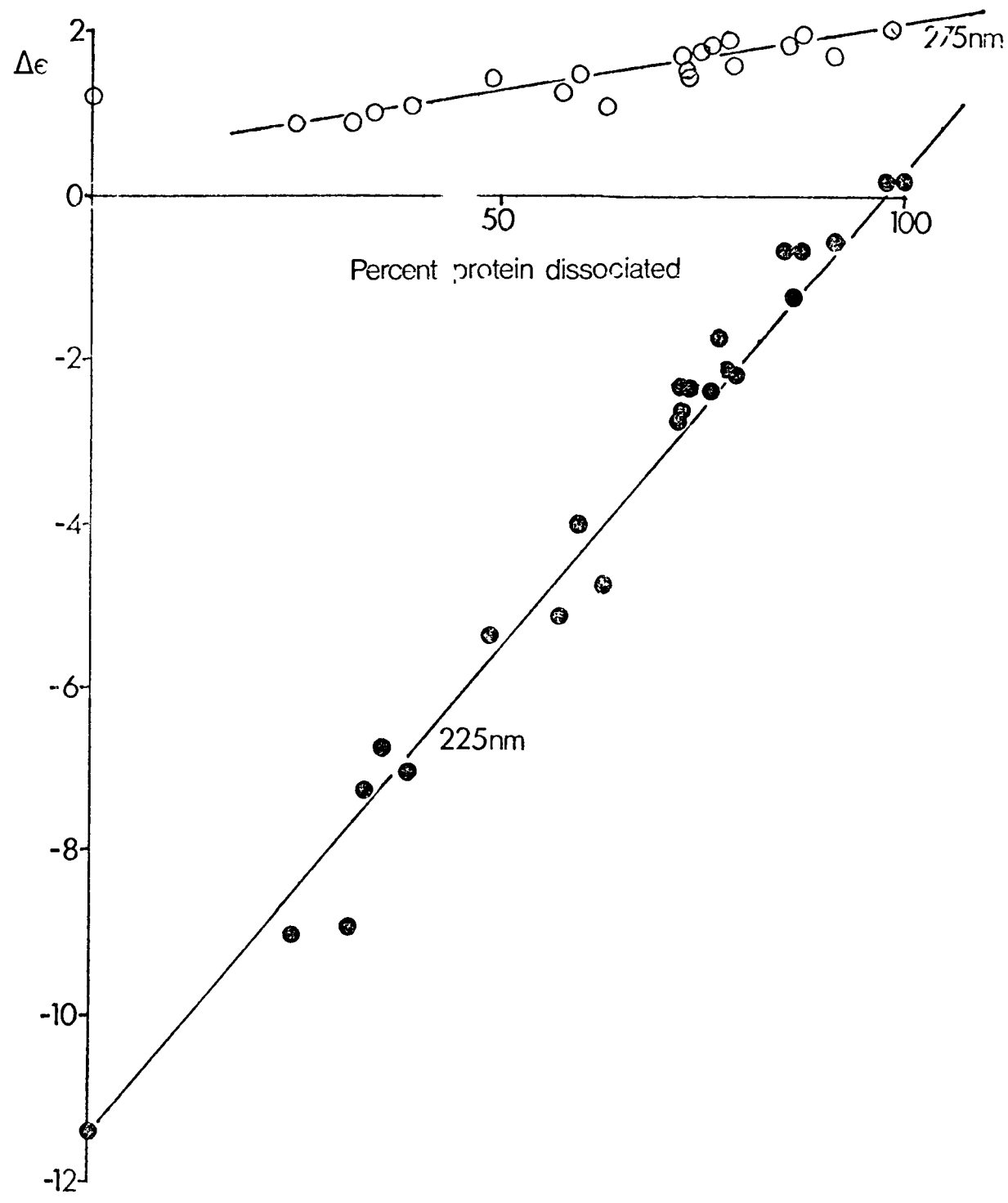


Fig 3.8 Variation of CD of nucleohistones with percentage protein dissociated



After the removal of f1, $\Delta\varepsilon_{275}$ also gives a good linear correlation with the percent protein dissociated (Fig 3.8). The change undergone by the DNA bases which causes the lowering of $\Delta\varepsilon_{275}$ is directly related to the amount of histone bound to the DNA. Binding of f1 histone either reverses this change or causes another change which has an opposite effect on the circular dichroism of the DNA.

It has been suggested that the lowering of $\Delta\varepsilon_{275}$ found in DNH is due to a high proportion of C-type structure in the complex.[79,101] However, the shape and magnitude of the positive CD band of DNA are very sensitive to solvent environment[25] and thus it is possible that it is simply a shielding effect of the bound protein which causes the change in $\Delta\varepsilon_{275}$.

It is attractive to propose that the reduction in $\Delta\varepsilon_{275}$ reflects changes brought about by the supercoiling of the DNA. This correlation is supported by the evidence reported above. Removal of f1 histone has no effect on the supercoil which is still present to a small extent in a depleted sample containing only 20% of its original protein. Whether there is more or less supercoiled structure in this latter sample than in DNH cannot be answered. However, if the CD-supercoil correlation is admissible we would expect there to be less.

The changes in $\Delta\varepsilon_{275}$ need not be related to DNA structure at all. It is possible that the changes in the CD spectrum are due to the induction of optical activity in the tyrosine residues of the histone when it is complexed with the DNA.

Certainly, the ionisation of the tyrosine residues removes these changes[83]. Further reduction in $\Delta\varepsilon_{275}$ is produced by treatment of DNH with formaldehyde, an effect which has been linked with the tyrosine residues[94]. Interactions do occur between tyrosine residues and DNA in model complexes[80 64].

Any of these hypotheses could be supported by a linear correlation between $\Delta\varepsilon_{275}$ and protein content of DNH.

b. Structure of the protein in DNH

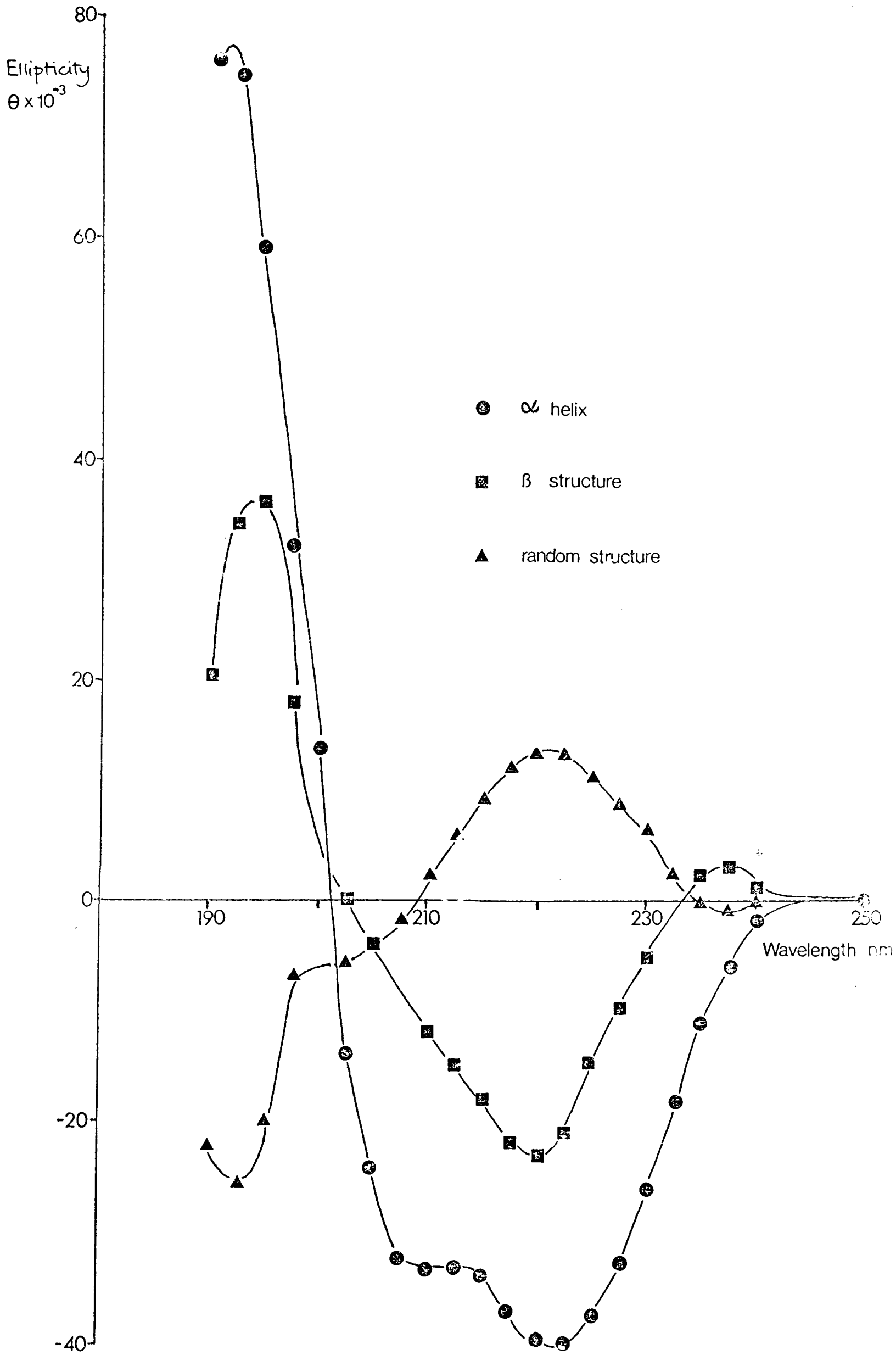
The observation that the $\Delta\varepsilon_{275}$ of depleted DNHs decreases linearly with the protein content implies that the structure of histones bound to DNA does not differ widely. Some variation can be envisaged but the sum of the circular dichroic effects of the components of each histone structure can only vary by a little.

Several attempts have been made to derive the secondary structure of a protein from its optical properties. In such cases, equations such as:

$$x = f_H x_H + f_\beta x_\beta + f_r x_r$$

are commonly used. Here f_H, f_β and f_r are the fractions, respectively, of the helix, β and unordered forms in the protein and the x are parameters representing a protein composed entirely of one form. The x-parameters were originally derived from observations on the model compound poly-L-lysine. Greenfield and Fasman[73] derived the CD parameters in this way and applied them to several proteins with limited success. Poly-L-lysine is unlikely to be a perfect model for globular proteins so this failure was hardly surprising. In particular, the 'random coil'

Fig 3.9 CD spectra of protein structural elements after Saxena and Wetlaufer[179]



structure of polylysine is not necessarily related to the 'unordered' structure of proteins - really a blanket term for all portions not having a recognisable structure.

A more promising approach was applied by Saxena and Wetlaufer[179] who used three proteins of known structure as the models from which to derive the ×-parameters. The CD curve derived for 100% α-helix with this technique differed little from that derived from polylysine. Considerable changes were found in the spectra for the β and unordered forms. These reference spectra are shown in Fig 3.9.

Henson, in her earlier work[81], applied Greenfield and Fasman's reference spectra to the CD of the protein moiety in DNH. Her protein spectra were derived by calculating the difference between successive depleted DNHs. A further attempt is made here to analyse the protein spectra using the spectra in Fig 3.9.

The protein CD spectra were derived by difference between the spectrum of the depleted DNH and a DNA spectrum generated on the Dupont Curve Analyser. The CD spectrum of DNA between 220 and 300nm was fitted with four gaussian curves. The height and width of these curves were then varied to give a good fit of each depleted DNH spectrum between 250 and 300nm. The envelopes of the generated DNA spectra were then traced out and subtracted from the DNH spectrum.

The resulting protein spectra were fitted at 5nm intervals by using a system of triangular coordinates to allow the solution for the three model spectra simultaneously[116]. The fit was not too good below 215nm where the data tailed off, nor above 235nm, an effect also noticed by Fasman[73].

Fig 3.10 Fit between protein CD spectra measured from depleted DNH and those calculated from data of Saxena and Wetlaufer

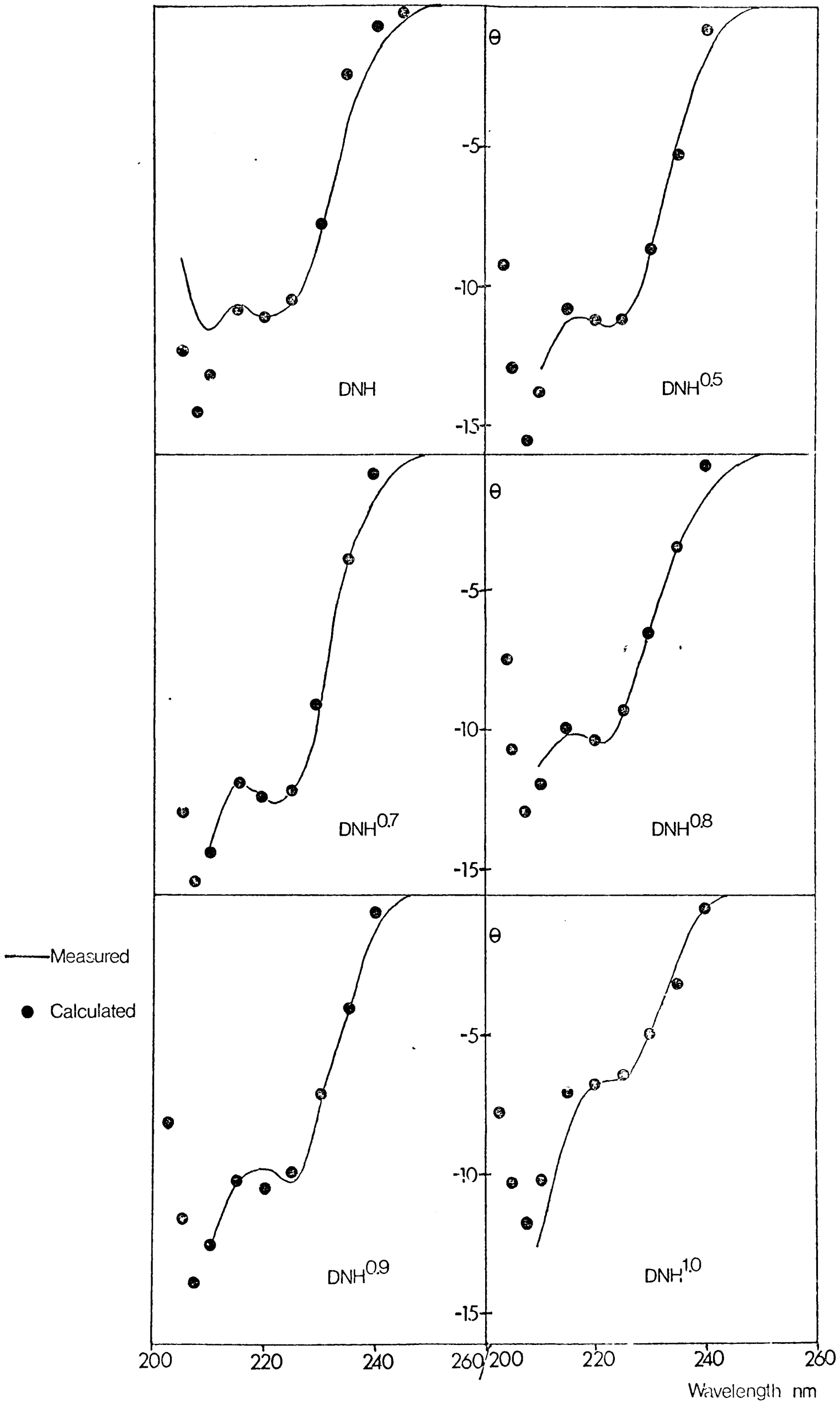
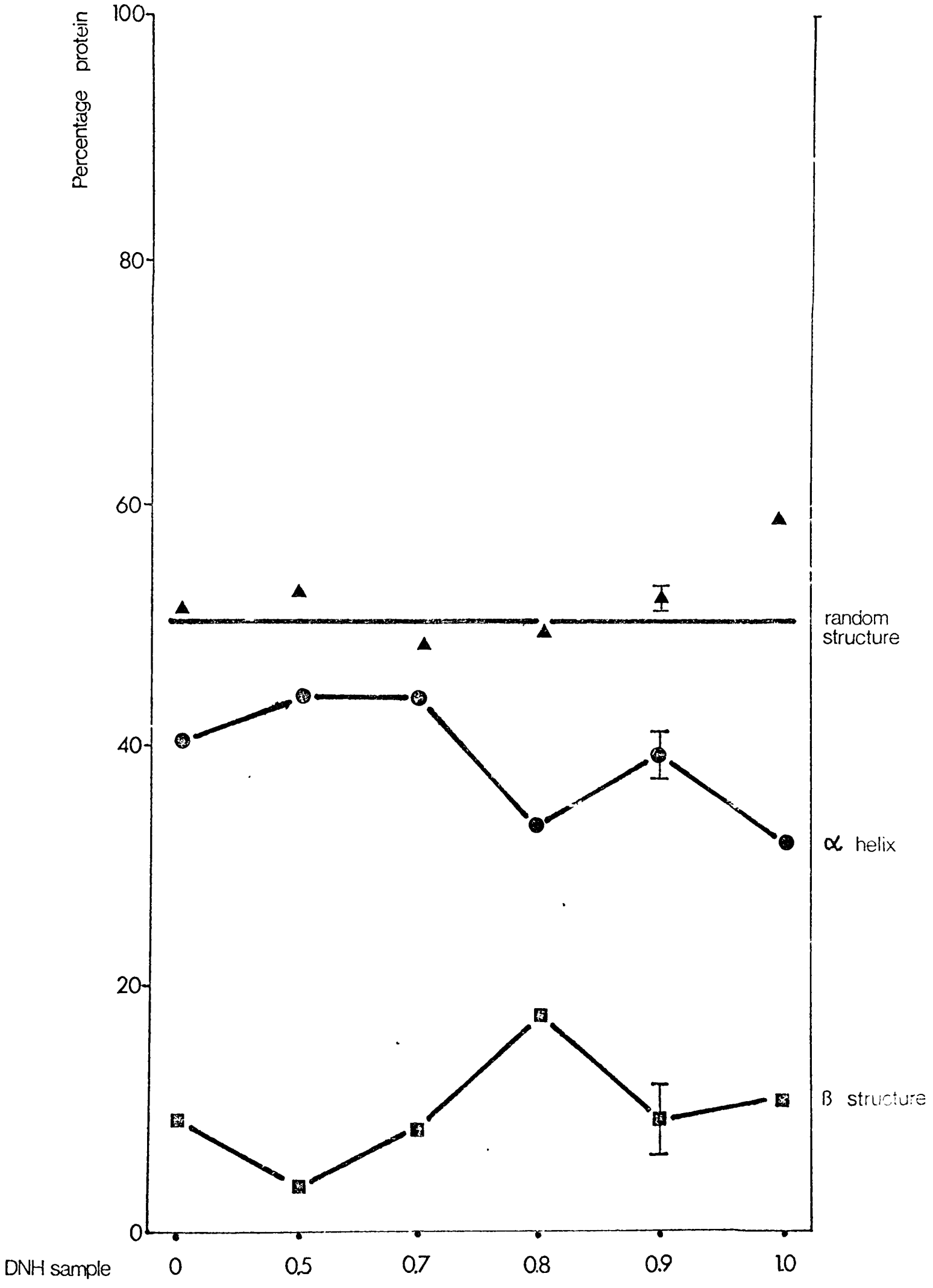


Fig 3.11 Protein structural parameters of depleted nucleohistones derived from CD data



However the fit was good between 215 and 235nm as can be seen from the spectra in Fig 3.10. The resulting structural analysis is shown in Fig 3.11.

The structure of the protein bound to DNA in the depleted DNHs, at least in the range 0.5M-1.0M salt, is shown as fairly stable, consisting of 35-45% α-helix, 5-15% β-structure and 50% unordered structure. This estimate of α-helix content is in very good agreement with studies of the IR spectra of DNH gels, and is well within the range obtained from analysis of ORD curves[23]. It contrasts with Henson's estimate of 30% α-helix, 20-30% β-structure and 40-50% unordered structure.

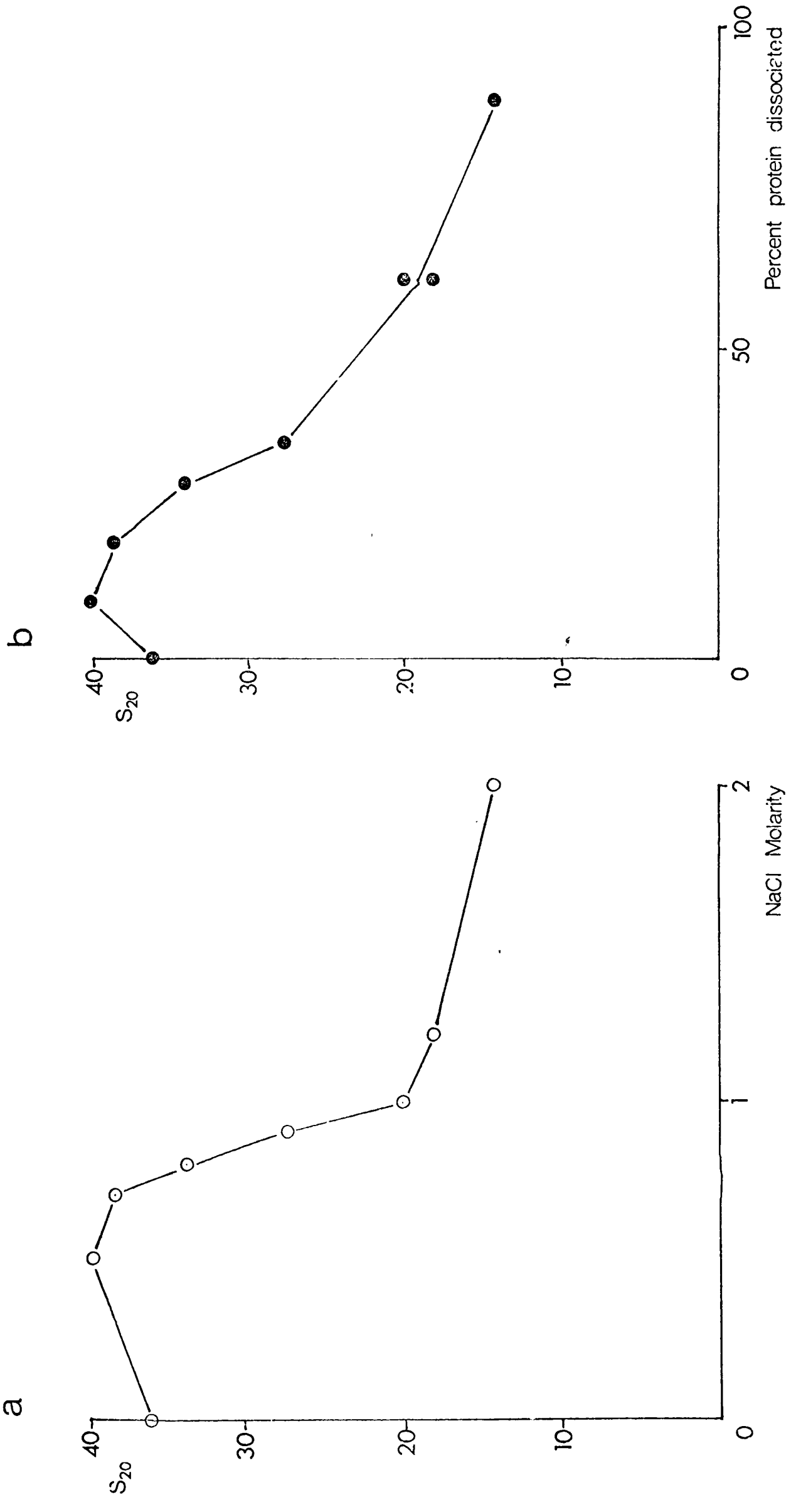
However, this approach to secondary structure is necessarily limited by the selection of suitable models. The low resolving power which derives from using a model with only three structural modes must be stressed, for all helices are not likely to be identical in their optical activity. A critique of such methods has recently been made along these lines[42].

A further attempt has been made to provide CD reference spectra, this time derived from five proteins of known structure[37]. The spectra for β and unordered structure are once again considerably changed and the overestimate of α-helical structure made by Saxena and Wetlaufer's data stressed. Obviously, the matter is still very much open.

4. Sedimentation velocity

The uncorrected s_{20} values for the depleted DNH samples from a typical preparation are plotted, in Fig 3.12, both against the percent protein dissociated and against the molarity of salt used in their preparation. In Fig 3.12a

Fig 3.12 Dissociation curve for DNH as shown by sedimentation velocity



the usual pattern is repeated - a rapid change in the property over the range 0.8M-1.0M salt. Once again, upon removal of the f1 fraction at 0.5M salt, an anomalous change in the property is observed. This increase in s_{20} on removal of f1 has also been reported by Bonner's group[198] and relates to the more compact structure of DNH that seems to form on removal of this fraction.

The general decrease in the s_{20} of DNH on removal of protein is a much reported observation.[138 139 198 21 142] The final s_{20} value achieved upon removal of all protein varies in these studies between 5 and 20, indicating the extent to which shearing varies during the preparation of DNH in various laboratories. The decrease in s_{20} marks the transition from the short rigid rod shape of DNH to the long flexible rod of DNA.

Fig 3.12b shows that, unlike $\Delta\varepsilon_{275}$, the s_{20} does not correlate linearly with the protein content of the DNH. A greater decrease in the sedimentation velocity is observed for the removal of the next fractions after f1 than for any subsequent fraction. This may perhaps be due to the importance for the sedimentation behaviour of the introduction of flexibility into the molecule. This takes place at a much earlier stage than that at which the compact DNH structure is rendered completely unstable.

4

EQUILIBRIUM STUDIES

If the histones are to play any active role in the structure-function relationships of chromatin, there must exist a mechanism for reversibly altering their interactions with DNA. Many such mechanisms have been proposed.

The enzymatic modification of histone primary structure with its consequent effects both on the structure and overall charge of the molecule has long been an attractive hypothesis. There is indeed a good body of evidence that this mechanism does apply during the replacement of histone by protamine that takes place during spermiogenesis in the trout.[128 206] There have been many reports of changes in the acetylation/phosphorylation of histone during the cell cycle.[200] Many other mechanisms have been proposed, including the displacement of histone by polyanions,[61] by interaction with acidic proteins[130] and even by proteolysis.[65]

The dissociation of histone by salt must also be considered as a possible mechanism. Reference to the dissociation curve will show that the sharp dissociation range and sigmoid shape of the curve give hints of a possible cooperativity. It does not of course have the specificity necessary to make it an acceptable mechanism of primary genetic regulation. However as a mechanism of general gene derepression it is quite admissible.

It was stressed, initially, that such a mechanism should be reversible. Is this true of salt dissociation? It has long been known that nucleohistone can be satisfactorily reconstituted from its components by gradient dialysis from high to low ionic strength.[186 187] It

has not been clear however whether this was a true reversal of the dissociation reaction. Urea appeared to be necessary for successful reconstitution. Urea does alter drastically both the extent and the order of the dissociation[106,10] so its role in reconstitution is unlikely to be passive and limited only to preventing the aggregation of the free histone.

Reconstitution in the absence of urea is possible if the final salt concentration is in excess of 0.7M i.e. if f1 remains free in solution. As reported in the last chapter, some of the properties of the original DNH are regained if the histone is completely removed in high salt and then replaced by dialysis to the appropriate salt concentration.

Several other results also indicate the possibility that in this system we are dealing with a reversible equilibrium. Henson[81] observed that during gel chromatography of DNH samples depleted at greater than 0.7M salt, the $^{P}/_{DNA}$ ratio across the leading peak is not constant but decreases slightly. This observation is consistent with the hypothesis that the equilibrium is being disturbed by passage through the column. Henson also found that repeated treatment of a single sample of DNH with aliquots of the same salt solution led to the removal of more protein with each successive treatment. Georgiev[68] has also reported this phenomenon with the added observation that $DNH^{0.6}$ is stable to such treatment. The interaction of histone f2b has been reported to be completely reversible.[36]

The variation between preparations, reported in the previous chapter, in the position of the major dissociation with respect to the salt axis can also be fitted into the reversible equilibrium model. Variations in concentration between the preparations can then be used to explain the variation in the position of the dissociation.

We determined to test this hypothesis by investigating to what extent the changes in the dissociation curve could be induced by variations in the initial concentration of the nucleohistone complex. It was also decided to apply to the data a simple physical model of a reversible equilibrium to observe:

1. how far the results could be adequately described by such a model,
2. whether any thermodynamic or mechanistic data could be obtained from such a system.

The model

The initial hypothesis of this model is that histone is bound to sites on the DNA backbone and that these sites are non specific, that is related only to the concentration of DNA phosphate groups. The dissociation by sodium chloride is then expressed as:-

$$P_{bound} + nNaCl \underset{}{\overset{K}{\rightleftharpoons}} P_{free} + xH \qquad 4.1$$

where P represents binding sites

n is the number of NaCl molecules required to free a single site

H is free histone

and x is that fraction of a histone molecule which binds to a single site, i.e. the reciprocal of the number of binding sites/histone molecule.

This expression is in the form of an equilibrium and hence makes no statement regarding the mechanism of the dissociation. However it is assumed that the sodium chloride molecules do take an active part in the dissociation, ending bound in some unspecified way to the product species.

This model will only be applied over that part of the dissociation curve in which f3, f2b and f2a2 are dissociated. It is an assumption of the treatment that all histone molecules involved are bound with the same binding energy. Such an assumption is only supportable if there is little or no fractionation of the separate histone fractions in the region of the dissociation treated. As we have shown in the previous chapter, f1 and f2a1 are removed from the DNH in two discrete steps. This model should not be applied to them. f2a2, f2b and f3 on the other hand, do not show any significant fractionation.

The model can be analysed thus:-

$$P_{bound} + nNaCl \underset{}{\overset{K}{\rightleftharpoons}} P_{free} + xH \qquad 4.1$$

$$K = \frac{\left[P_{free}\right]\left[H\right]^{x}}{\left[P_{bound}\right]\left[NaCl\right]^{n}} \qquad 4.2$$

It should be pointed out that n and x have descriptive significance for the dissociation. If x were significantly larger than unity then the reaction would be cooperative with respect to histone i.e. binding of one histone molecule would decrease the binding energy required to

bind the next. $^{n}/_{x}$ is the number of sodium chloride molecules required to dissociate one molecule of histone.

With one further assumption, we can express equation 4.2 in terms of experimentally determinable quantities. The assumption is that all the binding sites we have defined are saturated in native DNH and free in DNA. If we let the initial concentration of bound sites be a, the equilibrium concentration of free sites be p and the equilibrium activity of NaCl be c, we then have

$$K = \frac{p(xp)^{x}}{(a-p)c^{n}} \qquad 4.3$$

and

$$\ln K = \ln p + x\ln x + x\ln p - \ln(a-p) - n\ln c$$

$$(\ln K - x\ln x) = (x+1)\ln p - \ln(a-p) - n\ln c \qquad 4.4$$

Operating in conditions of salt excess, c can become the initial activity of salt. Now if this quantity is kept constant and the initial concentration of sites is varied,

$$\ln(a-p) = (x+1)\ln p + \text{constant} \qquad 4.5$$

and a plot of $\ln(a-p)$ against $\ln p$ will yield a straight line of slope $(x+1)$.

On the other hand, if the initial concentration of sites is kept constant and the salt concentration varied, as occurs in a normal dissociation experiment, equation 4.4 will give

$$(x+1)\ln p - \ln(a-p) = n\ln c + \text{constant} \qquad 4.6$$

and a plot of ((x+1)ln p - ln(a-p)) against ln c will produce a straight line of slope n.

We can obtain thermodynamic information from such a model by taking the relationship

$$\ln K = \frac{-\Delta G}{RT} = \frac{\Delta S}{R} - \frac{\Delta H}{RT}$$

and substituting it in equation 44to produce

$$\ln(a-p) - (x+1)\ln p = \frac{\Delta H}{R}\,\frac{1}{T} + \ln \frac{x}{C^n} - \frac{\Delta S}{R} \qquad 4.7$$

In a situation where the temperature is varied but the initial concentration of sites and that of sodium ions are kept constant, a plot of (ln(a-p) - (x+1)ln p) against $^1/_T$ will yield a straight line of slope $\Delta H/_R$ and with an intercept that can be related to ΔS.

Methods

When studying an equilibrium process what is ideally required is a method of measuring the concentrations of the participating species in the equilibrium mixture. A change in the structure or properties of one of the species on reaction would enable such measurements to be made.

If assumptions were made about the binding to DNA of the displacing salt molecules, then some spectrophotometric property of the Na^+ could be used. However such assumptions would be highly uncertain. In addition, the conditions of salt excess in which the dissociation takes place would render the measurement of smallamounts of bound cation very difficult.

No method of measuring free and bound histone

concentrations in situ was available when this work was started. However, a recent paper[36] has shown this to be possible by exploitation of the difference in the surface active properties of the free and bound proteins.

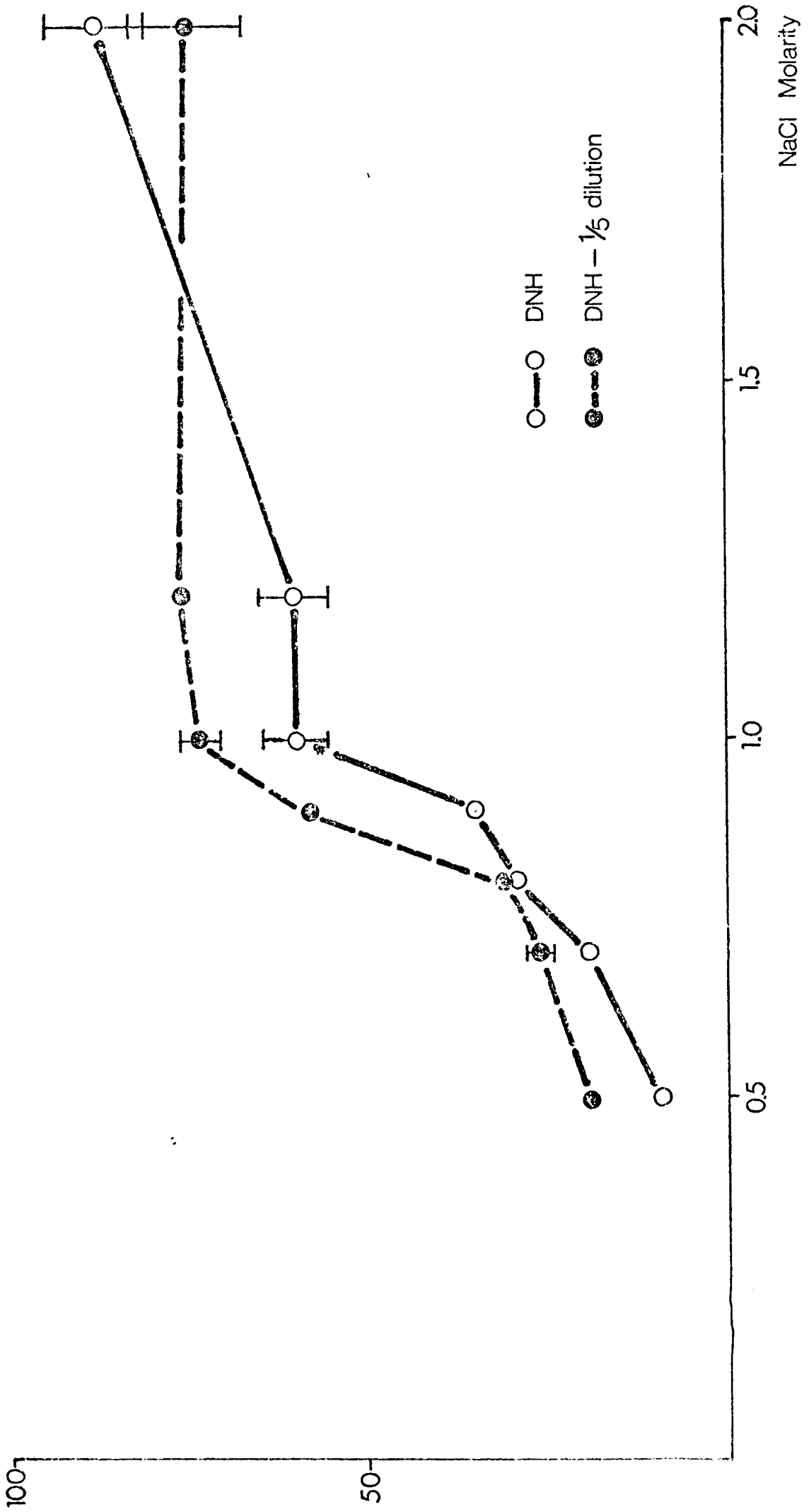
There remained only the option of separating the reacting species from the equilibrium mixture as quickly as possible and then measuring their concentrations. Despite the real objections there are to this procedure it was thought worthwhile to proceed in the hope of obtaining at least a semi-quantitative account of the reaction.

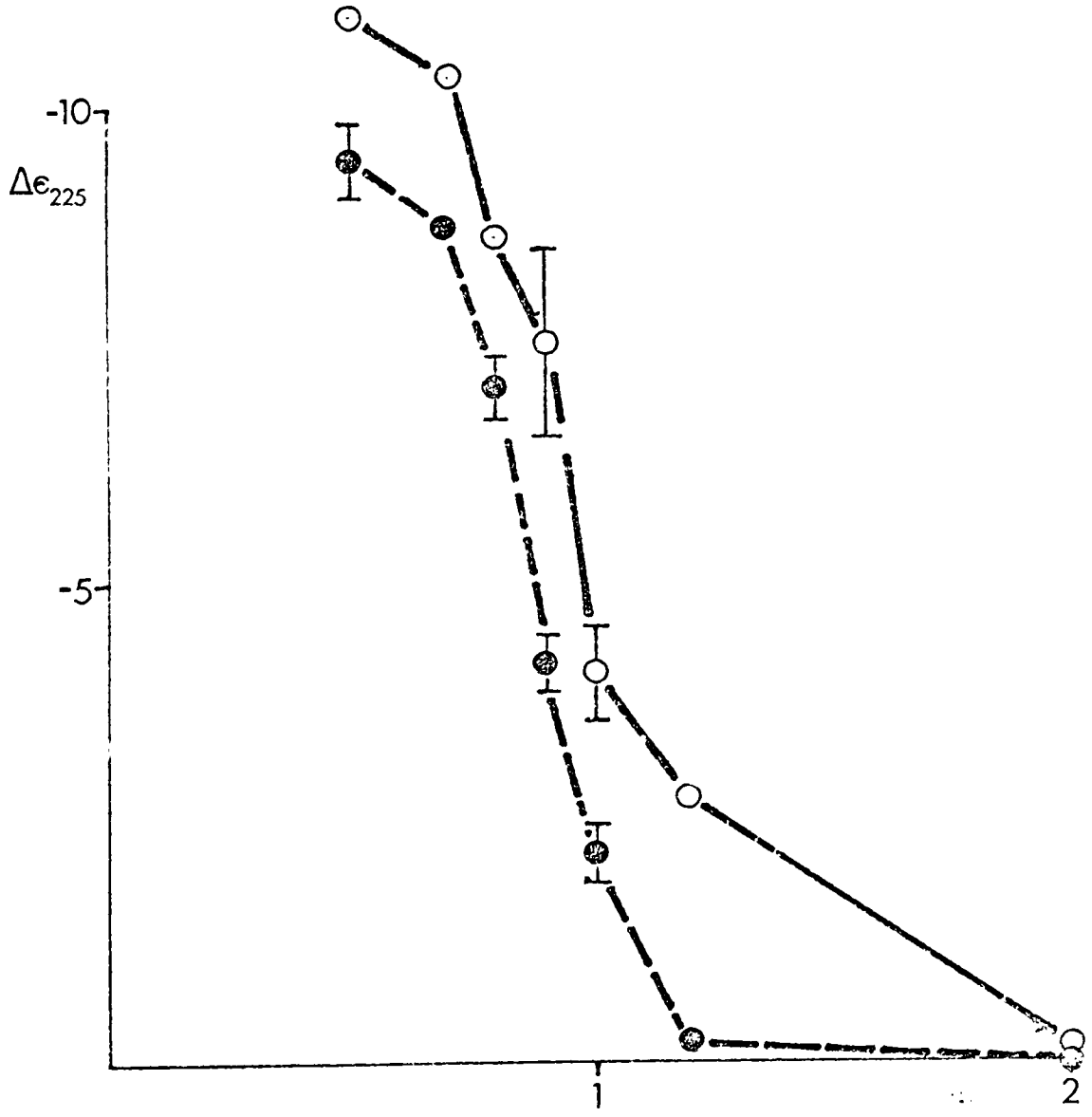
The method used was that, described before, of separating the equilibrium mixture on a column of Sepharose 4B. This is much less drastic than the ultracentrifuge separation favoured by some workers and in addition is much faster (3-4 rather than 16-18h). Nor does there seem to be any contamination of the depleted DNH samples with aggregated histone.

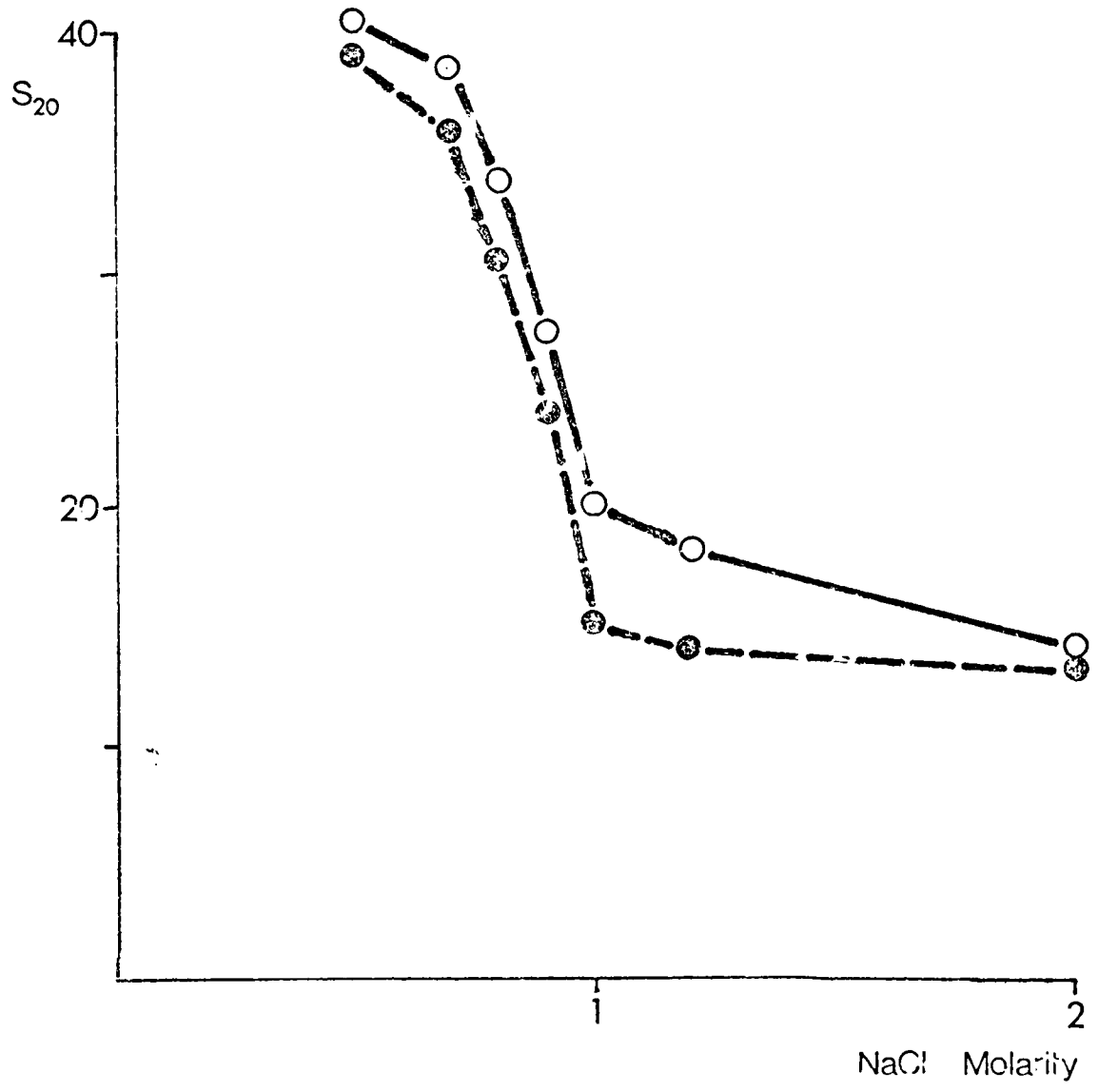
However there is a danger that the time taken to separate the components of the equilibrium mixture is sufficient for a considerable displacement of the equilibrium and reequilibration to take place. The variation of the $P/_{DNA}$ ratio across the peak observed by Henson could be due to such an effect. There is however an alternative explanation of this observation in the undoubted molecular heterogeneity of the DNH, which has often been reported to increase on partial dissociation.[34 199]

Two series of experiments were performed. The first examined the effect of variation of the initial concentration of DNH on the dissociation and the second

Fig 4.1 The variation of extent of dissociation with initial DNH concentration - first sample




-10
$\Delta\epsilon_{225}$
-5
1
2


40
S_{20}
20
1
2
NaCl Molarity

that of the variation of temperature. Comparisons between dissociation curves were only made where both were determined on the same preparation of DNH. These determinations were carried out wherever possible simultaneously to avoid complications arising from any variation in the integrity of the preparation with time. The determination of a single dissociation curve takes of the order of 7-10 days; the average preparation has a lifetime of 3-4 weeks.

Experiments were repeated in whole or in part on five different preparations. Only those readings which were contained within the central 80% of the dissociation curve were used in order to limit errors.

Results

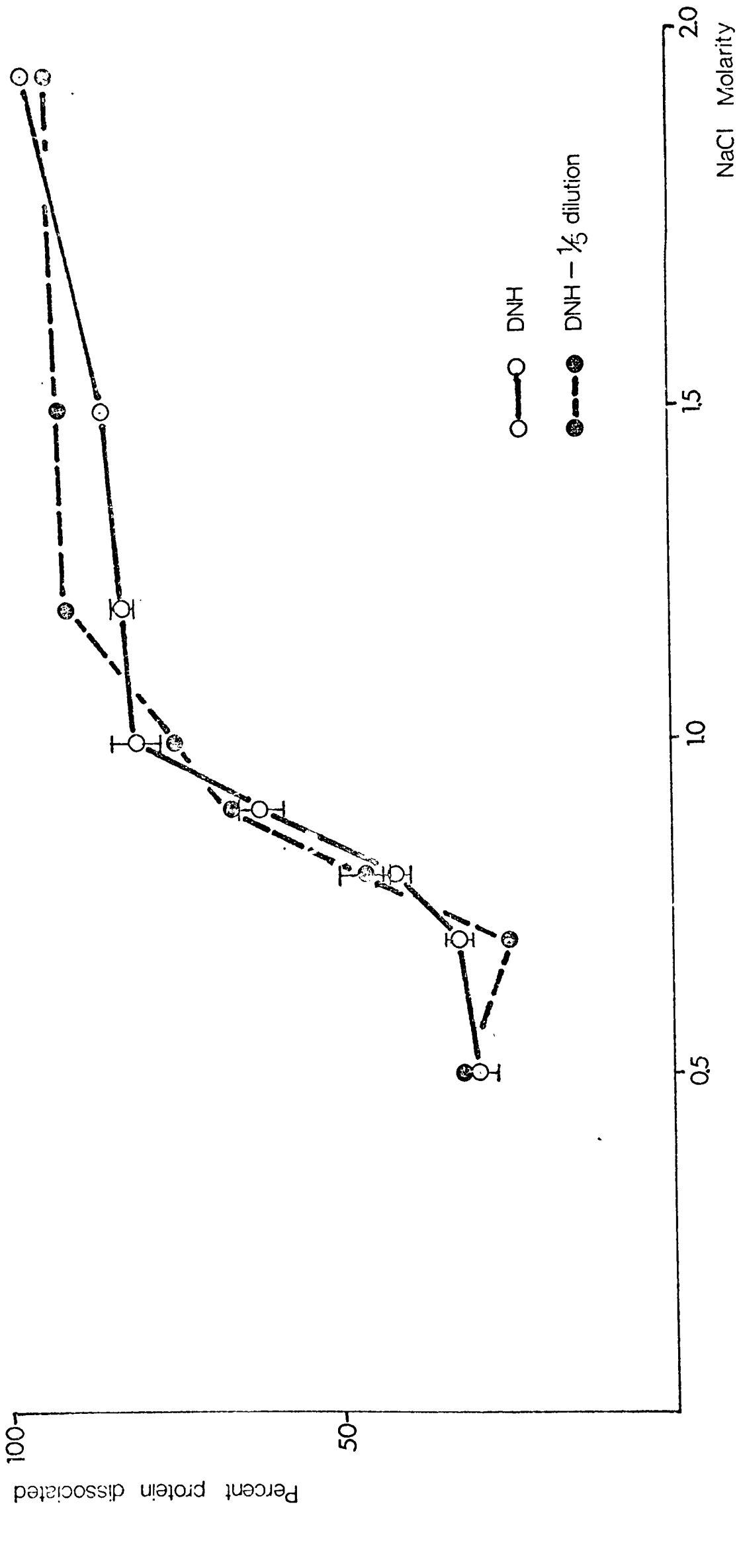
1. The effect of variation of the initial DNH concentration

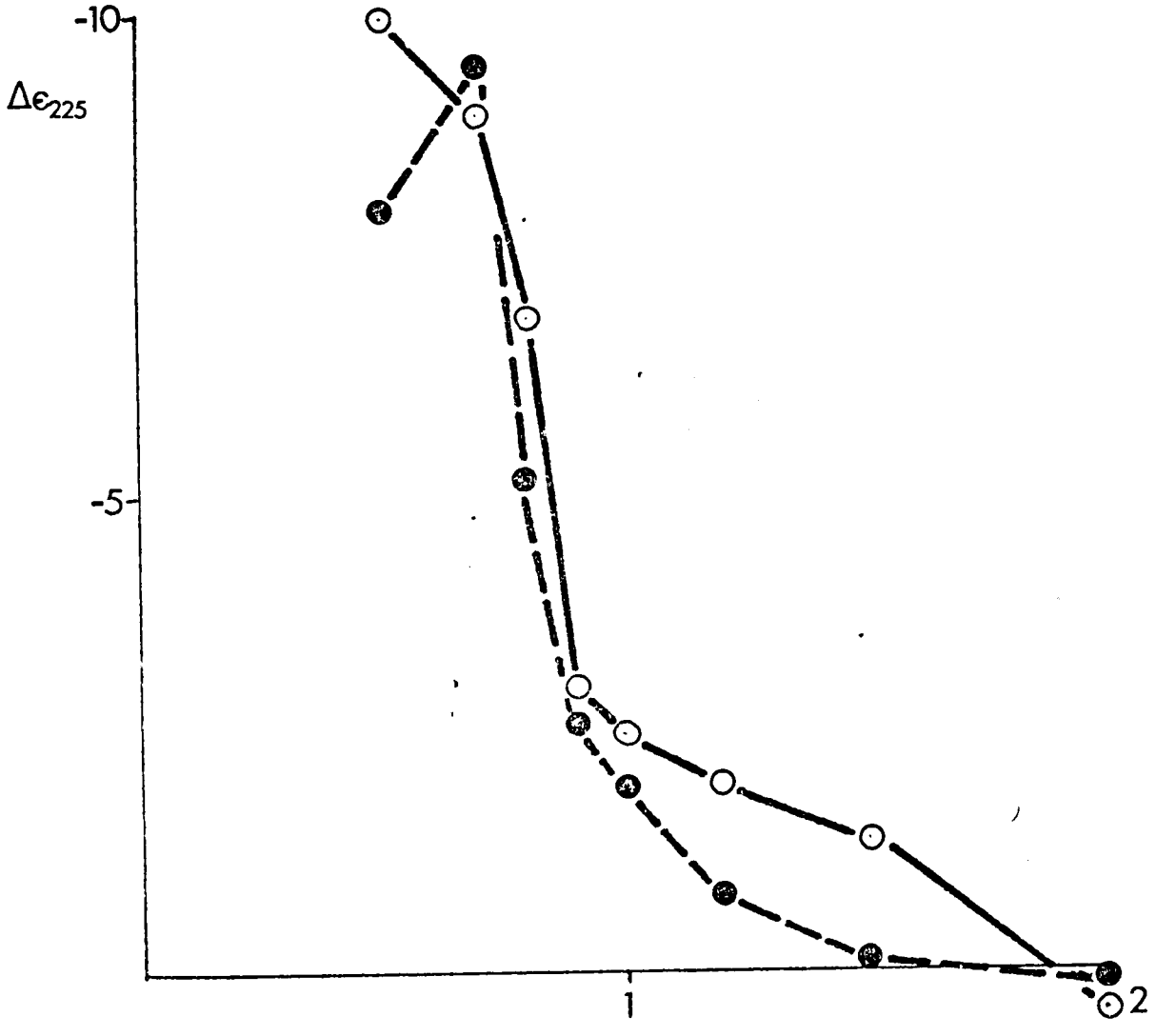
Experiments were carried out in the range of initial concentrations from 0.16 to 1.23 mM (with respect to DNA phosphate). The upper limit was imposed by the requiremcnt for a soluble, i.e. not gel-like, sample of DNH; the lower limit by the levels of accuracy of the various assay methods.

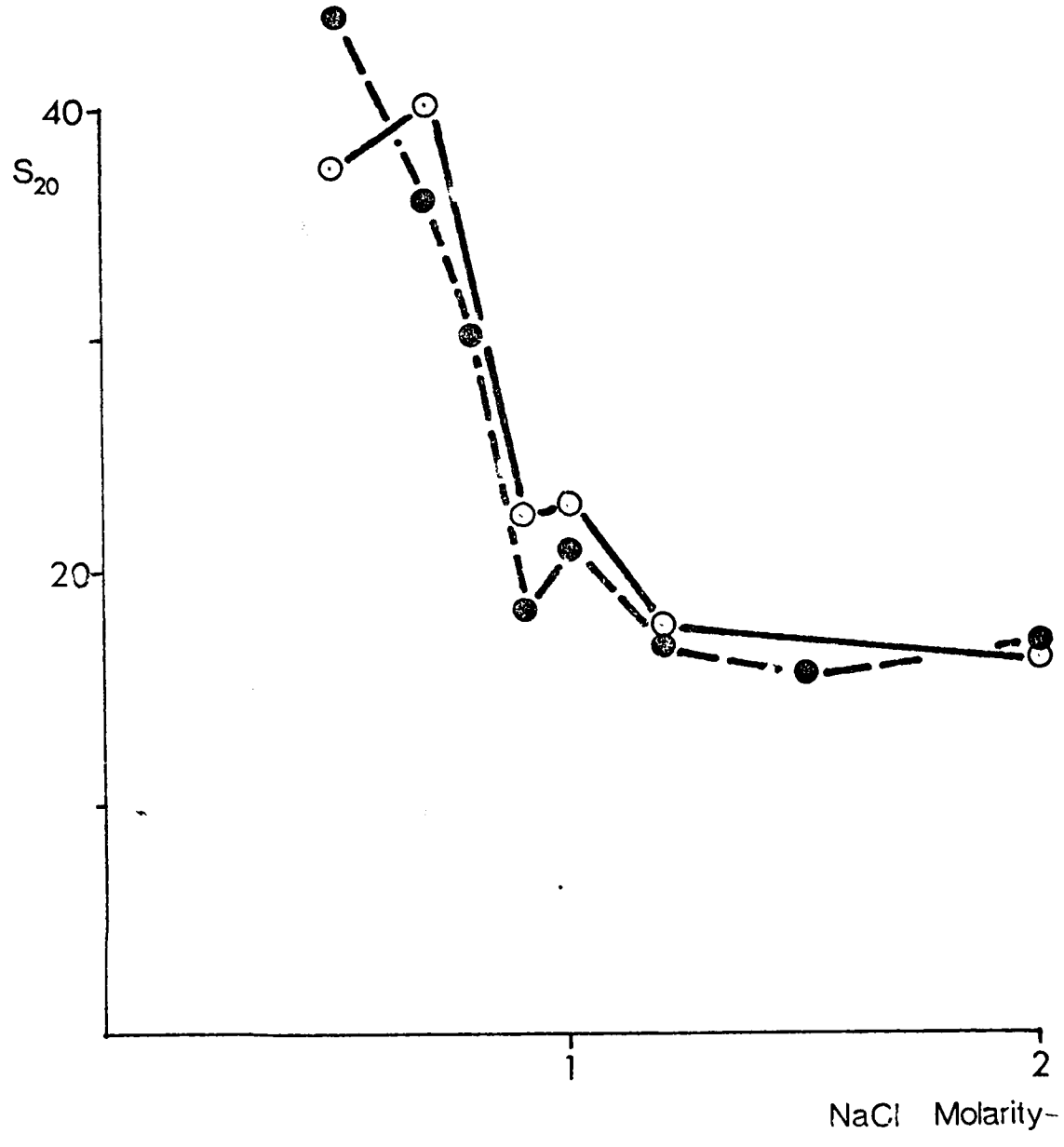
Figs 4.1 and 4.2 show the results from the two best preparations investigated. The extent of dissociation is expressed not only as the percentage histone dissociated, calculated from the Folin test, but also as the related quantities $\Delta\varepsilon_{225}$ and s_{20}.

The first thing that is worth stressing is the internal

Fig 4.2 The variation of extent of dissociation with initial DNH concentration - second sample




-10
$\Delta\epsilon_{225}$
-5
1
2


40
S_{20}
20
1
2
NaCl Molarity

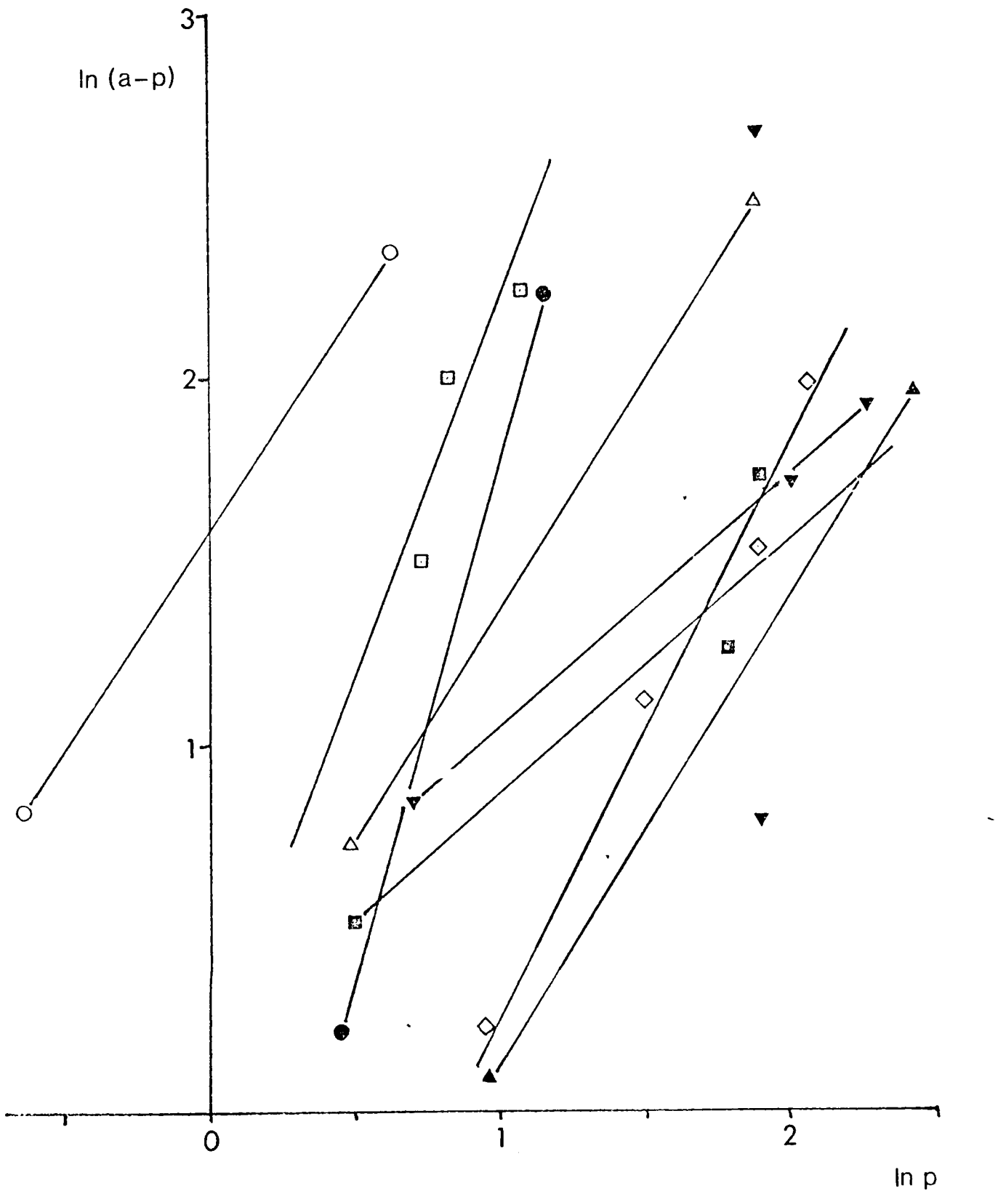
consistency of the two sets of data. The differences observed between the dissociation curves at the two concentrations are reflected in all the three measured quantities. Small variations do take place which can be ascribed to inaccuracies in the methods involved, but in general all three graphs tell the same story. However there are differences between the two sets of data.

The data of Fig 4.1 show a consistent and highly significant difference between the dissociation curves at the two concentrations. In Fig 4.2 there is also a difference between the two curves which is in the same direction as that in Fig 4.1, however it is not very consistent and is only just outside the limits of error.

The conclusion can be drawn that the dissociation curve is shifted to lower salt concentrations when the initial DNH concentration is reduced i.e. a given quantity of salt will have a greater dissociating effect at lower DNH concentrations. The shift in the curve can be as great as 50-100 mM in favourable conditions.

Even in their responses to a change in initial DNH concentration, preparations differ. While this experiment shows that indeed variation of initial concentration can lead to considerable displacement of the dissociation curve, it reasserts that there is still some unexplained variation between preparations which causes them to behave differently in this system. We can only assume that this variation resides in the molecular heterogeneity of preparations with respect to each other.

Fig 4.3 The effect on the dissociation of varying the initial DNH concentration - plot of ln(a-p) against ln p



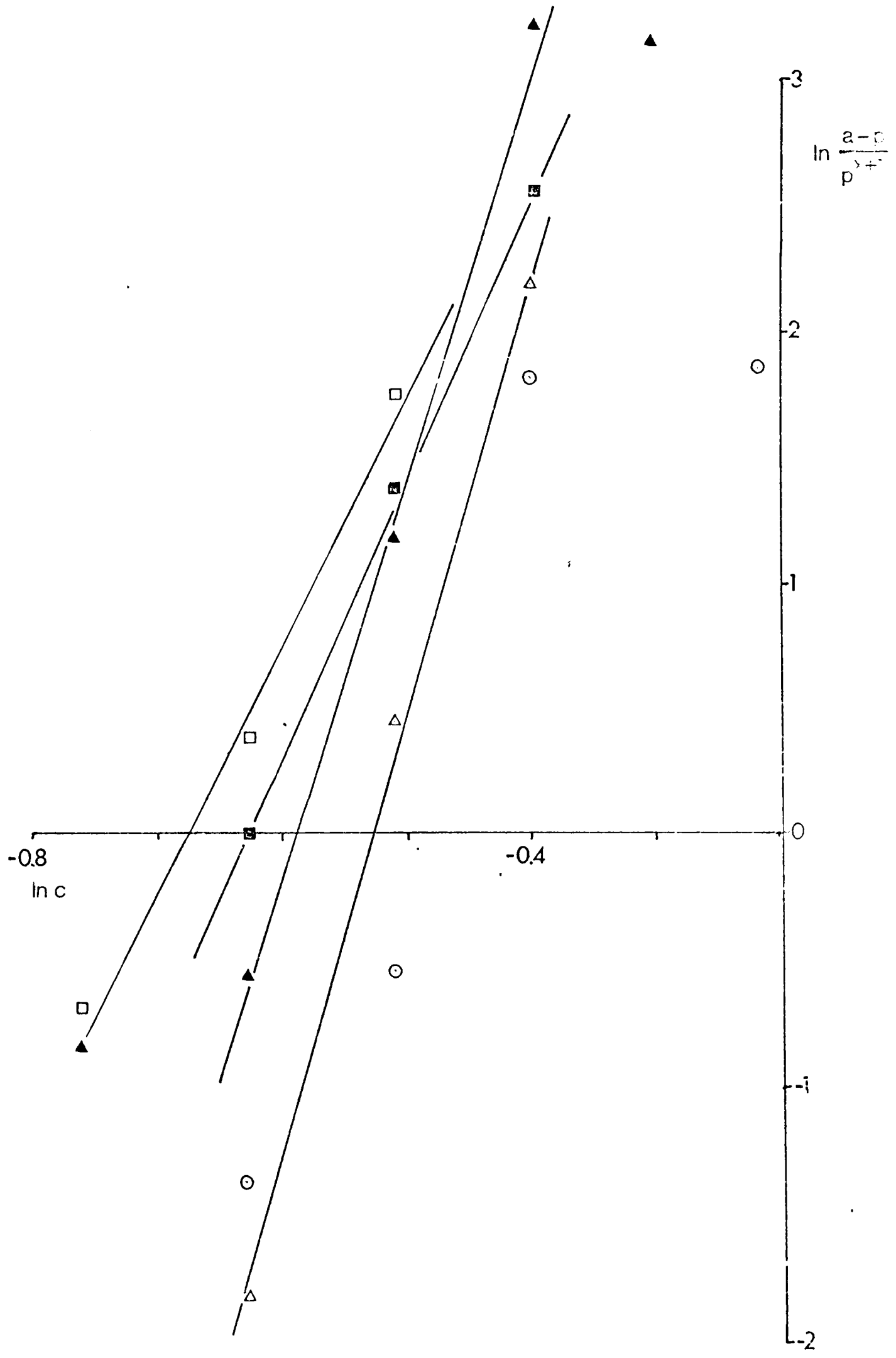
The immense variation in dissociation seen in Fig 3.1 can thus be partially explained by variations in initial concentration. The variation between preparations is only twofold. The fivefold variation in concentration used in Figs 4.1 and 4.2 can only produce a variation in dissociation which is equal to that observed between preparations.

Despite the confusion caused by the variations between the different sets of data, an attempt was made to apply the model to this and similar data from other preparations. Fig 4.3 shows the application of equation 4.5 to the data. Since variation of concentration at constant salt concentration is plotted, experiments such as shown in Figs 4.1 and 4.2 will only yield straight lines with two points. Experiments in which data was collected at three or more concentrations do give good straight lines however.

Little agreement is observed between the slopes of the lines even when they both derive from the same preparation. An average slope of 1.53±0.86 is obtained and hence the value of x, the histone exponent, lies between 0 and 1 with an average of 0.53. There is little or no cooperativity between histone molecules indicated for the reaction.

Equation 4.6 can now be applied to the available data using the value of n obtained above. The data processed from several experiments are shown in Fig 4.4. The value of n obtained is 14.7±3.5 indicating a greater measure of agreement than was observed in Fig 4.3. It should be noted that excellent agreement is observed between

Fig 4.4 The effect on the dissociation of varying the salt concentration at a fixed DNH concentration - plot of $(x+1)\ln p - \ln(a-p)$ against ln c



experiments carried out on the same DNH preparation and thus nearly all the error arises from differences between preparations.

$^{n}/_{x}$, the number of salt molecules involved in the dissociation of a single molecule of histone, is 27.7± 6.6. As had been deduced from the shape of the dissociation curve, the interaction is highly cooperative with respect to salt.

The proteins which dissociate in this region have the following numbers of basic residues:-[55,228]

f2a2	37 (calculated on the basis of a protein chain of 150 residues)
f2b	31
f3	32

of which some 80% are thought to be bound to the DNA by salt links[224], giving an average of 27 salt linkages per molecule.

2. The effect of variation of the temperature

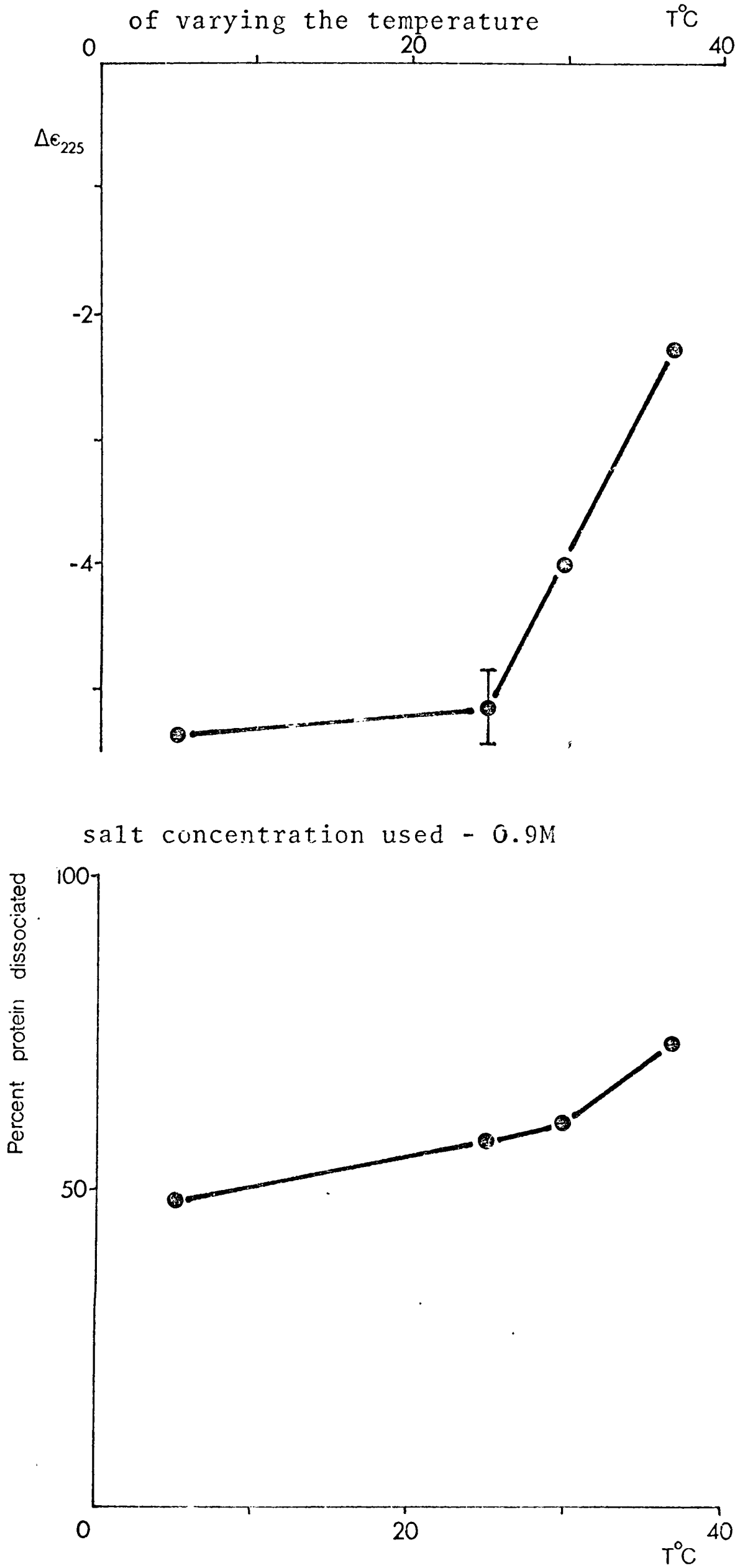
Whole or partial dissociation curves were determined with four different preparations at three temperatures - 4°, 30° and 37°C - in addition to room temperature. The entire experiments, dialysis of DNH and separation by agarose gel chromatography, were performed in rooms regulated at the temperature concerned.

There is a considerable amount of variation in these results. Table 4.1 shows that the observable trends can be divided into two sections. Differences in the dissociation at 4°C compared with that at room temperature

Table 4.1: The effect on the dissociation of varying the temperature

Preparation	Extent of dissociation at different temperatures	
a	$4^{o} = 22^{o}$	$22^{o} < 37^{o}$
b	$4^{o} > 20^{o}$	$20^{o} < 37^{o}$
c	$4^{o} < 20^{o}$	$20^{o} < 37^{o}$

Fig 4.5 The effect on the extent of dissociation of varying the temperature



do not give a coherent picture. The best set of results which is shown in Fig 4.5 shows little difference at all in the extent of dissociation at the two temperatures. This is corroborated by the CD data.

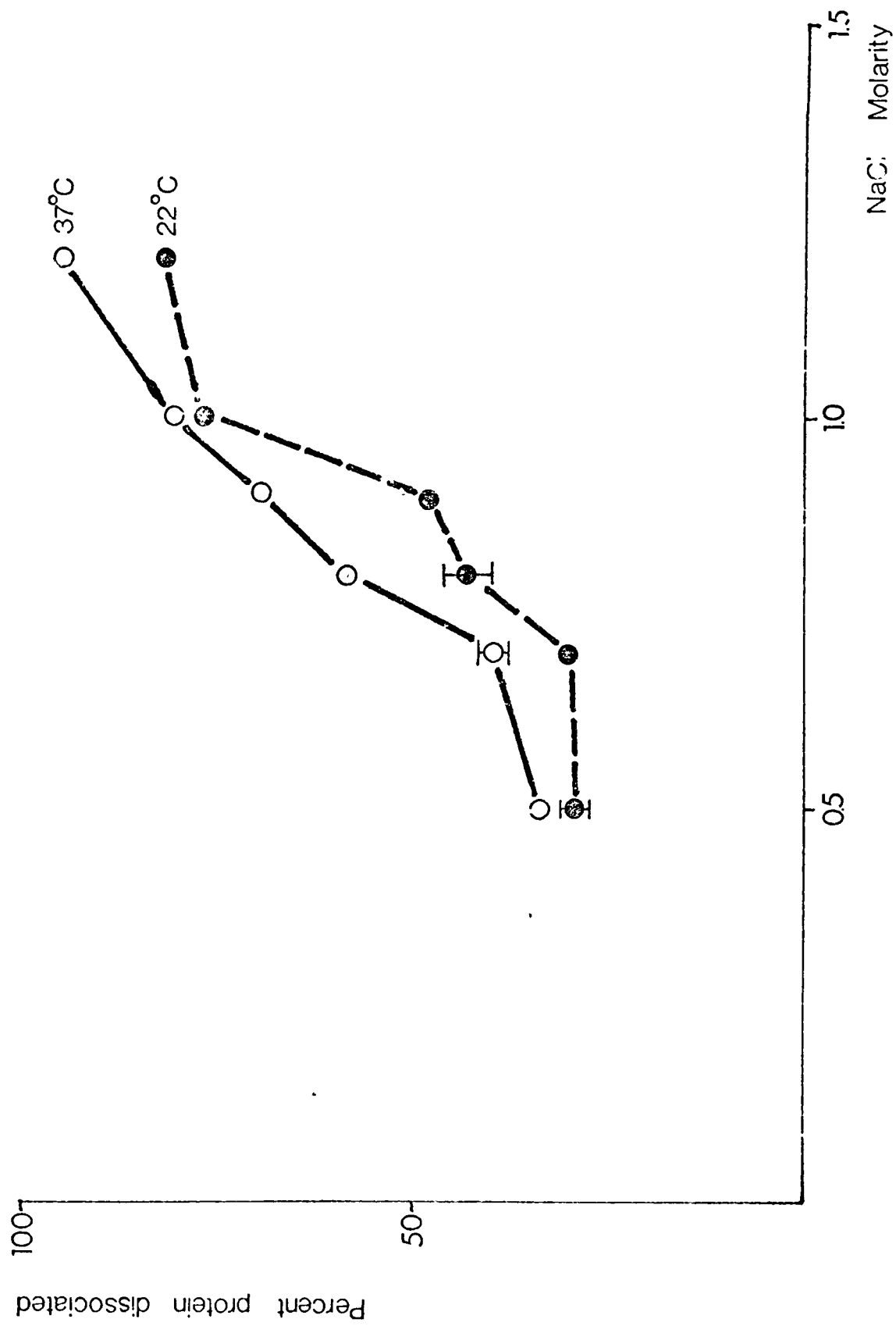
All preparations give results which agree in the region above room temperature. The extent of dissociation at 30° and 37°C is greater than at room temperature. This difference extends throughout the dissociation range as the data of Fig 4.6 show.

In the only other study to investigate the effect of temperature on the dissociation of histone from DNH, Bartley and Chalkley[10] used a mixture of urea and sodium phosphate as the dissociation agent. Raising the temperature from 4° to 20°C caused an increase in the dissociation of f1 histone. All the other fractions, however, decreased the extent of their dissociation. This, combined with other evidence was seen as arguing for the participation of hydrophobic bonding in the binding of all but the f1 fraction to DNA.

The results reported here do not confirm this observation. The solutions of sodium chloride used here have no direct effect on hydrophobic bonding comparable with that of the urea used in Bartley and Chalkley's work. Thus different mechanisms for dissociation are at work in the two cases. The breaking of both electrostatic and hydrophobic bonds takes place in both cases but the differing importance of each in the mechanism leads to different temperature effects.

It is not possible to rule out the possibility that this discrepancy is artefactual in origin. DNA-bound

Fig 4.6 The effect on the dissociation of varying the temperature



proteases are undoubtedly present in our preparations: they are responsible for the ultimate degradation of the preparations. Experiments carried out in this laboratory lead us to believe that proteolysis does not interfere with dissociation results even at elevated temperatures. However an effect of this nature cannot be completely ruled out.

This uncertainty coupled with the variability of the data of Fig 4.5 make the application of equation 4.7 a dubious procedure. The derivation of values for ΔH and ΔS on a semi-speculative basis would be more confusing than helpful. It seems reasonable to suggest, however, that, since there are no dramatic temperature variations in the dissociation, ΔH for this reaction is not large.

Estimates of ΔG can be obtained from the equilibrium constant. These also show considerable variation but are of the order of zero. Given this situation it is hard to derive any information about the effect of entropy on the reaction.

Discussion

Application of a simple thermodynamic model to the dissociation of histone from DNH by salt has produced a number of significant results. The thermodynamic origin of these results limits their mechanistic importance - they can really be held to indicate only differences between the initial and final states of the reaction.

The plots of $\ln(a-p)$ against $\ln p$ in Fig 4.3 are good straight lines. The lack of curvature stresses that the equilibrium constant for the reaction is related only

to the concentrations of the bound and free sites. The operation of any factor unrelated to the extent of protein binding is ruled out.

However, this structure does not eliminate any type of histone-DNA interaction. The effect that denaturing agents have on the dissociation of histone from DNH[106 10 199] would seem to suggest that other than electrostatic bonds are involved in the interaction. Hydrophobic histone-histone bonds may play a part as Bradbury et al.[22] have suggested for f1 in the formation of nucleohistone gel.

The value of approximately unity obtained for x, the histone exponent, is of great importance. The presence of any large degree of cooperativity in the binding of f2a2, f2b and f3 to DNA would seem to be ruled out. This means that the binding of one molecule has no effect on the binding of subsequent molecules. If there is a conformation of DNA which is favourable for the binding of histone, then the binding of the first histone molecule does not lock the DNA into that conformation. It is quite probable that f2a1 induces in the DNA a conformation which is suitable for the binding of the remaining histone molecules. The X-ray data of Chapter 3 would seem to support such a hypothesis.

Rubin and Moudrianakis[177] have claimed that the binding of DNA to histone is cooperative. This claim is based only on the sigmoid shape of the binding curve obtained when dialysing histone/DNA mixtures into low salt buffers. The histone molecules also appear to bind in clusters. These workers have not ruled out the possibility that histone aggregation may take place prior to binding to DNA.

Nor, indeed, have they shown that their complexes have any structural identity with extracted nucleohistone.

The data of Fig 4.4 havea most satisfying consistency. The derived figure for the number of salt molecules involved in the binding of one histone molecule is in good agreement with the theoretical number of salt links per histone molecule. This agreement supports the hypothesis that the dissociation is a direct displacement of histone molecules from the DNA by sodium ions. However the high cooperativity stresses that this is an all or nothing process. Histone molecules are either completely bound to the DNA or they are free in solution.

It cannot be stressed enough that the reasonable answers obtained with this model of the DNH dissociation do not help to prove the validity of the model. However, they do go some way to further illuminate the nature of the interactions between histones and DNA. The considerable deviations from linearity observed in places show that the model is not capable of encompassing the complexity of the system.

Indeed, the variations between preparations which continue to obtrude from the data emphasise our ignorance of the exact molecular composition of nucleohistone.

5

SALT SPECIFICITY

It is not clear what role sodium chloride plays in the dissociation of the DNH complex. However, it is becoming obvious that not all salts dissociate the complex to the same extent. Ohlenbusch et al[143] noted that sodium perchlorate was twice as effective a dissociating agent as sodium chloride. Sodium deoxycholate is 10-20 times more active in dissociation than the chloride[199]. Fredericq[59] has reported results from his laboratory that show that sodium salts can be arranged in order of decreasing dissociating power thus:-

$$NaClO_4 > NaCl > NaSCN > NaF$$

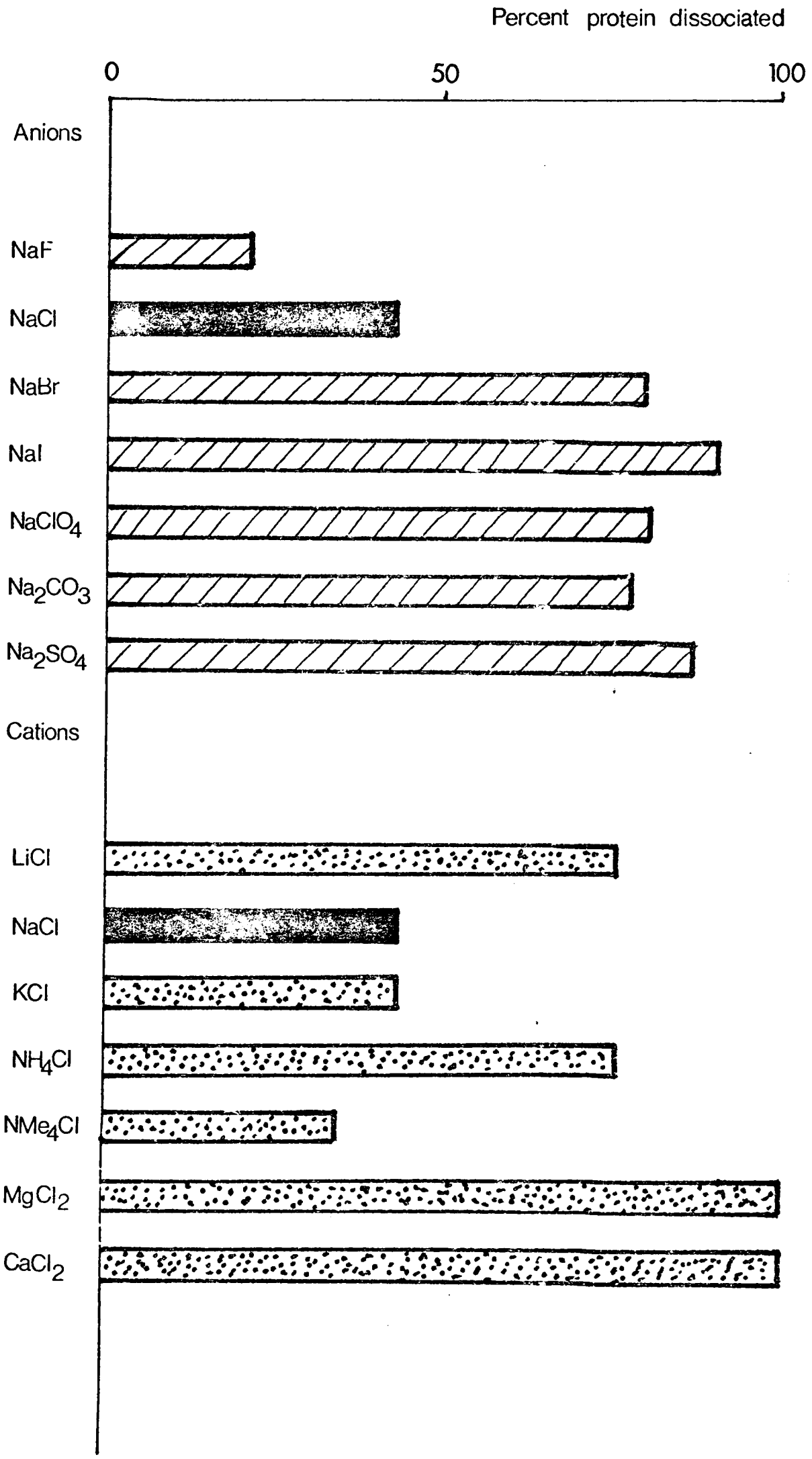
Sodium sulphate and sodium hydrogen phosphate are apparently very poor dissociating agents.

Variation of the cation also affects the extent of dissociation. Several workers have commented on the interchangeability of sodium and potassium chlorides[68]. Magnesium chloride is more active than either[59,103].

On the equilibrium model discussed in the previous chapter, some variation in the effectiveness of identical concentrations of different salts would be expected. For it is the activities of the various species which are paramount here. However, variations in the activity coefficients of the salts will not adequately explain the published results.

Further investigation of the variations in the dissociation brought about by different salt anions and cations was undertaken in the hope of obtaining some clue to the mechanism of the dissociation. It was also hoped to gain some insight into the interactions which may be

Fig 5.1 Extent of dissociation of DNH in 0.8M solutions of various salts



taking place in the physiological situation.

Results

Depleted DNH samples were prepared using the methods previously described. Comparisons are only made between experiments carried out on the same preparation. A single salt concentration was used for convenience - this was chosen as 0.8M, the mid-point of the sodium chloride dissociation curve.

Fig 5.1 shows the extent of dissociation of DNH induced by 0.8M solutions of various salts. Two series of experiments were performed; one comparing different sodium salts, the other comparing chlorides of various monovalent and divalent cations. The data are replotted in Fig 5.2, attention having been given to the variations in activity coefficient of the various salts. The percentage of protein dissociated is expressed as a function of the activity of the dissociating salt; the dissociation curve for sodium chloride is plotted here for comparison.

Of the salts used, all, with the exception of sodium fluoride, are better at dissociating than is sodium chloride. An attempt at quantitation can be made by comparing the extent of dissociation brought about by a particular salt with that caused by an equal activity of sodium chloride. These ratios are shown in Table 5.1 and lead to an ordering of ions in decreasing ability to dissociate DNH:-

$$Mg^{++} = Ca^{++} > NH_4^+ > Li^+ > K^+ > Na^+$$

$$SO_4^{=} > CO_3^{=} > ClO_4^- > I^- = Br^- > Cl^- > F^-$$

Fig 5.2 Extent of dissociation of DNH in 0.8M solutions of salts plotted against their activities

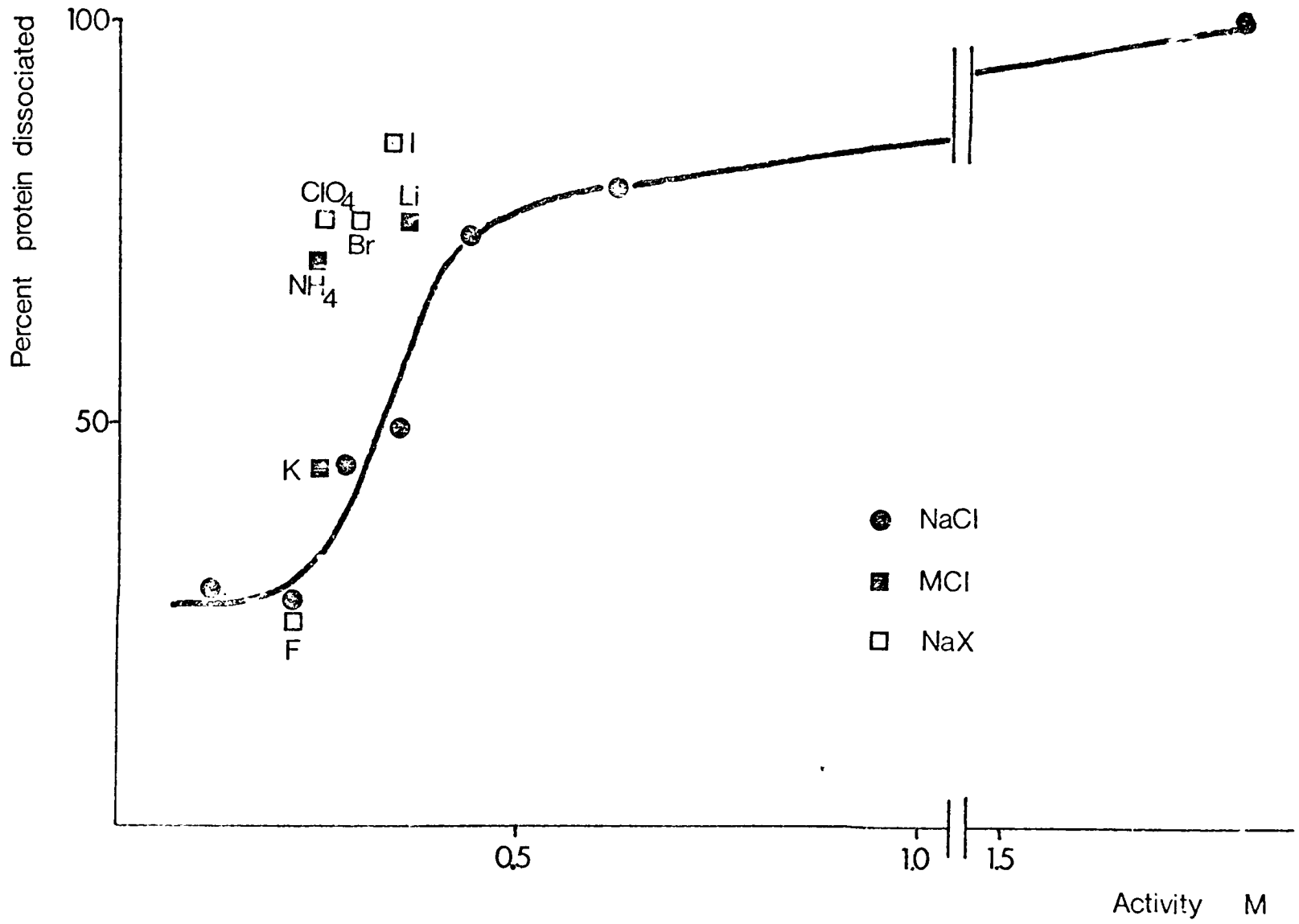


Table 5.1: Extent of dissociation in 0.8M solutions of salts compared to NaCl

Salt	Activity coefficient	Dissociation (%)	NaCl equivalent dissociation	Ratio
Sodium sulphate	0.180	87.5	30.0	2.9
Sodium carbonate	0.225	78.5	30.0	2.6
Sodium perchlorate	0.514	81.0	37.5	2.15
Sodium iodide	0.582	91.0	51.0	1.78
Sodium bromide	0.550	80.0	44.0	1.82
Sodium fluoride	0.475	21.0	31.5	0.67
Sodium chloride	0.530	43.0	40.2	1.06
Lithium chloride	0.605	76.0	58.0	1.32
Potassium chloride	0.495	43.0	34.5	1.24
Ammonium chloride	0.495	76.0	34.5	2.2
Magnesium chloride	0.417	100.0	30.0	$\geqslant$3.3
Calcium chloride	0.377	100.0	30.0	$\geqslant$3.3

The activity of tetramethylammonium chloride in the dissociation has also been measured on a different preparation. A 0.8M solution of this salt dissociates less protein from DNH than does an equivalent concentration of sodium chloride.

Discussion

Neutral salts vary in the effectiveness with which they dissociate DNH into DNA and protein. Since we are dealing with an equilibrium situation, the effect of the salts must be on the relative stabilities of the products or the reactants of the dissociation.

In general the addition of salts to solutions of macromolecules causes them to become less stable.[221] Such effects transcend details of macromolecular composition.[222] The activity of various ions in destabilizing proteins and nucleic acids is exemplified in the Hofmeister series. This was first described in relation to the salting out of euglobin[88] and gives an order of activity basically as follows:-

$$Ca^{++} > Mg^{++} > Li^{+} > Na^{+} > K^{+} > Me_4N^{+} \qquad [221]$$

$$CNS^{-} > ClO_4^{-} > I^{-} > Br^{-} > Cl^{-} > F^{-} > SO_4^{=}$$

Ions which are powerful destabilizers also promote the depolymerization of protein systems[221] by favouring increased exposure of the interaction sites to the solvent.

This chaotropic effect of added salts will have some small effect on the dissociation. It will cause the destabilization of the hydrophobic interactions involved, affecting both the products and the reactants.

This, however, is not the only effect operating. The products of the dissociation are polyelectrolytes and therefore are stabilized by the binding of counterions. Metal cations site bind to DNA phosphates. The strength of the binding varies with the cation as follows:-

$$Mg^{++} > Ca^{++} >>> Li^{+} > Na^{+} > K^{+} > Me_4N^{+}$$ [175 176]

At concentrations up to 1M, though, the stabilization of the DNA helix is independent of the cation concerned.[180]

The evidence presented in Chapter 4 suggests that the displacement of histone bound to DNA phosphate by metal cations does play a part in the dissociation. The greater the binding of a cation to phosphate then, the greater is the dissociation caused and this is what is found here. The hypothesis can be put forward that dissociation is brought about by the cooperative binding of metal cations to DNA phosphates, displacing the histone molecules.

Reference to Table 5.1 will show that the variation of the anion used has a greater effect on the extent of the dissociation - even if one is restricted to monovalent electrolytes - than variation of the cation. Sodium perchlorate is three times as 'effective' a dissociating agent as sodium fluoride.

Bonner and his associates have suggested that the activity of the deoxycholate ion in dissociation is due to its binding to DNH.[78] This lowers the stability of the DNH and allows dissociation to take place at lower ionic strength. However, this effect could equally well be described by the stabilization of free histone caused

by the binding of the anion. The large effects of certain anions on the dissociation are also likely to be due to binding of the anions to the histone moiety of DNH.

This emphasises that displacement of histone by cations is not the only mechanism operative in the dissociation. Binding of anion to the histone protein will also play an important part. This is not unreasonable. Histones are thought to contain concentrated polycationic regions in their structure[55,228] which would require considerable neutralisation by counterions to give stability in solution.

The dissociation of DNH into DNA and histone is aided both by the binding of cations to DNA phosphate and of anions to histone basic groups.

6

PRECIPITATION

All preparations of chromatin are based on its insolubility in physiological ionic conditions. In the case of nucleohistone the solubility minimum is at 0.15M-0.2M sodium chloride. At higher salt concentrations the complex becomes soluble once again.

Several observations on this system have been reported in the literature. The 'gel' fraction of chromatin is precipitated at a lower salt concentration than the soluble chromatin.[145 96] A fraction which behaves like euchromatin is more soluble in a given salt concentration than a heterochromatin-like fraction.[114] These results appear to link precipitation to the degree of condensation of chromatin visible in nuclei by light microscopy. Indeed changes in the degree of condensation of nuclear chromatin do take place when isolated nuclei are exposed to solutions of neutral salts.[114 144] These increases in granularity are accompanied by a corresponding reduction in nuclear volume. The changes are reversible on further increase of the ionic concentration. Nucleohistone gels undergo a reversible contraction when subjected to 0.2M sodium chloride.[22]

The appearance of nucleohistone in the electron microscope is altered by the presence of precipitating concentrations of salts.[172 163] Chromatin fibres prepared in the presence of chelating agents have 10nm diameter as opposed to the 25nm diameter typical of fibres from untreated nuclei.[172] Nucleohistone fibres from precipitated samples have diameters of up to 50nm dependent on the ionic concentration.[163]

Observations on the precipitation of soluble chromatin

have shown that the extent of precipitation is dependent on the initial concentration of chromatin.[100] Removal of the lysine-rich histones from the complex is reported to render the chromatin completely soluble even in 0.2M salt.[68 13 143] This together with other results has led to the suggestion that f1 has a specific role to play in the high level organisation of chromatin. A model involving f1-f1 cross-linking between DNH strands has been proposed.[22]

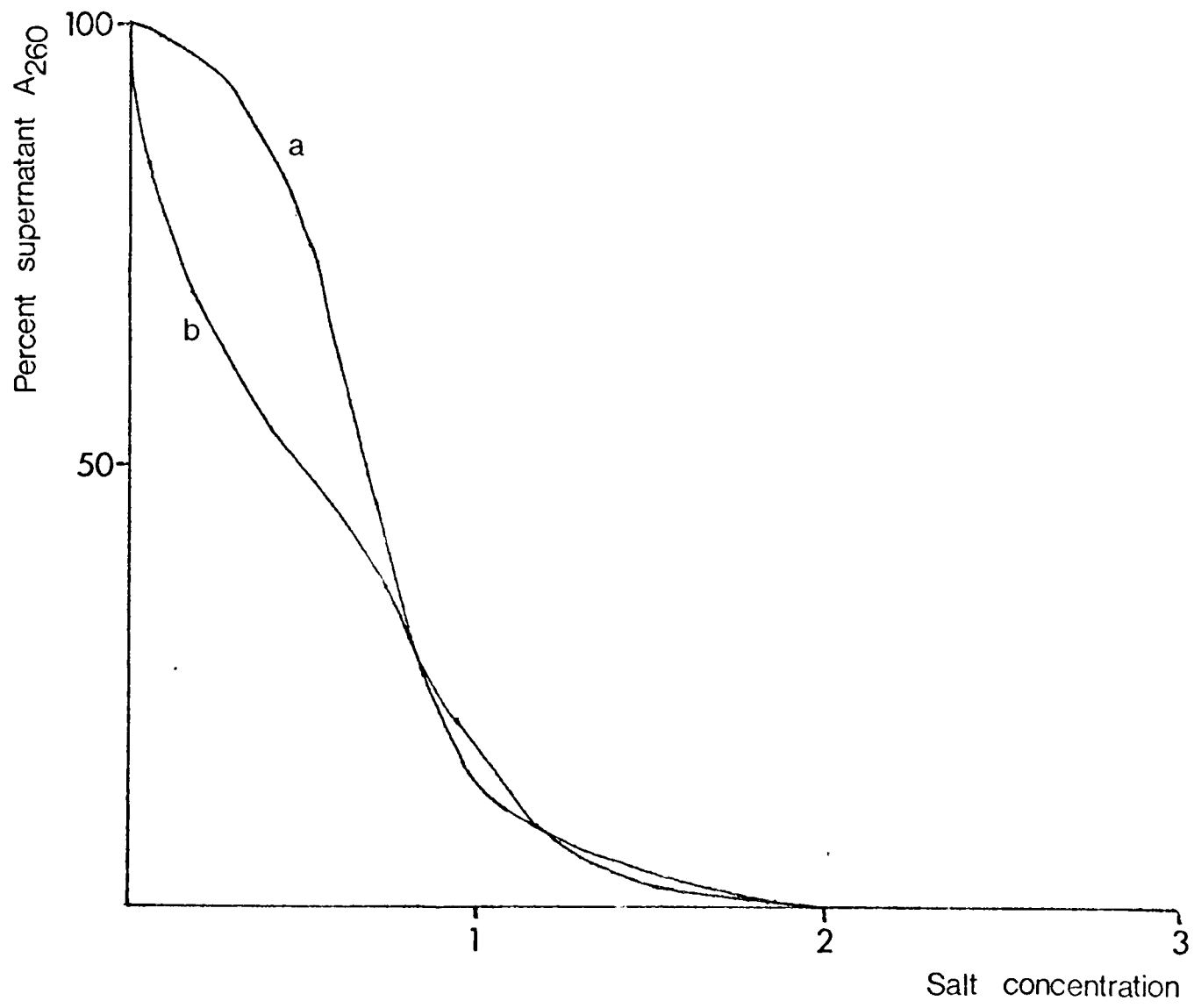
Not all salts give maximum precipitation of chromatin at the same concentration, alkaline earth salts being much more effective than salts of the alkali metals.[114 21] In particular, magnesium salts are effective at an hundredfold lower concentration than sodium salts.

These investigations have left a number of problems concerning the precipitation of chromatin unsolved. The most important of these is the mechanism of the precipitation itself. Is the solubility minimum caused solely by neutralisation of the DNA-phosphate groups? Leake[114] has suggested that the precipitation curve of chromatin is identical with the binding curve of the cation concerned to DNA phosphate.

Does the f1 fraction play a specific role in the mechanism of the precipitation? The effect produced on the precipitation by its removal could as well be explained by the change in total charge on the chromatin.

It was to answer questions like these that the work described below was carried out. The studies of precipitation by solutions containing more than one cation were undertaken both to throw light on the mechanism of precipitation and also to investigate the physical state

Fig 6.1 Typical precipitation curves of DNH



of chromatin under quasi-nuclear conditions. The physicochemical studies of nucleohistone structure in low ionic concentrations were carried out with the aim of providing a description of the precipitated state which could be related to the evidence from light and electron microscopy.

1. The solubility of DNH in solutions of single salts

As with the dissociation curves described in Chap. 3, the precipitation curves shown below vary slightly from preparation to preparation. Slight changes in precipitation become apparent during the lifetime of a preparation. Eventually, a specimen of DNH will become completely soluble at all salt concentrations. This loss of precipitability is presumably due to the digestion of considerable amounts of bound protein by the endogenous protease activity. No experiment has been used in which less than 80% of the DNA was precipitated at the maximum of the solubility curve.

The precipitation is dependent on DNH concentration.[100] Experiments were not all carried out at the same concentration. The range used was 0.003-0.006% DNH (50 to 100μM DNA phosphate). The extent of precipitation by 0.15M sodium chloride in this range varies by only 5% of the total A_{260}.[155] This variation is within the limits of accuracy of the experiment -±5% of the total A_{260}.

The shape of the precipitation curve also varies with the preparation. Fig 6.1 shows typical precipitation curves. Curve a is typical of the preponderant type, a sigmoid curve as reported by other

Table 6.1: The precipitation ranges of DNH with various cations

Cation	Precipitation		Redissolution
	Salt range	Half-precipitation	salt range
Na^{+}	0-200mM	85mM	300-600mM
NH_4^{+}	0-100mM	30mM*	130-220mM
Mg^{++}	0-2mM	760μM	100-200mM
Mn^{++}	0-1mM	500μM	

*one determination only

investigators.[145 96 114] However certain preparations are consistently less soluble at lower salt concentrations giving a curve of type b. There remains one unchanging feature of the precipitation and this is the range of salt concentrations over which it takes place. These ranges vary with the cation involved as shown in Table 6.1.

Typical curves for the precipitation of DNH in solutions of sodium and magnesium chlorides are shown in Figs 6.2 and 6.3 respectively. These data are of the same order of magnitude as those reported by Leake[114 113] for erythrocyte chromatin. The Na^+ precipitation reported here agrees very closely with his K^+ precipitation. The data for the other ions are not in such good agreement. The differences may be due to differences in procedure or in chemical differences between the two nucleoproteins. Leake's experiments were performed throughout in the presence of metal-free 0.25M sucrose, ours in 0.7mM sodium phosphate buffer pH6.8.

Samples of DNH become soluble again when the ionic strength of the medium is raised above the precipitation point. The ionic concentration at which this redissolution takes place is once again dependent on the cation concerned (Table 6.1). This salt range coincides with that over which histone begins to be dissociated from the complex. As has been remarked in Chap.3, f1 histone is already completely dissociated at 0.5M sodium chloride. A 0.1M solution of magnesium chloride will also dissociate this fraction.[103] A considerable quantity of protein is removed by 0.4M ammonium sulphate[29] - this is

Fig 6.2 Precipitation of DNH and f1-depleted DNH in solutions of sodium chloride

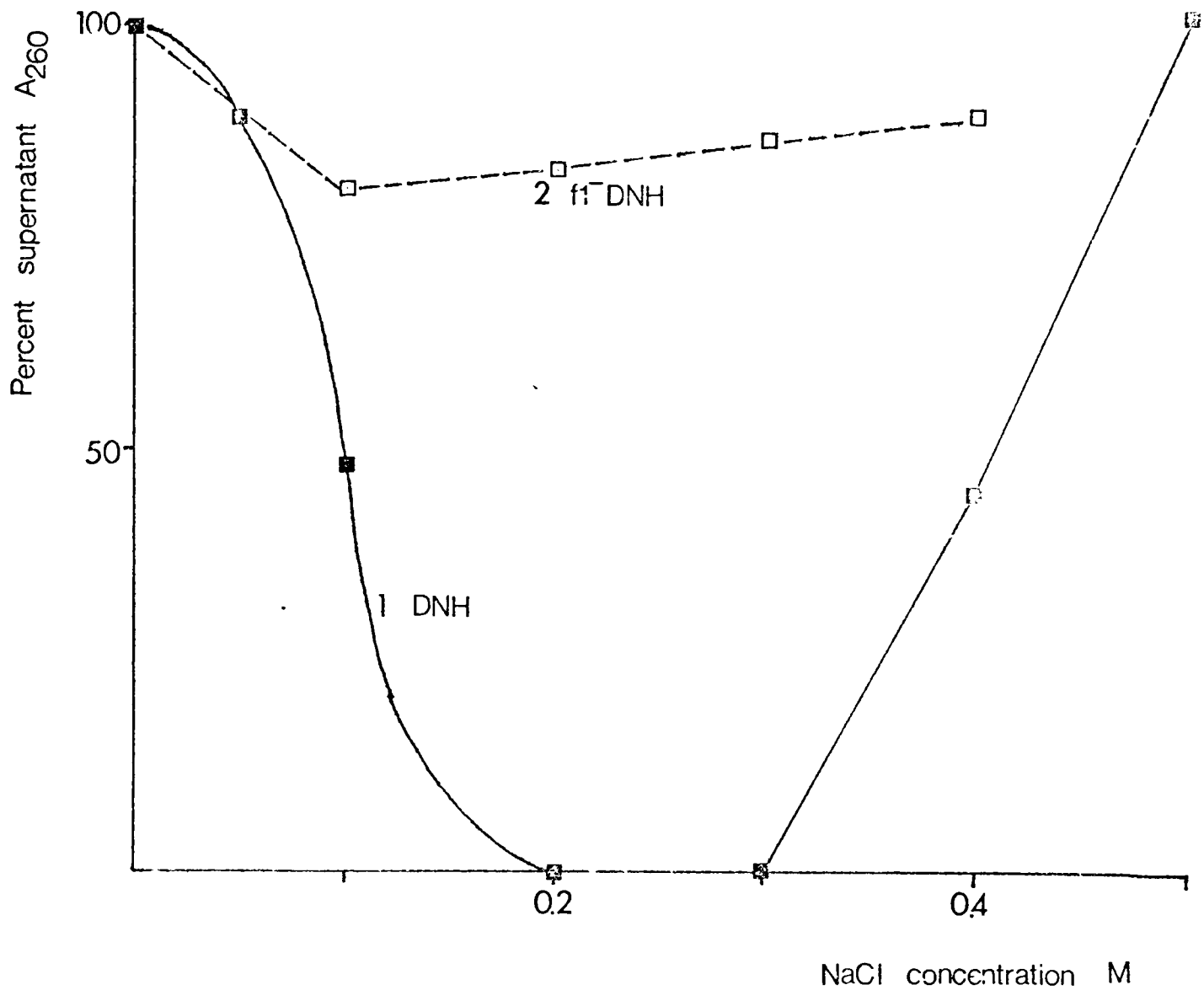
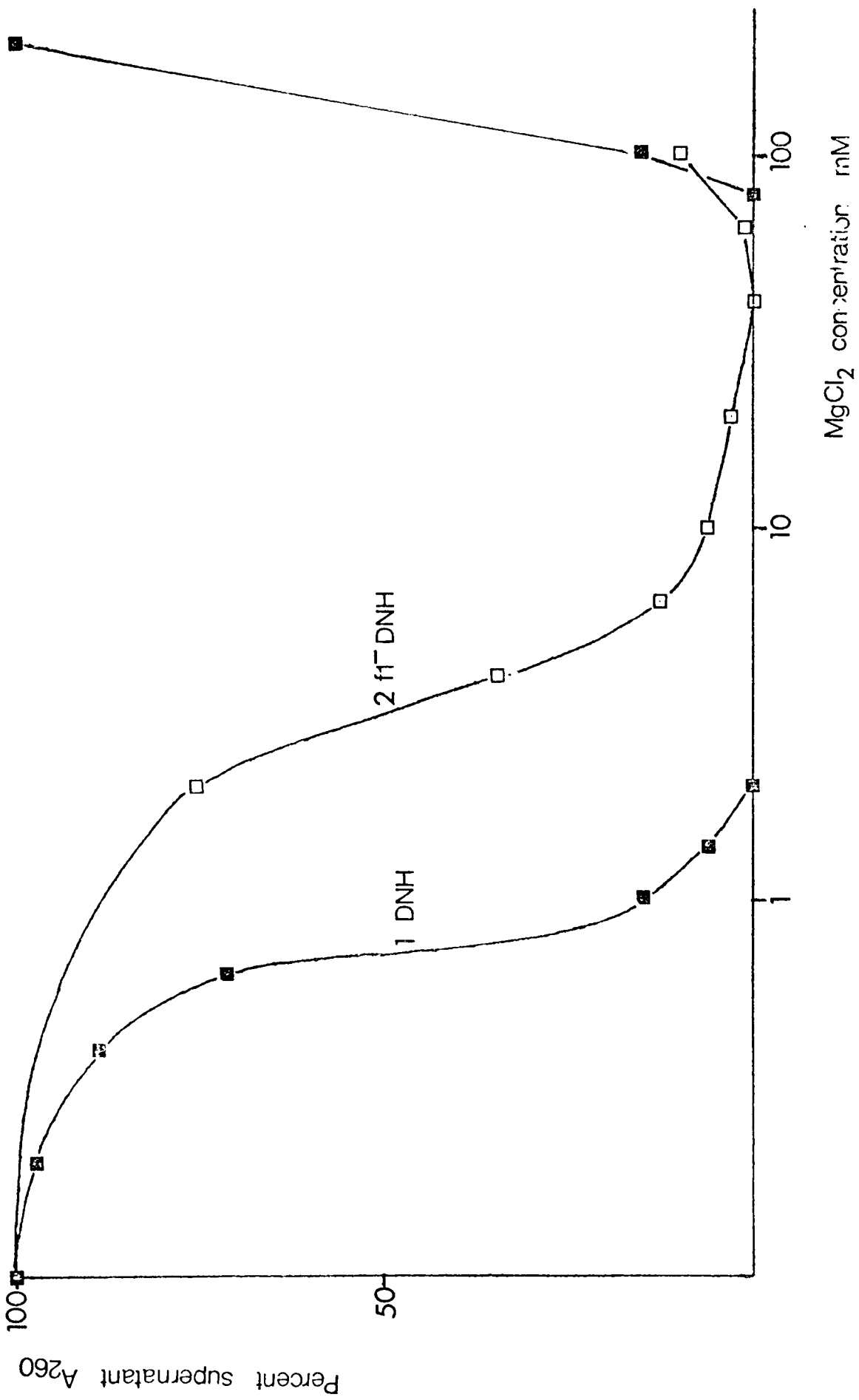


Fig 6.3 Precipitation of DNH and f1-depleted DNH in solutions of magnesium chloride



mainly f3.

Removal of the f1 histone renders chromatin soluble in physiological salt solution.[68,143] This observation is confirmed for our system by curve 2 of Fig 6.2. However, our f1-depleted DNH, prepared by the methods outlined in Chap.2, will precipitate at a sufficiently high molarity of magnesium chloride (curve 2, Fig 6.3). Greater than 90% of the A_{260} of such a sample is precipitated in 10mM Mg^{++}. f1-depleted DNH of this concentration will not precipitate in sodium chloride solutions of any molarity.

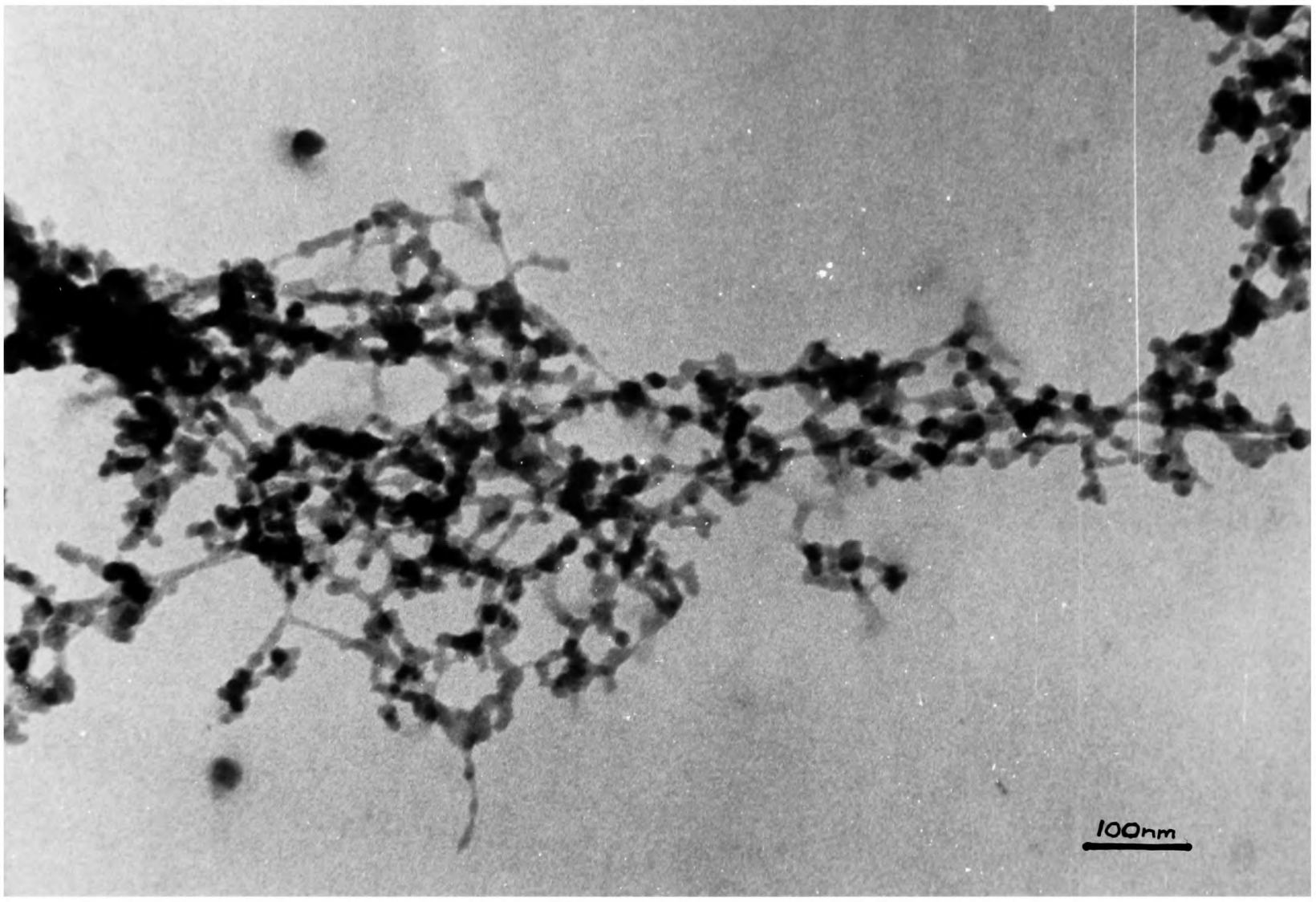
Further studies in this laboratory have shown that f1-depleted DNH can be induced to precipitate in 0.15M sodium chloride if a much higher initial DNH concentration is used. At an initial concentration of ~350μM in DNA phosphate, the f1-depleted DNH is precipitated to >70% under these conditions.[155]

Electron microscope studies, performed in collaboration with Dr. A. Pooley of Searle Research Laboratories, High Wycombe, have been carried out to investigate the precipitation of f1-depleted DNH.

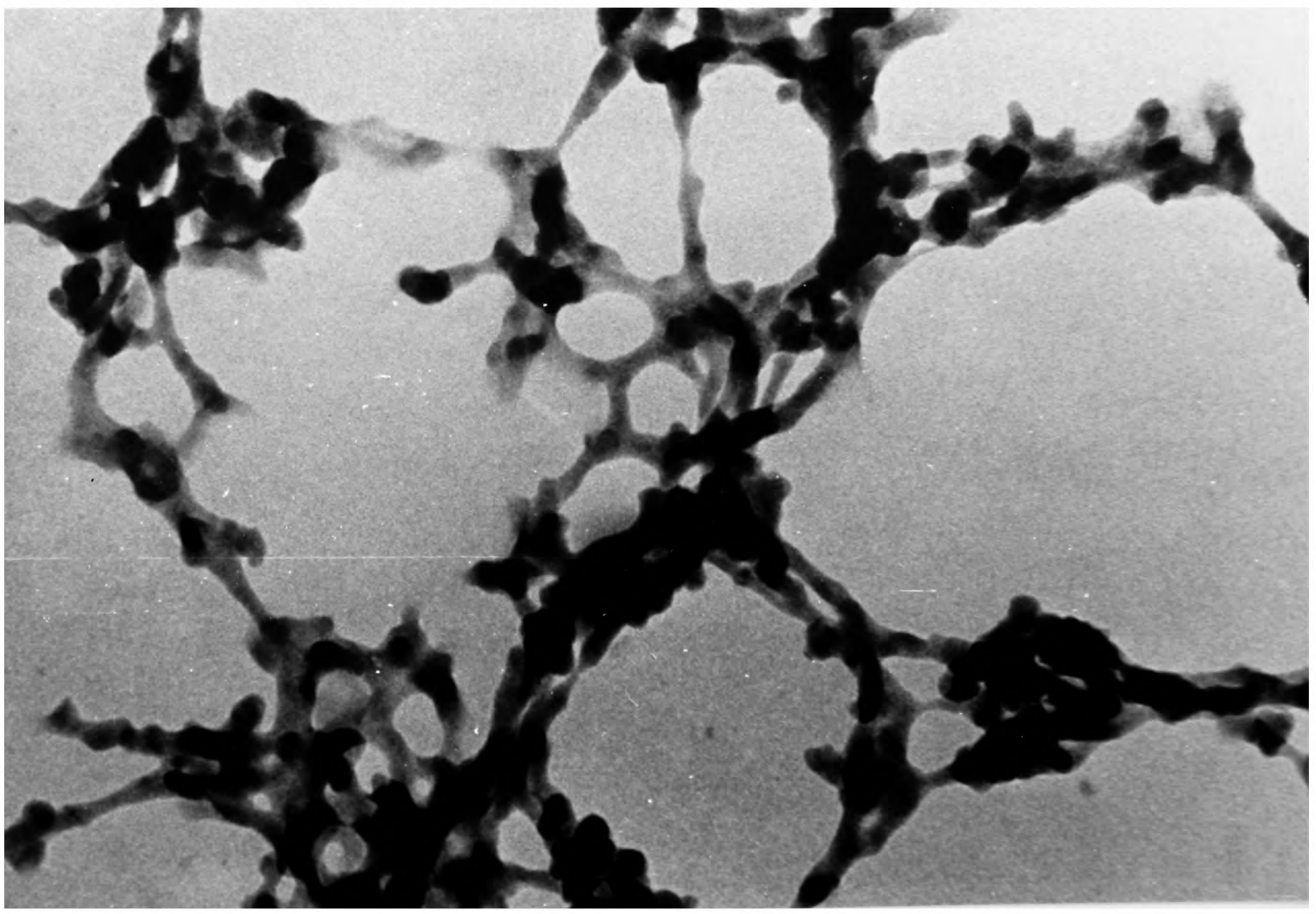
In a so-far unpublished investigation, Pooley and his colleagues have shown that the fibre diameter of DNH increases to the order of 50nm on the addition of precipitating concentrations of magnesium chloride. In the electron microscope, soluble DNH has an average fibre diameter of 8-10nm (Fig 6.4a). On pretreatment with Mg^{++}, the DNH shows diameters initially around 20-25nm (Fig 6.4b) but eventually, on further increasing the salt concentration becoming in excess of 50nm. The initial

Fig 6.4 Electron micrographs of DNH treated with solutions of magnesium chloride

a DNH

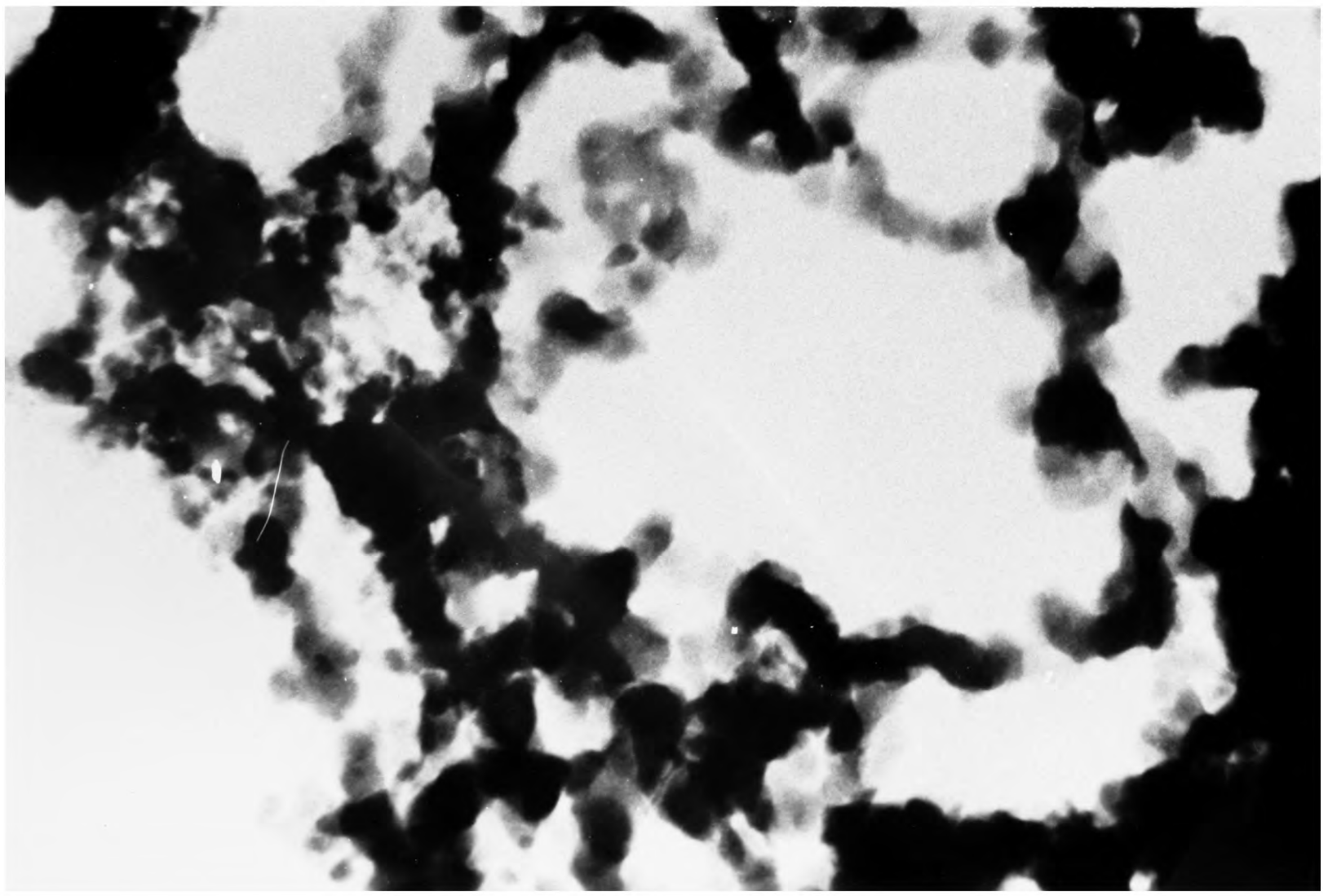


b DNH treated with 0.5mM $MgCl_2$



Magnification - 130,000

C DNH treated with 3mM $MgCl_2$



d Sample c washed extensively with EDTA

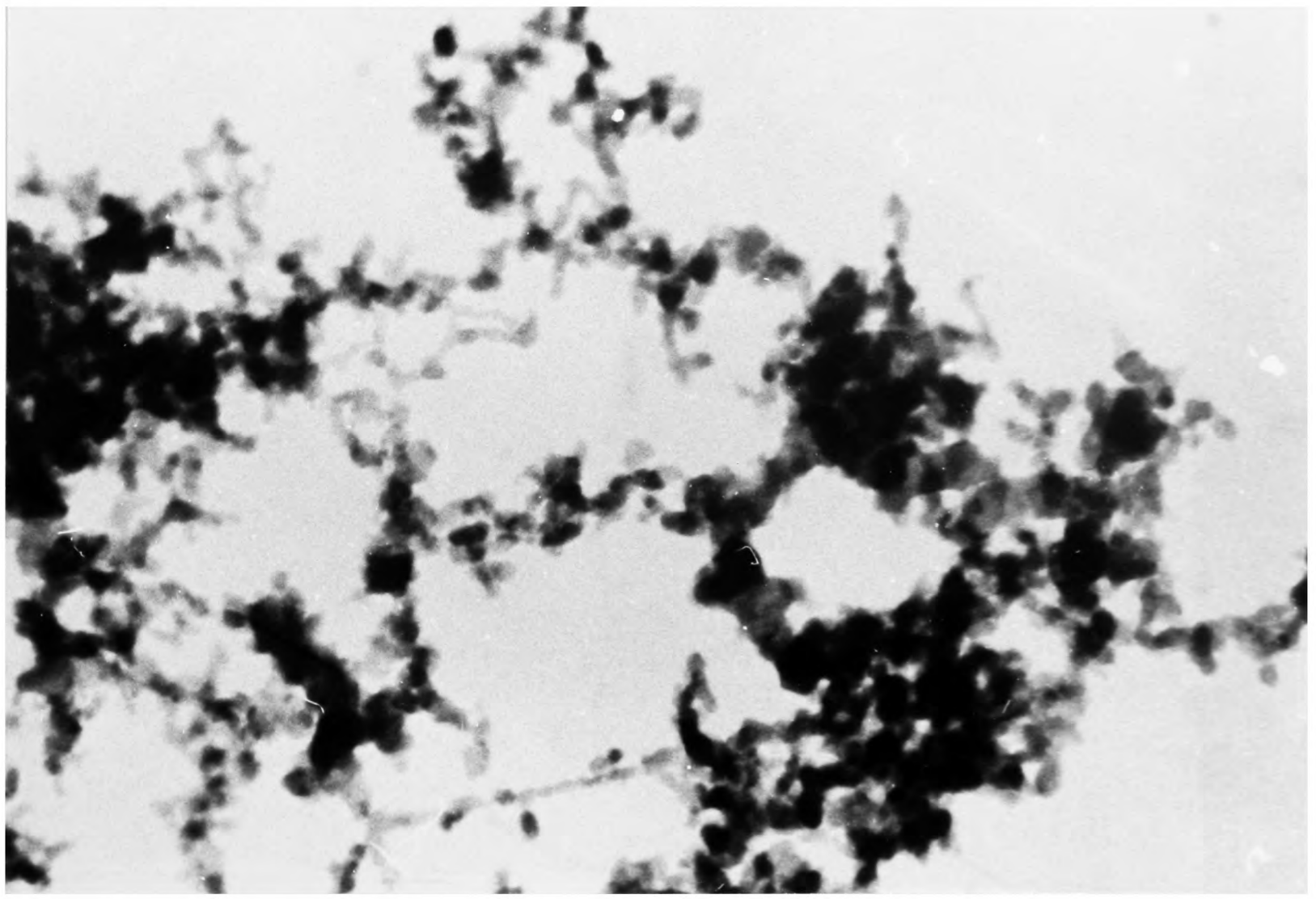
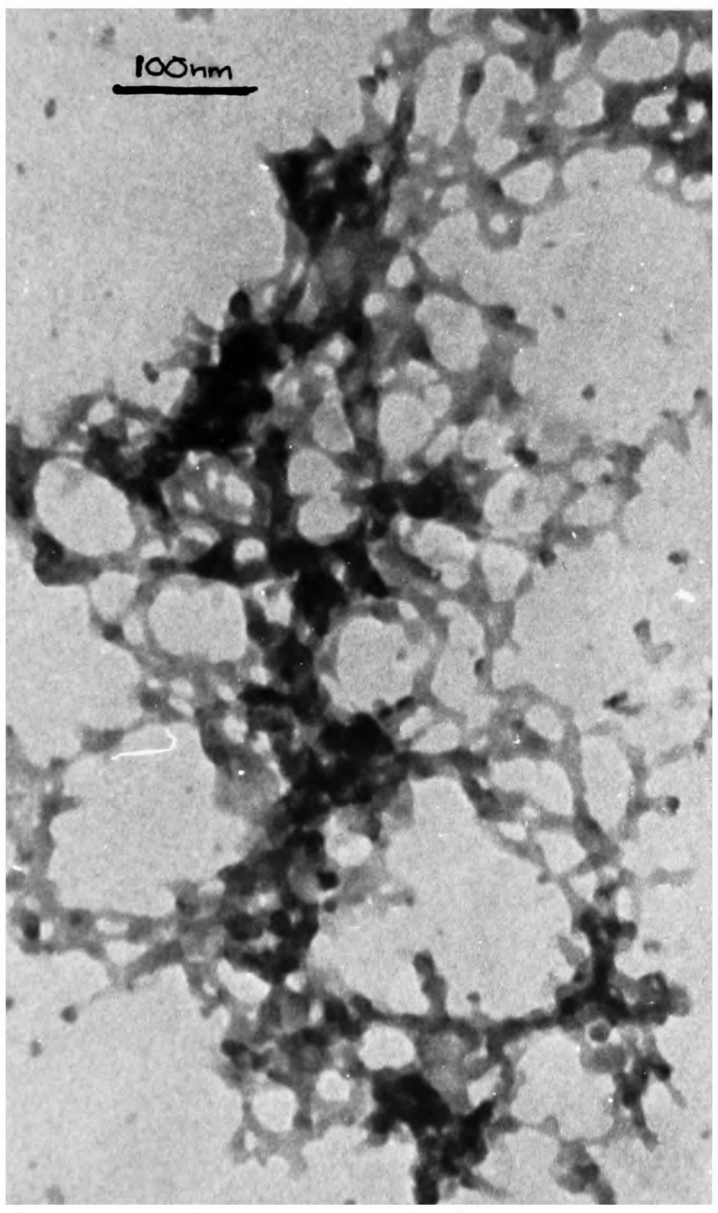
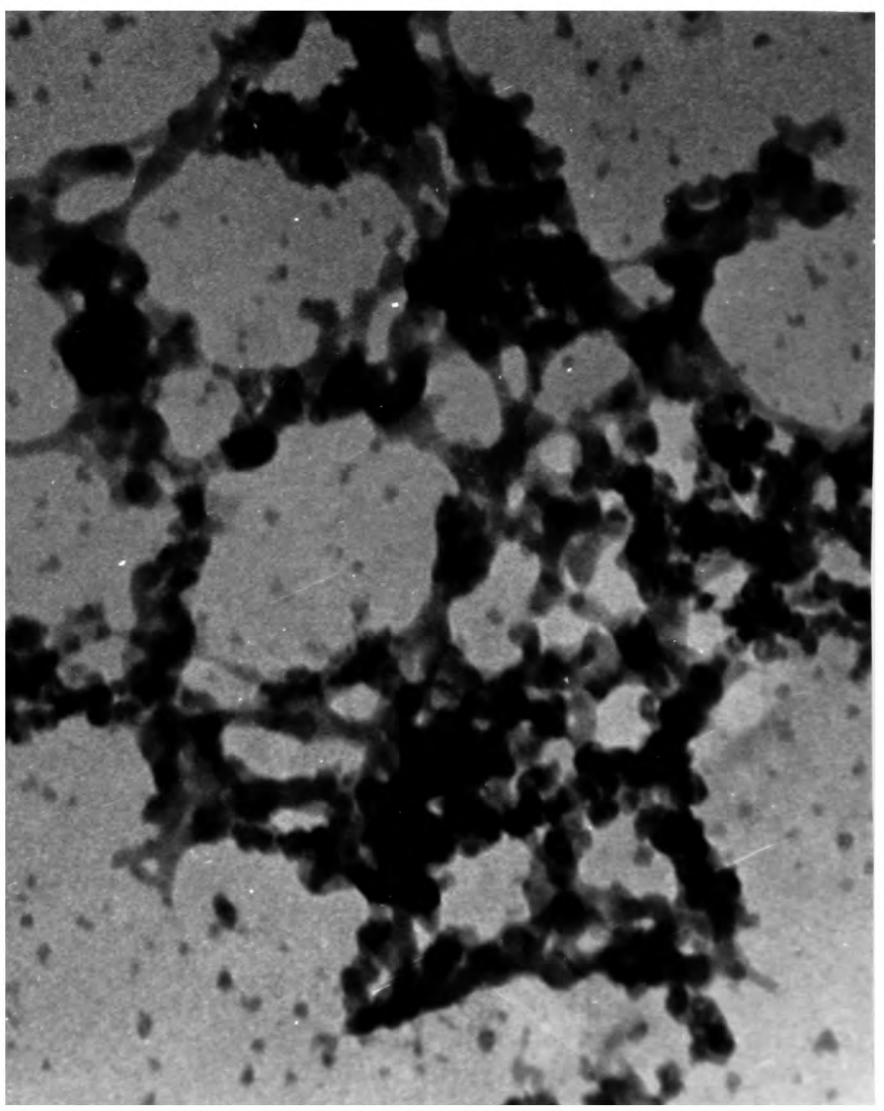


Fig 6.5 Electron micrographs of f1-depleted DNH treated with solutions of magnesium chloride

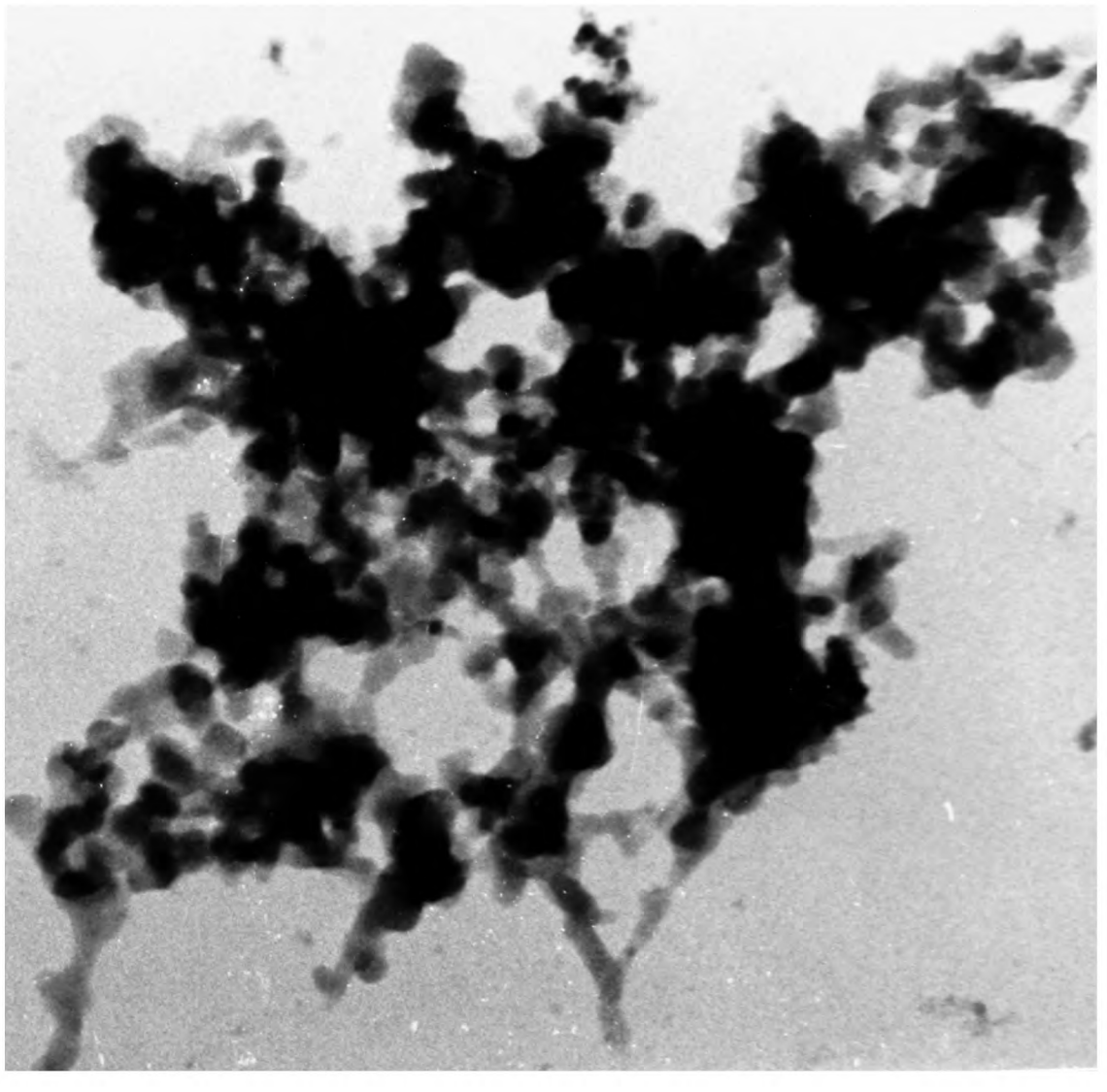
a $DNH^{0.7}$



b $DNH^{0.7}$ + 2mM $MgCl_2$



C $DNH^{0.7}$ + 50mM $MgCl_2$



increase takes place at less than 500μM Mg^{++}. The changes can be reversed by leaching out the magnesium ions with a suitable concentration of EDTA (Fig 6.4c).

The f1-depleted material showed a similar effect. The untreated material has few fibres with diameters in excess of 15nm (Fig 6.5a). In 2mM Mg^{++} the average fibre diameter has considerably increased but even in 50mM Mg^{++} it has not reached the order of thickness shown by the native DNH (Fig 6.5b and c).

Despite the limitations of electron microscopy, which were stressed in Chap. 1, this evidence is valuable. The precipitation of both native DNH and the f1-depleted material are accompanied by condensation of the fibres seen in the electron microscope. Precipitations of the two species are not entirely dissimilar processes.

The solubility of low concentrations of f1-depleted DNH in physiological salt solutions has been held to argue for a specific role for f1 in the precipitation. The analogue of precipitation with the condensation of nuclear chromatin has allowed this hypothesis to be extended.[68 13] f1 is postulated to play a large part in the high-level organisation of chromatin.[22]

An alternative hypothesis can be advanced. The precipitation of DNH in salt solutions depends on a non-specific neutralisation of charge. Binding of metal cations to DNA phosphate groups leads to removal of the complex from solution. The removal of protein from DNH will reveal more DNA phosphate sites and thus lead to precipitation at higher ionic strength. This is what

is observed for f1-depleted DNH in solutions of magnesium chloride. The removal of histone II by sodium deoxycholate gives a DNH which is soluble in 0.2M sodium chloride.[199] This is similar to the behaviour of f1-depleted material in sodium chloride. Depleted DNH samples do not precipitate in sodium chloride solutions because, before the ionic strength necessary for precipitation is achieved, further protein is dissociated from the complex thus releasing more DNA phosphate sites. The redissolution of precipitated native DNH also results from dissociation of protein from the complex thus increasing the charge.

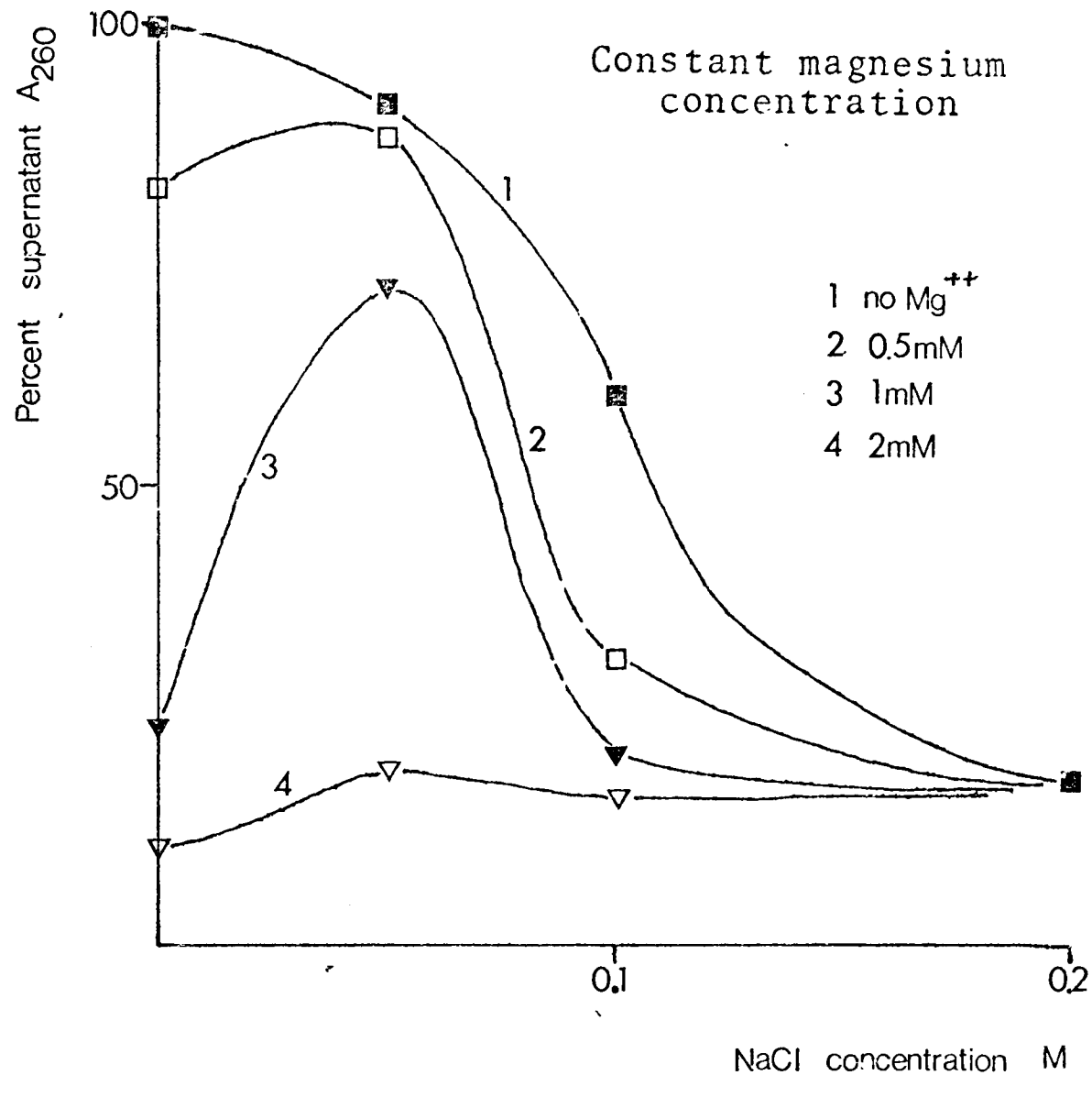
This hypothesis would predict that pure DNA can be precipitated from solution by addition of salts. Reports of the precipitation of DNA by lithium[79] and magnesium ions[181] can be found in the literature.

The hypothesis does not deny that f1 can play a role in the condensation of chromatin in vivo, it merely affirms that it does not play such a role in the precipitation of nucleohistone. The special conditions of the nucleus may well constrain f1 to play a specific role. This cannot be supported from evidence collected in vitro.

2. Precipitation of DNH in solutions of mixed cations

The ionic environment of the nucleus contains both monovalent and bivalent cations in considerable quantities.[191] If the condensation of chromatin in vivo is influenced by changes in this environment it will not be under conditions like those used in the previous experiments. For this reason, experiments were carried out to measure

Fig 6.6 Precipitation with mixtures of sodium and magnesium chlorides



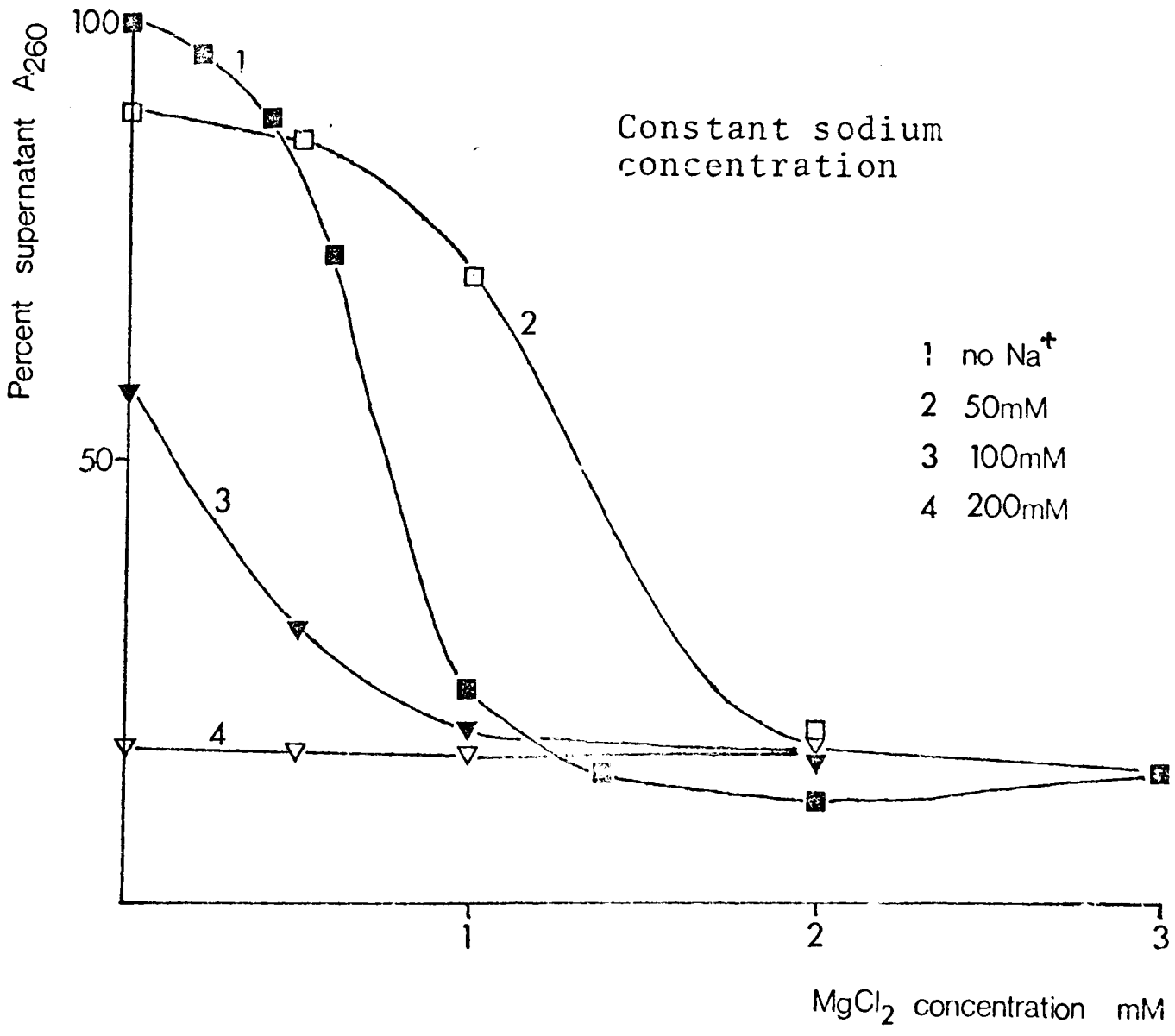
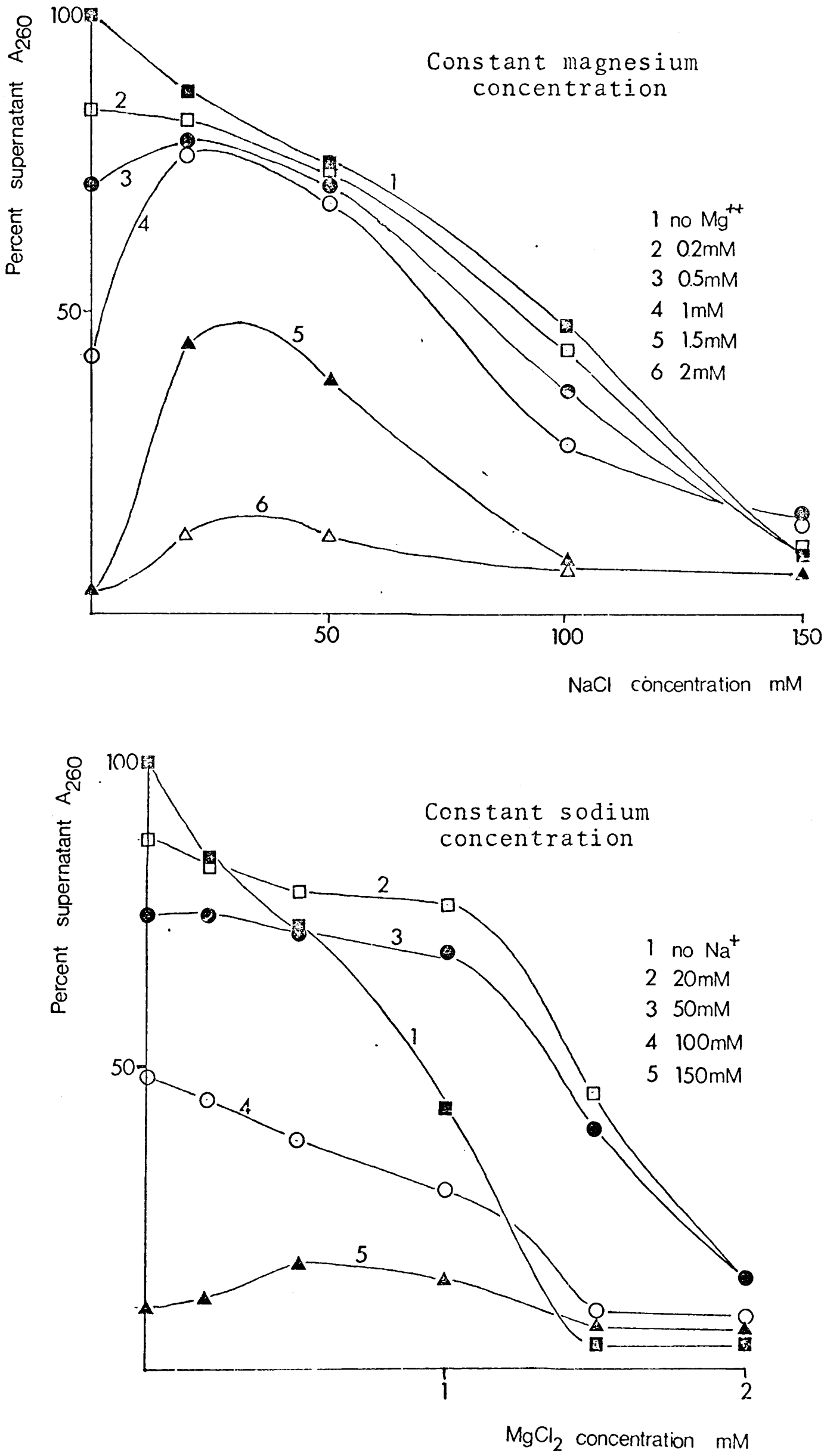


Fig 6.7 Precipitation with mixtures of sodium and magnesium chlorides



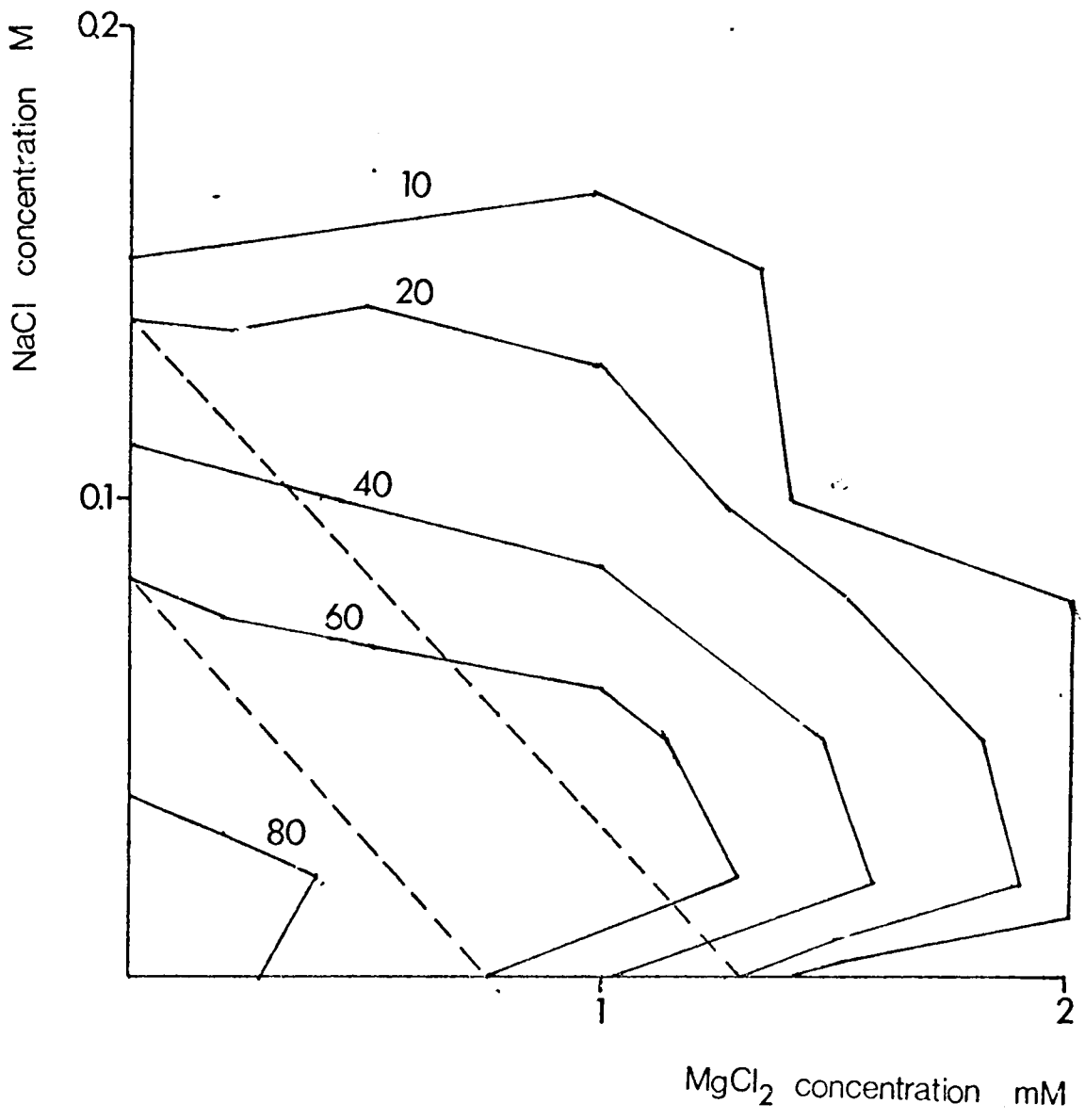
the extent of precipitation of DNH by bivalent cations in the presence of monovalent and vice versa. Such experiments also have diagnostic value when discussing the mechanism of precipitation.

The results obtained by precipitating DNH with mixtures of sodium and magnesium chlorides are shown, for two different preparations, in Figs 6.6 and 6.7. A similar effect can also be obtained by addition of concentrations of one ion to a DNH solution already treated - and presumably precipitated - with the other. These precipitation curves are not parellel, as would be expected if the cations had additive effects, but show definite indications of antagonism. The information from the curves can also be plotted as a phase diagram as shown in Fig 6.8 (from the data of Fig 6.7). The dotted contours are those that would be expected for an additive effect.

Fig 6.8 enables the formulation of a general rule to cover these data. When DNH is treated with a solution of xmM NaCl and ymM $MgCl_2$ then it will precipitate to an extent which is greater than the precipitation in an x mM solution of NaCl but less than in ymM $MgCl_2$. This applies for all x and y within the precipitation ranges of salt concentration except where $x>100y$.

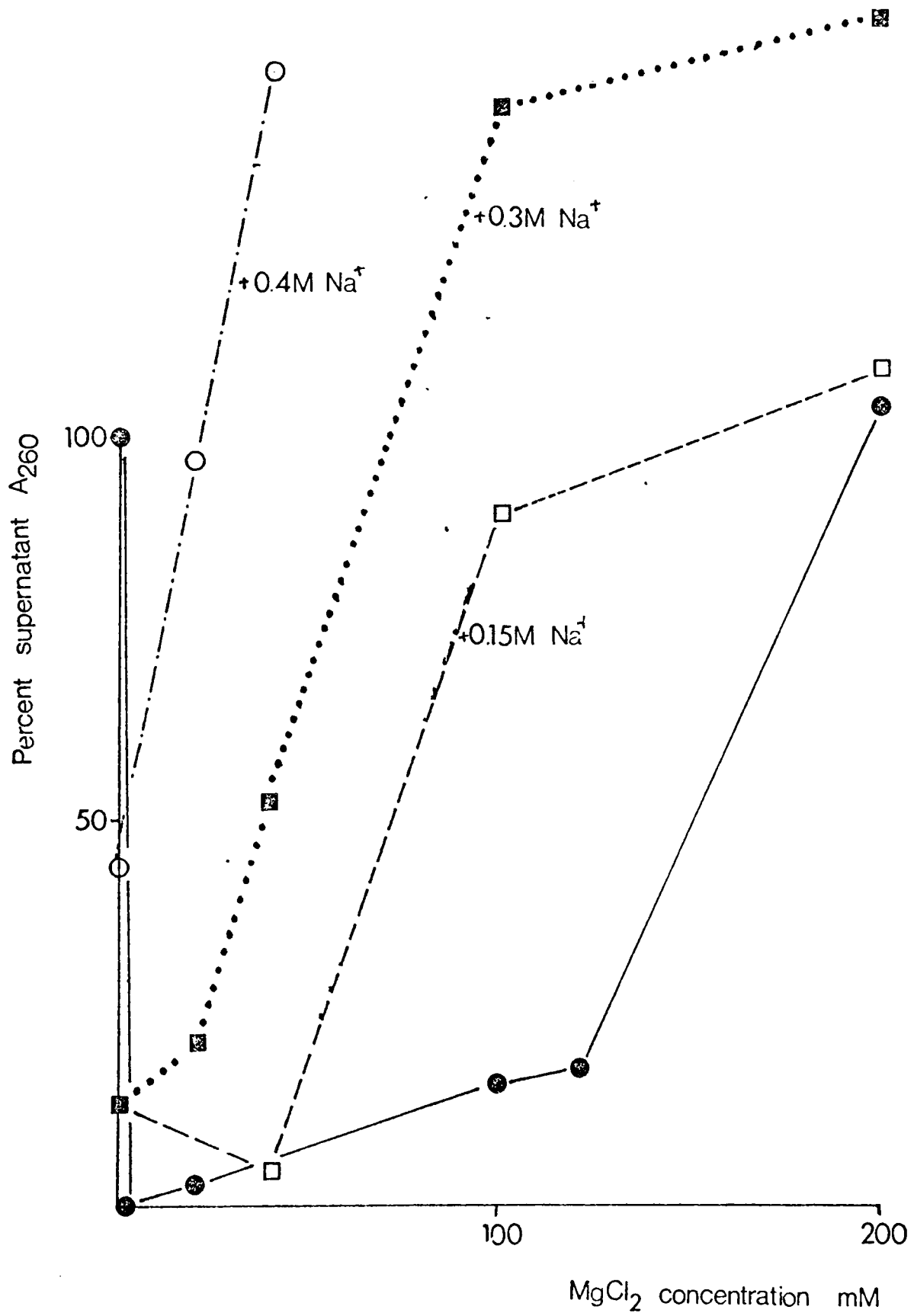
At the higher ionic concentrations required for the redissolution of the DNH the position is less anomalous. Fig 6.9 shows that addition of further ions to a solution of sufficient concentration to precipitate DNH will redissolve the DNH. No antagonism between mono- and bivalent cations is evident in the data. It would seem that any binding of cations which takes place during this

Fig 6.8 Phase diagram for precipitation in mixtures of sodium and magnesium chlorides



Contour lines join those solutions with the same extent of precipitation

Fig 6.9 Redissolution in mixtures of sodium and magnesium chlorides



process is not at the same sites as those involved in the precipitation. If it were, competition between the added cations and those already bound would be expected.

The hypothesis that redissolution is due to dissociation of histone would predict that more binding sites for cations would become free during this process.

3. Reversibility of precipitation

The reversibility of the precipitation of DNH by magnesium ions was investigated. A sample of DNH was subjected to a precipitating concentration of magnesium and then either diluted or treated with small amounts of sodium EDTA.[155] These attempts to reverse the precipitation were not wholly successful as shown in Fig 6.10. However some dissolution of the precipitated material did take place as shown by the hysteresis effect.

It is highly possible that the lack of reversibility shown here is a kinetic effect. Material precipitated in 0.2M sodium chloride can be redissolved by dialysing against 0.7mM phosphate buffer pH6.8. And indeed the addition of sodium chloride in certain concentrations to magnesium precipitated material results in a greater solubility (see previous section).

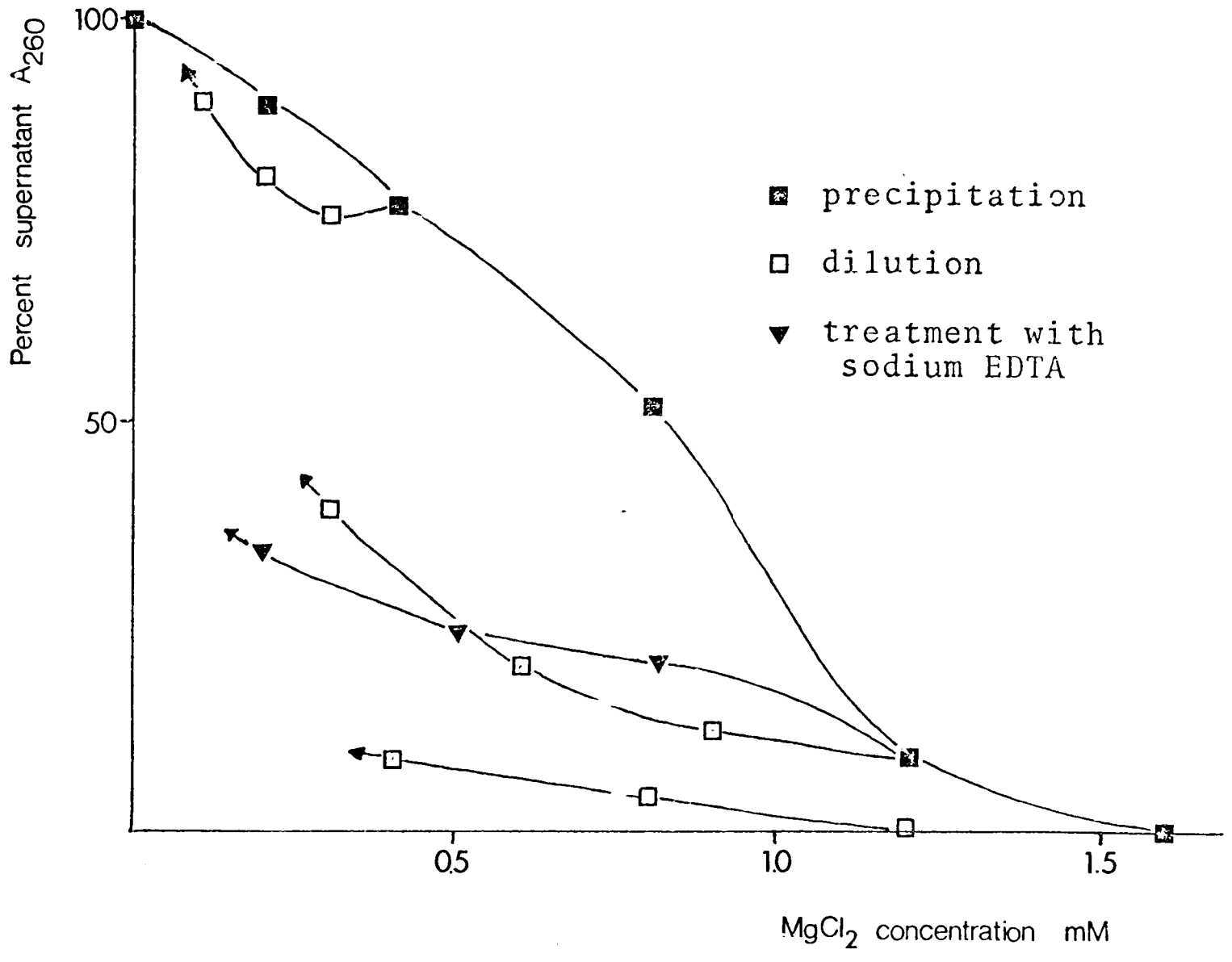
4. Conclusion

The principle features of the precipitation of DNH in low concentrations of salts can be outlined thus:-

a) The extent of precipitation is dependent on the concentration and nature of the cations present.

b) The extent of precipitation is dependent on the initial concentration of DNH and on the amount of histone

Fig 6.10 Attempt to reverse precipitation of DNH by magnesium chloride



bound in the DNH.

c) The effects of Mg^{++} and Na^{+} ions on the DNH are not additive.

It has often been assumed that charge neutralisation of the DNH is a necessary and sufficient condition for its precipitation. This assumption culminated in Leake's suggestion[113] that the precipitation could be expressed as a binding curve of the form:-

$$M + A \underset{}{\overset{K}{\rightleftharpoons}} MA \qquad 6.1$$

$$K = \frac{[MA]}{[M][A]}$$

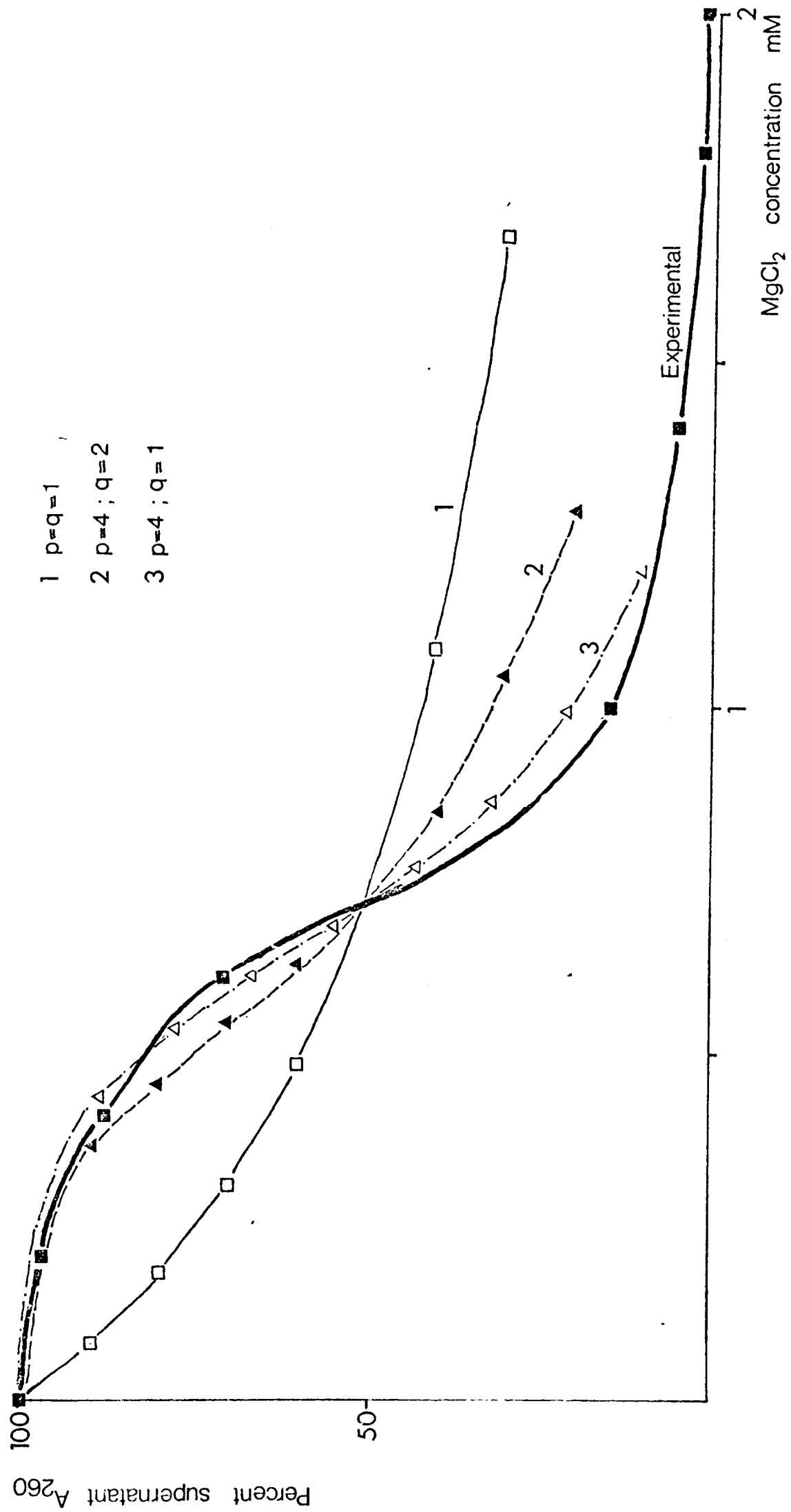
where M is the cation concerned and A are the binding sites. All the bound sites, MA, were assumed to be precipitated. Analysis of Leake's data by this model led to the conclusion that the binding sites could be identified with DNA phosphate[114].

However, this model is a most unsatisfactory one for a number of reasons. Firstly, the model will not produce a dependence of precipitation on DNH concentration. Secondly, the assumption that the precipitation curve is the binding curve for the appropriate cation is not confirmed by work on DNA. There is considerable evidence[184 50] to show that the binding of magnesium to nucleic acids is strong and occurs at magnesium concentrations of the same order as the DNH concentration. Concentrations of salt an order of magnitude higher than the DNH concentration are required to effect the precipitation.

Lastly, a binding curve as equation 6.1 would be a parabola asymptotic to the ordinate at infinity (Fig 6.11).

This is in marked contrast to the experimental

Fig 6.11 Precipitation curves derived from theoretical equation 6.2



precipitation curves which, even in Leake's own study, are sigmoidal. To mimic the experimental curve, an equilibrium of the form:-

$$pM + qA \overset{K}{\rightleftharpoons} M_pA_q \qquad 6.2$$

$$K = \frac{[M_pA_q]}{[M]^p[A]^q}$$

must be postulated. Several curves of this form are shown in Fig 6.11. When p is considerably larger than one, these quite closely approximate to the experimental curve. A model of this type will give good agreement with the experimental dependence of precipitation on DNH concentration, provided q >1.

A general equilibrium model can be used to describe the precipitation. The application of such a model, however, is dependent on the precipitation being thermodynamically reversible. This has not yet been proved although the evidence is suggestive of such a conclusion.

The high values of p necessary to obtain an empirical fit of the experimental precipitation curve need not imply a high cooperativity for the binding. The identity of binding and precipitation is not essential to this model. More than one equilibrium can go to make up the blanket equilibrium of equation 6.2.

Binding of metal cations to nucleic acid phosphate takes place with a stoicheiometry of one cation per phosphate even in the case of divalent ions such as magnesium.[50 196] It is likely that the sigmoid shape of the precipitation data is related to the presence of protein bound to the DNA.

Further insight into the mechanism of precipitation can be obtained by considering the effect on DNA of mixtures of cations.

A mixture of sodium and magnesium ions precipitates DNH less well than the same concentration of magnesium alone. This suggests that the two cations bind competitively to the same sites but effect the precipitation by different mechanisms.

If the binding of the two cations were at different sites then their effects would be additive. Binding to the same sites would result in competition. Less binding of each cation would take place than would be produced by that cation alone but the total charge neutralisation would be greater than the single cation case. Therefore, if the precipitation of DNH were produced non-specifically by charge neutralisation, a greater degree of precipitation would be expected.

Binding of magnesium ions to DNA is greatly reduced by the presence of sodium chloride.[50,183] In the competitive situation, considerably more sodium is likely to be bound than magnesium. If we postulate that the intermolecular interactions that mediate the precipitation can be better stabilised by the presence of magnesium ions than sodium ions, then the anomalous precipitation of the mixed cation case can be explained. The small amount of magnesium bound will cause much less precipitation than that bound in the presence of magnesium alone. The presence of bound sodium will not increase this precipitation much since it weakly stabilises the necessary interactions.

There is likely to be less precipitation than in the magnesium only case but more than in the sodium only case.

A potent modification of this hypothesis would be the assumption that magnesium ions stabilise the intermolecular interactions by cross-linking sites on different DNA strands. The presence of bound sodium ions would actually destabilise this situation since it would diminish the concentration of DNA phosphates available for cross-linking.

It is necessary to postulate that the precipitating cations have other effects than those of charge neutralisation in order to explain these data. The binding of cations to DNA phosphates and the attendant charge neutralisation is a necessary but not a sufficient condition for precipitation.

7

LOW SALT STRUCTURE

The variations in solubility, so typical of chromatin, which have been described in the previous chapter, have imposed corresponding limitations on the investigation of the solution structure of DNH. Almost without exception, workers have favoured a solvent of low ionic strength containing no divalent metal ions. Indeed, divalent ions are eliminated from samples of DNH during their preparation by constant washing with a chelating agent. This procedure has the aim of inhibiting the activity of endogenous lytic enzymes[233] but also has the effect of removing from the chromatin ionic species which may have a structural role to play in vivo.

While estimates of the concentration of ions in the nucleus are available[191], the ionic conditions within the nucleoplasm are harder to investigate. Considering the strength of the binding of magnesium to DNA[178] it seems likely that nuclear chromatin is closely associated with divalent ions. Nucleohistone extracted in the absence of chelating agents has been estimated to contain 10μM calcium ions[97].

The interaction of both monovalent and divalent cations with DNA and their effect on DNA structure is a subject well covered in the literature[58]. Similar studies on DNH however have been confined to the ranges of ionic concentration in which histone is dissociated from the complex. To obtain physicochemical measurements at lower cation concentrations in the absence of artefacts due to precipitation is by no means an easy matter and requires the use of concentrations well below the precipitation range.

The experiments reported below are confined largely to the effects of magnesium and, to a limited extent, sodium ions. It was hoped that the experiments would yield insights not only into possible *in vivo* structures for DNH but also into the processes which precede the gross precipitation effects described in the last chapter.

Samples for this work were obtained in one of two ways:-

a) by the addition to solutions of native DNH of small quantities of neutral salts. Such additions were made either by mixing or by dialysis;

b) by the preparation of fresh samples of DNH using methods not involving the leaching out of divalent metals. These methods are described in Chapter 2 and led to the DNH-Mg samples.

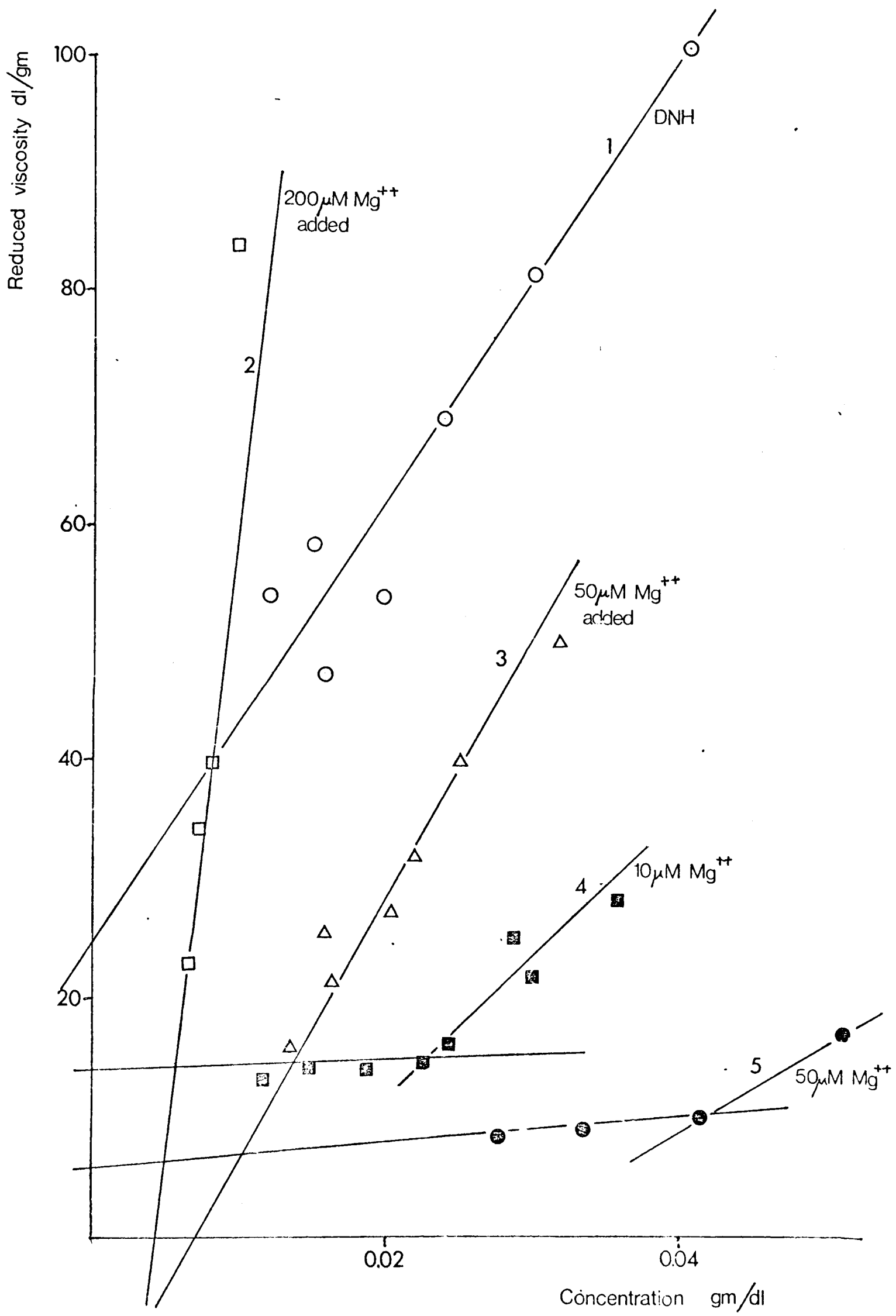
Some measurements were also made on samples that had been subjected to precipitating amounts of salt solutions. Artefacts stemming from this condition make the drawing of conclusions from such experiments very difficult.

As in Chapter 3, a variety of methods was used to probe various levels of DNH structure under these conditions. Hydrodynamic methods, such as viscosity and sedimentation velocity, gave information on the shape of the complex and on possible quaternary interactions. Circular dichroic spectra and thermal denaturation studies probed the secondary structure of the DNA backbone. Enzyme digestion studies yielded details of the arrangement of the histone molecules on the surface of the complex.

1. Viscosity

Native DNH has an intrinsic viscosity of about 20dl/gm[82]

Fig 7.1 Viscosity of DNH in solutions of magnesium chloride - reduced viscosity/concentration plot



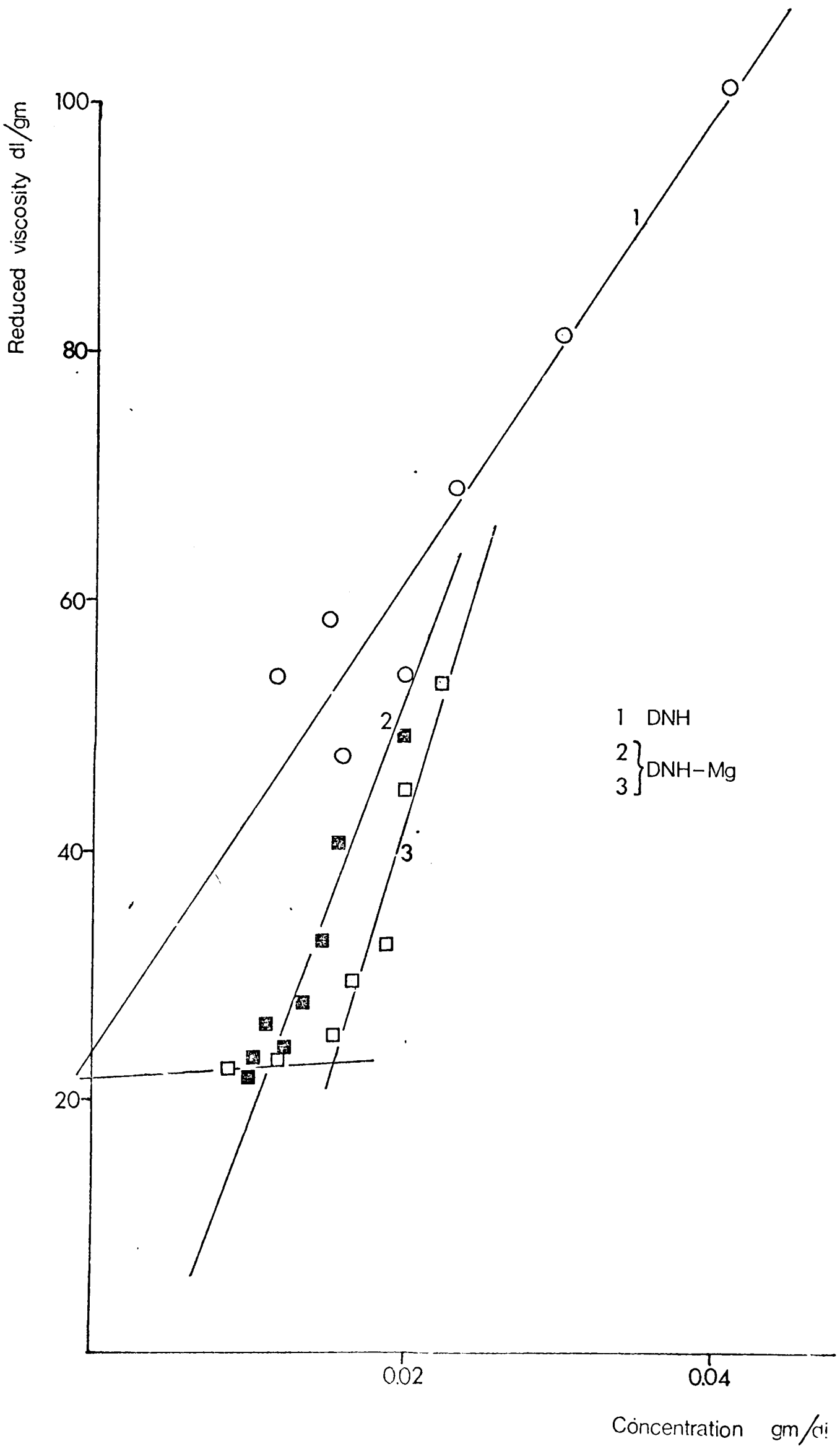
A typical plot of reduced viscosity against concentration is shown in Fig 7.1 as curve 1. When native DNH has been dialysed overnight against low concentrations of magnesium chloride, the plot of reduced viscosity against concentration changes radically (curves 4 and 5). The intrinsic viscosity has been reduced to about half its value and the plot, at low DNH concentrations has become virtually horizontal, although it reverts at higher DNH concentrations to a more normal slope. $DNH^{0.7}$ dialysed against similar concentrations of magnesium gives the same effect.

As precipitation becomes increasingly measurable at higher concentrations of Mg^{++} ions, and especially where the addition is made by mixing instead of dialysis, another type of plot is observed (Fig 7.1, curves 2 and 3). The slope of this plot is much greater than that for native DNH and the straight lines derived from the data by the least squares procedure give intercepts on the viscosity axis at values less than zero. Such intercepts are not interpretable.

Curves 2 and 3 in Fig 7.2 show the reduced viscosity/concentration plots obtained from DNH-Mgl preparations. As can be seen, these are similar to the two types shown in Fig 7.1. However those plots with a positive intercept on the abscissa now give values for the intrinsic viscosity of approximately 20 dl/gm.

The effects of magnesium ions on the viscosity of DNH may profitably be divided in two - the effects on the intrinsic viscosity and the effect on the shape and slope of the reduced viscosity/concentration plot. Scruggs and

Fig 7.2 Viscosity of DNH-Mg samples - reduced viscosity/concentration plot



Ross[181] have shown that the increase of ionic strength of a solution of DNA leads to a decrease in the intrinsic viscosity. The neutralisation of the polyelectrolyte charge leads to a collapse of the flexible structure of DNA to a more compact form. Even in DNH, already in a compact conformation[81], the neutralisation of charge still seems capable of producing a further compaction of the complex. Hence the reduction in intrinsic viscosity observed. DNH prepared in the absence of chelating agents has an intrinsic viscosity similar to that of DNH at the same ionic strength - it is unlikely that there is a radical difference in the shape of the molecules.

Plots of the type of curves 4 and 5, Fig 7.1 have been reported for solutions of DNA in the presence of magnesium ions[181]. There they were held to be indicative of cross-linking of DNA fibres by magnesium bridges. At low DNA concentrations intramolecular interactions are preferred, the collision frequency being low, but at higher DNA concentrations intermolecular interactions become predominant and the reduced viscosity explodes.

The high slope produced can be held as indicative of a high state of aggregation in the system. The Huggins constant can be split into two parts[63]:

$$A + \frac{B(M)}{[\eta]}$$

where A is $(k_1)_o$, relating to the long-range forces present - it is ultimately a shape factor,

and B(M) is a term proportional to the dissociation constant for dimerisation - an aggregation factor.

At high DNH concentrations it is this latter factor

Table 7.1: Properties of normal and DNH-Mg preparations*

	P:DNA ratio	Viscosity $[\eta]$ (dl/gm)	Sedimentation coefficient s_{20}	Circular dichroism $\Delta\epsilon_{225}$	$\Delta\epsilon_{275}$
normal	1.30±0.10	22.2±5.4	41.56±10.24	-10.44±0.54	1.32±0.11
DNH-Mg	1.26±0.24	19.3±2.1**	37.9±7.98	-11.36±0.87	1.25±0.05

*data from preparation DNH-Mg3 not included - digested when prepared

**Derived from the two preparations where the plot could be extrapolated to a positive intercept

which predominates and leads to a high slope for the reduced viscosity/concentration plot.

On this hypothesis, the anomaly in curves 2 and 3, Fig 7.1 can be explained in one of three ways:

a) The curves are limiting examples of the other type in which experimental considerations limit data collection to the region before the plot becomes horizontal.

b) They are examples of a situation in which tertiary and higher order interactions predominate so as to render the Huggins approximation totally inadequate, some higher term in the expression becoming the most important.

c) The experimental situation is such that the original gel matrix formed on adding magnesium ions to the DNH is so coherent that dilution fails to disperse the sample properly. Hence intramolecular interactions never become dominant.

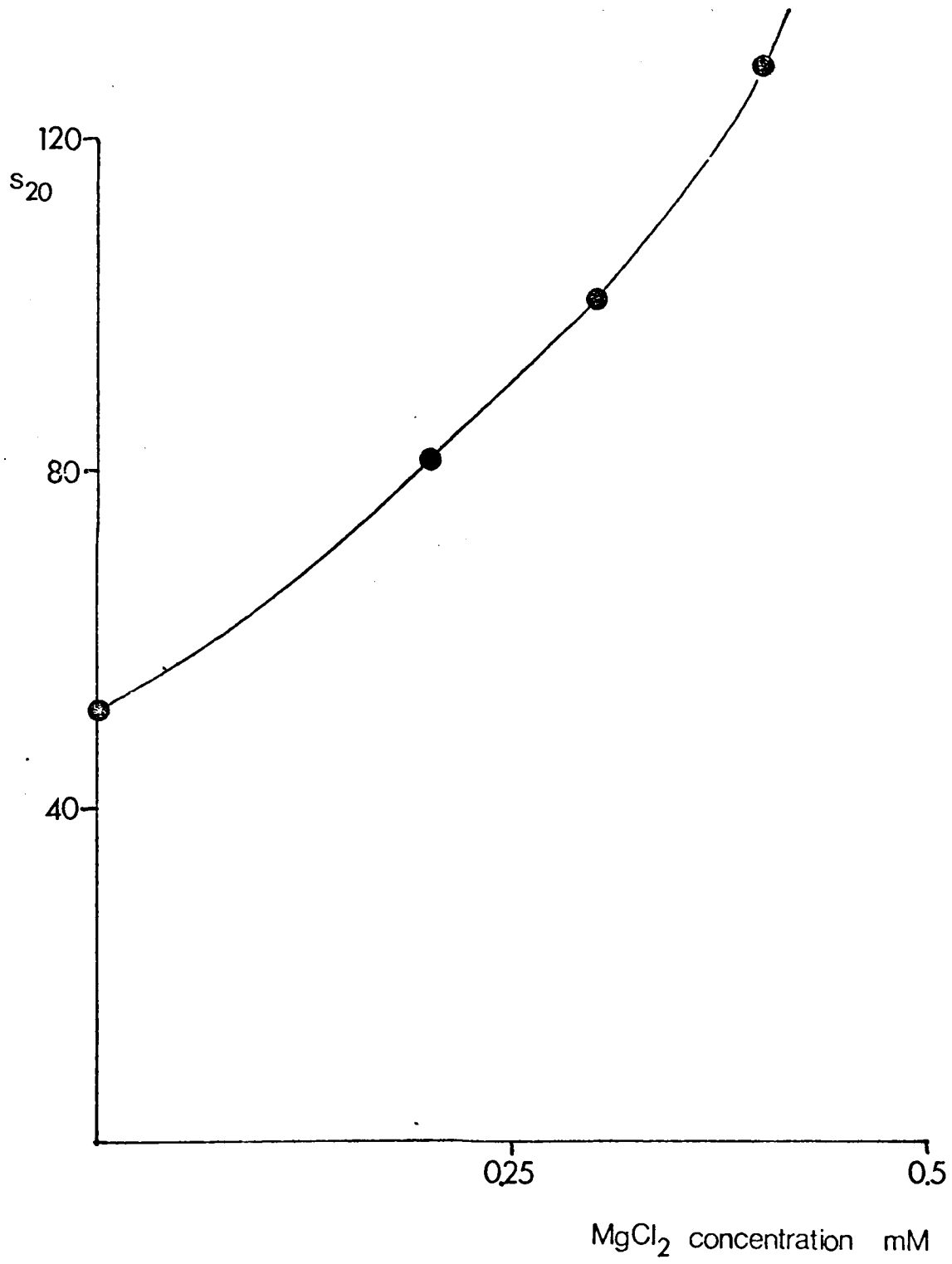
In the case where magnesium ions have been added directly to solutions of DNH, this last is the most probable explanation. The negative intercepts are artefactual and due to the system not being in equilibrium.

Other cases of this phenomenon are harder to diagnose. However, the good straight lines obtained for the reduced viscosity/concentration plots can be held to show that the Huggins approximation is not badly at fault. It would seem that experimental limitations are responsible for the anomalous intercepts in these cases.

2. Sedimentation velocity

As shown in Table 7.1, the sedimentation characteristics

Fig 7.3 Sedimentation velocity of DNH samples dialysed against low concentrations of magnesium chloride



of DNH-Mg1 and Mg2 preparations are indistinguishable from those of normal preparations. Dialysis of native DNH into low concentrations of magnesium chloride of the order of 10μM also has no measurable effect on the sedimentation coefficient.

However, dialysis of samples to higher magnesium ion concentrations does give rise to an increase in s_{20} (Fig 7.3). Even at the highest magnesium concentration used, no more than 20% of the A_{260} would have been precipitated. The boundaries observed were more diffuse than those of native DNH. This effect was undoubtedly caused by the low rotor speeds used.

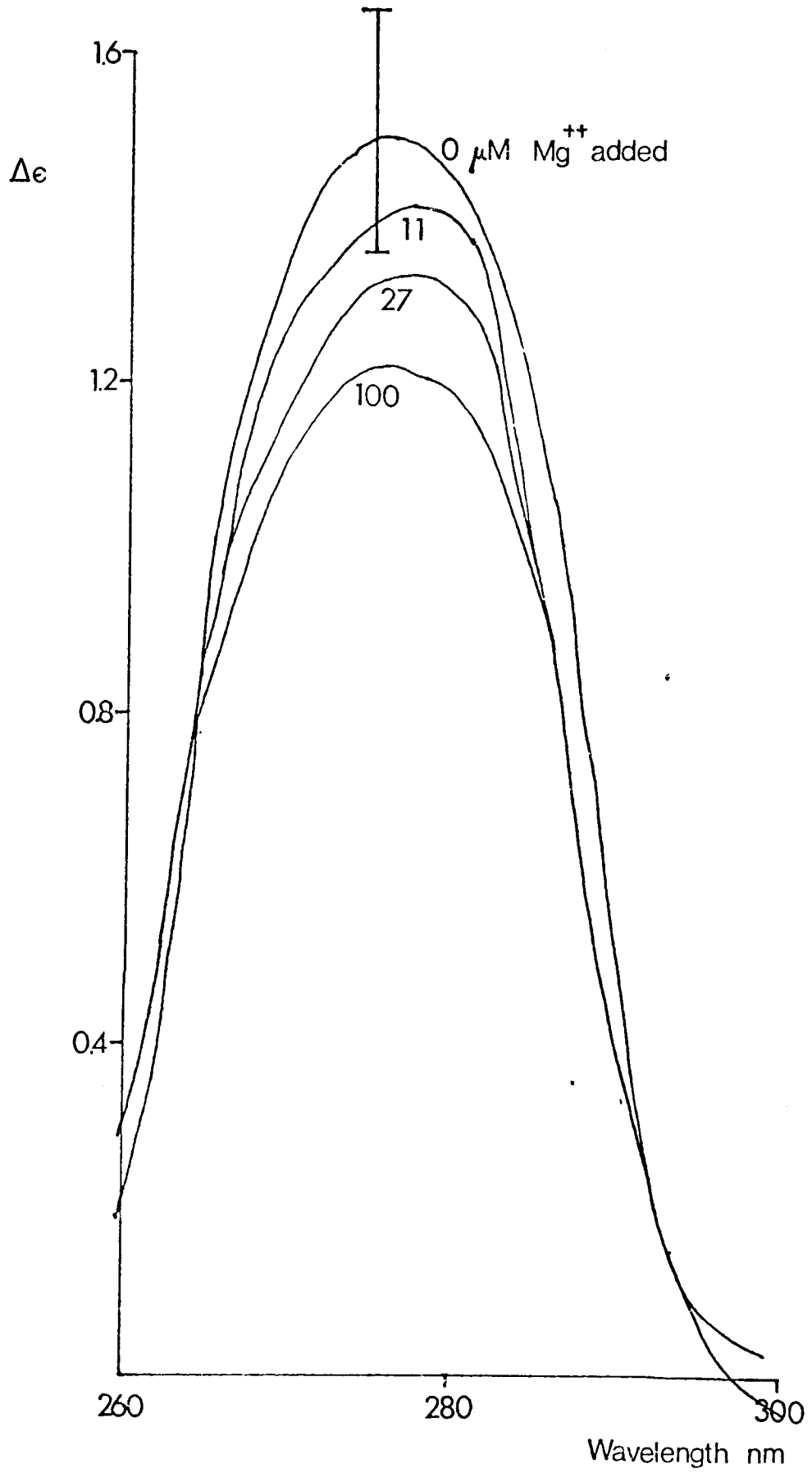
The sedimentation coefficient of soluble DNH is more than doubled on exposure to 400μM Mg^{++}. Whether this change is due to a polyelectrolyte effect or to an increase in molecular weight caused by aggregation is hard to say. The sedimentation coefficient of DNA increases on exposure to increasing ionic strength.[157 205] Typically, the s_{20} value doubles on going from distilled water to an ionic strength of 0.1M. Here a similar effect has taken place in the ionic strength range 0.7 - 1.9 x 10^{-3}M. Nor does our data show any sign of the plateauing of s_{20} which typifies the effect of ionic strength on DNA.

Aggregation of DNH molecules can be observed in the soluble DNH phase in this experiment.

3. Circular dichroism

The circular dichroic properties of DNH-Mg1 and Mg2 do not differ from those of an ordinary preparation (Table 7.1).

Fig 7.4 Change in the CD spectrum of DNH on the addition of magnesium ions



The addition of low concentrations of magnesium ions to solutions of DNH, however, has a significant effect on the spectrum. A reduction in intensity of the positive dichroic band at about 277nm of 20±5% takes place (Fig 7.4). This comes about in the concentration range 0-100μM Mg^{++} but a substantial reduction in the dichroism is already visible at 10μM Mg^{++}. A sample of DNH of high concentration, when dialysed into 10μM Mg^{++}, showed the completed effect. The reduction is consistent and outside the noise limits of the experiment. No change in the rest of the spectrum accompanies this loss of $\Delta\varepsilon_{275}$.

The DNA circular dichroism shown by DNH in 100μM Mg^{++} is very similar to that of $DNH^{0.7}$. Such an f1-depleted DNH exhibits no further change in $\Delta\varepsilon_{275}$ on addition of 100μM Mg^{++}. Nor does this concentration of magnesium ions have any effect on the CD spectrum of pure DNA.

The precipitation of DNH by the addition of further magnesium ions caused no further reduction in $\Delta\varepsilon_{275}$. However the positive CD peak is shifted approximately 5nm to higher wavelength. Such a red shift is a light-scattering artefact[70] (Fig 7.5).

DNH in 150mM sodium chloride solution has a similar CD spectrum to that in 1mM Mg^{++}.

The effect of 100μM Mg^{++} on the DNH CD spectrum is similar to those reported in the literature as caused by 250μM Ca^{++} and 140mM ammonium acetate[197]. The latter report, presumably describing precipitated DNH, commented on a change in the protein half of the spectrum which is not observed here. A similar CD spectrum to that observed

Fig 7.5 Circular dichroism of precipitated DNH

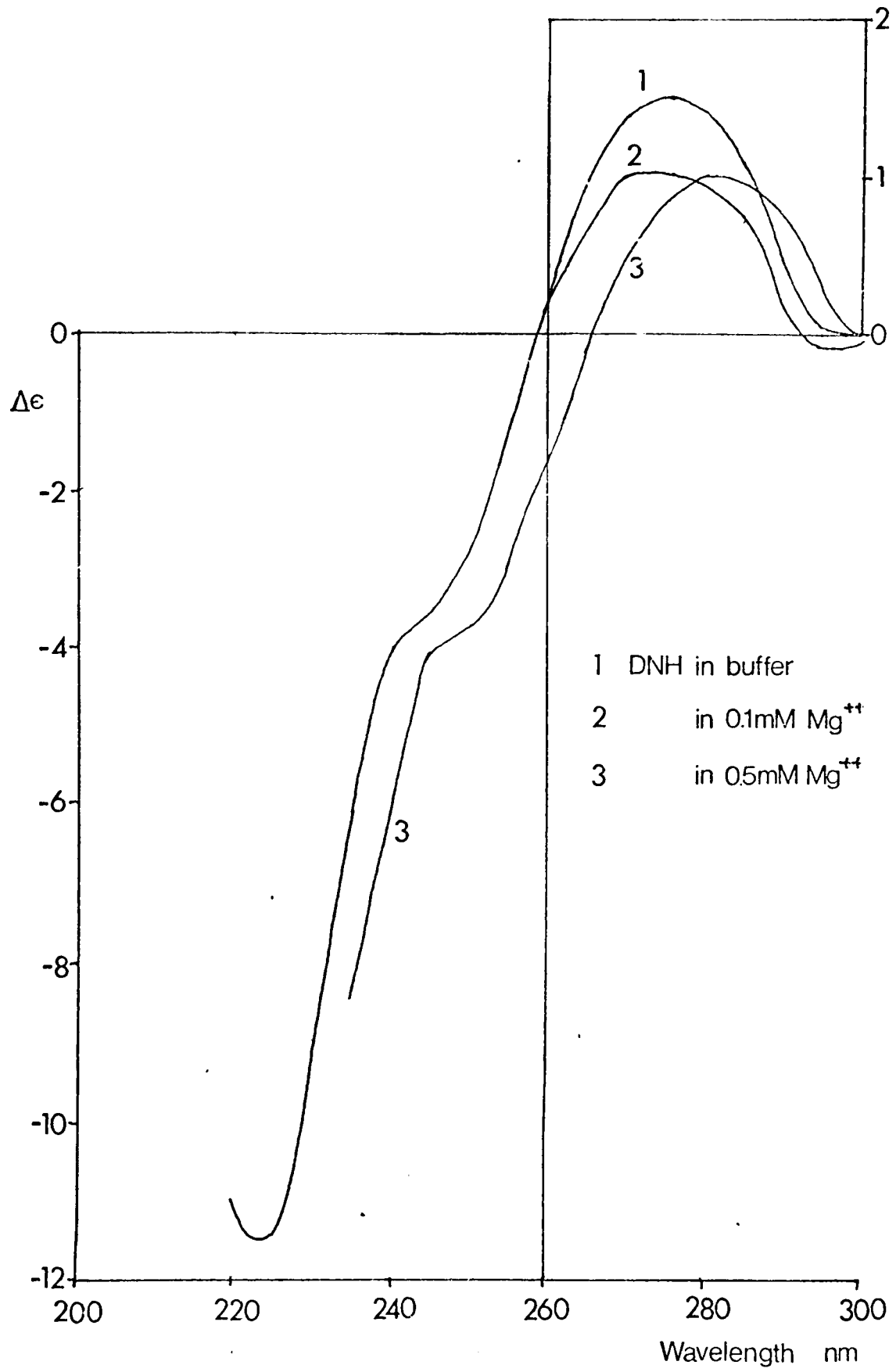
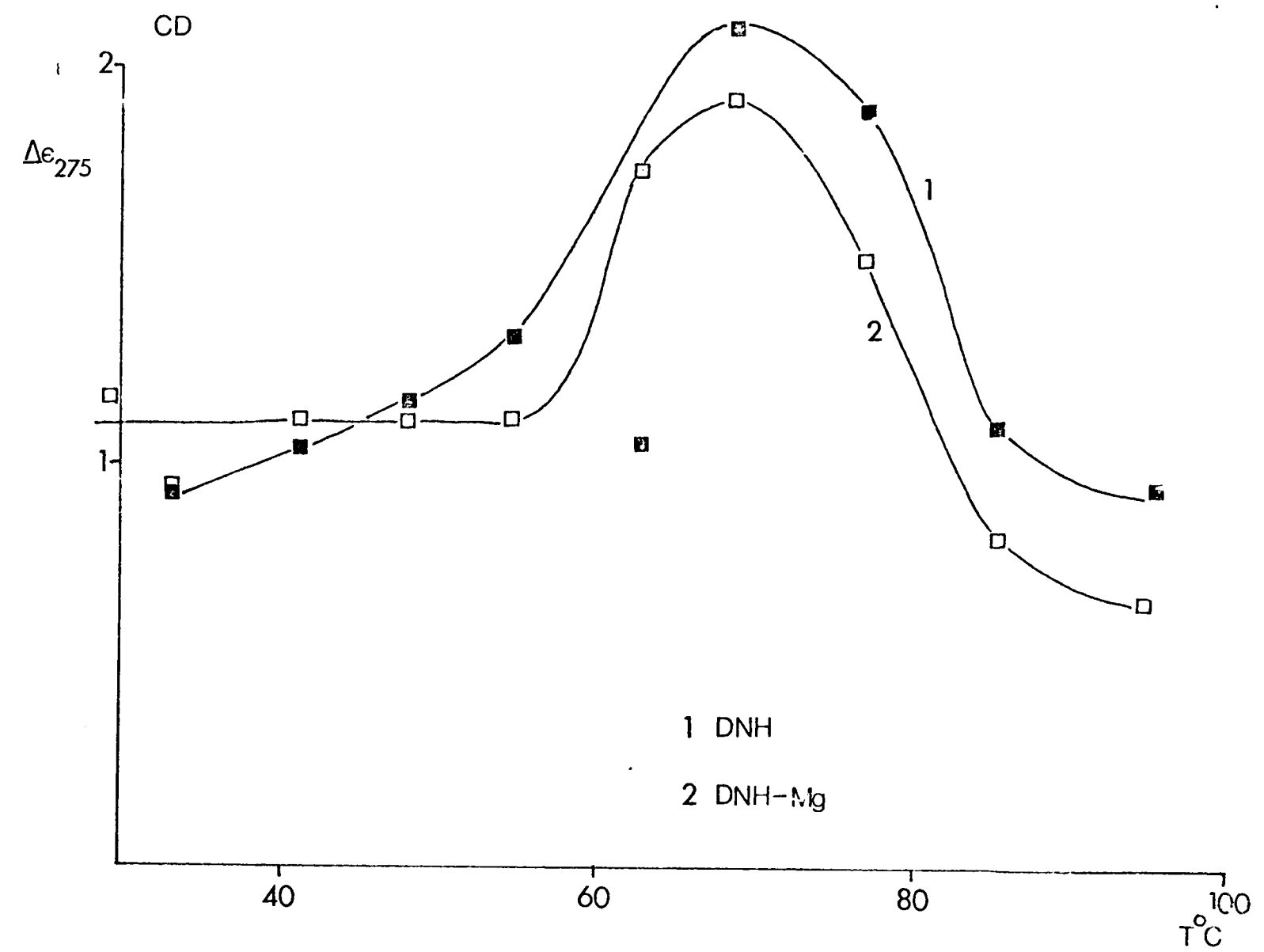
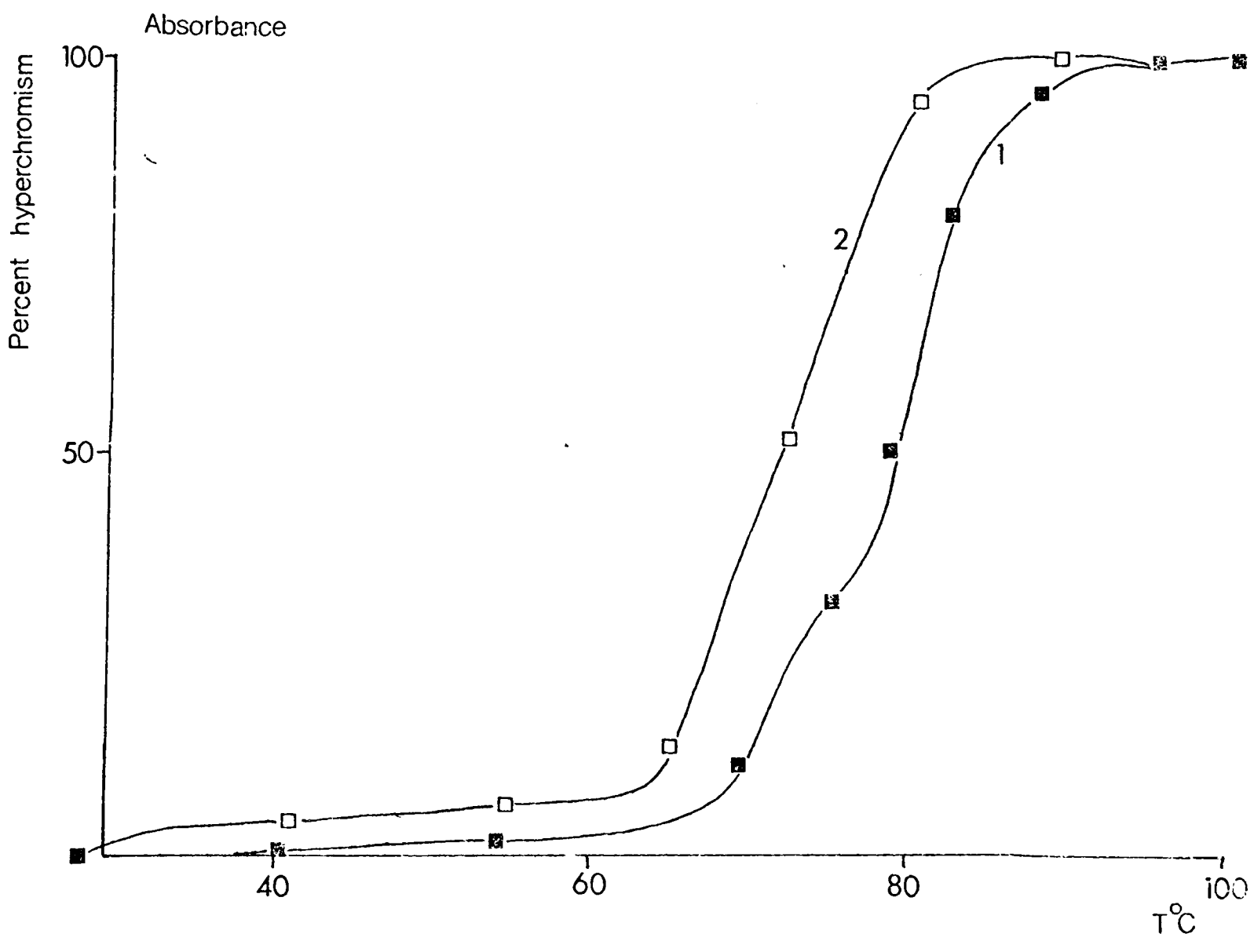


Fig 7.6 Melting profiles of DNH-Mg samples





in the presence of 100μM Mg^{++} is reported for a high-melting 'heterochromatic' fraction isolated from chromatin by ECTHAM-chromatography.[162]

4. Thermal denaturation

Fig 7.6 shows compared the circular dichroic and absorbance melting profiles of a preparation of DNH and of DNH-Mg1.

The absorbance melting profiles are both biphasic with approximately 40% of the hyperchromism in the first step. However DNH-Mg1 is measurably less stable to denaturation in both steps. The T_ms for the two preparations are shown in the following table.

Table 7.2: T_ms of DNH preparations (^oC)

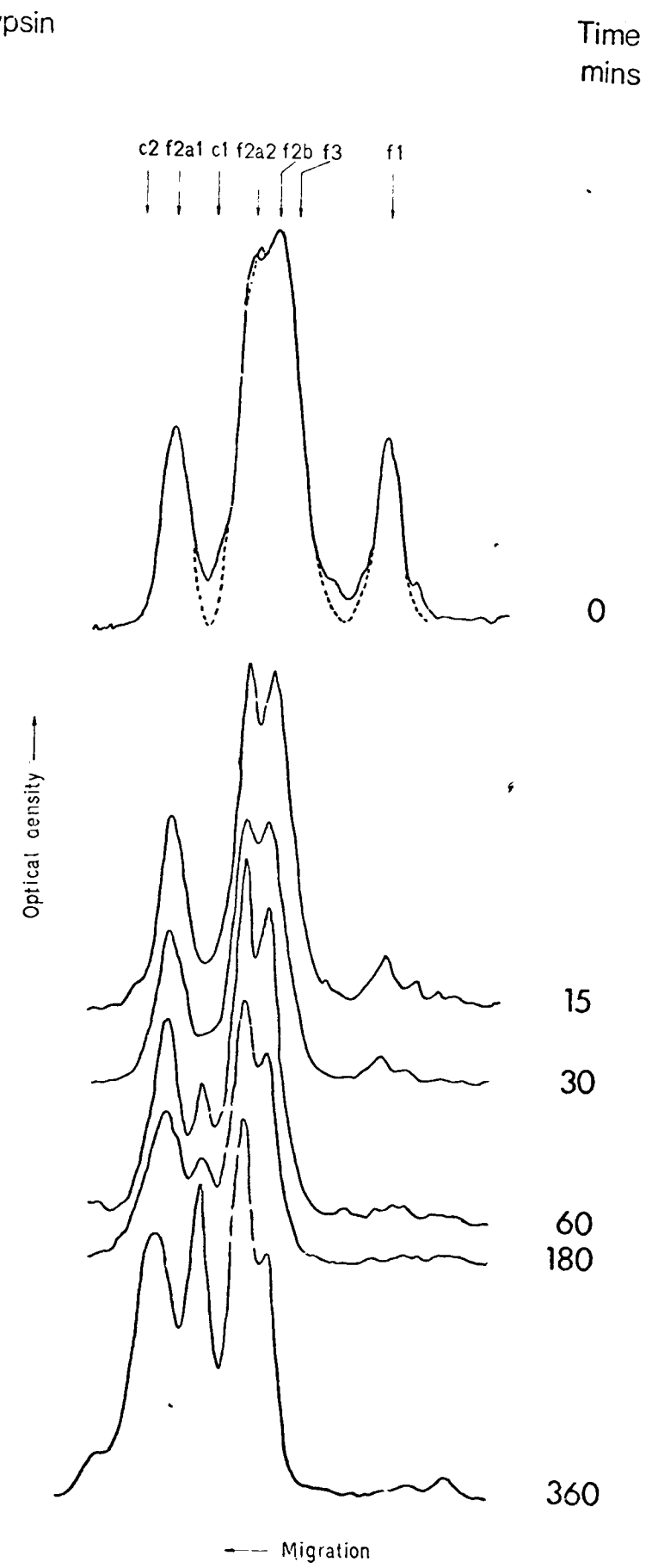
	1st step	2nd step
native DNH	70.9	81.2
DNH-Mg1	68.0	76.8

This situation is echoed by the circular dichroic profiles. The peak of the profile is shifted to lower temperatures in the DNH-Mg1 case.

Any loss of protein from the complex would cause a shift very similar to this.[82] However, in this case, the DNH-Mg1 sample had undergone no significant autolysis at the time of the experiment.

The binding of ions to DNA causes an increase in the T_m. This is thought to be due to a stabilization of the DNA helix by cation binding.[50,47] This stabilization is effected by magnesium at a much lower concentration than sodium. The replacement of sodium counterions by magnesium is likely to cause a stabilization of the helix. This will

Fig 7.7 Protease digestion of DNH after Chatterjee and Walker[35]



not account for the lowered T_m observed in DNH-Mg1.

It is possible that this could be brought about by a loosening of the histone-DNA electrostatic interactions caused by magnesium binding.

No measurements were made of the thermal stability of DNH-magnesium mixtures because of scattering artefacts due to the high temperatures involved.

5. The effect of controlled proteolysis

The incubation of DNH with the enzymes trypsin and chymotrypsin under controlled conditions leads to a well-defined pattern of proteolysis[35]. After three hour's digestion with trypsin, there is little or no change in the electrophoretic pattern of the extracted histone. In some preparations, there is some digestion of the f1 and f3 fractions. Chymotrypsin, however, has more effect. After the same period, f1 and f3 are completely digested, f2b is partially so but f2a1 and f2a2 are not attacked. Two well-defined degradation products appear in the electrophoresis pattern (Fig 7.7).

This proteolytic treatment was applied to samples of DNH Mg1 and to samples of native DNH treated with various concentrations of magnesium ions.

Fig 7.8 shows one such experiment in which samples of DNH, DNH in 2mM magnesium chloride and DNH-Mg1 were subjected to digestion by the two enzymes. Precipitation of the DNH with Mg^{++} does not have much effect on its susceptibility to chymotrypsin. The intensity of the c1 band has increased and hence some additional digestion must have taken place. Similarly DNH-Mg1 appears to have

Fig 7.8 Protease digestion of magnesium treated samples of DNH

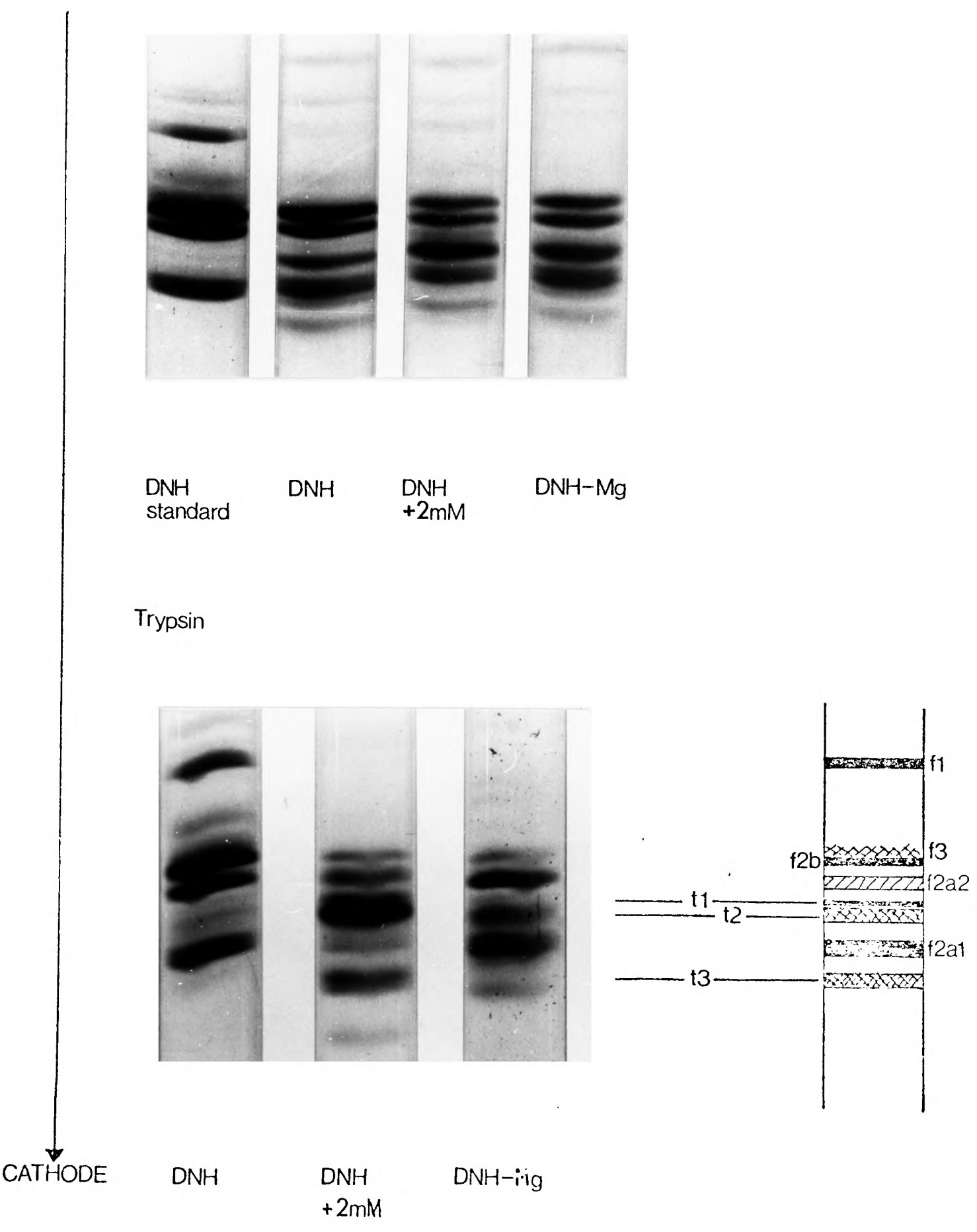
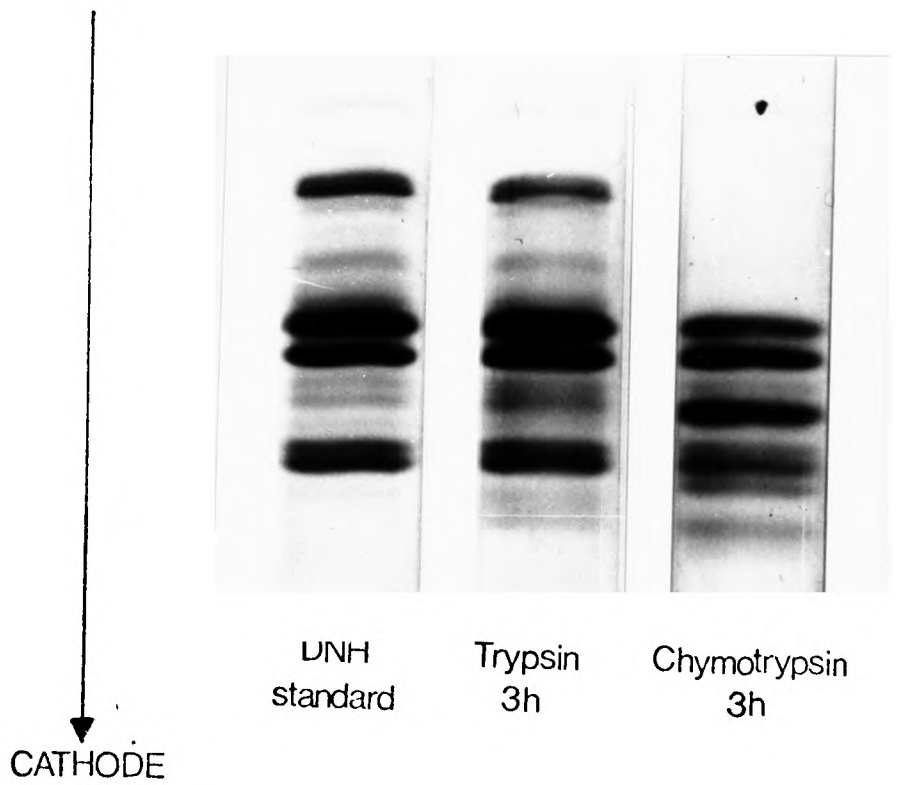


Fig 7.9 Protease digestion of a DNH-Mg sample



undergone some slight further digestion compared with the normal DNH.

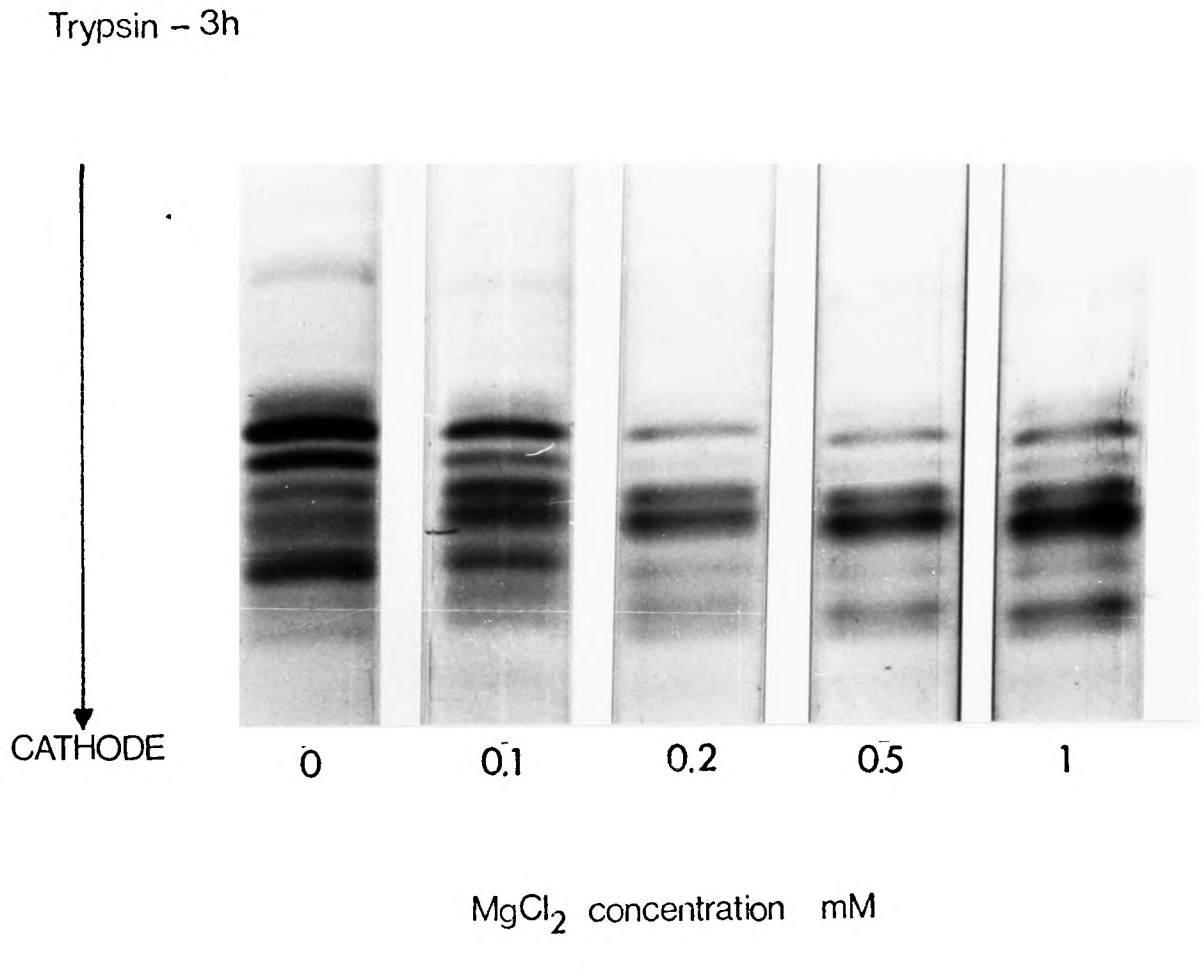
The situation with trypsin is very different. Whereas DNH in solution is hardly affected at all over 3h, the precipitated material appears to have undergone considerable proteolysis of all histone fractions. The electrophoretic pattern clearly shows the appearance of the fragment bands t1 - t3. The DNH-Mg1 sample too had undergone digestion of the entire f1 and f3 fractions together with the majority of the f2b. Two fragment bands have appeared.

This pattern of increased sensitivity to trypsin is typical for precipitated chromatin, whether in 2mM magnesium chloride or 150mM sodium chloride. However, the increased proteolysis of the DNH-Mg1 sample is not repeated in all preparations. Fig 7.9 shows the electrophoretic patterns resulting after proteolysis of another DNH-Mg1 preparation. Here the pattern of digestion is typical of a normal DNH sample.

The increased proteolysis of the DNH-Mg1 sample of Fig 7.8 may well have been due to prior digestion of the preparation by endogeneous protease. Such activity is higher in DNH-Mg1 preparations. Prior digestion could lead to exposure of more sites for trypsin action than are available on the native complex.

The changes in proteolysis brought about by precipitation could well be due to changes in DNH concentration brought about by the phase change. To test this hypothesis, the experiment shown in Fig 7.10 was performed. Samples of DNH treated with increasing

Fig 7.10 Magnesium dependence of protease digestion of DNH



concentrations of magnesium chloride were incubated for 3h with the usual concentration of trypsin. The digestion pattern typical of precipitated material exists at all concentrations of magnesium in excess of 200μM. At this concentration less than 20% of the A_{260} is found in the pellet after slow-speed centrifugation. The changes in proteolysis are mediated by increases in the salt concentration, not by the precipitation.

The experiment of Fig 7.10 also shows that in the presence of magnesium ions the susceptibility of the histone fractions to tryptic digestion is:

$$f1 > f3 > f2a2 = f2a1 > f2b$$

This is the same order reported for the susceptibility to trypsin under different conditions by Toczko and Jazwinski[216] but differs considerably from the order reported by Debabov and Rebentish[44].

There are three hypotheses which will explain the change in tryptic hydrolysis on the addition of subprecipitating amounts of magnesium. They are:-

a) activation or acceleration of trypsin activity by Mg^{++};

b) activation or acceleration of an endogenous protease activity by Mg^{++};

c) a conformational change in the histone-DNA complex caused by the addition of Mg^{++}.

Green and Neurath[72] observed that addition of magnesium to trypsin does increase its activity (expressed as a zero order reaction constant) but 1mM Mg^{++} only increases the activity by 9%. Chymotrypsin however is not affected

by the addition of magnesium[71]. Hypothesis a, although it would explain the difference between trypsin and chymotrypsin, would not provide an adequate explanation of the large increase in tryptic activity on addition of magnesium.

The endogenous protease of rat liver chromatin appears to be sensitive to increases in ionic strength, being maximally active at 0.3M NaCl[65]. However hypothesis b would predict an increase in digestion by both trypsin and chymotrypsin on the addition of magnesium. The addition of 2mM Mg^{++} to DNH does not produce any digestion on incubation for 3h - no endogenous protease is activated.

A change in DNH conformation leading to greater accessibility of histone basic groups provides a satisfactory hypothesis. The removal of the f1 fraction prior to trypsin digestion produces an increase in digestion but f2a1 and f2a2 remain untouched after 3h. Removal of f1 alone cannot explain the magnesium effect. 80% of the histone basic residues are inaccessible to the solvent, presumably linked by ionic bonds to the DNA phosphate groups[224]. The aromatic residues however are clustered in the non-basic parts of the histone primary structure and presumably form hydrophobic centres[55,228]. This distinction explains the difference in susceptibility of DNH to trypsin and chymotrypsin under normal conditions. A loosening of the ionic bonds between DNA and histone would lead to an increase in accessibility of histone basic groups to solvent and thus presumably to tryptic attack. The aromatic residues have no need to be affected by such a change in structure and thus the extent of chymotrypsin

digestion is not changed.

6. Discussion

A pattern emerges from these investigations of the solution structure of DNH in the presence of divalent metal ions. A change in the structure of the nuclechistone takes place accompanied by an increase in intermolecular interactions.

Preparations of DNH in the absence of chelating agents yield nucleohistones, e.g. DNH-Mg1 and 2, which are very little different from native DNH (Table 7.1). The similarity of the values of $[\eta]$ and s_{20} point to similarities of molecular shape and size. However, the increased slope of the reduced viscosity/concentration plots indicate an increase in the Huggins constant. This can be interpreted, together with the striking shape of the plot (Fig 7.2, curve 3), as due to intermolecular bridging[181] caused by the presence of magnesium ions. The interpretation of the differences in thermal denaturation and in the action of trypsin is made difficult by the uncertainties deriving from the variation in these preparations caused by autolysis. Indeed the action of trypsin on DNH-Mg1 as shown in Fig 7.8 is strikingly different from that on precipitated DNH and is much more like that on f1-depleted DNH - there is no attack of the f2a1 and f2a2 fractions.

The addition of small quantities of magnesium ions to normal DNH leads to changes in several physicochemical parameters. These changes do not all take place at the same magnesium concentration as Table 7.3 shows. None of these changes correlate with the precipitation. Two

Table 7.3: Effect of Mg^{++} on physicochemical parameters of DNH

Parameter	Experimental A_{260}	Magnesium concentration for completion of effect
Viscosity	~5	10μM
Sedimentation	~1	>400μM
CD	1-1.5	50μM
	~5	10μM
Trypsin digestion	~5	200μM
Precipitation	0.5	2000μM

of them take place when the material is still essentially 100% soluble - the changes in viscosity and circular dichroism. The two others, although visible at much lower magnesium concentrations are not completed until the precipitation of the material has become measurable.

The reduction of intrinsic viscosity on addition of magnesium is indicative of the formation of a more compact structure. This change correlates well with the lowering of $\Delta\varepsilon_{275}$. The increase in s_{20} also indicates a compaction of the molecule but as well suggests an increase in intermolecular interactions, especially at higher magnesium concentrations. The discontinuous reduced viscosity/ concentration plots also indicate increases in intermolecular interactions.[181]

These hydrodynamic effects mimic to some extent the behaviour of DNA in salt solutions. The addition of 50-100mM magnesium chloride to a sample of T4 DNA yielded a reduction of approximately 50% in the intrinsic viscosity but it was not until greater than 200mM concentrations were reached that the discontinuous plots of Fig 7.1 (curves 4 and 5) were produced.[181] The sedimentation coefficient of DNA doubles on going from distilled water to an ionic strength of 100mM.[205] The polyelectrolyte effects in DNH occur at concentrations some two or three orders of magnitude lower. This can hardly be accounted for simply by the difference in available phosphate binding sites between DNA and DNH. The proportion of free phosphates in DNH has been variously estimated - figures as large as 40% have been proposed.[38] It is likely therefore that these changes in DNH are not solely produced by the neutralisation

of charge but are also mediated by a conformational change in the complex involving the protein moiety as well.

The changes which take place in the circular dichroic spectrum of DNH and which accompany the compaction of the complex shown by the hydrodynamic studies can be interpreted in several ways. A reduction in $\Delta\varepsilon_{275}$ accompanies the binding of histone to DNA to form DNH. This correlates well with the amount of protein bound to DNA. As has been suggested earlier this may be due to removal of the DNA bases from the solvent environment. No further protein is bound however, during the magnesium-mediated $\Delta\varepsilon_{275}$ change. This change could be caused by the compaction of the molecule which it so closely follows. If the hydrodynamic changes were caused by a tightening of the supercoil, we might expect a change in the CD to take place. On the model proposed by Hanlon and her coworkers[79 101] it could be interpreted as an increase in the C-form DNA present.

However, f1-depleted DNH does not show the reduction in $\Delta\varepsilon_{275}$ on addition of magnesium. Nevertheless, this depleted DNH does become more compact as shown by the reduction in its intrinsic viscosity. If a structural change is responsible for the reduction in $\Delta\varepsilon_{275}$ it must involve f1. In fact $DNH^{0.7}$ has a very similar $\Delta\varepsilon_{275}$ value to that of magnesium-treated DNH. It is tempting to suggest that the binding of magnesium to the complex or the concomitant compaction of the molecule are causing some displacement of f1 from the complex. Since circular dichroic theory is not capable of stating whether or not the two states are structurally identical, this must remain merely a possible interpretation.

The evidence from controlled proteolysis of the magnesium-treated material adds to the picture here. As we have seen, by 200μM Mg^{++}, the digestion pattern with trypsin has changed radically and extensive proteolysis takes place. This indicates the availability of further sites for tryptic attack. These can only arise by the relaxation of the histone-DNA electrostatic bonds since it is the histone basic groups that are the substrates for trypsin action. Although this effect on trypsin activity is not complete until 200μM it is already visible at magnesium concentrations where the CD and viscosity effects take place.

The binding of magnesium to DNH causes two major effects. First a structural change takes place which makes the complex more compact. This compaction is due in some part to neutralisation of charge. Following this there is an increase in intermolecular interactions, possibly mediated by magnesium bridging which leads eventually to the aggregation of the DNH and its removal from solution.

The hydrodynamic data indicate that these two effects take place at very low magnesium concentrations. It is difficult to say with which effect the change in $\Delta\varepsilon_{275}$ is correlated - probably the former. The effect of magnesium on the trypsin digestion of DNH follows the second effect but it is not necessarily caused by it. Nevertheless it is possible to envisage a further structural change accompanying interchain interactions which would cause exposure of new sites for trypsin attack. Whether this structural change involves a general loosening of electrostatic bonds or merely, as suggested by the CD

change, a deprotection of the complex involving f1 is difficult to distinguish. However, if the latter is the case, the structural change does not yield the same deprotection of tryptic sites as does removal of f1 altogether.

8

DISCUSSION

1. The structure of extracted nucleohistone

The preparation of soluble nucleohistone by the procedure of Zubay and Doty[233] contains several processes which are potentially disruptive of nuclear chromatin structure. One of these is the removal of divalent metal ions by treatment with sodium EDTA.

Characterisation of nucleohistone preparations made in the absence of chelating agents - such as the DNH-Mg preparations of this study - provides a chance to examine the role of divalent metal ions in the structure of nuclear chromatin. The studies reported in Chap. 7 show that DNH-Mg preparations are indistinguishable from normal preparations in their protein:DNA weight ratio, circular dichroic spectra and hydrodynamic properties. However the variation of viscosity with concentration (Fig 7.2) showed that these preparations have an increased tendency to interact at higher DNH concentrations, probably via magnesium bridges.

The inclusion of sodium EDTA in the preparation appears to make little difference to the structure of extracted nucleohistone. Nevertheless its inclusion is amply justified by its effect on the action of the endogenous protease activity. Removal of divalent metal ions from the preparation doubles at least the lifetime of the DNH. The addition of only 20μM Mg^{++} ions to the preparation buffer ensured that the DNH did not even survive the period of the preparation undigested.

The DNH extracted by Zubay and Doty's method

is indeed a nucleohistone. The protein present contains little or no tryptophan by fluorescence analysis and can thus be assumed to be almost entirely composed of histone. This is in contrast to other preparations of nucleoproteins, especially from metabolically more active tissues, which contain up to 35% non-histone protein[19]. One non-histone protein has been detected in our preparations and that is the protease whose activity is responsible for their eventual degradation. However, there is no evidence to suggest that this activity is bound to the DNA. It could equally well be derived from bacterial contamination of the preparation.

The structure of the extracted nucleohistone in solutions of low ionic strength is more compact than that of the DNA contained in it. This study adds little to our knowledge of this structure with the exception of the discovery that DNH from which all histones have been removed save f2a1 still retains the 'supercoil rings'. Hence it must be concluded that if one fraction alone is responsible for the structure which gives rise to these rings then it is f2a1. This is in contrast to Henson and Walker's conclusion that the single structurally important histone fraction must be f2a2[84]. This earlier conclusion was derived from a study of the extensive hydrodynamic changes that take place on removal of all fractions except f2a1. The depleted nucleohistone containing only DNA and histone f2a1 behaves as a flexible polyelectrolyte rather than a rigid rod.

Hence it was concluded that its structure was DNA-like. Even so its intrinsic viscosity is still half that of completely protein-free DNA.[84 225]

Henson's deduction was perfectly correct as far as it went. If the hydrodynamic behaviour of DNH is to be assigned to the effect of a single histone fraction on DNA, then that fraction must be f2a2. However, the identity of the 'supercoil' structure with the conpact rod-like solution structure of DNH cannot be upheld in the face of our evidence. Even when a nucleohistone is substantially flexible in solution, a fibre drawn from that DNH will give the 'supercoil rings'.

These two structural elements- the rigid rod and the 'supercoil' - are not necessarily linked. Nor do they appear to be connected with the structure observed in the CD spectrum of DNH. We have shown that the increase in the DNA positive dichroism at 275nm on removal of protein from DNH is directly related to the amount of protein removed. The hydrodynamic changes are certainly not related to the protein bound. No data are available on the extent of supercoiling in depleted nucleohistones: the intensity of the X-ray diffraction rings is very difficult to measure.

The structural basis for the increase in $\Delta\epsilon_{275}$ cannot be ascertained due to the limitations of current CD theory. Possible causes include a change in the structure of the DNA, such as a tilting of the bases, or a solvent effect due to masking of the bases

by the histones. Whatever the mechanism of this effect, the f1 histone acts differently from the rest and gives an increase of $\Delta\epsilon_{275}$ on binding to the DNA.

Analysis of the CD spectrum of the depleted DNHs below 250nm shows that there is no great difference in the structure of the various histones when bound to DNA. They contain 35 - 45% α-helix, 5 - 15% β-structure and a considerable 50% of unordered residues. This estimate of α-helix content agrees well with that obtained in another CD investigation[194] and with the conclusions from the IR spectra of DNH gels.[204]

Several workers have held that extracted DNH is a metastable state of the DNA-histone system. On exposure to salt solutions, considerable exchange of histones is described as taking place.[100 38] The system is seeking the structure of minimum energy. If this hypothesis were true, then the depleted DNHs prepared by salt extraction would bear no structural relation to the original DNH but be merely artefactual. The most extensive test of this hypothesis[38] measured exchange of proteins between DNH and labelled native DNA in various salt solutions. The flaw in this argument lies in the extrapolation necessary in applying results from this system to the situation in a solution of DNH. Are there any sites on DNH to which histones from other DNH molecules are capable of binding? DNH will certainly exchange histone with many added polyanions - tRNA[95], protamine[218]

and polystyrene sulphonate[12] have been used successfully as well as DNA - but this does not mean that it will exchange with another DNH molecule.

The reconstitution of DNH structure which we are able to obtain by careful dialysis of DNH back from 2M salt argues against the metastable state hypothesis. The reconstituted structure at salt concentrations above 0.7M is identical with the original structure by three different criteria - species and amount of histone bound, circular dichroism and intrinsic viscosity. If the stable state - the reconstituted sample presumably - and the metastable state have identical structures then this hypothesis is a trivial one.

2. The interaction of DNH with neutral salts

The addition of salts to a polyelectrolyte solution results initially in a stabilisation of the polymer. This 'polyelectrolyte effect' is due to the masking of the electrostatic repulsions which take place between neighbouring groups on the polymer. Addition of further ions to the solution leads to progressive destabilization until eventually, at electroneutrality, the polymer is removed from solution.

DNH is a mixed polyanion consisting mainly of DNA phosphate and histone carboxylate anoins. However, the situation outlined above is here complicated by the fact that DNH is a complex of DNA and histone held together largely by electrostatic bonding.[224]

As the ionic strength of the solution increases

these bonds will become increasingly destabilized and eventually histone will become dissociated from the DNA. In addition the presence of the protein provides an extra degree of structural complexity which has its effect on the mechanisms of precipitation and dissociation.

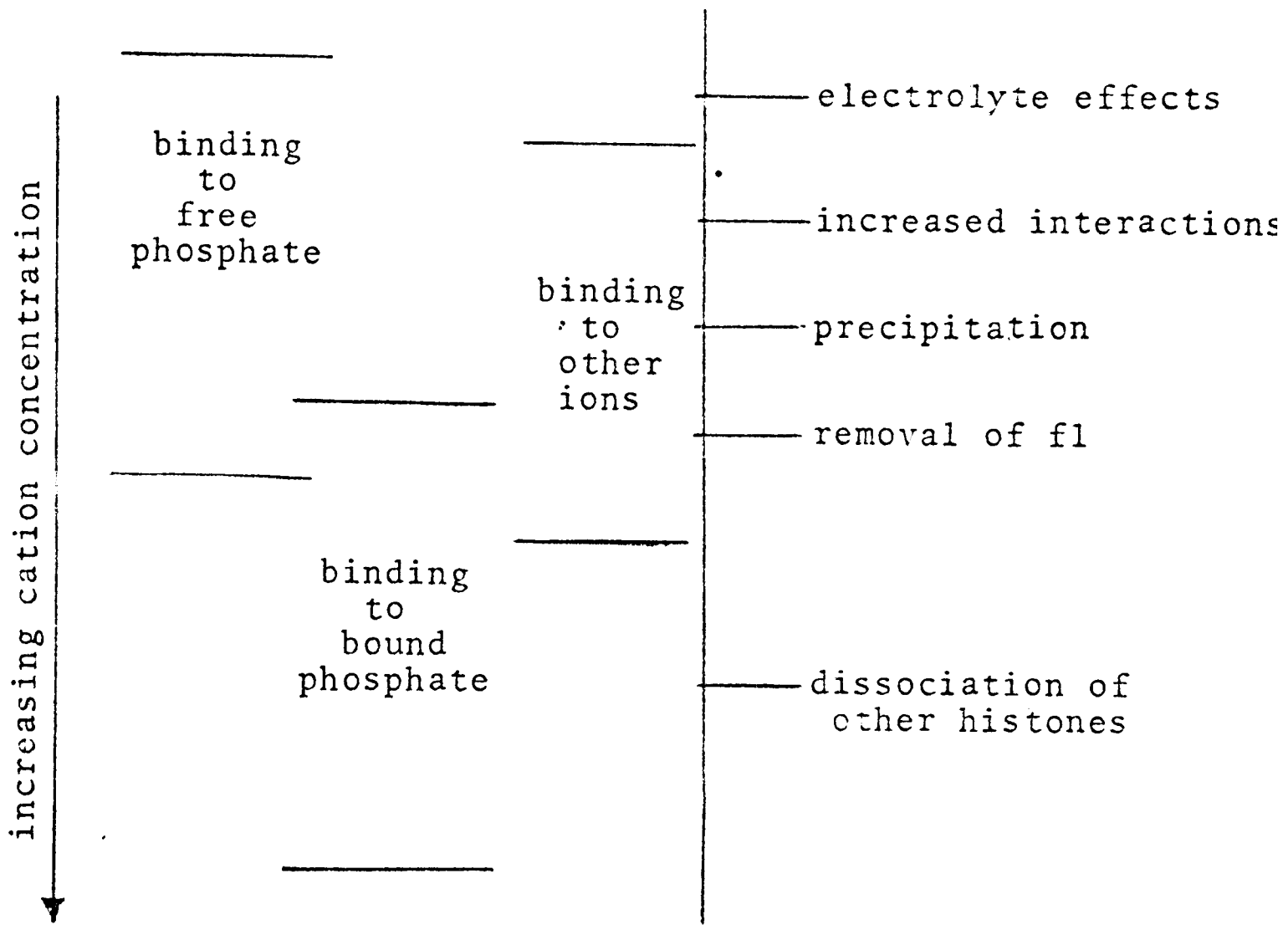
Nevertheless the interactions of DNH with neutral salts can be largely explained by reference to the interaction of metal cations with the anionic groups. The DNA phosphates can be usefully divided into free and bound phosphates - the latter being initially bound to histone basic groups. Binding of cations to these two sets can occur concurrently.

The behaviour of DNH in solutions of salts can be described in terms of the balance between binding to the various anionic groups. The importance of interactions specifically involving the DNA phosphates can be detected in two situations. The changes in DNH structure at very low magnesium concentrations, particularly the hydrodynamic changes, are so similar to the polyelectrolyte effects of DNA that they argue for involvement of the phosphate backbone. The identity between the number of salt molecules required to dissociate a molecule of histone and the number of salt-links between that histone and DNA argue that here direct displacement of the histone from phosphate by sodium ions is taking place.

Fig 8.1 outlines this model of DNH-salt interactions as they apply to our studies with sodium and magnesium chlorides.

DNH is kept stored in 0.7mM sodium phosphate

Fig 8.1 A model of DNA-salt interactions



buffer and its compact state under these conditions has been described previously.[84 82 83] Addition of small amounts of Mg^{++} (<50μM) causes a stabilisation of the molecule analogous to the polyelectrolyte effect of DNA. This leads to the more compact state observed by hydrodynamic methods. This change can be identified with binding of metal cations to the free phosphate groups.

Further addition of divalent metal ions causes an increase in intermolecular interactions which may be mediated by magnesium ions bridging from one DNA phosphate to another on a different molecule. Binding of cations to the remaining free DNA phosphates and the histone carboxylates leads to electroneutrality which is followed by complete precipitation of the DNH.

Addition of sodium ions of approximately 50mM concentration to a DNH sample already precipitated in magnesium chloride will reduce the amount of precipitation taking place. Sodium ions will displace magnesium ions from the DNA phosphate sites[50] but will not sufficiently stabilise the intermolecular interactions. In particular they will decrease the number of binding sites available for bridging.

Increase in the ionic strength will cause the precipitated nucleohistone to return to solution. This is accompanied by the dissociation of histone from the complex. Addition of any ion in sufficient concentration to a precipitated sample will result in redissolution. There is no competition between the ions responsible for the precipitation (bound to free phosphate and carboxylate) and those causing the dissociation

(binding to bound phosphate).

Normally precipitation is followed by dissociation. However, when bound phosphate is released for binding to cations before electroneutrality is reached then no precipitation takes place. $DNH^{0.7}$ at low concentrations in solutions of sodium chloride is a case in point. Here, the higher negative charge on the complex due to dissociation of f1 means that histones begin to dissociate before sufficient binding has taken place to give electroneutrality. In higher DNH concentrations electroneutrality is obtained at lower salt concentrations and dissociation occurs at higher salt concentration so that a precipitation phase can develop. No precipitation takes place in the DNH-sodium deoxycholate system but here the freeing of the bound sites is due to the effect of the salt anion on histone structure[78] rather than to cation displacement of histone.

3. The involvement of histone in DNH-salt interactions

The model developed above will satisfactorily account for most of the data on the ion specificity of precipitation. However a complete description of the dissociation and of the various structural changes involved in this and the precipitation requires reference to the histone moiety of the complex.

The reduction of intrinsic viscosity and increased intermolecular interactions of DNH on binding low concentrations of magnesium ions can be described by reference to the similar effects which take place in DNA.[181] However, they are accompanied by a reduction in the $\Delta\epsilon_{275}$ of DNH which only takes place in the

presence of f1. Removal of f1 from DNH does not affect the changes in hydrodynamic properties nor does it alter the changes in trypsin susceptibility which also appear on interaction with magnesium. f1-depleted DNH can also be induced to precipitate under the correct conditions.

The changes in trypsin digestion are indicative of a greater availability of the histone basic groups. These are arranged in the primary sequence in clusters, often disposed at the extremes of the molecule.[228] 80% of them are bound to DNA phosphate.

It seems likely that the magnesium-induced changes in compaction of the molecule and the increase in intermolecular interactions are accompanied by wider changes in the structure of DNH. These would involve a change in the binding of some of the histone basic clusters to DNA bringing them into greater contact with the solvent. No overall change in the structure of the histones themselves is likely since little change is observed in the chymotrypsin digestion patterns on addition of magnesium. Perhaps some basic clusters become involved in a loose interaction with histone molecules bound to another DNA molecule.

If we postulate that this change in DNH structure involves the quenching of whatever effect is responsible for the change in DNH CD on binding f1, we have an explanation of the absence of the $\Delta\epsilon_{275}$ change from systems not containing f1. It may be that the tyrosines of f1 histone are involved. An explanation of the $\Delta\epsilon_{275}$ effect can be obtained if the binding of f1 histone

to $DNH^{0.7}$ involves the trapping of these tyrosines in an asymmetric environment producing a positive CD at 275nm. The interaction of DNH with low magnesium ion concentrations releases these tyrosines into contact with the solvent and thus cancels their CD effect in a way similar to the removal of f1 from the complex.

As with the precipitation[22], so with this structural change which precedes it; the effect can be obtained in the absence of f1 but, if f1 is present, it will take an active part in the interaction. Nor should this be surprising since f1 is presumably situated near the surface of the DNH complex, witness its susceptibility to protease digestion and salt dissociation. It is in a position where it would be expected to be affected by intermolecular interactions such as are involved in precipitation.

Involvement of histones in DNH precipitation is twofold. They both reduce the electronegativity of the molecule thus allowing neutralisation to take place at low ionic strength and they provide increased possibilities for intermolecular interactions that would not be present in DNA alone.

The complex form required to describe the precipitation curve stresses that precipitation cannot be simply caused by charge neutralisation. Other effects are involved, the most important being the conformational changes that we have shown occuring even at very low magnesium ion concentrations. We can outline the precipitation as:-

$$DNH \underset{\text{conformational change}}{\overset{M^+}{\rightleftharpoons}} DNH^* \overset{M^+}{\rightleftharpoons} DNH_{agg}$$

where M^+ is any cation.

Precipitation is dependent on non-specific neutralisation of charge but does involve the proteins of DNH as well. No specificity for any one histone fraction has been detected in vitro.

The dissociation of histone from DNH by sodium chloride and its reverse, the binding of histone to DNA, can be considered in three steps. Not much is known of the first step, the dissociation of f1 histone. It takes place as a discrete step and appears to be concurrent with the redissolution of the precipitated material.

The second step, the dissociation of f2a2, f2b and f3, has been investigated in this study. Application of a simple model of a reversible equilibrium to the data gave the information that these fractions did not interact with the DNA cooperatively. Their dissociation in a single discrete step over a narrow salt range is the product of a high cooperativity with respect to salt. The number of salt molecules necessary to dissociate one molecule of histone agrees very closely with the number of salt linkages between a histone molecule and DNA phosphates. The dissociation is effected by a cooperative displacement of histone from DNA by sodium ions. Cooperative protein-protein interactions are not important to the binding of these

histones. Binding of salt anions to the histone can also affect the dissociation presumably by loosening the binding between histone and DNA.[78]

The third step, the dissociation of f2a1, does appear to be cooperative with respect to histone. The melting profile of a depleted nucleohistone containing only f2a1 is monophasic[82] and so f2a1 would seem to be uniformly distributed along the DNA. Such a nucleohistone has the 'supercoil rings' in its X-ray diffraction pattern. It also has an intrinsic viscosity which is midway between that of DNH and DNA.[84 82 225]

These data lead to a model of the histone-DNA system shown in Fig 8.2.

Binding of f2a1 to DNA occurs regularly along the DNA. The bound histones interact with each other to produce a structure which while being more compact than DNA is still flexible and which is presumably supercoiled.

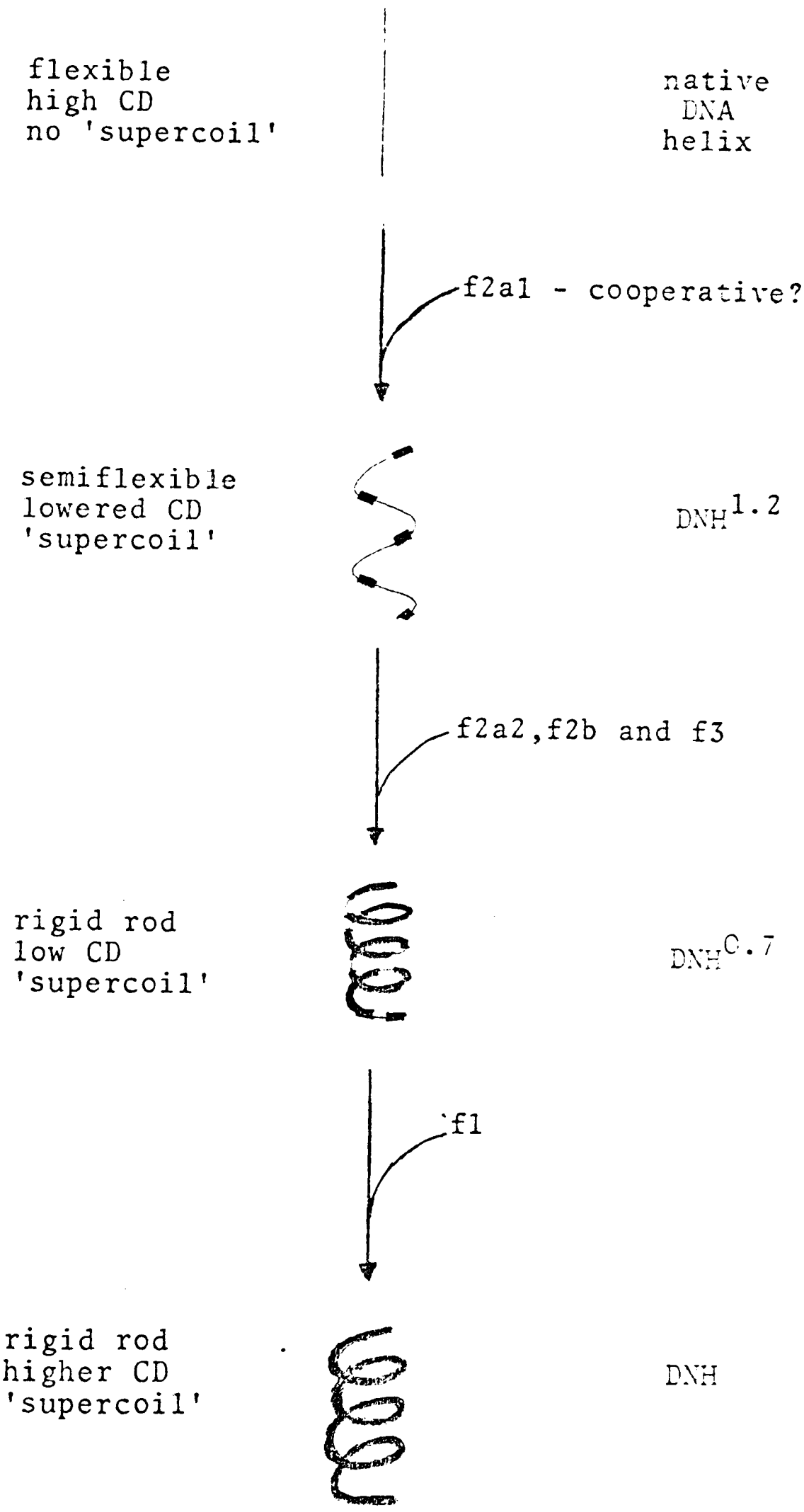
This structure then provides the template for the binding of f2a2,f2b and f3. As they are bound to the DNA they reduce the negative charge and mask the DNA bases from contact with the solvent so that a compact solution structure is produced which also has a low $\Delta\epsilon_{275}$. All the DNA is now in the supercoil configuration.

Binding of f1 to this nucleohistone ($DNH^{0.7}$) has no further effect on the structure of the DNH but causes an increase in the $\Delta\epsilon_{275}$ which can be ascribed to the f1 histone itself.

4. Ionic concentrations in the mammalian nucleus

We have described the structure of DNH in solution

Fig 8.2 A model of histone binding to DNA



'supercoil' refers to presence of low angle X-ray diffraction pattern

in a variety of ionic conditions. The relationship of these studies to the state of chromatin in the nucleus is dependent on the knowledge of the ionic composition of the nucleoplasm. Despite the practical difficulties which hinder measurement of such parameters, two systems have been extensively studied - the amphibian oocyte[140 167 33] and the rat hepatocyte.[188 190 191] Results from the amphibian system are highly contradictory and concern only monovalent ions. Data from Siebert's latest papers[191 189] on rat liver nuclei are shown in Table 8.1.

These data, the best we have at present, show two outstanding features. The total concentration of ions in the nucleus is greatly in excess of that in the cytoplasm. There is a marked difficiency of low molecular weight anions in both compartments. These presumably indicate that considerable quantities of cations in the cell are bound to macromolecular species. This is especially so in the nucleus for, in the absence of osmotic and electrophysiological phenomena at the nuclear membrane[189], the concentration of free ions must presumably equal that in the cytoplasm. Metal cations in the nucleus must have artificially low activity coefficients.

However, some nuclear membranes, e.g. in Drosophila,[120 121] have been reported to have a high resting potential. Nuclear ionic concentrations higher than those in the cytoplasm are perhaps possible, at least in this system.

Are the free ion concentrations in the nucleus dynamic or static? Muir and Whitley[135] have reported high

Table 8.1: Ionic concentrations in rat liver cells* after Siebert[191]

Ion	Nuclear	Cytoplasm
Sodium	121	11
Potassium	301	173
Magnesium	45	33
Calcium	7	5.7
Chloride	150	20
Sulphate (total su phur)	2.8	14

*concentrations are expressed in microequivalents per ml

both from rat liver and from chick erythrocytes. Condensation of the nuclei can be induced by washing with salt solutions. When mixed monovalent and divalent ions were used, a phase diagram for condensation was obtained which is very similar in outline to that given for the precipitation in Fig 6.8. Two of the parameters measured by the Olins are particularly illuminating in the context of this thesis.

The circular dichroism of the intact nuclei correlated well with their morphological appearance. Rat liver nuclei, containing considerable amounts of dispersed chromatin, gave a CD spectrum above 260nm which was very much like DNH[1.2]. The completely condensed chick erythrocyte nuclei on the other hand had a spectrum like precipitated DNH.

Even if we do not hold such spectral similarities as indicative of structural identity, dispersed and condensed chromatin appear to have different structures.

These differences do not extend to changes in the X-ray diffraction pattern. Nuclei under all salt conditions short of denaturation gave the low-angle 'supercoil' rings. This effect was not concentration dependent in the range 3.5-65%. This is in marked contrast to the results obtained by other workers with soluble nucleohistone. Gels of nucleohistone give reasonably good low-angle diffraction rings only at concentrations above 20% DNH.[152 67] Low concentrations of DNH only give the 'supercoil rings' in precipitating concentrations of divalent metal ions.[67]

Garrett has proposed that the 'supercoil rings' are dependent on there being sufficient divalent

metal ions present in the sample[67]. A sufficient concentration if such ions is present in the nucleus to explain this data. Perhaps the 'supercoil rings' are typical of the structure which DNH takes up in the presence of divalent cations. As we have shown this is a structure where intermolecular interactions are important. Indeed the original analysis[151] of the X-ray diffraction data based its findings on considerations of the packing of the supercoils into arrays.

These observations, together with those obtained in this study, lead to a model of chromatin structure in the resting nucleus. Binding of magnesium ions to the DNH produces a compact structure with considerable interactions between chains. These interactions are responsible for the the production of the low-angle X-ray diffraction rings. The histone basic clusters are more exposed to the solvent than in native DNH and may perhaps be interacting with histone molecules on other chains.

The transition between repressed and derepressed chromatin that takes place during the removal of coarse control, e.g. on the reactivation of erythrocytes on cell fusion or lymphocytes on PHA treatment, is brought about by the removal of some of the bound histone. Removal of histone and increase of acridine orange binding is a frequent initial step in the derepression of nuclei.[4 16 5] This is mediated by an ionic mechanism - derepression can be mimicked by increasing the ionic strength of nuclei[118 132 144] and chromosomes[174] - but not necessarily by increasing the ionic strength within

the nucleus. The increase in nuclear mass which takes place on the removal of coarse control[4,16,5] suggests that the binding of cytoplasmic polyanions has a part to play here.

The removal of some bound protein to produce derepression is an analogue of the removal of protein to produce a more soluble nucleohistone which occurs in precipitating ionic conditions in vitro. The relationship of such a change to the structure of chromatin seen in the electron microscope is seen in a study by Brasch[27] and his coworkers. Removal of lysine-rich histones from nuclei removed the 25nm fibres and replaced them with 10nm fibres.

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ABSTRACT

The chromatin of eukaryotic nuclei can be separated into two classes on morphological grounds. The heterochromatin is genetically inert and remains condensed while the euchromatin contains the genetically active DNA. Changes in the ionic strength of the medium can affect the balance between these two classes. The structure of chromatin in the electron microscope is also affected by ionic changes.

The ionic composition of the medium is critical for the structure of extracted nucleohistone. Increasing ionic strength will first precipitate the DNH and then dissociate the histone from the complex.

Nucleohistone was extracted from calf thymus glands by a standard procedure. The final product had a protein:DNA weight ratio of 1.30. It contained a full complement of histones. Fluorescence analysis of the DNH showed that there was little or no tryptophan present - denoting the absence of non-histone proteins. A protease activity was noted in the preparation which was responsible for its eventual degradation. f1 histone was completely digested from the complex in 4-6 weeks.

During the study of the effects of neutral salts on the DNH, several physicochemical methods were used to detect changes in the various structural levels of the complex. Polyacrylamide gel electrophoresis analysed the complement of histones present, circular dichroism examined the secondary structure of the DNA and hydrodynamic methods observed changes in the shape of the molecule. Controlled proteolysis was used to examine the arrangement of the histones on the surface

of the complex.

The dissociation of histone from DNH by salt was investigated by separating the products of the dissociation on a column of agarose gel. No equilibrium method was available for studying the extent of dissociation without separating the components. It was assumed that the equilibrium was not disturbed to any great extent by passage down the column.

The dissociation curve - the plot of amount of protein dissociated against salt concentration - varied from preparation to preparation. However, the main features remained constant. The first histone fraction to be dissociated was f1 at 0.4-0.6M sodium chloride - this dissociation correlates with the return to solution of the DNH. No further protein was removed until at greater than 0.7M salt, f2a2, f3b and f3 were dissociated together in a very narrow salt range. The position of this dissociation on the salt axis was variable. Finally f2a1 was removed in a discrete step at around 1.2M sodium chloride.

Removal of all the protein from DNH was accomplished in 2M salt. If a 2M salt sample was dialysed back to a lower salt concentration then a nucleohistone was obtained which was identical with a nucleohistone obtained by depleting the DNH at that salt concentration. This reversibility of the dissociation is held to argue for the view that DNH is the stable form of the DNA-histone system. Some workers have argued that DNH is metastable.

Some characteristics of the f1-depleted nucleohistone were investigated. It has a lower $\Delta\epsilon_{275}$ than DNH and a higher s_{20}. Fibres drawn from this DNH give the low-angle X-ray diffraction pattern described as the 'supercoil rings'. This degree of structure is also visible in a depleted nucleohistone containing only f2a1 and DNA. If only one histone is responsible for this 'supercoiled' structure then it is f2a1. This is in contrast with other reports that the compact hydrodynamic structure of DNH is dependent on f2a2 binding. The two structures are not identical.

After removal of f1, the CD of DNH increases at both 275 and 225nm, linearly with protein removal, until a protein-free DNA is obtained. The structure represented by $\Delta\epsilon_{275}$ is dependent non-specifically on binding of protein to DNA. Analysis of the CD spectra of depleted nucleohistones below 250nm using reference spectra derived from proteins of known structures shows that the extent of structure in the histone moiety of DNH does not vary from histone to histone. There is approximately 40% α-helix, 10% β-structure and 50% unordered residues. This estimate agrees well with other studies using different methods.

The dissociation of protein from DNH by salt was further investigated by treating it as a reversible equilibrium of the form

$$\text{DNH} + n\text{NaCl} \overset{K}{\rightleftharpoons} \text{DNA} + x\text{Histone}$$

The initial DNH concentration and the temperature

were varied independently and dissociation curves measured under the different conditions. A variation of DNH between 0.16 and 1.23mM (with respect to DNA phosphate) gave a shift in the position of the dissociation curves on the salt axis to higher salt concentrations. This was just outside the noise limits of the experiment and in a good experiment could be as much as 50-100mM. This shift was not sufficient to explain the heterogeneity of preparations with respect to dissociation. This must be due to a true structural heterogeneity.

Application of the model to the central portion of the dissociation concerning f2a2, f2b and f3, gave good straight lines but with a wide degree of variance. The value for x+1 obtained was 1.53 ± 0.86, leading to a value for the histone exponent of 0.53. There is little or no cooperativity in the binding of these histones to DNA. A massive cooperativity was found, however, with respect to salt. A value for n was obtained as 14.7 ± 3.5, leading to a value for the number of sodium chloride molecules required to dissociate a molecule of histone of 28. This closely agrees with the number of salt linkages between histone and DNA, thus strongly suggesting that the dissociation is mediated by the displacement of histone from DNA phosphate by sodium ions.

The variations in the extent of dissociation with temperature differ greatly from preparation to preparation. This lack of agreement together with the possibility of proteolytic artefacts when working at high temperature made the application of the model to these data dubious.

Other workers' claims to have observed cooperative

binding of histones to DNA are not borne out in this study. There are some indications that the binding of f2a1 may be cooperative.

We propose that the binding of f2a1 to DNA occurs cooperatively to form a template to which the following fractions can bind specifically.

Variation of the salt used to dissociate the histone from DNH can produce large differences in the extent of dissociation. A range of salts were used and the extent of dissociation at a single salt concentration was measured. The differences, which could not be accounted for by the differences in activity coefficients of the salts, were normalised with respect to sodium chloride and an order of effectiveness in dissociation was obtained:

$$Mg^{++} = Ca^{++} > NH_4^+ > Li^+ > K^+ > Na^+$$

$$SO_4^{=} > CO_3^{=} > ClO_4^- > I^- = Br^- > Cl^- > F^-$$

For cations this is the order of binding to DNA phosphate. This emphasises that displacement of histones by metal cations is an important part of the mechanism of dissociation. However, variation of the anion has quantitatively the greater effect on the dissociation. Binding of anions to the histone moiety of the complex can also affect the dissociation.

Estimation of the extent of precipitation of DNH in various salt solutions showed that the effect was cation dependent and related to the concentration of DNH present. The redissolution of precipitated complex on further increase of ionic strength is also cation

dependent.

Contrary to reports from other workers, f1-depleted DNH precipitates readily, although in low DNH concentrations only in solutions containing divalent metal ions. A higher salt concentration is required to precipitate the depleted sample. In the electron microscope, precipitating concentrations of magnesium affect both nor mal and depleted samples similarly. A gradual broadening of the fibre width is observed on increase of the magnesium concentration. f1 does not play an obligatory role in the precipitation of DNH. A non-specific charge neutralisation is likely to be the major mechanism.

Precipitation of DNH in mixtures of monovalent and divalent cations shows that the effects of these two classes are antagonistic and not additive. Sodium ions appear to be displacing magnesium ions from the binding sites and then not stabilising as effectively the structures involved in the precipitation. No antagonism is observed when mixtures of salts are used to redissolve the complex.

If charge neutralisation is a necessary and sufficient condition for the precipitation, this antagonism cannot be accounted for. The precipitation curve cannot be described in terms of such a model but only by a complex equation of the form:

$$pM + qA \underset{}{\overset{K}{\rightleftharpoons}} M_pA_q$$

$$K = \frac{[M_pA_q]}{[M]^p [A]^q}$$

q must be greater than one to account for the observed DNH concentration dependence,

p>4 gives a good fit to the experimental curve. Other changes are taking place than straightforward charge neutralisation. It is only a necessary condition for precipitation.

The effects of low concentrations of magnesium on the structure of DNH were investigated. Addition of the salts to normal DNH preparations was used, as were preparations made in the absence of chelating agents. These latter were shown to be little different from normal preparations except for an increased tendency to proteolysis.

Addition of 50μM concentrations of magnesium ions to DNH causes a reduction in the viscosity from 20 to 10 dl/gm. F1-depleted DNH gives the same effect. There is an increase in the Huggins constant which together with an increase in sedimentation coefficient indicates an increase in intermolecular interactions leading to aggregation. The flattening out of the reduced viscosity/concentration curves at low DNH concentrations has been reported elsewhere to stem from intermolecular magnesium bridging.

The $\Delta\epsilon_{275}$ of these samples of magnesium-treated DNH is reduced to approximately 80% of its normal value. It becomes similar to the value for f1-depleted DNH. The addition to magnesium ions to f1-depleted DNH does not further reduce the $\Delta\epsilon_{275}$.

The susceptibility of DNH to trypsin digestion increases markedly on addition of 200μM magnesium. No corresponding change in the digestion by chymotrypsin is observed. This indicates that a structural change is taking place which affects the accessibility of the

histone basic groups to the solvent.

All these changes take place before substantial precipitation of the DNH is observed. They indicate that considerable changes in DNH structure are taking place prior to the precipitation. The compaction and increased aggregation of the molecule are analogous to changes that take place in DNA structure under similar conditions. However, the changes in CD and in protease digestion indicate the the proteins and especially f1, if present, are affected.

It is proposed that the effects of neutral salts on DNH are caused by binding of metal cations to sites on the complex. In particular the balance of precipitation and dissociation is caused by the balance of binding between free and bound DNA phosphate sites.

Consideration of other results concerning the state of chromatin in the nucleus lead to the conclusion that it approaches the low magnesium structure observed here.