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The Solution and Chromatographic Behaviour of Denatured Deoxyribonucleic Acid

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Summary

The solution and chromatographic behaviour of denatured deoxyribonucleic acid

The effect of denaturing DNA by dilution below a critical concentration of DNA and salt, on the structure of DNA in solution has been shown to be very similar to that of thermally denaturing DNA. The change in relative absorbance with temperature of DNA so treated indicates that this causes the separation of the polynucleotide chains of both bacterial and mammalian DNA. On increasing the ionic strength of such a solution to the original conditions it is evident that strand recombination occurs, to produce "native-like" molecules. These results have been confirmed by sedimentation velocity measurements, density gradient equilibrium sedimentation measurements and electron microscopy. The density gradient measurements show, however, that only some of the mammalian DNA undergoes helix reformation. These measurements also show that the dilution denatured species are distinct from the heat denatured species in that they have a different bouyant density.

Fractionating DNA on columns of Ecteola-cellulose has been shown to result in the separation of molecules having different molecular weights. That the mechanism of this process depends on the physical heterogeneity of the sample, and not on the chemical heterogeneity, has been

shown by fractionating DNA that has been sonically degraded. The chromatographic profile shifts to weaker eluting conditions as the molecular weight of the DNA sample is decreased. This conclusion is supported by the correlation between fraction position and the size of the molecules, observed in this study.

The denaturation of DNA changes the chromatographic properties on this exchanger. The effect of thermal denaturation, which also gives rise to degradation, on the chromatographic properties has been compared with that of dilution denaturation. It was found that dilution denaturation has little effect on the chromatographic profile, resulting from this fractionation procedure, compared with native DNA. Thermal denaturation, however, produced significant changes in the chromatographic profile and these are interpreted in terms of degradation accompanied by aggregation of the low molecular weight species.

From these results it is concluded that dilution denaturation, which can be brought about by dissolving DNA in water at low DNA concentrations or by reducing the ionic strength of a dilute DNA solution, will result in the separation of the two polynucleotide chains of the DNA helix, uncomplicated by other effects such as degradation and aggregation. Increasing the ionic strength of such a solution of DNA will result in the recombination of these

polynucleotide chains. This recombination being essentially complete in the case of bacterial DNA but being incomplete in the case of mammalian DNA.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, nor any material previously submitted for a degree or diploma in any University, except where due reference is made in the text.

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Chapter I

Introduction

The model of deoxyribonucleic acid (DNA) proposed by Watson and Crick[1] has, with few modifications, become accepted as the basic structure for fibrous DNA. Confirmation of the general features of this model, together with precise details concerning the dimensions, have been supplied by X-ray diffraction data of fibrous crystals of the sodium salt of DNA[2-7] and of the lithium and rubidium salts.[8,9] This model is also used as the basis for discussion of the solution properties of DNA. The low angle X-ray scattering studies of Luzzati et al.[10,11] on solutions of mammalian and bacterial DNA have shown that the dimensions of the molecules in solution are in excellent agreement with those predicted by this model.

DNA molecules consist of polynucleotide chains, each chain being a copolymer of four, or possibly more, different nucleotides. The individual nucleotides are linked by phosphodiester groups which join the C(3')OH group of one nucleotide to the C(5')OH group of the adjacent nucleotide[12] (Fig. 1-I). The Watson-Crick model consists of two helical polynucleotide chains coiled about a common axis (Fig. 2-I), both of which follow right handed helices, the sequence of atoms in the sugar phosphate chains being in opposite directions in the two chains. The structure

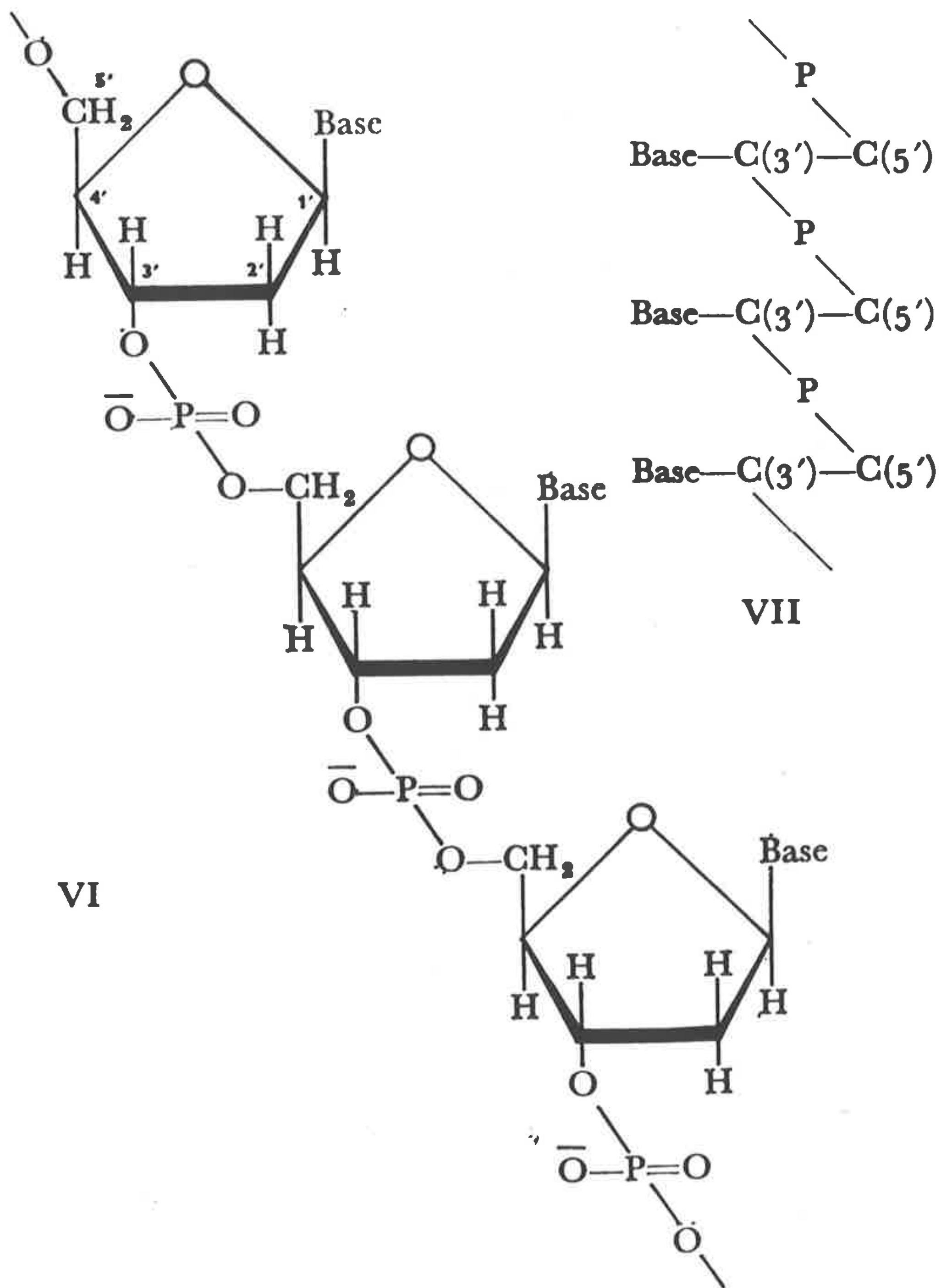

Fig. (1-I). The structure of a polynucleotide chain.

repeats itself every ten nucleotides, or 34A, the angle between adjacent nucleotides being assumed to be 36°. The polynucleotide chains are so arranged that the sugar and phosphate groups are on the outside of the helix and the purines and pyrimidines are on the inside.

The two chains are held together, according to the original Watson-Crick concept, by hydrogen bonds between the purines and pyrimidines of complementary chains. The planes of these bases are perpendicular to the molecular axis and the two bases, one from each chain, lie in the same plane and are held together by hydrogen bonds. The formation of these hydrogen bonds is found, on the basis of molecular models, to be very specific and only certain pairs of bases will yield a regular helix. It has been concluded that one member of the pair must be a purine and the other a pyrimidine base in order to bridge between the two chains. A bridge of two pyrimidines is not large enough to form the link and a bridge of two purines is too large. If the most probable tautomeric forms of the purines and pyrimidines are assumed it is found that the only pairs of bases that are possible are:

adenine with thymine

and guanine with cytosine.

According to Watson and Crick[13] 5-methylcytosine or 5-hydroxy methylcytosine may replace cytosine in the latter base pair. The base pairing proposed by Watson and Crick[13] and as

modified by Pauling and Corey[14] is shown in Figs. (3-I) and (4-I).

A given base can occur either way around, thus guanine can occur in either chain but when it does, its partner in the other chain, must always be cytosine, or one of the derivatives mentioned above. This structure places no restriction of the sequence of base pairs, but the specific pairing demands a definite relationship between the sequences of the two polynucleotide chains, namely that they must be complementary. It was originally thought that these hydrogen bonds constituted the sole forces holding the two polynucleotide chains together in solution, but more recently it has been postulated that in addition, hydrophobic interactions between the base pairs, may make a significant contribution to these forces.

In cells, genetic information is stored, in genes, in chromosomes which are components of the cell nucleus and which have been shown to consist of DNA in combination with protein. The information in the viruses and phages is also stored in the nucleic acid component, which although often DNA is RNA (ribonucleic acid) in some plant viruses. The idea that it is the nucleic acid which is responsible for transmitting genetic information stems from the discovery of Avery, Macleod and McCarty,[15] who found that the transforming principle, which induces the transformation of unencapsulated R variants of pneumococcus type II into fully

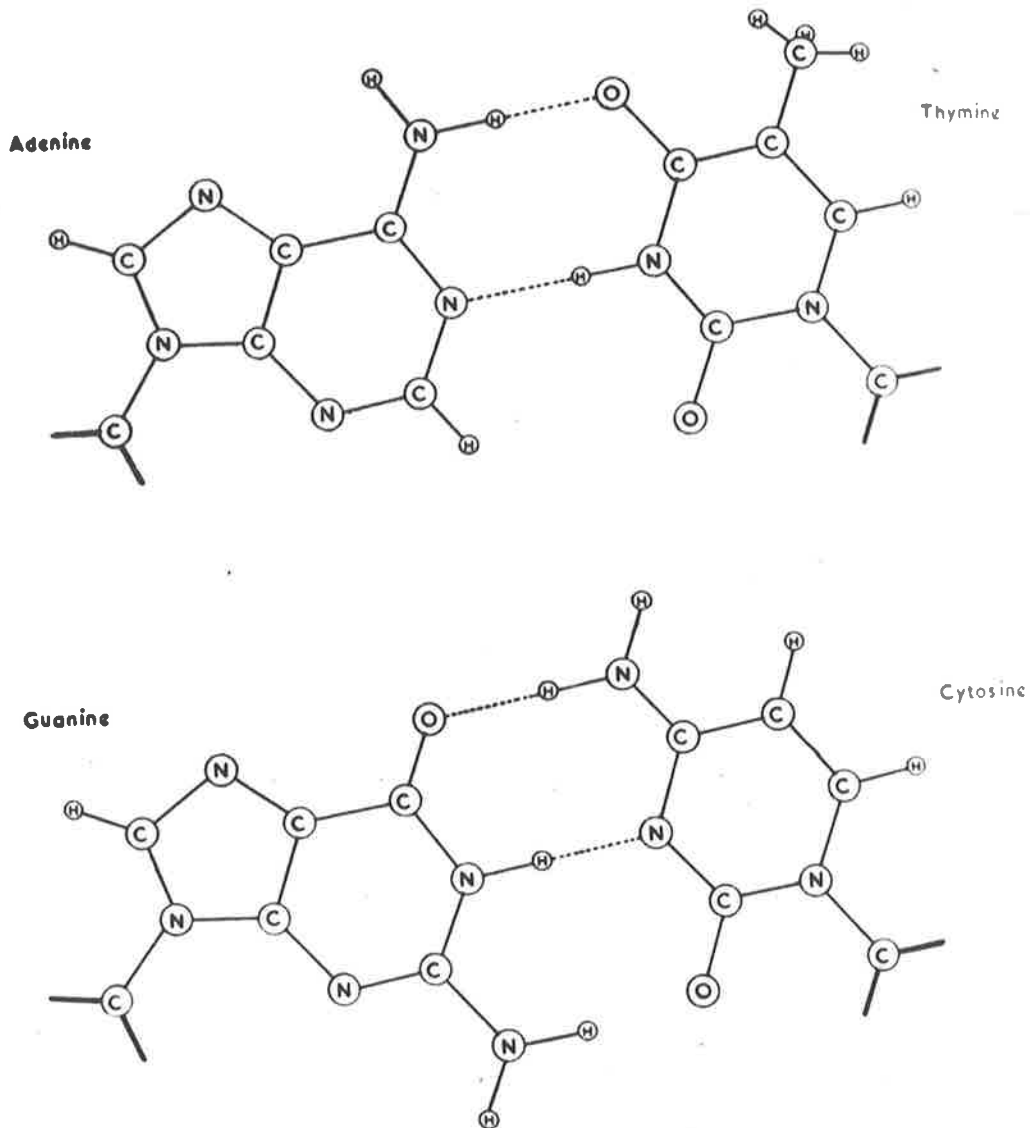

Fig. (3-I). Specific hydrogen bonding in the base pairs adenine and thymine, and guanine and cytosine according to Watson and Crick (ref. 19).

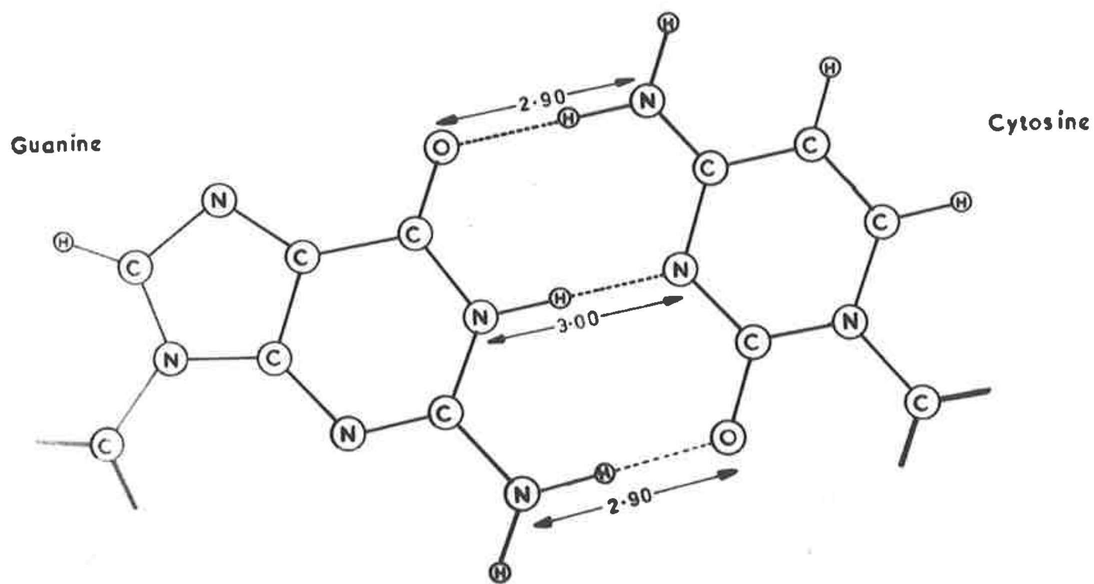

Fig. (4-I). Specific hydrogen bonding in the base pair guanine and cytosine according to Pauling and Corey (ref. 14).

encapsulated S cells of type III, was DNA. This was substantiated by the work of Hershey and Chase[16] who showed that when coliphage attacks E.coli cells the nucleoprotein constituting the phage dissociates and the nucleic acid and a very small amount (3%) of soluble protein enter the host cells and it is this that is responsible for phage reproduction. The premise that the nucleic acid is the genetic determinant is also supported by the observation[17,18] that RNA isolated from T.M.V. possesses virus activity which accounts for almost all the activity of the original virus or the reconstituted virus formed by the recombination of dissociated nucleic acid and protein.

In order that DNA may convey genetic information it must be capable of replication and of recording a vast quantity of information. A possible mechanism for these processes can be postulated on the basis of the Watson-Crick structure.[19] This mechanism postulates that the genetic information is recorded as a base sequence on the polynucleotide chains. Separation of the DNA helix with subsequent synthesis of two daughter molecules, the two halves of the original molecule being used as templates for the synthesis of the new polynucleotide chains, would result in two molecules holding the same genetic information as the parent molecule. This process presents two interesting fields of study, one concerned with the genetic principle and its relation to the base content of DNA molecules and

the other concerned with the mechanism of strand separation and reformation.

It has been suggested[20] that the genetic code may be determined by the length of nucleic acid molecules as well as by the sequence of purines and pyrimidines. This suggestion arose from evidence which showed that DNA from a given tissue was invariably physically heterogeneous as indicated by a wide distribution in the sedimentation coefficient.[21-23] Although it is possible that this heterogeneity may be the result of the preparative methods used, the fractionation of DNA on anion exchangers[24,25] shows a correlation between the composition of the eluting solution and the average sedimentation coefficient of the fraction which suggests that this heterogeneity is real. Thus the possibility arises that the DNA representing different genes may be of different length. In order to separate out a single genetic function it would be necessary to find a method of chromatography that fractionates DNA on the same basis as is involved in determining the genetic coding.

A promising method of fractionation for DNA is that of anion-exchange chromatography on an exchanger of Ecteola-cellulose.[26] The mechanism of this fractionating procedure is as yet uncertain, as at present there is evidence to suggest that fractions obtained differ both in base content and molecular size, although the evidence for the latter being the mechanism is more favourable. It

has been shown, however, that this method will not result in a single genetic principle occurring in one fraction.[27,28] Experiments will be described which are designed to investigate not only the mechanism of this fractionating process but also the fractionation of modified DNA and the effect of the chromatographic process on the resulting fractions.

The removal of the forces stabilizing the helix leads to a less ordered structure with markedly different physical properties. This process, which has become known as denaturation, may be brought about by several different ways including the addition of acid and alkali, thermal treatment and exposure to low ionic strength. It may be likened to the melting of an hydrogen bonded crystal[29] and occurs on raising the temperature over a narrow temperature range. The product may consist of either two separate strands or the two polynucleotide chains may remain entwined in a non-specific manner. The only denaturing process that has been shown to result in strand separation is that of thermal denaturation, however this must be carried out carefully in order to avoid degradation.

Denaturation is distinct from "degradation" which consists of scission of the two chains to produce two separate molecules which may, or may not, be denatured. Some processes which produce degradation are not accompanied by denaturation, for example, sonication and the direct shearing of the molecules. Degradation can also be produced by some

methods which are normally used to denature DNA, for example thermal treatment.

An investigation into the possibility that strand separation will result from the process of denaturing DNA by diluting it at low ionic strength will be described. The reverse process has also been investigated in order to find whether this will produce strand recombination.

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Chapter II

Denaturation of DNA

A. Heat Denaturation

i) Introduction

ii) Strand Separation

a) Preliminary evidence
b) Changes in hyperchromicity with different heat treatment
c) Change in molecular weight
d) Four stranded model
e) Effect of removing aggregation

iii) The nature of the bonding in the DNA helix

a) Hydrogen bonding
b) Hydrophobic bonding

iv) Dependence of strand separation on local inhomogeneities in bond strengths

a) All-or-none nature of denaturation
b) GC rich nuclei

v) Factors affecting denaturation and renaturation

a) GC content
b) DNA source and concentration
c) Effect of various reagents on T_m
d) Ionic strength
e) Time of heating

vi) Conclusions

B. Dilution Denaturation

i) Relation between ionic strength and melting temperature (T_m)

ii) Critical Concentration Phenomenon

a) Effect of lowering the DNA concentration
b) Properties of DNA below the critical concentration

iii) Suggested mechanisms

A. Heat Denaturation

i) Introduction

The effect of heat on DNA was first investigated by Cosgrove and Jordan[1] who found that on heating calf thymus DNA a titration curve could be obtained which resembled the back titration curve found by Gulland et al.[2] with the original unheated DNA. These results were confirmed by Cox and Peacocke[3,4] who showed by electrometric titration that various amounts of hydrogen bond breakage occurred when DNA was heated to different temperatures above 75°C. Goldstein and Stern[5] were able to show, by studying the viscosity of DNA as a function of temperature, that a critical temperature existed above which the viscosity of DNA fell markedly. This critical temperature was shown[6] to depend on the ionic strength of the solution as well as the native state and source of the DNA. Zamenhof et al.[7,8] further investigated this fall in viscosity with temperature and were able to show that both the intrinsic viscosity and the transforming activity of Hemophilus influenzae DNA fell precipitously at the same critical temperature, in the vicinity of 80°C.

The generally accepted explanation of the mechanism of heat denaturation is that the two polynucleotide strands of the DNA helix acquire sufficient kinetic energy to break some of the bonds holding them in the helical configuration. The sharpness of the transition in the

viscosity temperature curves suggested that this process resulted in a configurational change from rigid helices having a one-dimensional periodicity to disordered randomly coiled chains, with the characteristics of the melting of a periodic intra-molecular structure.[9,10,11] This temperature is now known as the melting temperature of the DNA sample.

ii) Strand Separation

a) Preliminary evidence

If, on denaturation, all the bonds between the polynucleotide chains were broken the two chains should separate with a resultant drop in the molecular weight. In order to investigate this possibility molecular weights of heated samples of DNA were determined by light scattering.[12-16] This method was chosen because it not only provides an absolute value for the molecular weight but at the same time provides a value for the radius of gyration, giving a measure of the dimensions of the macromolecule without any assumptions regarding its size and shape. These measurements showed that the molecular weight of the denatured DNA samples was the same as that of the native samples (provided the heating was not carried out for long periods when degradation occurred), but that the radius of gyration had decreased by a factor of three, corresponding to a decrease in the effective hydrodynamic volume by a factor of twenty-seven.[13] The light scattering results thus

confirmed the viscosity measurements which showed an extensive collapse in the secondary structure. From this Doty[12,13] concluded that the polynucleotide strands in the denatured state were held together by remaining or reformed bonds. This means that the sedimentation coefficient should increase due to a reduction of the frictional co-efficient. However it was found that the sedimentation coefficient of denatured DNA did not differ from that of the native sample, although there was a broadening of the distribution of sedimentation coefficients.[13,17-20]

b) <u>Changes in hyperchromicity with different heat treatments</u>

Goldstein and Stern[5] had found that on heating DNA to 100°C and then cooling the solution to 23°C there was a rise in the optical density of the solution. This increase in optical density (termed hyperchromicity) exhibited by DNA on denaturation was used by Thomas[6] to study the denaturation of DNA as a function of temperature. A curve was obtained which closely paralleled the rise in the proportion of hydrogen bonds broken with increase in temperature observed by Cox and Peacocke,[3] indicating, in agreement with viscosity results, that heat denaturation resulted in a collapse of the secondary structure due to breakage of interchain hydrogen bonds.

Both changes in optical density and specific rotation were used by Doty et al.[11] to study the thermal

denaturation of solutions of calf thymus DNA. The magnitude of these changes was shown to vary greatly depending on whether the measurements were made at the ambient temperature or after cooling the solution to room temperature, indicating a certain amount of reversibility in the hyperchromic shift on cooling to room temperature. The initial melting was found to be largely reversible, especially at low ionic strength, and even when the transition was complete substantial reformation of helical regions was found on lowering the temperature, particularly at high ionic strength. If a sample of DNA was heated to 100°C, then cooled and reheated the change in optical density with temperature became completely reversible in agreement with the results of Shack.[21] These results were explained[11] in terms of the reformation of short helical regions in the molecules on cooling. These regions must be short compared with the length of the molecule because they do not restore the high viscosity exhibited by native DNA. The broad nature of the curve obtained on reheating is also indicative of these short helical regions, since the analysis of Zimm et al.[22] indicates that the distributions would broaden and the mid-point of the curves (T_m) diminish as the chain lengths decrease.

On reheating bacterial DNA from D. pneumoniae, after heating a sample to 100°C and cooling it, it was found[23] that the change in optical density with temperature was

dependent on the method of cooling. If the sample was cooled slowly nearly all the original hypochromicity was regained and on reheating the sample had a definite melting point in the same region as the undenatured sample. This behaviour was attributed to the reformation of large complementary helical regions between the polynucleotide chains, resulting in a 'native-like' structure. This type of sample is henceforth referred to as 'renatured' DNA. Rapid cooling of the sample gave rise to a substance which had a very low transforming activity, in contrast to the slow cooled sample in which the transforming activity was nearly equal to that of the undenatured sample. The thermal denaturation curve for this sample showed that no long complementary helical regions had been formed during the cooling, instead non-specific hydrogen bonds had been formed between the chains.

The formation of renatured DNA has not been observed for calf thymus and other mammalian DNA's, their behaviour on slow cooling being the same as that for rapidly cooled bacterial DNA. This was thought to be due to the heterogeneous nature of mammalian DNA compared with the more homogeneous DNA from bacterial and viral sources. Thus if strand separation did occur on denaturation, it would be possible during the slow cooling of the more homogeneous bacterial DNA for two complementary strands to seek each other out. However, with heterogeneous mammalian DNA

there would be less chance of a given strand coming into contact with its complement and so non-specific hydrogen bonding would result. Rapid cooling of bacterial DNA would produce the same result, as the two complementary strands would have less chance to come in contact before the energy was lowered sufficiently for the hydrogen bonds to reform and hence non-specific hydrogen bonding results.

c) Change in molecular weight on denaturation

Early denaturation studies had shown that the expected halving of the molecular weight as a consequence of strand separation had not occurred, and so it was concluded that either all the bonds between the chains were not dissociated, thus preventing separation of the poly-nucleotide chains, or the potential bonding capacity between the two chains was sufficiently large that the two separated strands bonded non-specifically so entangling to form aggregates. These are thought to lead fortuitously to a molecular weight value approximately double that expected for the separated single strands. Although attempts were made to exclude aggregation as the basis of the failure to observe a halving of the molecular weight by light scattering[13,24] the methods were not successful, partially due to the fact that DNA from animal sources was used and partially due to the high concentration of DNA needed.

The experiment of Meselson and Stahl[25] was the first to show convincingly that the polynucleotide chains

separated during replication and denaturation. In their study on the mode of replication of E.coli DNA, cells were grown on a medium containing N^{15} as the sole nitrogen source and then transferred to an N^{14} medium. At various times after the medium transfer, samples were removed, lysed and centrifuged in a cesium chloride gradient in order to analyse the amounts of N^{14}, N^{15} and hybrid DNA. It was found that after one generation's time from the addition of the N^{14} media a hybrid $N^{14}N^{15}$ DNA was the only species found, this banded half way between the position of the N^{14} and N^{15} DNA's in the cesium chloride gradient. After the second generation of growth equal amounts of hybrid and unlabelled (N^{14}) DNA were found to be present. These results show that the replication of DNA in E.coli proceeds by a semi-conservative mechanism[26] in which half of the DNA is passed on to the daughter molecule. That the semi-conserved units were arranged side by side and not end to end was shown by ultra-sonically degrading the biological hybrid DNA,[27] this produced no change in the number of peaks in a cesium chloride density gradient, only a change in the spread of the peak.

Meselson and Stahl were also able to show that heating the biological hybrid DNA in the cesium chloride medium at 100°C for thirty minutes produced two species of DNA, equal in amount, which banded at different positions in the density gradient. Each of these samples had approximately half of the initial apparent molecular weight.

The positions of the bands were identical to those produced on heating an equal weight mixture of N^{15} labelled and N^{14} unlabelled DNA. Thus it was concluded that under these conditions the polynucleotide chains (or molecular subunits) had dissociated upon heating.

Marmur et al.[28] point out that the denaturing conditions of these experiments differed significantly in one respect from those previously reported, namely, the DNA concentration was at least forty-fold lower than that employed in earlier investigations. For example the DNA concentrations used in the earlier light scattering studies ranged from 20-200 μg/ml[13,19,24] whereas Meselson and Stahl[25] used only 1-2 μg/ml. This work was supported by that of Doty et al.[23] who showed that at a concentration of approximately 2 γ/ml, DNA from D.pneumoniae underwent strand separation on heating as shown by density gradient sedimentation and molecular weight measurements using intrinsic viscosity and sedimentation coefficient results.

Density gradient sedimentation was also used to show[23] that the separated strands could recombine to form a material resembling native DNA when samples were slow cooled under appropriate conditions. If the DNA concentration and ionic strength of the solution was too low then no renaturation took place and no aggregation occurred, that is the strands remained separate.

d) Four-stranded model for DNA

Cavalieri and his co-workers[29-34] put forward evidence to suggest that the subunits produced on strand separation did not represent single stranded DNA. They maintain that the DNA from rapidly dividing cells, such as *E.coli* in its logarithmic growth phase, is four stranded consisting of two Watson-Crick helices lying side by side and held together by "biunial" bonds. It was concluded that the drop in molecular weight found on denaturation is due to the disruption of these bonds and that the complementary strands of the double helix cannot completely separate although some separation, leading to a collapsed structure, can take place. To account for the absence of a change in the molecular weight of salmon sperm DNA when it was heated in cesium chloride,[29] it was postulated that DNA from non-dividing cells, e.g. calf thymus cells, was double stranded in accord with the Watson-Crick structure. The evidence for these conclusions was based mainly on a study of the change of light scattering molecular weight with time during degradation of DNA.

Molecular weights obtained by light scattering are almost certain to be of doubtful value as the measurements have to be made at concentrations which will result in aggregation of the DNA. Two further objections can be raised against these measurements of the light scattering molecular weight decay. Those samples that were to be treated with

the enzyme DNase II were first denatured and cooled rapidly to 25°C. This treatment, at the high concentrations of DNA used, would produce extensive aggregation and so would tend to complicate any interpretation of the kinetics of the enzyme degradation. Secondly, neither enzymatic nor X-ray degradation of DNA, the two methods used by Cavalieri and Rosenberg,[30,32] act in a random manner[35,36] thus invalidating the use of their kinetic results to determine the number of strands per DNA molecule.

Luzzati et al.[37,38] in order to test Cavalieri's model studied DNA from mammalian and bacterial sources by small angle X-ray scattering techniques. The results for both types of DNA were identical and showed that the dimensions of the molecules were in excellent agreement with those of the Watson-Crick model and that the molecules were independently distributed in the solution without any side-by-side association. This result is thus not in agreement with the conclusions of Cavalieri et al.

Cavalieri et al.[34] also carried out kinetic measurements on the renaturation of bacterial DNA which showed that renaturation in 1.0M sodium chloride is essentially first order at 60°C, but at 70 and 80°C there is an increasing amount of second order component, which at 85°C causes the reaction to become bimolecular. Thus it was concluded that renaturation results from the "zipping up" of collapsed and disordered, but not separate, double

stranded, denatured molecules. The bimolecular kinetics were interpreted as being due to the interaction between the ends of two DNA molecules, each of which possesses incompletely separated strands. This type of interaction is identified with the density hybrids of Schildkraut et al.[39] and the genetic hybrids of Herriott,[40,41] formed by heating and annealing DNA.

That this picture is incorrect has been shown by Rownd and Green,[42] who pointed out that the density hybrids of Schildkraut et al.[39] which have an intermediate buoyant density when observed in a cesium chloride density gradient, are not formed by non-specific, end-to-end aggregation of heavy isotope labelled and unlabelled DNA as the sonication experiments of Rolfe[27] had shown them to consist of different strands arranged side-by-side. Thus these biological hybrids could not be used to form DNA species possessing buoyant densities characteristic of either renatured fully labelled or fully unlabelled DNA, as found by Rownd and Green,[42] unless strand separation and subsequent recombination of similarly labelled strands occurs during the heating and annealing process. Heating and annealing hybrid molecules from both E.coli and B. subtilis with N^{15} and deuterium in one strand was shown to result in the formation of renatured fully labelled and fully unlabelled, as well as hybrid DNA molecules. The proportions being the same as if a mixture of labelled and unlabelled DNA was used as the starting material.

e) Effect of aggregation

Subirana et al.[43] found that if kinetic experiments were carried out at low bacteriophage and bacterial DNA concentrations (less than 10 μg/ml) in standard saline citrate in order to reduce aggregation phenomena the kinetics of renaturation were second order. The kinetics were shown to depend significantly on the DNA concentration and on the ionic strength. At high DNA concentrations and in 1.0M sodium chloride complex second order kinetics were found, possibly due to increased interaction between the renatured species to form "aggregate-renatured" DNA. In lower ionic strength solvents, even at high DNA concentrations, the kinetics remained second order.

Kit[44] showed that the thermal degradation of denatured DNA at 95°C was kinetically similar to that of RNA and hence reflected the depolymerization of a single polynucleotide chain. Since these measurements were made at an elevated temperature, at which intramolecular bonds are dissociated, the complications due to aggregation were eliminated. Thus it is concluded that if the possibility of aggregation is carefully avoided it is possible to show that both denaturation and renaturation do involve the separation of the two polynucleotide chains of a Watson-Crick helix.

This conclusion was further supported by the work of Thomas and Berns.[45,46] It had been shown[47,48] that

aggregation could be eliminated by denaturing in the presence of formaldehyde which reacts rapidly with the amino groups exposed by the dissociation of the inter-chain bonds. That inter- and intra-chain bonding is blocked by the formaldehyde treatment is shown by the fact that on cooling the denatured sample to room temperature very little of the original, native hypochromicity is regained. Thomas and Berns[46] denatured unbroken T_2 and T_4 bacteriophage DNA molecules by three methods in the presence of 1% formaldehyde. The molecular weight of the denatured whole molecules, as measured by the band width in density gradient equilibrium experiments, was found to be one half that of the native molecules, irrespective of the mode of denaturation.
Thus, denaturation of the T_2 and T_4 DNA molecules, which had been shown to possess a double-strand structure on the basis of mass per unit length considerations, results in a halving of the molecular weight. This could only have been accomplished by the separation of the polynucleotide chains under the denaturing conditions. If a few single chain breaks were produced by occasional enzymatic scission by an endonuclease the molecular weight of the denatured product was found to be more than halved, showing that there are no preformed breaks in the original single polynucleotide chains.

iii) <u>The nature of the bonding in the DNA helix</u>

a) <u>Hydrogen bonding</u>

The accumulative evidence suggests that on denaturation of DNA essentially all the inter-chain base pairs dissociate leading to the separation of the polynucleotide chains. Thus it is of interest to consider what forces contribute to the stability of the helix in native DNA. It was thought[3,6,13,49-55] until recently that the main forces holding the two chains of the DNA helix together resulted from the inter-chain hydrogen bonds. It was found that many protein denaturing agents could also denature DNA. Rice and Doty,[13] for example, found that urea which can compete with water for hydrogen bond formers and used as a protein denaturing agent could aid in the thermal denaturation of DNA. Although it had no effect at normal temperatures, the effect it had at high temperature was thought to be due to its aiding in the disruption of the hydrogen bonded base pairs. Similarly Ts'o et al.[56] have found that formamide can be used to denature DNA and their results indicate that in formamide solution nucleic acid polymers are devoid of all formal secondary structure.

The strongest evidence for helix stability being due to hydrogen bond formation comes from the work of Szer and Shugar[57] on synthetic polynucleotides. It was found that, in contrast to poly-uridylic acid (poly-U), poly-methyl-U exhibits no secondary structure and will not form

complexes with poly-adenylic acid. Thus by blocking the formation of a hydrogen bond in one of the aromatic rings inter-strand hydrogen bond interaction is prevented. The only other explanation of their results is that the introduction of the methyl group causes structural distortions due to steric hindrance.

It was suggested by Pauling and Corey[58] that the GC base pair could form three hydrogen bonds and the AT pair only two. Marmur and Doty[59-61] used this suggestion to account for their results which showed that DNA containing more GC than AT base pairs exhibited higher thermal stability. However, this result would not be expected on the basis of the different number of bonds, but only if the strength of a GC hydrogen bond differed from that of an AT hydrogen bond. If the bonds are of the same strength then when the temperature has been raised to a point where enough thermal energy has been added to break one bond they will all break and thus all DNA samples would have the same melting temperature. (The number of bonds will be important in the case of acid and alkali denaturation.) Therefore the greater stability of the DNA samples richer in GC than AT base pairs must mean that a single GC hydrogen bond is stronger than a single AT bond, if the stability of the helix is dependent on the hydrogen bonding forces. As the lengths of these hydrogen bonds are all nearly identical,[51,58] being 2.80 and 3.00 A in the case of the AT base pair and 2.90,

3.00 and 2.90 A in the case of the GC base pair, the difference in strength, if it exists, is unexpected.

b) Hydrophobic bonding

Recently evidence has been brought forward which suggests that forces other than that due to hydrogen bonding are involved in the denaturation of DNA. Sturtevant et al.[62] pointed out that the infrared spectral changes that accompany DNA denaturation[63,64] indicate that the purines and pyrimidines are more strongly hydrogen bonded in the denatured state and that the heat of denaturation of such a structure would have to be negative. It was suggested[61] that this discrepancy could be explained by postulating that the known stability of DNA was due to the fact that the differential solvation effects of water are so strong that hydrogen bonding makes only a relatively small contribution to the maximum obtainable stability.

Studies on the stability of the DNA secondary structure in organic solvents[65-67] and in concentrated solutions of 1:1 electrolytes[68,69] indicate that many substances not considered to be hydrogen bond breaking agents can promote the denaturation of DNA and bring about strand separation. It has also been shown that some of the usual protein denaturing agents are relatively ineffective in denaturing DNA.[13,70-72] When the destabilizing effects on DNA of a series of various organic reagents were stud-

ied[65-67,71,73,74] it was found that the effects were contrary to those expected on the basis of their hydrogen bonding potential. It was found[75] that purine and pyrimidine bases could act as destabilizing agents and hence lower the thermal denaturation temperature by several degrees. A similar result was found by Hamaguchi and Geiduschek[69] using anions such as trichloroacetate, trifluoroacetate, thiocyanate and perchlorate, these anions were found to lower the melting temperature of DNA by as much as 60°C at neutral pH. These authors were also able to vary the contributions of the AT and GC base pairs towards helix stability by using these anions. The slope of the straight line obtained in a plot of T_m versus G + C content for DNA in various salt solutions could be made to vary in either direction from that reported by Marmur and Doty[60,61] using saline citrate as solvent. Thus it was concluded that the differences in the stabilities of GC and AT rich DNA helices do not arise exclusively from hydrogen bond contributions. Some correlations were made between the effect of these salts on DNA denaturation and their effect on water structure as well as on the water solubility of electrolytes. From this Hamaguchi and Geiduschek concluded that the denaturation of DNA was due to the effect of these electrolytes on the structure of the water and thus referred to them as hydrophobic bond breaking agents. The statistical mechanical treatment of denaturation[76,77] also implies that forces

other than those due to hydrogen bonding are also involved in maintaining the stability of the DNA helix.

De Voe and Tinoco[78] have recently calculated what they consider to be the main base-base attractive forces in DNA, this work represents an initial step in the calculation of what may be the major forces holding the helices together. Although these authors did not take into account hydrophobic bonding forces their results could be described in terms of hydrophobic bonding if this term refers to the forces in DNA causing the bases in the native state to be attracted to each other more than they are to the water molecules which normally surround them in the denatured state. Dipole-dipole, dipole-induced dipole and London force interactions among the bases in the helix were shown to be large and result in the free energy of the helix being dependent on the base composition and sequence, although this dependence on the sequence must be small or the sequence would not be able to determine the genetic code carried by the DNA molecule. These calculations also indicate that the hydrogen bond interactions between the bases do not contribute significantly towards the helix stability although they may ensure specific base pairing. These results predict that the helix stability should be proportional to the G + C content which explains the results of Marmur and Doty[60,61] without having to postulate that

there is a difference in strength between the GC hydrogen bonds and the AT hydrogen bonds.

It is thus to be concluded that the main forces responsible for the stability of the entropically unfavourable helical conformation[79] of DNA are a combination of the base-base interaction forces and the hydrophobic bonding forces which cause these non-polar bases to interact with each other in preference to water. The hydrogen bonding is significant in that it ensures specific base pairing and also ensures that the energy stabilization gained from an enthalpic contribution due to the hydrogen bonding of the bases to water molecules is compensated for in the non-polar interior of the helix.

iv) Dependence of strand separation on local inhomogeneities in bond strength.

a) All-or-none nature of denaturation

Another concept of DNA denaturation that must be considered is whether the denaturation reaction is an all-or-none phenomenon.[13] It appears that those regions within an individual molecule which have a lower G + C content than other regions within the same molecule and hence a lower thermal stability, melt at temperatures insufficient to denature the entire molecule. Since the other regions of the molecule are still intact the base sequence remains potentially in register, thus if the denaturing conditions are removed the disrupted hydrogen bonds can reform.

This was shown by the work of Doty et al.[11] and Geiduschek[80] who measured the thermal denaturation curves for samples of DNA both at the ambient temperature and after the solution had been heated, rapidly quenched, re-equilibrated and its relative absorbance plotted as a function of the initial temperature to which the DNA had been exposed before cooling. The mid-point of the ambient temperature denaturation curve ($T_{\frac{1}{2},d}$) was found to be several degrees lower than the mid-point of the denaturation curve measured at 25°C ($T_{\frac{1}{2},i}$). The change in absorbance of DNA recorded at the ambient temperature is a measure of the disruption of the helical structure without necessarily involving strand separation. Thus Geiduschek[80] found that T_2 bacteriophage DNA could be heated to a temperature which causes a 37% increase in absorbance at the ambient temperature, and rapid cooling from this temperature, followed by absorption measurements at 25°C, indicated that very little, if any, denaturation had taken place. The absence of any overall viscosity change during this process[67] indicates that this lack of change in absorption on cooling the sample to 25°C is due to the recovery of the helical structure of the molecules.

Schildkraut et al.[81] using biologically active hybrid DNA from E.coli were also able to show that partial denaturation could be reversed. A DNA sample was heated

at its T_m for twenty minutes and then rapidly cooled. On equilibrating this sample in a density gradient the band profile remained the same as that of the native sample. From this it was concluded that the molecules remained in their helical form. In order to melt selectively the AT-rich molecules the same sample was heated to a temperature a few degrees above the T_m, where it was found that 25% of the molecules underwent strand separation. The molecules having the greater resistance to heat denaturation were found to have a higher density, in agreement with the hypothesis that they are GC rich. Thus it is possible to melt molecules having a high AT content leaving GC rich molecules undenatured.

b) GC-rich nuclei

The relation between strand separation in biologically active hybrid DNA from *E.coli* and the extent of thermally induced hyperchromicity was studied by Freifelder and Davison.[82] Samples of DNA in 0.01 M phosphate and 1% formaldehyde were heated and the percentage of the maximum hyperchromicity measured as a function of temperature. Solutions at a particular temperature were cooled and equilibrated in a cesium chloride density gradient. If during the heating process the strands underwent separation the formaldehyde would react with the amino groups exposed by the separated strands thus preventing the reformation of

the helical structure and giving rise to bands in the cesium chloride gradient corresponding to denatured DNA. Thus the percentage strand separation was determined as a function of both temperature and hyperchromicity. It was found that no strands separated until about 75% of full hyperchromicity had been obtained, and even at apparently full hyperchromicity a large fraction of the strands may be held together, possibly by GC rich regions.

Such regions as these Geiduschek[80] termed GC rich "nuclei". It was proposed that the existence of these thermally resistant nuclei, which can hold the base sequences of collapsed DNA molecules in register until the temperature is reduced whereupon the molecules can renature, could explain the difference between $T_{\frac{1}{2},i}$ and $T_{\frac{1}{2},d}$ ($\Delta T = T_{\frac{1}{2},i} - T_{\frac{1}{2},d}$). ΔT was shown to depend on the molecular weight of the DNA sample, its base composition and probably on the distribution of bases as well. The temperature absorbance curve of DNA re-equilibrated to 25°C after heating therefore reflects the existence of those denatured molecules which have suffered strand separation after the melting of the last base pair[82] or, to a smaller extent, those DNA molecules which are collapsed and in a partially denatured state.

These results[11,80-82] indicate that if DNA molecules are kept in the presence of denaturing conditions

such as elevated temperature, or when they are heated and cooled in the presence of formaldehyde, it is possible to obtain DNA molecules that are partially denatured. That is they resemble denatured molecules except that the chains are held in register by thermally resistant nuclei, presumed to be GC rich regions in the molecule.

The introduction of cross links by means of chemical or physical agents, such as ultraviolet light,[83-86] nitrous acid[86,87] and bifunctional alkylating agents,[87] will allow the DNA double helix to disrupt but, like the GC rich nuclei, will hold the two chains in register. Thus the DNA will return to a renatured configuration when the denaturing agent is removed.[80,83-87] Unlike the reversibility produced by GC rich nuclei these covalent links cannot be removed by normal denaturing action and the DNA containing them is permanently reversible.

These results show that it is possible to heat a sample of DNA and melt those molecules having a high AT content leaving those rich in GC undenatured. They also show that it is possible to break most of the hydrogen bonds in a molecule but provided a few stable nuclei are unbroken the molecule will not separate into two strands. Molecules consisting of denatured AT regions and native GC regions can also be obtained at elevated temperatures. Thus it is concluded that DNA denaturation is not an all-or-none reaction.

v) Factors affecting denaturation and renaturation

a) GC content

It has been shown above that the GC rich nuclei are responsible for the optimal renaturation temperature being GC dependent,[59,80] other than this it would appear that the renaturation process is independent of base composition. This is in contradistinction to the thermal denaturation process which mainly depends on the base composition. It has been shown that the higher the GC content of a DNA sample the higher is its thermal stability. For example in 0.15 M sodium chloride plus 0.015 M sodium citrate (S.S.C.) the T_m increased by 0.41°C per mole per-cent GC,[60,61] 0.56°C in 7.2 M sodium perchlorate, 0.35°C in 51% methanol containing 10^{-3} M sodium chloride and 10^{-3} M tris,[80] and 0.52°C in 0.1 M sodium perchlorate at pH 7.[88] De Voe and Tinoco[78] have shown that this result is a natural consequence of the interactions between the base pairs.

The nucleation hypothesis can also be used to explain the dependence of renaturation on molecular weight. It was found on measuring the extent of renaturation by the recovery of transforming activity and by spectroscopic methods[59,77] that the extent increased with molecular weight. This is to be expected on the basis of this hypothesis as the higher the molecular weight of a DNA sample the fewer are the nuclei required to renature a given amount of DNA.

It would appear,[61] however, contrary to the analysis of Zimm,[22] that the melting temperature of DNA measured at the ambient temperature ($T_{\frac{1}{2},d}$) is independent of the molecular weight of the DNA, although it is dependent on the molecular weight when measured after the heated DNA solutions have been cooled to room temperature ($T_{\frac{1}{2},i}$).[80] (Evidence is presented in Chapter IV which suggests that the ambient melting temperature may also to some extent be dependent on the molecular weight.) This can be attributed to the inhomogeneities in base sequence, as the higher the molecular weight the greater is the probability of the molecule possessing a greater number of more stable nuclei, thus more readily allowing their reversible denaturation compared to smaller DNA molecules.

b) <u>DNA source and concentration</u>

There are other factors which have different effects on denaturation and renaturation. For example it has been shown that the denaturation mechanism is generally unaffected by the source of the DNA[11,23,25,39,80,82] whereas thermal renaturation has been found by Marmur and Doty[23,59] to depend markedly on the source of the DNA. Mammalian DNA coming from cells with a very large content of heterogeneous DNA renatures only slightly, bacterial DNA with greatly reduced amounts of DNA per cell undergoes extensive renaturation and DNA from the very smallest

bacteria and bacteriophage, having the lowest DNA content, shows nearly complete renaturation. This dependence on homogeneity is expected since renaturation depends on the concentration of specific complementary strands.

Thus it is also expected that the amount of renaturation should increase with increase in the DNA concentration of a given solution of DNA. This was shown to be the case by Doty et al.[23] who found, in the case of pneumococcal DNA, that if concentrations much below 20 γ/ml were employed the amount of molecular recombination was quite low, in fact at a concentration of 1 γ/ml almost no recombination occurred. If the DNA concentration is too high the observation of renaturation becomes complicated due to the presence of aggregation, which starts to occur about the optimum concentration for renaturation. This can be overcome by specifically removing the aggregates, for example by the use of *E.coli* phosphodiesterase.[89]

This phenomenon of aggregation means that denaturation accompanied by strand separation will only occur at low DNA concentrations where aggregation does not occur or in the presence of reagents such as formaldehyde which prevent the reformation of hydrogen bonds thus preventing aggregation.

c) Effect of various reagents on T_m

Thermal denaturation is also affected by pH. At values of the pH between 5.5 and 8.5 the T_m varies

very little with change in pH but outside of this range the T_m falls rapdily with decrease (acid denaturation) and increase (alkali denaturation) of pH.[75,90]

The effect of the presence of many different reagents in lowering the denaturation temperature of DNA has been mentioned previously in section iii (b). The work of Freifelder and Davison[82] together with that of Stollar and Grossman,[91] Thomas and Berns[46] and Haselkorn and Doty[92] indicates the effect of formaldehyde in lowering the T_m and preventing reformation of denatured DNA. Denaturation of DNA without the introduction of undesirable side effects can be brought about by the addition of formamide. Ts'o et al.[56] have shown that formamide can denature DNA at both 25°C and 37°C without causing backbone scission. It was also shown that in formamide solutions nucleic acid polymers were devoid of all formal secondary structure. Mandel has shown that the T_m of DNA solutions increases when DNA interacts with spermine.[93] This type of behaviour is similar to that investigated by Lee et al.[94] who found that the T_m of calf thymus DNP was higher than that for DNA, possibly due to the effective screening of the phosphate groups on DNA by the protein.

The most extensive study to date on the denaturation of DNA by organic compounds is that of Levine et al.[69] who concluded from their results that amides, ureas, car-

bamates and alcohols act as denaturing agents, not because of their hydrogen bonding capacity, but rather because they stabilize denatured DNA relative to native DNA. This is accomplished by decreasing the ion-solvating power and increasing the hydrophobic nature of the solvent. The forces involved in denaturation are not necessarily considered to be the same as those which stabilize the native structure of DNA. Thus they believe the decrease in T_m of the DNA in the presence of organic denaturing agents cannot be used as evidence for the existence of hydrophobic interactions in the maintenance of helix stability. However, in the light of the work of De Voe and Tinoco[78] it would seem that it is these interactions that are responsible for maintaining the stability of the helix.

d) Ionic strength

Another factor which effects both denaturation and renaturation is the ionic strength. This will be discussed fully in the section on denaturation by dilution but a brief outline will be given here. It has been shown that the T_m of a DNA solution is nearly a linear function of the ionic strength of the solution.[75,88,95] However, different ions produce different effects on the T_m. For example, large anions, such as perchlorate at a concentration of 7.2 M, lower the T_m by approximately 40°C from the

value in 0.2 M sodium chloride[69,80] and the divalent cations magnesium, manganese, barium, cobalt, nickel and zinc increase the T_m of calf thymus DNA, whereas copper, cadmium and lead ions produce a decrease in T_m.[96] The first group of divalent cations probably act by neutralizing the charged phosphate groups[97] and the latter by coordinating to electron donor groups on the nucleotide bases so displacing hydrogen bonds.

It was found by Marmur and Lane[98] that renaturation of DNA, as measured by the increase in biological activity of D.penumoniae DNA, increased linearly with ionic strength up to 0.4 M sodium ion and levels off at this value. Thus they recommended that this value be used as the optimum ionic strength in order to produce maximum renaturation.

e) Time of heating

Heating, unfortunately, not only produces denaturation but is often accompanied by degradation. Doty et al.[23] showed that prolonged exposure of DNA to high temperatures produces considerable depolymerization as measured by the fall in molecular weight derived from the Mandelkern Flory equation. This degradation has also been demonstrated by the loss of biological activity caused by means other than the breakdown of secondary structure.[99,100] It has also been shown that this loss of biological

activity due to degradation caused by prolonged heating can occur at temperatures below the melting point.[101,102] Kinetic measurements have shown that the rate constants for this type of inactivation depend on whether it occurs above or below the melting point.[102,103] The inactivation appears to result from two mechanisms, chemical alterations in the DNA molecule such as depurination[99,102-104] and back-bone scission of either the single or double stranded polynucleotide chains.[99,100] Thus the interpretation of denaturation experiments that involve prolonged heating of DNA solutions will be subject to this complicating phenomenon.

vi) Conclusions

It has been clearly demonstrated that heat denaturation of DNA can produce strand separation under the appropriate conditions of ionic strength and concentration of DNA. It would appear that these strands are the two polynucleotide chains which constitute the Watson-Crick DNA helix. It has also been shown, in the case of bacterial and bacteriophage DNA, that these strands can recombine when denatured DNA is cooled slowly under appropriate conditions of temperature, ionic strength and DNA concentration, thus reconstituting the parent structure to a large degree. This behaviour is termed renaturation.

Cooling denatured DNA can also result in aggreg-

ation due to the formation of intra- and inter-strand non-specific hydrogen bonds. It was this aggregation phenomenon that had long prevented the elucidation of the mechanism of heat denaturation and strand separation for when avoided it became possible to demonstrate strand separation and obtain meaningful results from the kinetics of denaturation.

B. Dilution Denaturation

i) Relation between ionic strength and melting temperature (T_m)

Thomas[6] was the first to show that the critical temperature at which denaturation occurs was dependent upon the ionic strength of the solution. This was later confirmed by the work of Zamenhof et al.[8] and others[11,13,60,61,105] who found that decreasing the ionic strength gave lower values for this critical temperature. These results are to be expected in the light of the work of Miyaji and Price,[106] Sadron[107] and Coates[72] who have shown that the presence of electrolyte exerts a protecting effect on the denaturation of DNA by the action of heat. A linear relationship between log I (where I is the ionic strength) and T_m has been observed for DNA, at fairly low ionic strengths, by Ts'o et al.[75] and Dove and Davison,[88] and for dA$\bar{B}$U and dAT at low ionic strengths ($> 10^{-3}$ M in sodium ion) by Inman and Baldwin.[95] Marmur and Doty[60]

have also shown for <u>E.coli</u> and <u>D.pneumoniae</u> DNA that the T_m decreases with decrease in ionic strength down to a value of 0.01 M potassium chloride and their results if plotted also show this linear relationship between T_m and log I.

If the ionic strength is lowered sufficiently denaturation takes place at room temperature.[105] Thomas showed that if a solution of DNA in 1.0 M sodium chloride was diluted at constant nucleic acid concentration and at room temperature, an increase in the molar absorption coefficient (ε(P)) was obtained[6] and this increase is similar to that obtained by heat denaturation. On dilution no change in optical density occurred until a concentration of approximately 10^{-3} M sodium chloride was attained, at which point the optical density increased until it reached a maximum value, about 25% greater than the initial value, at a sodium chloride concentration of about 10^{-4} M.[6] This work was repeated using magnesium sulphate and it was found that the limiting value of the optical density obtained by removing the electrolyte was the same as for sodium chloride. However, the limiting concentration at which denaturation occurred was much lower for the divalent cation being $10^{-4.5}$ to 10^{-5} M.

The results of Thomas were confirmed and extended by the observations of Cavalieri et al.[108] who showed that

the rise in optical density which occurs on lowering the electrolyte concentration is dependent on the DNA concentration, the increase being greatest for the samples which have the lowest DNA concentrations. Jordan[55] suggests that this is due to the contribution of the nucleate counter-ions to the ionic strength and possibly also to the presence of a small amount of residual sodium chloride in the DNA as a result of the method of preparation.

ii) The critical concentration phenomenon

a) The effect of lowering the DNA concentration

The results of Cavalieri et al.[108] suggest that denaturation of DNA may also occur by dilution of the DNA itself in the absence of any added electrolyte. DNA was therefore dissolved[108] in water at various concentrations and a graph of the percentage increase in optical density versus the log of the counter-ion concentration plotted. This showed that at a concentration greater than 10^{-3} g DNA/ml no denaturation occurred and at a concentration of approximately 10^{-4} g DNA/ml the rise in optical density reached its maximum value. Poyet et al.[109] and Dhaussy[110] observed a similar phenomenon when they found a point of inflection in the curve relating optical density to DNA concentration in salt free solution.

This phenomenon has been studied by Inman and

Jordan.[111-114] Points of inflection have been found to occur, in the same concentration range, both in the graph relating optical density with DNA concentration and in that relating specific conductivity with DNA concentration. Dilution below this critical concentration was found to produce an irreversible change in the DNA of calf thymus. This change was considered to be a denaturation process in view of the similarity between DNA which has been dissolved in water below a critical concentration and heat denatured DNA. Similar graphs for optical density versus DNA concentration[113] and specific conductivity versus DNA concentration[112] are obtained for DNA denatured by either of the above treatments. When observed in the electron microscope[114] these samples appear as round blobs instead of long thin strands as is the case with native DNA. This latter result is in agreement with the findings of Doty et al.[23] for heat denatured DNA.

b) Properties of DNA below its critical concentration

Inman and Jordan found that the value of the critical concentration was not sharp,[113] reflecting heterogeneity in bond strengths between the molecules, similar to that which causes a broad melting range in the case of heat denaturation. The value of the critical concentration was also found to be very dependent on the ionic strength of the DNA solution,[112] the critical concentration increasing

with decrease in ionic strength. This indicates that denaturation by decreasing the ionic strength will only occur if the DNA concentration is below a certain critical value, which must be below the value for denaturation to be produced by dissolving DNA in water.

Below its critical concentration DNA was found to have a high charge fraction and a low mobility.[111] This low mobility was considered to be independent of the charge carried by the kinetic unit, and to be a true indication of the frictional force opposing the movement of the polyion unit in solution. It was suggested that strand separation might occur on denaturation by dilution below the critical concentration, however, a decrease in molecular weight was not observed to result from this treatment. As this was concluded from sedimentation experiments conducted in 0.2 M sodium chloride, the lack of change in the sedimentation coefficients (s) and the concentration dependence of s[114] is easily explained by non-specific hydrogen bond formation. This would give rise to a fortuitous result due to aggregation, similar to that obtained in light scattering experiments.

Thus if DNA is dissolved in water at reasonably high concentrations no denaturation takes place owing to the high counter-ion concentration. As dilution proceeds, the counter ions bound to the phosphate groups will dissociate and diffuse away leaving fully charged phosphate

groups. Denaturation is then thought to proceed by the same mechanism that produces denaturation on the removal of electrolyte.

iii) Suggested mechanisms

Mechanisms for this denaturation process have been put forward by Cavalieri et al.[108] and Inman and Jordan.[113] Cavalieri et al. suggested that the denaturation produced by the removal of electrolyte is the result of proton addition to the amino groups of the purines and pyrimidines of DNA. In the light of X-ray studies[115,116] which show that the proton is added to the N(1) or N(7) nitrogen of the ring system and not the amino group this mechanism can be disregarded. Inman and Jordan[113] consider the mechanism as a competition by the hydrogen bonded water structure for the hydrogen bonded sites on the DNA molecule.

It would appear that repulsion between the charged groups on the two polynucleotide chains is also a possible cause of this denaturation. Schildkraut and Jordan[117] have shown that the repulsive forces produced are more than sufficient to disrupt the double helix at, and below, the critical concentration, assuming that the bonding between the helices is due only to hydrogen bonds. In the light of recent evidence that hydrophobic bonding plays a large part in producing helix stability, it is

possible that the mechanism may depend on a change in the hydrophobic properties of the interior of the helix, or to a combination of all these effects.

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Chapter III

Reversibility of the Denaturation of DNA by Dilution

i) Introduction

ii) Evidence that changes in ionic strength can induce denaturation and renaturation of DNA

iii) Evidence to support the renaturation hypothesis

a) Sedimentation velocity measurements
b) Density gradient equilibrium measurements
c) Electron microscopy study

iv) Effect of ionic strength on the T_m

v) General conclusions

i) Introduction

Heating DNA above its denaturation temperature has been shown to produce strand separation, while cooling of the DNA solution results either in the reformation of the helical structure or the formation of random coils, depending on whether the cooling was slow or rapid respectively. The denaturation of DNA by dissolving it in electrolyte solutions at concentrations below critical values for both the DNA and electrolyte have already been discussed in the previous chapter. If strand separation occurs during this process it should be possible, having denatured DNA in water, to add electrolyte to the solution thus producing an environment in which native DNA would be stable, and which would thus favour the renaturation of solution denatured DNA.

Thomas,[1] in his study of the variation of the absorption of DNA with ionic strength for several different types of DNA, concluded that the denaturation produced on lowering the ionic strength was irreversible. These results showed, however, that there was some reversal in the hyperchromicity. Unfortunately the quality of the DNA samples used in this work, as shown by the magnitude of the hyperchromic shift, was poor. As the relative absorbance of renatured DNA has never been found, in any of the work so far reported by various authors, to be as

low as the original, native DNA, it is possible that the small amount of reversibility observed by Thomas could be real.

It is the aim of the work in this chapter to test the hypothesis that denaturation of DNA, brought about by decreasing the ionic strength, is reversible. A more extensive study of the effect of the variation in ionic strength of the solution on the denaturation of DNA will also be discussed.

ii) Evidence that changes in ionic strength can induce denaturation and renaturation of DNA

The extinction coefficient at the peak of the strong ultraviolet absorption band (at 259 mμ) is substantially less for a solution of DNA than for an equivalent mixture of its constituent nucleotides. This hypochromicity is thought to be due to the interactions that occur when the nucleotides are held in close proximity to each other.[2-6] It has been shown[5] that a large part of this hypochromicity disappears when the helical structure of the DNA sample is destroyed, for example by raising the temperature of the solution above its melting point. Hence the change in optical density of a solution of DNA relative to the value of the optical density of a native solution at 25°C (the relative absorbance)

can be used as a means of estimating the extent of denaturation and renaturation.

In Fig. (1-III) is given the variation of the relative absorbance with temperature for B.Megatherium. Curve a was obtained for native DNA in 0.15 M sodium chloride with 0.015 M sodium citrate (SSC) and shows a value for T_m of 88°C compared with that reported by Marmur and Doty[7] of 87°. On slow cooling a sample heated to 100°C, curve c is obtained on reheating, which is very similar to the result reported by Doty et al.[8] for DNA from D.pneumoniae. The material so obtained by slow cooling, i.e. renatured DNA, was regarded by these authors as containing substantial amounts of reformed complementary regions. Rapid cooling of a sample heated at 100°C (curve b) shows evidence of an almost complete lack of renaturation when reheated in contradistinction to the result of Doty et al.[8] They interpreted their result as showing that short, interchain, helical regions were formed.

That changes in ionic strength can induce denaturation and renaturation of DNA in a manner similar to that produced by heating and cooling is demonstrated for the DNA of B.Megatherium in Fig. (2-III). Curve a is analogous to curve a in Fig. (1-III) and represents the thermal denaturation curve of the native DNA in 0.2 M NaCl. Curve b shows the variation of the relative absorbance with temperature

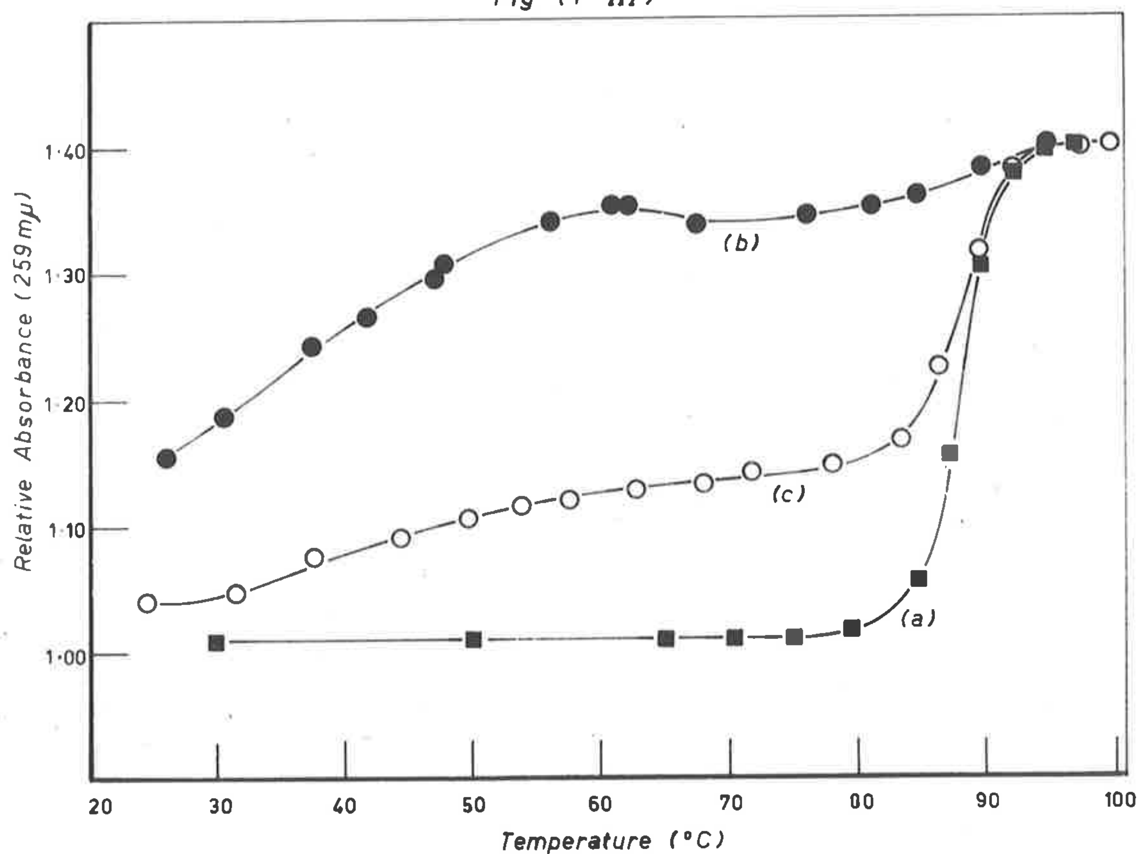


Fig. (1-III). Variation of relative absorbance with temperature for B.megatherium DNA in SSC. Curve a, native; Curve b, quick cooled; Curve c, slow cooled.

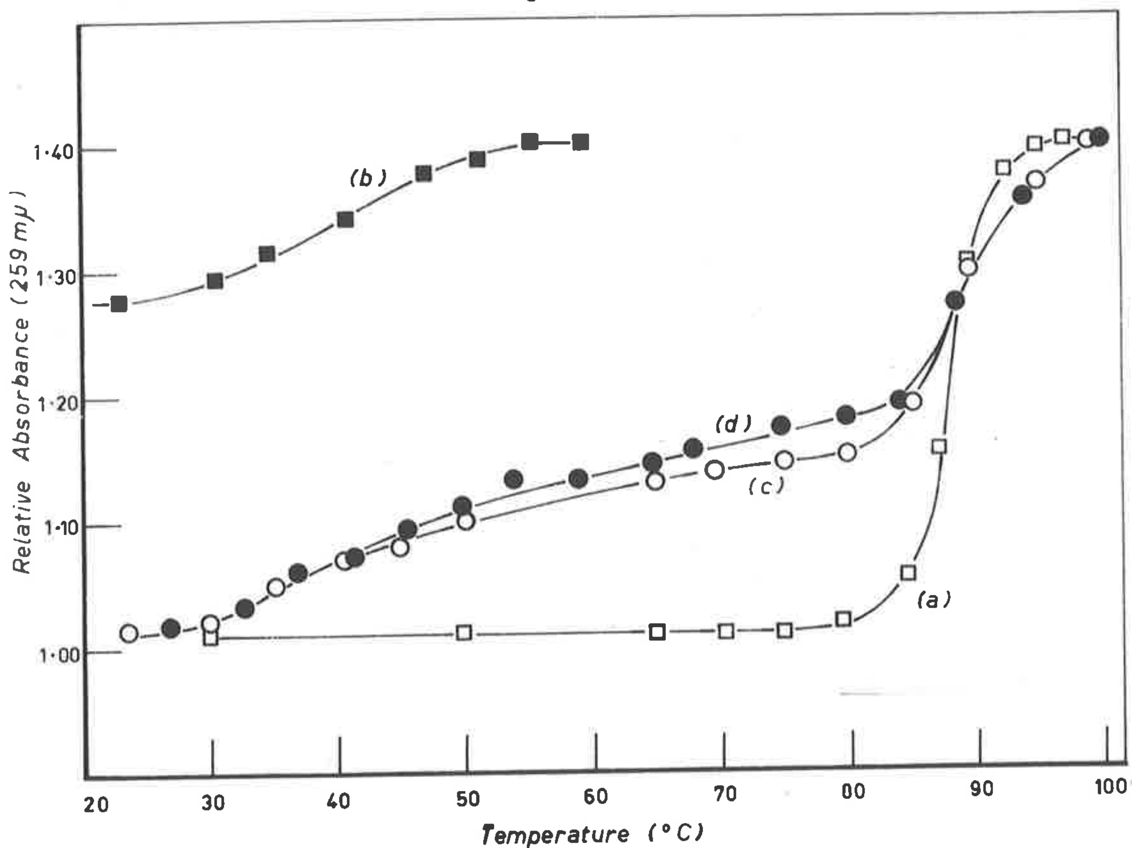


Fig. (2-III). Variation of relative absorbance with temperature for B.megatherium DNA. Curve a, native in 0.2 M NaCl; Curve b, DNA diluted below its critical concentration in H_2O ($k = 3.41 \times 10^{-7}$) sample b; Curve c, an aliquot of sample b made 0.2 M in NaCl by slow addition of NaCl; Curve d, an aliquot of sample b made 0.2 M in NaCl by rapid addition of NaCl.

for an 0.001% solution of <u>B.Megatherium</u> DNA in pure water. Although the value of the critical concentration could not be determined exactly for this sample of DNA it was found from extinction coefficient ($\varepsilon(P)$) measurements that it was somewhere above 0.01%. Thus this concentration of 0.001% is well below the critical concentration for denaturation as evidenced by the high value of $\varepsilon(P)$, 8330,[9] and the low hyperchromic shift on heating. This conclusion is in agreement with density gradient and electron-microscope results presented below and with the data of Inman and Jordan[10-13] for calf thymus DNA.

The small increase in the relative absorbance on heating this dilution denatured sample, Fig. (2-III) curve <u>b</u>, cannot be explained in terms of the reformation of non-specific bonds between the chains as the sample has not been removed from the denaturing conditions. As strand separation can be shown to have occurred in this sample (see section iii) this increase cannot be due to the breaking of residual bonds either. It can, however, be explained in terms of the hypochromic effect postulated for single strand DNA. Bolton and Weiss[14] point out that hypochromism is not necessarily confined to the helix-coil transformation and it has been shown[15] that even dinucleotides can exhibit some hypochromic effect (approx. 10%) which in some simple polynucleotides was found to reach a limiting value up to 35% at chain lengths of ten nucleotide units.

This hypochromicity was thought to arise due to interactions associated with the bases when they were arranged in an ordered structure. Hence when the temperature is raised these interactions are broken down giving rise to an hyperchromic effect.

In contrast the hyperchromic shift produced on reheating the quick cooled sample of DNA, Fig. (1-III) curve b, can be explained by the breaking of reformed, non-specific bonds, produced by the removal of the denaturing conditions. In addition there will be some effect due to the disruption of the base-base interactions postulated by Bolton and Weiss.[14] In both cases, 100% strand separation cannot be demonstrated, hence some contribution due to the breakage of residual bonds could be obtained, but this effect would be small as the proportion of unseparated molecules remaining is not large enough to be observed in a cesium chloride density gradient.

The addition of sodium chloride to solution of DNA in pure water to produce a concentration of 0.2 M produces an environment in which native DNA is normally stable at temperatures below the onset of heat denaturation (80°C) and thus should produce renaturation of this denatured DNA. Curve c (Fig. 2-III) shows the variation of the relative absorbance with temperature for the solutions of B.Megatherium DNA in pure water which was brought to 0.2 M

in sodium chloride by the slow addition of sodium chloride over approximately a 24 hour period. The curve is very similar to that obtained for the slow cooled, heat denatured DNA (Fig. (1-III), curve c) which was interpreted as indicating that the DNA had been largely renatured. Rapid addition of solid sodium chloride to the denatured solution produced a material showing a thermal denaturation curve given by curve d which is very similar, for this DNA, to curve c.

A similar cycle of operations has been performed with calf thymus DNA and the results are given in Figs. (3-III) and (4-III). In Fig. (3-III), the thermal denaturation curve in 0.2 M sodium chloride is shown (curve a) and yields a value for T_m of 87°C, which agrees with the value of 88°C recorded by Marmur and Doty.[6] For DNA heated to 100°C and cooled rapidly, the thermal denaturation curve, b, is obtained indicating little, if any, renaturation. Slow cooled material yields curve c on reheating, which suggests that under these conditions some renaturation may have occurred. This curve c is similar to that obtained by Doty et al.[16] The reason for the difference in behaviour between B.megatherium DNA and calf thymus has been discussed in the previous chapter where it was concluded that the extent of renaturation depends upon the homogeneity of a sample of DNA as it depends on the concentration of specific complementary strands.

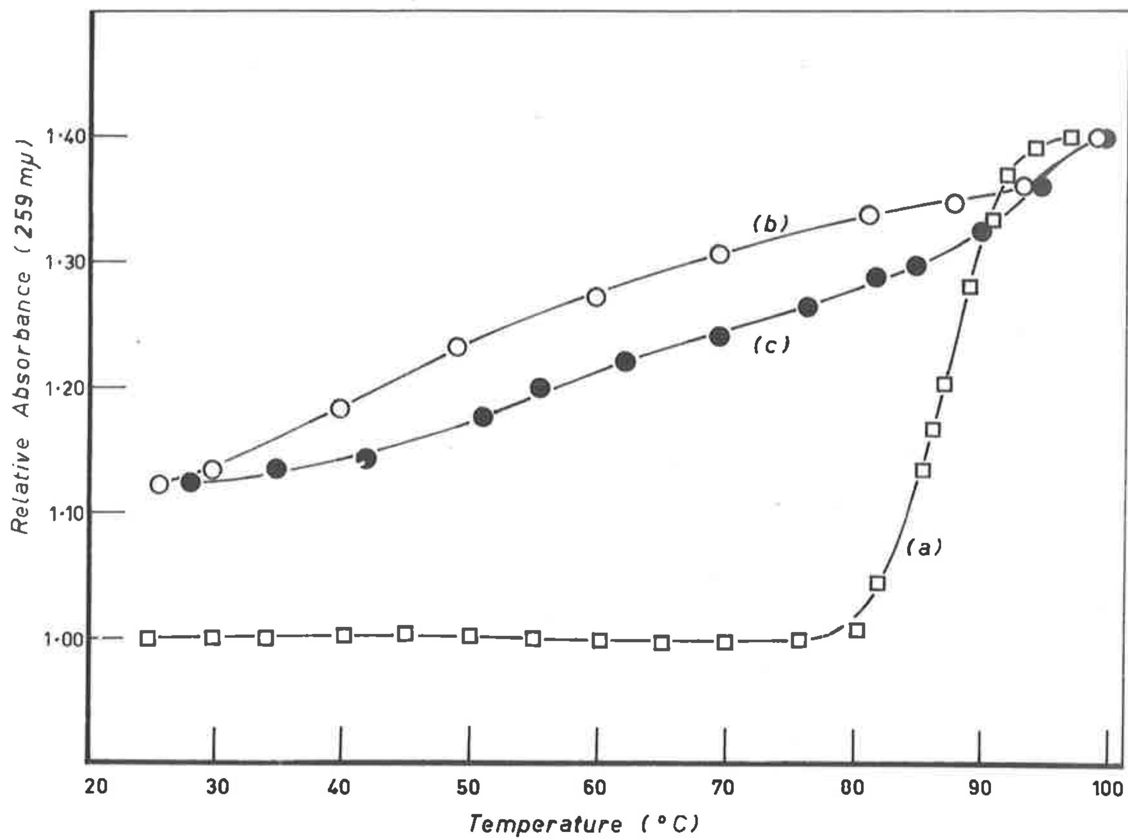


Fig. (3-III). Variation of relative absorbance with temperature for calf thymus DNA in 0.2 M NaCl. Curve a, native; Curve b, quick cooled; Curve c, slow cooled.

Solution of calf thymus DNA in pure water yields the variation of relative absorbance with temperature shown by curve _b_ (Fig. 4-III), a result which indicates, in agreement with the findings of Inman and Jordan,[10-13] that the calf thymus DNA has been denatured. Rapid addition of sodium chloride leads to a material with a thermal denaturation curve, _d_, which is similar to that obtained for the DNA from _B.megatherium_ and indicates that renaturation has occurred. Slow addition of sodium chloride to bring the solution to 0.2 M in sodium chloride produces renatured DNA for which the thermal denaturation curve is shown in _c_. This curve surprisingly shows a higher degree of renaturation than that shown, under similar conditions, by the DNA of _B. megatherium_.

This result is contrary to what is expected on considering the relative amounts of heterogeneity in the two samples. In fact it is expected that hardly any renaturation would occur with calf thymus DNA, as found on slow cooling thermally denatured DNA. This raises the question as to whether the percentage hyperchromicity and hypochromicity do give a measure of strand separation and reformation. The fact that many authors have been able to correlate these changes with changes in the number and position of peaks in cesium chloride density gradient equilibrium experiments indicates that this is the case. How-

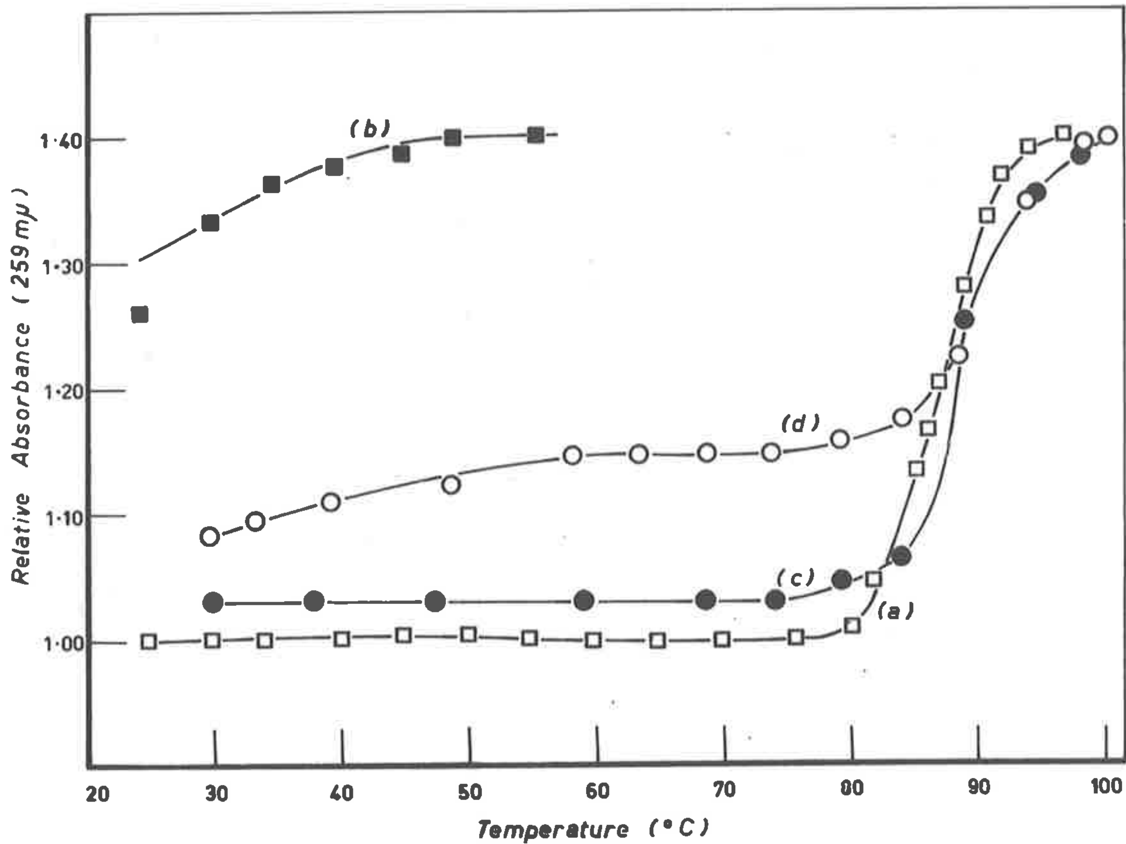


Fig. (4-III). Variation of relative absorbance with temperature for calf thymus DNA. Curve a, native in 0.2 M NaCl; Curve b, DNA diluted below its critical concentration in H_2O ($k = 3.89 \times 10^{-7}$) sample b'; Curve c, an aliquot of sample b' made 0.2 M in NaCl by slow addition of NaCl; Curve d, an aliquot of sample b' made 0.2 M in NaCl by rapid addition of NaCl.

ever the results of Freifelder and Davison[17] showed that 100 percent hyperchromicity did not necessarily correspond to strand separation. This suggests that the above treatment may not have resulted in strand separation as the hyperchromicity produced by dilution may not necessarily measure the amount of strand separation. Hence to test whether strand separation does occur on denaturation by dilution and whether the hypochromic effect observed on the addition of salt corresponded to renaturation recourse was made to various other techniques.

iii) Evidence to support the renaturation hypothesis

a) Sedimentation velocity measurements

A convenient method of studying strand separation has arisen from the technique of Thomas and Berns[18,19] in which formaldehyde is reacted with the denatured DNA, which by combining with the free amino groups prevents the reformation of a hydrogen bonded structure. This treatment should enable the separation of the effects of partial denaturation, where the strands are held in register by residual hydrogen bonds, from those of strand separation by sedimentation velocity measurements.

If the strands have not separated due to the residual helical regions the molecular weight of the partially denatured molecule should not have changed. However, those regions of the molecule which have been prevented from

reforming the rigid native helical structure, due to their reaction with formaldehyde, will cause the molecule to be more flexible and so it will assume a more compact shape in solution. Thus the frictional coefficient should decrease, and as the molecular weight remains constant, the sedimentation coefficient should correspondingly increase and it will rise to a maximum as more and more flexible regions are introduced into the molecule. However, when strand separation occurs, the molecular weight should fall to one half of its original value and this should be reflected in an abrupt drop in the sedimentation coefficient. This has been demonstrated by Freifelder and Davison who found that on reacting T7 DNA with formaldehyde the sedimentation coefficient fell precipitously over a very narrow temperature range when all of the inter-strand base pairs had been disrupted by thermal treatment.

In Table (1-III), the $s^o_{20,w}$ values are given for the native DNA of B.megatherium and calf thymus, together with the values obtained after heating in SSC in the presence of formaldehyde, to 100°C for 10 minutes, and also after dilution to a concentration below the critical followed by the addition of formaldehyde and then sodium chloride and sodium acetate to give SSC. The $s^o_{20,w}$ values for the native DNA are both high, as is normal for highly polymerized native DNA, and a considerable reduction in

Table (1-III)

Variation of the Sedimentation Coefficient of DNA (0.002%) on Heat and Dilution Denaturation in the Presence of 1% Formaldehyde

Treatment	$s^o_{20,w}$ B.megatherium	$s^o_{20,w}$ Calf thymus
Native DNA in SSC + 1% formaldehyde	24.6	23.8
Native DNA in SSC + 1% formaldehyde heated at 100°C for 10 min.	8.4_9	14.2
Native DNA dissolved in water, then made 1% in formaldehyde, and then brought to SSC	13.4	8.0_7

the values occurs on denaturation by heat and dilution. This indicates that separation of the polynucleotide strands of the double helix has occurred to a considerable degree with both methods of denaturation. That the values of $s^o_{20,w}$ for material produced by heating and dilution of B.megatherium DNA in the presence of formaldehyde are not identical is not surprising in view of the fact that thermal denaturation can give rise to degradation as well as denaturation. Why these values should be reversed for calf thymus DNA is not clear at present.

b) Density gradient equilibrium measurements

Another method that has been used to show the presence of separated and recombined strands is that of equilibrium density gradient sedimentation. Doty et al.[8] and Schildkraut et al.[21] have shown that equilibrating thermally denatured DNA in a cesium chloride density gradient enables native and single strand DNA to be identified, the latter having a higher density than the double stranded material. These authors also found that it was possible to identify renatured DNA by this method and that when this was treated with the enzyme E.coli phosphodiesterase, the density of the renatured material was almost identical with that of the native sample. Thomas and Berns[18,19] and Thomas and Pinkerton[22] have shown that the presence of formaldehyde does not interfere with this identification and that when the effects of aggregation are to be avoided its presence stabilizes the separated polynucleotide chains enabling their identification.

Fig. (5-III) shows the result of placing various samples of B.megatherium DNA used to obtain curves (1-IIIc) and (2-III b and c) in a cesium chloride density gradient, using native B.megatherium DNA as a marker its density being 1.697 g./cc.[23] Each of these mixtures was compared with a cesium chloride gradient containing just the appropriate sample, that is heat denatured DNA in 1% formaldehyde in

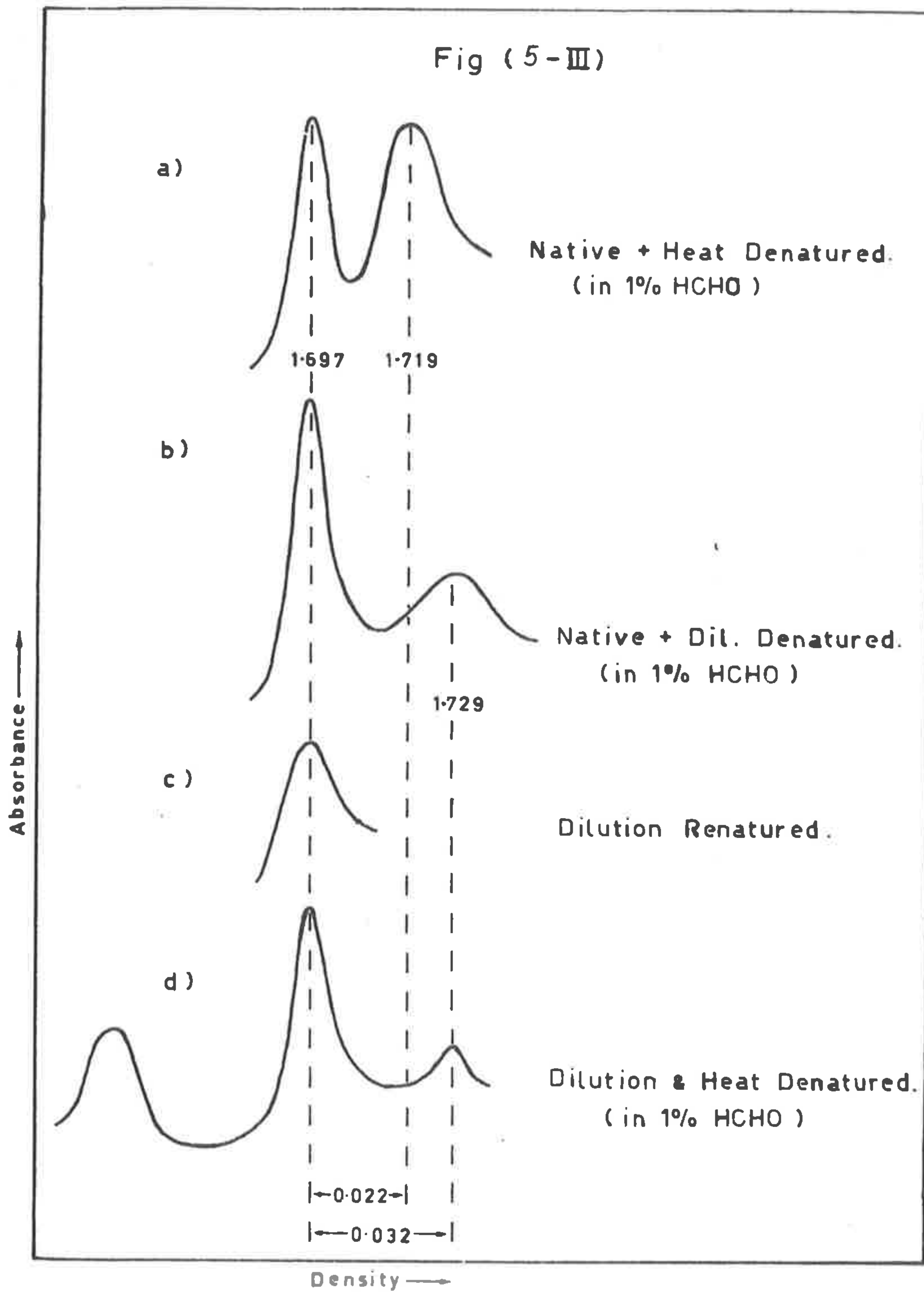


Fig. (5-III). Equilibrium concentration distributions of B.megatherium DNA samples banded in CsCl gradients.

a), dilution denatured DNA in 1% formaldehyde in b) (in both of these cases the cesium chloride solution was also made 1% in formaldehyde) and dilution renatured DNA in c), where this refers to DNA which has been diluted below its critical concentration in water and then made 0.2 M in sodium chloride slowly. The band obtained with just the dilution renatured DNA is shown in Fig. (5-III c) as this was identical with that also containing the native marker.

These bands show that on heat denaturation strand separation accompanied by an increase in density of 0.022 g./cc. occurs, while dilution denaturation also results in strand separation but produces an increase of 0.032 g./cc. in the density. Dilution renaturation, in contradistinction to the results of heat renaturation, produces a material having the same density as the native material without the use of E.coli phosphodiesterase.

An identical set of experiments was performed with calf thymus DNA and the results are summarized in Fig. (6-III). Due to the overlapping of some peaks the results of centrifuging the various samples and of centrifuging the mixture of these with the native marker calf thymus DNA have been shown, except in the case of dilution renatured DNA where this was essentially the same as that with the marker present. The marker density was taken as being 1.699 although Schildkraut et al.[23] reported a satellite

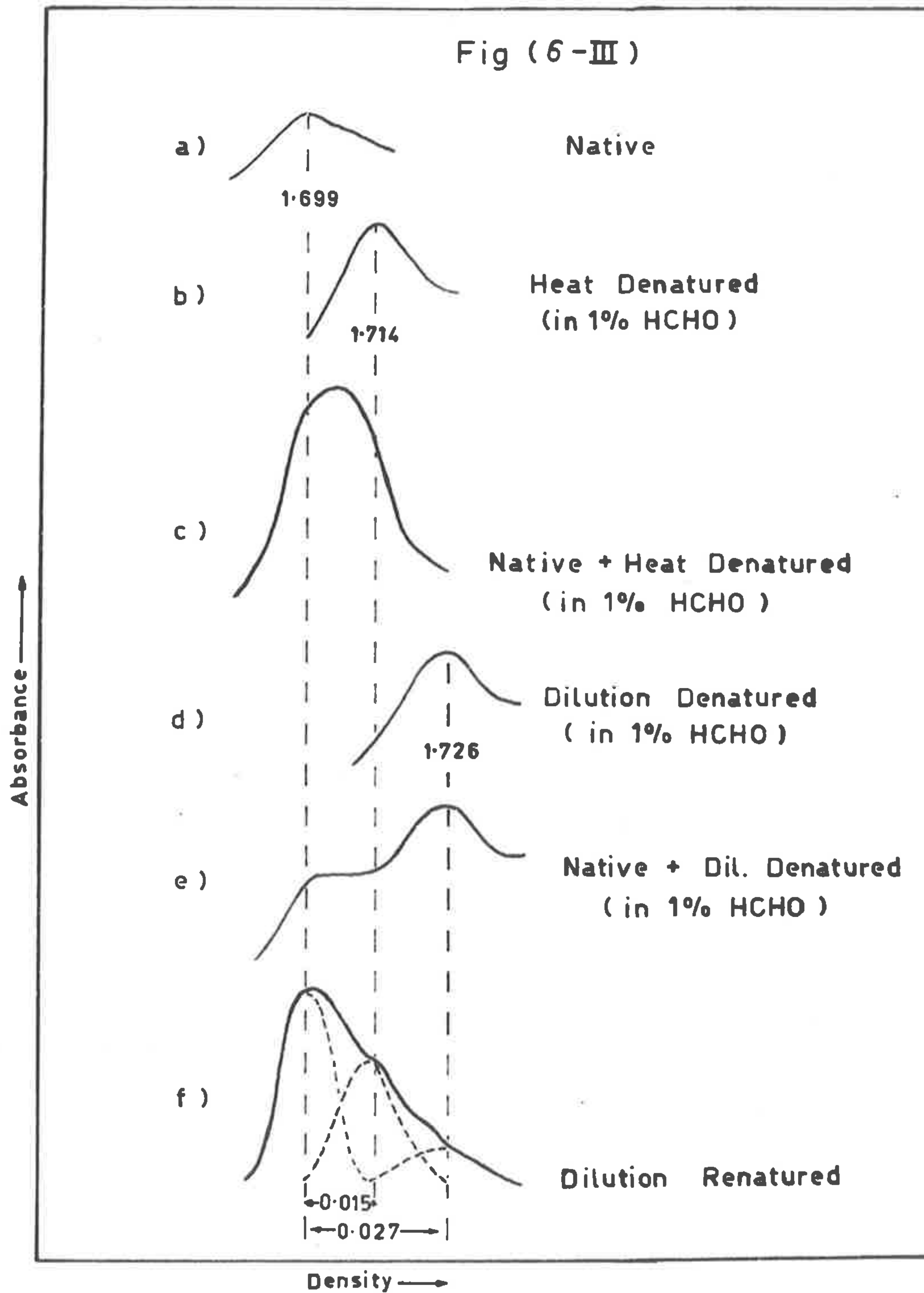


Fig. (6-III). Equilibrium concentration distributions of calf thymus DNA samples banded in CsCl gradients.

band at 1.713. This band was not observed in the sample studied here, but this may be due to the low concentration of native DNA used (Fig. 6-III a). It is interesting to note that the position of this satellite band corresponds with the position of the heat denatured band shown by curve b.

As with B.megatherium DNA, heat and dilution denaturation of calf thymus DNA gives rise to species having different densities, the density increase being 0.015 g/cc. in the case of heat denaturation and 0.027 in the case of dilution denaturation. Unlike the DNA of B.megatherium, calf thymus DNA on renaturation by the slow addition of salt to dilution denatured DNA does not produce solely a species having the density of native DNA. Instead a multiple peak is obtained, which appears to consist of three bands. On drawing symmetrical bands at the position of native and dilution denatured DNA and subtracting these from the original curve, a third symmetrical band is obtained having a density of 1.713 g/cc. which is almost identical with that of the heat denatured DNA.

The difference in the densities of heat and dilution denatured DNA in the presence of formaldehyde could be explained in terms of different reactions of the DNA with the formaldehyde in the two cases. For example if the formaldehyde did not react with the bases as readily at

high temperatures as at low temperatures then it is possible that some hydrogen bonds have been reformed in the case of the heat denatured DNA, or if at high temperatures formaldehyde gave rise to intra-strand crosslinking. Both of these effects would give rise to a slightly expanded structure and hence cause the density of that species to be lower. If this postulate is correct then heating a sample of dilution denatured DNA in 1% formaldehyde, at the same conditions used for thermal denaturation, should result in a lowering of the density.

As can be seen in Fig. (5-III) this treatment does not result in the production of a species corresponding to heat denatured DNA. What material that has not gone into the very low density band still has a density equivalent to dilution denatured DNA. The nature of this low density band cannot be explained at present. This result, together with that obtained on renaturing dilution denatured calf thymus DNA, indicates that heat denatured and dilution denatured DNA are distinct species, the reason for this difference being obscure at present.

c) Electron Microscopy Study

Electron micrographs of heat denatured DNA molecules show that the long fibres typical of native DNA break down into areas best described as irregularly coiled clusters.[8,24,25] When DNA is denatured thermally in the presence of 1% formaldehyde, which prevents bond reformation, Beer and Thomas[26]

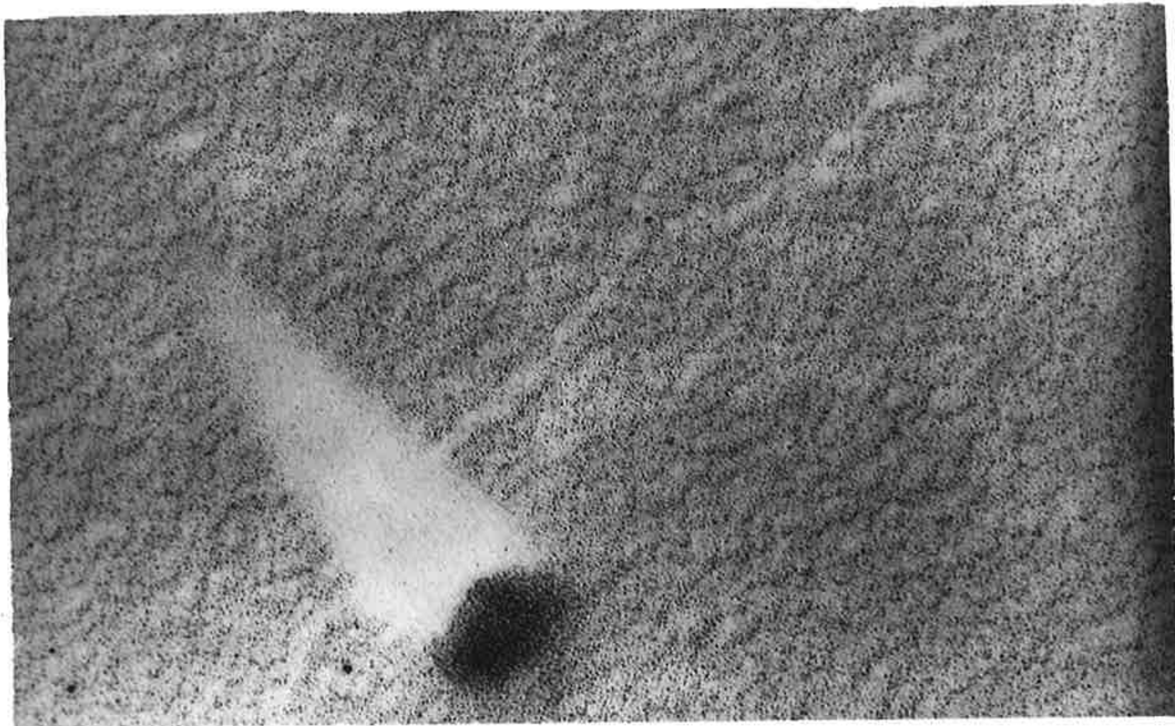
found that only flat patches were visible. Single stranded ϕX174 DNA was found[27] to exhibit these flat patches together with irregularly coiled material similar to that found in the micrographs of quickly cooled, heat denatured DNA, which showed[8] small clusters of irregularly coiled molecules similar to RNA.[27] Doty et al.[8] have shown that heat renatured DNA appears similar to native DNA in the electron microscope, except for the more frequent occurrence of irregular patches at the ends of the cylindrical threads. These patches were thought to be due to regions of denatured DNA arising from incomplete recombination or an inequality in the length of the two strands that are paired.

The evidence presented in the previous sections suggests that electron microscopy should reveal in the dilution denatured DNA the flattened, coiled patches that are characteristic of single stranded DNA. Similarly this technique should reveal in dilution renatured DNA the long cylindrical threads characteristic of native DNA. Accordingly a sample of native _B.megatherium_ DNA and a sample of this DNA which had been denatured by dissolving it in water and then renatured, by the slow addition of solid sodium chloride, were dialysed against 0.05M ammonium carbonate plus 0.1M ammonium acetate. These two samples, together with a sample of DNA which had been denatured by dilution in water, all at a concentration of 0.001% DNA,

were then sprayed onto freshly cleaved mica and electron micrographs obtained by the method outlined in chapter VII. Another sample of DNA in S.S.C. plus 1% formaldehyde, which had been denatured by heating to 100°C for ten minutes, was dialysed against 0.05M ammonium carbonate, 0.1M ammonium acetate plus 1% formaldehyde and micrographs obtained by the above method.

Electron micrographs of native B.megatherium DNA as shown in Plate (1-III) were obtained. The molecules display the typical rodlike structure reported previously[13,24,28] and the height to shadow ratio fixes the diameter at approximately 20 A.

The micrographs of dilution denatured DNA shown in Plate (2-III) show the same structure as that obtained with ΦX174, indicating that it is single stranded in agreement with the previous results. The size of the particles observed in these micrographs appear to be too small to have come from one molecule and therefore probably represent fragments of a molecule. This is in agreement with the results of Inman and Jordan[13] on dilution denatured calf thymus DNA and Maclean and Hall[27] on DNA from ΦX174. This fragmentation may be an artifact arising from the spraying technique used in preparing the micrographs and hence is not a true representation of the structure existing in solution. This is supported by the work of Maclean and Hall[27] who found that when micrographs of ΦX174 DNA were prepared by methods giving rise to less shearing some longer fibrils



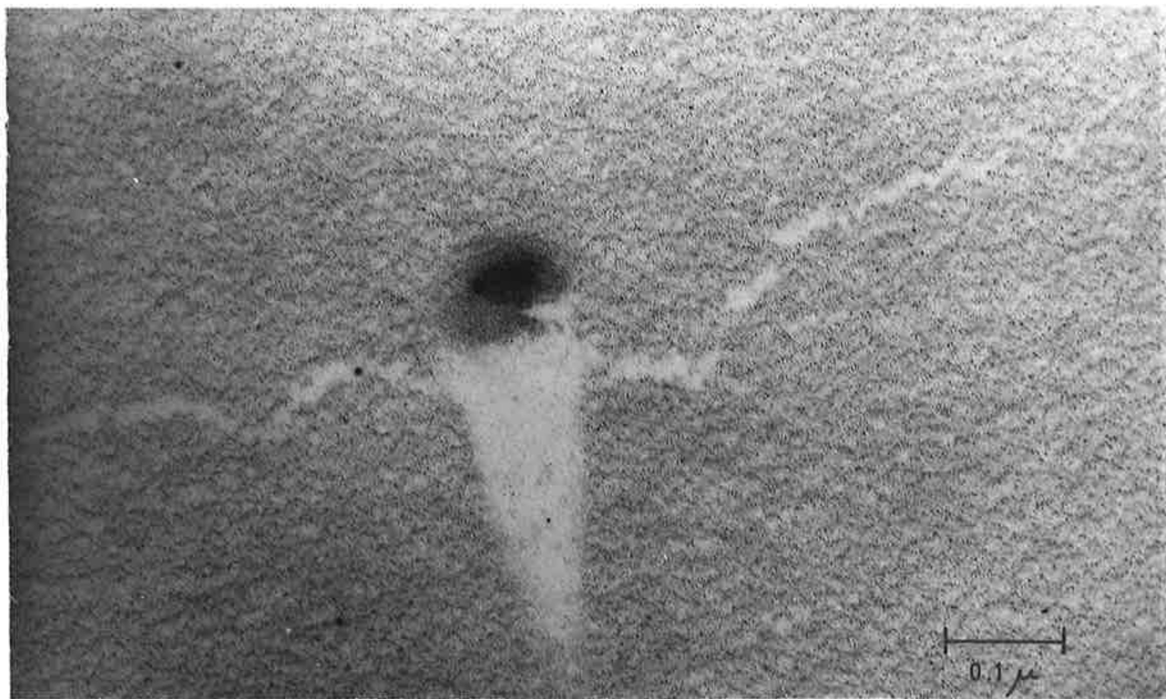
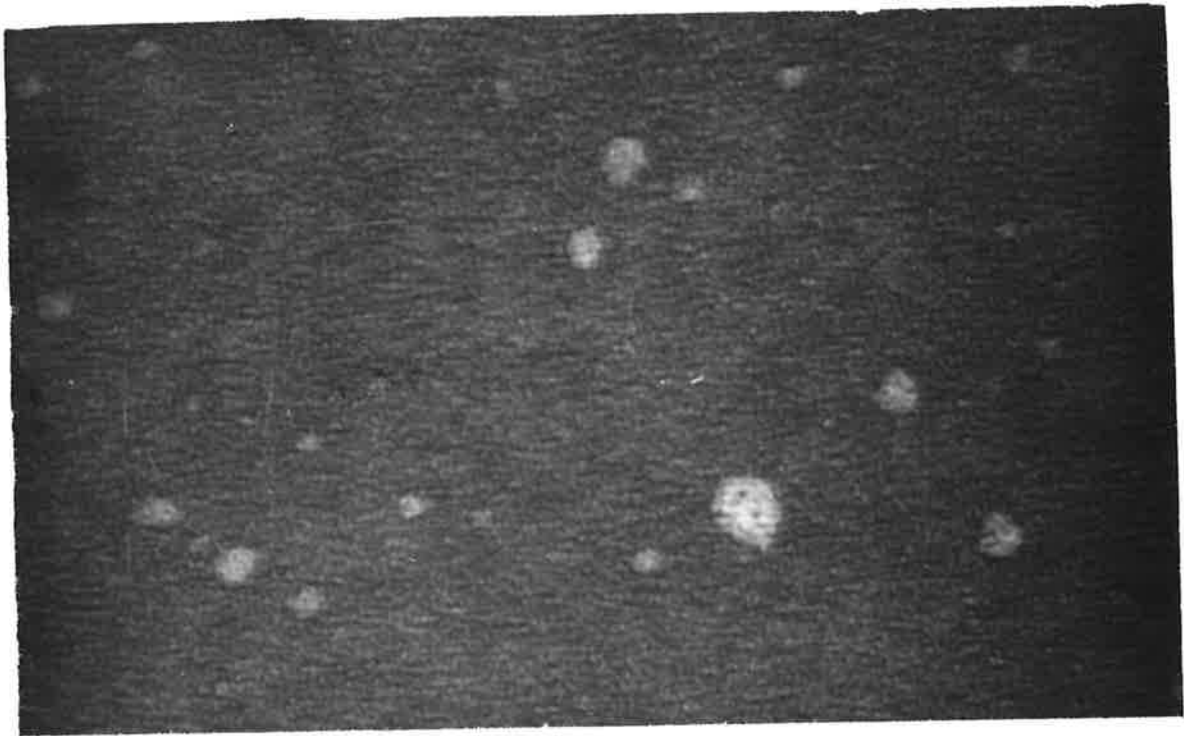


Plate (1-III). Native DNA from <u>B.megatherium</u>. Shadowed at 10:1, Magnification x160,000.

were observed.

The material resulting from heating B.megatherium DNA to 100°C in the presence of 1% formaldehyde can be seen in Plate (3-III). This is similar to that found by Beer and Thomas[26] with T4 bacteriophage DNA which has been subjected to the same treatment. The material in these micrographs appears to be more compact than that shown in Plate (2-III). These micrographs (Plate 2-III) show that the material obtained on dilution denaturation consists of loosely coiled clusters whereas Plate (3-III) shows that DNA denatured by thermal treatment in the presence of formaldehyde consists of smaller flattened patches of DNA. This difference between the material obtained by thermal and dilution denaturation was also indicated by the density gradient, which showed that these species had different buoyant densities in a cesium chloride gradient.

The micrographs of dilution renatured DNA, shown in Plate (4-III), are similar to those obtained with the native sample. That is native DNA, as shown in Plate (1-III), on being dissolved in water becomes a single stranded loosely coiled structure (Plate 2-III) and on the addition of sodium chloride to this sample these loosely coiled single strands recombine to form a "native-like" helical structure as shown in Plate (4-III). Unlike those obtained by Doty et al.[8] with heat renatured DNA these micrographs do not show the presence of irregular patches on the ends of the molecules.



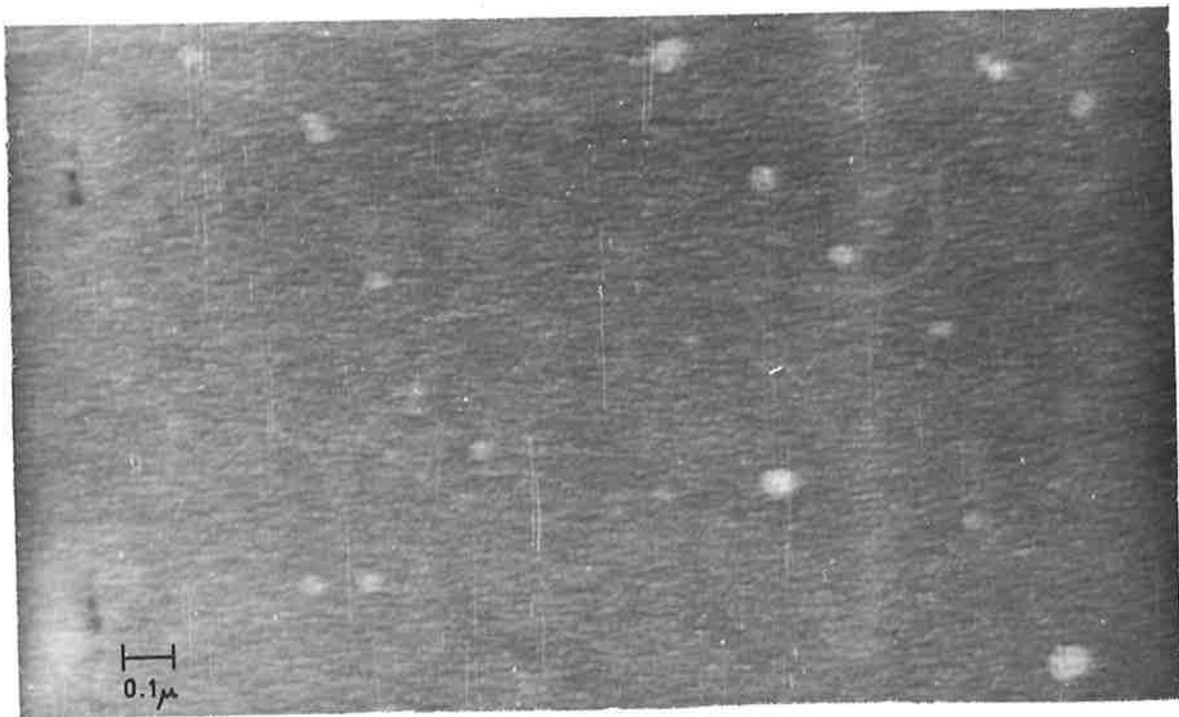


Plate (3-III). Thermally denatured DNA from B.megatherium in the presence of 1% formaldehyde. Shadowed at 10:1. Magnification x63,000.

This result is in agreement with the thermal denaturation experiments which show (Fig. (1-III) and Fig. (2-III)) that dilution renaturation results in a greater hypochromicity than does thermal renaturation. This result is also to be expected from the density gradient equilibrium results which show that the dilution renatured species has the same buoyant density as the native species, without being treated by E.coli phosphodiesterase.

Thus the identification of dilution renatured DNA as being similar to native DNA and denatured DNA as being a loosely coiled single strand structure is justified by these results.

(iv) Effect of ionic strength on the T_m

In Figs. (2-III) and (4-III), curves a and b represent the thermal denaturation curves of the two different specimens of DNA in high, a, and low, b, ionic strength solutions. It appeared important that the change in the thermal denaturation curve with ionic strength should be examined. Marmur and Doty[7] have previously studied the variation of the thermal denaturation curves of the DNA of *D.pneumoniae* and *E.coli* with ionic strength. However, their measurements were made at relatively high ionic strengths (0.01 and above) whereas it is the low ionic strength range which is of interest in the present study.

The results for the DNA of *B.megatherium* and calf thymus are given in Figs. (7-III) and (8-III) where the concentrations of the solvent (sodium chloride solution) are given as specific conductivities. It is necessary to record the concentrations in this way since in pure water and very low sodium chloride concentrations the conductivity and hence the ionic concentration and the thermal denaturation curve are very dependent upon the quality of the conductivity water used. The conductivities recorded for the various solutions are those of the solvent immediately prior to the addition of the stock solution of DNA. These results show that as the conductivity of the solvent is decreased the T_m of the resulting solutions also decreases,

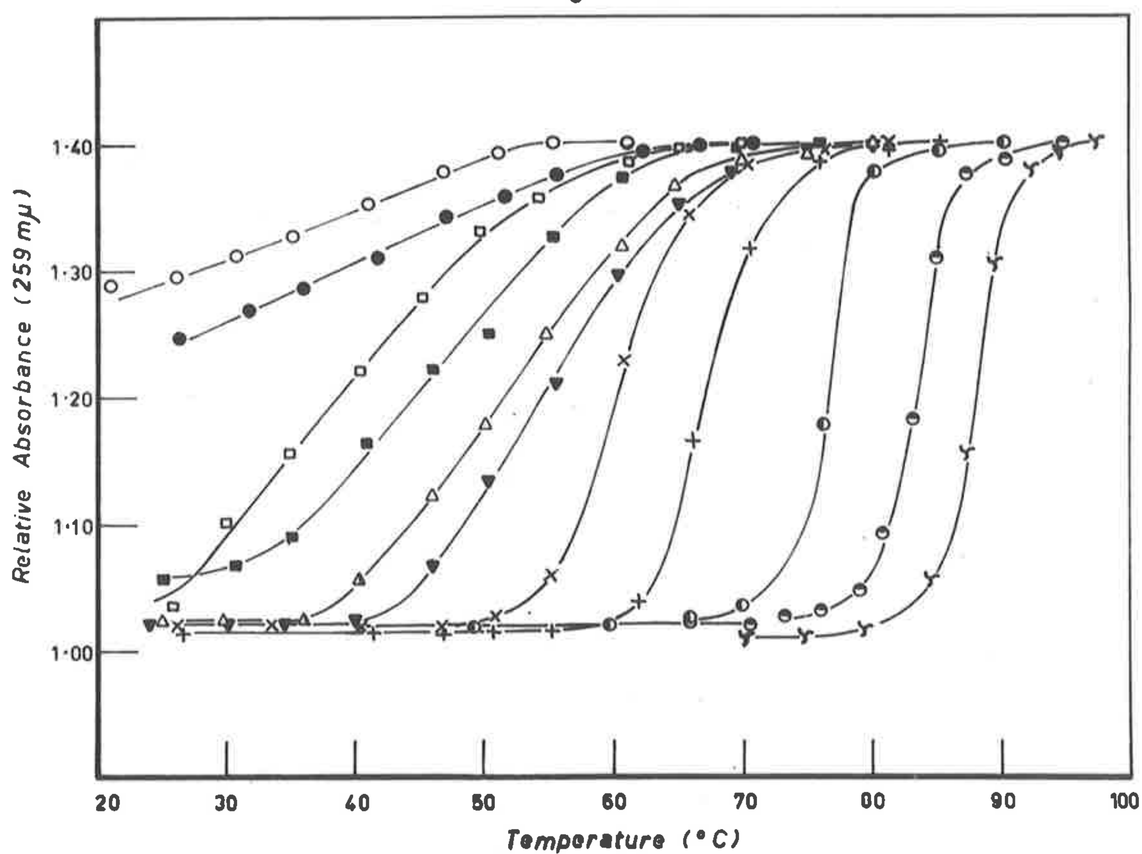


Fig. (7-III). Variation of relative absorbance with temperature for B.megatherium DNA (0.003%), in aqueous solvents of varying specific conductivities (ohms^{-1} cms^{-1}).

○ 2.88×10^{-7}	● 1.43×10^{-6}	□ 5.00×10^{-6}
■ 1.08×10^{-5}	△ 2.27×10^{-5}	▲ 1.07×10^{-4}
✖ 3.30×10^{-4}	✚ 1.00×10^{-3}	◑ 3.23×10^{-3}
⊖ 1.00×10^{-2}	⅄ 2.14×10^{-2}	

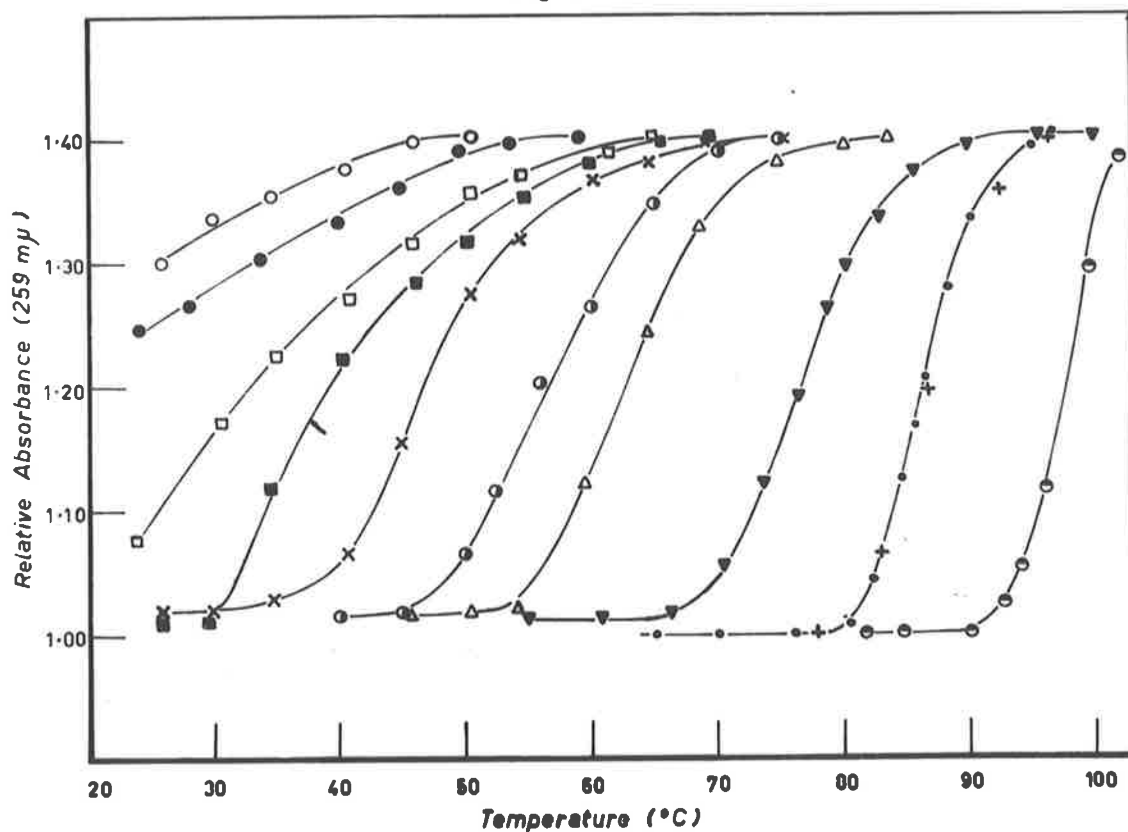


Fig. (8-III). Variation of relative absorbance with temperature for calf thymus DNA (0.004%), in aqueous solvents of varying specific conductivities (ohms^{-1} cms^{-1}).

○ 9.6×10^{-7} ● 3.75×10^{-6} □ 1.00×10^{-5}
■ 3.80×10^{-5} × 9.40×10^{-5} ◑ 3.36×10^{-4}
△ 8.00×10^{-4} ▼ 3.14×10^{-3} + 1.70×10^{-2} (0.2M NaCl)

• native in 0.2 M NaCl ⊖ 7.10×10^{-2}

until at very low conductivities (of the order of 10^{-5} $ohms^{-1}$ $cms.^{-1}$) the solutions become partially denatured at room temperature.

The influence of the ionic strength of the solvent on T_m may be attributed to the existence of two denaturation mechanisms operating simultaneously. Denaturation by heat will be produced by the two strands acquiring sufficient kinetic energy to break the bonding forces between the strands. This mechanism is quite different from that produced by decreasing the ionic strength. This may be due to the increased repulsion between the strands due to the increase of charge on the DNA ion as dilution proceeds, as shown by Inman and Jordan,[10] to a change in the water structure surrounding the macro-ion, or to a change in the hydrophobic properties of the interior of the helix or to a combination of all these effects. At very low ionic strengths, where the DNA is denatured at room temperature, the latter mechanism is operative, whereas at high ionic strengths, the DNA only becomes denatured at high temperatures and the former mechanism occurs. The increase in the breadth of the transition at low ionic strength has been interpreted by Dove and Davison[29] as showing that the transition is less cooperative under these conditions.

A linear relationship between log I (where I is the ionic strength) and T_m has been observed by Ts'o et al.,[30]

Dove and Davison,[29] and Inman and Baldwin[31] for DNA and for polynucleotides. The results of Marmur and Doty[7] when plotted at T_m versus log I also show this linear relationship. A linear relationship between log k (where k is the specific conductivity) and T_m down to very low conductivities has been observed in this study (Fig. 9-III). However, at low values of log k, the linear relationship is not strictly obeyed and there would appear to be a discontinuity in the straight line (Fig. 9-III). By plotting the temperature at which the relative absorbance reaches the value 1.3 ($T_{1.3}$), compared with 1.2 for T_m, the thermal denaturation curves at lower ionic strengths can be included and the discontinuity becomes more evident as seen in Fig. (9-III). The existence of this discontinuity is not unexpected in view of the two mechanisms for denaturation that are operating simultaneously. At ionic strengths below the discontinuity, considerable denaturation has already occurred by the second of the two mechanisms given above, whereas above the discontinuity, this mechanism is much less important and heat denaturation predominates.

The basis of this logarithmic relationship is not obvious[29] and still awaits theoretical explanation. The explanation suggested by Ts'o et al.[30] in terms of the distribution of counter ions with reference to the work of

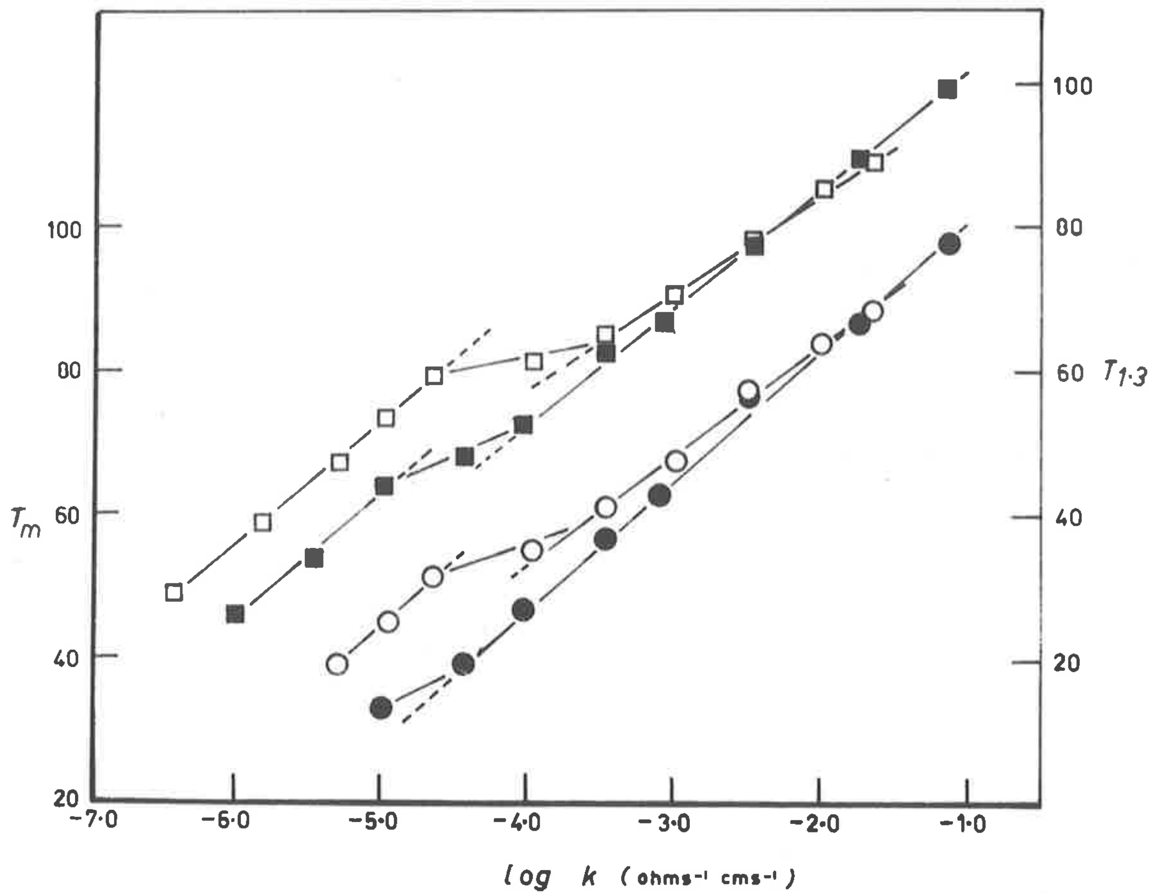


Fig. (9-III). Variation of T_m and $T_{1.3}$ with log specific conductivity.

Calf thymus DNA: ● T_m ■ $T_{1.3}$

B.megatherium DNA: ○ T_m □ $T_{1.3}$

Fuoss et al.[32] would appear to be incorrect as the relationships given by Fuoss et al. refer to the macro-ion and not the counter ion.

v) General Conclusions

From the results described above it is evident that denaturation of DNA by solution in low ionic strength solvents, or by dilution of concentrated solutions below a critical concentration, is a reversible procedure. Renaturation occurs on increasing the ionic strength either slowly or rapidly and there is evidence for a high degree of complementary double helix formation in the renatured DNA. Furthermore the sedimentation studies and density gradient equilibrium studies, together with the electron microscopy results, give evidence that denaturation by dilution can lead to strand separation. The behaviour of the DNA of B.megatherium on solution in pure water followed by the addition of sodium chloride appears to be quite analogous to that exhibited on heat denaturation and renaturing by slow cooling. There is, however, no analogous behaviour corresponding to quick cooling of heat denatured DNA, since the thermal denaturation curves for material obtained on increasing the ionic strength slowly and rapidly overlap (Fig. 2-III). With calf thymus DNA, denaturation by dilution is also reversible, although there is a marked difference between the behaviour of the material

prepared by slowly and rapidly increasing the ionic strength (Fig. 4-III). This result was unexpected in view of the previous observation of Doty et al.[16] that heat denaturation of calf thymus DNA was irreversible, a conclusion in agreement with our own results (Fig. 3-III). The behaviour of the material prepared by the slow addition of sodium chloride in a density gradient shows that complete renaturation has not taken place in the case of calf thymus DNA, and two other species appear to be present. One of these has a density corresponding to dilution denatured DNA and the other has a density corresponding to heat denatured DNA (Fig. 6-III). The difference in density of these two species, observed with both B.megatherium and calf thymus DNA, has been interpreted as indicating that the two treatments give rise to distinct species and the presence of both of them in this sample indicates that they could be interconvertible. An experiment using B.megatherium DNA (Fig. 5-III d) shows that this interconversion cannot apparently be brought about by different formaldehyde reactions at 100°C and room temperature.

The difference between the heat and dilution denatured samples can be clearly seen from the electron micrographs of these species (Plates (2-III) and (3-III)). The high degree of double helix formation on renaturation is also shown by electron microscopy (Plate 4-III).

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Chapter IV

Chromatography of DNA

i) Early fractionation

ii) Mechanism of chromatography using Ecteola

iii) Results of Bendich and co-workers

iv) Sonic degradation

v) Effect of size on chromatography

vi) Denaturation produced by the chromatographic process

vii) Heterogeneity in chromatographic fractions

i) Early Fractionation

The attempt to fractionate DNA was motivated by the desire to separate molecules having different genetic functions, together with the need for a sample of DNA having little or no physical heterogeneity. The need for such homogeneous samples arises from the difficulty experienced in interpreting the results of physical measurements when performed on heterogeneous samples.[1,2]

In order to obtain such a separation a wide variety of methods have been employed. The first separation of DNA into fractions was achieved by making use of its differential solubility in various solvents. The original suggestion that this process could be used to fractionate DNA probably came from Mirsky and Ris[3] who observed that DNA from chromosomes could possibly be separated into two fractions. It was found that most of the DNA was soluble in 1 M sodium chloride but that a small fraction remained in the chromosomes which was insoluble in this solution. It was originally thought that this residual nucleic acid was a contaminant, but it is also possible that a fractionation, based on the solubility in 1 M sodium chloride had been carried out. The work of Schneider and Hogeboom[4] also suggests this possibility, as only a part of the DNA of liver nuclei was found to be soluble in strong sucrose solution.

The first real attempt to fractionate DNA by this method was made by Bendich and co-workers.[5,6,7] Nucleic acid preparations extracted from tissues at very high temperatures (85°C) could be separated into two fractions on the basis of their differential solubility in 0.87% sodium chloride. Besides differing in their solubility in physiological saline these fractions differed in the extent to which previously administered C^{14} had been incorporated into the various purines and pyrimidines. This result was also shown to hold for calf thymus DNA which had never been exposed to temperatures above 5°C.[6]

A more successful procedure involved the extraction of DNA-histone complexes with sodium chloride. Chargaff et al.[8,9,10] studied the effect of different salt concentrations on the properties of the DNA-histone of calf thymus, which led to the discovery that DNA fractions of different, though regularly graded, composition could be prepared by this procedure which involves the fractional dissociation of the conjugated protein.[9] These studies were extended to artificial complexes between DNA and histone and other basic proteins.[10] The procedure of extracting with a series of sodium chloride solutions of rising concentration was found to yield a series of successive fractions with diminishing concentration of guanine and cytosine and rising concentrations of adenine and thymine.

Brown and Watson[11] used the same procedure as Chargaff et al. only they suspended the DNA on a histone coated kieselguhr column and separated the fractions by chromatography. The DNA once adsorbed on the columns was eluted by passing sodium chloride through the column, increasing the sodium chloride concentration stepwise. It was found, in agreement with the results of Chargaff et al.,[8] that with each increase in sodium chloride concentration a definite fraction of DNA was eluted at the concentration boundary between one eluting solution and that preceding it, thus demonstrating that the DNA preparation was heterogeneous with respect to the strength of the salt linkages binding it to the basic protein. It was also observed that the DNA in the fractions showed an increase in the ratios of adenine to guanine and thymine to cytosine with increase in the sodium chloride concentration of the eluting solution.

Lucy and Butler[12] have repeated the work of Chargaff et al. and although fractionation was achieved by extracting DNA-histone with sodium chloride, their results demonstrate that the essential variable in the extraction of the gel is not the sodium chloride concentration, since a similar fractionation was achieved by repeatedly extracting the gel with solutions of the same concentration.

The experiment of Brown and Watson was the first involving the use of chromatography to fractionate DNA, a

technique that has proven to be the most successful as a basis for separation. Brown and Martih,[13] in place of kieselguhr as the supporting agent for the histone (as used by Brown and Watson[11]), used columns of phosphorylated cellulose. T4 bacteriophage DNA could be separated into two fractions on eluting this column with a concentration gradient of sodium chloride. It was found that the two fractions had quite distinct adenine to guanine ratios and that the fractionation process was independent of the method of extracting the DNA from the bacteriophage. Lerman[14] used a technique similar to that of Brown and Watson to fractionate the transforming principle of pneumococcus. A column of methylated bovine serum albumin and celite was used, and eluted with a gradient of sodium perchlorate. This technique has been improved by Mendell and Hershey, who used it to separate DNA and RNA[15] as well as to isolate phage T2 DNA and to identify fragments produced from this DNA.[16] It was also used by Sueoka and Cheng to fractionate crab DNA and to distinguish between native and denatured DNA.[17]

Calcium phosphate gel, which has been satisfactorily used for the chromatography of proteins,[18] has been employed by Main and Cole[19] and Semenza[20] for the fractionation of DNA. Solutions of DNA were eluted stepwise[20] or by linear gradient elution using a gradient of phosphate.[19]

Reproducible fractionation into three elution peaks was observed by Semenza, but Main and Cole obtained no multiple peaks and observed no significant fractionation. However, separation of DNA from a mixture of degradation products was achieved[19] using this column. The smaller the nucleotides the lower was the concentration of phosphate required to elute them. A further method of fractionation has been used by Frankel and Crampton[21] who fractionated DNA on magnesium polymethacrylate, using a gradient of magnesium acetate to elute the column.

One of the most promising fractionation techniques so far developed is that of Bendich et al.[22-28] Since DNA carries a net negative charge due to the ionized phosphate groups (the isoelectric point is below pH 1) they have treated DNA as a mixture of polyanions and accordingly used a method of anion-exchange chromatography.

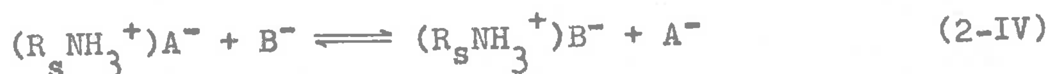
Sober and Peterson[29] have prepared an anionic exchange resin, which consists of a cellulose base that has been reacted with epichlorohydrin and triethanolamine. It was this exchanger, termed Ecteola-cellulose, that was used by Bendich and co-workers to fractionate DNA by means of gradient elution. The chemical structure of this resin is not fully known but it contains a variety of nitrogen functions of weak and medium basicity and possibly even some quaternary ammonium groups.

ii) Mechanism of chromatography using Ecteola

The exchange mechanism by which this exchanger acts is thought to arise from the fact that weakly basic exchangers are to a large extent undissociated in an alkaline or neutral medium.[30] The dissociation equilibrium being written thus:

$$R_sNH_2 + H_2O \rightleftharpoons (R_sNH_3^+) + OH^- \qquad (1\text{-}IV)$$

The fact that these anion exchangers are inert in alkaline medium led to the concept of regarding the reaction as an adsorption of the whole acid molecule. Thus the ion exchange reaction in acid medium is an equilibrium reaction of the type:



$$(R_sNH_3^+)A^- + B^- \rightleftharpoons (R_sNH_3^+)B^- + A^- \qquad (2\text{-}IV)$$

From the foregoing considerations it is obvious that the hydroxyl ion has an extremely high affinity for the resin, i.e. other anions are easily displaced by hydroxyl ions. This means that as the pH of an eluent is increased, whatever is adsorbed on the column will be eluted probably by displacement analysis.

Wiklander[31] found that concentrated solutions of chloride ions possessed a greater affinity than sulphate ions for a resin of the weakly basic type, which means that next to hydroxyl ions chloride ions have the highest affinity for this resin provided the chloride ions are present in high

concentration. Therefore another means of displacing the adsorbed material is to slowly increase the chloride ion concentration. The simple phosphate ion affinity for these resins is greater than that of the chloride ion at low chloride ion concentrations but less at high chloride ion concentration. Kunin and Myers[32] report the following sequence for the affinity between ions and weakly basic anion exchange resins:-

Fluoride < Chloride < Bromide = Iodide = Acetate < Molybdate < Phosphate < Arsenate < Nitrate < Tartrate < Citrate < Chromate < Sulphate < Hydroxyl.

A similar series has been obtained by Veder and Pascha[33] for the affinity between anions and Ecteola-cellulose.

Provided the DNA molecules are considered to be attached to the resin column by means of the phosphate backbone of the molecules (that this is a reasonable hypothesis will be shown later in this chapter), the elution of DNA from columns of Ecteola-cellulose can be considered to occur in the following manner. The resin is washed firstly with hydroxyl ions in order to remove any unwanted ions from the resin by displacement analysis, and then with excess phosphate ions in order to remove the hydroxyl ions, probably by a type of elution analysis. When the column is loaded with DNA an equilibrium between simple phosphate ions and macromolecular phosphate ions is set up. Provided only about one

quarter of the total DNA capacity is loaded on the column all the macromolecular phosphate ions are adsorbed and they are not displaced by washing with excess phosphate buffer. Thus it appears that the resin has a greater affinity for macromolecular phosphate ions than for the simple phosphate ions. Development is then by means of displacement analysis using a gradual increase in the concentration of the developers, chloride ions, hydroxyl ions or a combination of both. Thus in equation (2-IV) A^- represents $(PO_4^-)_n$, i.e. the phosphate backbone of a DNA molecule, and B^- represents either Cl^- or OH^-.

This mechanism involves two types of chromatographic separation, elution and displacement analysis. These two separation procedures act in the following manner.[34]

Elution Analysis

The developer liquid passes through the column desorbing the adsorbed material. As it passes over fresh adsorbate it picks up more and more solute until eventually, somewhere in its passage through the zone, the liquid may reach equilibrium with the adsorbate at a maximum concentration which is maintained almost to the leading edge of the zone. As this is a flowing system equilibrium may not be reached, however a steady state approximating to equilibrium may be reached. At the leading edge of the zone the concentration in the adsorbate decreases and so de-

position of solute from solution occurs and the liquid rapidly becomes depleted of solute. Thus the developer liquid starts with zero concentration of solute, picks up solute as it passes through the zone until maximum concentration is reached, and then returns to zero concentration after depositing the substance in a lower portion of the column. This causes the zone to migrate in the direction of flow with a trailing edge.

The rate of movement of the zone depends on the rate of flow of the liquid and the strength of adsorption of the substance involved. The more strongly a substance is adsorbed the slower it moves, since it is less completely desorbed by the developer in its passage through the zone. This constitutes the basis of the separation procedure.

The way in which phosphate ions replace hydroxyl ions will differ from the above procedure as the hydroxyl ions are not adsorbed onto the column in a zone at the top of the column but are adsorbed throughout the resin. As excess phosphate ions are passed through the column the equilibrium between bound and unbound hydroxyl ions

$$R_sNH_3^+ + OH^- \rightleftharpoons (R_sNH_3^+)OH^-$$

will be upset as the unbound hydroxyl ions are removed and so phosphate ions will be able to replace hydroxyl ions on the resin so giving rise to an effect similar to elution analysis.

Displacement Analysis

In this form of chromatographic separation the developer is adsorbed more strongly than the eluted zone. The developer displaces all less strongly adsorbed species from the front and the more strongly adsorbed of these displaces less strongly adsorbed material from the next region and so on down the column. Thus the developer acts as a kind of piston.

The differential effectiveness of a developer can be enhanced by gradually increasing its concentration so that its displacing effect steadily rises. This forces a segregation of the components by gradually increasing the eluting power of the developer, which in turn results in the zones moving consecutively out of the column, without the presence of any trailing edges. Once a component has been separated from the rest of the material with which it was initially adsorbed, it may travel down the column with an R_f of one, or very nearly one, and be little influenced by the remainder of the column packing. This assumes that the capacity of the column is sufficient to hold all of the initial sample. Actually, gradient elution, not being a countercurrent multistage procedure, can theoretically[35] be carried out without a column, although placing the exchanger in a column has mechanical advantages. Increased resolving power is obtained, not by increasing the length of

the column as in elution analysis but by using a more gradual change in the composition of the eluent.

iii) <u>Results of Bendich and co-workers</u>

Bendich and co-workers chose Ecteola-cellulose as a chromatographic medium in preference to Deae-cellulose and C.M.-cellulose because of its favourable capacity for DNA, low shedding blank, essential reproducibility of elution pattern and high recovery obtainable without the use of drastic eluting conditions. On fractionating DNA from various sources on this exchanger, either by discontinuous or gradient elution, multiple fractions could be obtained. As gradient elution is in general a more satisfactory technique it was used in preference to stepwise elution. However, a satisfactory resolution could not be obtained using a single mixing chamber gradient elution system.[22] This was overcome by the use of a double mixing chamber system which produces a more gradual change in eluent concentration. This system[28] produces fractions which fall into four main regions, namely: 1) 0 to 0.5 M sodium chloride; 2) 0 to 0.1 M ammonium hydroxide in 2.0 M sodium chloride; 3) 0.1 M to 1.0 M ammonium hydroxide in 2.0 M sodium chloride; and 4) 0.5 M sodium hydroxide. The first region consists of a gradient in neutral sodium chloride, the second and third consist of pH gradients and the last region is one in which any DNA

remaining on the column is eluted at high pH.

Ward and Putch[36,37] have also used Ecteola-cellulose to fractionate DNA. However, the resin used had a much lower capacity than that used by Bendich and co-workers, in order to obtain greater resolution during fractionation. This method has also been used by Kit[38,39,40] and Klouwen and Weiffenbach.[41]

Bendich et al.[23] found that DNA from different sources, such as calf thymus, human leucocytes, E.coli, B.cerus and pneumococcus, or from closely related sources such as T6r and T6r$^+$ bacteriophage or the brain, spleen, intestine and kidney of the rat, give rise to different chromatograms. It was suggested[23] that these differences are a reflection of the intrinsic differences within the samples of DNA as it had been shown[23,26,28] that chromatograms obtained with the DNA of any given species were very reproducible. Ward and Putch[37] have also reported that chromatography of DNA from various organs of the golden hampster, on their sample of Ecteola-cellulose with its low degree of substitution, gave rise to different chromatograms. A test as to whether a fraction is a real entity or just an artifact arising from the procedure is to rechromatograph the fraction. Bendich et al. have shown[22,23] that when a selected portion of a narrow peak is rechromatographed, the large bulk of the DNA contained therein appears at that place in the chromatogram (in terms of a particular ionic strength

and pH) at which the original sample was eluted.

The high degree of resolution possessed by this process was shown by the separation of DNA molecules containing the thymine-analogue 5-bromouracil from molecules containing thymine.[24,28] The separation of DNA from polyadenylic acid has also been demonstrated.[28] Fractionation of pneumococcal DNA produced further evidence of this resolution.[23] The individual fractions obtained as a result of the chromatographic separation showed considerable biological activity for transformation to streptomycin resistance, compared with that present in the original preparation, the increase being more than fifty-fold in some cases. A similar partial separation of the streptomycin, penicillin and mannitol activities has also been reported.[42]

Fractions of calf thymus DNA obtained using this process were found[23,28] to contain different relative amounts of base pairs. Contrary to the predictions of Watson and Crick these authors found that the ratios of adenine to thymine and guanine to cytosine for many of the fractions differed significantly from the value of 1.00. Jordan[43] has postulated that this might be due to a breakage of the specific hydrogen bonds between the polynucleotide chains of the Watson-Crick model as a result of binding the molecule to the exchange resin, which might thus yield some single polynucleotide chains. However, Bendich et al. have reported evidence which suggests that the DNA removed from the column is still native,[26]

although evidence is presented later in this chapter which suggests that denaturation may have occurred to a small extent.

Bendich and his collaborators consider that the basis on which the separation of DNA into fractions on columns of Ecteola is achieved is a result of a combination of chemical and physical heterogeneity. The separation of chemically heterogeneous species is indicated by the difference in base content for the different fractions, however there is no relation between the base content and the position of the fraction as was the case in the work of Chargaff et al.,[8,9,10] Brown and Watson[11] and Lucy and Butler.[12] Experiments arising from an attempt to isolate a fraction of DNA which represents a particular genetic function also indicate that the basis of the fractionation process is not one of chemical heterogeneity. Different genetic functions are thought to correspond to different arrangements or sequences of the purine and pyrimidine bases. Bendich and co-workers[23,42] have shown that DNA having the ability to transform receptor cells to streptomycin resistance does not fractionate at any particular position in the chromatogram of transforming DNA. Early fractions (No. 25) as well as middle (No. 173) and late ones (No. 260) possess transforming activity, thus there is again no correlation between a specific base sequence, which constitutes the

genetic properties of the molecule, and the fraction position.

A variety of techniques have been used to demonstrate the separation of physically heterogeneous samples. The first evidence for this type of separation came from experiments using very low molecular weight polynucleotides. Bendich et al.[22,23] demonstrated that monodeoxyribonucleotides were quantitatively eluted by means of 0.1 M phosphate buffer, pH 7, whereas a deoxyribonuclease digest of calf thymus DNA containing a large proportion of oligonucleotides required increases in sodium chloride concentration up to 0.2 M (in phosphate buffer) for quantitative elution. As the largest molecules in this digest have a molecular weight in the region of 2,400,[44] these authors concluded that fractions eluted with solutions of higher ionic strength or pH would be of higher molecular weight.

On the basis of these results ultracentrifugal analysis of the fractions resulting from the chromatography of calf thymus DNA was carried out[26,45] to investigate the possibility of a correlation between the sedimentation coefficient, hence the molecular weight, of DNA and the ionic strength or pH of the eluents. The original calf thymus DNA showed the usual[1,46-51] wide spread in sedimentation coefficients. Sedimentation boundary analysis was also performed on dilute solutions of eight fractions obtained by combining fractions produced by chromatography.

These fractions amounting to 56.5% of the DNA recovered from the column with eluents of increasing ionic strength and then increasing pH, showed progressive increases in $s_{50\%}$ from 10.9 to 24.1 s compared with 15.8 s for the original. The spread of sedimentation coefficients in the boundary also changed, the early fractions had very narrow distributions and this broadened as the ionic strength and pH of the eluent increased. Some of the later fractions appear to contain molecules having sedimentation coefficients greater than are present in the original sample. The explanation of this behaviour suggested by these authors is that the total amount of DNA with values of s in these high ranges is so small that it would not be detected in the original DNA.

These results imply that it is the size of the molecules which governs their behaviour on this ion-exchange resin. A consequence of the Watson-Crick structure is that DNA can be considered as a cylinder covered with charged phosphate groups. Thus when DNA is adsorbed onto an anion-exchange resin it must be attached through these phosphate groups as they are the only groups in juxtaposition to the surface of the resin matrix. Therefore fractionation by chromatography must be the result of a difference in the ease with which these bonds may be ruptured for different DNA molecules. The strength with which a molecule is bound

to the ion-exchange agent is a reasonable consequence of the number of charged groups per molecule in contact with the exchange agent at any given time.[42] Thus the more charged groups on a DNA molecule, i.e. the higher its molecular weight, the stronger will it be bound to the column matrix and the stronger will the eluting solution have to be to rupture all such salt linkages in order to obtain elution. In this discussion the eluents sodium chloride and 0.1 M ammonia will be referred to as weak eluents and 1.0 M ammonia and 0.5 M sodium hydroxide as strong eluents.

A direct means of confirming the hypothesis that it is the physical heterogeneity that is governing the fractionation of DNA on Ecteola would be to fractionate a sample of DNA that has been degraded. Bendich et al. attempted this using DNA degraded by heating,[52] which will be discussed in detail in the next chapter. It was concluded from this work that the changes produced in the chromatogram of heated calf thymus DNA were a direct result of the changes in molecular weight produced by the effect of heat. Some of the results were, however, complicated by the presence of denaturation. If a sample of DNA is sonically degraded it is possible to bring about a decrease in the size of the molecules without affecting their tertiary structure. Thus if the above hypothesis is correct the chromatographic profile for the degraded samples should show a shift towards weaker eluting conditions,

compared to the profile for native DNA. This will occur because the molecules in these samples will still possess the double stranded helical structure but will be reduced in length, i.e. the number of phosphate groups binding these molecules to the column will be less than in the case of the original native molecules. Therefore the eluting conditions required to rupture these bonds will be milder than the conditions required for the undegraded molecules.

A brief account of the mechanism of sonic degradation, together with a brief physical study of the 'sonicates', produced by subjecting calf thymus DNA to sonic vibrations for various lengths of time, is presented before discussing the results obtained on chromatographing these sonicated samples of DNA.

iv) Sonic Degradation

A mechanism for sonic degradation was postulated by Gooberman[53] who suggested that the degradation occurred through the subjection of the molecules to shearing stresses produced by solvent flow. This flow results from the shock wave radiating from a cavitation. It was shown,[53] on the basis of this mechanism, that the bond most likely to be broken was that near the centre of mass of the molecule. Thus in an initially monodisperse sample of macromolecules a fraction of the molecules will meet stresses capable of degrading them to halves, while much smaller fractions will

be further subdivided. Owing to the fact that DNA is not a rigid linear macromolecule the bond must likely to break may not be at the centre of the chain and so a small increase in polydispersity is to be expected. Rigid molecules such as T.M.V. have been shown to break exactly at the middle of the chain during sonic degradation.[54]

If sonic degradation does proceed by this mechanism of shear degradation then we would expect strand breakage to occur near the centre of the chains. Hershey and Burgi[16,55] have shown, for T2 DNA, that shear degradation produced by stirring solutions of DNA does produce breaks which have a strong tendency to occur near the centre of the molecule. The results of Freifelder and Davison[56] on the sonic degradation of DNA for short periods agree with this picture. Sonication was shown to proceed by a mechanism which resulted preferentially in the successive halving of the molecules.

The above results appear to be somewhat different from those of Doty et al.[57] who concluded that the breaks, produced by sonically degrading calf thymus DNA, were distributed randomly along the original molecular length. This resulted in a very broad distribution of lengths whose mean depended on the time of treatment. Hershey and Burgi[16] found that they obtained a narrow distribution of lengths whose mean depended chiefly on the speed of stirring. These differences can be explained if the effect found by Doty et al.[57] is considered as the result of weak shearing

forces acting for a long time and the latter effect the result of strong shearing forces acting for a short time.

The final product produced by a given rate of shear must consist of fragments too small to be broken by that shearing force. This implies that the production of fragments much shorter than the maximum length resistant to breakage is prohibited. Thus in the case of sonic degradation with its weak shearing force, the molecular weight distribution obtained after long periods of treatment will be very different from that obtained after intermediate periods.

Fig. (1-IV) shows the effect on molecular weight of degrading calf thymus DNA for various times at different frequencies. Curve a results from degrading DNA with 50 watt, 9 K.cycle sonic vibrations and curve b from using 11.5 watt, 500 K.cycle vibrations. The rapid fall in molecular weight has ceased after five minutes in the case of degradation by sonic vibrations, curve a, which is very similar to the curves presented by Freifelder and Davison[56] for T7 DNA and Doty et al.[57] for calf thymus DNA (both presented in reference 56) obtained on degrading DNA by the same method. In the case of degradation produced by the high frequency, curve b, the fall in molecular weight is less marked and does not taper off until after approximately 15 minutes. This suggests that the method of degradation

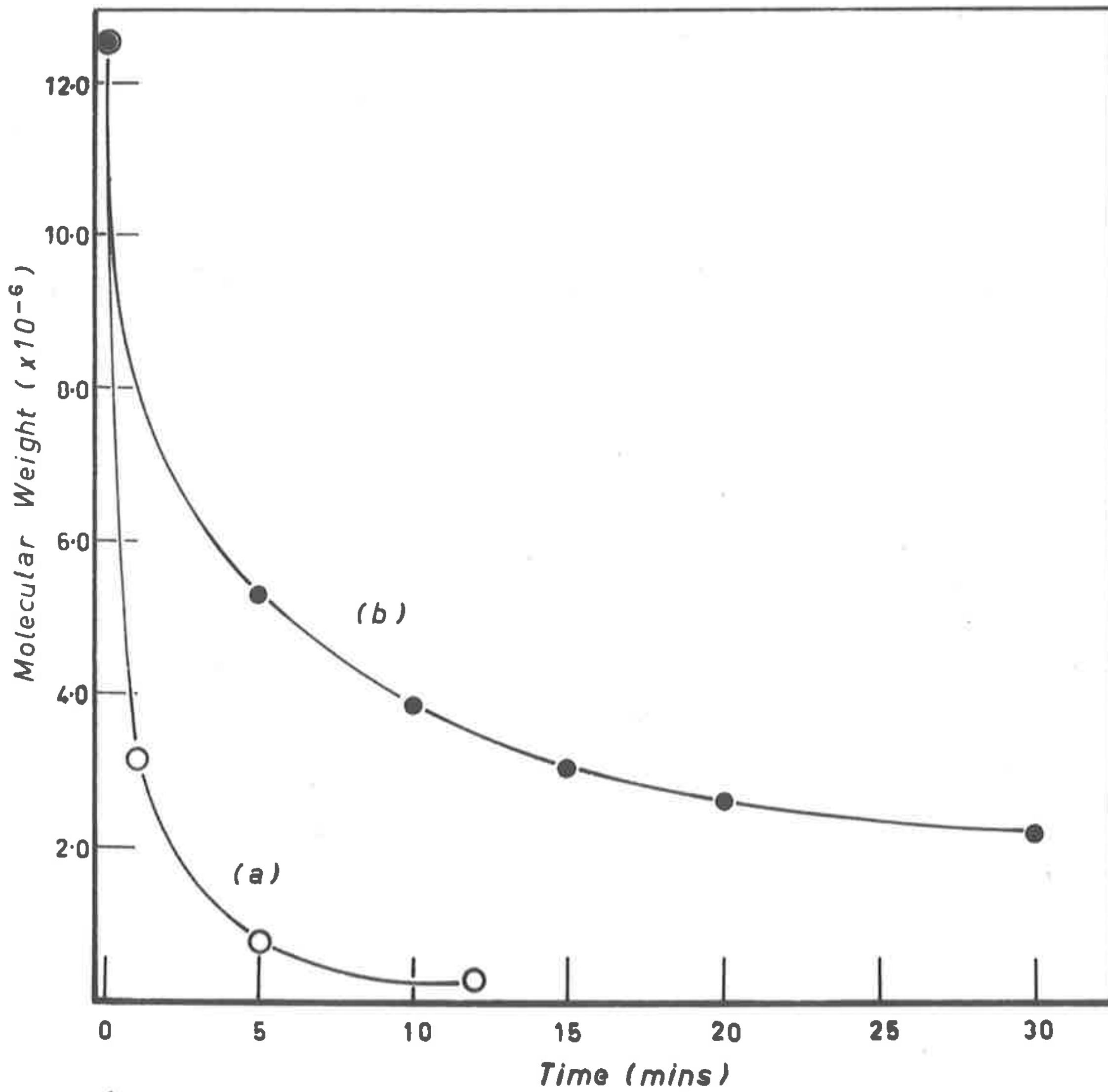


Fig. (1-IV). Variation of molecular weight of calf thymus DNA with exposure time. Curve a, exposed to sonic vibrations. Curve b, exposed to ultra-sonic vibrations.

giving rise to curve **a** involves stronger shearing forces than the method giving rise to curve **b**.[16] Neither method appears to cause denaturation, as evidenced by the lack of any change in the optical density of the solution on degradation. This conclusion is supported by the results obtained on thermally denaturing the samples produced by sonic degradation, referred to as 'sonicates' (Fig. 2-IV). These show that the maximum hyperchromicity produced on heating the sonicates is the same as that produced on heating the original native DNA.

These results also show, in agreement with the predictions of Zimm et al.,[58] that as the molecular weight of the sample of calf thymus DNA is decreased so its T_m decreases. Marmur and Doty[59] concluded from their results that a decrease in the molecular weight of calf thymus DNA from 8×10^6 to 6.2×10^5 produced no change in T_m. If the graph on which they base this conclusion is compared with the curves for native, one and five minute sonicates in Fig. (2-IV) it can be seen that the two sets of results are almost identical. Thus it appears that the T_m only changes slightly with molecular weight provided this is not below some minimum value, however, if the molecular weight is decreased sufficiently, as in the case of the twelve minute sonicate, the expected fall in T_m with molecular weight is observed.

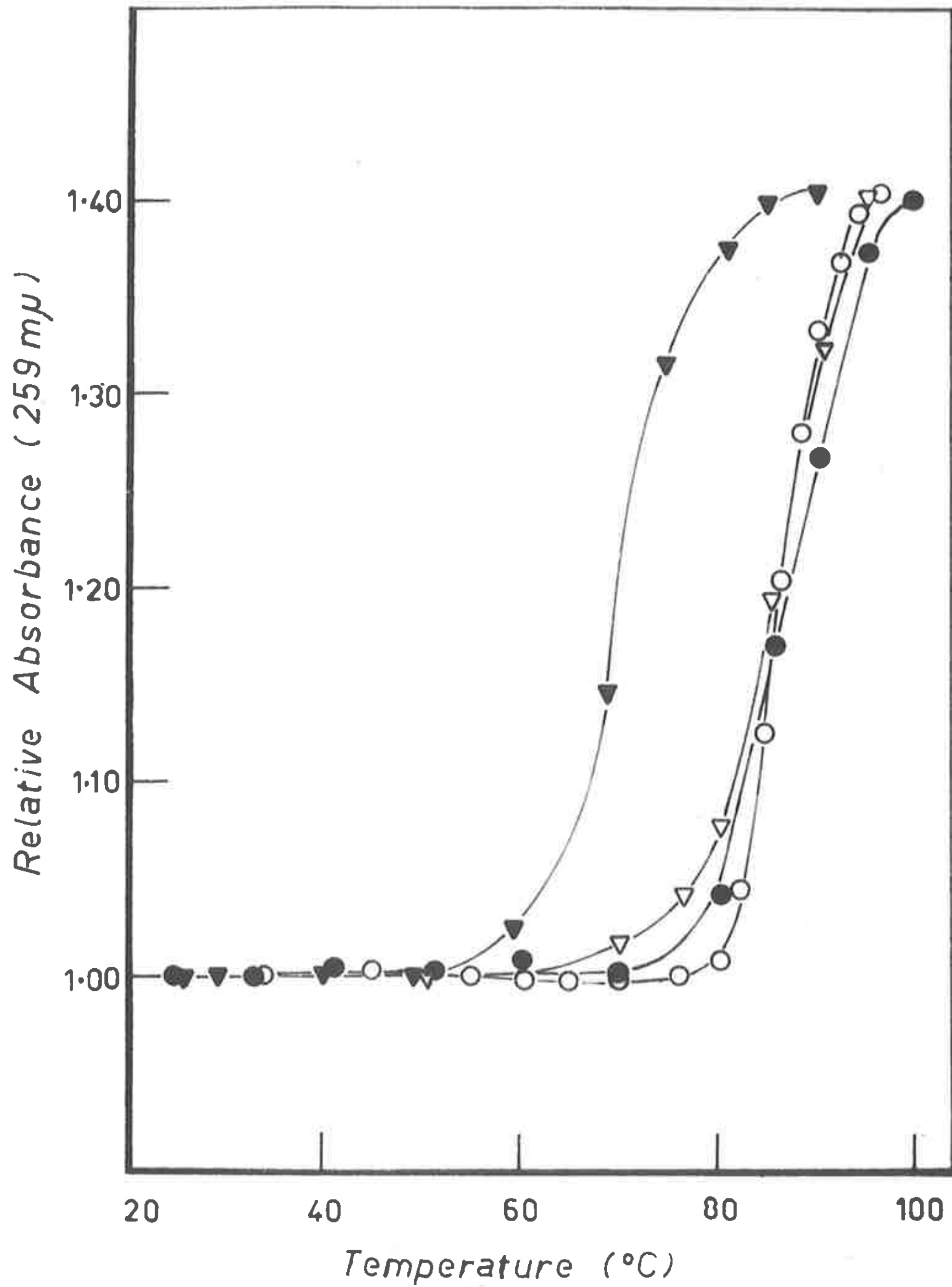


Fig. (2-IV). Variation of relative absorption with temperature for samples of calf thymus DNA.

○ native, ● 1 min. sonicate, ▽ 5 min. sonicate, ▼ 12 min. sonicate.

As the samples produced by sonic degradation show the larger decrease in molecular weight and are essentially native, it was decided to use them for the chromatography experiments. Hence further experiments were conducted on these sonicates in order to estimate the variation of the molecular weight distributions from that of the original sample.

This was achieved by using the method of sedimentation boundary analysis discussed by Schumaker and Schachman[49] (see chapter VI) to determine the sedimentation coefficient distribution of the native sample and the three sonicates. The results, presented in Fig. (3-IV), clearly show the fall in molecular weight with time of sonication. The curve for the original native sample (curve a) shows the usual wide spread of sedimentation coefficients previously reported.[1,46-51] Curves c and d, i.e. the distributions of the five and twelve minute sonicates, indicate that these samples have a high degree of homogeneity. The mean sedimentation coefficient (s) for the one minute sonicate (curve b) is approximately half of that for the native sample (curve a), but the spread of sedimentation coefficients is still very large. It is obvious from curve b that only the larger molecules have been reduced in size, which is in agreement with the hypothesis that the shearing forces will only reduce the size of the molecules to a given minimum size

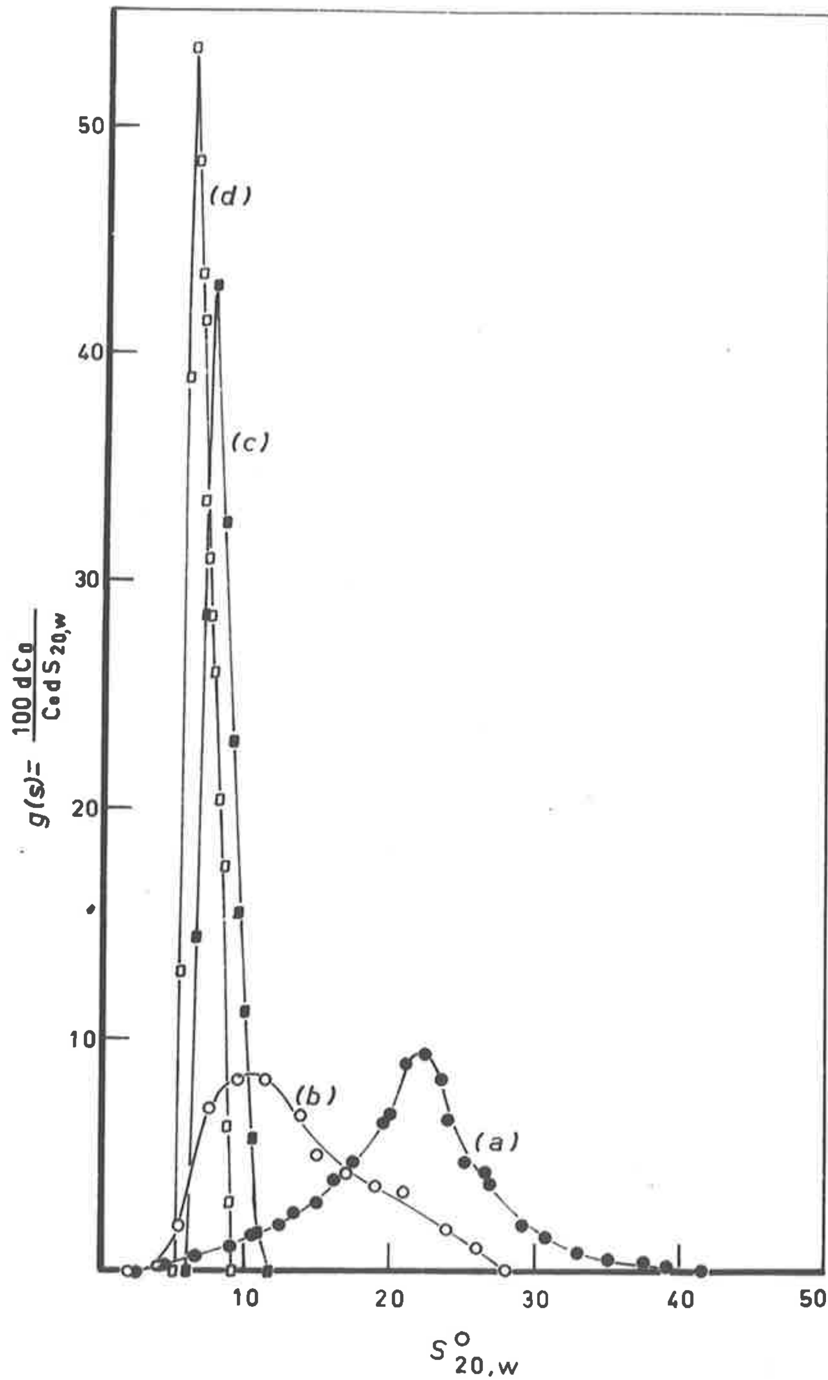


Fig. (3-IV). Sedimentation coefficient distributions for samples of calf thymus DNA. Curve a, native; Curve b, one minute sonicate; Curve c, 5 minute sonicate; Curve d, 12 minute sonicate.

governed by the size of the force. This is borne out by the fact that there is little drop in the mean s value from the five minute to the twelve minute sonicate. The apparent absence of molecules having very low sedimentation coefficients in these two samples is probably due to the fact that these molecules are present in such low proportions as to be undetected by the analysis procedure.

The results of Doty et al.[57] showed that the sonicated samples each had a molecular weight distribution similar to the original, whereas Freifelder and Davison[56] showed that for T7 DNA the distribution of degraded molecules was unlike that of the original, being essentially bimodal, but skewed on the side of lower $s_{20,w}$, as expected theoretically. This difference can most probably be attributed to the fact that DNA isolated from any but viral sources is very heterogeneous and also probably represents the mechanically ruptured fragments of one or more initially long chains.[60,61] Thus the starting sample of calf thymus DNA used by Doty et al.[57] probably had a molecular weight distribution essentially similar to that produced by sonication. The results presented in curve b, Fig. (3-IV), show that after sonicating for one minute the distribution does tend to become bimodal, only the large degree of heterogeneity in the original sample appears to stop complete separation into two distinct peaks. After five minutes, curve c, it

is obvious that most of the original molecules have disappeared and the molecules are nearing the minimum size below which the shearing force has no effect.

It is therefore concluded that this sample of DNA on degradation by sonication behaves in the manner predicted from the proposed mechanism of sonic degradation, except where the expected resultant distribution must be modified due to the high degree of heterogeneity present in this sample.

v) Effect of size on chromatography

The method of chromatography used by Bendich et al.[28] has been modified in some aspects (see chapter VII) in order that low molecular weight samples could be fractionated on the columns and the possibility of having extraneous peaks is removed. The chromatogram obtained on fractionating native calf thymus DNA in phosphate buffer on a column of Ecteola-cellulose is shown in Fig. (4-IV). No DNA is eluted with phosphate buffer and very little with sodium chloride at a concentration less than 2.0 M, unlike the chromatogram obtained by Bendich et al.[28] As can be seen from Table (1-IV), most of the DNA (61.7%) is eluted in the 1.0 M NH_3/2 M NaCl region (region 5), with 26.7% being eluted in the 0.1 M NH_3/2 M NaCl region (region 4).

The change in chromatographic profile with degradation is shown in Figs. (5-IV) and (6-IV). Sonic-

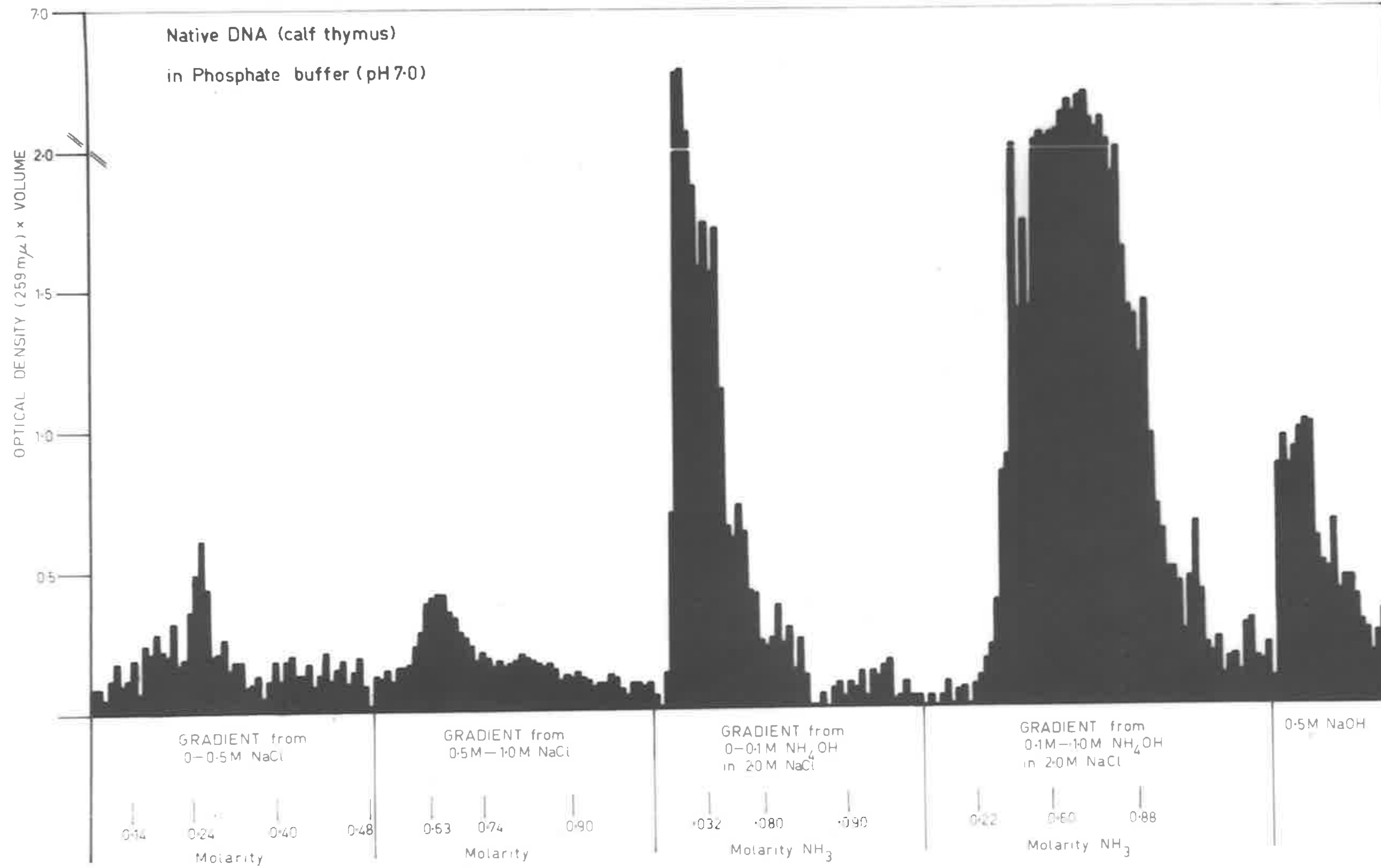


Fig (4-IV)
Native DNA (calf thymus)
in Phosphate buffer (pH 7·0)
OPTICAL DENSITY (259 mμ) × VOLUME
7·0
2·0
1·5
1·0
0·5
GRADIENT from
0 – 0·5 M NaCl
0·14
0·24
0·40
0·48
Molarity
GRADIENT from
0·5 M – 1·0 M NaCl
0·63
0·74
0·90
Molarity
GRADIENT from
0 – 0·1 M NH4OH
in 2·0 M NaCl
·032
·080
·090
Molarity NH3
GRADIENT from
0·1 M – 1·0 M NH4OH
in 2·0 M NaCl
0·22
0·60
0·88
Molarity NH3
0·5 M NaOH

Table (1-IV)

Sample	Percentage recovery in different regions					
	Region 1 phosphate buffer	Region 2 0.5M NaCl	Region 3 1.0M NaCl	Region 4 0.1M NH_3/2M NaCl	Region 5 1.0M NH_3/2M NaCl	Region 6 0.5M NaOH
Native	-	2.0	-	26.7	61.2	10.2
1 Minute sonicate	-	4.6	52.3	10.3	29.2	3.5
5 Minute sonicate	1.0	15.5	54.1	18.5	8.5	2.3

ating DNA for one minute results in a general shift of the chromatographic profile to the left, i.e. region of weaker eluting conditions (Table 1-IV). There is a substantial decrease in the amount of DNA in the sodium hydroxide and 1.0 M ammonia/2 M sodium chloride regions and a complete loss of the peak in region 4. Most of this DNA appears to be eluted in the 1.0 M sodium chloride region (region 3), the rest appearing in a new peak in region 4, being eluted at a concentration of 0.09 M ammonia instead of approximately 0.02 M ammonia. The amount of DNA eluted in region 2 is approximately the same as for native DNA. This shift is produced by a change in molecular weight from 12.6×10^6 to 3.2×10^6 (Fig. 1-IV). In the case of the five minute sonicate the molecular weight is reduced to 8.8×10^5. This reduction produces even greater changes in the chromatographic profile on fractionation. Very little DNA is eluted in either region 5 or 6. Some is eluted in the position of the origin peak in region 4, but again over 50% of it is eluted in region 3. There is an increase in the amount eluted with 0.5 M sodium chloride and a very small amount is eluted with phosphate buffer.

The chromatogram of native DNA (Fig. 4-IV) shows the largest amount of DNA to be in region 5, in agreement with results of Bendich et al.,[28] while the largest amounts of the one and five minute sonicates are in the third region.

Fig (5-IV)

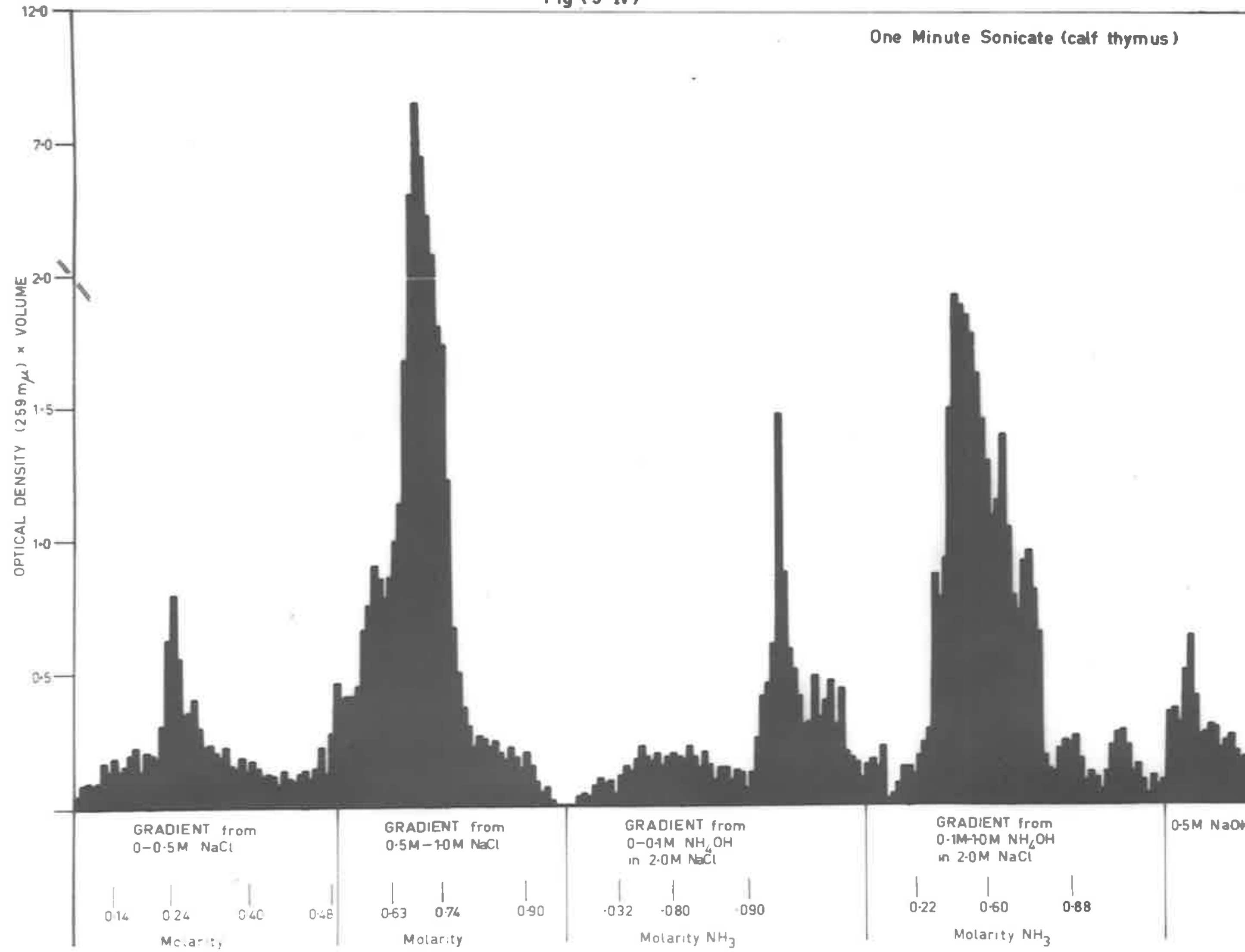
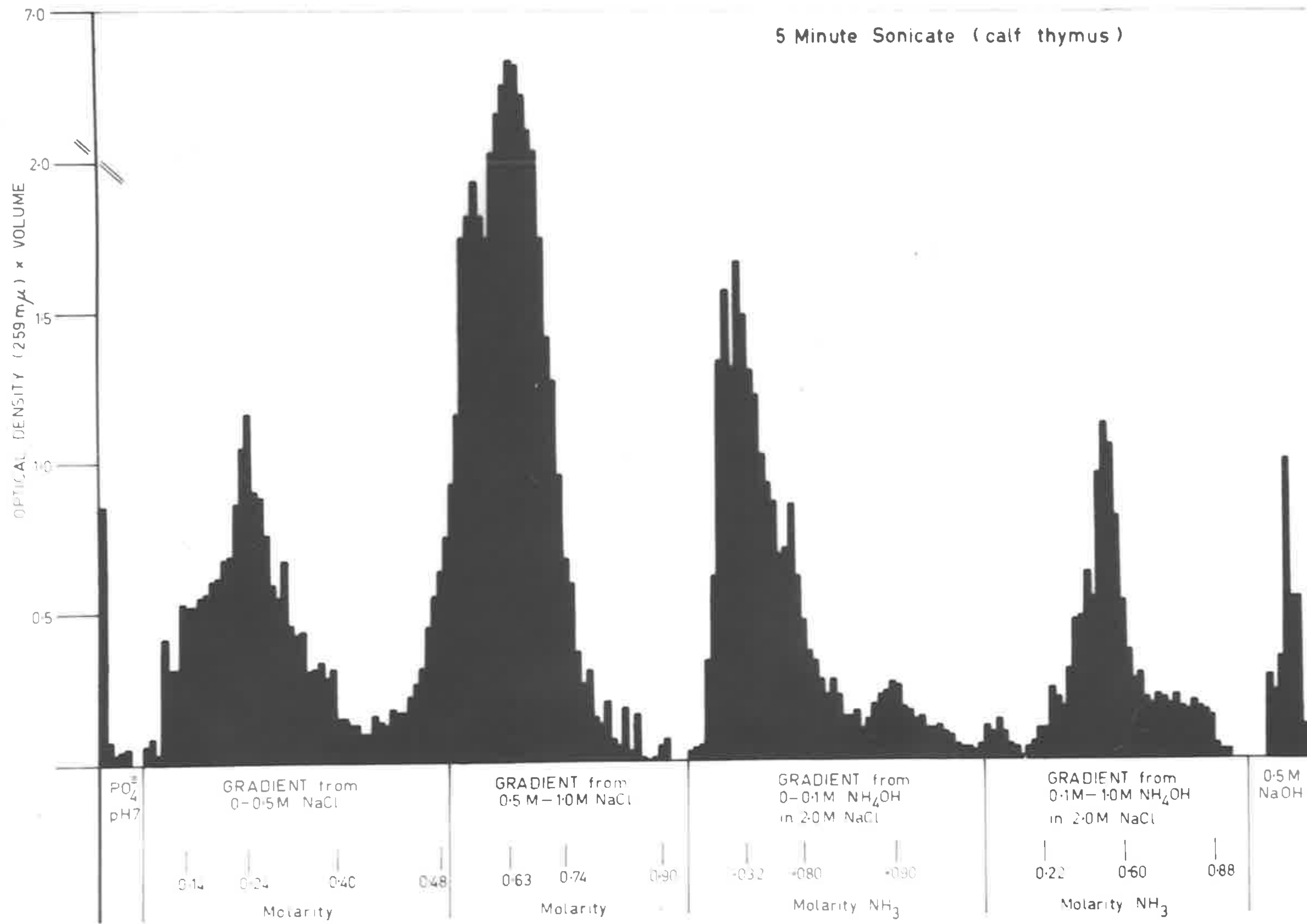


Fig (6-IV)



In the case of the one minute sonicate (Fig. 5-IV) all but 4.6% of the rest of the DNA is in regions above this, whereas in the chromatogram of the five minute sonicate (Fig. 6-IV) 16% of the DNA is eluted prior to region 3. This clearly shows that as the molecular weight of the DNA sample is decreased the elution profile shifts to regions of weaker eluting conditions, a result which had been predicted earlier from the postulate that the fractionation procedure involved the separation of molecules attached to the column by different numbers of phosphate groups. Thus it appears that the mechanism of this fractionation process involves the separation of molecules of different length.

vi) Denaturation produced by the chromatographic process

Bendich et al.[26] have concluded that the column fractions are probably no more degraded or denatured than an unfractionated sample of DNA. Rechromatography of the column fractions was found[23] to yield a chromatogram in which the bulk of a given fraction appears at the same position in the elution profile as in the original fractionation. A structural change in the DNA, such as occurs when it is subjected to heat treatment, had been shown[26,52] to cause a marked change in the chromatographic profile. Thus it was deduced[26] that, if alteration of the DNA had attended its initial fractionation, this effect was slight as it did not materially influence the sample's chromatographic

properties when it was rechromatographed. Further evidence was obtained from fractionation studies on pneumococcal transforming DNA. Pahl et al.[42] found that the combined fractions from the column have almost the same biological activity as the original material which indicates the absence of denaturation, as the loss of biological activity is used as a sensitive test for denaturation.[62] Evidence was also obtained from the activity-response curves for both unfractionated and fractionated transforming DNA which suggested that the quality of the DNA in the fractions was the same as that in the unfractionated sample.[42]

The results of rechromatographing samples of DNA used in this study were the same as those found by Bendich et al.[23] However, it has been found (see chapter V) that denaturation alone does not result in large changes in the chromatographic profile. Therefore it is quite possible for samples which have been slightly denatured, by their removal from the column, to be eluted in the same position in the elution profile on rechromatography as they were in the original fractionation.

It has been shown, in the previous chapter, that the amount of hyperchromicity produced on heating a solution of DNA above its T_m gives a measure of the amount of denaturation that has occurred in the sample. Doty et al.[63] have shown that raising the temperature of a solution of calf

thymus DNA sufficiently above its T_m and holding it there for fifteen minutes produces a 40% increase in the extinction coefficient. This suggests that a very sensitive test for denaturation would be to measure the amount of hyperchromicity produced on heating a sample of DNA to a temperature, above its T_m, at which the optical density ceases to increase. This can then be compared to the increase obtained on heating an undenatured sample.

Fig. (7-IV) shows the variation of the relative absorbance with temperature for native DNA (Fig. 7(a)-IV), the one minute sonicate (Fig. 7(b)-IV) and the five minute sonicate (Fig. 7(c)-IV). This method of presentation shows directly the amount of denaturation present in each sample, as it results in all samples having the same maximum hyperchromicity. It can be seen from Fig. (7(a)-IV) that fractionation of native DNA in the presence of 0.5 M sodium chloride produces no denaturation, while 0.1M ammonia produces approximately 10% and 1.0 M ammonia approximately 12.5% denaturation. Fig. (7(b)-IV) shows that 1.0 M sodium chloride and 0.1 M ammonia both produce approximately 15% denaturation and 1.0 M ammonia approximately 30% denaturation, when used to fractionate one minute sonicate DNA. Fig. (7(c)-IV) shows that when 1.0 M ammonia is used to fractionate the five minute sonicate DNA, about 34% denaturation is produced.

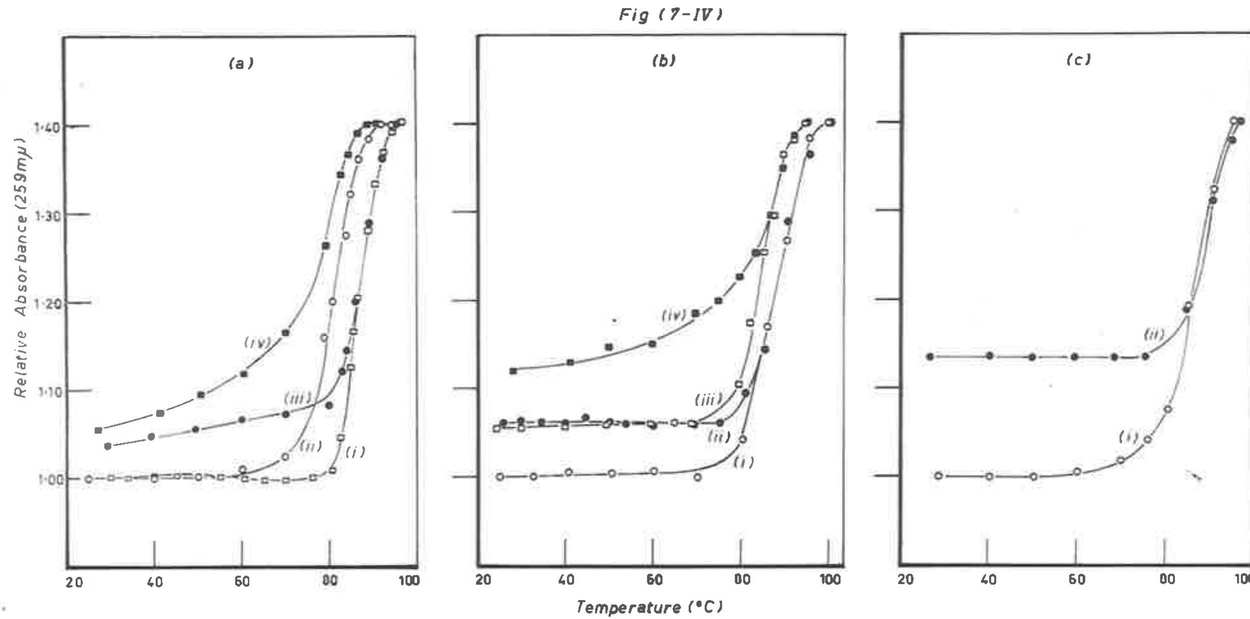


Fig. (7-IV). Variation of relative absorption with temperature for samples of calf thymus DNA and fractions.

a, native: Curve i), unfractionated; Curve ii), 0.5M NaCl fraction; Curve iii), 0.1M NH_3/2M NaCl fraction; Curve iv), 1.0M NH_3/2M NaCl fraction.

b, one minute sonicate: Curve i), unfractionated; Curve ii), 1.0M NaCl fraction; Curve iii), 0.1M NH_3/2M NaCl fraction; Curve iv), 1.0M NH_3/2M NaCl fraction.

c, 5 minute sonicate: Curve i), unfractionated; Curve ii), 1.0M NH_3/2M NaCl fraction.

It would appear from these results that, as the strength of the eluting conditions is increased, the amount of denaturation produced also increases. This is thought to be due to the mechanics of removing the molecules from the column rather than from the presence of the eluting solution. Bendich and Rosenkranz have shown[45] that dissolving DNA in the solutions used for elution does not cause a change in the sedimentation coefficient distribution. 1.0 M sodium chloride does not normally produce denaturation, however the high pH's used to elute fractions in the 1.0 M ammonia and 0.5 M NaOH regions can produce denaturation. Only those fractions held fairly firmly on the column appear to undergo denaturation, thus it is possible that the forces required to break the bonds holding the molecules on the column are also sufficient to produce denaturation.

These results also suggest that as the molecular weight of the sample is decreased the sample becomes more susceptible to denaturation during chromatography. The reason for this is difficult to understand in the light of the results described in Fig. (2-IV). These show that the reduction in molecular weight produced by sonicating a sample of DNA for five minutes has very little effect on the T_m of the DNA. A decrease in the T_m is only evident when the molecular weight has been reduced to the order of 3×10^5, as in the twelve minute sonicate.

vii) Heterogeneity in Chromatographic Fractions

Evidence that the position in the chromatogram, at which a molecule is eluted, depends on the size of the molecule, is given in Figs. (8-IV), (9-IV) and (10-IV). Differential sedimentation coefficient distribution curves, described by Schumaker and Schachman,[49] were calculated for various samples of calf thymus DNA and fractions obtained from the chromatographic fractionation of each of these DNA's on Ecteola. Fractions eluted with eluents of increasing ionic strength or increasing pH show a progressive increase in the sedimentation coefficient at the 50% concentration point in the sedimenting boundary ($s_{50\%}$). The spread of sedimentation coefficient distributions is very narrow for early fractions but broadens considerably for later fractions.

Fig. (8-IV) shows the sedimentation coefficient distribution for various fractions of native DNA. Curve a is the distribution obtained with unfractionated DNA, while curves b, c and d represent the distribution of sedimentation coefficients of the molecules in fractions eluted by sodium chloride, 0.1 M ammonia and 1.0 M ammonia. The sedimentation coefficient distributions of the one minute sonicate are shown in Fig. (9-IV) and those of the five minute sonicate in Fig. (10-IV), the curves represent the distributions of the unfractionated and fractionated samples.

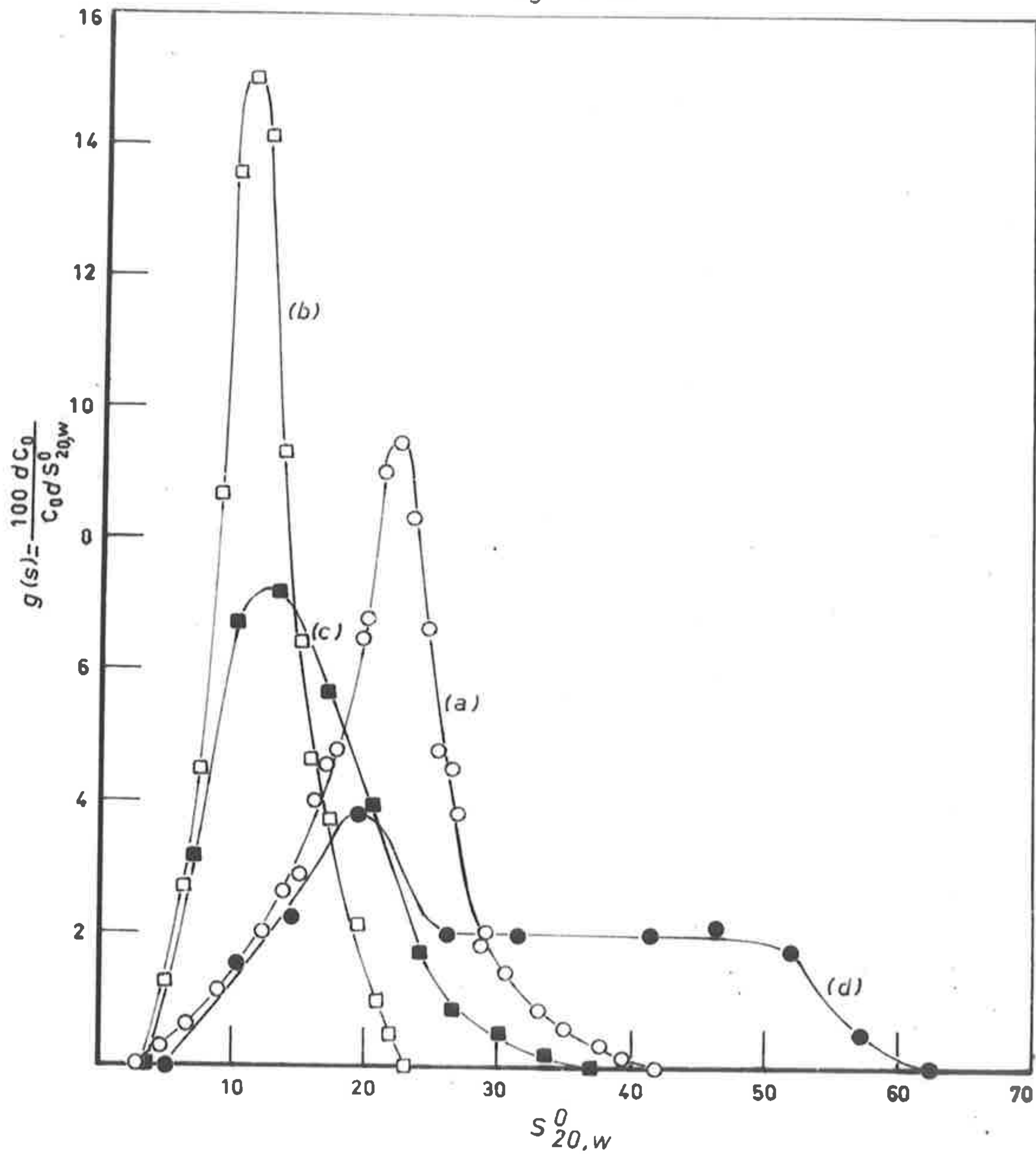


Fig. (8-IV). Sedimentation coefficient distribution for native calf thymus DNA and its fractions. Curve a, unfractionated; Curve b, 0.5M NaCl fraction 20; Curve c, 0.1M NH_3/2M NaCl fraction 6; Curve d, 1.0M NH_3/2M NaCl fraction 27.

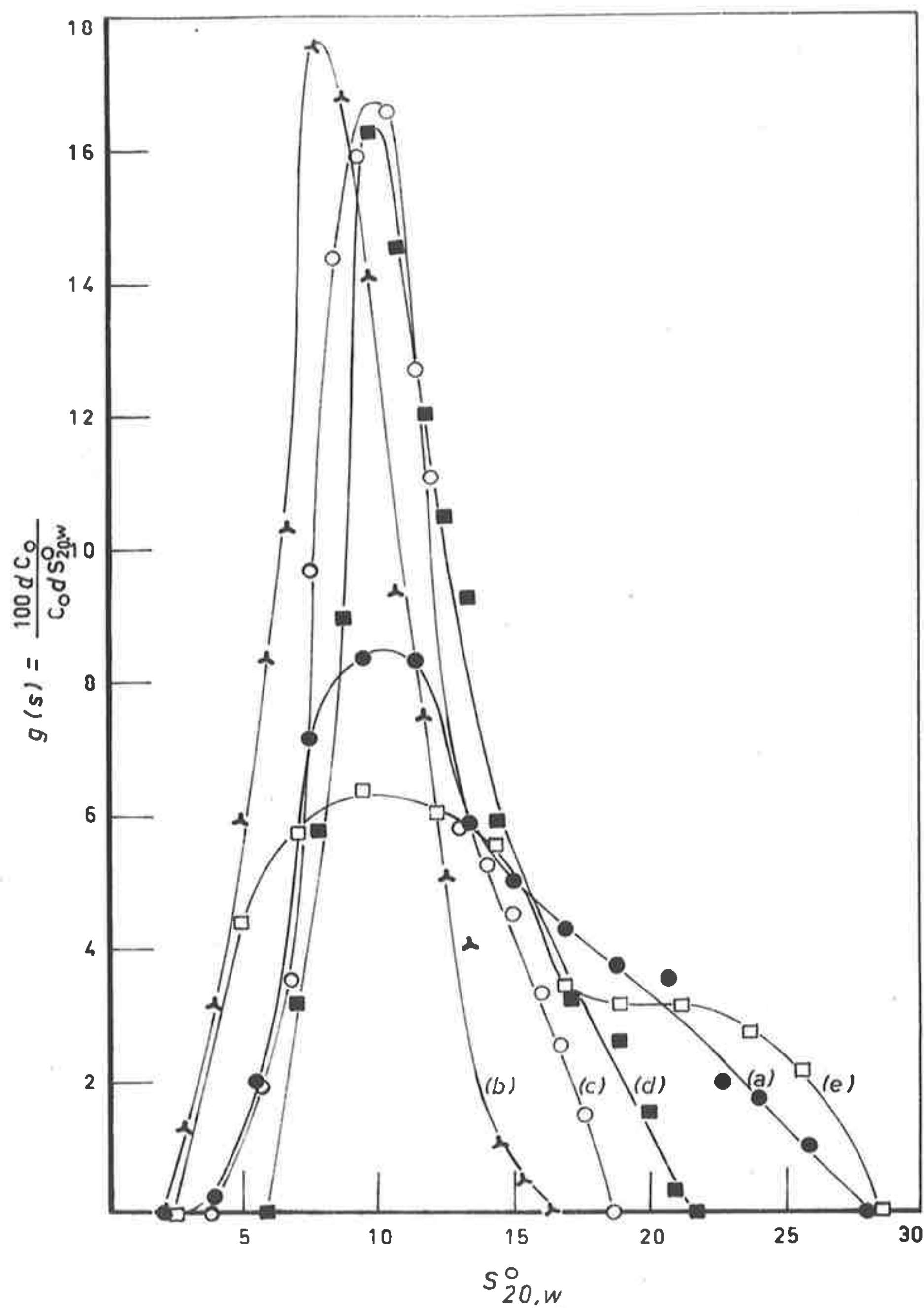


Fig. (9-IV). Sedimentation coefficient distribution for calf thymus one minute sonicate DNA and its fractions.
Curve a, unfractionated; Curve b, 0.5M NaCl fraction 19; Curve c, 1.0M NaCl fraction 8; Curve d, 1.0M NaCl fraction 9; Curve e, 1.0M NH_3/2M NaCl fraction 18

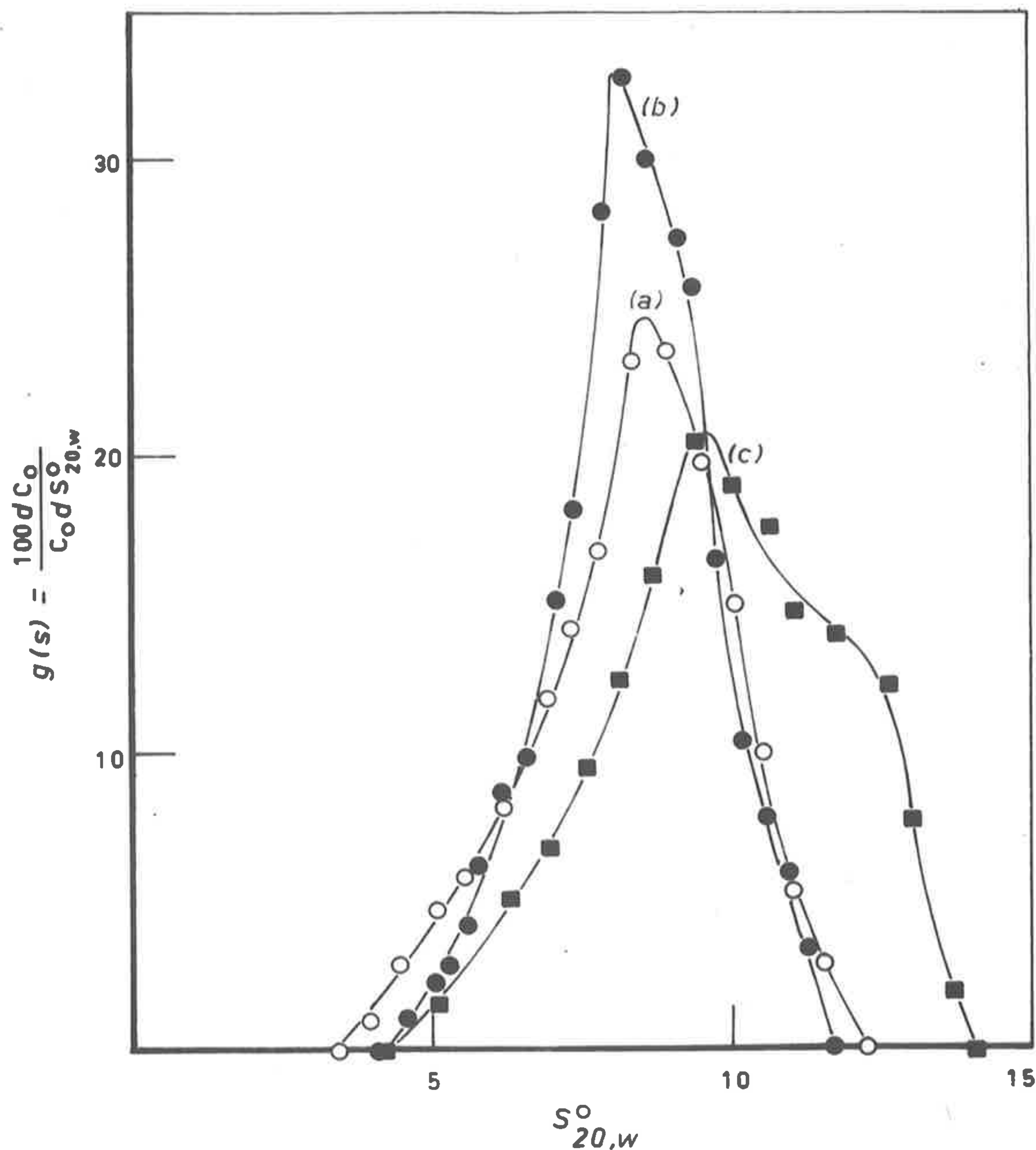


Fig. (10-IV). Sedimentation coefficient distribution for calf thymus 5 minute sonicate DNA and its fractions.
Curve a, unfractionated; Curve b, 1.0M NaCl fraction 11; Curve c, 1.0M NH_3/2M NaCl fraction 21.

From these results it can be concluded that the weaker the eluent used to remove a fraction from the exchanger, the smaller is the sedimentation coefficient of the molecules that are present in the highest proportion in that fraction, and the narrower is the distribution of the sedimentation coefficients of the molecules in the fraction. It is not clear why the later fractions, which contain the larger molecules, should also contain a high proportion of the smaller molecules.

These results are similar to those obtained by Bendich and Rosenkranz[27,45] only the method of presentation is slightly different. Instead of the differential boundary analysis, these workers used an integral method. Bendich and Rosenkranz found it necessary to combine fractions in order to obtain sufficient DNA for sedimentation analysis, whereas, since concentrations of approximately 0.002% DNA were used for this analysis, individual fractions could be used in this study. Thus the fractions represented in Figs. (8-IV), (9-IV) and (10-IV) are much narrower than those discussed by Bendich and Rosenkranz. The distribution of sedimentation coefficients for unfractionated DNA was shown by these authors to be unaltered by exposing DNA to the solvents used during elution. Thus it was concluded that the different distributions found for the various fractions was not a result of exposure to the different eluents. It

was also demonstrated that the molecules in the fractions, having these distributions, pre-existed in the original DNA and were not artifacts arising from the chromatographic process. This was done by calculating the sedimentation coefficient distribution curve that would have been obtained had the fractions been mixed together in the proportion of their occurrence in the original DNA and comparing this reconstructed curve with that of the unfractionated DNA.

Fig. (8-IV) curve e, shows, in agreement with the results of Bendich and Rosenkranz, that some of the later fractions contain molecules having larger sedimentation coefficients than appear to be present in the original sample. Bendich suggested that this was due to the fact that the total amount of DNA, with values of the sedimentation coefficient (s) in this range, is so small that it could not be detected in the original, unfractionated DNA. In drawing this conclusion Bendich was influenced by his earlier postulate that the molecules in latter fractions were not in any way affected by the fractionating process. Evidence discussed above suggests that this may be incorrect and that these fractions may be to some extent denatured. If this is the case then non-specific bonding can give rise to some form of aggregates which will have a reduced hydrodynamic volume and, as previously shown, this will cause the sedimentation coefficient of the molecules to increase. Thus the presence

of these high sedimentation coefficient species in some of the fractions may be due to a combination of both these effects. Some doubt is thrown on this latter explanation by the fact that several of the fractions which to some extent appear to be denatured (Fig. 7-IV), do not apparently contain molecules with these high sedimentation coefficients. This can be clearly seen in the case of the 1.0 M ammonia fraction of the one minute sonicate. However, this does not exclude this mechanism as the distribution for this 1.0 M ammonia fraction (Fig. 9-IV) does suggest the presence of two distinct species, as do the 1.0 M ammonia fractions of the native sample (Fig. 8-IV) and the five minute sonicate (Fig. 10-IV), and the 1.0 M sodium chloride fraction of the one minute sonicate. This distribution was not included in Fig. (9-IV) but is compared with the 0.5 M sodium chloride fraction in Fig. (11-IV).

The major inference to be drawn from these boundary analysis experiments is that there is a direct correlation between fraction position and the size of the molecules (as measured by their sedimentation coefficients) and the heterogeneity of the molecules in the fraction. As the eluent conditions become stronger so the average size of the molecules in the fraction and the amount of heterogeneity of the molecules increases. From these results, together with those obtained on fractionating

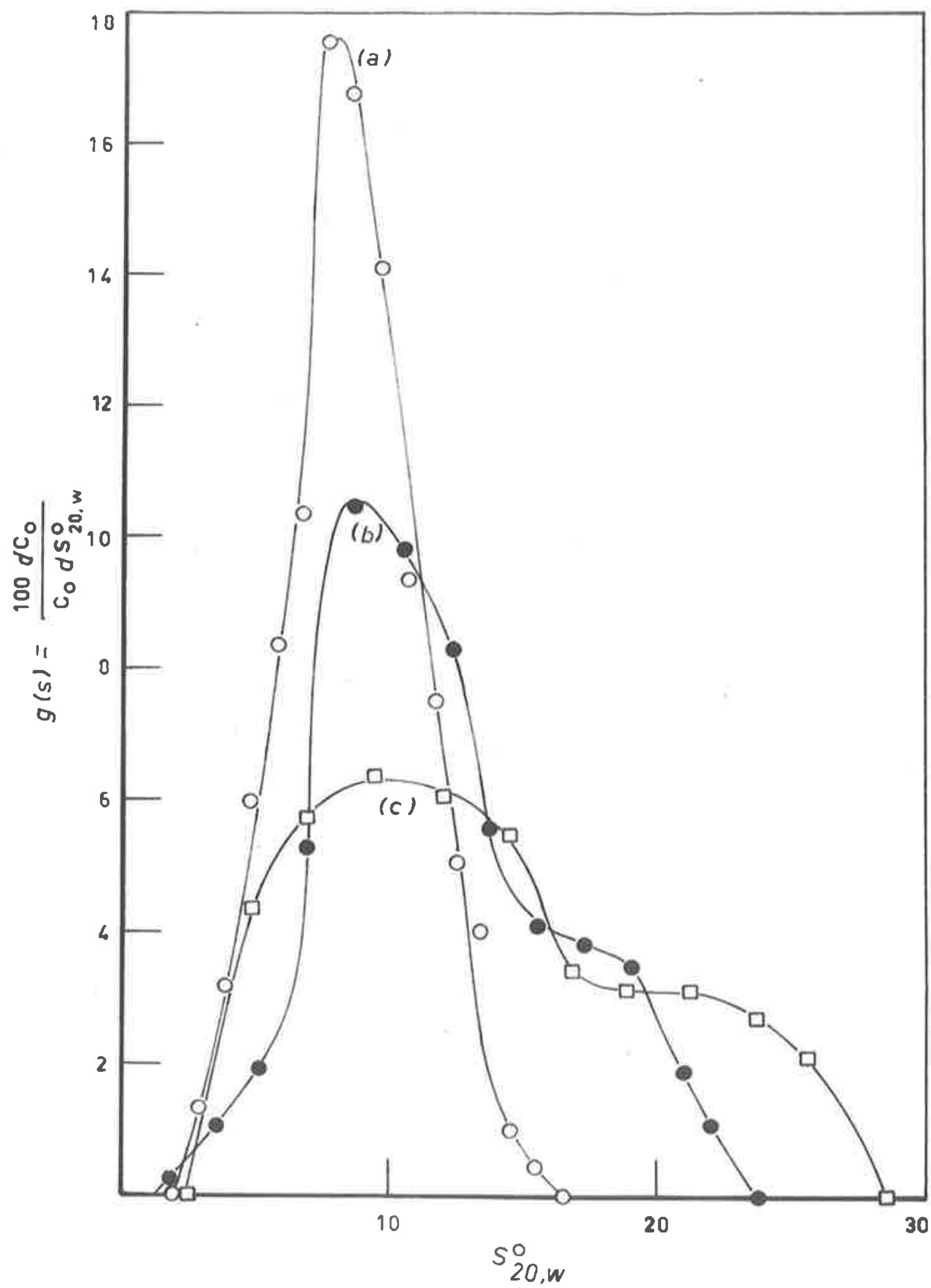


Fig. (11-IV). Sedimentation coefficient distribution for fractions of calf thymus one minute sonicate DNA.

Curve a, 0.5M NaCl fraction 19; Curve b, 1.0M NaCl fraction 16; Curve c, 1.0M NH_3/2M NaCl fraction 18.

sonically degraded DNA, it can be concluded that it is the physical heterogeneity of a sample of DNA that determines its behaviour on an ion-exchange column of Ecteola-cellulose and not its chemical heterogeneity.

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Chapter V

Chromatography and Denaturation of DNA

i) Changes in DNA produced by thermal treatment

ii) Previous results obtained on fractionating thermally treated DNA

iii) Separation of the effect of denaturation from that of degradation

iv) The effect of reducing the molecular size of a DNA sample

v) Conclusions

i) Changes in DNA produced by thermal treatment

The effect of heat on the physical properties of DNA solutions has been discussed in chapters II and III. The following conclusions can be drawn as to the configuration of the DNA molecules after different treatments. When a sample of DNA is heated in salt solution for short periods of time at temperatures above its T_m (i.e. of the order of 100°C) the molecules will be denatured. This denaturation will result in the separation of the polynucleotide chains. On slow cooling bacterial DNA these strands will recombine and form a 'native-like' structure. If the bacterial sample of DNA is cooled quickly, at a concentration greater than $20 \mu g/ml$, the two strands will bond non-specifically, producing loosely coiled aggregates of DNA. Loosely coiled aggregates of DNA are also produced when mammalian DNA is quick, or slow, cooled.

If the sample is heated for a long time at 100°C these results will be complicated by the presence of degradation. Doty et al.[1,2] have shown that heating DNA in 0.2 M sodium chloride at 100°C for sufficiently long times and heating DNA at lower temperatures, 84°C for E.coli DNA, in hundredth molar phosphate produces a marked decrease in molecular weight.

Solution of DNA in water, at a concentration of DNA below its critical value, has been shown also (Chapter

III) to result in the separation of the polynucleotide chains. Equilibrium density gradient measurements have shown (Fig. 6-III), for calf thymus DNA, that the addition of sodium chloride to this solution results in the formation of some 'native-like' material and some other species which are thought to be loosely coiled. A similar treatment with B.megatherium DNA produces only the 'native-like' material. Heating and cooling a sample of calf thymus DNA which has been treated in this manner should therefore result in the formation of loosely coiled aggregates.

Dekker and Schachman[3-5] have shown that heating an 0.005% solution of DNA in water for fifteen minutes at 100°C reduces the sedimentation coefficient from s = 20 to s = 6, which they interpret as indicating a large decrease in molecular weight. Butler et al.[6,7] also found that on heating DNA in water extensive degradation occurred and the sedimentation coefficient dropped to a value between 4 and 6.5 s. The sedimentation results of Bendich and Rosenkranz[8] show a sharp fall on heating DNA both in water and in 0.01 M phosphate which is indicative of degradation having occurred in both cases. That the degradation effects are greater for solutions of DNA in water, or at low ionic strength, is probably associated with the protective effect salt has been shown to have on thermal denaturation.[7,9-12]

ii) Previous results obtained on fractionating thermally treated DNA

Information, having direct bearing on the relationship between molecular size and the position of a fraction in the chromatogram, has arisen from fractionation studies on heated solutions of DNA. Bendich et al.[8,13] fractionated calf thymus DNA heated in water and in 0.01 M phosphate on Ecteola-cellulose and compared the resulting chromatograms to that obtained with native, untreated DNA, interpreting the changes in the chromatograms in terms of the molecular weight change resulting from thermal degradation. It was found that on heating DNA in water most of the DNA originally eluted in the last two regions was eluted with a sodium chloride gradient, the dilute ammonia peak appearing to remain unchanged. Since two of the sodium chloride fractions had $s_{50\%}$ values of 1.8 s and 3.8 s it was concluded that the fractions eluted in this first region had very low molecular weights.

Heating in salt produced a slightly different result. Most of the DNA originally eluted in the third (1.0 M ammonia/2 M sodium chloride) region was transferred into the second region along with the DNA originally in the first region. The first of these steps was explained as the result of degradation and the second as the result of aggregation. The latter interpretation was based on the results of Doty and Rice,[14] who reported an increase

in the sedimentation coefficient on heating DNA in salt. These authors have since amended this result[9] and report no change in the sedimentation coefficient on heating. However, as has been shown in chapter II, increasing the ionic strength of a solution of DNA not only has a protective effect against heat denaturation but on denaturation aids the formation of aggregates due to non-specific hydrogen bonding if the concentration of DNA is fairly high. Since Bendich and Rosenkranz used a concentration of 0.1% DNA it is possible that aggregation, as well as degradation, did accompany the thermal denaturation of this sample of DNA.

iii) Separation of the effect of denaturation from that of degradation

In order to elucidate what is occurring during the chromatography of heat denatured DNA the effect of denaturation on the fractionation process, uncomplicated by the effect of degradation, was studied. This can be achieved by using the denaturing process studied by Inman and Jordan[15-18] and discussed in chapter II. This process involves the denaturation of DNA by dissolving it in water below a critical DNA concentration. Cavalieri et al.[19] found that this process was unaccompanied by a molecular weight change. However, as mentioned above if the denaturing conditions are not altered the process will result in the separation of the polynucleotide chains. If the conditions are changed, in that the ionic strength is

raised above its critical value, it was found that a certain amount of helix reformation does take place (Chapter III). Thus a sample of DNA, which has been dissolved in water below its critical concentration and then placed on an ion exchange column equilibrated with 0.01 M phosphate (pH 7.0), can be pictured as a mixture of molecules. Some of these molecules will have long double helical regions in them and others will consist of loosely coiled, non-specifically bonded polynucleotide chains. As the bonding of the molecules to the column does depend largely on the number of phosphate groups free to interact with the column, as shown in the previous chapter, fractionating this sample of DNA should result in a shift of the chromatographic profile towards weaker eluting conditions. This is to be expected as the loosely coiled regions will result in the shielding of some of the phosphate groups from the column and so bring about a slight decrease in the strength with which these molecules are bound to the exchanger. If the process of placing the DNA on the ion-exchange column brought about an increase in the DNA concentration this would result in more of the molecules consisting of loosely coiled polynucleotide chains. This would result in a slightly greater shift of the chromatographic profile to regions of weaker eluting conditions.

The result of fractionating a sample of DNA

dissolved at a concentration of 0.003% in water, which is below the critical concentration for calf thymus DNA, on Ecteola-cellulose is shown in Fig. (1-V). It can be seen by comparison with the chromatogram for native DNA (Fig. 4-IV) and from Table (1-V) that there is a shift of the chromatographic profile to regions of weaker eluting conditions as expected. This shift can be compared with that resulting from fractionating the one minute sonicate, with two notable exceptions. Firstly there has only been a slight change in the peak eluted with sodium hydroxide from that for native DNA and secondly instead of obtaining a peak with the 1.0 M sodium chloride gradient (region three), as in the case of the one minute sonicate, two peaks are obtained on eluting with a gradient of dilute ammonia (region four).

A sample of DNA that had been thermally denatured and degraded was then fractionated and the resultant chromatogram compared with that obtained above. In order to obtain the maximum possible amount of degradation a sample of DNA dissolved in water at a concentration of 0.1% was heated to 100°C for one hour and quenched quickly. This sample was then made 0.01 M in phosphate (pH 7.0) in order to produce the same amount of non-specific hydrogen bonding and hence the same amount of possible aggregation as achieved by Bendich and Rosenkranz[8,13] when they heated calf thymus

Fig (1 - V)

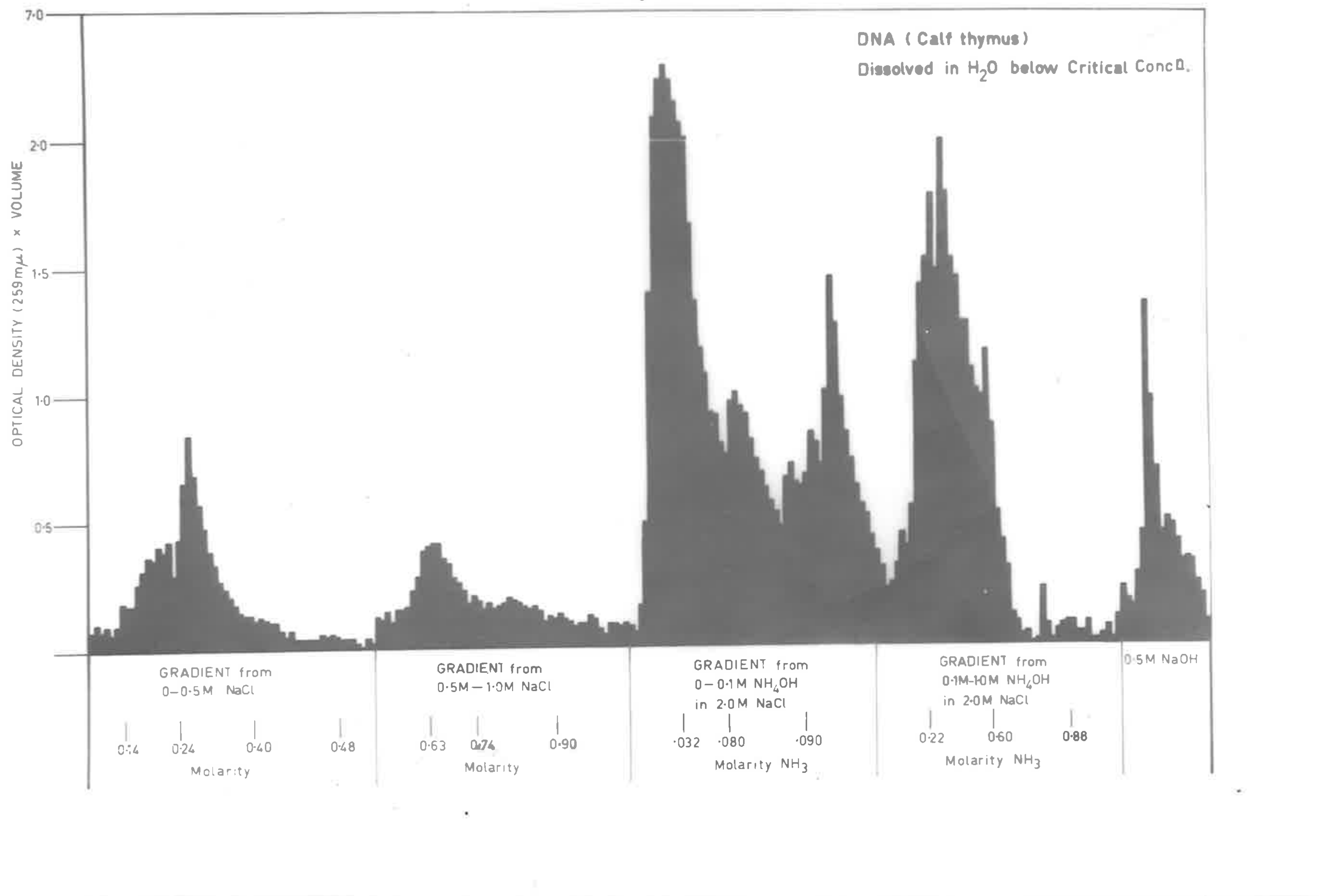


Table (1-V)

DNA Sample	Percentage recovery in different regions					
	Region 1 phosphate buffer	Region 2 0.5M NaCl	Region 3 1.0M NaCl	Region 4 0.1M NH_3/2M NaCl	Region 5 1.0M NH_3/2M NaCl	Region 6 0.5M NaOH
Native[a]	-	2.0	-	26.7	61.2	10.2
5 Minute sonicate[a]	1.0	15.5	54.1	18.5	8.5	2.3
Native diluted in water	-	8.8	-	44.3 and 13.7	26.2	7.0
Native heated in water	-	←30.2→		63.1	4.1	2.5
5 Minute sonicate diluted in water	0.9	11.2	40.4	23.6	9.9 and 5.1	8.9
5 Minute sonicate heated in buffer	7.2	19.9	-	50.0 and 5.6	8.9	8.4

[a] Reproduced from Table (1-IV).

DNA in 0.01 M phosphate. The result of this treatment was to reduce the molecular weight, as measured by the Flory-Mandelkern[20,21] equation, to a value of 3.0×10^5 (Table 2-V), which on fractionation gave rise to the chromatogram shown in Fig. (2-V). If this chromatogram is compared with the chromatograms obtained by Bendich et al.,[8,13] for DNA heated in water and for DNA heated in 0.01 M phosphate, it can be seen that Fig. (2-V) incorporates the changes found in both of these chromatograms. Namely on heating in water Bendich et al. found that the peak normally eluted with the gradient to 1.0 M ammonia and 2 M sodium chloride had disappeared and on heating in 0.01 M phosphate the peak normally eluted with the gradient to 0.5 M sodium chloride was found to have disappeared. Fig. (2-V) shows that the peaks normally eluted in regions five and six (1.0 M ammonia/2 M sodium chloride and 0.5 M sodium hydroxide) have disappeared and that very little DNA is eluted in the second region (0.5 M sodium chloride). In fact the only significant peak is that eluted by dilute ammonia, although as can be seen from Table (1-V) the broad peak stretching over regions two and three contains 30% of the total DNA eluted from the column.

It is of interest to note (Table 2-V) that the molecular weight of this sample of heated DNA is less than that of the five minute sonicate (8.8×10^5) (If a value of 2.0 is used for β in the Flory-Mandelkern equation for the

Fig (2 -V)

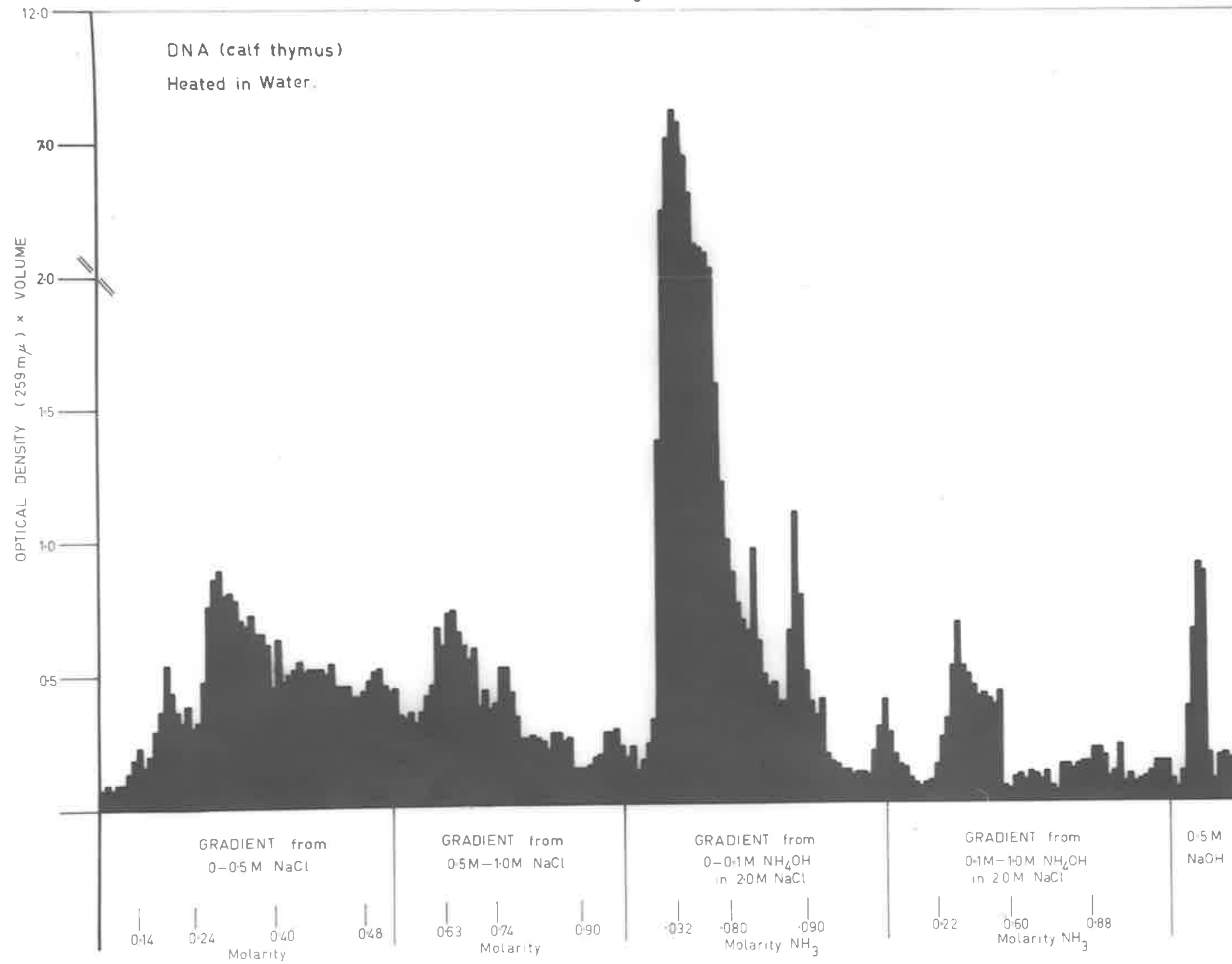


Table (2-V)

DNA Sample *	Molecular weight from F-M equation[20,21]
Native	12.6×10^6
One minute sonicate	3.1×10^6
Five minute sonicate	8.8×10^5
Twelve minute sonicate	3.1×10^5
Native heated for 1 hr. at 100°C in water and then made 0.01 M in phosphate	3.0×10^5
0.1 M ammonia fraction of the heated native sample	1.5×10^5
Five minute sonicate sample diluted in water below its critical concentration	1.0×10^6
1.0 M sodium chloride fraction of the diluted five minute sonicate	9.2×10^5
Five minute sonicate sample heated for 1 hr. at 100°C in 0.01 M phosphate	8.5×10^5
0.1 M ammonia fraction of the heated five minute sonicate	5.6×10^5

* All samples made 0.2 M in sodium chloride before sedimentation coefficient and intrinsic viscosity measurements were made.

heated sample the molecular weight only increases to 4.5 x 10^5.) and yet the shift of the chromatographic profile to weaker eluting conditions is far more noticeable in the case of the five minute sonicate. The experiments discussed in chapter IV showed a definite correlation between molecular weight and the position of the general chromatographic pattern. Thus it would appear that although a large proportion of the original DNA has undergone a decrease in molecular weight on heating, some of the smaller molecules have undergone aggregation, probably as a result of inter-molecular non-specific hydrogen bonding as suggested above. It has been shown (Table 2-V) that the weight average molecular weight for the whole sample has been decreased to approximately a third of that for the original native DNA on heating, which is in agreement with the results of Shooter et al.[22] and Doty et al.[2] It should be remembered that this value represents a weight average of molecular weights of all species present in the thermally degraded sample, and considerable variation between fractions may be expected. For example the molecular weight of a sample comprising some of the 0.1 M ammonia/ 2 M sodium chloride fractions (numbers 8-13) is a half that of the unfractionated material (Table 2-V). This sample comprises about 30% of the total DNA eluted from the column and 30% of the remaining DNA will have a lower molecular

weight than this sample as it is eluted by weaker eluents (sodium chloride). Thus most of the remaining 40% must have a molecular weight much greater than the average for the unfractionated DNA and could be aggregates of low molecular weight material.

It can be concluded from these experiments that thermal denaturation results in a shift to weaker eluting conditions as a result of the accompanying degradation. A shift of part of the chromatographic profile to stronger eluting conditions also occurs on thermal denaturation. This is thought to occur due to the aggregation of low molecular weight material.

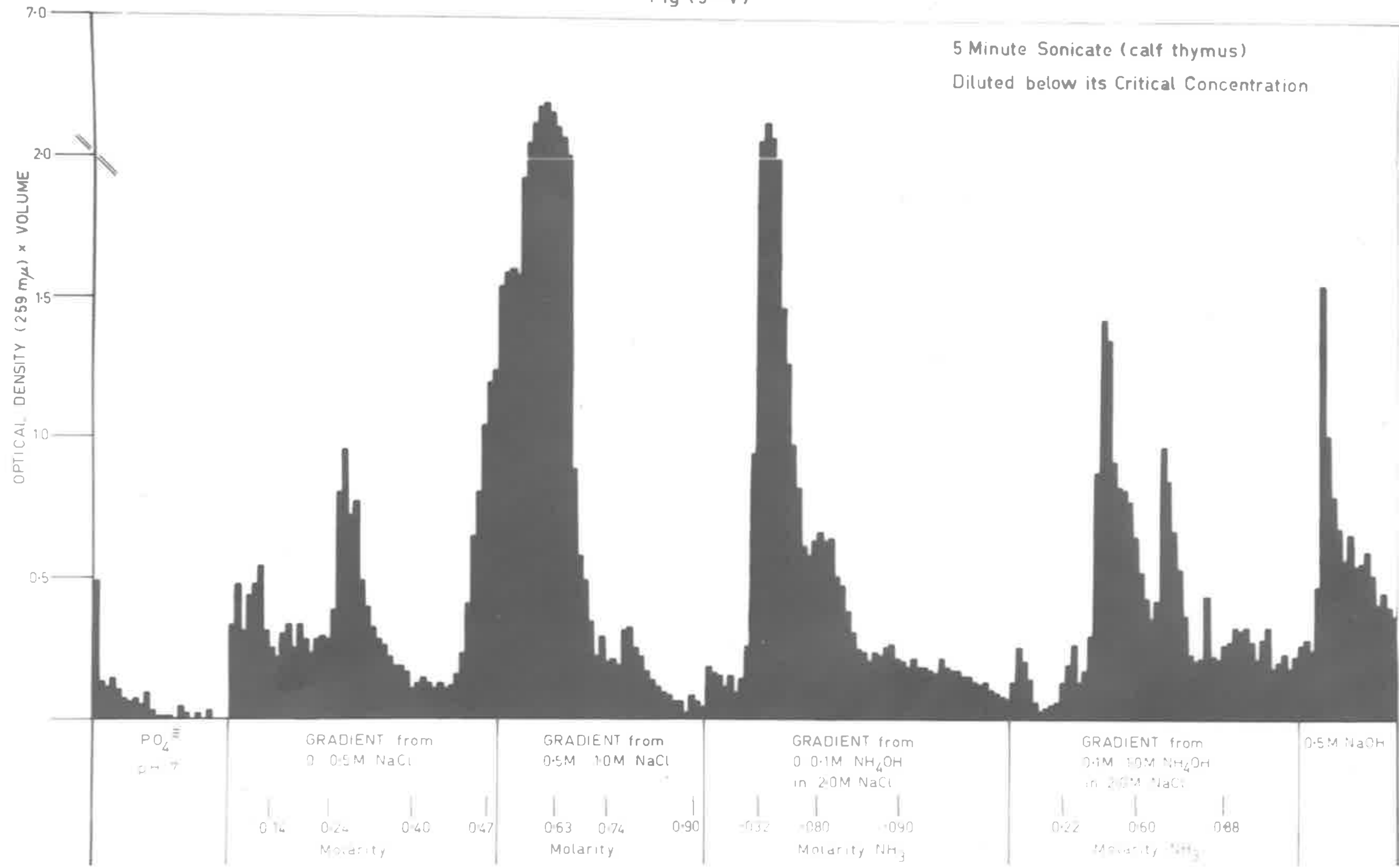
iv) The effect of reducing the molecular size of a DNA sample

It was shown above that dilution denaturation, possibly accompanied by renaturation, results in a slight shift of the chromatographic profile to weaker eluting conditions when a sample treated in this manner is fractionated on Ecteola-cellulose. If aggregation of low molecular weight material does occur during denaturation, then dilution denaturation of a sample of DNA, the molecular weight of which has been decreased, should result in a shift of the chromatographic profile to stronger eluting conditions on fractionation. Therefore an aliquot of a DNA sample that had been sonicated for five minutes was diluted below its critical concentration in water and then fractionated.

The resultant chromatogram (Fig. 3-V) can then be compared with that obtained on fractionating the five minute sonicate (Fig. 6-IV). It can be seen from this comparison that denaturation by dilution below the critical concentration again produces only a little change from the chromatogram obtained by fractionating the original DNA. Both chromatograms show that some DNA is eluted with phosphate buffer. There is slightly less DNA eluted in the sodium chloride regions in the denatured sample and slightly more DNA eluted by the pH gradients (regions four, five and six). Thus denaturation of this low molecular weight sample has given rise to a slight shift of the elution profile to regions of stronger eluting conditions, whereas denaturation of native DNA produced a slight shift to weaker eluting conditions. This suggests that a slight amount of aggregation is possibly occurring in this sample.

The suggestion, supported by the above evidence, that the aggregation which follows denaturation occurs with those molecules which are originally of low molecular weight, can be investigated further by heating a sample of the DNA that has been reduced in size by sonication. If the suggestion is correct we would expect to find much more aggregation in this case than is found on heating native DNA. In order to test this a further aliquot of the sample of DNA sonicated for five minutes was taken and heated to 100°C for one hour in 0.01 M phosphate (pH 7.0).

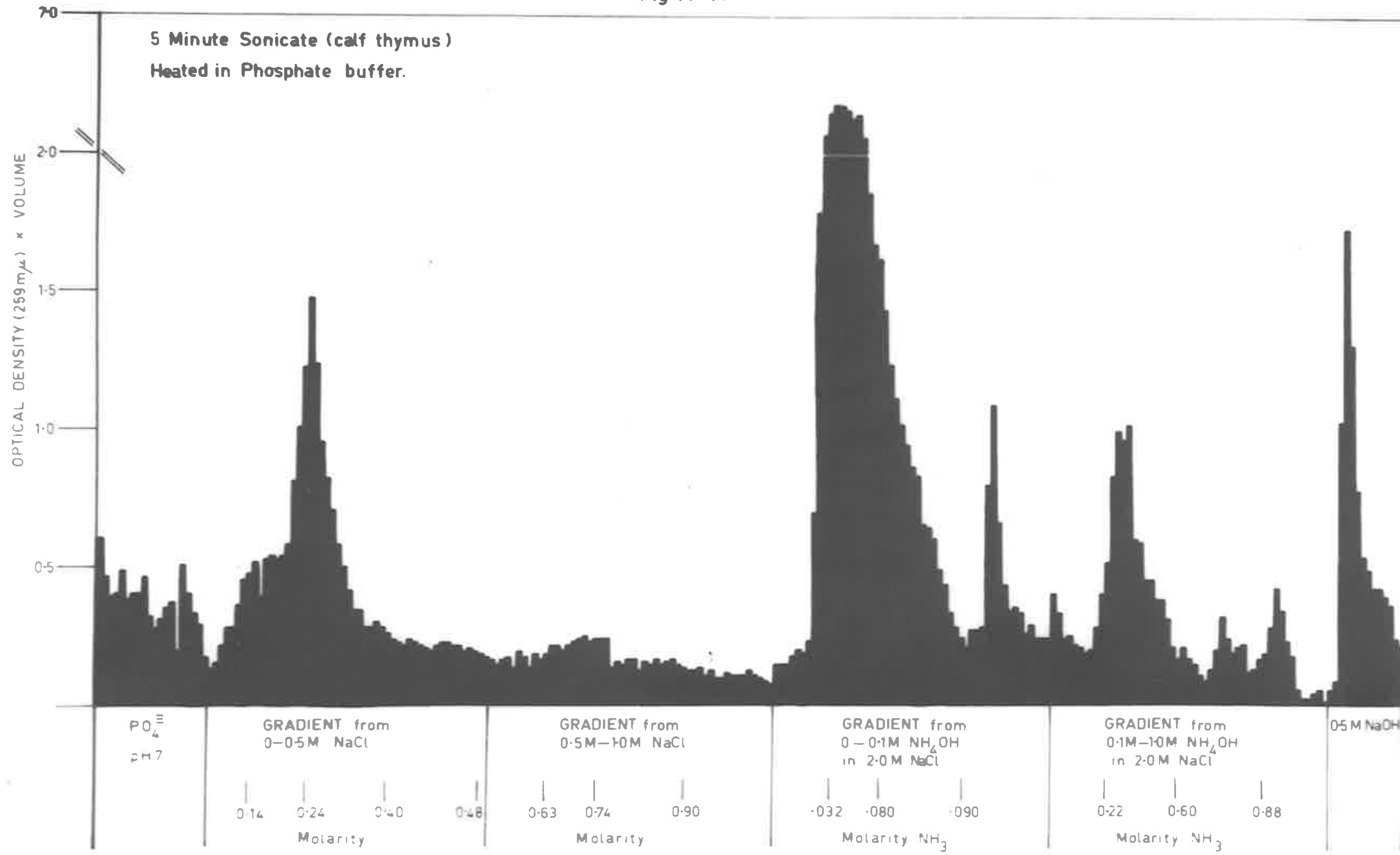
Fig (3 - V)



The result of this treatment on the chromatographic behaviour of the DNA can be seen in Fig. (4-V) and Table (1-V). The amount of DNA eluted in regions one and two indicates that this sample contains molecules of lower molecular weight than any other sample that has been fractionated. However, it can be seen from Table (2-V) that this sample has a weight average molecular weight in excess of that of the heated native sample and essentially the same as that of the five minute sonicate, which indicates that this sample has undergone aggregation to a large extent. This is borne out by comparing this chromatogram with that obtained on fractionating the five minute sonicate. As can be seen from Figs. (4-V) and (6-IV) the peak eluted by 1.0 M sodium chloride (region three), in the chromatogram of the five minute sonicate, is eluted by dilute ammonia (in region four) when the heated sample is fractionated and no DNA is eluted in region three with this sample. The molecular weight of some of the fractions from region four (numbers 11-16), shown in Table (2-V), although lower than the value of the unfractionated sample, is higher than that of the unfractionated, heat denatured, native sample. This suggests that all the material eluted from this region upwards consists of aggregated molecules.

It is interesting to note the resemblance between the chromatogram of this heat denatured sample (Fig. 4-V) and that for the sample of native DNA which has been thermally

Fig (4 -V)



denatured (Fig. 2-V). The former does differ in that there is slightly more DNA eluted by very weak and very strong eluents. Since sonication has been shown (Chapter IV) to produce a shift in the chromatographic profile to weaker eluting conditions, the presence of more species eluted by strong eluents in the chromatogram of the heated sonicate sample than in the chromatogram of the heated native sample indicates that it is the low molecular weight molecules which undergo aggregation. The molecules eluted by the very weak eluents have probably been degraded to such an extent that the number of phosphate groups holding them on the column is so few that even free phosphate groups can compete with some of them for adsorption sites on the resin.

The chromatography results indicate that the heated five minute sonicate has undergone more aggregation than the diluted five minute sonicate. However, this would not be deduced from the molecular weight results for the two samples (Table 2-V), which suggest that the diluted sample has undergone more aggregation than the heated sample as its molecular weight is higher than that of the five minute sonicate and the heated sample. Table (2-V) shows that there is very little difference between the molecular weight of the unfractionated, diluted five minute sonicate sample and the 1.0 M sodium chloride fraction of this sample. This result, coupled with the presence of molecules in the heated five

minute sonicate sample which are fractionated under very weak eluent conditions and therefore are presumably of very low molecular weight, suggests that the amount of heterogeneity in these samples differs significantly. It would appear that the heated sample was far more heterogeneous, especially in that it contained much more low molecular weight DNA than the diluted sample. That this is the case is shown by the sedimentation distribution measurements made on the two samples (Fig. 5-V). Thus it can be concluded that the heated five minute sonicate sample has undergone more aggregation than the diluted five minute sonicate sample, as suggested by the chromatographic results. The low weight average molecular weight being due to the presence of more low molecular weight material as well as more aggregated molecules.

v) Conclusions

It has been shown that solely denaturing calf thymus DNA by dissolving it in water below its critical concentration does not give rise to aggregation. This is concluded as the chromatogram obtained on fractionating this sample, when compared to that obtained on fractionating native DNA, shows that only a slight shift of the profile towards weaker eluting conditions has occurred. This shift is indicative of the presence of some loosely coiled molecules in agreement with the findings in chapter III.

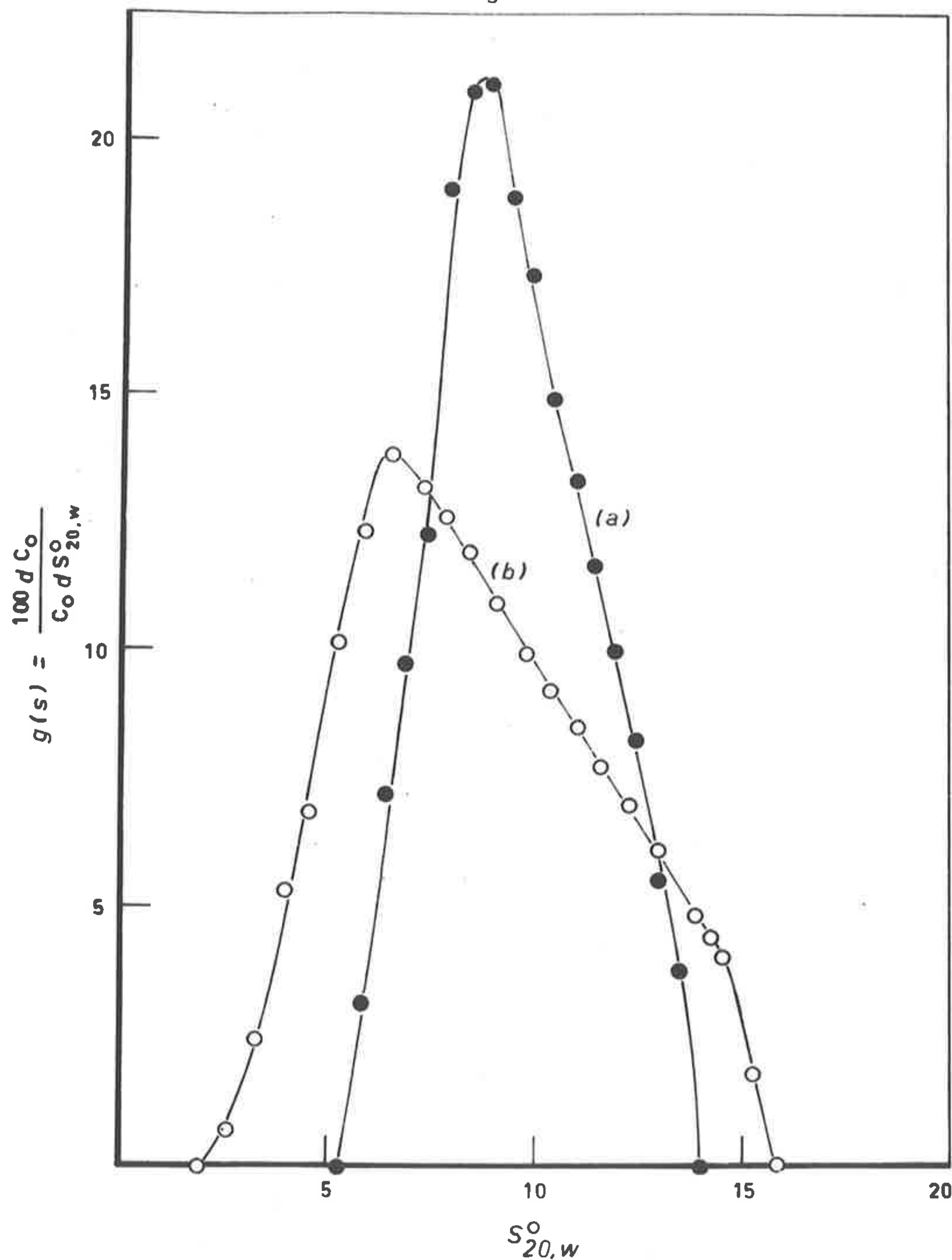


Fig. (5-V). Sedimentation coefficient distribution for calf thymus DNA. Curve a, 5 minute sonicate diluted below its critical concentration; Curve b, 5 minute sonicate heated in phosphate buffer.

Denaturing and degrading a sample of calf thymus DNA by heating it to 100°C in water and then making the solution 0.01 M in phosphate does appear to produce aggregation. Fractionating this sample produces a chromatogram which has no material eluted either by strong or by very weak eluents. This is thought to correspond to a reduction in molecular weight accompanied by aggregation of the low molecular weight species. This is consistent with the result for the five minute sonicate, which has a higher molecular weight than this sample, and which, when fractionated, shows a much greater shift of the chromatographic profile to weaker eluting conditions, compared with that of native DNA.

If an aliquot of this five minute sonicated sample is denatured by diluting it in water, below its critical concentration, a slight shift of the chromatographic profile to stronger eluting conditions occurs, compared with that of the five minute sonicate. This shift is also thought to be due to aggregation which occurs as the size of the molecules has been decreased. Chromatography and molecular weight results for an aliquot of this five minute sonicate, which has been thermally denatured and degraded, indicate that an appreciable amount of aggregation has occurred. The amount of heterogeneity in this sample has also increased due to the presence of very low molecular weight species as shown by chromatography and sedimentation

distribution experiments.

In the previous chapter it was found that more denaturation was produced by the chromatographic process at lower molecular weights. Sedimentation distribution results indicated that this denaturation resulted in aggregation. Thus it would appear from these results, together with those discussed above, that low molecular weight material is more susceptible to denaturation, and aggregation resulting from this denaturation.

The lack of aggregation as a result of dilution denaturation of native DNA, as indicated by chromatography, suggests that dilution denaturation followed by an increase in ionic strength is far less likely to produce aggregation than is thermal denaturation followed by quick cooling. This agrees with the findings in chapter III.

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Chapter VI

The Validity of Molecular Weight and Heterogeneity Measurements

A) Molecular weight from sedimentation and viscosity measurements

- i) Introduction
- ii) Mandelkern-Flory equation
- iii) Scheraga-Mandelkern equation
- iv) Comparison of $M_{\eta,s}$ with M_L for DNA

B) Measurement of heterogeneity in samples of DNA

- i) Heterogeneity in sedimentation coefficients of DNA
- ii) Boundary analysis
  - a) Definition of g(s)
  - b) Application to ultra-violet absorption optics with DNA
- iii) Estimation of the relative degree of heterogeneity in DNA samples

A) Molecular weight from sedimentation and viscosity measurements

i) Introduction

During the study of the chromatographic properties of DNA, discussed in chapters IV and V, it was necessary to measure both the molecular weight of the various samples and, in some cases, the relative degree of heterogeneity. In this chapter the methods used to find these values will be discussed.

There are three methods available for the measurement of the molecular weight of DNA. It can be determined from the Svedberg equation,[1] which involves a combination of sedimentation velocity measurements and diffusion measurements. Sedimentation velocity measurements can also be combined with viscosity measurements to give molecular weights from the Flory-Mandelkern equation.[2,3] The third method makes use of light scattering measurements.

The first of these methods can be discarded since a reliable value for the diffusion coefficient of DNA has not been obtained. Coates[4] has shown that the practical difficulties associated with forming a stable boundary, due to the high viscosity of DNA, makes an accurate determination of this coefficient impossible. The remainder of this section will therefore be devoted to a discussion of the validity of the two remaining methods when applied to DNA.

ii) Mandelkern-Flory equation

Flory et al.[5-12] have shown that the frictional coefficient (f_o) of a hydrodynamic sphere, equivalent to a long chain molecule, in terms of its viscosity is given by the equation

$$f_o = P\phi^{-1/3}(M[\eta])^{1/3}\eta_o \qquad (1\text{-VI})$$

where P and ϕ are universal constants, M is the molecular weight of the polymer, $[\eta]$ is the intrinsic viscosity of the polymer solution and η_o is the viscosity of the medium. An equation for the frictional coefficient of the equivalent hydrodynamic sphere, at infinite dilution, has also been obtained in terms of the sedimentation coefficient (s^o), measured at infinite dilution.[1] Namely

$$f_o = \frac{M(1 - \bar{v}\rho)}{Ns^o} \qquad (2\text{-VI})$$

where N is Avogadro's number, $\bar{v}$ is the partial specific volume of the solute and ρ is the density of the solution. Although equation (2-VI) was derived for a binary system, it has been shown to hold approximately for a three component system,[13-15] the type generally used when making sedimentation measurements.

Mandelkern and Flory[2,3] have shown that by combining these two equations, an equation is obtained which enables the molecular weight of a polymer species to be calculated from intrinsic viscosity and sedimentation data, at infinite dilution ($M_{\eta,s}$). The equation obtained by

substituting for f_o from equation (2-VI) in equation (1-VI) is

$$M = \left(\frac{s^o [\eta]^{1/3} \eta_o N}{\beta (1 - \bar{v}\rho)} \right)^{3/2} \qquad (3\text{-VI})$$

where $\beta = P^{-1} \Phi^{1/3}$ and should, according to the above theories, be a universal constant the same for all randomly coiled polymers.

Mandelkern et al.[3] when summarizing the results of several workers[16-19] found that the value of β for several polymers in various solvents ranged from 2.31 to 2.75×10^6. Mandelkern and Flory reported two calculated values for β.[2] One is obtained from Einstein's equation for the viscosity of a solution of spheres and Stokes' law for the frictional coefficient of a sphere, assuming that the two equivalent spheres are identical, and gives a value of 2.1×10^6. The other, calculated from the hydrodynamic theories of Kirkwood and Riseman,[20] yields a value of 3.0×10^6. The experimental average value of 2.55 ± 0.2 lies between these two values.

The value of Φ given by the second of these theoretical calculations is 3.6×10^{21}, which Mandelkern and Flory[2] suggest to be in error since when the value of Φ found experimentally[8] (2.1×10^{21}) is combined with the calculated value of P (5.1) a value of 2.5×10^6 is obtained for β, which is the average of the experimental values. However, Flory et al.[21] then found that the experimental

value of Φ , given above, was in error and when corrected for heterogeneity in the polymer sample gave a value of approximately 2.5×10^{21}. Krigbaum and Carpenter[22] collected the results of various authors[21,23-25] which showed that Φ was not a constant as predicted by Flory and Fox but varied to a slight extent depending on the nature of the polymer-solvent interaction. These results showed that Φ could vary from about 2.0×10^{21} to 2.8×10^{21}, which could indicate a slight discrepancy in the derivation of equation (2-VI).

iii) Scheraga-Mandelkern equation

The same equation as that of Mandelkern and Flory has been derived by Scheraga and Mandelkern for globular proteins.[26] This derivation differs in that, although they have used essentially the same model, they have assumed that the viscosity and friction coefficient depend on a shape factor as well as the apparent volume of the model.

Thus they represented a molecule by an equivalent hydrodynamic ellipsoid of axial ratio p. The size and shape of this effective ellipsoid are defined as those which allow the experimental hydrodynamic observations to be treated by the hydrodynamic equations of Simha[27] and Perrin.[28,29] for rigid ellipsoids of revolution, even though the actual molecular configuration may not be a rigid one. That is the size and shape of this rigid ellipsoid are such as will take into account possible

flexibility of the molecule and permeation of the solvent. This procedure is analogous to the use of the effective hydrodynamic sphere by Fox and Flory for flexible polymers in consideration of the Einstein-Stokes relations for spheres.

This derivation results in β of equation (3-VI) being of the form

$$\beta = \gamma F \nu^{1/3} \qquad (4\text{-VI})$$

where γ is a constant, ν is a shape factor which depends on the axial ratio p as does F, a function defined by Perrin,[28,29] and p = b/a where a is the semi-axis of revolution and b is the semi-equatorial axis of the equivalent ellipsoid.

Whereas the Mandelkern-Flory equation predicts that $\Phi^{1/3}P^{-1}$ should be a universal constant, which agrees well with experimental measurements, this derivation predicts that β should depend solely on p. This dependence has been calculated from the functions of Simha[27] and Perrin[28,29] and the results show that as p varies from 1 to 300, β only changes from 2.12 to 2.15 x 10^6 and as p varies from 1 to 1/300 β varies from 2.12 to 3.60 x 10^6. Thus for oblate ellipsoids β is essentially independent of the axial ratio whereas for prolate ellipsoids it varies as the axial ratio changes.

iv) <u>Comparison of $M_{\eta,s}$ with M_L for DNA</u>

The value of β used in calculating molecular

weights for DNA will depend on the model that is chosen to represent the DNA molecule. Doty et al.[30-32] have shown, on the basis of light scattering experiments, that DNA molecules have a highly extended, gently coiled configuration. The persistence length for DNA was found to be of the order of 500 A, indicating a very low degree of coiling.[31] Butler et al.[33] and Doty et al.[32,34,35] calculated molecular weights of DNA assuming a β value of 2.6×10^6, which is the value found for flexible synthetic polymers and the highly extended cellulose nitrate molecules which have a persistence length of 117 A.

It has been shown that molecular weights derived from equation (3-VI) using this value of β agree with those obtained from light scattering measurements up to values of 4 to 5×10^6.[33] However it was found[31-35] that above these values light scattering molecular weights (M_L) were much lower than those obtained from viscosity and sedimentation results ($M_{\eta,s}$). This could mean that the value of β used in the equation was too small, however, Butler et al.[33] have pointed out that values of β as high as 6×10^6 would be required to eliminate the difference. The difference is thus either due to a failure of the equivalent hydrodynamic sphere or ellipsoid model to fit the DNA molecule in solution, or to an underestimation of the molecular weight due to the failure of the light scattering theory to hold for the higher molecular weights.

Doty[31] suggests that the former is the case, and this appears to be borne out by the reported relations found by Doty et al.[34] between molecular weight and viscosity, namely

$$[\eta] = 1.45 \times 10^{-6} M^{1.12} \qquad (5\text{-VI})$$

Fox and Flory[5,8] predict that the maximum possible value of the exponent of M in this relation is 0.80, therefore it appears that the model on which they based their derivation does not apply to solutions of DNA. The molecular weights used to calculate this relation were M_L values not $M_{\eta,s}$ values, which are possibly higher. If the value of $M_{\eta,s}$ calculated from the results of Doty et al.[34] for their untreated sample of calf thymus DNA is put into the above empirical equation, relating intrinsic viscosity to molecular weight, the viscosity is found to be proportional to $M^{1.09}$. Although this is far greater than the maximum value of the exponent predicted by Fox and Flory[8] it is only just greater than the maximum value of 1.00 predicted by Debye and Bueche[36] and Kirkwood and Riseman.[20] The slope obtained from a plot of log $[\eta]$ vs. log $M_{\eta,s}$ (Fig. 1-VI) for the samples used in the chromatographic studies of this work was found to be 0.96. The results of Butler et al.[33] are also plotted in Fig. (1-VI) and a line of slope 1.00 is drawn through them, and the least squares slope for this set of data was found to be 0.97. Peacocke and Preston[37] and Schachman[38] have found that the intrinsic

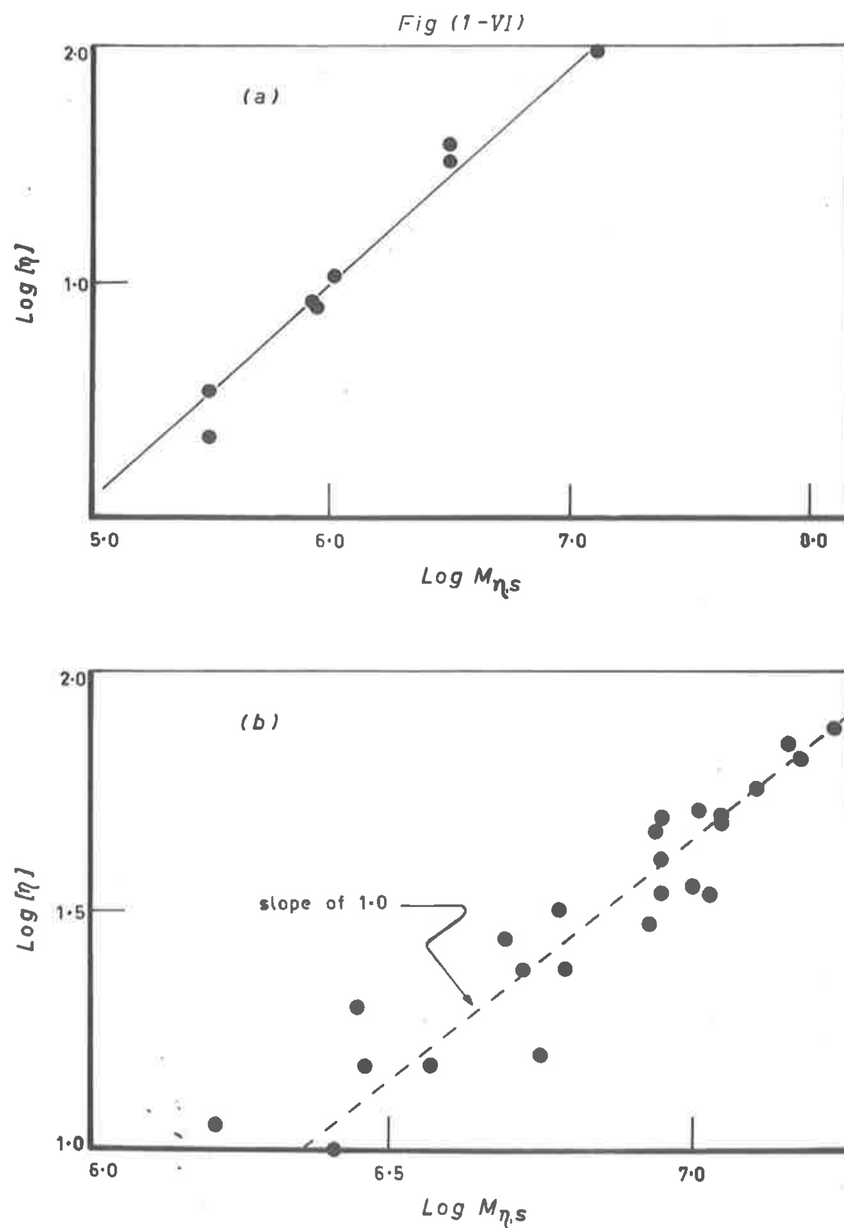


Fig. (1-VI). Plots of log molecular weight versus log intrinsic viscosity.

(a) For samples of calf thymus DNA used in the chromatographic studies presented in this work. Least square slope = 0.96.

(b) From the results presented by Butler et al. (ref. 33). Least square slope = 0.97.

viscosity was proportional to $M^{1.00}$.

Thus the relation predicted by Fox and Flory between the viscosity and the molecular weight of coiled polymers does not hold for DNA. This suggests that the dependence of $\bar{\alpha}$ on molecular weight derived by them is incorrect, one of the assumptions employed in its derivation not applying to DNA solutions. It may be that the model employed is incorrect, as suggested by the variation in the values of Φ reported by Krigbaum and Carpenter,[22] but this is not thought to be so since equation (3-VI) does appear to apply to DNA solutions as shown by the following results.

Butler et al.[33] and Eigner and Doty[39] have shown that

$$s^{o} \propto [\eta]^{1/3}$$

which together with the relation

$$[\eta] \propto M$$

shown above, satisfies the equation, as from these relations the following relation can be deduced

$$s^{o} [\eta]^{1/3} \propto M^{2/3}$$

which is the relation derived in equation (3-VI). Butler et al.[33] and Schachman[38] have also shown that the other relation resulting from this equation, namely $s^{o} \propto M^{1/3}$ holds fairly well for calf thymus DNA.

The value of 2.6×10^{6} assumed for β when using this equation for DNA has been shown by Scheraga and Mandelkern[26] to correspond to an axial ratio of 0.02 for a prolate

ellipsoid. This means that F is approximately proportional to $p^{2/3}$. Now Simha et al.[40] have calculated the dependence of ν on p and have shown it to be approximately of the form

$$\nu = kp^{-1.8} \qquad (6\text{-VI})$$

when $p \simeq 0.02$. Hence $\beta = \gamma F \nu^{1/3}$ is approximately proportional to $p^{0.06}$, that is β is effectively independent of axial ratio provided it is greater than, or equal to, 0.02. This agrees well with the predictions and findings of Mandelkern et al.[2,3] Butler et al.[33] have also reached this conclusion by calculating β from the equations of Kirkwood and Riseman[20] and Peterlin.[41]

It would appear from these considerations that in calculating $M_{\eta,s}$ the same value of β may be used for all samples. Since M_L and $M_{\eta,s}$ show reasonable agreement when M_L is less than 5×10^6 it would seem that the value of β used (2.6×10^6) is substantially correct. Rice and Geiduschek[42] have shown that the effect of heterogeneity on the molecular weight obtained from viscosity and sedimentation measurements must be to make it less than the weight average molecular weight (M_w). Butler et al.[33] have estimated that $M_{\eta,s}$ will be less than M_w by a factor of 0.9, hence this method will possibly underestimate the molecular weight by approximately 10%.

When $M_{\eta,s}$ is greater than 5×10^6, light

scattering results give a lower molecular weight as previously mentioned, in fact M_L appears to reach a constant value in the region 5 to 6 x 10^6 while $M_{\eta,s}$ continues to increase. Butler et al.[33] examined the equations of Debye for a random coil and for a rod-like molecule and concluded that the light scattering intensity in the range of angles available for measurement should reach a limiting constant value at high molecular weight. It has been shown also, by considering two possible transitions between a coil and a rod, namely Peterlin's stiffened coil and the zig-zag coil, that overestimates of the intercept at $\theta = 0$ would be obtained in either case if the molecules had a high degree of stiffness. However, the values of the dissymmetry observed are difficult to reconcile with the view that sufficient stiffness exists to produce this effect, unless these values are anomalously high.

It has been suggested above that the value of $M_{\eta,s}$ is approximately 10% below the true value of the weight average molecular weight for a DNA sample. It follows therefore that the light scattering method must be underestimating the molecular weight when $M_{\eta,s}$ is greater than 5 x 10^6. That this is possibly so can be seen by examining the extrapolation procedure used to obtain the molecular weight from the scattering envelope. The Rayleigh ratio can be expressed in the following form

$$\frac{K_\theta c}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2 c + \cdots \qquad (7\text{-VI})$$

where R_θ is the Rayleigh ratio, which is a measure of the angular dependence of the scattered-light intensity, c is the total concentration, K_θ is a constant for the particular solvent-polymer system and $P(\theta)$ is the ratio of the Rayleigh ratios for large and small molecules and can be written in the form

$$P(\theta) = \frac{2}{r^2u^2}(e^{-ru} - 1 + ru) \qquad (8\text{-VI})$$

where $u = \left\{(4\pi/\lambda)\sin(\theta/2)\right\}^2 b^2/6$ and r is the number of statistical elements in the chain of length b. If u is small, i.e. θ is small, equation (8-VI) can be expanded and $P(\theta)^{-1}$ can be written in the form

$$P(\theta)^{-1} = 1 + \frac{1}{3}ru + \frac{1}{36}r^2u^2 \qquad (9\text{-VI})$$

hence at c = 0

$$\frac{K_\theta c}{R_\theta} = \frac{1}{M_w}(1 + \frac{1}{3}r_z u + \cdots) \qquad (10\text{-VI})$$

The graph of this equation approximates very closely to a straight line and at $\theta = 0$ the equation becomes

$$\frac{K_\theta c}{R_\theta} = \frac{1}{M_w} \qquad (11\text{-VI})$$

Whereas if u is large, i.e. θ is large, e^{-ru} tends to zero and $P(\theta)$ tends to the value $2/ru - 2/r^2u^2$. Hence

equation (7-VI) becomes at c = 0

$$\frac{K_\Theta c}{R_\Theta} = \frac{1}{M_n}\left(\tfrac{1}{2}\, r_n u + \tfrac{1}{2} + \cdots\right) \qquad (12\text{-VI})$$

where M_n is the number average molecular weight. The graph of this equation will also approximate to a straight line and at $\Theta = 0$ the equation becomes

$$\frac{K_\Theta c}{R_\Theta} = \frac{1}{2M_n} \qquad (13\text{-VI})$$

Thus if $K_\Theta c/R_\Theta$, for c = 0, is plotted against $\sin^2\Theta/2$ the curve shown in Fig. (2-VI) can be expected.[43]

Doty et al.[30,32,44] have shown that the scattering envelope is approximately a straight line down to the lowest angle measured ($\Theta = 30^\circ$). Doty[31] points out that in order to be able to extrapolate this graph to $1/M_w$, measurements would have to be made down to 10° in order to overcome the effects due to heterogeneity. However practical difficulties prevent measurements being made below an angle of 30°. This problem was overcome by assuming a random distribution of molecular weights,[31] such that $M_w : M_n = 2:1$, which allowed them to ignore the change of slope at low angles as $1/2M_n$ equals $1/M_w$. Extrapolation of this straight line therefore gave them M_w. Doty[31] reports that a value of 8.0×10^6 was obtained for calf thymus using this method, whereas viscosity and sedimentation data yielded a value of 12.4×10^6. If a distribution of the

Fig (2-VI)

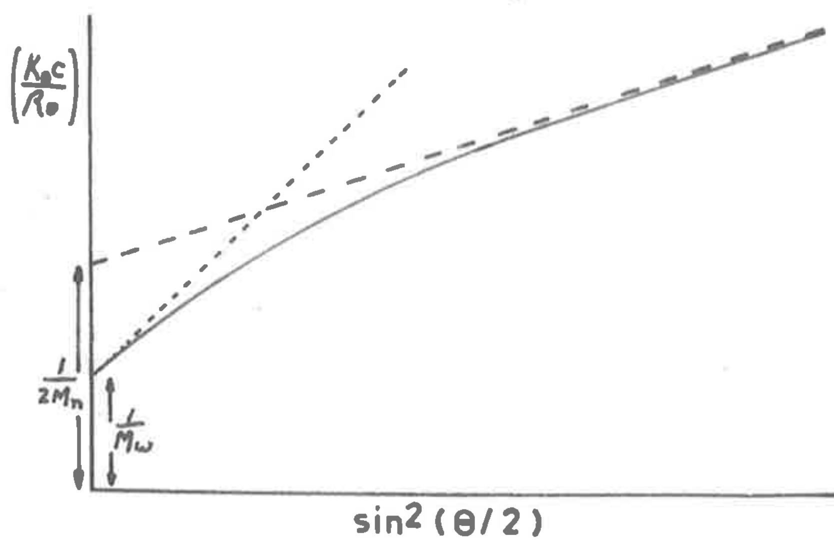


Fig. (2-VI). Schematic plot of $K_\theta c/R_\theta$ for $c = 0$ versus $\sin^2(\theta/2)$ showing quantities obtainable from such a plot. From Benoit, Holtzer and Doty (ref. 43).

form $M_w:M_n$ = 3:1 had been assumed, taking the value of 8×10^6 to be M_n would have given a value of 12.0×10^6 for M_w. Thus if they had assumed a slightly broader distribution of molecular weights the values of M_L and $M_{\eta,s}$ would have agreed very well. That this would have been a reasonable assumption is shown by the results of Schachman[38] who reported just such a distribution of molecular weights for a sample of calf thymus DNA. The reported agreement of the two methods for low molecular weight DNA[33] can be explained if there is a change in the type of molecular weight distribution for these samples. The low molecular weight material used by Butler et al.[33] was prepared by irradiating native DNA which is likely to result in a more random distribution of molecular weights. This has been shown to be so by Peacocke and Preston[37] and Charlesby.[45]

Benoît et al.[46] have recently obtained light scattering results at very low angles, the scattering of DNA solutions being measured at angles as low as 16°. When molecular weights obtained from these measurements were compared with those obtained from measurements made between 30 and 150° it was found[46] that both methods resulted in the same molecular weight, up to values of 6×10^6. However, at values greater than this the measurements made in the usual angular region ($30-150^o$) were found to give results which underestimated the molecular weight.[46]

It is therefore concluded that molecular weights derived from intrinsic viscosity and sedimentation coeff-icient measurements, at infinite dilution, for undenatured samples of DNA are within 10% of the correct weight average value; and the value of β used in the Mandelkern-Flory or Scheraga-Mandelkern equation for these samples should be 2.6×10^6. Denatured DNA was referred to by Laurence[47] as an almost solid coil and has been shown by Rice and Doty[32] to have a shape resembling a flexible coil, similar to that of ordinary polyelectrolytes. Thus denatured DNA should also obey this equation. The value of β for such a molecule should lie somewhere between 2.1 and 2.6×10^6. On the basis of the Mandelkern-Flory equation a value of 2.6×10^6, the value found for coiled polymers like poly-styrene, is expected as they predict that β should be a universal constant. The equivalent ellipsoid model of Scheraga and Mandelkern however, predicts a value less than 2.6×10^6 since the axial ratio should be less than that of native DNA, but the value should be greater than 2.1×10^6, the value β would have if the axial ratio were one.

Thus using this equation it is possible to compare the molecular weights of native-like molecules and to compare those of denatured molecules. Since a decrease in β from 2.6 to 2.1×10^6 will not make a great difference in the molecular weight it is probably valid to compare the mol-ecular weights of native samples of DNA with those of

denatured samples.

B) Measurement of heterogeneity in samples of DNA

i) Heterogeneity of sedimentation coefficients in DNA

The adaption of ultraviolet absorption optics to ultracentrifuges enabled the sedimentation coefficient of molecules, such as DNA, which adsorb strongly in the region 259 $m\mu$ to be measured at very low concentrations. When DNA was centrifuged at concentrations of the order of 0.001% it was found that the resulting sedimentation boundary was very broad.[38,48-56] Shooter and Butler[50] carried out an extensive investigation in order to show that this broad boundary corresponded to a real heterogeneity in the DNA sample. In order to show that it was not an artifact due to density gradients in the cell, sedimentation coefficients at different concentration levels in the boundary were found by plotting log x/t for three different points in the boundary, where x is the distance of a point in the boundary from the centre of rotation at time t. It was found that the sedimentation coefficient did not vary from point to point and all three lines extrapolated back to the same point, showing that the boundary was infinitely sharp as it left the meniscus. The sedimentation coefficient distribution curves were calculated for various times during the experiment and these showed that the distribution did not change with time. Hence it was concluded that changes due to

convection and diffusion were negligible. It was also shown that the distribution did not change if the analysis was carried out on solutions that had been allowed to dissolve for different times, thus they concluded that the heterogeneity was not due to the presence of random aggregates resulting from incomplete dispersion of DNA.

This distribution of sedimentation coefficients may not necessarily mean a polydispersity in molecular weight, as it can correspond to a polydispersity in shape. For example a species may have low sedimentation coefficients because the molecules are more elongated and therefore experience a greater frictional resistance to motion in the centrifugal field. In an attempt to resolve this problem Schachman and co-workers[55] performed some fractionation experiments by zone centrifugation in sucrose gradients. From preliminary experiments they concluded that the slower material was of lower molecular weight since it was less viscous than the leading material. However as they found that the exponent in the equation relating viscosity to molecular weight was not a constant, but tended to decrease with increase in molecular weight, they felt that the polydispersity was possibly not exclusively due to a polydispersity in molecular weight.

Doty[56] reported a similar investigation in which DNA was fractionally separated in a swinging bucket rotor and the viscosity of the supernatant measured. They

found, in agreement with Schachman, that the intrinsic viscosity of the supernatant was lower than that of the original solution but it was not as low as expected. Thus it was again concluded that the sedimentation distribution was not entirely due to molecular weight polydispersity.

Despite the dependence of the sedimentation coefficient distribution of a sample of DNA on a slight polydispersity of shape, it is felt that the distribution sufficiently reflected a polydispersity in molecular weight to enable it to be used to give a measure of the relative amounts of molecular weight heterogeneity between different DNA samples, as used by Rosenkranz and Bendich.[57-59]

ii) Boundary analysis

(a) Definition of g(s)

Analysis of the boundary gradient curves observed in sedimentation velocity experiments is, in principle, capable of giving a distribution of the sedimentation coefficients of the molecular species present in terms of the proportion of each species in the sample.[60] In practice the determination of such a distribution is complicated by the necessity of using a sector-shaped cell, in which the cross-sectional area is proportional to the distance from the centre of rotation, x, which causes radical dilution and by the fact that the centrifugal field strength,

$\omega^2 x$, also depends on this distance. It is further complicated by the effects of diffusion and the concentration dependence of the sedimentation coefficient.

Baldwin et al.[60] have defined an apparent distribution function which is free from the first two of these effects. They represented the distribution on a refractive index basis by g(s), where

$$g(s) \equiv \frac{1}{\Delta n_o} \frac{d(\Delta n_s)}{ds} \qquad (14\text{-VI})$$

Δn_o is the total refractive increment and Δn_s is the refractive increment of material with sedimentation coefficient less than s. Thus the value of $g(s_1)$ is the relative frequency within a sample, on a refractive index basis, of material with sedimentation coefficient s_1.

(b) Application to ultraviolet absorption optics with DNA

The photodensitometer tracing obtained from ultraviolet absorption ultracentrifugal analysis gives a direct measure of the concentration of a species at different distances from the centre of rotation. Therefore g(s) is redefined in terms of the initial concentration of the DNA solution,

$$g(s) \equiv \frac{1}{c_o^{obs}} \frac{dc_o^{obs}}{ds^o_{20,w}} \qquad (15\text{-VI})$$

which is a normalized distribution function, where

c_o^{obs} is the observed concentration corrected for radial dilution[61] and $s^o_{20,w}$ is the sedimentation coefficient at infinite dilution, corrected to the value it would have in a solvent with the viscosity and density of water at 20°C.[1] If the effects of diffusion are negligible and the concentration dependence of the sedimentation coefficient can be ignored, and the molar absorption coefficient is the same for all molecules in the boundary, the relative frequency, on a weight basis,[62] of material having a sedimentation coefficient s can be obtained by a simple transformation of coordinates.[63] To plot the new curve the ordinate becomes

$$g(s) = \frac{\omega^2 xt}{c_o^{obs}} \left(\frac{\Delta c^{obs}}{\Delta x}\right) \left(\frac{x}{x_o}\right)^2 \qquad (16\text{-VI})$$

where x_o is the distance of the meniscus from the centre of rotation, Δc^{obs} is the change in concentration measured over the interval $x - \Delta x/2$ to $x + \Delta x/2$ and the value of $s_{20,w}$ corresponding to this value of g(s) is given by the expression

$$s_{20,w} = \frac{\ln x/x_o}{\omega^2 t} \left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho}\right) \left(\frac{\eta}{\eta_{20,w}}\right) \qquad (17\text{-VI})$$

It has been shown[50,54] that for DNA the diffusion effects are negligible. Shooter and Butler[50] have also shown that the distribution curve at infinite dilution differs little from that observed at 0.001%, while Schumaker

and Schachman[54] have reported that the agreement between the distributions calculated for runs at 0.001, 0.002 and 0.004% is excellent. Thus it is concluded that below 0.004% the sedimentation coefficients obtained are essentially those at infinite dilution. Therefore the above transformation does apply to the ultracentrifugal analysis of DNA solutions below a concentration of 0.004%.

If the distribution described by equation (15-VI) is normalized by summation and division, the following equation is obtained[54]

$$g(s_i) = \frac{\Delta c_o^{obs}/\Delta s^o_{20,w}}{\sum_i (\Delta c_o^{obs}/\Delta s^o_{20,w})_i (\Delta s^o_{20,w})_i}$$

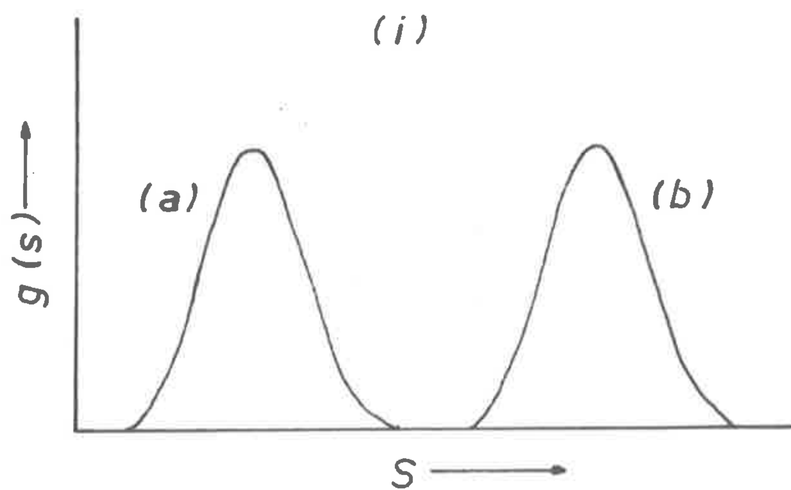
$$= \frac{\Delta c_o^{obs}/\Delta s^o_{20,w}}{\overline{\Delta s}^o_{20,w} \sum_i (\Delta c_o^{obs}/\Delta s^o_{20,w})_i} \qquad (18\text{-VI})$$

where $\overline{\Delta s}^o_{20,w}$ is the average of the largest and smallest values of $\Delta s^o_{20,w}$. If the transformation described by equation (16-VI) is then applied to this expression the following relation results

$$g(s_i) = \frac{(\Delta c_o^{obs}/\Delta x)(x/x_o)^3}{\overline{\Delta s}^o_{20,w} \sum_i (\Delta c^{obs}/\Delta x)_i \ (x/x_o)^3} \qquad (19\text{-VI})$$

The $s^o_{20,w}$ values corresponding to each $g(s_i)$ value being

Fig (3-VI)



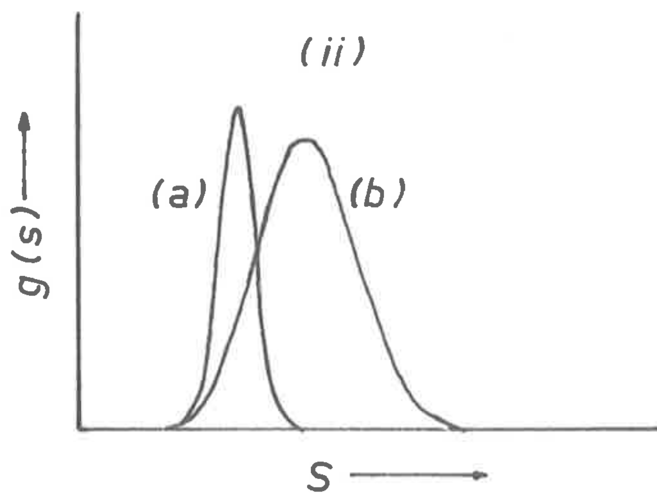


Fig. (3-VI). Schematic plots of sedimentation coefficient distributions.

calculated from equation (17-VI), using concentrations less than 0.004% for the analysis.

iii) Estimation of the relative degree of heterogeneity in DNA samples

The distribution obtained from equation (19-VI) represents the relative frequency, on a weight basis, of the molecules in the sedimenting boundary of a sample of DNA. From such distributions the relative amounts of heterogeneity in various samples of DNA can be determined. Heterogeneity is understood to mean the spread in the relative sizes of the molecules in a polydisperse sample. This is consistent with the practice of obtaining an estimate of heterogeneity from the ratio M_w/M_n, the larger this ratio the greater being the heterogeneity. Thus the standard deviation cannot generally be used to estimate the heterogeneity from sedimentation coefficient distributions, as the absolute spread in sedimentation coefficient values, for a given degree of heterogeneity, will be smaller the smaller is the average sedimentation coefficient of the sample. For example in Fig. (3-VI)(i) curves (a) and (b) both have the same standard deviation but the degree of heterogeneity is greater in the case of (a) than in (b), M_w/M_n being much nearer to 1 for curve (b) than it is for curve (a). Therefore some such function as the coefficient of variation (Standard deviation/Mean) which involves taking

into account the mean value would have to be used to compare distributions of this type.

The distributions obtained in chapters IV and V, with calf thymus DNA, have all approximately a common lower value, similar to the type of distributions shown in Fig. (3-VI)(ii). Although the mean of curve (b) is higher than that of curve (a) the smallest molecules present in the distribution represented by curve (a) are also present in the other distribution, represented by curve (b), and this distribution obviously also contains much larger molecules than are present in the distribution represented by curve (a). Therefore for distributions of this type, a comparison between two, or more, distributions made by drawing the distributions and inspecting them should give as valid an estimate of the relative amounts of heterogeneity present, as calculating the coefficient of variation. Thus the relative degree of heterogeneity, in the distributions presented in chapters IV and V, is estimated by inspection, as has been done for these two distributions.

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Chapter VII

Experimental

i) Preparation of samples

  a) Calf thymus DNA
  b) *B.megatherium* DNA

ii) Preparation of water

iii) Determination of concentrations

  a) Phosphorus analysis
  b) Direct determination

iv) Hydrodynamic measurements

  a) Viscosity
  b) Sedimentation velocity
  c) Sedimentation equilibrium in a density gradient

v) Denaturation techniques

  a) Measurement of thermal denaturation
  b) Cooling methods
  c) Slow and quick addition of sodium chloride

vi) Chromatography

  a) Apparatus
  b) Exchange resins
  c) Eluents

vii) Degradation

viii) Ultraviolet spectroscopic measurements

ix) Electron microscopy

i) Preparation of samples

a) Calf thymus DNA

This was prepared by the method of Kay, Simmons and Dounce[1] from 100 g of calf thymus gland, frozen approximately 3 minutes after removal from the animal. The method was modified slightly in that 0.0014M sodium chloride was used instead of water at all stages in the preparation and the final fibres were washed with increasing concentrations of ethanol and air dried. This yielded 1.8 g of DNA having a T_m of 87°C, an $\varepsilon(P)$ value of 6520 and a broad sedimentation coefficient distribution with a value for $s^o_{20,w}$ at the 50% concentration point of 22.4. The ultraviolet spectrum of this sample is shown in Fig. (1-VII).

b) B.megatherium DNA

The parent culture of B.megatherium (Univ. of Penn.) from which the cells were grown was a gift from Dr. J. Marmur of Brandeis University. The DNA was extracted from cells in the logarithmic growth phase using his procedure,[2] with the modification that the cells were left in the presence of lysozyme at 37°C for 24 hrs. in order to achieve a reasonable amount of lysis. 11 mg of DNA were obtained from 22 g. (wet weight) of freshly harvested B.megatherium cells, and this material had a T_m of 88°C, a narrow sedimentation coefficient distribution

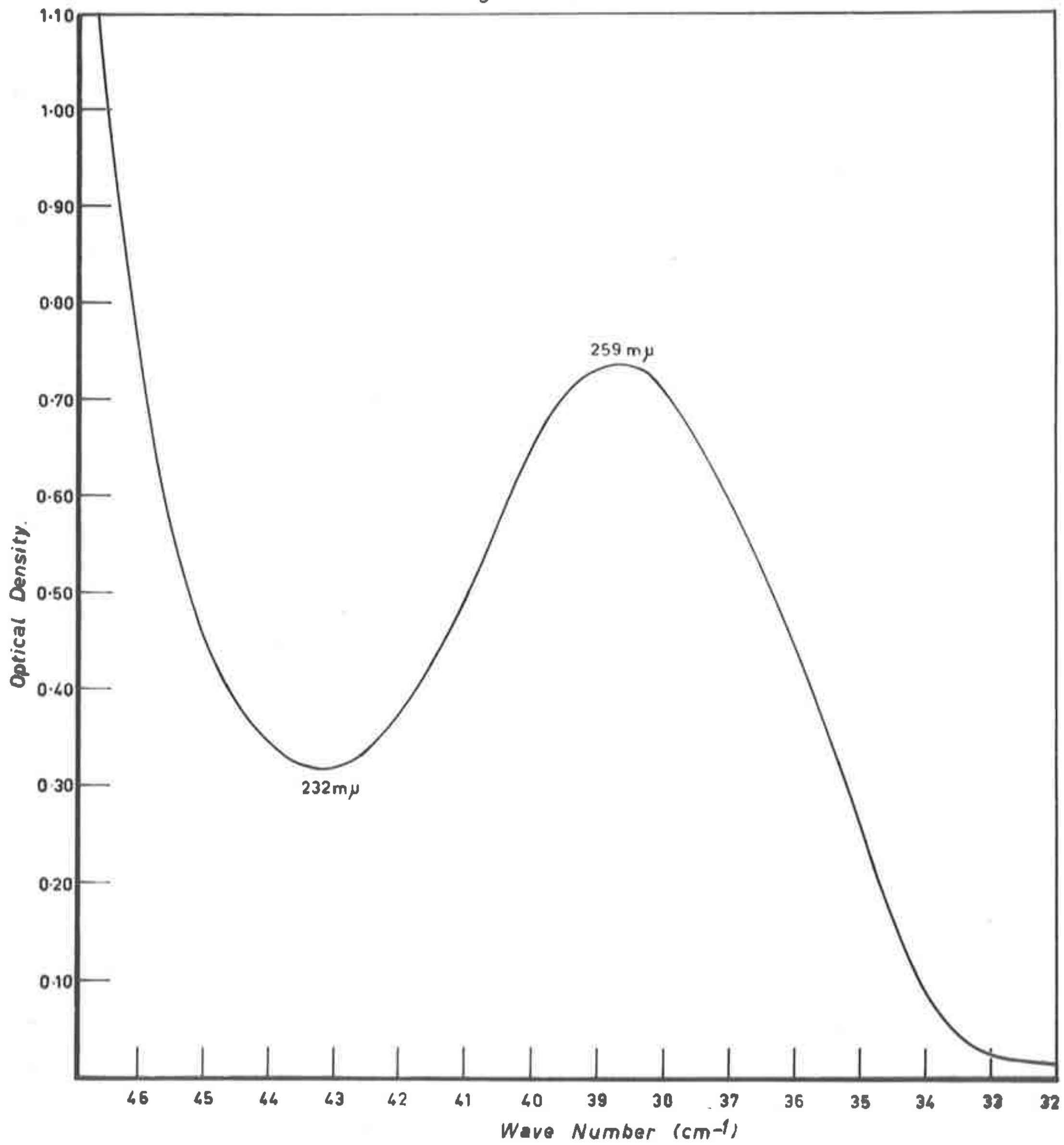

Fig. (1-VII). Ultraviolet spectrum of B.megatherium DNA.

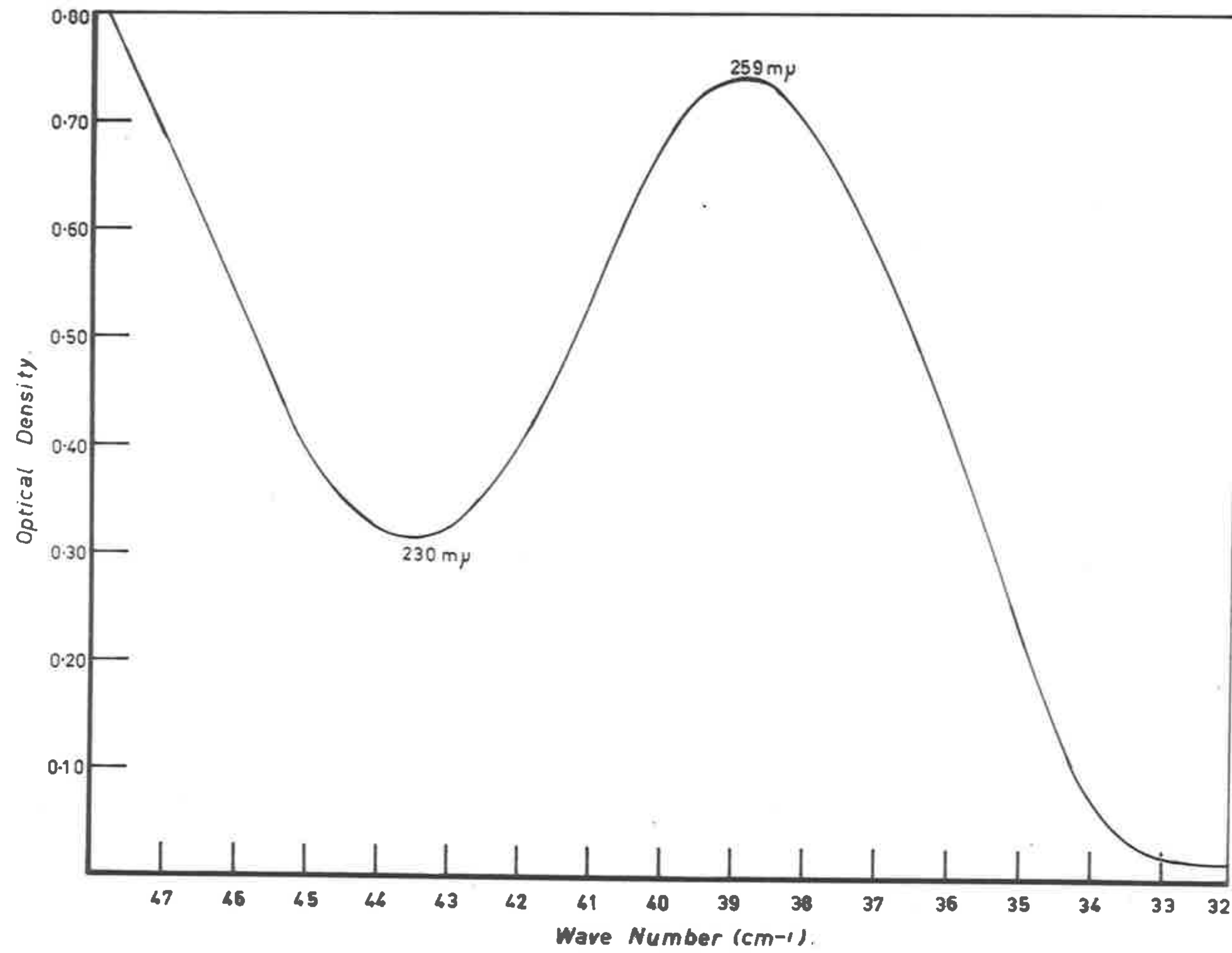

Fig. (2-VII). Ultraviolet spectrum of calf thymus DNA.

with an $s^{o}_{20,w}$ (50%) value of 23.3 and gave rise to a 40% increase in hyperchromicity on heating to 100°C. Its ultraviolet spectrum is shown in Fig. (2-VII).

ii) Preparation of water

In order to bring about a reproducible amount of denaturation on dissolving DNA in water, at a concentration below the critical value, it was necessary to use extremely pure water. Water was therefore prepared in the following manner: deionized water was distilled in an all-glass apparatus and then redistilled from alkaline potassium permanganate. The middle fraction was then distilled against a stream of pure nitrogen in an apparatus with a silica condenser and receiving flask. The conductivity of the resulting water was approximately 4×10^{-7} $ohms^{-1}$ cm^{-1}, when measured under purified nitrogen in the conductivity bridge described previously by Inman and Jordan.[3] It was necessary to obtain water of higher purity than this and so instead of distilling the water in silica the middle fraction from the alkaline potassium permanganate still was cycled through a mixed bed resin, consisting of Amberlite IRA-400(Cl), which had been converted to the hydroxide form, and IR-120(H). The conductivity of this water, measured in the same manner as above, was approximately 1×10^{-7} $ohms^{-1}$ cm^{-1}.

iii) <u>Determination of concentrations</u>

a) <u>Phosphorus analysis</u>

Concentrations were determined in terms of the number of moles of phosphorus using a modification of the methods of Jones et al.[4] and Griswold et al.[5] to determine the phosphorus content of a sample of DNA. This then enabled the extinction coefficient in terms of the number of moles of phosphorus $\varepsilon(P)$ to be determined. It was found that unless a large number of samples and standards were used in the phosphorus analysis it was possible to have a 10% discrepancy between samples. It was therefore decided to use an alternative method to measure actual concentrations of DNA.

b) <u>Direct determination</u>

In this method the concentration was determined in terms of the weight of dried DNA in 100 g of solution. In order to determine this three clean weighing bottles were heated to 105°C under vacuum for 24 hrs and then weighed. This process was then repeated until they reached constant weight. The following procedure was used for all weighings: the bottles were allowed to cool in a desiccator, the tap of which was left open to the atmosphere, in a room held at constant humidity. The bottles were then transferred to the balance case with a pair of long tongs and left for several hours. Their weights were then measured to the nearest micro-gram on

a Mettler model M5 microbalance.

An aliquot of a concentrated stock solution of DNA in pure water (approximately 0.1% DNA) was then placed in each of these bottles and the bottles plus solution reweighed. They were then heated to 105°C under vacuum for 48 hrs and then weighed. This process was then repeated for 12 hr periods until they reached constant weight. From these measurements the concentration of the stock solution was found to better than 1%. The concentrations of other solutions were then found by diluting this stock solution by weight.

That this stock solution consisted of native molecules was shown by diluting with sodium chloride to give a solution 0.2 M in sodium chloride and comparing the value of T_m and the magnitude of the hyperchromic shift of this solution with that of a fresh solution prepared by dissolving DNA directly in 0.2 M sodium chloride.

iv) Hydrodynamic measurements

a) Viscosity

Solution viscosities were determined using a Couette type, co-axial rotating cylinder, variable shear, viscometer described by Kurucsev and Jordan,[6] similar in design to that of Ogston and Stanier.[7] This enabled intrinsic viscosities at zero DNA concentration and zero shear to be measured by carrying out a Zimm type plot in order to extrapolate to zero shear and concentration.

All viscosities were determined in 0.2 M sodium chloride. In the case of chromatographic fractions the solutions were dialysed against 0.2 M sodium chloride for 96 hrs, the dialysate being renewed every 24 hrs.

b) Sedimentation velocity

A Spinco model E ultracentrifuge equipped with ultraviolet optics was used to measure sedimentation coefficients and sedimentation coefficient distributions. The majority of measurements were made at 47,660 r.p.m. using a 30 mm aluminium wide window cell in a B rotor thermostated at 20.0°C (± 0.05°C). The filling hole of this cell had been enlarged to take a 1.2 mm internal diameter syringe needle, thus reducing the risk of degrading the DNA in high shear gradients. A few measurements, on very low molecular weight samples, were made at 59,780 r.p.m. in a 12 mm cell, in a D rotor.

All runs were performed on DNA dissolved either in 0.2 M sodium chloride or in S.S.C. in the concentration range 0.003% to 0.001% DNA, these concentrations being low enough to yield s^o values.[8] These values were then corrected to $s^o_{20,w}$ values using the corrections suggested by Svedberg.[9]

Photographs were taken on Kodak transparency film and developed for three minutes at 20°C in Kodak D19 developer. The films were then fixed in Kodak acid fixer for ten minutes and then left in Kodak hypo clearing solution

for 30 minutes, before washing in distilled water.

These experimental photographic records were converted into plots of concentration versus distance in the cell with a Spinco Analytrol photodensitometer with a microanalyser attachment.

Calculation of the distribution of sedimentation coefficients from the absorption photographs requires that the height of the tracing above the baseline be directly proportional to the concentration of absorbing material at that point. The validity of this relationship was tested using the method described by Schumaker and Schachman.[8] The plot of optical densities of DNA solutions against distance of pen travel above the baseline for the representative tracings was found to be linear below an optical density of 1.0.

The position in the cell of the 50% concentration point was measured by superimposing a millimetre net on the photodensitometer tracing. The measurements were used in calculating the sedimentation coefficient distribution were also made using this millimetre net to measure the increase in concentration at equal intervals across the boundary. The method of calculating this distribution has been discussed in the previous chapter, as has the method of combining sedimentation coefficients, at zero concentration, with intrinsic viscosity measurements, at zero shear and concentration, to yield a value for the

molecular weight of a DNA sample.

c) Sedimentation equilibrium in density gradients

"Optical grade" cesium chloride, obtained from L. Light and Co., was used to bring the density of the DNA solution to a value corresponding to a refractive index of the order of 1.4008. In those cesium chloride solutions that contained 1% formaldehyde, the measured refractive index was 0.0012 units higher. These solutions were prepared by weighing out 2.58 g of cesium chloride and adding to this sufficient DNA to give a final concentration of 4 γ/ml, and the solution made up to a final volume of 2 ml by the addition of water, and in some cases formaldehyde. In some experiments native DNA, at the same concentration, was added to act as a marker. The refractive index of the solution was then adjusted by the addition of either solid cesium chloride or water. From the refractive index the density of the solution can be calculated making use of the relation reported by Ifft et al.[10]

$$\rho^{25.0°C} = 10.8601\, n_{D,25.0°C} - 13.4974 \qquad (1\text{-VII})$$

Approximately 0.7 ml of the final cesium chloride solution was placed in a 12 mm cell containing a plastic Kel-F centrepiece and centrifuged in the above-mentioned ultracentrifuge at 44,770 r.p.m. at 25.0°C. After 24 hrs of centrifugation, ultraviolet absorption photographs were taken on Kodak transparency film. That equilibrium had been obtained by this time was shown by comparing the

resulting density distribution with that obtained after 48 hrs, no difference in the density of the resulting bands being found.

The densities of these bands were calculated by using the position of the native DNA as a reference.[11] The cesium chloride gradient was obtained from the data of Ifft et al.[10] The compositional density gradient was then used in the following equation to determine the buoyant density of the DNA at a distance r from the centre of rotation.[12,13]

$$\rho = \rho_o + \tfrac{1}{2} k\omega^2 (r^2 - r_o^2) \qquad \text{(2-VII)}$$

where ρ_o = density of the reference DNA

ω = speed of rotation in radians $sec.^{-1}$

r_o = distance of the reference DNA from the centre of rotation

and k is a constant whose value depends on the value of ρ. A graph of k versus ρ was plotted from the values quoted by Thomas and Berns[12] and the appropriate value obtained from this graph for the various samples.

The density values of the native samples of DNA were obtained from the tabulated results of Schildkraut et al.,[13] namely, 1.697 g/cc for B.megatherium and 1.699 g/cc for calf thymus. The absolute accuracy of these values is not important as they are only used in order

to identify the various denatured and renatured species.

v) Denaturation techniques

a) Measurement of thermal denaturation

The denaturation temperatures (T_m) were measured using a Unicam SP500 spectrophotometer. The temperature of the solutions was raised by placing the spectrophotometer cells in an electrically heated and thermostatically controlled cell holder. The temperature of the holder was regulated by a thermistor incorporated in the body of the cell holder in conjunction with an Eilco, Type 2, Temperature Controller, and the actual temperature was measured with a chromel-P-alumel thermocouple, calibrated in terms of the temperature inside of the spectrophotometer cell. The cells were fitted with tight-fitting polythene stoppers which prevented loss of water by evaporation.

The absorbance ratio is defined by $O.D._T/O.D._{(Native)25^o}$ at 259 mμ, where $O.D._T$ has been corrected for the thermal expansion of the solution. In the graphs showing the variation of absorbance ratio with temperature, the curves have been arbitrarily adjusted so that the maximum values of the absorbance ratio at high temperatures correspond to the value obtained with native DNA. The melting temperature T_m is defined as that temperature at which the absorbance ratio has a value corresponding to half the maximum value for native DNA. In the case of the

two samples used in this study this corresponds to a value of 1.200 for the absorbance ratio.

b) Cooling methods

Quick cooling was achieved by taking the stoppered spectrophotometer cell from the heated cell holder, at a given temperature, and placing it in an ice bath. A drop in temperature from 100°C to 20°C was generally attained in 1-2 minutes. Slow cooling was achieved by placing the stoppered cell containing the solution in a large, closed, Dewar flask filled with boiling water. The rate of cooling was approximately 30° in the first 12 hours and 40° in the following 24 hours. Samples were, in general, left in the vacuum flask for 48 hours by which time they had cooled to room temperature.

c) Slow and quick addition of sodium chloride

Renatured samples in which the sodium chloride was said to be added quickly refer to samples that were made 0.2 M in sodium chloride by the addition of an appropriate amount of solid sodium chloride. This was all added at the one time and quickly dissolved by stirring.

Slow addition of sodium chloride was achieved by taking 10 ml of DNA solution and adding the appropriate amount of solid sodium chloride in the following manner: ½ mg was added every 15 minutes for 2 hours, 1 mg every 15 minutes for 2 hours, then 1 mg every 5 minutes for 2 hours.

At this stage the solution was allowed to stand for 12 hours and then 2 mg were added every 5 minutes for 2 hours and 3 mg every 5 minutes for an hour. The whole process taking approximately 21 hours.

vi) Chromatography

a) Apparatus

A double mixing chamber, gradient elution apparatus similar to that described by Bendich et al.[14] was used with the modification that much larger columns, the dimensions being 34 x 2½ cm, were used and only one mixing chamber was used to produce the gradient from 1.0 M sodium chloride to 2.0 M sodium chloride and 0.1 M ammonia. 10 ml samples were collected using a constant volume, Tower automatic fraction collector (model A) and these were transferred manually to spectrophotometer cells in order to measure the optical density of the effluent.

The gradient produced by this apparatus was tested by using 0.5 M sodium chloride in the upper reservoir and analysing the samples collected during a "dummy" run by determining the concentration of chloride ion present in each tube by titration. It was found that the effluent concentration could be given by the formula

$$[NaCl]_{eff.} = [NaCl]_{stock} - [NaCl]_{stock} \left(\frac{V+1}{e^V}\right) \qquad (3\text{-VII})$$

where $V = \frac{\text{cumulative volume passed through}}{\text{volume in mixing chamber}}$

as found by Bendich[14] and that the gradient was reproducible to within one tube.

b) Exchange resins

Ecteola-cellulose was prepared by the method of Peterson and Sober[15] using Whatmans cellulose powder. This exchanger was found to have a pK' value, in 0.5 M sodium chloride, of 9.5; 0.37 m.moles of ionizing groups per gram and a DNA capacity of 7.6 mg DNA/g exchanger. It was found, however, that after approximately twelve months this resin started to lose its capacity for DNA. Therefore another sample was prepared using Solka-Floc SW-40B as the cellulose base, this proved to be a very stable sample. It had a pK' value, in 0.5 M sodium chloride, of 7.0, the number of ionizing groups on the resin was 0.32 m.moles/g and the adsorption capacity was 8.9 mg DNA/g.

Ward and Putch[16] suggested that a resin having few charged groups available within the molecular contact volume of the resin was advisable so that mild elution techniques could be used. In contrast Bradley and Rich[17] suggest that an exchanger with a high number of functional groups per contact volume would be desirable so that a given eluent could demonstrate greater selectivity and so elute sharper fractions. On the grounds of this latter

suggestion an exchanger with a large number of charged groups per contact volume has been used. As it was intended to fractionate low molecular weight material the number of charge groups was made larger than that of the resin used by Bendich et al.[14] Due to its high ionic capacity this exchanger, when used to fractionate native DNA, resulted in the chromatographic profile being shifted, compared to the results obtained by Bendich et al., to regions of stronger eluting conditions as desired. It was concluded that the number of charge groups was not too high, since very little DNA was left on the column to be eluted by 0.5 M sodium hydroxide and approximately all (102%) of the DNA loaded on the column was recovered.

In order to avoid differences in resulting elution profiles from this column due to overloading, as found by Bendich et al.[18] only one-fifth of the total capacity/g was loaded on the column. Thus 6 mg of DNA, in the form of a 0.1% solution, were loaded onto 3 g of resin, which is the reason that much larger columns than those described by Bendich et al.[14] have been used. This modification, together with that of using a stronger resin, was found to remove the "breakthrough" peak obtained on washing the column with phosphate buffer prior to elution.

c) Eluents

The same elution technique as that described by Bendich et al.[14] has been used in this work except for

the following modifications. A gradient to 1.0 M sodium chloride was introduced as it was found that fractionation could occur in this region with many of the samples used. At no stage during the fractionation process was liquid allowed to remain stationary in the column, as it was found that this gave rise to an artifact peak on re-starting the flow of eluent. This modification meant that when the eluent was changed it did not result in a sudden elution of DNA giving rise to a concentrated DNA solution in the first few fractions collected after the change.

All fractionations were performed in duplicate, the two columns being eluted simultaneously, so that both fractionations were carried out under the same conditions. In order to find whether small changes in temperature ($\pm$ 4°C) would affect the chromatogram, native DNA was fractionated at various temperatures in this range and in all cases the chromatograms were found to be reproducible to within one tube, which was the limit of reproducibility of the gradient.

vii) Sonic degradation

Solutions of 0.1% DNA in 0.01 M phosphate buffer were degraded by placing them in the steel cup of a Raytheon, 9 kilocycle magnetostriction oscillator (model S-102A) and irradiating them for periods ranging from one to twelve minutes. The generator was operated on 120 volts, with the plate voltage being set to a maximum. Oxygen free

nitrogen was bubbled through the solutions prior to their irradiation and ice water was circulated through the outer jacket of the cup before and during the irradiation.

viii) Ultraviolet spectroscopic measurements

The optical densities of the various fractions resulting from chromatography and those values used in constructing thermal denaturation curves (at 259 mμ) were obtained using a Unicam SP500 spectrophotometer, placing the solutions in one centimetre silica cells. This instrument was adapted in that it was fitted with a Labgear spectrophotometer power supply (Type 115D) instead of using lead accumulators to supply the power.

The ultraviolet spectra were also measured in one centimetre silica cells, using a Unicam SP700 Recording Spectrophotometer, the results being obtained as a graph of optical density versus wave number.

ix) Electron microscopy

The instrument used in these investigations has been described by Inman and Jordan,[19] and is equivalent in performance to the Philips EM-100 electron microscope. The methods used in this investigation are very similar to those used by Hall.[20,21,22]

All the solutions, except that containing DNA denatured by dilution, were dialysed against two changes of 0.1 M ammonium acetate/0.05 M ammonium carbonate in order

to remove non-volatile salts. In the case of the heat denatured DNA the dialysate was made 1% in formaldehyde so that the denatured molecules would remain in the presence of formaldehyde. The concentration of DNA in these solutions is approximately 0.001%. Although this means that the concentration of DNA relative to the concentration of impurities may be small, it is necessary to use a concentration in this range to overcome the tendency of the molecules to aggregate as the droplets of solution evaporate. An aqueous solution of polystyrene spheres (880A) is added to these solutions to aid in the location of droplets and in focussing.

The solutions are sprayed onto freshly cleaved mica, which is very smooth and also has the advantage of being hydrophilic, using a low pressure "atomiser". The DNA solution is sprayed onto the mica in such an amount that 50% of the surface is covered with evaporated droplets. The mica is placed at such a distance from the sprayer that a steady state is set up between droplets hitting and droplets drying on the slide. If the population of undried droplets becomes too large the droplets merge.

The slides are then shadowed with platinum. 5.0 cm of 0.1 mm wire is used, at a distance of 7 cm and a shadow to height ratio of 10:1. A 0.5% collodion solution is then spread on the slides and allowed to drain off.

This leaves a thin supporting film of collodion over the platinum replica. This film, plus the replica, is then floated off the mica and lowered onto 300 mesh grids which are used in the electron microscope.

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