THE PHYSICO-CHEMICAL BEHAVIOUR OF POLYNUCLEOTIDES IN NON-AQUEOUS SOLUTION.

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ABBREVIATIONS

The following abbreviations have been used in this Thesis:

deoxyribonucleic acid	DNA
ribonucleic acid	RNA
polyriboadenylic acid	poly-A
polyribouridylic acid	poly-U
polyriboinosinic acid	poly-I
polyribocytidylic acid	poly-C
polyriboguarylic acid	poly-G
polyribothymidylic acid	poly-T
copolymer of adenylic and uridylic acid	poly-AU
copolymer of the four major nucleotides	poly-AGUC
copolymer of deoxyribo-adenylic and thymidylic acids	poly-dAT
complexes formed between poly-A and poly-U	poly-(A+U) poly-(A+2U)
ribonucleoside monophosphates	XMP
ribonucleoside diphosphates	XDP
ribonucleoside triphosphates	XTP

where X represents a purine or pyrimidine ribonucleoside,

e.g. A, adenosine
U, uridine
I, inosine
C, cytidine
G, guanosine
T, thymidine

hexadecyltrimethyl ammonium ion	Q
hexadecyltrimethyl ammonium salts of	
DNA	DNA-Q
RNA	RNA-Q
poly-A	poly-A-Q
poly-U	poly-U-Q
poly-I	poly-I-Q

Enzymically prepared synthetic polyribonucleotides have been referred to as "polynucleotides" as distinct from naturally occurring nucleic acids.

tris(hydroxymethyl)-aminomethane	Tris.
ethylenediaminetetraacetic acid	E.D.T.A.

SUMMARY

The recognition of the biological importance of nucleic acids, both ribo- and deoxyribo-nucleic acids, has prompted the investigation of their physico-chemical properties *in vitro*, in attempts to elucidate the configuration, the size and the mode of biological activity of these biopolymers. The discovery of several enzyme systems, capable of promoting polynucleotide synthesis, greatly assisted in these studies, since synthetic polynucleotides having limited compositions could then be studied. Examination of the helical structures formed by synthetic homopolyribonucleotides has provided considerable insight into the problem of the specificity and interactions of naturally-occurring nucleic acids.

Although the biological activity of nucleic acids is intimately related to the configuration and interactions of the polymer under the conditions existing *in vivo*, physico-chemical investigations have largely been carried out using aqueous salt solutions which do not necessarily resemble the biological conditions. Even in these simplified systems it is difficult to obtain accurate quantitative data on polynucleotides or nucleic acids. This results, not only from the variability in the configuration, as exemplified by helix-coil transitions or denaturation, and from the macromolecular nature and the frequent heterogeneity of these compounds, but is also due to their polyelectrolyte behaviour arising from the charged nature of the polymeric backbone. This behaviour severely hampers the characterization of differing polynucleotide or nucleic acid samples, and virtually precludes an unambiguous assessment of polymer shape and size or correlation of physico- chemical properties with biological function.

The possibility of using non-aqueous solutions for characterization of polymer samples has been further investigated in the present work, with particular reference to the hexadecyltrimethyl ammonium salt of polyadenylic acid (poly-A-Q) in ethanol solution. Although these systems are, in principle, more easily reproduced and defined, many of the problems encountered in aqueous systems are also evident under non-aqueous conditions. This is found to apply particularly to the uncertainty of the extent of the helix-coil transition and to polyelectrolyte behaviour. In general, the results

In general, the results obtained from sedimentation, viscosity and optical measurements for poly-A-Q and DNA-Q in ethanol are consistent with the polymer chains existing in a collapsed random coil configuration. However the physico-chemical properties of polynucleotides and nucleic acids in non-aqueous solution were found to be sensitive to the presence of low concentrations of ionic impurities. Thus it has been concluded that non-aqueous solutions of these biopolymers, either in the form of the simple inorganic salts or as the quaternary ammonium salts, are not suitable for use in the characterization of various samples.

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

Since the discovery of nucleic acid material in cells by Mieschner in 1871,[1] research into the physico-chemical and bio-chemical nature of nucleic acids has become an important part of biological research. The occurrence, nature and biological importance of these compounds *in vivo* have been studied in conjunction with their properties *in vitro* in an attempt to establish the basic contributions these compounds make to the continuity and maintenance of life.

Two distinct groups of nucleic acids are known, (i) the deoxyribonucleic acids, (DNA), in which 3'-5' phosphodiester linkages join the monomeric nucleotide units containing purine (adenine, guanine) and pyrimidine (thymine, cytosine) bases attached to the C'_1 of the deoxyribose, and (ii) ribonucleic acids (RNA) which have a similar primary structure, but which contain a ribose sugar and have uracil rather than thymine bases. The secondary structures of the two groups of nucleic acids are dissimilar. Native DNA is considered to be a double-stranded α-helix, in which the two polynucleotide chains are anti parallel and are held together by bonding between bases of the complementary chains, similar to that initially proposed by Watson and Crick.[2] In contrast to the well defined structure of DNA, RNA exists as single strands and is incompletely organized, probably consisting of short helical regions separated by unorganized, random sequences.[3] The DNA, which occurs predominantly within the cell nuclei, is considered to be the material responsible for the transmission of hereditary factors from one generation to the next. The RNA has a more general distribution through the cells and it appears to be involved in the expression of these hereditary factors, particularly as a result of its participation in the biosynthesis of proteins.

The investigation of the molecular biochemistry of nucleic acids has often been hindered by the complexity of the biological systems involved, however the isolation of various enzyme systems capable of synthesizing polynucleotides in vitro made the simplification of the studies possible. The synthetic polymers may be prepared with comparable, or even identical, chemical composition, structure and properties as the naturally occurring nucleic acids; the enzyme systems can also synthesize homopolymers containing a single type of monomer and copolymers with restricted compositions rather than with a mixture of at least four different nucleotide monomers as found in the naturally occurring species. Thus, in the same way that the study of synthetic polypeptides aided in the elucidation of protein chemistry and structure, so the investigation of the properties of synthetic polynucleotides has aided the understanding of nucleic acid chemistry.

The earliest work in the field of polynucleotide synthesis was carried out by Ochoa et al. (1955)[4,5] who observed the synthesis of polyribonucleotide from nucleoside diphosphates during a study of the biological phosphorylation mechanisms in bacterial extracts. The enzyme, which was called polynucleotide phosphorylase, was initially observed in crude extracts of Azotobacter vinelandii but has subsequently been shown to occur in a large number of bacterial species and also in some plant and animal cells.[6] This enzyme is considered to probably be involved in the degradation rather than the synthesis of RNA in the living cell.[6] Synthesis of RNA by a polymerase enzyme, which utilizes nucleoside triphosphate as substrate, has been recognized as the more probable synthetic pathway. Such RNA polymerase enzymes have been observed in and isolated from many bacterial, plant and animal sources.[7,8] The polymerase systems require the presence of DNA primer to initiate and direct the polymerization, whereas polynucleotide phosphorylase may or may not require priming, depending on the source and state of purity of the enzyme. The base sequence in polynucleotides synthesized by RNA polymerase appears to be determined by the sequence in the primer DNA and to be random as in the polymers produced by the phosphorylase system. It is assumed that information contained within the DNA molecule can be transmitted to the RNA by this mechanism.[8] Another class of polymerase, requiring the presence of a RNA primer to initiate and direct the synthesis of polyribonucleotides from nucleoside triphosphates, has also been reported.[9]

Progress in the field of synthetic polydeoxyribonucleotides followed the discovery and isolation, in 1956 by Kornberg _et al_.,[10,11] of an enzyme system in _Escherichia coli_ which utilized deoxyribonucleoside triphosphates as substrates, in the presence of magnesium ions and a DNA primer, in the synthesis of DNA-like polymers. As in the case of polyribonucleotide synthesis, polymers having a more limited base sequence than naturally occurring DNA may be synthesized. Enzyme systems capable of the synthesis of polydeoxyribonucleotides have been isolated from a variety of sources by Bollum and others.[12]

Polynucleotides may be synthesized by chemical, as distinct from enzymic, methods. The specific chemical synthesis of the 3'-5' phosphodiester linkage has been accomplished for both polyribonucleotides and polydeoxyribonucleotides[13,14], however the degree of polymerization obtained has been low, the maximum obtained in good yields being only 15 nucleotide units. This is due, at least in part, to the limited solubility of the higher polymers in the organic solvent of the reaction mixture. Homopolymers of the five major ribonucleotides, and several copolymers have been synthesized by chemical means. This method permits considerable control over the base sequence in the polynucleotide product.

Synthetic polynucleotides having molecular weights comparable to those of naturally occurring nucleic acids can thus only be prepared by enzymic systems. Unfortunately this method of preparation yields polynucleotides which may be contaminated by enzymes, by protein and in some cases by primer polynucleotide. Similar impurities also occur in samples of nucleic acid isolated directly from biological materials. Although many purification techniques have been employed to remove contaminants,[15,16,17] it is difficult to obtain polynucleotide samples which are free from foreign matter. The presence of contaminating enzyme material may lead to the instability of the polymer, particularly in the case of polyribonucleotides[18].

Most nucleic acid samples show heterogeneity of composition and of degree of polymerization due to the innate heterogeneity of the original material but also due partly to degradation or denaturation during isolation procedures. Synthetic polynucleotides also tend to exhibit heterogeneity and this interferes with accurate investigations of the physico-chemical properties of these polymers; fractionation of polymer samples may be necessary. However a more serious problem, encountered during the physico-chemical characterization of nucleic acid and polynucleotide samples, is the dependence of the properties on the environment of the polymer.

The conformation in solution, and hence the properties of all classes of polynucleotides are determined by the nature, the pH and the ionic strength of the solvent, by the temperature and by the nature and concentration of other components of the solution.[19,20]

Under conditions causing the breaking or weakening of the secondary forces within the molecules, specific, organized structure is destroyed. This is exemplified by the helix-coil transitions, (or denaturation) experienced by DNA and other polynucleotides when the temperature is increased above a critical value, by altering the pH of the solution, by the addition of reagents known to destroy hydrogen bonds, by exposure to low ionic strength or by changing the dielectric constant of the solvent. This helix-coil transition, which is reversible in the case of most polyribonucleotides but only partially reversible in DNA, is reflected by changes in the optical, hydrodynamic and electrical properties of these polymers. In incompletely organized polynucleotides (e.g. RNA, "alkaline" poly A, denatured DNA) some variation of the properties is observed when the temperature, pH or solvent is altered. This is attributed to a loss of order in the small, non-random regions.

The charged nature of the macromolecular nucleic acids and polynucleotides leads to the polyelectrolyte behaviour observed, particularly in incompletely organized polymers.[21,22] The properties of these polymers in aqueous solutions show a marked dependence on the ionic strength of the supporting electrolyte, and similar behaviour has been noted in non-aqueous solutions of nucleic acids.[23,24] The dependence of the properties of polynucleotides and nucleic acids in solution, on the solvent emphasizes the need for an easily defined, reproducible system in which the polymeric material can be characterized. Such a system is essential if data concerning these compounds are to be correlated and interpreted.

Some aspects of the behaviour of nucleic acids and polynucleotides in non-aqueous solutions have been studied[23,24] but complete physico-chemical investigations have been precluded by limited solubility and other difficulties. Aubel-Sadron _et al_.[25] have utilized the solubility of longchain quaternary ammonium salts of nucleic acids in polar organic solvents to prepare non-aqueous solutions, and this procedure has now been used to obtain non-aqueous solutions of homopolynucleotides. The behaviour of these systems, and particularly of the hexadecyltrimethylammonium salt of poly-A in ethanol have been studied.

The enzymic synthesis of polyribonucleotides, using polynucleotide phosphorylase systems, is discussed in Chapter II, together with the general structure of these polymers. The physical chemistry of polynucleotides, particularly of poly-A, is considered in Chapter III and the examination of the properties and behaviour of polynucleotides in non-aqueous solutions is described in Chapter IV. The main emphasis of this study has been on polyribonucleotides, particularly enzymically prepared poly-A, however some aspects of the behaviour of DNA have been investigated, and these data are discussed in conjunction with those for polynucleotides.

Chapter II

ENZYMIC SYNTHESIS AND THE STRUCTURE OF POLYRIBONUCLEOTIDES.

1. Polynucleotide Phosphorylase
    - (i) Reactions promoted
    - (ii) Distribution
    - (iii) Enzyme properties
    - (iv) Specificity
    - (v) Primer requirements
    - (vi) Isolation
        - a) from *Azotobacter agilis*
        - b) from *Micrococcus lysodeikticus*
2. Synthesis and isolation of polynucleotides
3. Polynucleotide structure
    - (i) Primary
    - (ii) Secondary

1. Polyribonucleotide Phosphorylase.

There are two distinct groups of enzymes capable of promoting the synthesis of polyribonucleotides, namely the polynucleotide phosphorylases and the ribonucleic acid polymerases. The preparative work described in this investigation refers only to the former class of enzyme, which was discovered by Ochoa and coworkers.[4]

(i) Reactions promoted.

Polynucleotide phosphorylase promotes the synthesis of polyribonucleotides from nucleoside-5'-diphosphates, in the presence of magnesium ions, with the elimination of inorganic orthophosphate. (Equation (II)-1)[4,5]

$$\text{nNPP} \xrightleftharpoons{Mg^{++}} (\text{NP})_n + \text{nP}_i \qquad \text{(II)-1}$$

where N is a purine of pyrimidine nucleoside, PP is pyrophosphate and P_i inorganic orthophosphate. The composition of the polymer, $(NP)_n$, formed is determined mainly by the type and relative concentration of the nucleoside diphosphate in the incubation mixture. Most phosphorylase enzymes catalyze other reactions in addition to the polymerization.[4,26] These include (a) phosphorolysis of polynucleotides, the reverse of the polymerization reaction, (b) the exchange between inorganic orthophosphate and the terminal phosphate of a nucleoside diphosphate, as illustrated by the use of P^{32} labelled orthophosphate (Equation (II)-2)

$$NPP + P_i^{32} \xrightleftharpoons{Mg^{++}} NPP^{32} + P_i \qquad (II)\text{-}2$$

and (c) arsenolysis,[26] in which arsenate replaces the orthophosphate as substrate in a reaction analogous to the phosphate exchange (Equation (II)-2). A fourth reaction, involving the transfer of nucleoside monophosphate units from a polynucleotide donor to a polynucleotide acceptor has also been postulated.[27] Several of these reactions have been utilized in assaying the enzyme activity.

(ii) Distribution.

Polynucleotide phosphorylases are widely distributed in nature, occurring among both aerobic and anaerobic bacteria.[6] They have also been reported to occur in some plants[28] and may occur in animal tissue.[7,29,30]

The enzyme has been purified to varying extents, and its properties studied, the most common sources being Azotobacter agilis,*[4,31] Micrococcus lysodeikticus,[32,33] and Escherichia coli.[34,35] The enzyme isolated from various sources appear to differ in specificity,

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* Azotobacter agilis (American-Type Culture Catalogue, 5th. edition, No. 9104(1949)) is also referred to as Azotobacter vinelandii.

requirements and stability. The behaviour of the enzymes from *E.coli* and *A.agilis* is similar,[6] but is unlike that of the enzymes from *M.lysodeikticus* and *Clostridium perfringens*[36]. These differences may be inherent to the enzymes, or may be due to contaminants in the various preparations.

(iii) Enzyme properties.

In many cases, crude extracts from enzyme sources are capable of producing polynucleotides, however the activity of the enzyme is affected by the presence and concentrations of metal ions, salts, other contaminating enzyme systems, nature and concentration of the substrate, organic and inorganic phosphates, proteins and polynucleotide material.[37] The specificity of the enzyme, and its requirements may be considerably altered after extensive purification, as exemplified by the "lag period" and primer requirements observed in highly purified *A.agilis*, *E. coli* and *M.lysodeikticus* polynucleotide phosphorylase.

The conditions for optimum enzyme activity vary, depending on the source of the enzyme and the reaction under consideration. Polynucleotide phosphorylases show an absolute requirement for magnesium ions, and an alkaline pH is required for optimum activity for all the types of reactions promoted. However, the value of the optimum pH is dependent on the concentrations of magnesium ions, salts and substrate.[37] The enzyme from A.agilis exhibits a pH optimum between pH 7.5 and 9.0 for polymerization and phosphorolysis, but has a sharp optimum at pH 8.1 for the phosphate exchange reaction.[5] Similarly the M.lysodeikticus enzyme has an optimum for polymerization at 9.0-9.5, while arsenolysis and phosphorolysis have a pH optimum at about 8.5.[37] In the case of the E.coli enzyme, the optimum for all three reactions is 7.4.[35]

Extensive studies of the enzyme kinetics have provided data concerning the influence of various factors on polymerization and phosphorolysis. These have been reviewed by Grunberg-Manago[6] and Steiner and Beers.[37]

(iv) Specificity

Polynucleotide phosphorylase enzymes are specific with respect to the nature of the sugar moity and the number of phosphate groups esterified to the terminal position of the nucleoside. However, the specificity toward substrate is less well defined, and varies with the enzyme source and often with the degree of purification of the enzyme. For instance, the M.lysodeikticus enzyme becomes specific for ADP at high degrees of purification, and no longer polymerizes CDP and UDP,[38] however no separation of the activity towards the bases could be shown in the phosphorolytic reaction.[39] Enzymes from A.agilis and E.coli show constant relative activities towards all the nucleotides during extensive purification[31,40] suggesting that there is a single enzyme present.

In addition to catalyzing the polymerization of homopolymers, including poly-A, poly-U, poly-I, poly-C and poly-G from the corresponding nucleoside diphosphates, some types of polynucleotide phosphorylase can produce copolymers containing a mixture of bases, e.g. poly-AU, poly-AGUC if the appropriate bases are incubated with the enzyme under suitable conditions. The linear distribution of nucleotides in such polymers appears to be essentially random, and is probably governed by purely statistical considerations.[41] Some enzyme preparations, particularly those from A.agilis and E.coli, can also utilize other, less common or substituted nucleoside diphosphate analogues.[6]

(v) The effect of Primers on Polymerization.

The purification of crude enzyme preparations, particularly the removal of protein and nucleotide - containing material, often results in a lag period prior to the polymerization of nucleotides. This lag period can be eliminated by the addition of small amounts of polyribonucleotide (both naturally occurring and synthetic) or of certain types of oligonucleotides which act as a primer to the synthetic reaction. The instantaneous polymerization observed with crude enzyme extracts is probably due to the presence of suitable primer material in these extracts. The synthesis of poly-G is very dependent on the presence of primer, and even crude enzyme extracts require an added primer which is incorporated into the polymer.[31,42-44]

The particular requirements for priming of polymerization depends largely on the enzyme source, the A.agilis and E.coli enzymes being similar but differing from those of M.lysodeikticus and Cl.perfringens. The synthesis of poly-A by the enzyme from Cl.perfringens is stimulated by, and when the enzyme is highly purified, dependent on the presence of basic polypeptides; polynucleotides have no effect.[36]

The main classes of primers are:

a. Oligonucleotides with a free C-3' hydroxyl group on the terminal nucleoside residue. These molecules, having a degree of polymerization of 2-4, are incorporated into the polymer produced, each molecule apparently initiating a polymer chain. There is no indication of base specificity.[39,42,45]

b. Oligonucleotides with an esterified C-3' hydroxyl group. These have a very limited specificity with respect to substrate base, and are not incorporated into polymers.[42,45] These oligonucleotides cannot overcome the lag period for enzymes from all sources[39] and in some cases tend to inhibit polymerization.

c. Long chain Polyribonucleotides.

Polymers of this type, either naturally occurring or synthetic, have been shown to overcome the lag period and to stimulate polymerization for most enzyme systems; the polymer may or may not be incorporated into the product, depending on the particular system. Priming of this type shows a considerable degree of specificity as illustrated in Table (II)-1.

The actual mechanism of priming by oligonucleotides and long chain polynucleotides, and the interpretation of the lag period have not been completely elucidated. It appears unlikely that a replication mechanism is involved since deoxyribonucleotides are not active as primers, the primer does not appear to have a significant influence on either the base composition or sequence of the polymer,[31] and the synthesis of homopolymer tends to be stimulated by the addition of the corresponding and not the complimentary homopolymer. Heppel[48] suggested that inhibition of polymerization resulted when the polymeric primer and the substrate nucleoside diphosphate contribute a pair of bases known to be involved in highly ordered, multistranded complexes. This inhibition is manifested by a temperature dependent lag period followed by an autocatalytic time dependence of polymer formation. Eventually the rate of reaction becomes equal to that observed in an uninhibited system.[48] Inhibition may also be overcome by the addition of certain polymers or oligonucleotides.

TABLE (II)-1

The effect of long chain polyribonucleotides on the polymerization of nucleoside diphosphates by polynucleotide phosphorylase of *A.agilis*. [31,42,46-48]

	Synthesis of						
Primer	poly-A	poly-U	poly-I	poly-C	poly-G	poly-AU	poly-AGUC
Poly-A	+	-H	-	-	o		o
poly-U	-H	+	o	-			o
poly-I	-H	o	+	-H			
poly-C	+	+	-H*	+	-H*		+
poly-G				-H	+		
poly-AU	+	+				+	
poly-AGUC	+	+		-			+
RNA (bact.)	+	+		-			+
RNA (yeast)	+	+		-			+

\+ priming, o no effect, - inhibition

H inhibition is due to base pairing.

* Reference 48.

Priming, activation and inhibition do not necessarily occur under the same conditions for reactions other than the polymerization, catalyzed by the same enzyme.[39]

(vi) Isolation from bacterial cells.

The preparation of enzyme samples suitable for use in polynucleotide synthesis has two main phases, viz. the preparation of cell free extracts from the enzyme source, and the removal of non-enzyme material such as nucleic acid, protein and polysaccharide.

a) The procedure used for the isolation of polynucleotide phosphorylase from *Azotobacter agilis* was essentially the same as that described by Ochoa *et al*.[5,49] (See Chapter V). Enzyme solutions obtained after the calcium phosphate gel fractionation had specific activities* of approximately 10-20 units per mg. protein, and these solutions were used in polynucleotide synthesis. Polynucleotide phosphorylase samples of much higher specific activity can be prepared by additional purification steps.[31,50] Even in these samples, some polynucleotide material was found

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* Specific activity, enzyme units per mg. protein, was determined by the phosphate exchange assay. See Chapter V.

to remain closely associated with the enzyme protein.

Although the method of preparation of the Azotobacter enzyme has been described in detail in the literature, the purification is not very reproducible and many difficulties were experienced during attempts to prepare enzyme samples suitable for polymer synthesis. Similar difficulties have also been reported by other workers.[39,51]

b) Although the polynucleotide phosphorylase from Micrococcus lysodeikticus has not been obtained in highly purified states[39] some features of the partially purified enzyme - particularly with respect to specificity, activators and inhibitors - differ from those of the crude extracts. However these enzyme preparations are suitable for the synthesis of polynucleotides having high molecular weights, and can be easily and reproducibly obtained. Polynucleotide phosphorylase was prepared from spray-dried cells using the method of Steiner and Beers,[52] and was used in the synthesis of poly-A.

2. Synthesis and isolation of Polynucleotides.

The conditions used in the synthesis of polynucleotides (See Chapter V, 5) were adapted from conditions cited in the literature[5, 11, 37, 53], and the kinetics of the enzyme preparations used were not studied specifically, since the object of this work was the preparation of polynucleotides for physico-chemical studies. In the case of the Azotobacter enzyme, the substrate nucleoside diphosphates were buffered at pH 8.1 with Tris buffer in an aqueous solution containing magnesium ions and the reaction solution, containing enzyme, was incubated at 30°C. The reaction mixture used with the Micrococcus enzyme was similar, but it was buffered at pH 9.5, and maintained at 37°C. In each case, sufficient enzyme was used to produce maximum synthesis in 3-7 hours.

The progress of the synthesis was followed viscometrically, using an Ostwald viscometer with a flow time of about 15 seconds for water. Although the viscosity of the reaction solution will be altered by changes in ionic strength and concentration as well as by the degree of polymerization of the forming polymer, this method provides a simple, semiquantitative method of gauging the extent of the synthesis.

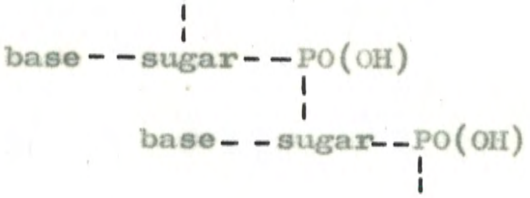
The lag period prior to polymerization discussed previously, was evident in some preparations of the Azotobacter enzyme. However no primer was added to the reaction mixture. In several enzyme solutions, particularly when the specific activity was less than 15, the flow time for the reaction mixture increased to a maximum value and then fell away. This behaviour has been observed previously,[54] and may be due in part to contaminating nuclease.

Polynucleotide material was isolated from the reaction mixture by precipitation with ethanol in the presence of salt. (See Chapter V, 6.) A variety of precipitating agents have been used to isolate polynucleotides and nucleic acids from synthesis mixtures or from biological material; these have been reviewed by Razzell,[55] Jordan[15] and others. The purification of polynucleotide samples used for physico-chemical studies, is discussed in later chapters.

3. Polynucleotide Structure.

(i) Primary Structure

The basic primary structure of naturally occurring nucleic acids, both DNA and RNA, was shown to be



$$\begin{array}{l} \quad\quad\quad\ \vdots \\ \text{base}--\text{sugar}--\text{PO(OH)} \\ \quad\quad\quad\quad\quad\quad\quad\quad\ \vdots \\ \quad\quad\quad\quad\ \text{base}--\text{sugar}--\text{PO(OH)} \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\ \vdots \end{array}$$

despite the differences in base compositions and the sugar moiety.[56] The phosphodiester linkage between adjacent nucleotides is between the C'_3 and C'_5 of successive sugar molecules for both classes of nucleic acid.[57] This was established by means of exhaustive enzymic and chemical studies of the polymer structure.[57,58] In general these polymers are considered to be long unbranched polynucleotide chains.

The mode of linkage between nucleotides has also been investigated for biosynthetic polynucleotides using techniques of controlled chemical and enzymatic degradation under suitable conditions to give identifiable products.[41,58,59] Although most of this work has been carried out on polymers prepared using the A.agilis polynucleotide phosphorylase, all enzymically prepared polynucleotides are considered to contain the 3'-5' phosphodiester linkage found in naturally occurring nucleic acids.[41]

In the synthetic copolymers, there is a question of the randomness of base structure to be considered. Ribonuclease, which shows a specificity with regard to purine and pyrimidine bases, has been used in sequence studies. Heppel <u>et al</u>.[41] showed that the distribution of adenine and uracil bases in the poly-AU copolymer prepared with the Azotobacter enzyme was essentially random. It was subsequently suggested that the ratio of adenine to uridine nucleotides in the polymer roughly parallels the ADP to UDP ratio in the reaction mixture.[60,61] Similar studies on the randomness of distribution of the nucleotides in copolymers prepared with polynucleotide phosphorylase from other sources has further substantiated these findings.

In contrast to the essentially random base sequence in those polynucleotides already discussed, polymers synthesized with ribonucleic acid polymerase systems have both their base composition and base sequence dictated by the primer.[62-65] Homopolymers, including poly-A, poly-U and poly-C, can also be synthesized if the appropriate nucleoside triphosphate is included in the reaction mixture.[65-68] Poly-AU, containing a regularly alternating sequence of adenylic and uridylic nucleotides, is synthesized if the synthetic polydeoxyribonucleotide, poly-dAT is used as primer.[16,69]

(ii) Secondary Structure.

Although the primary structure of all classes of polynucleotides are similar, considerable differences occur in the secondary structure. This varies from the ordered helical structure of DNA to the randomly orientated structures of some synthetic polynucleotides. The particular conformation of the polynucleotide is determined by the nature, state and the environment of the polymer.

The generally accepted structure of native deoxyribonucleate ions in aqueous solution is essentially the structure by Watson and Crick[2] and modified by Wilkins et al.[70,71] This model consists of two helical, antiparallel polynucleotide chains coiled about a common axis in such a way that the purine and pyrimidine bases be inside the helix. The molecular structure is maintained by means of secondary valence forces, bonding between the specific base pairs of the complementary chains. Both hydrophobic and hydrogen bonds are considered to be important for the stability and specificity of the helix.[24,72-75] Although the usual base pairings found in naturally occurring nucleic acids are adenine-thymine and cytosine-guanine, other pairings are sterically feasible.[76] The physico-chemical properties of DNA, the results of enzymic degradation, and studies of the behaviour of synthetic polydeoxyribonucleotides are consistent with the double stranded helical structure postulated, however some degree of flexibility does exist in the macroion.[77]

In contrast to the well defined structure of native DNA in the solid state and in solution, denatured DNA in solution has properties approaching those expected for a randomly coiled polymer.[72] Although denaturation, which involves the removal of the forces stabilizing the double stranded helix, can result in strand separation,[78] partial renaturation often occurs when the denaturing force is removed. This yields molecules containing some ordered regions and some randomly oriented areas which is consistent with the observed behaviour of denatured DNA in aqueous solutions.[72]

The secondary structure of naturally occurring RNA is not as well defined as that of DNA. X-ray diffraction studies of RNA have indicated that there is an underlying three dimensional configuration common to all types of RNA, despite differences in base content and molecular size.[79] Physico-chemical properties and x-ray diffraction data are in agreement with the concept of a single stranded, interrupted helix in which short helical regions occur.[3,80] These are probably intramolecular double helical regions stabilized by hydrogen and hydrophobic bonding between the bases.[3,80] Similar configurations are considered to occur in many synthetic copolymers, including the poly-AU, poly-AC, poly-IC and poly-AGUC prepared with polynucleotide phosphorylase.[81,82] In contrast, poly-AU prepared using the RNA polymerase of E.coli with poly-dAT copolymer as primer, can exist as a rigid rod-like helix.[18] The configuration of the copolymers having a random base sequence is at least partially determined by the base ratio.[81]

Although poly-U has been shown to exist in an ordered state at low temperatures (below 8°C.), at ordinary temperatures the physico-chemical and x-ray evidence all suggest a randomly coiled configuration essentially devoid of any ordered secondary structure.[83] Similar random structure appears to be present in poly-I in solutions which are alkaline to the zone of titration of the hypoxanthine group. This can be explained since the loss of the proton from the N_1 position leaves the hypoxanthine base without any potential hydrogen bond donor group, and also would lead to electrostatic stresses great enough to prohibit the formation of inter- or intra-molecular bonding.[84] In solutions of pH below approximately pH 9, there is strong evidence for the existence of some degree of ordering. A triple stranded helical structure was proposed, by Rich,[85] for poly-I in the solid state, and this configuration is thought to persist in neutral and acidic solutions.

Polyadenylic acid fibres drawn from concentrated gels have been used in x-ray and birefringence studies. The data obtained are consistent with a double stranded, helical structure existing in the fibres;[86] a similar structure has been proposed for poly-A in acid solutions.[87,88] Hydrogen bonding between the adenine bases of adjacent polynucleotide strands is sterically feasible[76] (See Fig. (II)-1), and this bonding is considered to contribute to the stability of the helix. In aqueous solutions which are more alkaline than the pK of the adenine group, (pH $>_{\text{ca.}}$ 6), poly-A possesses many characteristics of a relatively unorganized system. The physico-chemical properties of poly-A in the alkaline form are in agreement with the concept of a randomly coiled configuration for the polymer. There may be some regions containing some degree of helical content within the overall randomness.[89]

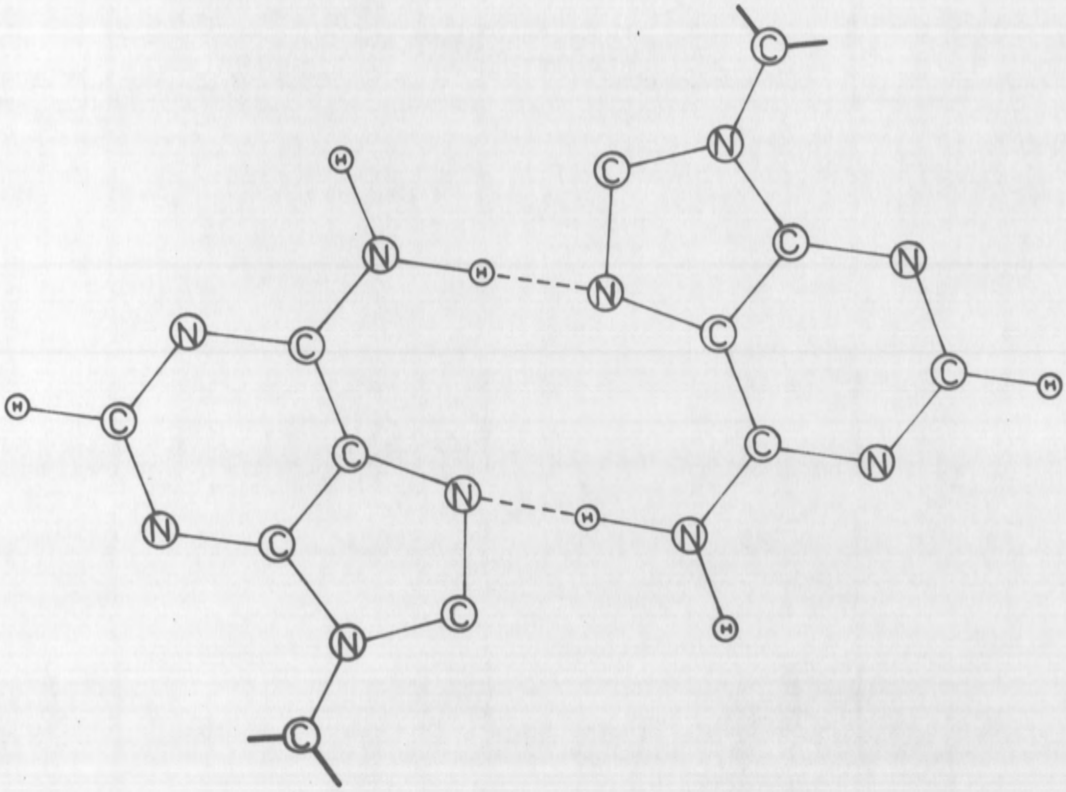


Fig.(II)-1. Hydrogen bonds linking pairs of adenine residues in poly-A

The tendency to exist as multistranded helical structures or as ramdomly coiled single strands, depending on the conditions, has been observed in all homopolynucleotides available, and has also been demonstrated by the formation of multistranded complexes containing dissimilar strands. If solutions containing poly-A and poly-U in the random form are mixed, the physical properties of the resulting product are best explained on the basis of either two or three stranded helices, depending on the conditions.[90] X-ray diffraction patterns of poly-(A+U) fibres suggest a double stranded, helical structure, which probably is stabilized by adenine-uracil bonding similar to that postulated for adenine and thymine in the Watson-Crick model for DNA.[91] It is proposed that a second strand of poly-U could be bonded in the wide helical groove of the poly-(A+U) helix. Other highly ordered, multistranded complexes have been observed, including poly-(A+I), poly-(A+2I),[92] poly-(I+C),[93,94] poly-(G+C), poly-(2G+C),[95] poly-(AI+U),[96] poly-(A+AU) and poly-(U+AU).[97] Complexes involving both polyribonucleotide and polydeoxyribonucleotide strands have also been observed (e.g. poly-(dG+C),[98] and poly-(dT+A)[99].) Studies of these multistranded complexes have been useful in the elucidation of the structure and physico-chemical properties of naturally occurring nucleic acids.

The structural studies have been carried out using polynucleotides and nucleic acids in solution and in the solid state, and the resulting data are not necessary applicable to these polymers _in vivo_. However, the biochemical behaviour _in vitro_ of some nucleic acids and polynucleotides is comparable to their biochemical action _in vivo_. For example, poly-U and poly-T promote the incorporation of phenylalanine into protein by cell-free _E.coli_ extracts;[17] this behaviour is analogous to the role of soluble RNA in protein synthesis _in vivo_. Studies of the biochemical behaviour of synthetic polyribonucleotides and synthetic polydeoxyribonucleotides have been important in investigations of the biological role of nucleic acids.

Chapter III

PHYSICO-CHEMICAL BEHAVIOUR OF POLYNUCLEOTIDES IN AQUEOUS SOLUTION WITH PARTICULAR REFERENCE TO POLY-A

1. Introduction

2. The shape and size of polynucleotides, from light scattering data.

3. Hydrodynamic properties.
    - (i) Viscosity.
    - (ii) Sedimentation.
    - (iii) Molecular weights from hydrodynamic data.

4. Optical properties.
    - (i) Ultraviolet absorption spectra.
        - a) Molar absorbance.
        - b) Hypochromicity.
        - c) Effect of pH.
        - d) Effect of temperature.
        - e) Effect of ionic strength.
    - (ii) Optical rotation and optical rotatory dispersion.
    - (iii) Infrared spectra.
    - (iv) Nuclear magnetic resonance.

5. Conclusions.

1. Introduction

The ability of polynucleotides to exist in widely differing configurations, depending on the conditions, has already been mentioned. It is essential that any correlation or interpretation of experimentally observed data concerning polynucleotides must include the consideration of polymer conformation. In the case of poly-A, it is convenient to anticipate the concept of the helix coil transition, and to discuss the behaviour of the helical and the coiled forms separately. The physical chemistry of poly-A in aqueous solution will be reviewed in this chapter, and data concerning the particular poly-A samples studied here will be presented. In the relevant sections the properties of other polynucleotides and of DNA and RNA will be briefly discussed.

The concept of order-disorder transitions in polynucleotides was initially proposed to explain the anomolous changes observed in the titration behaviour, the ultraviolet absorption spectrum and the hydrodynamic and light-scattering behaviour as the pH, temperature and/or ionic strength of their solutions changed.[100] The transition had previously been postulated to occur in DNA, where it constitutes denaturation of the native, double stranded helix, and has been observed in homopolymers, copolymers and polynucleotide complexes. In the latter case, the transition is reversible,[84] but in DNA it is largely irreversible. This has been attributed to the more complex base sequence in DNA reducing the probability of the specific bonding between the base pairs being reformed.[72]

Since the configuration and consequently the physico-chemical properties of the polynucleotides is determined by the experimental conditions, and since these factors are interdependent, a precise definition of the conditions is necessary before any experimental data are discussed.

2. Size and Shape, from Light Scattering Data.

Light scattering techniques have been widely applied in the field of nucleic acid research, and have also been used in the study of synthetic polynucleotides. The radius of gyration, R_g, defined as the weighted mean distance of sub-elements of the molecule from the centre of mass, can be determined directly from light scattering data without assuming any model configuration. This quantity is usually quoted as a parameter of molecular size, and if the general configuration of the molecule is known or can be assumed, it is possible to estimate the molecular dimensions from R_g.[101] Values of the average molecular weight can be obtained from light scattering data, although this may necessitate the use of some model structure (e.g. rigid rod, sphere, random coil, stiff coil.)[101,102]

Beers and Steiner[100] have obtained data which are consistent with the concept of poly-A existing in solution as a polydisperse system of random Gaussian coils at neutral or alkaline pH and ionic strengths of 0.01M or greater. This was shown by the linearity of the limiting curve of c/R_θ as a function of $\sin^2(\theta/2)$, where c is the concentration of the polymer, R_θ is the reduced scattering intensity at an angle θ to the incident light, and also by the low value calculated for the (light-scattering mean) radius of gyration $\langle R_g^2 \rangle_z^{\frac{1}{2}}$. The mean end-to-end distance of the poly-A molecule, as calculated from R_g on the basis of a random coil configuration, was of the order of 1/13 to 1/18 of the length calculated for a completely extended helix. The radius of gyration of poly-A in this coiled form increased markedly at low ionic strength,[100] as in the case of most flexible polyelectrolytes,[101] in contrast to the relative invariance of R_g for native DNA under similar conditions.[103] The effect of ionic strength on the radius of gyration of RNA appears to vary, depending on the source of the nucleic acid.[104]

Early light scattering investigations on the acid form of poly-A were interpreted as in terms of the formation of aggregates which were considered to be more rigid than the coiled, alkaline form of poly-A. The changes in the radius of gyration observed tended to vary depending on the ionic strength and polymer concentration at which acidification was affected.[89]

The light scattering behaviour of native DNA has been examined by many workers.[72,103,105] The configurational model considered to be most compatible with the observed data is that of a stiff coil with a large persistence length, thus exhibiting behaviour between that of a random coil and a rigid rod.[106,107] Denatured DNA has a much more compact conformation, and its properties resemble those of a random coil much more closely than do those of native DNA. The radius of gyration drops during denaturation, and shows a dependence on ionic strength for denatured material.[72]

3. Hydrodynamic Properties.

While light scattering data can be used to obtain some information about the size and shape of the essentially static polymer chain, hydrodynamic methods are widely used to give further information about the configuration, size and shape of the molecules. Polynucleotides and nucleic acids exist in solution as charged macro-ions, and hence tend to behave like the synthetic, linear polyelectrolytes. Hence the theories used to deduce the size, shape and polydispersity of these polymers have been modified to include the contribution made by electrostatic interactions to the configuration and the effective hydrodynamic volume of the polymer. Polyelectrolyte theories in general, combine the configurational theories of uncharged polymers[108-110] with electrostatic interaction theories.[109,111] The latter contribution is largely determined by the potential of, and the ionic atmosphere around, single polyions. Since there is no general way in which these can be evaluated for varying configurations, most theories are based on an overall model including defined shape, symmetry and charge distribution.

In solution complete separation of the polyions and their oppositely charged counterions does not usually occur; some fraction of the counterions remain essentially fixed to the polymer chain. The distribution of the "free" counterions in the solution largely depends on the local charge density of the polyion,[109] and must be considered in the electrostatic interaction theory. In the presence of added electrolyte, electrostatic interactions of the polymer chain will tend to be suppressed, and in the presence of sufficient added electrolyte, the hydrodynamic data from polyelectrolytes can be treated in the same way as for that of uncharged polymers. Since the treatment of intermolecular interactions is not well developed, it is usual to extrapolate results obtained experimentally to infinite polymer dilution to obtain information concerning the isolated molecule.

(i) Viscosity.

Viscosity data have been reported for most polynucleotides and nucleic acids, over a range of experimental conditions. Particularly under the conditions where the polynucleotides are considered to be in an incompletely organized form, there is a marked dependence of the intrinsic viscosity on the ionic strength of the solution, similar to that observed for polyelectrolytes. In the latter, this behaviour has been attributed to the decreased Coulombic repulsion between similarly charged segments of the polymer chain when the ionic strength of the supporting electrolyte is increased.[112] At moderate or high electrolyte concentrations, the concentration dependence of polyelectrolytes is similar to that of uncharged polymers. In the absence of added electrolyte, maxima may be observed in the concentration dependence of viscosity at low polymer concentrations.[113]

The viscosity of macromolecular solutions is described in terms of the reduced viscosity, $\eta_{sp/c}$, defined by the equation

$$\eta_{sp}/_{c} = (\eta - \eta_{o})/\eta_{o}c.$$

where η_o and η are the viscosity coefficients of the solvent and solution respectively, and c is the polymer concentration expressed as weight per unit volume of solution. The reduced viscosity of uncharged polymer solutions can be related to molecular parameters if the hydrodynamics of continuous media are applied to some suitable model representing the dissolved polymer. Equations are available for the viscosity of solutions of various model polymer configurations, for example spherical particles,[114] and ellipsoids of rotation.[115,116] However, such theories require modification before they can be applied to real polymer systems, since rigid, impermeable models cannot fully describe the flexible, coiled polymer chains usually encountered.[117] When the viscosity of polyelectrolyte solutions in the absence of added electrolyte is being considered, further modification of the viscosity theory is required.[118]

In practice it is usual to eliminate the effects of intermolecular interactions in the viscosity of polymers by the extrapolation of experimental data to infinite dilution to give the intrinsic viscosity, $[\eta]$, which is defined either by

$$[\eta] = \lim_{c \to o} \left(\frac{\eta_{sp}}{c} \right)$$

or by
$$[\eta] = \lim_{c \to o} \left(\frac{\ln \eta_{rel}}{c} \right)$$

where $\eta_{rel} = \eta / \eta_o$.

This extrapolation does not eliminate the shear dependence observed in the viscosity of some polymeric materials and a separate extrapolation of experimental data to zero shear may be necessary before experimental data can be interpreted with viscosity theories.[119,120] This is usually necessary if the polymer conformation is highly asymmetric, but is not important for randomly coiled chains.

A marked decrease in the intrinsic viscosity as the ionic strength of the solution is increased has been noted in many polynucleotide systems, including neutral and alkaline solutions of poly-A,[121] poly-U,[122] poly-C in its neutral form[123] and RNA.[21,104] In the case of poly-U, since no apparent change in the molecular weight was observed as the ionic strength was decreased, the increase in $[\eta]$ under these conditions was attributed to the diluting out of the counterion concentration, thus decreasing the shielding of the electrostatic repulsions between polymer elements and allowing expansion of the polymer chain.[122] The linearity of the double logarithmic plot of approximate intrinsic viscosity against total cation concentration, and the slope of this line (0.38), were considered characteristic of highly permeable polymer chains.[122] Cox[124] has shown, on the basis of the Flory theory of polyelectrolyte viscosity, that for a randomly coiled polymer, the viscosity is inversely proportional to the 0.6 power of the counter ion concentration if it is permeable to solvent, but only to the 0.4 power if it is free draining. Richards <u>et al.</u> considered that the proposed randomly coiled single chain configuration was supported by the virtual absence of shear dependence of the viscosity of poly-U in aqueous solution.[122]

In contrast to the ionic strength dependence of $[\eta]$ discussed above, changes in ionic strength have little effect on the viscosity of poly-A in acid solutions[87,100,121] or of other polynucleotides which have ordered configurations. However, there is some evidence of a variation in $[\eta]$ for native DNA as the electrolyte concentration changes, and Scruggs and Ross have suggested that the apparent invariance previously observed was due to trace protein contaminants in the DNA preparations.[125]

When some degree of secondary structure is present, for example in native DNA, RNA etc., the viscosity is usually shear dependent. Littauer and

Eisenberg[21] have shown that in pure water the viscosity of RNA is strongly shear dependent, and that both this dependence and the value of the reduced viscosity decreased as the ionic strength increased. In addition the plot of ηsp/c against concentration had a large maximum, with ηsp/c decreasing rapidly as infinite polymer dilution is approached. Littauer and Cox[126] have shown an analogous dependence of hydrodynamic and optical properties of RNA on the ionic strength and on temperature. They consider that these changes are at least partly due to changes in the secondary structure of the molecules.

The denaturation of DNA and of other multistrand polynucleotide complexes, is accompanied by a marked lowering in the intrinsic viscosity. The viscosity of denatured DNA, in contrast to that of native DNA, is almost independent of shear rate, but it is dependent on ionic strength,[72] This behaviour is consistent with an essentially randomly coiled configuration for denatured DNA.

The investigation of the viscosity behaviour of polynucleotides, and the interpretation of the experimental data obtained has often been hindered by the instability of samples[127] particularly in salt free solutions, by polydispersity of the samples and by the difficulty in obtaining reliable and reproducible data at the low concentrations necessary for accurate extrapolation to infinite dilution. However, as a means of following changes in polymer configuration, and in the estimation of molecular weight in conjunction with other physical chemical data (See III-3-(iii)) viscosity provides useful information.

(ii) Sedimentation.

The sedimentation properties of nucleic acids and polynucleotides are of limited use in the elucidation of size, shape and polydispersity of samples. As in the case of many synthetic polyelectrolytes, the sedimentation coefficient is dependent on polymer concentration and the ionic strength of the solution as well as on the molecular weight of the polymer. The data are further complicated by the considerable changes in polymer configuration associated with the helix-coil transition.

In many cases, particularly for the more rigid polymers such as native DNA and the acid form of poly-A, boundary sharpening resulting from the dependence of the sedimentation coefficient on polymer concentration, tends to obscure the polydispersity in the sample.[77,100]

(See Fig. (III)-1). This is particularly serious at the concentration necessary if a schlieren optical system is used to follow the rate of sedimentation, but can be largely avoided by the use of ultraviolet optics.[128] Thus, solutions of low concentration can be studied, essentially eliminating the concentration dependence of the sedimentation coefficient. Much of the available sedimentation data has been obtained by extrapolating, to infinite dilution, data from schlieren patterns. However, the necessary extrapolation over a large concentration range tends to make such data rather unreliable. The effect of diffusion on the distribution of sedimentation is usually neglected, since this should be small when the molecular weight is greater than 10^5.[129]

The sedimentation data for poly-A under alkaline conditions can be interpreted by assuming a randomly coiled model configuration for the chain. However, the behaviour observed for poly-A in acid solutions is more in keeping with an interrupted helical model.[87,89] The actual nature of the acid form cannot be unambiguously deduced from hydrodynamic data, owing to the aggregation of the polymer.

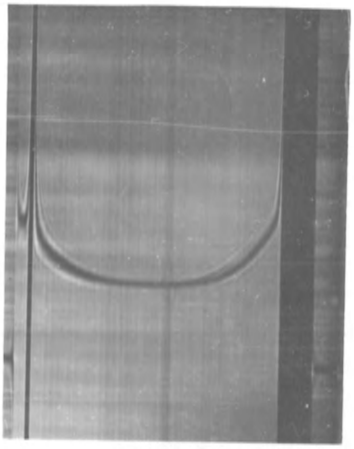


Fig.(III)-1. Hypersharp sedimenting boundary for poly-A, concentration 0.25 g/dl, in 0.15M sodium chloride, 0.015M sodium citrate pH = 7.5

(iii) Molecular weights from hydrodynamic data.

Little quantitative data can be derived solely from either sedimentation or viscosity data. Sedimentation equilibrium methods have been employed to gain some knowledge of polynucleotide molecular weights under specified conditions. However, the more usual procedure is to combine sedimentation coefficient data with viscosity or diffusion data obtained using identical conditions. The values of $[\eta]$ and s^o can be used to calculate an average molecular weight for a polynucleotide sample from the Flory-Mandelkern equation,[130]

$$\frac{N\,\eta_o\,s^o_{20,}[\eta]^{1/3}}{\beta\,(1-\bar{v}\rho_o)} = M^{2/3}$$

where N is Avagadro's number, η_o and ρ_o are solvent viscosity and density at 20°C respectively, $\bar{v}$ is the partial specific volume of the polymer and β is a constant, which depends on the shape of the polymer chain and hence on polymer-solvent interactions. The value of β varies from 2.12×10^6 for a spherical molecule to 3.6×10^6 for a rigid rod of very high axial ratio.[77] For a system of random coils of narrow molecular weight distribution, $\beta = 2.5\times10^6$, and this value is expected to decrease by about 10% with polydispersity[131]; values of $(2.25\pm0.1)\times10^6$ have been estimated for RNA and poly-A.[122] In general, the polymer molecule is represented by a model configuration, and the value of β , appropriate to that model, is used to estimate the molecular weight.

Although this method has been widely used to obtain molecular weight data for nucleic acids and polynucleotides, the approximations necessary due to the obscure and varying nature of polynucleotide configuration with changes in the environment, together with the poorly defined nature of the average molecular weight obtained ($\bar{M}_{\eta,s}$), limit the usefulness of the method.

It is theoretically possible to combine sedimentation and diffusion data to estimate polymer molecular weights from the Svedberg equation.[128] However the practical difficulties encountered in attempting to obtain reliable diffusion data virtually preclude the use of this equation.

4. Optical properties.

The light scattering behaviour and the hydrodynamic properties of polynucleotides in aqueous solutions are consistent with and often suggestive of particular configurations and transitions between these configurations. However, in most cases comprehensive data are not available, and the precise configurations of the polymer chains cannot be obtained. Typically, the properties of poly-A in alkaline solutions have been shown to be consistent with an essentially randomly coiled polymer, but certain aspects of this form suggest that a considerable and varying degree of secondary structure exists. The optical properties of polynucleotide chains have provided a simple and useful method for the investigation of this secondary structure, and have been widely used as a method of following changes in polynucleotide and nucleic acid configuration.

(i) Ultraviolet spectra.

All the purine and pyrimidine bases which occur in nucleic acids and synthetic polynucleotides exhibit very strong ultraviolet absorption, resulting particularly from the ($\pi \longrightarrow \pi^*$) and the ($n \longrightarrow \pi^*$) electronic transitions.[132,133] The main absorption bands in nucleic acids generally occur near 190-210 mμ and 250-265 mμ, however the actual wavelength of maximum absorption and the corresponding extinction vary with the composition of the polymer chain. In addition these quantities vary with the pH, the temperature, the ionic strength and frequently on the previous treatment of the sample. The precise positions and extinctions of the maxima and minima are dependent on the state of ionization of the purine and pyrimidine bases, even for mononucleotides.[134]

a. Molar absorbance.

Since nucleic acid and polynucleotide samples usually have varying water contents, "dry weight" has little significance, and hence it has become usual to define an extinction coefficient per gram atom of phosphorus per litre of polynucleotide solution, ϵ_P.[135] Since the monomeric nucleotide unit contains one phosphorus atom for every purine or pyrimidine base, the value of ϵ_p is the mean absorbency per mole of nucleotide per litre. The values of ϵ_p for poly-A and <u>E.coli</u> DNA have been determined (see Chapter V - 9) and these data together with the conditions used are shown in Tables (III)-1 and (III)-2 respectively.

Poly-A in both the acidic and alkaline form was found to obey Beer's law over a 100-fold concentration range (1.4×10^{-6} - 1.4×10^{-4}M). Thus the polynucleotide concentration of solutions used for physico-chemical measurements was estimated from the optical density of a suitable dilution of the solution, together with the experimentally determined ϵ_p value.

TABLE (III)-1.

Molar extinction coefficients, ϵ_p, for some polynucleotides in aqueous solution.

Polynucleotide	Solvent	λ mμ	$\epsilon_p \times 10^{-4}$*
poly-A	5×10^{-3}M. NaCl, pH 8.0	257.1	1.03 ± 0.05
poly-A	saline citrate,** pH 7.0	257.1	$0.99_8 \pm 0.03$
poly-A	saline citrate,** pH 4.6	252.5 257.1	$0.87_2 \pm 0.05$ $0.84_5 \pm 0.05$
poly-A	0.1M NaCl, 0.01M sodium cacodylate, pH 6.85-6.90	257	1.00 ± 0.02[94]
poly-I	0.1M NaCl, 0.01M sodium cacodylate, pH 6.85-6.90	248	1.04 ± 0.02[94]
poly-U	0.1M NaCl, 0.05M Tris. pH 7.5	260	0.92[24]
poly-A	10^{-3}M. sod. cacodylate pH 7.0	257	0.99_5[51]

* ϵ_p cm.^{2} moles(P)$^{-1}$ litres^{-1}.

** 0.15M sodium chloride, 0.015M sodium citrate buffer.

TABLE (III)-2.

Molar extinction coefficients, ϵ_p, at 259mμ for E.coli DNA in 0.15M sodium chloride, 0.015M sodium citrate buffer.

Sample	pH	ϵ_p*
native DNA	9.8	7,250
	7.0	7,100
quick-cooled, heat denatured DNA	9.8	7,870
	7.0	8,000
heat denatured DNA (measured at 98°C)	7.0	9,940

* Values of ϵ_p are averages from 20-30 separate determinations. Percentage error is approximately ±3%.

b. Hypochromicity.

The discrepancy between the observed molar extinction coefficient of nucleic acids and that extinction coefficient calculated assuming summation of the extinctions of the component nucleotides, is also characteristic of synthetic polynucleotides.[136] The diminution of the extinction, called hypochromicity, may also be accompanied with an appreciable hypsochromic shift of the absorption maxima at about 260 mμ . The degree of hypochromicity in polynucleotide systems varies considerably with the nature of the polymer, and is sensitive to changes in the ionic strength, the pH, the temperature and to other components of the solution.

Hypochromicity has been observed in all synthetic polynucleotides prepared. At room temperature, poly-U exhibits the lowest (about 9%) and this is not altered by pH, ionic strength or increased temperature. However at lower temperatures, below about 6°C., the hypochromicity is increased.[83,137] Alkaline poly-A shows a high degree of hypochromism, amounting to about 40% at 20°C. and ionic strength 0.1,[136] and also shows an hypsochromic shift of about 2 mμ in the absorption maxima compared to its mononucleotides under the same conditions. When poly-A solutions are acidified ($pH < ca. 6$) an additional hypsochromic shift of about 5 mμ occurs in $\lambda_{max.}$, and the hypochromicity is increased by approximately 15% (See Fig. (III)-2). The changes accompanying acidification can be abolished by pretreatment of the polymer with formaldehyde,[100] but are not inhibited by concentrated urea.[138]

Hypochromicity has been considered to have two component parts, (i) a "residual" hypochromism, such as that exhibited by poly-U at room temperature and by alkaline poly-A at elevated temperatures, which appears to be associated with polymer formation, and (ii) hypochromism resulting from the secondary structure within the polynucleotide chain. Several theories have been proposed to explain the hypochromic effect,[139-142] but a complete explanation has not yet been achieved. Considerable hypochromicity has been noted in oligonucleotides of short chain length.[143,144] The hypochromism and the hypsochromic shift in $\lambda_{max.}$ going from mononucleotides to the alkaline form of poly-A is essentially complete in tri- and tetra- nucleotides, and is appreciable even for dinucleotides. Furthermore, the changes in ϵ_P and $\lambda_{max.}$ observed on acidification of poly-A solutions are comparable with those changes observed

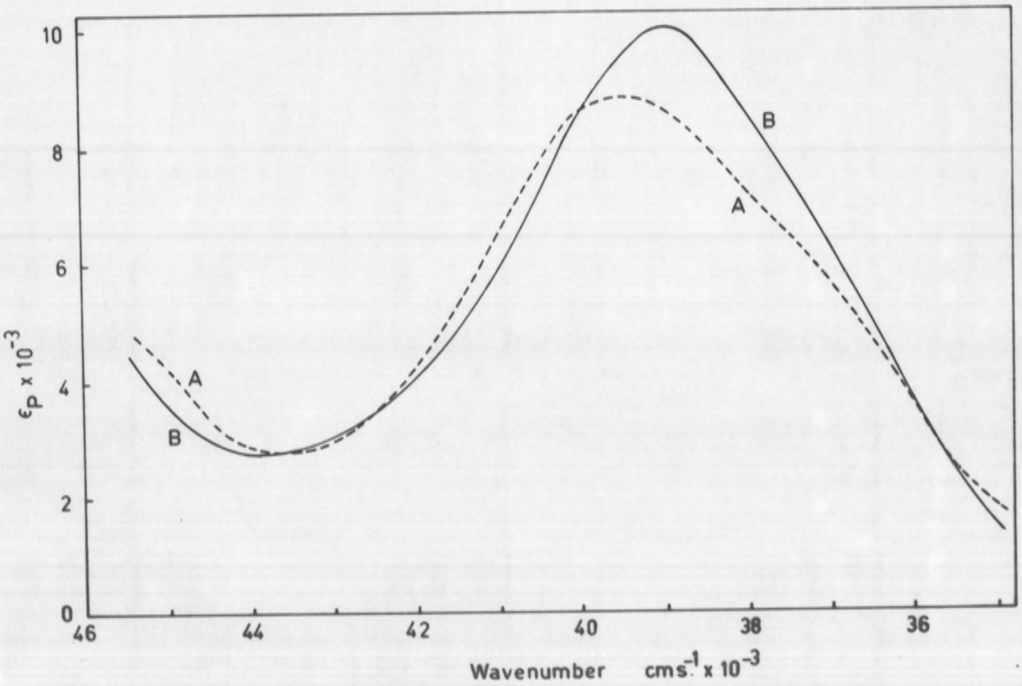


Fig. (III)-2. Ultraviolet absorption spectra for poly-A in 0.15M sodium chloride, 0.015M sodium citrate buffer. A, pH = 4.5; B, pH = 7.5.

for oligonucleotides containing seven or more monomer units. This evidence suggests that hydrogen bonded structures are not necessarily prime causes of hypochromicity. Michelson[145] considers that the basic cause of hypochromicity is probably the direct interaction between heterocyclic bases, and that the effects of hydrogen bonds on the absorbance result from the increased interaction of the purine and pyrimidine rings.

The considerable hypochromism associated with the formation of polynucleotide complexes, and the loss of hypochromism on disruption of the secondary structure as indicated by other methods, has lead to the use of the degree of hypochromicity as an empirical index of the relative extent of secondary structure in the polynucleotide chain, and particularly of the helix-coil transition.[146]

c. The effect of pH

The effect of pH on the ultraviolet absorption spectra of poly-A in aqueous solution is demonstrated in Figs. (III)-2 and (III)-3. The main features of this effect are the marked change in the hypochromicity and the shift in $\lambda_{max.}$ which occur at pH values of

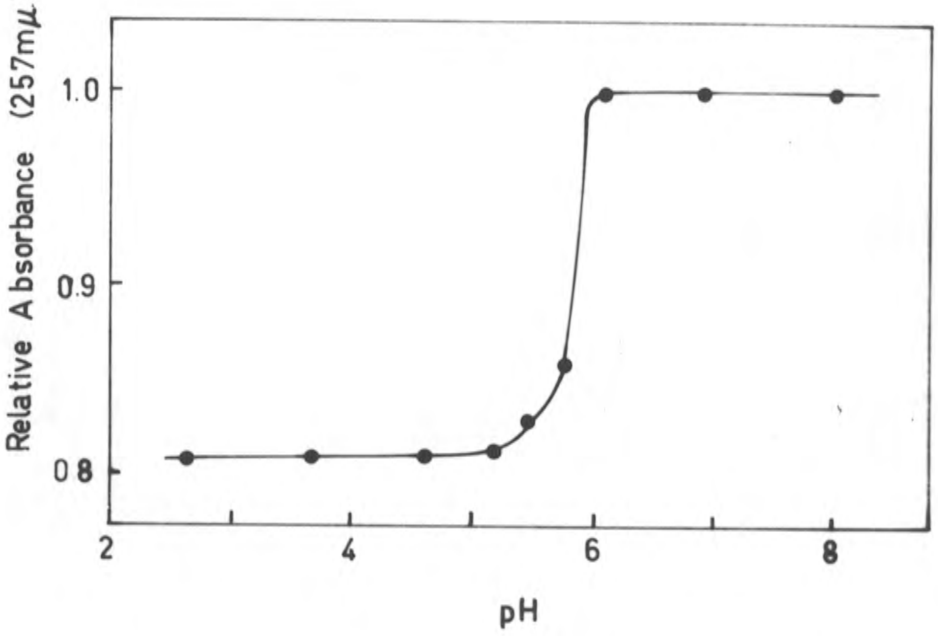


Fig.(III)-3. The pH dependence of absorbance, relative to absorbance at pH 7.0, for poly-A in 0.15M sodium chloride, 0.015M sodium citrate buffer. T = 20°C.

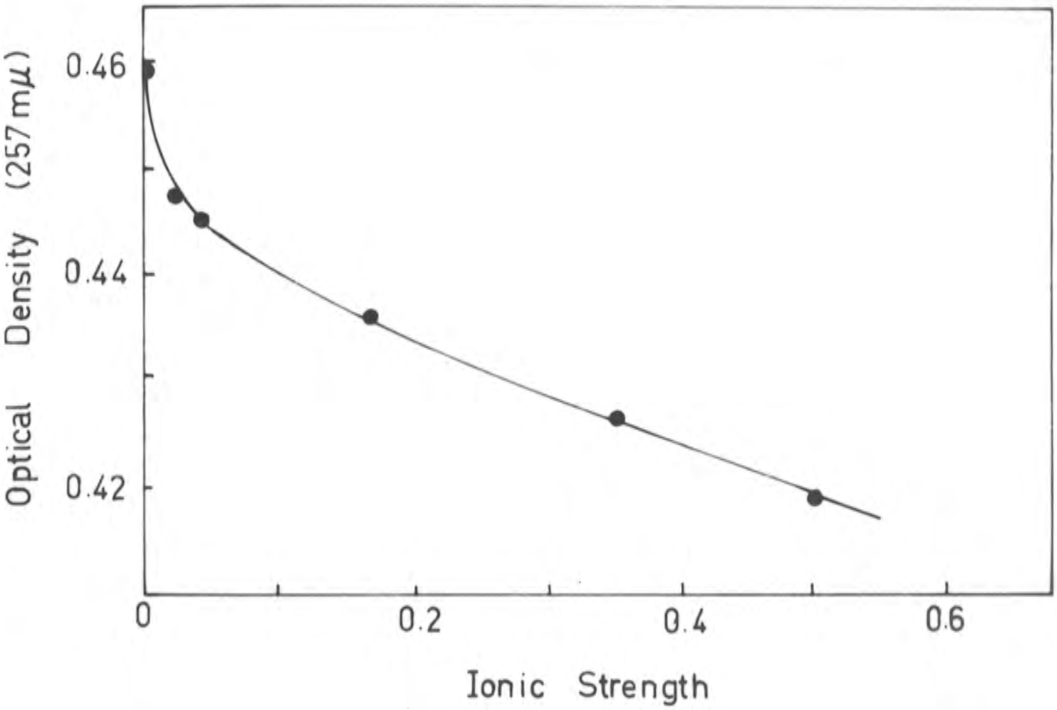


Fig.(III)-4. Optical density of poly-A in aqueous solution pH 7.0, as a function of ionic strength. T = 20°C.

approximately 5-6, depending on the temperature and ionic strength of the solution.[100] The pH_m has been defined as the pH at which the change in absorbance is 50% complete, and this value is used to compare experimental data referring to different conditions. Other physico-chemical methods have shown that these changes in absorbance occur under conditions corresponding to the helix-coil transition. Thus the relative change in absorbance has been equated to the helix stability.

Changes in absorption, similar to those indicated for poly-A, also occur in other polynucleotides, polynucleotide mixtures and in naturally occurring DNA and RNA. However, the degree of reversibility of the transition differs for different polymers.

d. The effect of temperature.

The temperature dependence of the absorbance of polynucleotides and nucleic acids can readily be observed, and is probably one of the most extensively used indices of secondary structure. The similarity between the melting out of a crystallite and the loss of ordered structure during the helix-coil transition of poly-nucleotide has resulted in the concept of the melting temperature,[147] T_m, for the latter. T_m is defined as the temperature at which the relative increase in absorbance, or the relative change in some other quantity, (compared with the value at 25°C) is 50% complete. The value of T_m varies with the polynucleotide system; in the case of DNA a linear relationship has been found between the melting temperature and the cytosine-guanine content.[148,149] A similar relationship has been demonstrated between ϵ_p and the C-G content.[150] T_m depends to some extent on the wavelength employed, owing to the different absorption characteristics of the adenine-thymine and cytosine-guanine base pairs,[151] however, for DNA it is usual to measure T_m at 259mμ .

In native DNA and in helical polynucleotides (e.g. poly-A at pH $<$ ca. 6) the thermal transition as observed via the absorbance changes, is sharp, but the transition is much more gradual in incompletely ordered systems such as alkaline poly-A (See Figs. (III)-5 and (III)-6). The sharpness of the transition and the value of T_m are used as a measure of the amount of denaturation of a sample. In these cases melting curves are constructed from absorbance measurements at the ambient temperature.

The lowering of T_m when the ionic strength is decreased has been demonstrated for DNA and other polynucleotides.[152-154] Zimmer and Venner[155] showed an inverse relation between T_m and the ionic radius of the cation in the solution, whereas Kotin,[156] introducing

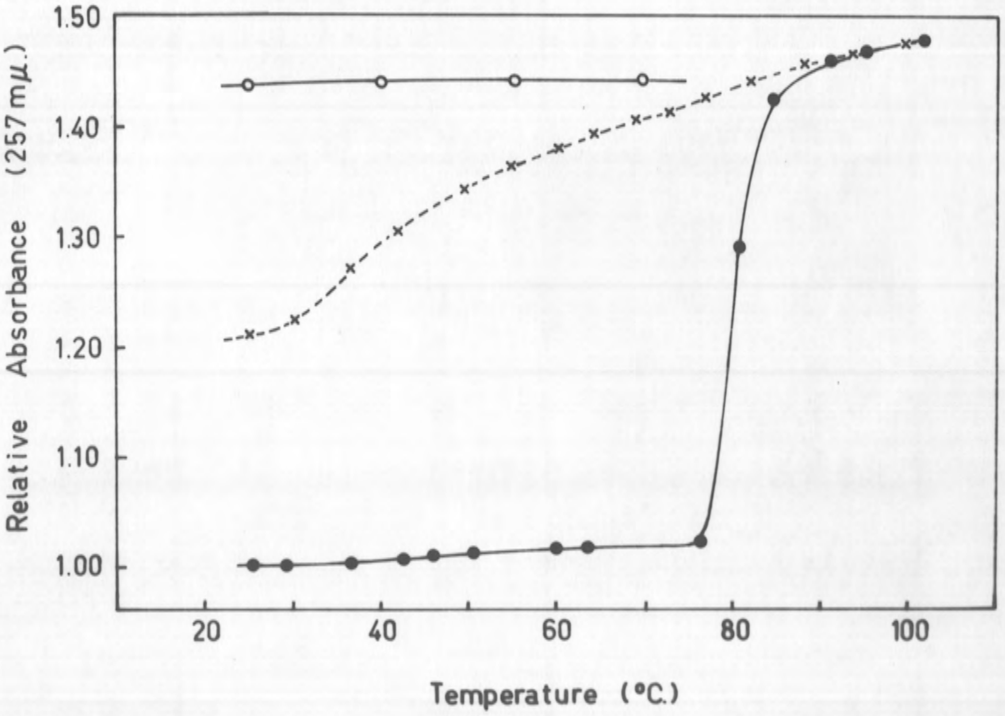


Fig.(III)-5. Temperature dependence of the relative absorbance of poly-A in 0.15M sodium chloride, 0.015M sodium citrate, ● pH = 4.6; × pH = 7.5; and of poly-A-Q in ethanol (o).

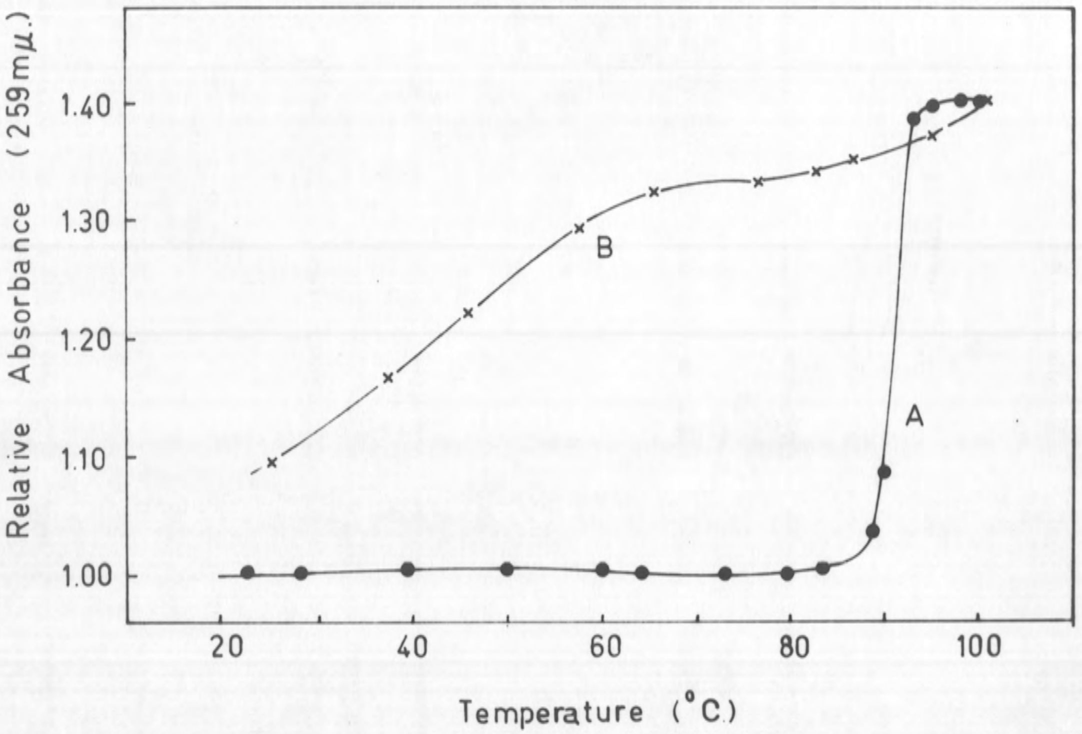


Fig. (III)-6. Temperature dependence of the relative absorbance of E. coli DNA in 0.15M sodium chloride, 0.015M sodium citrate, pH = 7.0. Curve A, native; curve B, quick cooled, heat denatured.

polyelectrolyte theory, explained the linear relation observed between T_m and $\log[Na^+]$.[153] These observations have been associated with the concept that the stability of polynucleotide and DNA helices requires the neutralization or screening of a large proportion of the charged groups on the polynucleotide chains.[157]

The effect of pH on the T_m and the nature of the temperature dependence of the absorbance largely arises from pH induced helix-transition. This is illustrated in Fig. (III)-5 for poly-A. In alkaline solution poly-A exhibits a gradual rise in extinction as the temperature increases. This has been explained on the basis of the melting of the regions containing some degree of secondary structure, and is in accord with the increase in absorbency of poly-A in the presence of concentrated urea.[136] The loss of a more highly organized structure is indicated by the melting curve of poly-A under acid conditions. ($T_m = 81^\circ$ in 0.15M NaCl + 0.015M sodium citrate). Fresco and Klemperer[121] have demonstrated the variation of the T_m for the helix-coil transition of poly-A on the pH, the ionic strength and to a lesser extent on the chain length.

e. The effect of ionic strength.

The influence of ionic strength on the on-set of the helix-coil transition is reflected in the dependence of T_m and pH_m on the ionic strength as previously mentioned. In addition, at constant temperature and pH, the absorbance of polynucleotides may depend on the ionic strength of the solution. Fig.(III)-4 shows the effect of salt concentration on the optical absorbance of poly-A in the alkaline form.

(ii) Optical Rotation and Optical Rotatory Dispersion.

Research into the optical rotatory power of synthetic polypeptides and in proteins reflected configurational regularities in the polymer chain.[158] The existence of a similar relation between optical activity and secondary structure has been investigated for polynucleotides and nucleic acids. It was predicted that a polynucleotide chain with a completely random configuration should exhibit a specific rotation at 5,890 Å, $[\alpha]_D$, close to 0^o.[159] When the chain had a helical configuration a substantial specific rotation should occur, due to the interaction of the polarized light with the ordered array of optically active centres in the helically wound backbone chains. Thus, disruption of the secondary structure by heat, pH changes or by denaturing agents should be reflected by changes in $[\alpha]_D$.

The thermally induced helix-coil transition of many helical polynucleotides has been examined via optical rotation and the results obtained have been consistent with the predictions above.[24,80,121,159,160] In addition, the specific rotation-temperature profile is usually identical to the optical density-temperature profile, although some departures are observed with DNA.[80]

Close similarity also exists between the temperature dependence of the optical rotation and the optical density of incompletely ordered polynucleotides and nucleic acids, including the alkaline form of poly-A,[121] tobacco mosaic virus RNA, and heat denatured calf thymus DNA.[161] The invariance of the optical density and the optical rotation of poly-U with temperature, the low hypochromicity and the value of $[\alpha]_D$ which is very close to the specific rotation of the monomers, are all evidence of the completely random configuration already proposed for poly-U at temperature above ca. 10°C.[122]

In addition to the temperature dependence of the optical rotation of polynucleotides and nucleic acids, other factors known to modify polymer configuration have also been shown to change the optical rotatory power. Thus, the specific rotation, $[\alpha]_D$ has been used as a useful, but empirical, index of helical content in polynucleotides and nucleic acids.[146]

The specific rotation of poly-A under various experimental conditions are summarized in Table (III)-3.

TABLE (III)-3.

Optical rotation of poly-A in aqueous solutions.

Solvent	pH	T^0C	$[\alpha]_D$	Reference
0.1M NaCl, 0.1M sodium acetate	4.85	20 80	374° 11.2°	24
0.15M NaCl	7	22 90	155° -8°	159
0.15M NaCl, 8M urea	7	22	75°	159
10^{-3}M sodium cacodylate, $2x10^{-4}$M $MgCl_2$	7.5	20	84°	50
10^{-2}M NaCl	4.0	20	ca.180°	This work
0.1M NaCl, 0.1M sodium acetate	4.85	20	-42°*	24

* AMP

The optical rotatory dispersion of polynucleotides and nucleic acids and its relation to configuration, has been studied in attempts to examine the molecular and electronic interactions involved in polymer structure. For most synthetic polynucleotides and native nucleic acids, the dispersion data fit one term Drude equations,

$$[\alpha] = \frac{K}{\lambda^2 - \lambda_c^2}$$

over a considerable range of wavelengths.[24,122-3,159,160] This is true for poly-A irrespective of the polymer conformation, however λ_c values calculated, (λ_c is supposed to indicate the wavelength of an optically active transition) varies in a regular manner with the degree of helical content.[159]

Thus, changes in the formal helical conformations of polynucleotides and nucleic acids can be followed by changes in the specific rotation and by changes in λ_c. Examination of the anamolous dispersion data obtained in the near ultraviolet absorption band (230-300 mμ)[160,161] also indicate a dependence on configuration, and these data are consistent with RNA and DNA having common element of helical structure.[159,161] However ORD measurements in this region present experimental difficulties owing to the high absorbance of the purine and pyrimidine residues, and hence the potential value of this region in the elucidation of polynucleotide chain configuration has not been fully realized.

(iii) Infrared Spectra.

Some infrared spectral data has been obtained for poly-A using films prepared by evaporation of polynucleotide solutions onto silver chloride discs and by using solutions of polynucleotide in D_2O. Morgan and Blout[162] found that films prepared from acidic solutions of poly-A had sharper bands, particularly in the 1,240 and 1080 $cm.^{-1}$ regions, than those prepared from alkaline solutions. The four main absorption bands observed, with band centres at about 3,300, 1,650, 1,240, and 1,080 $cm.^{-1}$ were characteristic of nucleic acids. The adenine ring absorbs near 1,650 $cm.^{-1}$, the cyclic furanose rings at about 1080 $cm.^{-1}$ where some contribution from ionic phosphate groups is likely, and the NH and OH groups are expected to absorb at 3,300 $cm.^{-1}$ However the spectrum may be modified by other interactions and secondary structure.

Miles and coworkers[94,163,164] have studied the infrared spectra of polynucleotides in D_2O solution, and used this method in the determination of the kinds and amounts of different polymer species and complexes in solution. In particular they have utilized the region 1,750-1,550 $cm.^{-1}$

(poly-A bands in this region are 1,628 cm.$^{-1}$ and 1057cm.$^{-1}$) to follow the formation and to determine the strandedness of polynucleotide complexes.

(iv) Nuclear Magnetic Resonance Spectra.

High resolution NMR techniques have been utilized by several groups in attempts to obtain information about the hydration of nucleic acids, and there have also been structural studies on mononucleotides, mononucleosides and on nucleic acids and polynucleotides.[165] The technique has yielded detailed information on inter- and intra-chain interactions such as the fractional helical content and the rate of fluctuation between the ordered and disordered states.

The NMR spectra of poly-A, poly-U and poly-(A+U) complexes in D_2O solution, and the pH and temperature dependence of the spectra, have been reported,[165] and the results are in accord with data from other investigations. For example, the fractional disordered content estimated for poly-A at pH 6.1 agreed within 5% with the estimates from optical density measurements. When a completely ordered structure is present, as in poly-A under suitable conditions, and in poly-I, poly-(A+U), poly-(I+C), no high resolution NMR spectrum is observed.[165] In contrast, poly-U at temperatures above 20°C exhibits NMR behaviour indicative of the complete absence of helical structure, with free internal rotation about the ribose-phosphate and ribose-base linkages.

5. Conclusions.

The physico-chemical investigation of polynucleotides leads to some general conclusions. The data obtained by most techniques available can only be interpreted in terms of a transition from a more ordered to a less ordered state or vice versa, depending on the energetically favoured state for the conditions prevailing. In addition to this helix-coil transition, polynucleotides also exhibit configurational changes under certain conditions which are typical of linear polyelectrolytes. The configuration of polynucleotide chains is, therefore, very sensitive to changes in environment and hence it is difficult to assign a model configuration to a real polymer system in order to evaluate various molecular parameters. This is particularly true in the case of incompletely organized forms such as poly-A in neutral or alkaline solution, but even the ordered forms cannot be simply represented by a model shape. Evidence has been cited which shows that poly-A under acidic conditions exists in a doubly stranded helical structure, but that the size of the complex varies depending on the conditions during acidification. It is considered that aggregates containing more than two poly-A molecules can form.

The general problem in polymer chemistry, of obtaining reproducible data at low concentrations in order to be able to extrapolate experimental data accurately to infinite dilution and so effectively eliminate intermolecular interactions, is also encountered with polynucleotides and nucleic acids. This problem is sometimes accentutated, as in the case of DNA, by the denaturation of the native structure at high dilution.

The study of nucleotide-containing polymers has been further complicated by the frequent polydispersity of the polymer samples and by the difficulty in obtaining completely pure nucleic acid or polynucleotide samples. Contamination by protein can seriously affect physico-chemical data since nucleoprotein complexes do not exhibit purely the nucleic acid properties, and if certain enzymes are present in this protein, enzymic degradation of the polymer may occur. Various treatments can be used to remove these contaminating proteins (these are discussed in Chapters IV and V).

Thus although a general concept of the size, shape and polydispersity of polynucleotides, and an idea of the way these factors depend on the environment of the macromolecule, can be obtained from physico-chemical studies of their aqueous solutions, the quantitative characterization of these polymers is difficult. Any interpretation or correlation of data is strictly dependent on the knowledge of the particular conditions existing when such data were obtained.

Chapter IV

THE PHYSICO-CHEMICAL BEHAVIOUR OF POLYNUCLEOTIDES IN NON-AQUEOUS SOLUTIONS, WITH PARTICULAR REFERENCE TO POLY-A.

1. Introduction.

2. The solubility of polynucleotides and nucleic acids in non-aqueous solvents.
    (i) Alkali metal salts.
    (ii) Quaternary ammonium salts.

3. Macromolecular properties.
    (i) Viscosity.
    (ii) Sedimentation.

4. Optical properties.
    (i) Ultraviolet absorption spectra.
    (ii) Optical rotation and optical rotatory dispersion.

5. The effect of solution in non-aqueous solvents on the nature and properties of the recovered polynucleotides and nucleic acids in aqueous solutions.

6. General Discussion.

1. Introduction.

Investigations into the effect of non-aqueous solvents on the properties of nucleic acids have been undertaken in an attempt to understand, more fully, the factors involved in the formation and maintenance of a regular secondary structure in these polymers. Particular emphasis has been placed on the study of DNA, although RNA and some polynucleotides have also been studied under non-aqueous conditions. Such systems have been considered as providing a possible means for estimating heterogeneity in polynucleotide and nucleic acid samples, and as a basis for comparing various preparations *in vitro*. However, information concerning these macromolecules in non-aqueous solutions cannot be expected to bear any direct relation to the configurations which exist under biological conditions.

In general, the native configurations of the more ordered polynucleotides and nucleic acids do not appear to persist in non-aqueous solutions, and thus model configurations appropriate to denatured material have been used in the interpretation and discussion of their physico-chemical properties. These properties have been studied using pure, non-aqueous solvents and solvent mixtures and the resulting data have been instrumental in modifying the earlier ideas that hydrogen bonding was the only dominant stabilizing force in the native, double-stranded DNA helix. These data, together with data concerning the behaviour of quaternary ammonium salts of polynucleotides (particularly poly-A) and nucleic acids will be discussed in the following sections. The effect that solution in organic solvents has on these polymers will also be considered.

2. The solubility of polynucleotides and nucleic acids in non-aqueous solvents.

(i) Alkali metal salts.

The direct solubility of simple salts of DNA (e.g. sodium, and potassium salts) is limited to a very small number of pure solvents, including water, ethylene glycol, formamide and hydrazine,[166,167] all of which are highly polar, reactive or hydrogen bonded liquids. Geiduschek and Gray[168] showed that solution of DNA in 98% ethanol could be achieved by dialyzing electrolyte-free aqueous solutions of DNA against increasing concentrations of ethanol in water. Subsequently this procedure has been used to prepare solutions or dispersions of DNA, RNA and some polynucleotides in various organic solvents.[23,169,170] In some cases this is only possible at very low polymer concentrations and often aggregation of the polymer material occurs. Solution in non-aqueous solvents is very dependent on the electrolyte content of the original aqueous solution. The dialysis method has been used to obtain solution of nucleic acids in solvents varying widely in polarity, hydrogen bonding capacity and other properties.[23] The most commonly used solvents have been formamide, ethanol and methanol; dimethylformamide (DMF) and dimethylsulphoxide (DMS) have also been used.

The changes occurring in the configuration of polynucleotides and nucleic acids as the relative concentration of non-aqueous solvent increases have been illustrated by Herskovits.[171] $S_{\frac{1}{2}}$ has been defined as the concentration of non-aqueous solvent in a water-solvent mixture which produces one half of the maximum absorbance increase associated with denaturation (e.g. at 259 mμ for DNA), and has been used as a convenient measure of the denaturing power of various solvents.[171]

(ii) Quaternary ammonium salts.

Polyanions can be precipitated from aqueous solutions by a variety of cations. Quaternary ammonium cations are particularly efficient, and have been used in precipitating a variety of polyanions from aqueous solutions, including proteins[172,173] polyphosphates,[173,174] polyacrylic acids,[173] polysaccharides[175] and nucleic acids.[176-178] Jones et al.[176,177] used the quaternary ammonium salts in the isolation of nucleic acid from biological material, and utilized the differing solubilities of the quaternary ammonium salts of DNA and RNA, in sodium chloride solutions of varying concentrations, to affect separation of these nucleic acids. Aubel-Sadron and coworkers have studied the precipitation of nucleic acid material from aqueous solutions, by a variety of quaternary ammonium cations, and the subsequent solubility of the precipitates in a variety of organic solvents.[25,179-181]

It has been shown that the precipitating power of the quaternary ammonium cations is determined mainly by the nature of the longest alkyl chain. For the precipitation of nucleic acids, the maximum power tends to be associated with the C_{16} side chain.[25,180] Hexadecyltrimethyl ammonium cations have been most widely used as a precipitant for nucleic acids, and have been particularly useful since there is virtually no absorption in the ultraviolet region where nucleic acids absorb. It has been shown in this work that in addition to the precipitation of DNA and of most types of RNA, hexadecyltrimethyl ammonium cations also precipitate synthetic polynucleotides, particularly poly-A, poly-U and poly-I. The conditions under which precipitation of polynucleotide occurs are the same as for naturally occurring nucleic acids.

Aubel-Sadron <u>et al</u>. have shown that the wet precipitated or freshly lyophilized quaternary ammonium salts of DNA (DNA-Q) and of RNA (RNA-Q) are soluble in many polar organic solvents. Methanol (dielectric constant is 32.6 at 25°C[182]), ethanol (24.3), propanol

(20.1) and DMF (36.7) are excellent solvents for DNA-Q, RNA-Q and S-RNA-Q,[25,180] and have been found to be good solvents for poly-A-Q, poly-I-Q and poly-U-Q. Non-solvents include benzene (2.28), dioxane (2.21), chloroform (4.81) and ethylacetate (6.02). In general, solvents in which the quaternary ammonium salts are soluble are also solvents in which Herskovits <u>et al</u>.[23] were able to obtain solutions of nucleic acids by the dialysis method.

The solubility of RNA-Q and DNA-Q in ethanol-water and methanol-water systems has also been studied.[25] RNA-Q is soluble in methanol only if the water content is less than 25%, and its solubility in ethanol drops rapidly if more than 35% of water is present. Similar results have been obtained for the solubility of poly-A-Q in alcohol-water mixtures.

The conditions for optimum precipitation between hexadecyltrimethly ammonium bromide and nucleic acids have been shown, by Aubel-Sadron <u>et al</u>., to occur when the mole ratio of quaternary ammonium salts to nucleotide is between one and two. The resulting complex has a nitrogen to phosphorus ratio (N:P) in good agreement with the value calculated on the basis that one hexadecyltrimethyl ammonium ion is attached to every phosphate group in the nucleic acid[25] (see Table (IV)-1). The N:P ratios obtained for the quaternary ammonium salt of poly-A are also in accord with the calculated value for the ratio (see Table (IV)-1).

TABLE (IV)-1.

The experimentally determined and calculated values of the N:P ratio for the sodium and the hexadecyltrimethyl ammonium salts of nucleic acids and poly-A.

Sample	N:P (exp.)	N:P (calc.)
poly-A-Q	2.73 2.76	2.71*
DNA-Q[25]	2.02	2.15*
RNA-Q[25]	2.20 2.22 1.92	2.15*
poly-A	2.20	2.26
Calf thymus DNA		1.67

* calculated for one hexadecyltrimethyl ammonium ion per nucleotide.

Although the accuracy of the nitrogen and phosphorus analyses is limited, these results justify the assumption that the complexes are formed by a salt-type bond between a hexadecyltrimethyl ammonium cation and the phosphate anion of each nucleotide. Molecular weight determinations, by light scattering, are also in agreement with this model.[181]

Desiccation of poly-A-Q and poly-U-Q over phosphorus pentoxide or with other drying agents rendered these precipitates insoluble in ethanol, propanol and DMF. Analogous behaviour was observed by Aubel-Sadron _et al_. in that DNA-Q and RNA-Q which had been dried with 2-ethoxyethanol was not soluble in organic solvents.[25]

3. Macromolecular properties.

The polyelectrolyte behaviour of polynucleotides and nucleic acids in aqueous solutions is particularly expressed in the dependence of their hydrodynamic properties on the ionic environment and in the anomolous viscosity behaviour observed. These factors have been considered in Chapter III. Some modification of the hydrodynamic properties of these polymers in non-aqueous solutions is observed as the salt concentration in these solutions is changed. The polyelectrolyte nature of nucleic acids in non-aqueous solution has been shown by Herskovits et al.[23] A small but significant equivalent conductance was observed for poly-A-Q in ethanol in the absence of added salt (Fig. (IV)-1). Since aggregation and precipitation of nucleic acid from non-aqueous solutions occurs in the presence of low concentrations of salt, only limited physico-chemical investigations have been possible in non-aqueous solvents and the effect of added electrolyte on the properties of these polymers has been studied for water-solvent mixtures.[25,169]

The degree of ionization of polynucleotides and nucleic acids, and the consequent electrostatic inter- and intra- molecular interactions which modify the chain

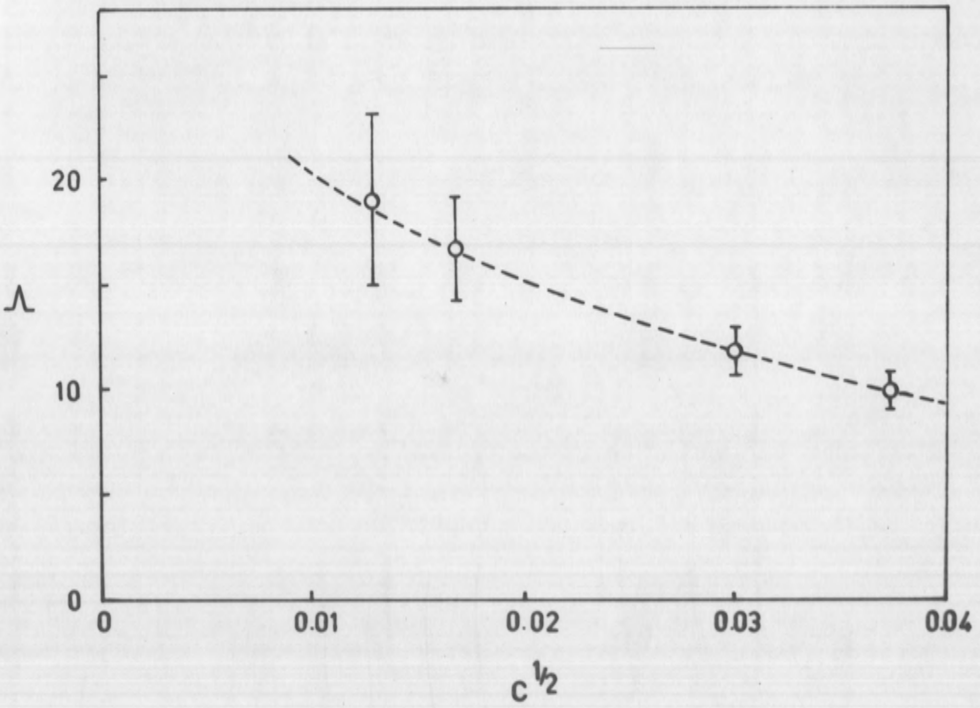


Fig.(IV)-1. Equivalent conductance, Λ_o, of poly-A-Q in ethanol at 20°C, as a function of concentration. C is molarity of poly-A-Q phosphorus.

configuration, are strongly influenced by the dielectric constant of the solvent medium.[171] The degree of dissociation of the sodium salt of DNA in ethanol has been estimated to be less than 0.2,[23] compared with a value of approximately 0.4 in water;[183] value close to 1.0 would be anticipiated for DNA in formamide. The concentration of electrolyte required to suppress the electrostatic interactions of the polynucleotide chains would be greatest for solvents of high dielectric constant (e.g. formamide, 110[182]), and some correlation between the effective size of the polymer molecule and the solvent dielectric constant can be expected. For example as the dielectric constant is decreased, the tendency for the polymer chain to collapse should increase. However, this is not the only factor operating since, even under conditions where electrostatic interactions have been eliminated, various solvents cause differing degrees of change in the configuration and properties of the polymer chain.[171]

(i) Viscosity.

Preliminary investigations by Ts'o _et al._[24] indicated that DNA and microsomal RNA in pure formamide had very high reduced viscosities ($\eta_{sp}/_{c} > 100$ dl/g. and $\eta_{sp}/_{c} > 10$ dl/g respectively) which were relatively independent of concentration. This suggested that the polynucleotide chains were highly extended under these conditions, a view which is compatible with the high electrostatic repulsion between the charged groups on the polynucleotide backbone. This was further substantiated by the marked lowering in the viscosity when electrolyte was added to the formamide solution.[24]

In ethanol, DNA displays properties characteristic of collapsed high-polymer chains.[23] The viscosity, under salt free conditions, is considerably lower in ethanol, and to a lesser extent in methanol, than in aqueous solutions. In the presence of copper ions or at low electrolyte concentrations, the viscosity of DNA in ethanol is further reduced, however the tendency towards aggregation, and the configurational heterogeneity of the polymer, limits the reproducibility of measurements.[23] Further evidence of the decreased viscosity of nucleic acids in non-aqueous solutions has been accumulated from studies using water-solvent mixtures. For example, the intrinsic viscosity of DNA in methanol-water mixtures shows a sharp drop at approximately 80-85% methanol (v/v) depending on the ionic strength. This drop parallels the onset of marked changes in other polymer properties.[169]

Steiner[170] has found a comparable drop in the viscosity of poly-A when it is dissolved in methanol. In water, containing 0.001M sodium acetate $[\eta]$ = 16.8 dl/g for poly-A while in methanol (0.001M sodium acetate) $[\eta]$ = 0.26 dl/g.

The viscometric behaviour of the quaternary ammonium salts of poly-A and of E.coli DNA in ethanol, have been studied in an attempt to investigate their configurations, and the effect, if any, of the quaternary ammonium ion on the properties of these polymers.

a) Time dependence of viscosity.

Solutions of poly-A-Q in ethanol, which had been prepared from poly-A samples synthesized or those obtained commercially (see Chapter V - 5) without special treatment, showed a decrease in viscosity with time. (Fig. (IV)-2.) Similar behaviour was observed for high molecular weight calf thymus RNA-Q in ethanol, and in aqueous poly-A solutions. No significant decrease in viscosity was found for a degraded sample of RNA-Q in ethanol, or for either calf thymus DNA-Q or E.coli DNA-Q (Fig. (IV)-3) in ethanol. The rate of decrease in viscosity was at a maximum in freshly prepared ethanolic solutions, but decreased essentially to zero after several days.

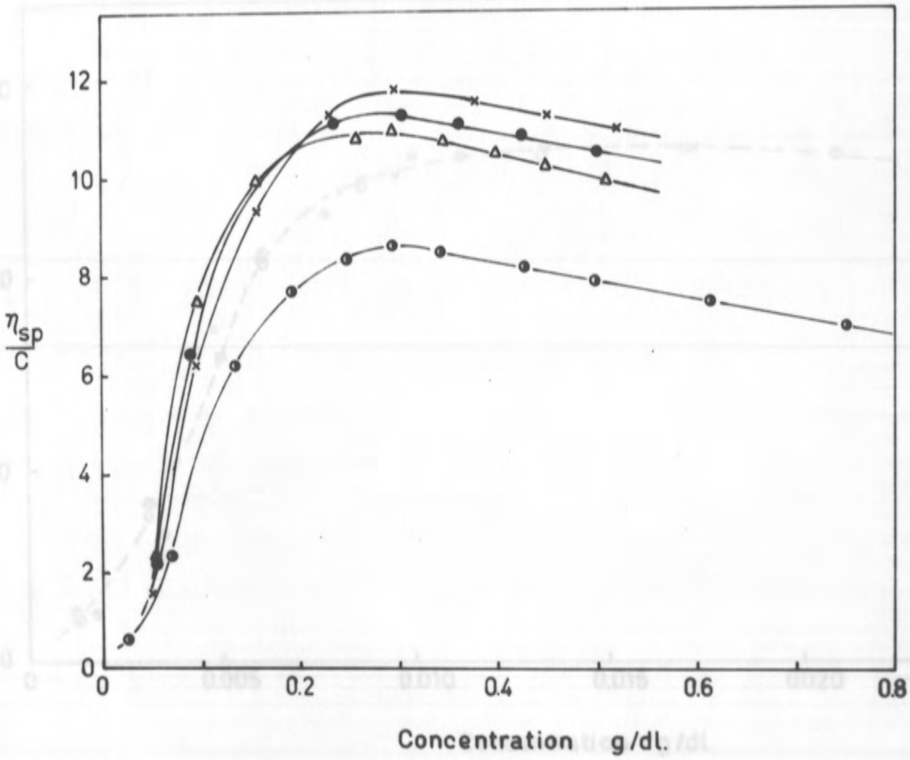


Fig. (IV)-2. The variation of reduced viscosity, (η sp/c, dl/g) with concentration for poly-A-Q in ethanol, at 25.0°C.

- × freshly prepared;
- ● after 24 hours at 25°C ;
- △ after 4 days at -15°C;
- ⊙ freshly prepared, for poly-A sample having lower molecular weight.

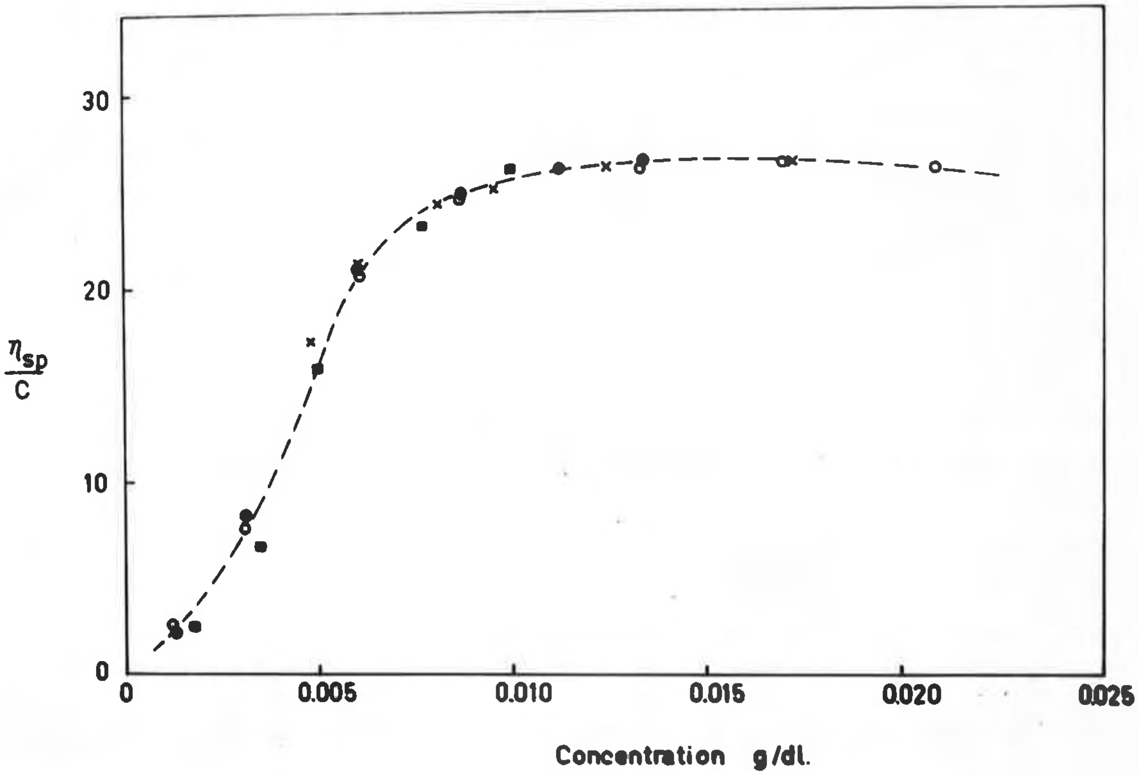


Fig.(IV)-3. Reduced viscosity (η sp/c, dl/g) of DNA-Q in ethanol as a function of concentration. T = 25.0°C

- ● freshly prepared;
- × after 1 day;
- o after 2 days;
- ▪ after 10 days.

Similar time dependence of viscosity has been observed in many aqueous polynucleotide systems,[127] where it has, in general, been attributed to nuclease contamination of the polymer. Such contamination may arise from nucleases, remaining in the polynucleotide preparation or may be introduced by handling[184] or released by trace bacterial contamination.[18] The presence of traces of metal ions has also been suggested as a contributory cause.[21] Extensive deproteinization of samples only slightly improves the stability of polynucleotides in aqueous solution, but reproducible data have been obtained following treatment with bentonite or hectorite clays[17,185] or in the presence of certain polymeric materials.[122,186] Szer and Ochoa[17] stabilized poly-T by brief heating of its solution to 85°C. Chamberlain et al. have recommended that handling of polyribonucleotides should be carried out under sterile conditions, using gloves to prevent contamination of samples.[18]

In an attempt to eliminate the time dependence of the viscosity of poly-A-Q in ethanol, the poly-A samples used were subjected to phenol deproteination using sterile conditions, and the polymer solutions were lyophilized after extensive dialysis (See Chapter

V-6). Freshly prepared solutions of the lyophilized poly-A were treated with quaternary ammonium bromide (See Chapter V-8), again using sterile conditions. Solutions of poly-A-Q in ethanol prepared using this procedure were more stable than solutions prepared from untreated poly-A. No significant decrease in viscosity occurred over a period of one day, during which viscosity experiments could be carried out. The relative stability of DNAQ solutions in ethanol did not necessitate a special purification for the DNA.

Although the activity of ribonuclease tends to be reduced by organic solvents, including ethanol,[187] the relative time dependence of the viscosity of aqueous poly-A and of poly-A-Q in ethanol were not significantly different if the polymer had been treated by the procedure above. The time dependence of viscosity of ethanolic poly-A-Q solutions was not changed by addition of small quantities of water and was not dependent on temperature at which the solution was kept in the temperature range -15°C to 20°C. Shear degradation and absorption of the polymer were also shown to be unimportant.

b) Concentration dependence of viscosity.

In the absence of added salt, the viscosity-polynucleotide concentration curves for both poly-A-Q and DNA-Q in ethanol exhibited maxima at concentrations of 0.01-0.03 g/dl. (see Figs.(IV)-2,3,4). This behaviour is similar to viscosity maxima, observed for many polyelectrolytes and for salt-free solutions of RNA,[126] which have been mentioned previously (Chapter III-3-(i)). It has been suggested that the viscosity maxima which have been observed for polyelectrolyte, at low concentrations of added electrolyte or in salt-free solutions, result from the presence of ionic impurities or from the absorption of atmospheric carbon dioxide.[113] The latter possibility was eliminated in this examination of the viscosity of poly-A-Q in ethanol since all measurements were carried out under an atmosphere of nitrogen in the viscometer (see Chapter V-12), and solutions in the viscometer were mixed by gently passing a stream of ethanol-saturated nitrogen through the solution. However, the viscosity maxima observed for poly-A-Q and for DNA-Q in ethanol may well reflect the presence of traces of quaternary ammonium, bromide, sodium or chloride ions, or other impurities introduced, or not eliminated during the preparation of the quaternary ammonium compounds or from the ethanol used for the solutions. The presence

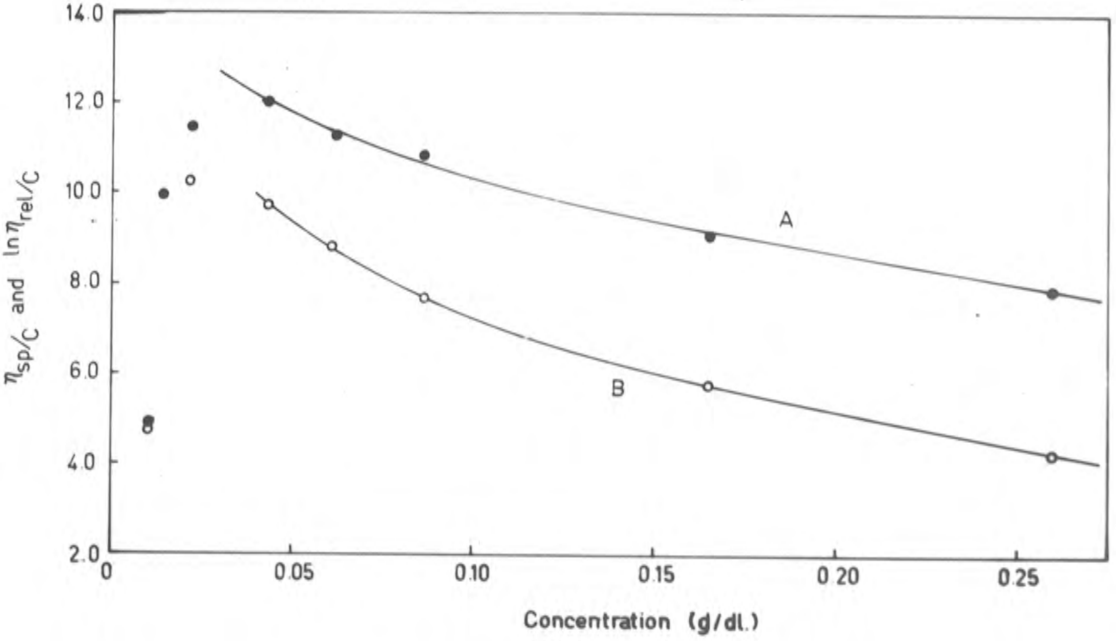


Fig.(IV)-4. Viscosity of poly-A-Q in ethanol as a function of concentration at 25.0°C.

Curve A, η sp/c

Curve B, ln $\eta_{rel/c}$

of small amounts of water, necessary to ensure solution of the DNA-Q or poly-A-Q precipitate, may also be significant in this respect.

The viscosity data obtained using "salt-free" solutions of poly-A-Q in ethanol (see for example, Fig. (IV)-4) can be reasonably fitted by the Fuoss equation[118] at concentrations greater than where the viscosity maxima occurred. (Fig. (IV)-5). This plot gives a value of $[\eta] = 18.5$ dl/g, a value of the same order as that obtained by extrapolation to infinite dilution of the $\eta_{sp}/_{c}$ against c for concentrations greater than 0.03 g/dl. This portion of the viscosity concentration curve is relatively insensitive to small amounts of added electrolyte which give rise to the anomalous viscosity behaviour at low polymer concentrations; differing small amounts of electrolytes change the concentration at which the maximum occurs, but not the slope of the curve at concentrations above this maximum.[113] However, estimation of an unambiguous value of the intrinsic viscosity of poly-A-Q in ethanol is clearly difficult.

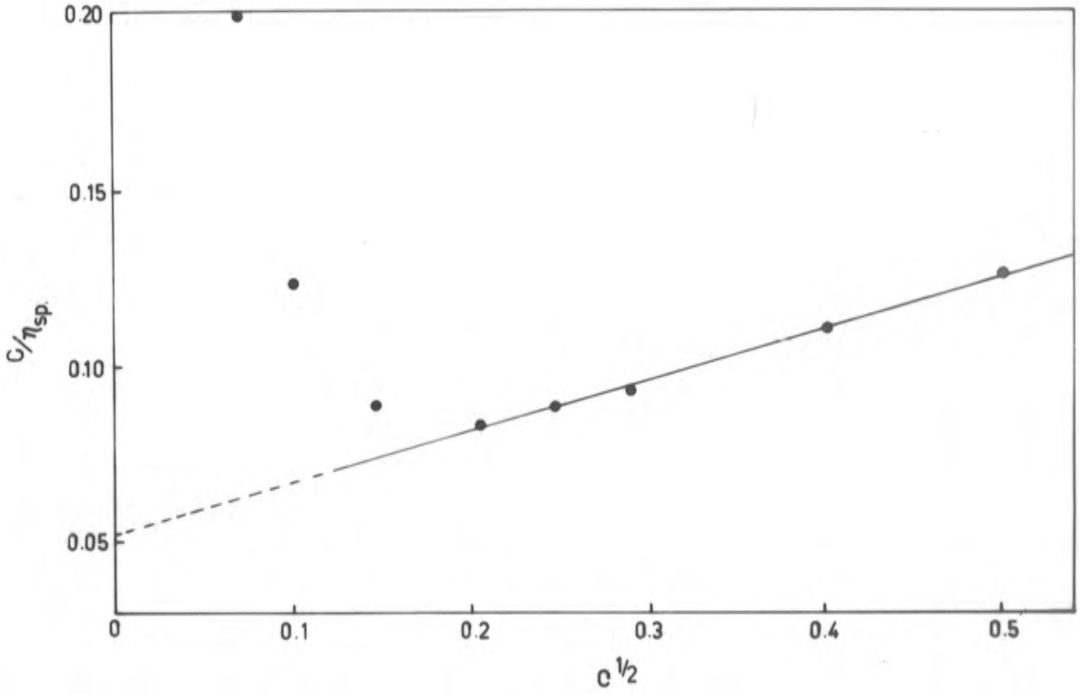


Fig.(IV)-5. Reciprocal reduced viscosity against $C^{\frac{1}{2}}$ curve (Fuoss plot) for poly-A-Q in ethanol.

c) The dependence of viscosity on the concentration of added salt.

The lowering of the viscosity and the suppression of the maxima in the viscosity-concentration curves in the presence of added electrolyte has already been mentioned with reference to aqueous solutions of nucleic acids and polyelectrolytes (Chapter III-3-(i)). A fall in the viscosity of non-aqueous solutions of nucleic acids occurs when electrolyte is added, and this has been interpreted as indicating that in these solutions nucleic acids behave as a polyelectrolyte.[23,24,190] Analogous behaviour has been observed for poly-A-Q in ethanol (Fig.(IV)-6). The addition of salt, either sodium bromide or hexadecyltrimethyl ammonium bromide, to the ethanolic solution causes a large decrease in viscosity and virtually eliminates the viscosity maximum discussed above. (Fig. (IV)-7.) The salt concentration, $[Q]$, used in these figures refers only to the concentration of added quaternary ammonium bromide. When the salt concentration is greater than approximately 0.03M, precipitation of the poly-A-Q compound occurs.

The marked decrease in viscosity at low concentrations of added electrolytes (i.e. below about 0.004M) is compatible with the idea of shielding of the electrostatic repulsive forces on the polymer chain,

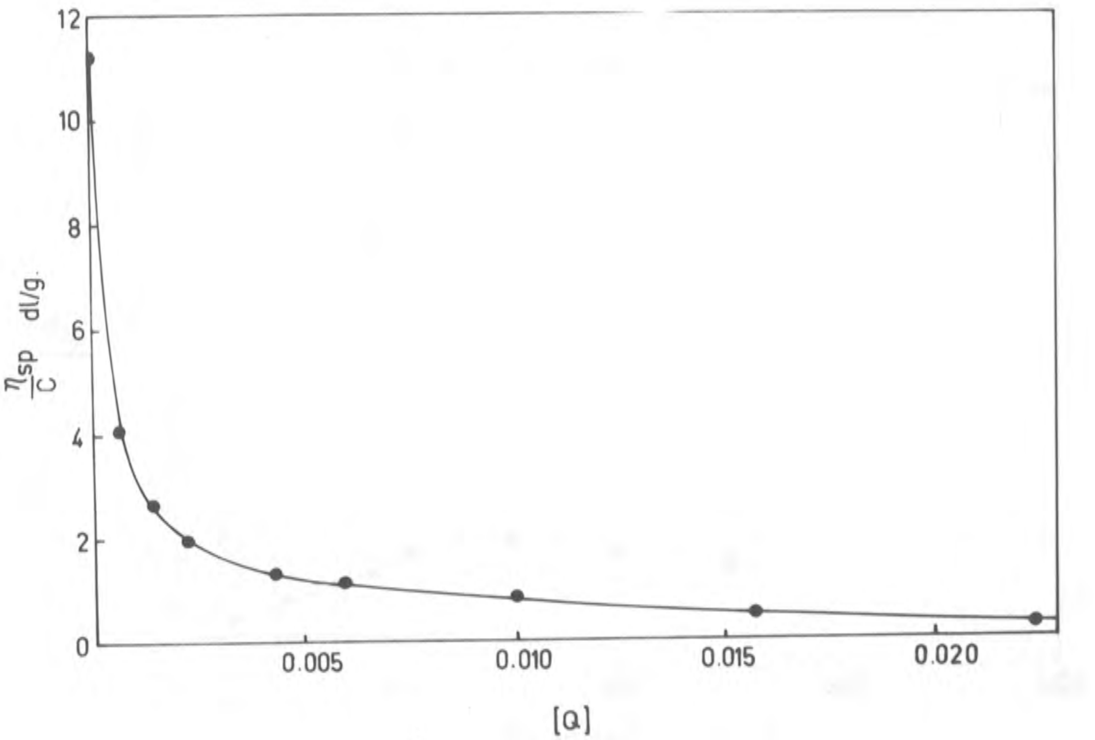


Fig.(IV)-6. Reduced viscosity of poly-A-Q in ethanol. ($C = 0.21_6$ g/dl) as a function of the molar concentration of hexadecyltrimethyl ammonium bromide, **[**Q**]**, at 25.0°C.

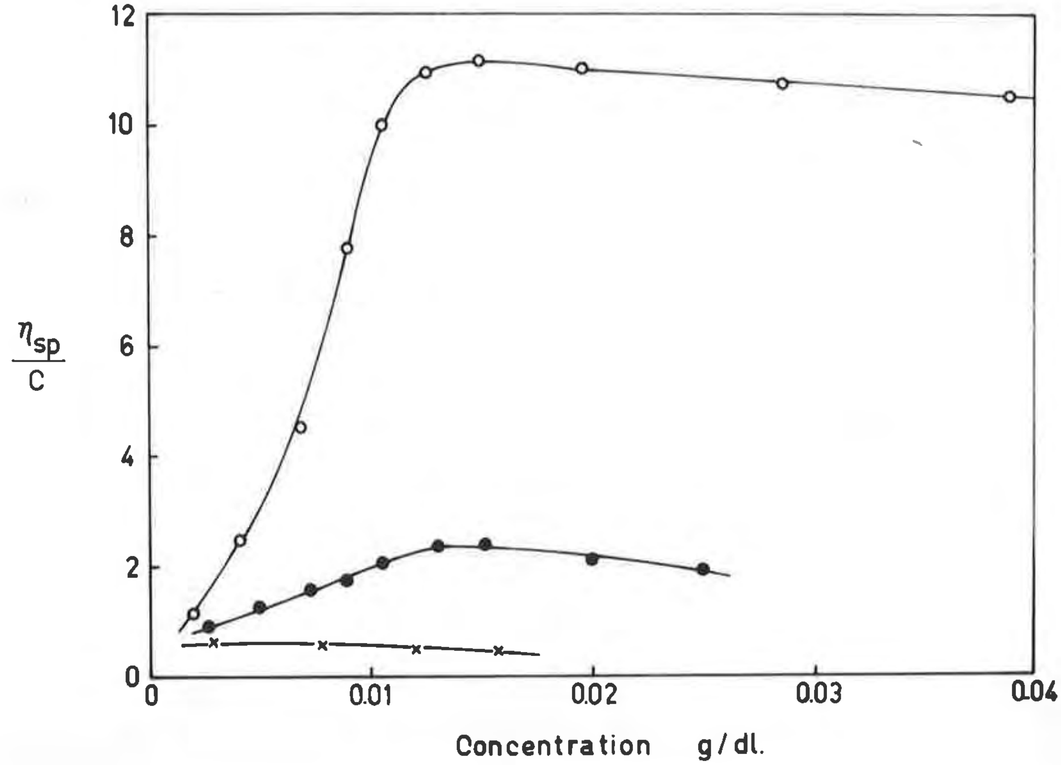


Fig.(IV)-7. Reduced viscosity (dl/g) of poly-A-Q as a function of concentration

- ○ in pure alcohol;
- ● in 0.002M quaternary ammonium bromide in ethanol;
- × in 0.03M quaternary ammonium bromide in ethanol.

causing a change in polymer configuration. Owing to the lower dielectric constant of ethanol, compared to aqueous solvents, the association of gegen-ions would be greater in ethanol, thus the polymer will have a lower total charge and consequently the influence of charge changes on the polymer configuration, in the presence of salt, will be less. The linearity of the double logarithmic plot of approximate intrinsic viscosity against cation concentrations, for cation concentrations in the range 10^{-2} - $10^{-3.5}$M, (Fig.(IV)-8), and the slope of this plot (0.58), can be interpreted on the basis of the Flory theory, as considered by Cox,[124] to indicate that poly-A-Q molecules in ethanol exist as random coils which are virtually impermeable to solvent. This configuration is in agreement with the collapsed polymer configuration proposed for DNA in ethanol.[23] Under these conditions there should be no significant shear-dependence in the viscosity, it would therefore be of interest to study the viscosity of poly-A-Q in ethanol under varying rates of shear.

(ii) Sedimentation.

The sedimentation behaviour of nucleic acids in non-aqueous solvents differs from that observed in aqueous systems. The sedimentation coefficients of

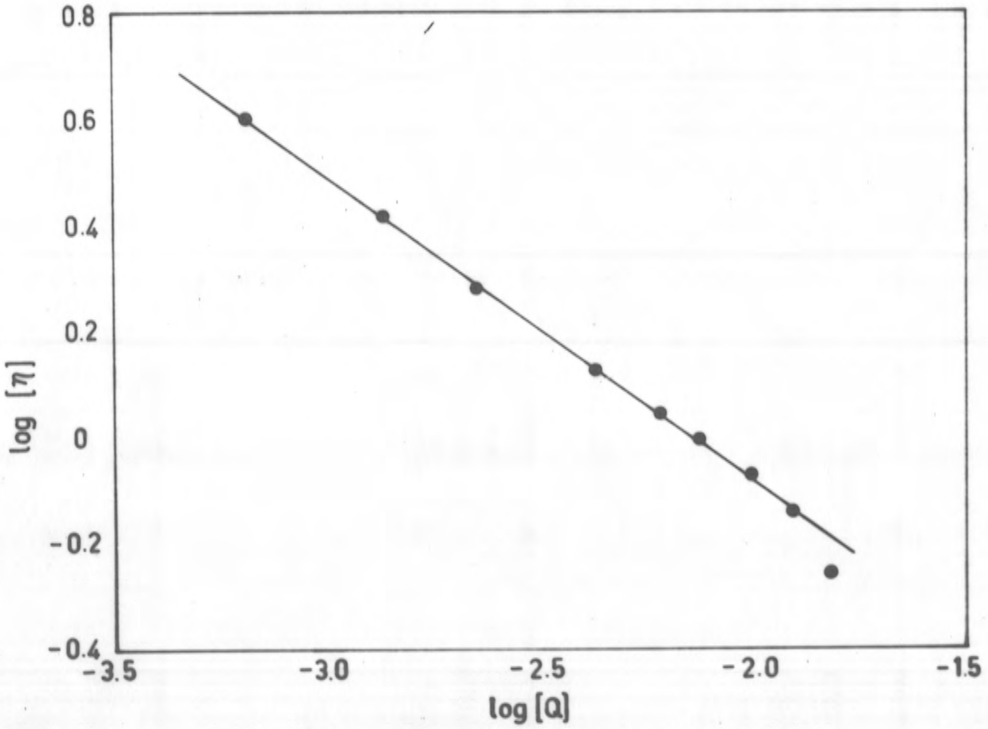


Fig.(IV)-8. A double logarithmic plot showing the dependence of [η] dl/g, (approximate intrinsic viscosity = η sp/c for C = 0.021_6 g/dl) of poly-A-Q in ethanol, on the cation concentration, [Q]. T = 25.0°C.

DNA and of microsomal RNA in formamide are very low (<2) and are very dependent on the concentration of added electrolyte.[24] This is consistent with the viscosity data already cited, and is in agreement with the highly extended configuration proposed.

The sedimentation boundaries found for DNA in ethanol solutions are not hyper-sharp, as in the case of DNA in aqueous salt solutions, and spread rapidly with time yielding normal, symmetrical boundaries.[188] The sedimentation coefficients observed are very high, and although aggregation might occur in some cases, these high values are considered to reflect a collapsed structure. Similarly, high values for the sedimentation coefficient have been observed for poly-A in methanol. Coates and Jordan found that S^o for calf thymus DNA in ethanol was more dependent on molecular weight than for dilute aqueous salt solutions, and hence they studied the sedimentation of DNA in ethanol in an attempt to gauge the heterogeneity of the DNA samples. High values for the sedimentation coefficient have also been obtained for DNA in other non-aqueous solvents, and in many cases well defined sedimentation boundaries have been recorded.[23] However the absence of traces of metal ions is necessary if aggregation of the nucleic acid is to be avoided.

A sharp change in the average sedimentation constants of nucleic acids in water-solvent mixtures, occurs when the concentration of non-aqueous solvent reaches a particular value. However this transition occurs at higher alcohol concentrations than do the changes in viscosity and ultraviolet absorption. Geiduschek and Herskovits[169] have proposed that the sedimentation change probably reflects an end point of the transition from an extended to a more compact configuration.

Comparison of the sedimentation coefficients of nucleic acids in different solvents requires the consideration both of the different characteristics of the solvent (i.e. density, viscosity) and the relative structure of the polymer. In addition to the inherent difficulties, associated with the interpretation of polymer and of polyelectrolyte sedimentation data which were considered in Chapter III, there may be a greater uncertainty of the polymer configuration of nucleic acid and polynucleotides in non-aqueous solutions. The aggregation tendencies also tend to obscure the true sedimentation properties in these systems.

Sedimentation data for ethanolic solutions of DNA-Q have not been very consistent[181] and the results obtained indicated that the configuration of DNA-Q molecules in ethanol solution differed from one treatment to another, even using the same DNA sample. These differences have been attributed, in part, to the presence of water in the organic solutions.[181]

The sedimentation of poly-A-Q in ethanol has been studied, using a schlieren optical system to observe the sedimenting boundary. Interference optics could not be used owing to the distortion of the interference fringes in high centrifugal fields. The sedimentation boundaries observed in the absence of added electrolyte (Fig.(IV)-9) are not as hypersharp as in the case of aqueous poly-A solutions (Fig.(III)-1), and boundary spreading was not excessive in the course of a sedimentation run. Under these conditions, the experimental plots of lnr against time (where r is the distance from the sedimenting boundary to the axis of rotation) was not strictly linear over the time interval considered. The approximate S values evaluated from these data are shown in Fig.(IV)-10. Values of the sedimentation coefficient have also been computed from these data, using the method of Billick,[189] so that any pressure dependence of the sedimentation rate was eliminated. These values are also shown in Fig.(IV)-10.

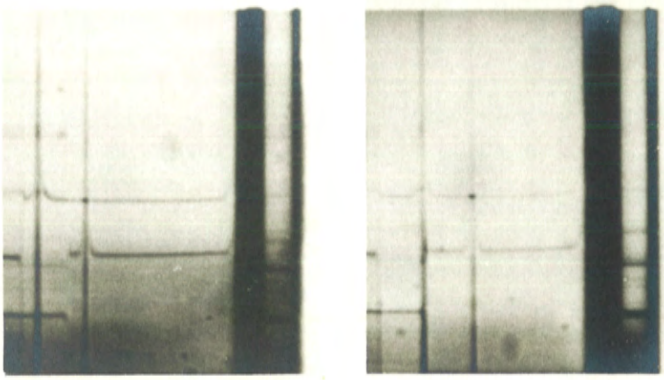
Fig.(IV)-9. Sedimentation of poly-A-Q in ethanol, $T = 25^{o}C$, speed = 59,780 r.p.m., photographs taken at interval of 56 mins. The direction of sedimentation is from left to right.

A. Leading boundary, conc. = 0.43_1 dl/g
Following boundary, conc. = 0.34_5 dl/g.

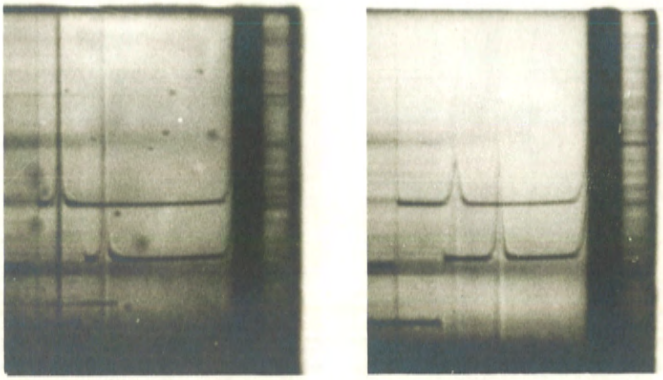
B. Leading boundary, conc = 0.25_8 dl/g
Following boundary, conc. = 0.17_3 dl/g.

C. Leading boundary, conc. = 0.08_6 dl/g
Following boundary, cond. = 0.06_5 dl/g.

A



B



C

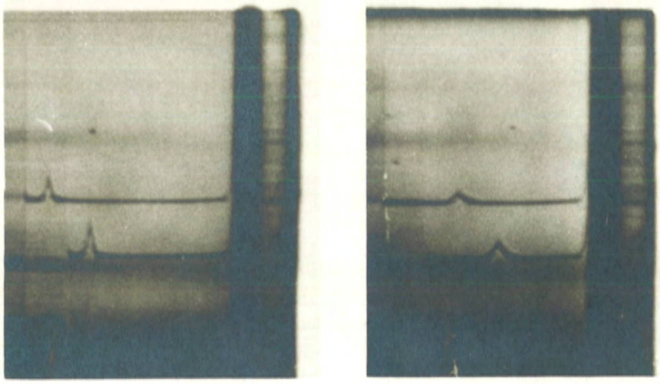


Fig.(IV)-10

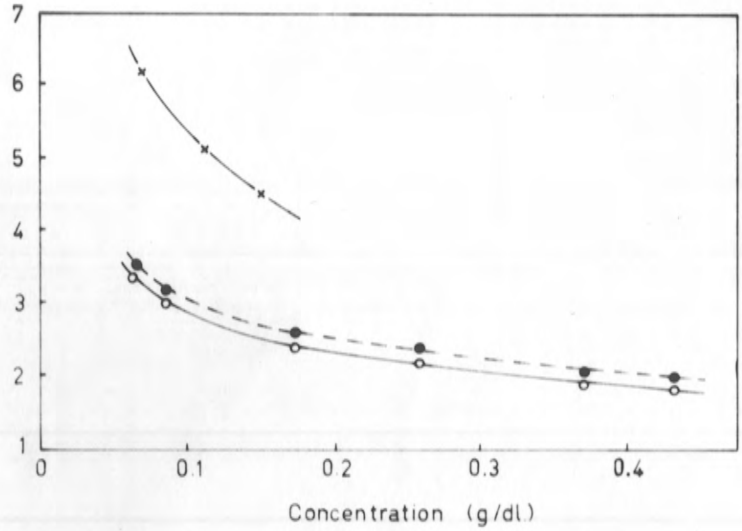
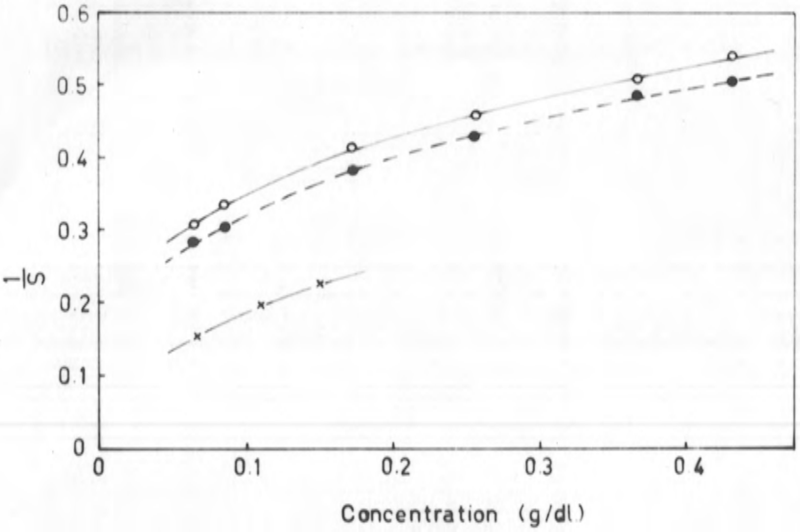


Fig.(IV)-11.



Figs.(IV)-10 and (IV)-11.

Concentration dependence of the sedimentation coefficient, S, and of 1/S for poly-A-Q in ethanol.

- ○ values calculated (see text)
- ● values of S_o computed by method of Billick[189]
- × S values for higher molecular weight sample.

The sedimentation coefficients measured for poly-A-Q in ethanol cannot be used to obtain an accurate value for S^o, the sedimentation coefficient at infinite dilution, due to the curvature of the $\frac{1}{S}$ versus concentration curve (Fig. (IV)-11). Such an extrapolation would be more reliable if sedimentation data for lower poly-A-Q concentrations were available, e.g. those obtained using ultraviolet absorption techniques to measure the rate of sedimentation. However, at these low polymer concentrations boundary spreading is more serious, and this would limit the precision of the value of S obtained.

The polyelectrolyte behaviour of poly-A-Q in ethanol which is indicated by the anomalous viscosity behaviour at low polymer concentrations and by the dependence of the viscosity on the concentration of added salt, is also evident in the sedimentation behaviour. Both the degree of boundary spreading and the sedimentation coefficient (corrected for changes in solvent viscosity) are significantly increased as the concentration of added salt is increased (see Figs.(IV)-12, (IV)-13). The increase in sedimentation rate is similar to that observed

Fig.(IV)-12. The effect of added salt on the sedimentation of poly-A-Q in ethanol. Poly-A-Q concentration = 0.18 g/dl; speed = 59,780 r.p.m., Sedimentation is from left to right.

A. Leading boundary, pure ethanol

following boundary, [Q] = 0.001M

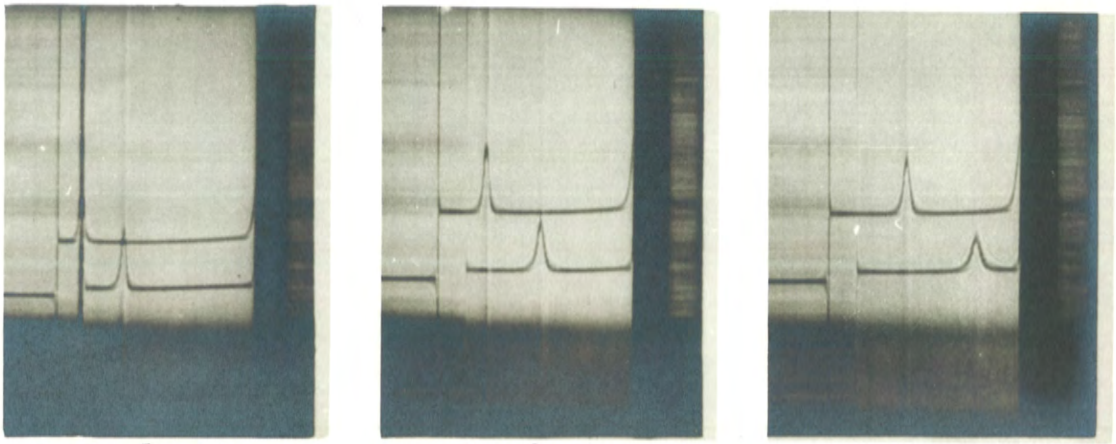
Photographs 2 and 3 were taken 32 and 72 mins. respectively after photograph 1.

B. Leading boundary, [Q] = 0.005M

following boundary, [Q] = 0.010M

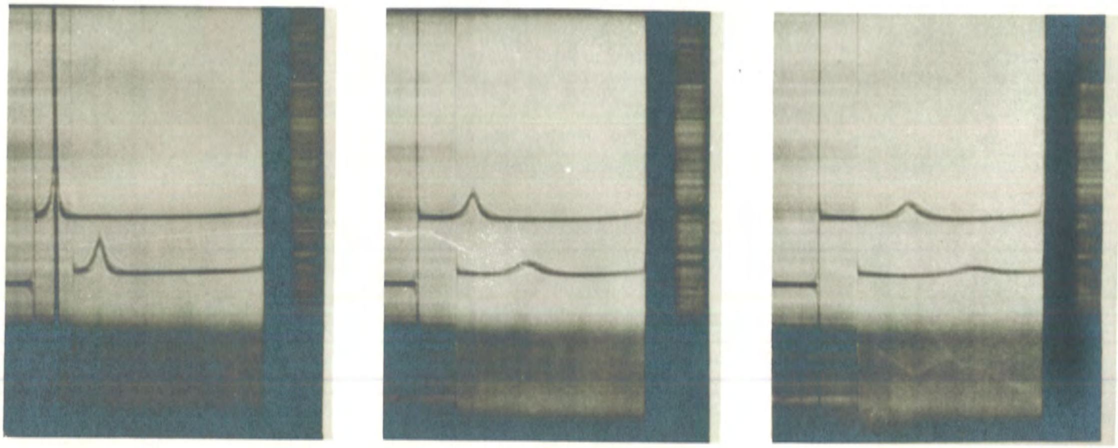
Photographs 2 and 3 were taken 20 and 40 mins. respectively after photograph 1.

A



1. 2. 3.

B



1. 2. 3.

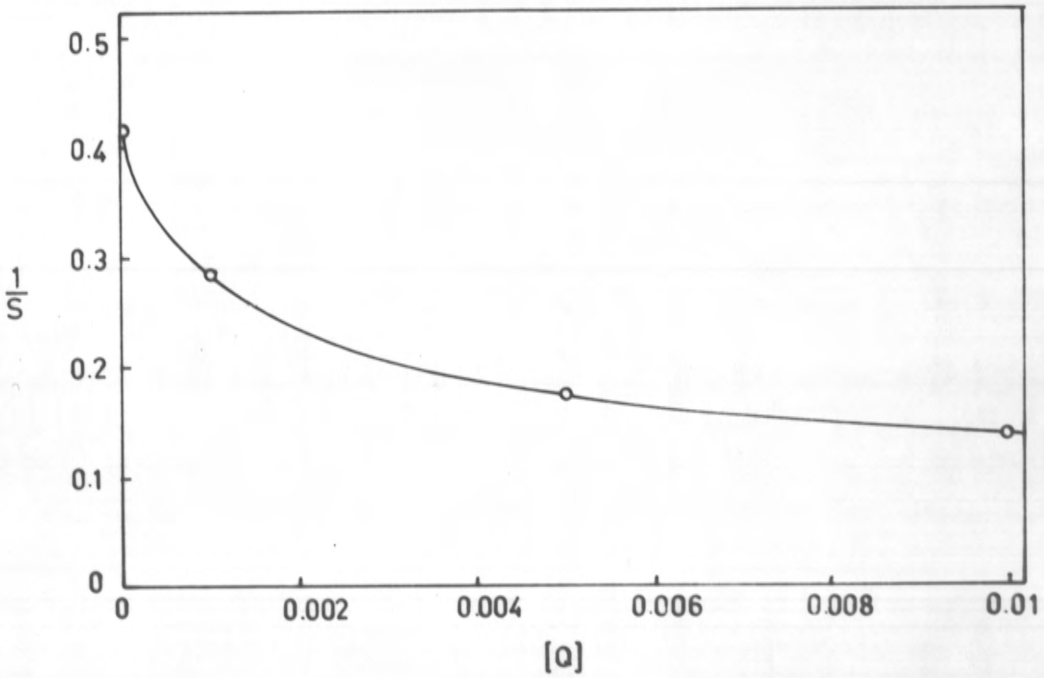


Fig.(IV)-13. Variation of the reciprocal of the sedimentation coefficient for poly-A-Q in ethanol (conc. = 0.18_1 dl/g) with the molarity of added hexadecyltrimethyl ammonium bromide, [Q].

for aqueous solutions of poly-U as the ionic strength increases.[122]

4. Optical Properties.

The optical properties of nucleic acids and polynucleotides in non-aqueous solvents have been extensively studied, particularly with respect to the changes in the configuration of the polymer chain on going from aqueous to non-aqueous conditions. Since low concentrations are required for most optical measurements, they are particularly useful in cases where only limited polymer solubility occurs. However, measurements, in the ultraviolet wavelength region, are restricted to a few organic solvents including ethanol, methanol and formamide. Many other organic solvents absorb strongly at wavelength below approximately 270 mμ and thus obscure the absorption bands of interest in nucleotide-containing materials.

(i) Ultraviolet Absorption Spectra.

The ultra-violet spectral properties of nucleic acids and polynucleotides, either as inorganic salts or as the quaternary ammonium salts, in non-aqueous solvents (Fig. (IV)-14) differ from those of aqueous solutions of native polymer. This is particularly evident in the higher ϵ_p values, (i.e. low hypochromicity) in non-aqueous solvents as shown in Table (IV)-2.

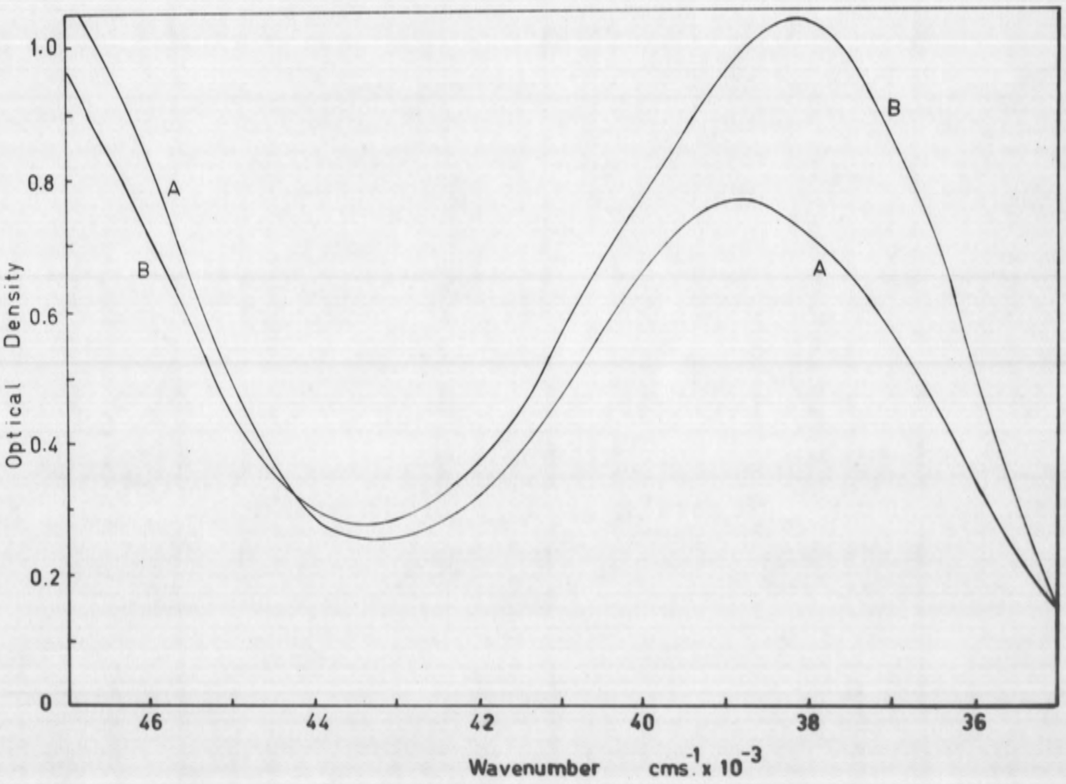


Fig.(IV)-14. Ultraviolet absorption spectra of A. poly-A-Q, and B. poly-U-Q in ethanol at 25°C.

TABLE (IV)-2

Spectral properties of some nucleic acids and polynucleotides in non-aqueous systems.

Material	Solvent	T^{o}C	λ max. mμ	$(\epsilon_P)_{max.}$ x10^{-3}	λ min. mμ	Ref.
poly-A	formamide	26	260	13.8		146
poly-A	methanol (0.001M sodium acetate	20	260	13.6		170
poly-A	aqueous s.c.* pH 7.5	25	257.1	9.9_8	229	this work
poly-A	aqueous s.c.* pH 7.5	90		12.2		this work
poly-A	ethanol	25	259	11.9	230	this work
poly-A-Q**	ethanol	25	257	11.5	231	this work
AMP	formamide	26	260	14.8		146
AMP	0.1M NaCl, 0.05M Tris pH 7.5	20-80	260	15.4		146
poly-U-Q**	ethanol	25	261.8		230.4	this work
poly-I-Q	ethanol	25	247		228	this work
E.coli DNA	aqueous s.c.* pH 7.0	25	259	7.1_0	230	this work
	aqueous s.c.* pH 7.0	98	259	9.9_4		this work
E.coli DNA-Q	ethanol	25	260	9.9_0	232	this work
Calf thymus						
DNA	0.1M NaCl		259	6.8_4		181
DNA-Q	ethanol		260	9.1_0	230	181
DNA	0.2M NaCl		259	6.7_0		23
DNA	ethanol			9.5_0		23
Yeast RNA	0.1M NaCl		259	7.8_9		181
RNA-Q	ethanol		260	9.9_7		181

TABLE (IV)-2 - contd.

* 0.15M sodium chloride, 0.015M sodium citrate buffer.

** See Fig. (IV)-14.

Herskovits[171] has observed an increase of 35-50% in ϵ_p associated with the denaturation transition of most DNA samples in water-solvent mixtures. Helmkamp and Ts'o[146] cite a difference of 32% between the ϵ_p values of pea microsomal RNA in 0.1M sodium chloride and in formamide. Similar increases have been observed by Aubel-Sadron *et al*. for calf thymus DNA going from 0.1M sodium chloride to DNA-Q in ethanol[181] and in this work for *E.coli* DNA under similar conditions. The ϵ_p value for *E.coli* DNA in ethanol is almost identical with the value for the heat denatured material in neutral saline citrate when the absorbance is measured at a temperature above T_m.

The value of ϵ_p for poly-A-Q in ethanol determined experimentally (11,500) is approximately 15% greater than that for poly-A in aqueous saline citrate, pH = 7.5. This increase is similar to the increment of 11-26% cited for yeast RNA.[181] If the ϵ_p value for poly-A in acidic solutions (helical configuration) is used as the basis, the increase in ϵ_p is comparable to that observed for DNA. Since the quaternary ammonium bromide has a very low extinction near 260 mμ, the changes in ϵ_p observed can be related to the changes in the configuration of the polymer chains.

Thus the extinction coefficients measured for DNA-Q and poly-AQ are in agreement with the loss of ordered structure in these polymers which was suggested by their macromolecular properties.

In contrast to the temperature dependence of the ultraviolet absorbance of aqueous solutions of nucleic acids and poly-nucleotides, discussed in Chapter (III)-4-(i), there is no significant temperature dependence shown by solutions of these polymers in salt-free, non-aqueous solvents.[24] Studies on DNA in water-solvent mixtures have shown that the T_m values in salt free methanol and ethanol are below room temperature,[23,169] but in both water-methanol and water-ethanol mixtures, T_m increases with increasing ionic strength. As the relative concentration of organic solvent is increased, the dependence of T_m on ionic strength became less pronounced.[23] A variation of T_m, and hence extinction, of nucleic acids on the ionic strength has been shown for formamide[190] and other solvents.[171] In general these effects have been attributed to the "electrolyte" effect, in that the presence of added electrolytes tends to reduce the intramolecular electrostatic forces by shielding the charged groups, and thus modifies the configurational changes. This is consistent with the insensitivity of T_m values to ionic strength, above a particular ionic strength, which is largely determined by the dielectric constant of the medium.[171] The effect of temperature on the absorbance of E.coli DNA and poly-A-Q, at 259 mμ and 257 mμ respectively, has been examined. No significant change in absorption could be measured over a temperature range of 20-75°C (See Figs. (III)-5 and (IV)-16.).

The optical absorbance of poly-A-Q in ethanol was relatively insensitive to the addition of low concentrations of sodium bromide, hexadecyltrimethyl ammonium bromide, or water. When the water content was less than 10% (v/v) the molar extinction was constant, within experimental accuracy, but increased gradually at higher water contents. (ϵ_P increased by 3% as water content was increased to 30%). When the water content exceeded 35%, precipitation of the poly-A-Q occured. The water content in the ethanolic solutions of poly-A-Q and DNA-Q used in these studies is considered to be less than 5-6%, and hence the use of optical absorption measurements to estimate polymer concentrations is justified, particularly as separate measurements have shown that poly-A-Q in ethanol obeys Beer's law over the concentration range 10^{-6}-10^{-4}M at $\lambda_{max.}$ = 257 mμ , λ = 230 mμ and λ = 265 mμ . The optical density of salt-free solutions of DNA-Q and poly-A-Q in ethanol remained constant for periods of at least one month, regardless of concentration or temperatures (below 30°C), provided that evaporation was eliminated.

(ii) Optical rotation and Optical rotatory dispersion.

The convenient, but empirical, relation between optical rotation and optical rotatory dispersion with the degree of secondary structure has been discussed with reference to aqueous solutions of polynucleotides and nucleic acids (Chapter III-4-(ii)). The optical rotatory properties of these polymers in non-aqueous solvents have been intepreted on a similar basis, and have, in fact, provided much of the evidence indicating the absence of secondary structure in the polynucleotide chains under such conditions.

In general, the specific rotation, $[\alpha]$, of RNA, DNA and polynucleotides in salt free non-aqueous solutions differ markedly from those observed in aqueous solutions. $[\alpha]$ values are very low or negative, compared to the high positive rotations of polymers with high helical content, and $[\alpha]$ is essentially independent of temperature.[24,169,191] Free nucleotides, in formamide or dimethyl sulphoxide, also have $[\alpha]$ values which are lower than their values in aqueous solution, however this decrease is much less than the decrease observed for the polymers. (See for example Table (IV)-3.)

TABLE (IV)-3.

The specific rotations, $[\alpha]$, of poly-A and the adenine mononucleotide in various solvents.

Material	Solvent	T^{o}_{C}	$[\alpha]_{589m}$	Ref.
AMP	acetate buffer* pH4.85	20	-42°	20
AMP	formamide	20	-47°	20
Poly-A	acetate buffer* pH4.85	20	+374°	20
Poly-A	acetate buffer* pH 4.85	80	+11.2°	20
Poly-A	saline citrate pH7.5	20	+154°	20
poly-A	saline citrate pH7.5	80	+8°	20
Poly-A	formamide	20	-38°	20
Poly-A	formamide	80	-44°	20
Poly-A	0.001M sodium acetate	20	+100°	6
Poly-A	methanol**	20	-25°	6
Poly-A	10^{-2}M NaCl, pH 4	22	ca. +180°	this work
Poly-A-Q	ethanol	22	ca. -20°	this work

* 0.1M NaCl, 0.1M sodium acetate.

** methanol containing 0.001M sodium acetate.

The optical rotations, at $\lambda = 436$ mμ and $\lambda = 589$ mμ, of polynucleotides and nucleic acids in various water-solvent mixtures have been studied[169,191] and the values were found to decrease as the solvent content increased, with the breadth of the transition varying with the nature of the solvent and the type of nucleic acid. In the case of RNA a gradual transition occurred.

A marked lowering of $[\alpha]$ occurs when poly-A goes from aqueous to non-aqueous solutions, as shown in Table (IV)-3. Irrespective of whether the polymer is in the form of its sodium salt or of its quaternary ammonium salt in the non-aqueous medium, the value of $[\alpha]$ decreases to a value close to that observed for the free nucleotides, or for poly-A in aqueous solutions at elevated temperatures. The optical rotatory dispersion of poly-A-Q in ethanol, (See Fig.(IV)-15), is also consistent with the concept that the poly-A chain, in this system, has virtually no helical content, being similar to that observed for poly-A in formamide or in aqueous solutions at high temperatures.[191].

When added salt is present in the non-aqueous solutions of nucleic acids, such as formamide solutions containing potassium chloride, $[\alpha]$ is higher than in salt-free solutions, and shows a significant temperature dependence.[167,190] This observation is consistent with

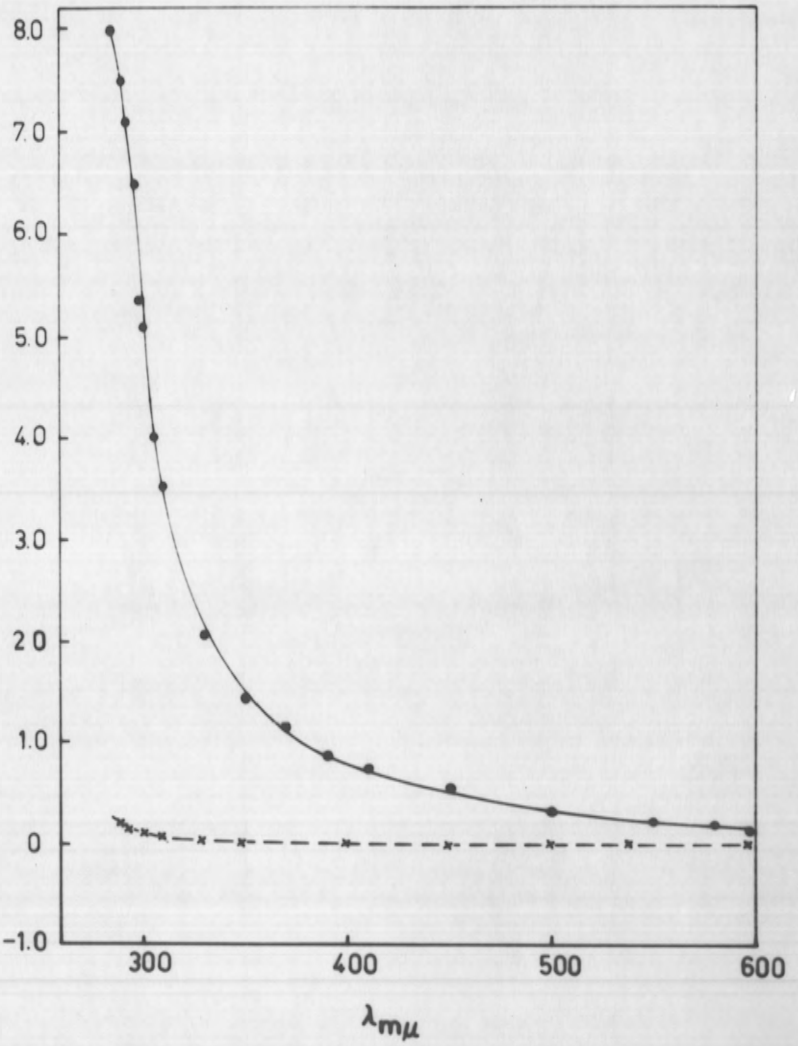


Fig.(IV)-15. Optical rotatory dispersion of poly-A in 10^{-2}M sodium chloride, pH = ca. 4 (●), and of poly-A-Q in ethanol (×), at 22°C.

the macromolecular properties of these polymers in non-aqueous solvents containing added salt, (particularly in formamide) and indicates an incomplete loss of secondary structure.

5. The effect of solution in non-aqueous solvents.

In the preceding sections, the macromolecular and optical properties of two groups of salts of polynucleotides and nucleic acids in non-aqueous solutions have been discussed. The available data has been interpreted in terms of a loss of the formal secondary structure of the polymer chain in these systems, leading to collapsed, random coils. Furthermore, the actual conformation of the polymer chain depends on the nature of the solvent and on the concentration of any other component in the solution, and is considered to be, at least in part, due to the relative electrostatic inter- and intramolecular interactions. The extent of the denaturation and of strand separation, and the reversibility of the transition when the organic solvent is removed, are of considerable interest. The study of this behaviour has yielded information concerning the formation and stability of secondary structure in nucleic acids.

The extent of denaturation, indicated by optical criteria, depends on the solvent used. Herskovits[171,192] has concluded that both the ionizing power and the hydrogen bonding capacity are important in complete denaturation of DNA since the denaturing ability of solvents increases with increasing hydrocarbon content. For example, propanol and ethanol are more effective than methanol, and the alkyl substituted solvents DMS and DMF are more effective than formamide.[171] Similar findings, on the relation between structure and effectiveness as denaturing agents for DNA, have been reported by Levine <u>et al</u>.[193] There is some evidence that the base composition of DNA has some effect on the efficiency of non-aqueous solvents as denaturing agents. Nucleic acids, and polynucleotides in non-aqueous solutions as their quaternary ammonium salts appear to be completely denatured, as judged by optical criteria.

Although a collapsed, denatured configuration has been assigned to nucleic acids and polynucleotides in non-aqueous systems, there is little unambiguous evidence of strand separation except in the case of formamide denaturation. In formamide, the DNA molecule exists in a highly extended configuration, as shown by its macromolecular properties. Marmur and Ts'o have demonstrated strand separation in the $^{14}N^{15}N$ hybrid <u>E.coli</u> DNA after formamide denaturation, by caesium chloride gradient sedimentation,[194] however, DNA isolated after solution in formamide containing 0.4M potassium chloride, at 25°C is essentially identical to the initial native material.[190] In contrast to this, the average molecular weight ($\bar{M}_w$) determined for nucleic acid samples in alcohols and aqueous solution have been very similar,[23] and the values evaluated from light-scattering data for DNA-Q in ethanol and for the same DNA in 0.2M sodium chloride also suggest that no strand separation has occurred.[181] Geiduschek and Herskovits, on the basis of reversibility studies, have also concluded that no strand separation in DNA occurs in methanol solutions.[169]

The reversibility of the denaturation, caused by organic solvents, is usually considered in terms of the macromolecular, optical and biological properties of the material recovered from the non-aqueous solution, relative to the properties of the initial native material. In most cases, the partial denaturation at relatively low solvent concentrations in water-solvent mixtures is quite reversible. For example, the tranforming activity of Diplococcus pneumonia is not effected until more than 55% formamide is present, but at higher formamide contents the activity falls rapidly.[194] This transition corresponds to the change in optical absorbance as the formamide content increases.[194] Partial renaturation can be achieved if the formamide denatured material is heated at 65°C for some time and then slowly cooled. Formamide treatment does not destroy the biological activity of TMV RNA or soluble RNA, and evidence accumulated indicates that no scission of the polynucleotide backbone occurs.[24] In general, the degree of reversibility of formamide denaturation depends on the nature of the nucleic acid, the concentration of added salt and the temperature used.

Herskovits has demonstrated that except for dimethyl sulphoxide, DNA denaturation by organic solvents can largely be reversed by water, in the presence of electrolytes. Alcohol denaturation can be almost completely reversed, while the reversal of the denaturation caused by ethylene glycol, formamide and DMF may be appreciable but is not as extensive as for alcohols.[161,171] The denaturation of homopolynucleotides or copolymers of regular sequence, by organic solvents is completely reversed when these polymers are recovered in aqueous solution.

Polynucleotides and nucleic acids can be recovered as their sodium salts, from non-aqueous solutions of the quaternary ammonium compounds,[25] and the properties of the regenerated material in aqueous solution have been examined in attempts to assess any effect due to the quaternary ammonium cation. Data obtained for E.coli DNA, in this work, have clearly demonstrated that the denaturation produced during the preparation of ethanolic solutions of DNA-Q results from the treatment with the organic solvent. DNA which was converted to

DNA-Q, but then recovered as the sodium salt of DNA without being dissolved in ethanol, (this material is referred to as "cycled" DNA) had optical properties (ϵ_p, T_m) identical to those of native DNA. (Fig. (IV)-16, Table (IV)-4.)

TABLE (IV)-4.

Optical properties of E.coli DNA in 0.15M sodium chloride, 0.015M sodium citrate pH=7.0

ϵ_p and T_m refer to 259 mμ.

Sample	ϵ_p	T_m °C.	OD_{280}/OD_{260}	$OD_{max.}/OD_{min.}$
native	7,100	91.6	0.51_6	2.3_2
"cycled"	7,150	91.4	0.50	2.4
"regenerated"	9,280		0.50	2.1
quick-cooled heat denatured	8,000		0.5_2	2.2_3

The decrease in the ratio $OD_{280\ m\mu}$: $OD_{260\ m\mu}$ and the increase in $OD_{max.}$: $OD_{min.}$ are attributed to some purification of the DNA. This treatment is essentially the same as that used for isolating and purifying nucleic acid materials.[176-178] In contrast to the "cycled" DNA, "regenerated" DNA, which had been recovered from DNA-Q in ethanol solution, has optical properties (ϵ_p and melting profile) consistent with a partially renatured

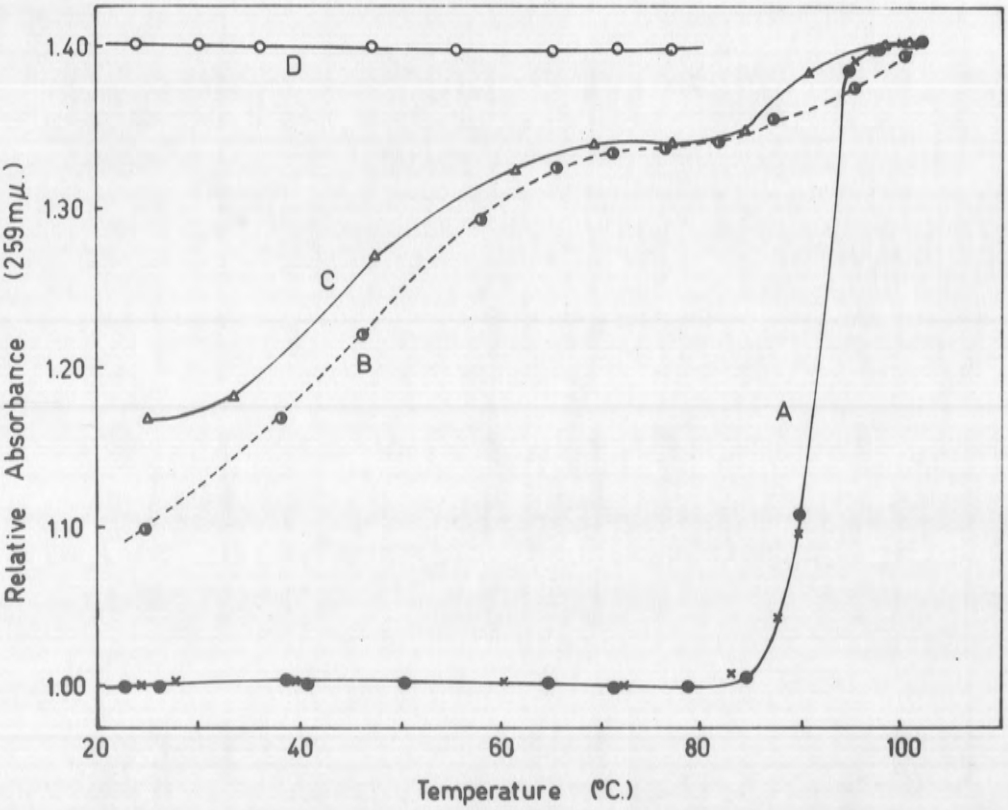


Fig.(IV)-16. Temperature dependence of the relative absorbance of E.coli DNA in 0.15M sodium chloride, 0.015M sodium citrate pH = 7.0

A. ● native, × "cycled"

B. quick cooled, heat denatured

C. regenerated

D. DNA-Q in ethanol

configuration, and similar to those of quick cooled heat denatured DNA in aqueous solution. The denaturation which is associated with the dissolution of DNA-Q in ethanol tends to be less reversible than that observed for the sodium salt of DNA, however this difference may reflect the differences in the methods used to recover the nucleic acid. The dialysis procedure used in the latter case would permit a slower change from non-aqueous to aqueous conditions, and this may facilitate the partial renaturation.

Poly-A samples were not significantly changed by the solution of their quaternary ammonium salts in ethanol, or their subsequent regeneration, except in those polymer samples known to contain some protein contaminant. In the latter samples the optical properties of the regenerated material were comparable to the properties displayed by corresponding samples which had been deproteinized by phenol extractions.

The preparation of the quaternary ammonium salt, solution in organic solvent (particularly DMF and ethanol), and regeneration into aqueous solution did not alter the physico-chemical properties or the biological activity of several RNA samples.[180,181,195] Weil and

Ebel [180] have used this treatment in attempts to fractionate S-RNA. The denaturation of DNA is not completely reversible, and results in a significant decrease in the transforming activity of Bacillis subtilis following regeneration from non-aqueous solutions.[195]

6. General Discussion

Although it is possible to prepare solutions of nucleic acids and polynucleotides in non-aqueous solvents, both as their quaternary ammonium salts and as inorganic salts, the examination of their macromolecular and optical properties has shown that such systems are not suitable for the routine characterization of the samples. This is mainly due to the varying extent of the helix-coil transition, which depends on the solvent and on the conditions used, and to the dependence of the properties and conformation of the polymer chain on the concentration of added electrolyte. However further complications arise from the tendency of the polymeric materials to aggregate, and from the presence of traces of ionic contaminants which tend to cause anomalous hydrodynamic behaviour at low polymer concentrations.

Sedimentation and viscosity data for nucleic acids and poly-A in non-aqueous solvents, other than formamide, have been indicative of a collapsed polymer structure. More direct evidence of this conformation has been obtained from light scattering investigations, which have given very low values for the radius of gyration for nucleic acids in ethanol and methanol.[23] However, aggregation of the nucleic acid material may occur, leading to an under-estimation of the extent of collapse. Aggregation has not been so serious in the case of RNA-Q and DNA-Q in ethanol, and considerably lower values for the radius of gyration have been measured, compared with the values for the initial nucleic acid material in aqueous salt solution.[181] The dissymmetry is also decreased for the DNA-Q in non-aqueous solution but to a greater extent in ethanol than in DMF.[181] The effect of added salt on the light scattering behaviour of the quaternary ammonium salt of nucleic acids has not been studied, but it would be of interest to compare such data with the increased sedimentation coefficients and the decreased viscosity which has now been observed for poly-A-Q in ethanol. This would give a better indication of the extent of the possible aggregation which could cause these changes in the hydrodynamic properties. However, for the most part, this dependence of hydrodynamic properties on the concentration of added salt and the low conductivity which have been observed for poly-A-Q in ethanol, have been interpreted on the basis of poly-A-Q behaving as a polyelectrolyte. Thus, careful definition of the solvent is necessary before this system could be used as a basis for comparing different polynucleotide samples. This requirement is essentially the same as that found for the case of polynucleotides and nucleic acids in aqueous solutions.

In the case of the quaternary ammonium salts of nucleic acids, additional uncertainty arises from the small amounts of water present in the organic solution. Nevertheless, some estimates of their molecular weight have been made. Aubel-Sadron *et al.*[181] have evaluated the molecular weights for DNA-Q and RNA-Q from light scattering data, and have found good agreement between these and the molecular weights calculated from the light scattering molecular weight of the initial nucleic acid material in aqueous salt solution, on the basis of one quaternary ammonium cation per nucleotide. Tsvetkov *et al.*[196] have studied the properties of soluble RNA-Q in organic solvents, including methanol, butanol and benzyl alcohol, and have reported that the dynamic and optical properties are in agreement with the molecules being in the form of statistical coils. They have estimated the molecular weights of their samples from sedimentation, diffusion and viscosity data.[196] A value for the molecular weight of poly-A-Q in ethanol has been calculated from the hydrodynamic data obtained during the present work, using the Flory-Mandelkern equation[130] (see Chapter III - 3-(iii)). The value of $\bar{v} = 0.92$ ml/g used was evaluated from density data for poly-A-Q in ethanol, and values for $[\eta]$ and S^o (18.5 dl/g and 5.5×10^{-13} secs. respectively) were obtained from viscosity and sedimentation data at 25°C.

(Figs. (IV)-5 and (IV)-11.) Since the available evidence suggests a collapsed, coiled structure, β was taken as 2.4×10^6. The molecular weight calculated from these data was 1.8×10^5, however this is an approximate value only, due to the necessary assumption of a shape factor and the uncertainty associated with the estimation of the $[\eta]$ and S_o values, which has been discussed in the relevant sections of this Chapter. A comparison between the average molecular weights obtained from hydrodynamic data and that obtained from light scattering data for the same polymer samples would be of considerable interest.

The difficulties and the uncertainty involved in the measurement and interpretation of the macromolecular and optical properties of nucleic acids and polynucleotides in non-aqueous systems preclude a quantitative determination of size and shape of the polymers in these systems. Many of these difficulties are similar to those encountered during the examination of the physico-chemical behaviour of nucleic acids and polynucleotides in aqueous solution.

Chapter V

EXPERIMENTAL.

1. Bacteriological methods.

2. Preparation of polynucleotide phosphorylase
    - (i) from A.vinelandii
    - (ii) from M.lysodeikticus

3. Enzyme assay

4. Protein estimation
    - (i) Biuret method
    - (ii) Spectrophotometric method
    - (iii) Folin-Ciocaulteu method

5. Polynucleotide synthesis

6. Isolation and purification of polynucleotides

7. Purification of solvents.

8. Preparation of quaternary ammonium salts of polynucleotides and nucleic acids.

9. Spectrophotometry

10. Optical rotatory dispersion

11. Sedimentation

12. Viscometry

1. Bacteriological methods.

The Azotobacter strain used for the preparation of polynucleotide phosphorylase was received from the University of Wisconsin (Azotobacter vinelandii strain O-p, non gummy) and was maintained on agar slopes stored at 1-5°C. Bulk cultures were grown in sterilized, inorganic Burk's nitrogen-free medium, pH 7.3[49,197] using sucrose (2%) as an energy source; slopes were prepared from the same medium but replacing the sucrose by mannitol (1.5%). Solutions other than those containing sucrose were sterilized by autoclaving; sucrose solutions were steamed for 1 hour per day for three days and then added to the sterile inorganic solution. The sucrose and all inorganic reagents were B.D.H. "Analar" grade, and the mannitol used was from May & Baker Ltd., Laboratory chemicals.

Inocula for bulk cultures were prepared by inoculating small shake cultures (50 or 100 mls) directly from the agar slopes. While these cultures were still in the logarithmic growth phase* they were used to inoculate the bulk cultures (400 mls inocula per 6 litres

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* As indicated by measurements of optical density and cell counts on the culture as a function of time.

bulk culture). All growing cultures were incubated at 30°C, and bulk cultures were continuously aerated with air filtered through a sterile cotton wool plug. The bacteria were harvested by centrifugation after about 24 hours. The wet cells were washed with distilled water, centrifuged (about 7,000 g for 10 mins. at 0-5°C), and stored at -15°C until used for enzyme preparation. The yield of wet cells was 2-4 gms per litre.

2. Enzyme Preparations

(i) From Azotobacter.

The procedures of Ochoa and his coworkers[5,49] have been adopted. The efficiency of several abrasives and of sonication in rupturing bacterial cells has been examined and the results shown in Table (V)-1. Sonication was affected in a 9.0 Kc. Raytheon magnetostriction oscillator, model S-102A, operated on 120 volts with plate voltage set to a minimum. The oscillator cup was continuously cooled with ice water to keep contents at 0-4°C.

TABLE (V)-1.

Preparation of cell free extracts from A. vinelandii

(5 gm. samples of wet cells)

Method	Total protein (mg.)[b]
Grinding[a] with silicon carbide (240 mesh)	170
Grinding with silicon aloxite (240 mesh)	200
Grinding with silicon aloxite (400 mesh)	130
Grinding with alumina A 301[c]	350
Sonic oscillation[d]	480

a. Cells ground with 10 gms. abrasive with pestle and mortar, at 2-8°C, extracted with 25 mls 0.15M KCl, 0.001M. EDTA, 0.02M tris buffer pH 7.5 and then centrifuged.

b. Protein estimated by biuret method.

c. Alcoa Alumina A301 (325 mesh) was donated by the Aluminium Co. of America.

d. Cells suspended in 10 mls water and sonicated for 30 mins, and then extracted with additional 10 mls. water.

Polynucleotide phosphorylase solutions were prepared from bacterial extracts, obtained after sonic oscillation, by the method Ochoa.[49] The purification steps, including ammonium sulphate, zinc-ethanol and calcium phosphate gel fractionations, removed much of the contaminating protein (as shown by the increased specific activity), polysaccharide and polynucleotide (as indicated by the changes in the ratio $OD_{280\ m\mu} : OD_{260\ m\mu}$). These are shown in Table (V)-2.

The enzyme eluted from the calcium phosphate gel was used for polynucleotide synthesis. These solutions were not completely free from polynucleotide material, however some preparations did exhibit a lag period prior to polymer synthesis. Enzyme solutions were stored at -18°C in small aliquots, and retained their activity for a period of at least three months.

Although some preparations yielded satisfactory enzyme samples, in general the procedure was not very reproducible. Similar difficulties have been reported by other workers.[39,151] In addition, some samples appeared to be contaminated by some nuclease enzymes, as indicated by the apparent decrease in viscosity in polymer synthesis.

TABLE (V)-2.

Purification of polynucleotide phosphorylase from *A.vinelandii*

(40 gms. wet cells)

Step	Volume mls.	Protein[a] mg.	S.A.[b] units/mg.prot.	OD_{280}/OD_{260}
crude extract	205	3580	-	0.66
ammonium sulphate (35-46%)	30	900	1.7	0.8
zinc-ethanol (15-20%)	18	315	4.5	0.93
calcium phosphate gel	8.4	17	18.0	0.91

a. Specific Activity, enzyme units per mg. protein was determined by phosphate exchange (see V - 3)

b. Protein in initial extract was determined by the biuret method, in subsequent solutions it was estimated spectroscopically (V - 4).

(ii) From Micrococcus lysodeikticus

Polynucleotide phosphorylase was isolated, according to the method of Steiner and Beers,[52] from spray-dried bacterial cells obtained from Calbiochem, Los Angeles, California, U.S.A. Enzyme solutions were stored at -18°C as above. Enzyme samples obtained after the second ammonium sulphate fractionation step were used for enzyme synthesis.

3. Enzyme Assay

The enzyme activity was assayed after each stage during the purification of polynucleotide phosphorylase from A.vinelandii using an assay base on the phosphate exchange reaction catalyted by the enzyme. (See Chapter II equation (2).) The method used was essentially that of Ochoa et al.[5,198] using adenosine diphosphate* as substrate, with the modification that the final aqueous phase containing the "organically bound" radioactive phosphorus was diluted to 8 mls and the radioactivity counted directly using a scintillation counter (EKCO Type N550A) with an automatic scaler (EKCO Type N530D).

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* Sigma Chemical Company, St. Louis, Miss.

The specific activity of the enzyme was expressed as units of enzyme activity per mg. protein, where one enzyme unit is defined[5] as that amount of enzyme which catalyses the incorporation of one micromole of tracer labelled phosphate under the conditions of the assay ($T = 30^{\circ}C$, $pH = 8.1$) in 15 minutes. The number of micromoles phosphate incorporated is given by

$$\text{micromoles } P^{32} \text{ incorporated} = \frac{\text{c.p.m. incorp. } (\mu \text{ moles} P_i + \mu \text{ moles ADP})}{\text{c.p.m. phosphate (initial)}}$$

4. Protein Estimation

The method used to estimate protein concentrations in experimental solutions was dictated by the nature of the particular solution.

(i) Biuret method[199]

When solutions were not completely clear, or if they contained relatively high amounts of nucleic acids, such as during the initial stages of the preparation of enzyme from <u>A. vinelandii</u>, the protein content was estimated by the biuret method. The crystalline ovalbumin used as a protein standard was prepared from fresh egg white by the method of La Rosa[200] and its purity examined electrophoretically, according to Creeth _et al_.[201,202] Ovalbumin concentration was determined by both dry weight and refractive index increment measurements.

(ii) Spectrophotometric method.

In solutions containing only small amounts of nucleic acids (in later stages of the enzyme preparations) protein concentration was estimated from optical density measurements using the data given by Warburg and Christian.[203] Good agreement, between results from this and from biuret estimations, was obtained for clear solutions when the nucleic acid content was less than about 20%. The spectrophotometric method was particularly useful in late stages of the enzyme preparations because of the small amount of material required.

(iii) Folin-Ciocalteu Method.

When small amounts of protein were present in solutions containing large amounts of nucleotide material the method of Lowry _et al_.[204] using Folin-Ciocalteu reagent was preferred. Salt free, crystalline ovalbumin was used as protein standard.

5. Polynucleotide synthesis.

Reaction mixtures used in the synthesis of poly-A and poly-I by the Azotobacter enzyme had the following composition:-

100 mg. nucleoside diphosphate (Sigma) in aqueous solution adjusted to pH 8.1 with 1M KOH.

200	micromolecules	Tris buffer pH 8.1
20	micromolecules	Magnesium Chloride
1	micromolecule	E.D.T.A. (pH 8)
10	units enzyme	

volume about 10 mls.

and were incubated at 30°C.

Large scale preparations, based on 1 gm of nucleoside diphosphate were also carried out. Small scale preparations, or samples from large scale syntheses, where carried out directly in a viscometer thermostatted at 30°C and the extent of the synthetic reaction was estimated from the increase in viscosity of the reaction mixture.

A reaction mixture similar to that used by Steiner and Beers,[53] was incubated at 37°C for poly-A synthesis with the <u>M.lysodeikticus</u> enzyme. The extent of the synthesis was again measured viscometrically, and the amount of enzyme solution used was chosen to give maximal increase in viscosity in 3-4 hours.

The poly-U and one sample of poly-A used in this study were obtained from Calbiochem, Los Angeles, California, and a second poly-A sample was obtained from the Miles Chemical Co., Clifton, New Jersey. The general physico-chemical behaviour of these samples was essentially the same as that observed for polynucleotides samples prepared above, provided all the material had been purified by the same procedure.

6. Isolation and purification of polynucleotides.

Initially polynucleotide material was precipitated from the rapidly chilled reaction mixture by the addition of 2 volumes of cold ethanol (0-2°C).[5] After standing for 1 hour at 0°C, the precipitate was collected, dissolved in a small volume of cold water and reprecipitated with ethanol. The precipitate was again dissolved in water and dialysed exhaustively at 0-4°C against distilled water or 5×10^{-3}M NaCl prior to lyophilization. However some difficulty was encountered in obtaining good precipitation of polynucleotide by this method, and all subsequent polymer samples were precipitated from solution by ethanol in the presence of 0.5M NaCl or KCl.[53]

The removal of contaminating proteins from synthetic polynucleotides is necessary, as in the case of nucleic acids from biological sources, if stable samples are to be obtained. Many deproteinization methods have been used[16,18,38,51,94,205] and in polynucleotides used in this work have been deproteinized with the phenol method. The phenol (B.D.H., Laboratory grade) was redistilled immediately before use. An aqueous polymer solution (approximately 5% solution in 0.01M sodium citrate, 10^{-4}M E.D.T.A. pH 6.0) was shaken vigorously with an equal volume of 90% (w/v) aqueous phenol for 5 minutes at room temperature. After centrifugation the aqueous layer was isolated and the phenol extraction repeated twice. Traces of phenol were removed from the final aqueous layer by extraction with ether and the polynucleotide precipitated from solution as above. The polymer was then dissolved in distilled water and dialysed in the cold against successive changes of 0.01M E.D.T.A., pH8, 1.0M NaCl and 0.005M NaCl prior to lyophilization. This procedure was carried out under essentially sterile conditions.[18]

The protein content of polymer samples treated by this method was less than 0.3% as estimated by the method of Lowry.

The pH values of all solutions were measured at 25°C with a Radiometer pH meter, with an accuracy of ±0.01 pH units.

7. Purification of solvents.

All aqueous solutions were prepared using water which had been deionized prior to distillation in an all-glass still. When sterile solutions were required, as during the purification of polynucleotides, freshly boiled, glass distilled water was used.

The ethanol used in this study was prepared by refluxing S.V.R. alcohol over potassium hydroxide pellets followed by careful fractional distillation. The fraction boiling at 78-79°C was collected, under nitrogen, and this was found suitable for ultraviolet absorption spectrophotometry.

n-Propanol (B.D.H. Laboratory grade) was dried by treatment with magnesium and was then fractionally distilled, collecting the fraction of boiling point 97-98°C.

Dimethylformamide (B.D.H. Laboratory grade) was dried by fractional distillation at reduced pressure under dry nitrogen. The central fraction (boiling point 73.5-74.0°C at a pressure of 4.2 cms. of mercury) was collected.

8. Preparation of Quaternary ammonium salts.

The quaternary ammonium salts of poly-A, poly-U, poly-I, DNA (calf thymus and *Escherichia coli*) and RNA (calf thymus) were prepared by the method of Aubel-Sadron *et al.*,[25] using approximately 1-1.5 mg. quaternary ammonium bromide per mg. polynucleotide of RNA and 1-2 mg. per mg. DNA. Freshly distilled organic solvents were used to dissolve the partially dried precipitates.

Polynucleotides and nucleic acids were recovered as their sodium salt by the addition of aqueous sodium chloride to the non-aqueous solution,[25] however after dissolution in dilute sodium chloride the polymer was collected by salt-ethanol precipitation as described in section (V)-6.

The hexadecyltrimethyl ammonium bromide (B.D.H. Laboratory grade) was recrystallized from water, as suggested by Scott[175] prior to the preparation of the nucleic acid or polynucleotide complexes.

9. Spectrophotometry.

The spectrophotometers used in these studies were a Unicam SP700 recording spectrophotometer, a Unicam SP500 (in which the power source was a Lab-gear spectrophotometer power supply, Type 115D) and a

Beckman D.U. spectrophotometer. The wavelength or wavenumber scales were checked as recommended by the manufacturer, and the necessary corrections applied, to experimental data. The photoelectric responses of the instruments were tested by measurement of the extinction coefficients of standard potassium chromate solutions.[205] Silica curvettes used (cell lengths 1.00, 0.50, 0.20cm.) either matched exactly, or the necessary correction was applied to data obtained.

The temperature dependence of the extinction of polynucleotides and nucleic acids was measured with the Unicam SP500 which was fitted with an electrically heated cell holder. The temperature of the holder was regulated by an incorporated thermistor in conjunction with an Eilco Type 2 Temperature controller. The actual temperature was measured with a chromel-p-alumel thermocouple, calibrated in terms of the temperature of a solution within a curvette placed in the cell holder. Tight-fitting polythene stoppers prevented evaporation of solutions at elevated temperatures.

Melting curves for polymer samples were constructed using the absorbance ratio $OD_{T^{o}C} : OD_{25^{o}\mathrm{native}}$ for the appropriate wavelength (259 mμ for DNA, 257 mμ for poly-A) OD_T was corrected for the thermal expansion of the solution. In the figures presented the curves have been adjusted so that the maximum values of the ratio at high temperatures correspond to the values obtained for the native nucleic acids and polynucleotides.

In studies of the concentration dependence of the extinction coefficients of both aqueous and non-aqueous polynucleotide solutions, successive increments of a stock solution were added into the solution in the curvette, using an Agla micrometer syringe. The solutions were mixed with a small platinum stirrer. The accuracy of the method was verified by measurements on separately diluted solutions. This procedure was also used to study the effect on the extinction when water was added to poly-A-Q in ethanol.

The extinction coefficient per mole phosphorus per litre, ϵ_p, was determined for use in estimating polynucleotide or nucleic acid concentrations in experimental solutions. The optical density of a suitable dilution of the polymer solution was measured at the required wavelengths and the phosphorus content of the same sample was estimated. Initially the phosphorus determinations of Jones and Peacocke,[206] and Griswold,[207] were used, however the majority of estimations were by the method of Ames and Dubin[208] in which inorganic phosphate is determined by the procedure of Chen.[209] The latter method had the advantage that much smaller amounts of polymer solution were necessary. The average of 20-30 individual determinations was taken as the ϵ_p value, for a given sample, and most cases there was an uncertainty of approximately 3-5%.

10. Optical Rotatory Dispersion.

Optical rotation and optical rotatory dispersion measurements were made using a Bellingham and Stanley (London) polarimeter in conjunction with a Unicam monochromator, over a wave length range from 600 mμ to 285 mμ . The polarimeter cells used (10 cms.) were fitted with quartz windows.

11. Sedimentation.

A Spinco model E ultracentrifuge equipped with schlieren optics was used to measure the sedimentation rates of polynucleotide samples. Most measurements were made at 59,780 r.p.m., a constant acceleration rate being applied to attain this speed. 12 m.m. cells were used in a D rotor in which the temperature was controlled at 25.0°C.

Sedimentation experiments were carried out on poly-A in aqueous 0.15M sodium chloride, 0.015M sodium citrate buffer and on poly-A-Q (0.06-0.45 gm/dl.) in ethanol. The latter system exhibited pressure dependence of the sedimentation coefficient and values of S, which were corrected for this dependence, were computed by the method of Billick,[189] using an I.B.M. 1620 computer. S^o values were estimated graphically in the usual way.

The partial specific volume of poly-A-Q in ethanol was estimated from density data, obtained at 25°C using a pycnometer with a volume of approximately 5 mls. The approximate $\bar{v}$ value calculated was 0.92 ml./gm.

12. Viscometry.

All viscosity measurements were made using an Ubbelohde viscometer, in which concentration changes could be affected directly.

The dimensions of the viscometer were such that the kinetic energy and end effect corrections could be neglected, for the purpose of the present investigation. The solvent (ethanol) flow time for the viscometer was 181.10 ± 0.05 seconds at 25.00°C.

The viscometer was thermostatted at 25.00°C and the temperature controlled to within ± 0.005°C. Measurements were made on polymer solutions under an atmosphere of nitrogen, and solutions in the viscometer were mixed and manipulated by oxygen free nitrogen which had been passed through a liquid air trap, a sodium hydroxide wash and through 2 or 3 washes of ethanol (at 25°C). The concentration of experimental solutions was either calculated for dilutions of the stock solution used, or estimated directly from optical density measurements on aliquots of solution withdrawn from the viscometer.

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