Biophys. Struct. Mechanism 1, 203—219 (1975)

The Significance of the 2′ OH Group and the Influence of Cations on the Secondary Structure of the RNA Backbone

K. Kölkenbeck and G. Zundel

Physikalisch-Chemisches Institut der Universität München

Received February 28, 1974 / Accepted October 10, 1974

Abstract. In the IR spectra, the coupling of vibrations leads to band splitting and/or bands shifting in opposite directions which provides information on the mutual orientation of groupings. From such band shifts in the range 1800 to 1500 cm^{-1} one can draw conclusions on the double helix formation of polynucleotides. These band shifts are caused either by vibrational coupling of stretching vibrations within pairs of base residues or by coupling of stretching vibrations with the bending (scissor) vibration of the $-NH_2$ groups; the latter is indicated by band shifts after deuterium substitution within the amino groups. Couplings of phosphate and ribose vibrations in the range 1300 to 1000 cm^{-1} provide information on the secondary structure of the backbone.

In order to obtain information on the structure of the RNA backbone, the IR spectra of poly(ribonucleotides) were studied in neutral media in which they were single-stranded. The shift due to coupling of the band of the 2′OD bending vibration and that of the antisymmetric stretching vibration of the ether group of the ribose residue proves that ribose residues of the backbone are cross-linked via hydrogen bonds. These are formed between the 2′OD or 2′OH groups, respectively, and the O atoms of the ether group of the neighboring ribose residues. This is the reason for the difference between DNA and RNA as regards the 2′OH group. The structure formation caused by these hydrogen bonds results in a stiffening of the RNA backbone. The tendency to form these hydrogen bonds increases in the order poly(U), poly(C), poly(A). This order of secondary structure stabilization is due to an interplay between the influences of (1) the 2′OH hydrogen bonds and (2) the base residues' stacking. Furthermore, the coupling of the antisymmetric stretching vibration of the $>PO_2^-$ groups with a vibration involving the 2′OH group can result in a doublet structure of the band at about 1240 cm^{-1} if cations with strong fields are present. This probably shows that these cations can turn the $>PO_2^-$ groups — which are usually turned outward at the backbone, as shown by construction of molecular models — toward the base residues. Thus they cause stiff monohelices which are right-handed screws.

Key words: RNA Backbone — Secondary Structure — 2′OH Group — Cations — IR Spectra.

I. Introduction

Natural RNA — with the exception of double-helical virus RNA — usually contains non-base-paired, that is, single-stranded sections. The question arises as to whether these sections have a secondary structure and, if so, of what nature this structure is ? It would seem that the 2′OH group at the ribose residues of the backbone are of decisive importance for a secondary structure formation of the single strands. It is also known that the presence of Mg^{++} ions is essential for the biological activity of RNA, as already postulated by Watson (1964) and verified by various experiments (Maruta *et al.*, 1969; Wacker, 1969; Römer *et al.*, 1970; Mc Quillen, 1962). Thus one can assume that cations play an important role in

structure formation, too. These questions were studied by means of IR spectroscopic investigations of poly(ribonucleotides).

Poly(A) (Rich *et al.*, 1961; Beers and Steiner, 1957; Holcomb and Tinoco, 1965; Witz and Luzzati, 1965; Adler *et al.*, 1969; Janik *et al.*, 1972) as well as poly(C) (Akinrimisi, 1963; Langridge and Rich, 1963; Fasman *et al.*, 1964) form double helices in a weak acidic medium. ORD measurements show (Holcomb and Tinoco, 1965; Adler *et al.*, 1969; Fasman *et al.*, 1964) that these helices "melt" within a rather small temperature interval, that is, this structure exhibits a high degree of cooperativity.

In contrast to the above, ORD (Holcomb and Tinoco, 1965; Fasman *et al.*, 1964), UV (Leng and Felsenfeld, 1966), and Raman spectroscopic measurements (Small and Peticolas, 1971) in neutral medium showed with regard to poly(A) and poly(C) that these polynucleotides form under these conditions a secondary structure which melts at temperatures higher than 20° C. This occurs over larger temperature intervals. Cooperativity accordingly plays a less significant role for this structure. This is confirmed, in particular, by measurements of di-, tri-, and hexanucleotides of the adenosine where the melting behavior depends only to a slight degree on the chain length (Leng and Felsenfeld, 1966). Small angle X-ray scattering investigations showed that *poly(A) and poly(C)* are single-stranded in neutral medium. According to these investigations, these polymers contain relatively stiff, rod-like regions (Witz and Luzzati, 1965; Gulik *et al.*, 1970). This seems to contradict a flow birifringence investigation of the poly(A) (Wada, 1967). The X-ray small angle scattering already indicates ordered regions at 100 Å, the flow birifringence, however, only indicates ordered regions above 500 Å. Hence the length of the structured regions probably lies between these limits. It is one of the main purposes of this paper to investigate the structure formation causing the stiffening of the RNA backbone, observed by X-ray scattering investigations.

The UV spectroscopic measurements (Richards *et al.*, 1963) as well as the Raman spectroscopic investigations (Small and Peticolas, 1971) of the *poly(U)* showed that the latter forms no secondary structure above 25° C. On cooling down to 0° C, however, with Mg^{++} ions a secondary structure is formed (Szer, 1965) and slight hypochromicity is found even when Mg^{++} ions are absent. The scattering curves obtained at 3° C are similar to those of the poly(A) and poly(C) but show that the poly(U) molecules are somewhat more flexible (Witz and Luzzati, 1965).

The nature of the double helices formed by the poly(A) and poly(C) in the acidic medium differ fundamentally from one another. This double helix formation is determined to a large extent by the nature of the bases. In contrast, the structures of poly(A), poly(C), and poly(U), formed in the neutral medium, appear to resemble each other. It therefore appears that backbone properties determine the formation of these structures.

II. Coupling of Vibrations and Structure

Band splitting and/or bands shifting in opposite directions as a result of coupling between vibrations (Fermi resonance) are observed in the IR spectra of macromolecules and may provide information on the secondary structure.

Usually, such coupling effects are strongest when the coupling vibrations are caused by one and the same group. Coupling is, however, also observed between

vibrations of neighboring groups. Howard, Frazier and Miles (1969), for example, showed that the two C=O stretching vibrations of guanine and cytosine couple in the (G+C) pairs.

Coupling is caused by an interaction of the vibrational transitions. This interaction is either electromagnetical or mechanical.

Electromagnetical coupling (see Dawydov, 1965, p. 522ff.) is a dipole-dipole interaction between the transition dipole moments, i.e., an interaction via the electromagnetical fluctuating fields. Hence electromagnetical coupling only occurs when the transition moments of both vibrations are largely parallel or antiparallel.

Mechanical coupling (see Herzberg, 1945, p. 215ff.) is a coupling via the anharmonic terms of the potential. This coupling is induced directly via the electron system by a contact of the neighboring groups with the coupling vibrations via intermolecular bonding, for instance, a hydrogen bond.

When observing band splitting and/or bands shifting in opposite directions, it can in most cases not be decided whether electromagnetical or mechanical coupling is the reason. Therefore one considers the system under the assumption of electromagnetical as well as mechanical coupling. When assuming *electromagnetical coupling*, one receives information on the mutual orientation of the groupings since with this type of coupling the transition moments must be either parallel or antiparallel. Under the assumption of *mechanical coupling*, information on the intermolecular bonding is obtained directly. As we shall see in the following, often the same results regarding the secondary structure are obtained under both assumptions. Only then unambiguous results are obtained on the structure of the system investigated.

Compared to X-ray analysis, IR spectroscopic structure investigations are of decisive advantage when macromolecules cannot be crystallized.

III. Helix Formation

With D_2O hydrated polynucleotides, stretching vibrations, which have more or less C=C, C=N, and C=O character, are observed in the range 1800 to 1500 cm^{-1} (Tsuboi, 1969). With H_2O hydrated samples, stretching vibrations as well as the $—NH_2$ scissor vibration are observed. Furthermore, the H_2O scissor vibration is superimposed at 1640 cm^{-1}.

When the double helix forms, the following band shifts are observed: (1) Band shifts largely independent of deuteration due to coupling between the stretching vibrations. The double helix formation with the (G+C) pair, for example, was studied in detail with the aid of such a splitting of the C=O stretching vibration bands of (G) and (C) (Howard *et al.*, 1969). (2) Band shifts sensitive to deuteration, caused by coupling between in-plane stretching vibrations and the $—NH_2$ scissor vibration. This may happen when the $—NH_2$ groups are fixed in the ring planes in connection with the double helix formation, as studied and discussed in detail, for example, with the semi-protonated poly(C) (Zundel *et al.*, 1972). The effect, however, disappears on deuteration.

Let us now discuss the coupling of the C=O stretching vibrations in the (G+C) pairs: Assuming that the band splitting is caused by *electromagnetical*

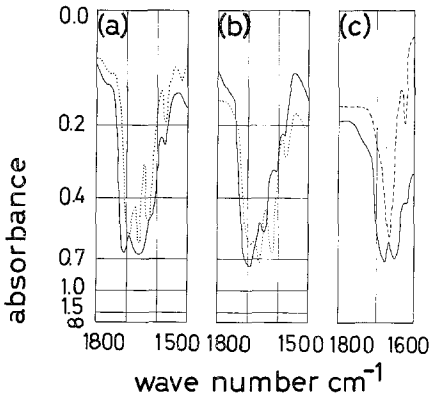

Fig. 1

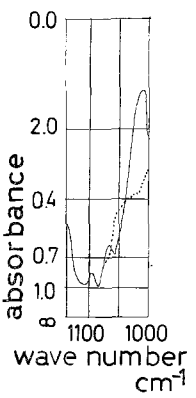

Fig. 2

Fig. 1a—c. IR spectra of calf thymus DNA. a) films hydrated at 90 % relative humidity of the air ——— H_2O-hydrated; ····· D_2O-hydrated; b) the same films thoroughly dried; c) solutions (6 mg/ml) in D_2O ——— at 25° C, - - - - - at 92° C

Fig. 2. IR spectra of the K^+ salts of poly(C) at 75 % relative humidity of the air; ——— H_2O-hydrated; ····· D_2O-hydrated

coupling, this coupling occurs after the secondary structure formation since the transition moments become oriented antiparallel when the double helix forms. Hence the band splitting provides information on the mutual orientation of the C=O groups of (G) and (C), thus showing the double helix formation. Assuming that the band splitting is caused by *mechanical coupling*, this coupling must be induced by bonding between the base pairs. Thus the hydrogen bonds formed between the base pairs, and hence the double helix formation is shown by the band splitting. This means that even if it cannot be decided whether electromagnetical or mechanical coupling is the case the same conclusion can be drawn under both assumptions.

On the basis of a *typical "structure band"* occurring at about 1700 cm^{-1} with DNA (Kyogoku *et al.*, 1961; Shimanouchi *et al.*, 1964; Fritsche, 1972), double-helical RNA (Tsuboi, 1969), and RNA-DNA hybrids (Higuchi *et al.*, 1969) double helix formation is discussed in the publications mentioned. The nature of this band can now be understood better: Band splitting (1680, 1645 cm^{-1}) is observed in the D_2O-hydrated samples (Fig. 1a and c) which disappears on melting (Fig. 1c) and decreases on drying (Fig. 1b), that is, when the structure breaks down. With respect to the foregoing considerations, this splitting is caused by a coupling of in-plane stretching vibrations of the base residues, especially by a coupling of the C=O stretching vibrations within the (G + C) pairs. The peak at the larger wave number (1680 cm^{-1}) is the "structure band". This "structure band" appears with H_2O-hydrated samples at somewhat larger wave numbers than with D_2O-hydrated ones (Fig. 1a). Probably due to coupling with the $-NH_2$ scissor vibration, it is shifted even further toward larger wave numbers. Another reason for this shift

on H → D exchange could, however, be a slight participation of D or H of the NH or ND groups with the in-plane stretching vibrations of the base residues.

The couplings of in-plane stretching vibrations of the base residues observed in the range 1800 to 1550 cm^{-1} indicate the double helix formation, as shown above.

IV. The Structure-Promoting Effect of the 2′OH Group

Couplings of vibrations of the ribose residues and the phosphate groups, observed in the range 1300 to 1000 cm^{-1}, supply information as to the secondary structure of the backbone. The melting behavior of the backbone of DNA and RNA was studied earlier, utilizing these bands (Tschirgadze *et al.*, 1972; Thomas and Hartmann, 1973; Thomas *et al.*, 1973).

Information concerning the structure-promoting effect of the 2′OH group is given by shifts which occur due to coupling of a skeleton vibration of the ribose residue and the bending vibration of the 2′OD group in the range 1100 to 1000 cm^{-1}. This effect is studied in the following with homopoly(ribonucleotides).

Let us first consider the assignment of the most important bands in this region (Fig. 2). The symmetrical stretching vibration of the $>PO_2^-$ group is expected at about 1080 cm^{-1}, for it is observed there with other phosphorous-organic substances of the type $R_2PO_2^-$ (Bellamy, 1958). The band at 1085 cm^{-1} is accordingly assigned to this vibration (Shimanouchi *et al.*, 1964; Tsuboi, 1963).

Several ribose vibrations are likewise expected in this region. In the spectra of the H_2O-hydrated samples a broad shoulder is observed at the slope of the symmetrical stretching vibration band toward smaller wave numbers. Sometimes, for instance with the K^+ salt of the poly(C), this appears as a sharp band at 1060 cm^{-1} (Fig. 2). This band is to be ascribed to the antisymmetric stretching vibration of the C—O—C ether grouping in the ribose, for with diethyl ethers the antisymmetric C—O—C stretching vibration is found at 1062 cm^{-1}, and tetrahydrofurane absorbs at 1076 cm^{-1} (Bellamy, 1958).

A more or less intense band, depending on the nature of cations present (Fig. 4), is found with all poly(ribonucleotides) at the slope of the symmetrical $>PO_2^-$ stretching vibration toward larger wave numbers at about 1130 cm^{-1}. Tsuboi (1963) and Tsuboi *et al.* (1963) ascribe this to the antisymmetric stretching vibration of the $\begin{matrix} C \\ C \end{matrix}\!>\!C-O$ grouping of the ribose. The intensity of this ban depends strongly on the cations present. This shows that the conformation of the ribose changes as a function of the cations. To clarify the conformation occurring with the different cations normal coordinate treatments for the various conformations must be performed. The conformation of the ribose, varying from case to case, can, however, explain the finding that with the H_2O-hydrated samples the vibration of the ether group is either a band or only a more or less marked shoulder on the slope of the band complex at about 1060 cm^{-1} (Fig. 4, H_2O-hydrated samples).

The band observed with the D_2O-hydrated samples in the range 1050 to 1000 cm^{-1} as a more or less marked shoulder (Fig. 4, D_2O-hydrated samples) is assigned to the in-plane bending vibration of the 2′OD group (Tsuboi, 1963). In the region 1450 to 1000 cm^{-1} with secondary alcohols, two bands are observed which are affected by deuteration. These bands are assigned to the in-plane OH bending vibration which couples with another vibration, for instance, with the C—O stretching vibration (Bellamy, 1958). With polynucleotides Tsuboi (1963) assigned corresponding bands at 1412 and 1311 cm^{-1} to an analogous band pair which involves the 2′OH bending vibration. In the following we shall see that the 2′OH in-plane bending vibration must be involved in a doublet structure, sometimes shown by the broad band at about 1240 cm^{-1}.

In Fig. 2 the spectrum drawn with a continuous line is the H_2O-hydrated K^+ salt of poly(C), that drawn with a dotted line represents the D_2O-hydrated salt. With the H_2O-hydrated sample the vibration of the ether group of the ribose residue

Table 1. Assignment of the bands and splitting of the band at about 1240 cm^{-1}, dependent on the cations present

Position of band cm^{-1}	Assignment
1300	probably glycosidic ν N—C
with H_2O hydrated poly(U) at 1266	shift caused by coupling with the NH bending vibration
1250 to 1220 sometimes doublett	$\nu_{as} > PO_2^-$ sometimes coupled with a vibration in which the 2′OH bending vibration is involved
1130	ν_{as} of the C>C<C—O groups of the ribose residues
1080	$\nu_s > PO_2^-$
1060 to 1095 sometimes coupled	ν_{as} C—O—C of the ether groups of the ribose residues
1050 to 1000	ν_{OD} of the 2′OD groups

Substance	Wave number cm^{-1}		
	H_2O		D_2O
Poly(A)			
Mg^{++}	1242	1224	1236
Ca^{++}	1242	1222	1235
Ba^{++}	1241	1227	1236
Li^+	1245	1220 sh	1237
Na^+	1241	1220 sh	1238
Poly(U)			
Mg^{++}	1242	1219 sh	1235

is observed at 1060 cm^{-1}. On H → D exchange the 2′OD bending vibration emerges at about 1030 cm^{-1}. Further one can see — and this is particularly important — that the ether vibration is shifted toward larger wave numbers by coupling with the 2′OD bending vibration. Hence the vibration of the ether group merges with this band complex. Due to coupling the bands shift in opposite directions. The 2′OD bending vibration is shifted all the more toward smaller wave numbers, the stronger the coupling is. In the case of strong coupling, as for instance, with almost all poly(A) samples, it becomes an isolated band (Fig. 4, D_2O-hydrated samples).

Frequently, however, the band of the ether vibration is not pronounced. It is only observed as a broad shoulder on the side of this band complex (Fig. 4, H_2O-hydrated samples). Not in all cases one can decide clearly whether this shoulder shifts toward larger wave numbers when the 2′OD bending vibration emerges in the region 1050 to 1000 cm^{-1}. However, the coupling — if present — can always be clearly recognized by the shift of the 2′OD bending vibration to smaller wave numbers (cf. in Fig. 4, the H_2O- and the D_2O-hydrated samples).

The finding that the ether vibration and the 2′OD bending vibration couple proves that the 2′OH group is linked with the neighboring ribose residue via a hydrogen bond. This conclusion is independent of the nature of the coupling. In the case of *mechanical* coupling, the coupling would be induced by the hydrogen

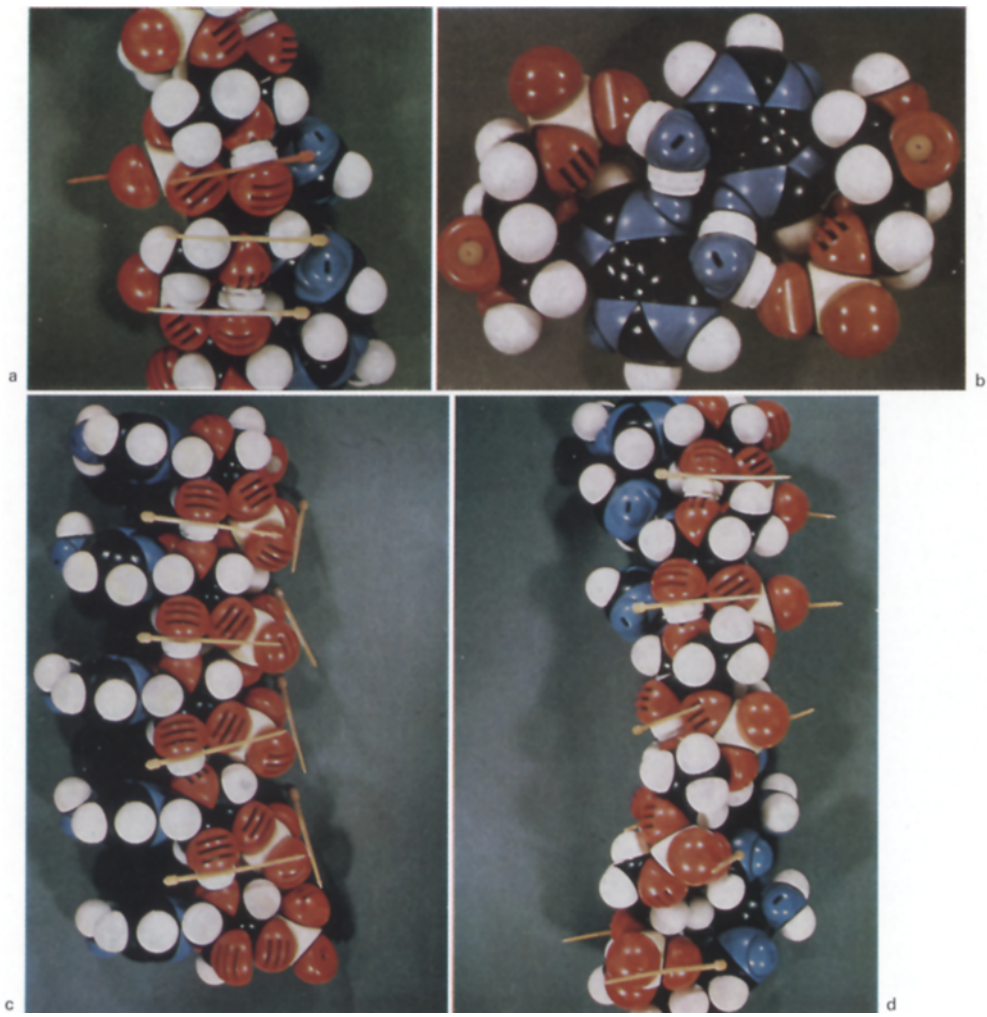

Fig. 3a—d. Models of poly(ribonucleotide) molecules. a) Model to demonstrate the coupling of the antisymmetric stretching vibration of the C—O—C group and the bending vibration of the 2′OD group (yellow sticks). This indicates the formation of the hydrogen bond by the 2′OD group on the backbone. b) Poly(A) double helix formed in acidic medium; c) poly(A), $>PO_2$ groups turned away from the base residues; d) poly (A) monohelix, $>PO_2$ groups turned to the base residues as shown clearly in the case of the upper nucleotides

bond formed. Assuming, however, *electromechanical* coupling, the orientation of the transition moments must be considered. The transition moment of the antisymmetric skeleton vibration of the ether group is in the C (1′)—C(4′) direction. That of the 2′OD bending vibration is perpendicular to the OD bond. The construction of molecular models shows that these transition moments are oriented parallel to one another only when the 2′OD groups are linked via a hydrogen bond with the O-atom of the ether groups of the neighboring ribose residues (Fig. 3a).

Differentiating between the mechanical and the electromagnetical mechanisms is not possible considering the present results. However, the formation of the hydrogen bond (Fig. 3a) is a necessary prerequisite to both mechanisms and is therefore indicated by the band shifts observed. This type of 2′OH group hydrogen bonding was postulated by Rabczenko and Shugar (1971 and 1972) for the structure formed by Mg^{++} poly(U) at low temperatures.

The fact that these hydrogen bonds formed by the 2′OH group are of importance for the secondary structure formation is not in contradiction to the observation by Zmudzka and Shugar (1970), who found that the 2′—O-methylated Mg^{++} poly(U) also forms a secondary structure. The melting behavior of this structure is, however, completely different from that of the non-2′—O-methylated compounds. It melts at higher temperatures and the melting curve is not steep. Thus the latter shows that in contrast to the structure formed by the non-methylated compound the cooperativity is not large. Hence the stabilizing forces of the structure of the 2′—O-methylated compound must be quite different from those which stabilize the structure of the non-methylated ones. Rabczenko and Shugar (1971) suggested that hydrophobic interactions between the methyl groups and the base residues are of importance for the stabilization of the structure formed by the methylated compounds.

The observed backbone structure conforms well with the observation made by Melcher (1970) on NMR investigations, namely that the 2′H of the ribose interacts with the π-electron system of the base, this as a result of hydrophobic interaction. Fig. 3a shows that this 2′H group is turned toward the aromatic π-electron system.

The backbone of the RNA, *in contrast to that of the* DNA, can accordingly become more rigid through the formation of hydrogen bonds between their 2′OH groups and the O atom of the neighboring ribose residues. This result is of great significance for it shows that the difference between DNA and RNA, as far as this is due to the 2′OH group, is caused by the structure promotion of these hydrogen bonds.

IV.1 Dependence on the Type of Base Present

Fig. 4 shows these bands for poly(A), poly(C), and poly(U) in the presence of various cations. The spectra of the H_2O-hydrated samples are shown in the top left of the figure, respectively, those of the D_2O-hydrated sample below. The corresponding samples which have been extensively dried are represented on the right-hand side.

In the case of all D_2O-hydrated salts of the poly(A), the 2′OD bending vibration appears shifted toward smaller wave numbers as a separate band at 1030 cm^{-1}. That is, the hydrogen bonds, which form the 2′OD groups with the ether O-atom of the neighboring ribose residues, are well formed with all salts of the poly(A), thus stiffening the backbone.

With the poly(C) the 2′OD bending vibration can only be recognized as a shoulder, that is, the band is no longer shifted so markedly toward smaller wave numbers, due to the coupling. Furthermore, the band is somewhat broader, i.e., the tendency for the hydrogen bonds to form in the backbone has decreased from poly(A) to poly(C)[1].

[1] In the case of the double helices formed by poly(A) and poly(C) in acidic medium, this 2′OH group hydrogen bond in the backbone is found with poly(A) but not with poly(C) (Kölkenbeck and Zundel). Due to the considerable electrostatic repulsion of the excess protons in the double helix formed by semi-protonated poly(C), this double helix is extended strongly, thus preventing the formation of 2′OD hydrogen bonds.

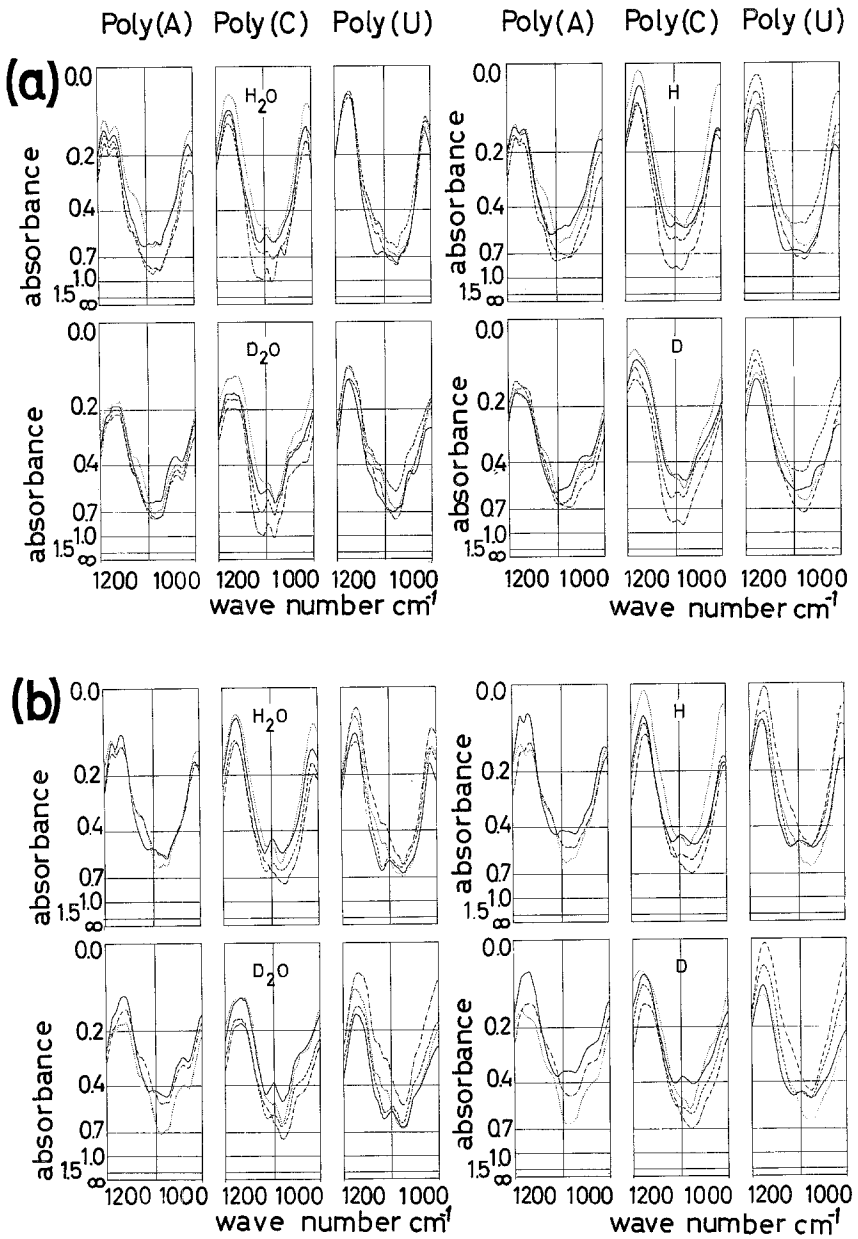

Fig. 4a—b. IR spectra of poly(A), poly(C), and poly(U), region 1200 to 1000 cm^{-1}. In each figure the upper spectra show the H, the lower the D forms. *Left side:* Films hydrated at 75% relative humidity of the air; *right side:* the same films after thorough drying. a) ——— Li^+, - - - - - Na^+, -·-·-· K^+, ····· Cs^+ salts; b) ———Mg^{++}, -·-·-· Ba^{++}, ····· Cs^+ salts

With poly(U) this shoulder is usually even weaker, but can be clearly recognized with the Mg^{++} salt. Li^+ salt, which exhibits a marked band at 1040 cm^{-1}, is an exception. Accordingly with poly(U) usually only few 2′OD

hydrogen bonds are formed in the backbone, that is, with most salts of the poly(U) the stabilization of the backbone due to these hydrogen bonds plays only a minor role.

The tendency of the 2′OH group to form hydrogen bonds accordingly increases in the order poly(U), poly(C), poly(A). This is understandable on considering that, in accordance with NMR investigations by Wagner (1972), *base residue stacking* increases in the above order. This base residue stacking favors formation of the secondary structure and thus, too, formation of the hydrogen bonds in the backbone. Structure stabilization due to the hydrogen bonds observed and stabilization due to *base stacking* favor each other mutually. This leads to the observed sequence of structure formation as a function of the type of base residues present.

IV.2 Dependence on the Degree of Hydration

Let us now compare the hydrated samples with the thoroughly dried ones in Fig. 4. The band or shoulder, respectively, of the 2′OD bending vibration is far less marked and has sometimes disappeared completely in the thoroughly dried samples. The shift of this band toward smaller wave numbers due to the coupling is accordingly less, that is, the structure of the backbone breaks down on drying.

V. Phosphate Group Orientation at the Backbone

Fig. 5 shows the range at about 1240 cm^{-1} for poly(A), poly(C), and poly(U) in the presence of various cations. The spectra of the H_2O-hydrated samples are shown top left, below those of the D_2O-hydrated ones. On the right-hand side the corresponding dried samples are illustrated.

The intense band at about 1240 cm^{-1} is ascribed to the antisymmetric stretching vibration of the $>PO_2^-$ groups (Shimanouchi *et al.*, 1964).

In the case of the poly(U) an additional band is observed on H_2O hydration at the slope of the former band toward larger wave numbers (Fig. 6). This is probably the glycosidic C—N stretching vibration, which on coupling with the NH bending vibration is shifted from 1300 cm^{-1}, where it is observed with the D_2O-hydrated sample, toward smaller wave numbers, that is, to 1266 cm^{-1} (see Bellamy, 1958) (Fig. 6). The NH bending vibration emerges on the transition from D_2O to H_2O hydration at 1422 cm^{-1}. The C(4)=O stretching vibration (Miles, 1964) is likewise shifted due to coupling with the NH bending vibration on transition from D_2O to H_2O hydration from 1658 cm^{-1} toward larger wave numbers, and thus appears only as a shoulder at the band complex at about 1690 cm^{-1}.

The band complex at 1240 cm^{-1} sometimes exhibits doublet structure, especially in the case of H_2O-hydrated poly(A). This doublet structure is never observed with the D_2O-hydrated samples[2].

[2] The band observed with the D_2O-hydrated Li^+ salt of the poly(U) at 1217 cm^{-1} is not involved with this doublet structure and is probably not the D_2O scissor vibration. A corresponding band is also found with this Li^+ salt at the slope toward large wave numbers of the symmetrical O—P—O stretching vibration complex at 820 cm^{-1}, this in contrast to all other samples. The origin of these bands is not clear. However, it appears conceivable that it is connected with the structural peculiarity of the Li^+ poly(U), which is also seen in the hydrogen bond formation in the backbone (band at 1030 cm^{-1} in Fig. 4, D_2O-hydrated sample).

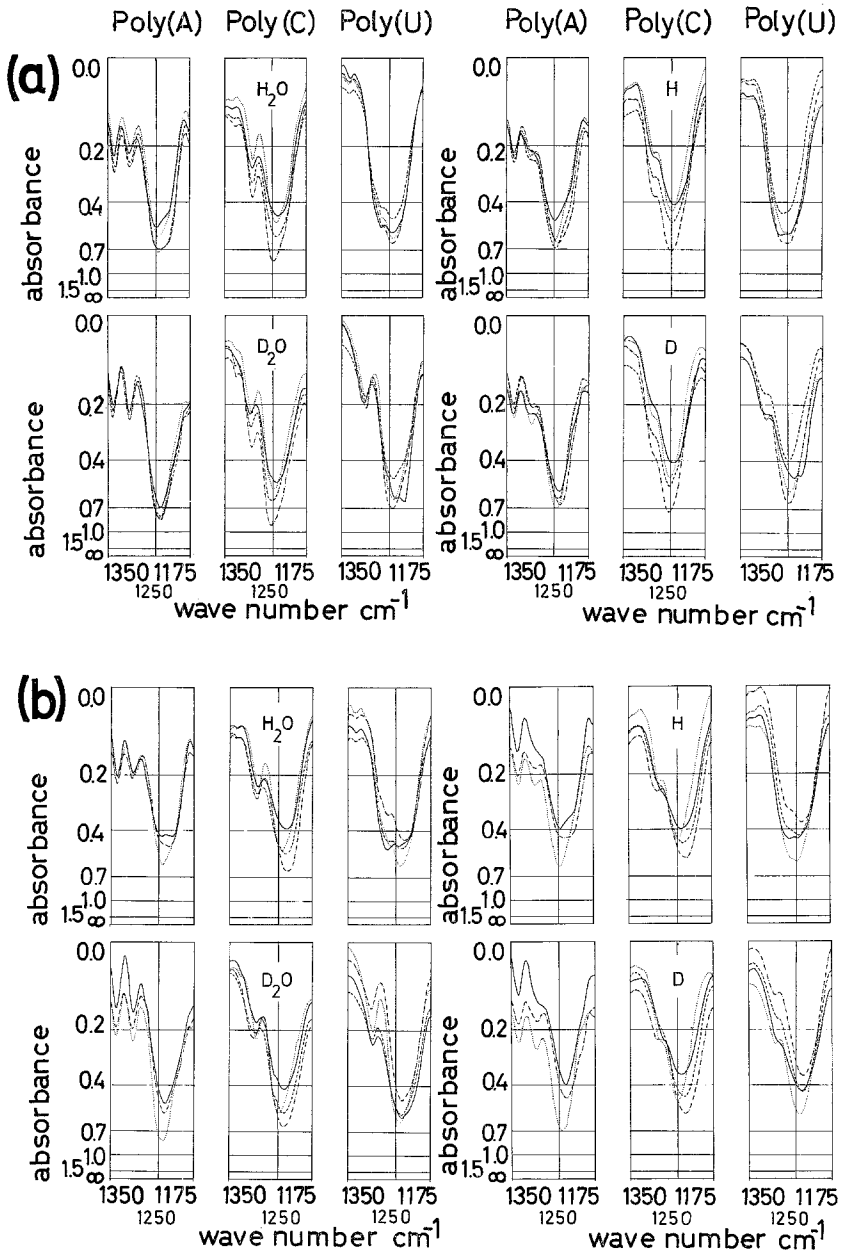

Fig. 5a—b. IR spectra of poly(A), poly(C), and poly(U), region 1350 to 1175 cm^{-1}. In each figure the upper spectra show the H, the lower the D forms. *Left side:* Films hydrated at 75% relative humidity of the air; *right side:* the same films after thorough drying. a) ——— Li^+, - - - - - Na^+, ·-·-·- K^+, ····· Cs^+ salts; b) ——— Mg^{++}, ·-·-·- Ba^{++}, ····· Cs^+ salts

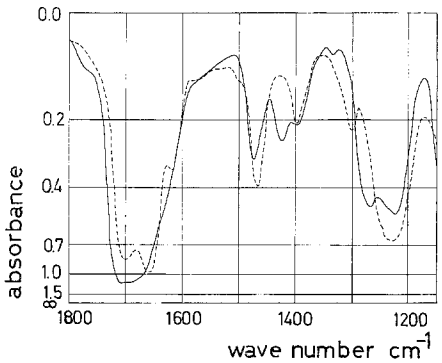

Fig. 6. IR spectra of poly(U), films hydrated at 75 % relative humidity of the air. ——— H_2O-hydrated, - - - - - D_2O-hydrated

Table 1 shows that the band observed on D_2O hydration lies between the two doublet maxima found on H_2O hydration. Thus no significant shift of the overall band complex is involved with the splitting. A vibration which disappears on deuteration must participate in the coupling causing this splitting. In this range only OH and NH bending vibrations come into question. The only band common to all poly(ribonucleotides) and especially, too, to poly(A), is the in-plane bending vibration of the 2′OH group. It is known from investigations with secondary alcohols that in the region 1450 to 1000 cm^{-1} a band pair is observed in which the OH in-plane bending vibration participates (Bellamy, 1958). Analogous bands in which the 2′OH in-plane bending vibration is involved are expected with polynucleotides. Thus the deuteration-sensitive splitting indicates that one of these two vibrations in which the 2′OH bending vibration is involved is contained in the band complex observed at about 1240 cm^{-1}.

Hence the doublet structure shows that the antisymmetric stretching vibration of the $>PO_2^-$ group can couple with a vibration with which the 2′OH bending vibration is involved. This doublet structure, however, is only observed with some salts of the poly(ribonucleotides). Hence two different backbone conformations must exist, one whereby these two vibrations couple and another whereby they cannot couple.

A comparison with the spectra of the double helix formed by poly(A) in acidic medium show sthe following: When this double helix is formed a similar doublet structure of the band near 1240 cm^{-1} is found. With this structure, however, the $>PO_2$ groups are turned toward the base residues (Rich *et al.*, 1961), as illustrated in Fig. 3b. The transition moment of the antisymmetric PO_2 stretching vibration is parallel to the line connecting the two non-ester-bonded oxygen atoms. The transition moment of the in-plane bending vibration of the 2′OH group is perpendicular to the OH bond and fixed in the furyl ring plane when — as previously discussed — the 2′OH groups cross-link the ribose residues. Consideration of the double helix of poly(A) shows that in this structure, i.e., if the $>PO_2$ groups are turned toward the base residues, the transition moments of these two vibrations are largely parallel. Hence electromagnetical coupling of

these two vibrations is probably the reason for the splitting of the band at about 1240 cm^{-1}.

The same should be true if an analogous splitting is observed with the spectra of the polynucleotides in neutral medium. The molecular model (Fig. 3d) shows that the transition moments of the two vibrations become parallel if the $>PO_2$ groups are turned toward the base residues (this is clearly seen with the two upper nucleotides). Hence, the observed band splitting probably indicates this secondary structure of the backbone. This structure is a relatively stiff mono-helix as the molecular model shows (Fig. 3d). This mono-helix is a right-handed screw. This structure of the backbone influences the structure formation of the double or triple helices formed by poly(ribonucleotides) (Kölkenbeck and Zundel, Herbeck and Zundel).

The $—CH_2—$ group in 5′ribose position and the phosphate group can be turned about their diester bond in opposite directions. In this way the phosphate group may be turned away from the base residues. The result of this conformation change is shown in Fig. 3c. With this structure the transition moments of the antisymmetric stretching vibration of the $>PO_2$ group and those of the vibration with which the 2′OH bending vibration is involved are oriented perpendicular to one another. Hence, these two vibrations can no longer couple. Thus when no splitting of the band at about 1240 cm^{-1} is observed, probably the conformation of the backbone shown in Fig. 3c is present.

Similar band splitting at 1240 cm^{-1} was observed by Sato *et al.* (1966) and by Tsuboi (1969) on investigating the double helical rice dwarf virus RNA. These authors were able to orient the macromolecules in the films and then to study the samples with polarized IR. They likewise found a band doublet on H_2O hydration. The one component appeared preferentially when the polarization plane was oriented parallel, the other when this plane was oriented perpendicularly to the helix axis. These authors assume that this splitting is the result of a coupling of the antisymmetric stretching vibration of all phosphate groups at the backbone. For Miyazawa's theory (Miyazawa, 1960 and 1961; Miyazawa *et al.*, 1963) states that due to such coupling one vibration parallel to the helical axis and one vibration perpendicular to the helical axis is to be expected. As with this investigation, the band splitting, however, disappears on $H \rightarrow D$ exchange (Sato *et al.*, 1966; Tsuboi, 1969). This finding does not conform with the explanation given. A further problem arises, since Miyazawa's theory regarding the polynucleotides predicts not only a splitting but a considerable shift of the whole band complex, which is observed neither in the case of the rice dwarf virus nor with the poly(ribonucleotides) (Table 1). On the other hand it can be assumed that dichroism would also be observed with the polymers we investigated if these could be oriented in the film.

The question now arises as to the possibility of interpreting these observations homogeneously and free of contradictions.

A splitting is observed in the case of the DNA, too. The latter, however, is so slight that it appears merely as an extremely small band shift on investigation with an IR light oriented perpendicular or parallel to the helix axis (Shimanouchi *et al.*, 1964). This splitting can be caused by the coupling of the antisymmetric stretching vibrations of the $>PO_2^-$ groups, since, according to Miyazawa's theory in the case of polynucleotides, if the splitting is small the shift of the whole band complex is small, too.

The observations regarding the RNA could then be interpreted as follows: The relatively large splitting with RNA is caused by the coupling of the antisymmetric stretching vibration of the $>PO_2^-$ groups and of the vibration with which the in-plane bending vibration of the 2′OH groups is involved. In addition, these coupled vibrations of the individual nucleotides are, according to Miyazawa's theory, coupled mutually, too. This coupling causes additional splitting of the individual components, which, however, is not observed, since — as with the DNA — it is small. This additional coupling, which is increased by the previously discussed

backbone stiffening in the monohelices, results, however, in the single doublet components obtaining a preferential direction. This interpretation would explain all experimental findings. Further studies, especially theoretical investigations, however, are necessary to provide a final interpretation of these coupling effects.

V.1 The Dependence of the Orientation of the $>PO_2^-$ Groups at the Backbone on the Cations Present and on the Degree of Hydration

Fig. 5 shows that the splitting of the band at 1240 cm^{-1} is observed with the Mg^{++} and Ba^{++} salt of the poly(A). The second band appears as a marked shoulder at the slope of the band toward small wave numbers with the Li^+ salt of the poly(A), with the Mg^{++} salt of the poly(U), and finally as a somewhat less marked shoulder with the Na^+ and K^+ salt of the poly(A). According to the above, cations with strong fields probably turn the $>PO_2^-$ groups toward the base residues, thus inducing the stiff monohelical structure illustrated in Fig. 3d. This is understandable, for these cations can interact in this structure with the phosphate groups. With this structure free enthalpy is gained by the cation-phosphate group interaction, and especially since the negative charges of the phosphate group are screened to a large extent and do no longer repulse each other. In the case of cations with strong fields these free enthalpy gains evidently compensate for the lower free hydration enthalpy of the phosphate groups and the cations involved with this conformation. It is striking that this splitting is never observed with poly(C). According to this, the cations with poly(C) can never turn the $>PO_2^-$ groups toward the base residues.

The doublet structure disappears on drying. This is understandable, for, as we already know, the backbone structure is disturbed on removal of the hydration water.

VI. Experimental

The synthetic polynucleotides poly(A), poly(C), and poly(U) were obtained from Boehringer, Mannheim, W. Germany. They were first passed through a K^+-loaded ion exchanger in order to remove the contamination caused by divalent ions. Since poly(C) and poly(A) protonate in hydrous solutions depending on the concentration of CO_2 in the air, all procedures to which these solutions were subjected had to be carried out in a dry box. The solutions were first set to pH 9 with KOH, then passed through the ion exchanger, subsequently dialyzed against distilled water of pH 7 and then lyophilized. The atom absorption analysis of the cleaned substances indicated contamination of less than 1 mole% of divalent cations.

A solution of 3 mg/ml in water of pH 7 was prepared from the K^+ salts. One ml of this was dialyzed against the solutions of the salts desired, normally for 24 hrs against 0.5 N solutions. The earth alkaline salts of the poly(A) and poly(U) tended to precipitate and for this reason they should only be dialyzed versus 0.05 N solutions. Slight turbidity usually persisted, but vanished during the next step. The dialysis lasted 12 hrs against distilled water of pH 7, the outer solution being replaced several times.

Films of reproducible thickness are made from these solutions on Ge sample carriers with the aid of a centrifugation drying procedure (Hofmann and Zundel,

1971). The water was removed during the centrifugation at 7% air humidity (saturated hydrous NaOH solution).

The samples are then hydrated in the cells described by Zundel (1969) at 75% rel. air humidity, investigated and subsequently investigated again after thorough drying. All investigations were conducted in cells thermostated to 25° C. The DNA spectra were plotted with a liquid cell (layer thickness 50 μ). The concentration of the aqueous solution was 6 mg/ml. The ordinate was expanded three times.

The spectra were plotted with the IR spectrophotometer model 325, supplied by Bodenseewerk Perkin Elmer, Überlingen, W. Germany, except for the spectra in Fig. 1a and 1b which were plotted with the model 221. All spectra were plotted with the slit program 6.5 with response 3 and recording speed 1 wave number/s. The sensitivity was checked several times during spectra plotting and the amplification adjusted accordingly.

Acknowledgements. We should like to express our thanks to Dr. W. D. Lubos for the spectra of the DNA film, and to Dr. R. Herbeck for those of the solutions. Our thanks are also due to the Deutsche Forschungsgemeinschaft and to the Fonds der Chemischen Industrie for providing the facilities for this work.

References

Adler, A. J., Grossman, L., Fasman, G. D.: Polyriboadenylic and polydesoxyadenylic acids optical rotatory studies. Biochemistry **8**, 3846—3858 (1969)

Akinrimisi, E. O., Sander, C., Ts'o, P. O. P.: Properties of helical, polycytidylic acid. Biochemistry **2**, 340—344 (1963)

Beers, R. F., Jr., Steiner, R. F.: Titration and spectrophotometric studies upon polyadenylic acid. Nature (Lond.) **179**, 1076—1077 (1957)

Bellamy, L. J.: The infra-red spectra of complex molecules. 2nd ed. London: Methuen 1958

Dawydov, A. S.: Quantum mechanics. Oxford: Pergamon Press 1965

Fasman, G. D., Lindblow, C., Grossman, L.: The helical conformations of polycytidylic acid: Studies on the forces involved. Biochemistry **3**, 1015—1021 (1964)

Fritsche, H.: Infrarotspektroskopie und kernmagnetische Resonanz in der Nucleinsäureforschung. Z. Chem. **12**, 1—18 (1972)

Gulik, A., Inoue, H., Luzzati, V.: Conformation of single-stranded polynucleotides: Small-angle X-ray scattering and spectroscopic study of polyribocytidylic acid in water and in water-alcohol solutions. J. molec. Biol. **53**, 221—238 (1970)

Herbeck, R., Zundel, G.: Conformation of Mg^{2+}-poly(U) at low temperatures — IR investigations (in preparation)

Herzberg, G.: Molecular spectra and molecular structure, vol. II. New York: D. van Nostrand Co. 1945

Higuchi, S., Tsuboi, M., Iitaka, Y.: Infrared spectrum of a DNA-RNA hybrid. Biopolymers **7**, 909—916 (1969)

Hofmann, K. P., Zundel, G.: Quantitative spectroscopy — reproducible production of thin layers on supports from solutions. Rev. Sci. Instr. **42**, 1726—1727 (1971)

Holcomb, D. N., Tinoco, I., Jr.: Conformation of polyriboadenylic acid: pH and temperature dependence. Biopolymers **3**, 121—133 (1965)

Howard, F. B., Frazier, J., Miles, H. T.: Interbase vibrational coupling in G:C polynucleotide helices. Proc. nat. Acad. Sci. (Wash.) **64**, 451—458 (1969)

Janik, B., Sommer, R. G., Bobst, A. M.: Polarography of polynucleotides. II. Conformations of poly(adenylic acid) at acidic pH. Biochim. biophys. Acta (Amst.) **281**, 152—168 (1972)

Kölkenbeck, K., Zundel, G.: Ca^{2+}-ion induced conformation of polyriboadenylic acid (in preparation)

Kyogoku, Y., Tsuboi, M., Shimanouchi, T., Watanabe, I.: Nucleic acids in deuterium oxide solution. Nature (Lond.) **189**, 120—122 (1961)

Langridge, R., Rich, A.: Molecular structure of helical polycytidylic acid. Nature (Lond.) **198**, 725 to 731 (1963)

Leng, M., Felsenfeld, G.: A study of polyadenylic acid at neutral pH. J. molec. Biol. **15**, 455—466 (1966)

Maruta, H., Natori, S., Mizuno, D.: Protein synthesis with Escherichia coli ribosomes altered in conformation by monovalent cations. J. molec. Biol. **46**, 513—522 (1969)

Mc Quillen, K.: Ribosomes and the synthesis of proteins. In: Progress in biophysics, vol. 12, pp. 67—106. London: Pergamon Press 1962

Melcher, G.: The stabilization of nucleic acid structures. Biophysik **7**, 29—32 (1970)

Miles, H. T.: The structure of the three-stranded helix, poly(A+2 U). Proc. nat. Acad. Sci. (Wash.) **51**, 1104—1109 (1969)

Miyazawa, T.: Perturbation treatment of the characteristic vibrations of polypeptide chains in various configurations. J. Chem. Phys. **32**, 1647—1652 (1960)

Miyazawa, T.: Molecular vibrations and structure of high polymers. I. General method of normal coordinate treatment by internal coordinates and infrared frequencies and conformations of $(-CH_2-)_n$, $(-CH_2-O-)_n$, and $(-CH_2-O-CH_2-)_n$. J. Chem. Phys. **35**, 693—713 (1961)

Miyazawa, T., Ideguchi, Y., Fukushima, K.: Molecular vibration and structure of high polymers. IV. A general method of treating degenerate normal vibrations of helical polymers and infrared-active vibrations of isotactic polypropylene. J. Chem. Phys. **38**, 2709—2720 (1963)

Rabczenko, A., Shugar, D.: Studies of the conformation of nucleosides, dinucleoside monophosphate and homopolynucleotides containing Uracil or Thymine base residues and ribose, desoxyribose or 2′—O-methylribose. Acta biochim. pol. **18**, 387—402 (1971)

Rabczenko, A., Shugar, D.: Hydrogen bonding scheme involving ribose 2′-hydroxyls in polyribouridylic acid. Acta biochim. pol. **19**, 89—91 (1972)

Rich, A., Davies, D. R., Crick, F. H. C., Watson, J. D.: The molecular structure of polyadenylic acid. J. molec. Biol. **3**, 71—86 (1961)

Richards, E. G., Flessel, C. P., Fresco, J. R.: Polynucleotides. VI. Molecular properties and conformation of polyribouridylic acid. Biopolymers **1**, 431—446 (1963)

Riley, M., Maling, B., Chamberlin, M. J.: Physical and chemical characterization of two- and three-stranded Adenine-Thymine and Adenine-Uracil homopolymer complexes. J. molec. Biol. **20**, 359—389 (1966)

Römer, R., Riesner, D., Coutts, S. M., Maass, G.: The coupling of conformational transitions in alanine specific transfer ribonucleic acid from yeast studied by a modified differential absorption technique. Europ. J. Biochem. **15**, 77—84 (1970)

Römer, R., Maass, G.: Wechselwirkung von Mg^{2+}-Ionen mit verschiedenen Konformationen von Transfer-Ribonucleinsäuren. Hoppe-Seylers Z. physiol. Chem. **351**, 125 (1970)

Sato, T., Kyogoku, Y., Higuchi, S., Mitsui, Y., Iitaka, Y., Tsuboi, M., Miura, K.: A Preliminary investigation on the molecular structure of rice dwarf virus ribonucleic acid. J. molec. Biol. **16**, 180—190 (1966)

Shimanouchi, T., Tsuboi, M., Kyogoku, Y.: Infrared spectra of nucleic acids and related compounds. In: Advances in chemical physics, vol. VII (ed. J. Duchesne), pp. 435—498. New York: Interscience Publ. 1964

Small, E. W., Peticolas, W. L.: Conformational dependence of the Raman scattering intensities from polynucleotides. Biopolymers **10**, 69—88 (1971)

Szer, W.: Secondary structure of poly-5-methylcytidylic acid. Biochem. biophys. Res. Commun. **20**, 182—186 (1965)

Thomas, G. J., Jr., Hartmann, K. A.: Raman studies of nucleic acids. VIII. Estimation of RNA secondary structure from Raman scattering by phosphate-group vibrations. Biochim. biophys. Acta (Amst.) **312**, 311 (1973)

Thomas, G. J., Jr., Chen, M. C., Hartmann, K. A.: Raman studies of nucleic acids. X. Conformational structures of Escherichia coli transfer RNAs in aqueous solution. Biochim. biophys. Acta (Amst.) **324**, 37 (1973)

Tschirgadze, Ju. I., Sche, M., Charitonenkov, I. G.: New data on the structure of the Shugar phosphate skeleton of nucleic acids in aqueous solution. Dokl. Akad. Nauk SSSR Biophysika **203**, 959—960 (1972)

Tsuboi, M., Matsuo, K., Shimanouchi, T., Kyogoku, Y.: On the 1120 cm^{-1} band of ribonucleic acids. Spectrochim. Acta **19**, 1617—1618 (1963)
Tsuboi, M.: Infrared spectra of a few synthetic polyribonucleotides. J. Polymer Sci. C, 125—137 (1963)
Tsuboi, M.: Application of infrared spectroscopy to structure studies of nucleic acids. Appl. Spectr. Rev. **3**, 45—90 (1969)
Ts'o, P. O. P.: The physicochemical basis of interactions of nucleic acids. In: Molecular association in biology (ed. B. Pullman), pp. 39—75. New York: Academic Press 1968
Wacker, W. E. C.: The biochemistry of magnesium. Ann. N. Y. Acad. Sci. **162**, 717—723 (1969)
Wada, S.: Conformation of biopolymers. New York: Academic Press 1967
Wagner, K. G.: Zur Stacking-Spezifität der Nucleobasen. Hoppe-Seylers Z. physiol. Chem. **353**, 765 (1972)
Watson, J. D.: Abstracts of Proceedings of 6th International Congress of Biochemistry, New York, I—S 15, 1964
Witz, J., Luzzati, V.: La structure des acides polyadenylique et polyuridylique en solution: Etude par diffusion centrale des Rayons X. J. molec. Biol. **11**, 620—630 (1965)
Zmudzka, B., Shugar, D.: Role of the 2'-hydroxyl in polynucleotide conformation. Poly 2'—O-methyluridylic acid. FEBS Letters **8**, 52—54 (1970)
Zundel, G., Lubos, W. D., Kölkenbeck, K.: Proton dispersion forces. Secondary structure stabilizing forces between the hydrogen bonds of the polynucleotides. Biophys. J. **12**, 1509—1514 (1972)
Zundel, G.: Hydration and intermolecular interaction. New York: Academic Press 1969 and Moscow: Mir 1972

Prof. Dr. G. Zundel
Physikalisch-Chemisches Institut
der Universität München
D-8000 München 2
Theresienstr. 41
Federal Republic of Germany