Hydrogen Bonding in Derivatives of Adenosine and Uridine*

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ABSTRACT: Infrared spectra of carbon tetrachloride solutions of derivatives of uridine and adenosine, mono- and disubstituted on the ribose moiety, were studied. Compounds with both 2'- and 3'-hydroxyls substituted show intramolecular hydrogen bonding 5'-hydroxyl–base, and so are at least partially in the syn conformation. Compounds with the 5'-hydroxyl substituted show the 2'-hydroxyl–base intramolecular hydrogen bonding, which is sterically possible only in the anti conformation; they also show bonding between the 2'- and 3'-oxygens. An unsubstituted 3'-hydroxy group was shown to bind intramolecularly only to the 2'-oxygen. In addition, it is shown that the hydrogen bonding 2'-hydroxyl–uracil in the uridine derivatives increases the base pairing of the uracil–uracil type.

The study of nucleoside derivatives in nonpolar solutions has revealed some biologically important properties, nonexisting or completely masked in water solutions, such as base-pair formation (Felsenfeld and Miles, 1967). In this work we use nonpolar solutions to study intramolecular hydrogen bonding between base and ribose in nucleoside derivatives. The results are pertinent to an understanding of the conformation of these compounds and the modification of base-pairing ability brought about by such hydrogen bonding (Ts'o *et al.*, 1966). On extrapolation the results can give us some information about the nature of the well-known differences between polyribonucleotides and polydeoxyribonucleotides (Adler *et al.*, 1969; Bobst *et al.*, 1969; Brahms *et al.*, 1969; Bush and Scheraga, 1969; Zmudzka *et al.*, 1969, and references therein) and on the role of the hydroxy group in the aminoacyl-transfer reaction during peptide synthesis (Rychlik *et al.*, 1969; Neumann *et al.*, 1969).

The present knowledge of the ribose–base interaction in nucleosides is mainly derived from crystallographic, optical rotatory dispersion, nuclear magnetic resonance, and chemical studies. Infrared spectroscopy, used in the present study, can complement these results. It enables us to study very dilute solutions in nonpolar solvent, where intermolecular perturbations are minimal; in addition, compared to nuclear magnetic resonance results, every species present, irrespective of its lifetime, has its own absorption and so detection is easier. On the other hand, in the measurements described the substituted nucleoside derivatives and weakly solvating media are used. Consequently, when extrapolating the conclusions to other compounds and media, it must be kept in mind that both these factors can influence the conformational equilibria.

Experimental Section

Di-O-trityluridine. 2',5' and 3',5' isomers were prepared by tritylation of uridine (Zemlicka, 1964). They had infrared spectra identical with those of compounds kindly supplied to us by Drs. Zemlicka, Ogilvie (Ogilvie and Iwacha, 1969), and Moffatt (Cook and Moffatt, 1967). Furthermore both isomers gave single spots with the expected R_F values on chromatograms.

N^3-Methyl-5'-O-trityluridine. To a solution of 250 mg of 5'-*O*-trityluridine in 10 ml of 2-methoxyethanol was added at 0° 100 ml of an ethereal solution of diazomethane (prepared from 5 g of *N*-nitroso-*N*-methylurea). After 1 hr at 0° the solution was evaporated *in vacuo;* the residue was dissolved in carbon tetrachloride, precipitated by cyclohexane, and dried *in vacuo* (0.1 mm) at 60°. This gives the amorphorus *N*-methyl derivative. Upon chromatography (thin-layer chromatographic silica gel 6060, Eastman Kodak) it forms a single spot in six different solvent systems. The compound was identified by hydrolysis, achieved by heating in 80% acetic acid for 3 hr at 100°. After evaporation the hydrolysis mixture was partitioned between water and carbon tetrachloride. The water extract was evaporated and the residue had, without any purification, an infrared spectrum (KBr pellet) identical with the one of 3-methyluridine.

N^6-Methyl-5'-O-trityladenine was prepared by a slight modification of the procedure used by Jones and Robins (1963) for N^6-methyladenosine, but using 5'-*O*-tritylad enosine as the starting material. Methylation was performed in *N,N*-dimethylacetamide using methyl iodide, and the Dimroth rearrangement was performed in 1 N sodium hydroxide–ethanol (1:2, v/v) solution. The compound was recrystallized from ethanol, mp 208–210. *Anal.* Calcd for $C_{30}H_{29}N_5O_4$ (523.6). C, 68.82; H, 5.58. Found: C, 68.68; H, 5.79. *O^2*-Ethyl-2',3'-*O*-isopropylideneuridine was kindly given to us by Dr. D. M. Brown (Brown *et al.*, 1957); 2',3'-*O*-isopropylidenepseudouridine by Dr. R. W. Chambers; other compounds were commercial samples or were prepared by previously described procedures.

Spectral Measurements. A Beckman IR12 infrared spectrophotometer, flushed by dry air, was used. The wave number scale was calibrated using gaseous NH_3 and H_2O; the transmittance scale was rechecked using solutions of trichloroethylene in carbon tetrachloride (Schimozava and Wilson, 1966). Measurements were performed with infrasil cells (4, 1, and 0.2 cm long), the sample cell being placed in a thermostated jacket (30°). Carbon tetrachloride (Reagent ACS grade) was used as a solvent. This solvent is very hygroscopic; there-

* From the Gerontology Research Center, National Institute of Child Health and Human Development, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland, and the Baltimore City Hospitals, Baltimore, Maryland. *Received March 25, 1970.*

TABLE I: Absorptions of the Stretching Vibrations (ν) of OH and NH Bonds in Nucleoside Derivatives; Dilute ($C < 2 \times 10^{-3}$ M) Solutions in Carbon Tetrachloride.

No.	Compound	Band Position (cm^{-1}) and Assignment[a]
1	2′,3′-*O*-Isopropylideneuridine	3490 (HB), 3405 (NH)
2	2′,3′-*O*-Isopropylidene-6-azauridine	3502 (HB), 3386 (NH), 3204 (DIM)
3	O^2-Ethyl-2′,3′-*O*-isopropylideneuridine	3245 (HB)
4	2′,3′-*O*-Isopropylideneadenosine	3537 (NH), 3421 (NH), 3243 (HB)
5	2′,3′-Di-*O*-acetyladenosine	3537 (NH), 3421 (NH), 3250 (HB)
6	2′,3′-*O*-Isopropylidene-5′-*O*-acetyladenosine	3536 (NH), 3421 (NH)
7	5′-*O*-Trityluridine	3544 (diol), 3407 (OVL, NH), 3375 (OVLS, HB), 3224 (DIM)
8	N^3-Methyl-5′-*O*-trityluridine	3555 (diol), 3442 (HB)
9	5′-*O*-Tritylthymidine	3409 (NH)
10	2′,5′-Di-*O*-trityluridine	3567 (diol), 3407 (NH)
11	3′,5′-Di-*O*-trityluridine	3562 (diol), 3440 (OVLS, HB), 3407 (OVL, NH), 3201 (DIM)
12	N^6-Methyl-5′-*O*-trityladenosine	3562 (diol), 3450 (NH), 3250 (HB)

[a] Absorption in the range from 3600 to 3100 cm^{-1} given; OVL, overlapped; NH, ν_{NH} of groups not involved in the hydrogen bonding; DIM, ν_{NH} of groups involved in hydrogen bonding; diol, ν_{OH} of the groups involved in 2′–3′ hydrogen bonding; HB, ν_{OH} of groups involved in other hydrogen bonding; OVLS, overlapped strongly, band position approximate.

fore, whenever water absorption bands were noted in any of the spectra, drying procedures (isothermal distillation of water to phosphorus pentoxide) were invariably applied; but in no case did any significant spectral change result. Of the compounds examined, the solubilities of the following proved to be too low to obtain good spectral data: 2′,3′-*O*-isopropylidene-pseudouridine, 2′,3′-*O*-isopropylidenecytidine, 2′,3′-*O*-isopropylideneinosine, 2′,3′-*O*-isopropylidene-6-mercaptopurine riboside, and 5′-*O*-trityladenosine. Compounds **1**–**5**, **7**, **9**, and **12** (Table I) were only slightly soluble, so saturated solutions of the secompounds were prepared in the following way. The sample was boiled with stirring in carbon tetrachloride for about 3 min and left for about 15 min at room temperature, and the solution filtered through sintered glass. Solutions were further diluted to check the intramolecular character of the bands. Final concentrations were estimated in these cases by infrared absorptions. Compounds **10** and **11** were quite soluble and were studied in detail. Solutions were prepared by weighing; that of **10** decomposed upon prolonged standing, with separation of a sediment. Data were processed by linearization procedures. Compound **10** does not have an overlapped ν_{NH} (monomer) band,[1] so the common procedure (*e.g.*, Kyogoku *et al.*, 1967) was used. Compound **11** has an overlapped ν_{NH} (monomer); therefore the absorption of ν_{NH} (dimer) was used. Coordinates $X \equiv C_{total}/(A_{dimer})^{1/2}$, $Y \equiv (A_{dimer})^{1/2}$ were used; K_{dimer} = slope/(2 × intercept^2). In the calculation of apparent molar absorptivities for this compound the corresponding ϵ values of **8** and **10**, for correction at 3407 cm^{-1}, were used. The results of analyses of ir absorption data are known to be sensitive to the type of processing (*e.g.*, Kyogoku *et al.*, 1967; Drozdova *et al.*, 1969), but the difference in association of **10** and **11** is fortunately apparent even upon qualitative comparison of the spectra (Figure 1), and so the validity of our conclusions does not rely exclusively on the numerical values of K_{dimer}.

[1] ν_{XH} denotes stretching vibration of XH bond.

Results and Interpretation

Absorptions between 3700 and 2400 cm^{-1} were recorded. These bands belong generally to the stretching vibrations of XH bonds, except for some weak overtones which can be also present. Assignment of the majority of bands is straightforward. Absorptions of hydroxy groups not involved in hydrogen bonding occur above 3600 cm^{-1} (Tichy, 1965). CH bonds in trityl groups absorb in the 3100–3000-cm^{-1} region; in ribose and aliphatic substituents, in the 3000–2750-cm^{-1}

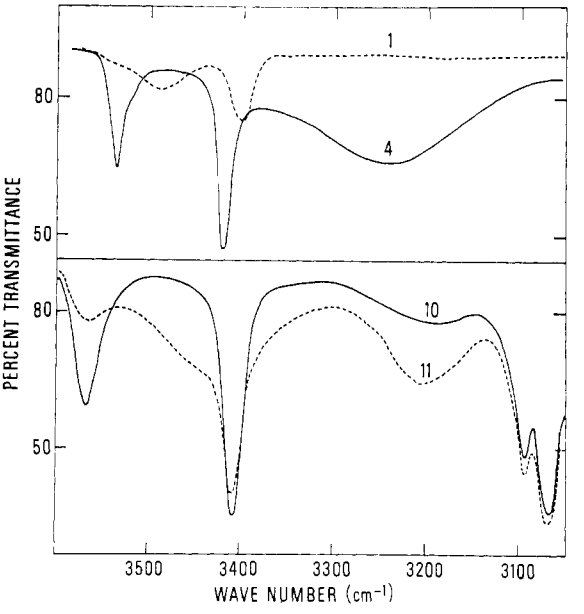

FIGURE 1: Infrared spectra of carbon tetrachloride solutions in a 1-cm cell without base-line correction: 2′,3′-*O*-isopropylideneuridine (**1**) (saturated, $<10^{-3}$ M); 2′,3′-isopropylideneadenosine (**4**) (saturated, ~4 × 10^{-4} M); 2′,5′-di-*O*-trityluridine (**10**) (2.1 × 10^{-3} M); 3′,5′-*O*-trityluridine (**11**) (2.0 × 10^{-3} M).

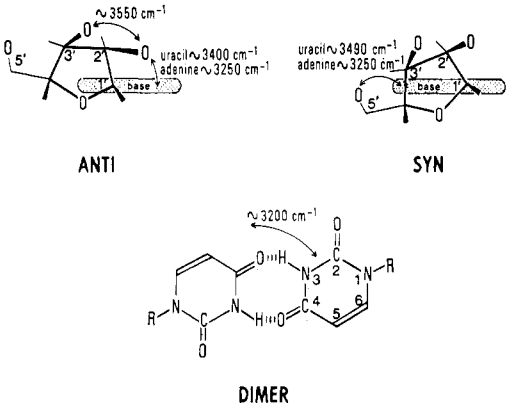

FIGURE 2: Scheme of hydrogen bonding observed in the adenosine and uridine derivatives.

region. Heterocyclic CH absorptions are usually weak and in the 3100–3000-cm^{-1} range (Kyogoku *et al.*, 1967).

All other strong bands are due to the stretching vibrations of NH bonds and to vibrations of OH bonds involved in hydrogen bonding. Only these absorptions and those from the 3600–3000-cm^{-1} region will be discussed further; their wave numbers are given in Table I, and the representative spectra are in Figure 1. The assignment of stretching vibrations of NH bonds not involved in hydrogen bonding is simple: NH bonds in uridine derivatives absorb in the 3410-cm^{-1} region (*e.g.*, Pitha and Vasickova, 1965; Küchler and Derkosch, 1966), the NH bond in the N^6-methyladenosine (**12**) derivative, at 3450 cm^{-1}; adenosine derivatives (**4**, **5**) with an amino group show two NH bands, around 3540 cm^{-1} for the asymmetrical mode and at 3420 cm^{-1} for the symmetrical mode (*e.g.*, Kyogoku *et al.*, 1967). The remaining bands belong to the bonds involved in hydrogen bonding.

Compounds **1**–**5** (Table I) have only one unsubstituted hydroxy group, that in position 5′. Previously we found (Pitha *et al.*, 1963) from a study of the infrared spectra of a number of such compounds in chloroform solutions that the hydroxy group is involved in intramolecular hydrogen bonding. The position of the corresponding absorption did not depend appreciably on the nature of the substituents on the ribose, but was very different when the base was altered. Thus, the base must be the proton acceptor in hydrogen bonding. In purine derivatives, N-3 is indicated from purely geometrical reasons. For pyrimidines it was observed that aza substitution in position 6 does not produce any drastic change in the band position, and so it was concluded that the oxygen of the carbonyl group is involved. Thus, both pyrimidine and purine derivatives must exist partially in the syn conformation (Donohue and Trueblood, 1960; compare Figure 2).

The present results are in Figure 1 (1 and 4) and in Table I. They were obtained with better resolution than the previous ones; also, the use of more transparent solvent, carbon tetrachloride, made it possible to measure the spectral region completely. Nevertheless, the conclusions are the same. Comparison of data on **1** and **9** proves that the band in question (HB in Table I) is due to the hydroxy group and not to the carbonyl overtone, which may also be expected in this region. Substitution of the nitrogen atom in position 6 in uridine derivatives (compare substances **1** and **2**) or change of substitution in positions 2′, 3′ (**4** and **5**) does not produce any appreciable shift. On the other hand, change of the base from uracil to adenine (**1** and **4**) or replacement of the carbonyl group in uracil by ethoxyl (**1** and **3**) produces large shifts. Thus, the proton acceptors are the bases generally, and in the uridine derivatives the acceptor must be the carbonyl group. Further support for this conclusion can be gained by a study of C-riboside derivatives. In such compounds the carbonyl group is in geometrically the same position as in uridine derivatives, but the ribose-bearing atom of the heterocycle, which is also within reach of the hydroxy group, is different. Pseudouridine derivatives proved to be highly insoluble, but fortunately the position of the 5′-hydroxy band was described for 6-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)-2-methyl-3-methylthio-2,5-dihydro-1,2,4-triazin-5-one by Bobek *et al.* (1969). The band positions, for this compound 3448 cm^{-1}, and 3490 cm^{-1} for the corresponding uridine derivatives, are close enough to give further evidence that carbonyl groups act as proton acceptors in both cases.

The bands just discussed were tested as to their intramolecular character. Characteristically, the absorptions persist to concentrations as low as 5×10^{-4} M in carbon tetrachloride without significant change. This, on the basis of general experience in the field (Bennet *et al.*, 1967), practically excludes the possibility that the absorptions are due to intermolecular associations.

The role of hydroxy groups in positions 2′ and 3′ was studied in **7**–**12**. The simplest patterns are obtained for compounds having only the 3′-hydroxy group unsubstituted. This hydroxy group can then apparently form an intramolecular hydrogen bond only to the substituent in position 2′, if any is present. Thus, the 2′-deoxy derivative **9** does not show any hydrogen bonding at all, and the 2′,5′-*O*-dityrityl derivative **10** has hydroxyl absorption at 3567 cm^{-1} (Figure 1). A comparison of other compounds having possible hydrogen bonding between 2′- and 3′-oxygens, **7**, **8**, and **10**–**12**, shows that bands in the range 3540–3570 cm^{-1} are invariably present. This range is comparable to the one found in *cis*-cyclopentane-1,2-diol derivatives, 3580–3540 cm^{-1} (Tichy, 1965), which have similar geometry. Thus all these bands (denoted diol in Table I; compare Figure 2) must be assigned to such hydrogen bonds.

Compounds which have an unsubstituted hydroxy group in position 2′ show additional bands (Table I). In uridine derivatives **7**, **8**, **11**, the bands are in the region 3375–3450 cm^{-1} (Figure 1); in the adenosine derivative **12**, the band is at 3250 cm^{-1}. Clearly, the corresponding hydrogen bond must be between the 2′-hydroxy group and the base. The 3250-cm^{-1} band given by the adenosine derivative is present even at concentrations as low as 2×10^{-4} M, which testifies to the intramolecular character of the bond. For the uridine derivatives **7** and **8** the situation is the same.

On the other hand, the uridine derivative **11** gives a more complex spectral pattern. The band corresponding to the ribose–uracil hydrogen bond is hardly detectable at very high dilution but increases strongly with concentration. Furthermore, the spectrum, compared to the one of the isomer **10**, has a more prominent 3200-cm^{-1} band at the same concentrations (Figures 1 and 3). Since the intensity of that band corresponds to the amount of base-paired dimer, the formula of which is shown in Figure 2 (Küchler and Derkosch, 1966), the two isomers **10** and **11** must differ in their ability to form

this type of dimer. Analysis of data from Figure 3 confirms this qualitative conclusion. The value K_{dimer} for isomer **10** is 93 l. mol^{-1}, which is comparable to the association of the fully substituted uridine derivative (Küchler and Derkosch, 1966). On the other hand, for isomer **11**, which has a ribose–uracil hydrogen bond, the processing gives a higher value of K_{dimer}, 194 l. mol^{-1}, thus indicating a connection between these hydrogen bonds. Further it may be noted that increasing concentration of **11** also increases the extinction coefficient of the band assigned to the ribose–uracil hydrogen bond (denoted as ◐ in Figure 3 on the right). The magnitude of this increase (approximately 3-fold; Figure 3) suggests that there is a mutual reinforcement between the corresponding uracil–uracil and uracil–ribose hydrogen bonds. But the entire increase is not necessarily attributable to such a mechanism. The extinction coefficients inherent in different species may vary; *e.g.*, dimers absorb more strongly than monomers. On the other hand, attempts to account for the phenomena observed solely by such variations leads to unreasonably large differences in extinction coefficients of the individual species.

Uridine derivatives **7** and especially **8**, nevertheless, show that the ribose 2′-hydroxyl–uracil hydrogen bonding persists even without any assistance from uracil–uracil pairing. The bonding in these cases may be supported by two other factors. One is the decreased steric strain, since one bulky trityl group is missing. Another may be the presence of an additional hydrogen bond: simultaneous bonding from hydroxyl 3′ to 2′ from 2′ to base; such a scheme must be energetically favored and, indeed, all the corresponding bands are seen in the spectra. A summary of our assignment of the bands of bonded hydroxy groups is given in the scheme in Figure 2.

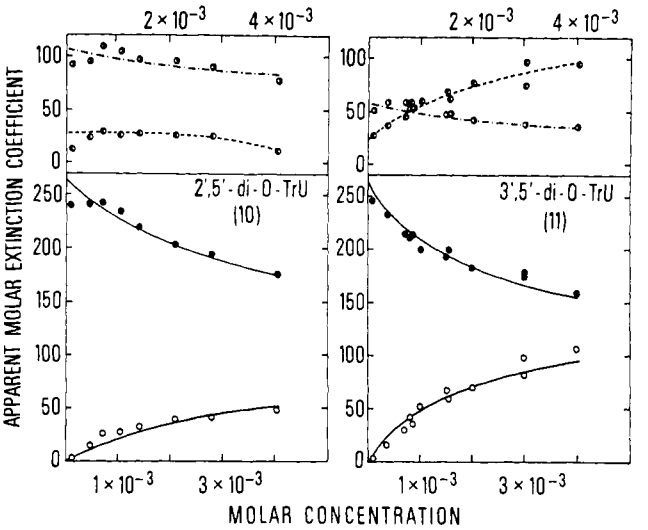

FIGURE 3: Concentration dependence of apparent molar extinction coefficients of 2′,5′-di-*O*-trityluridine (**10**) and 3′,5′-di-*O*-trityluridine (**11**). Full lines denote the calculated dependences, points denote the observed ones: (●) 3407 cm^{-1} (NH *cf.* Table I); (◑) 3567 and 3562 cm^{-1}, respectively (diol); (◐) 3440 cm^{-1} (HB in **11**); (○) 3200 cm^{-1} (DIM).

Discussion

Of the two plausible conformations of the ribose moiety in nucleoside derivatives the anti form was the only one found in a number of cases, both in crystals and solutions. This prevalence is stressed in many articles, but it should be noted that the other form, syn, occurs in at least a few crystal structures. A recent review by Sundaralingam (1969) notes 37 instances of anti conformation compared to 7 syn, the latter all being found in purine derivatives; but the syn form has subsequently been found in crystals of 4-thiouridine (Saenger and Scheit, 1969), and some evidence exists for the presence of syn forms in solutions (Chambers, 1966; Guschlbauer and Courtois, 1968; Pitha *et al.*, 1963; Ward and Reich, 1968). There are no clear steric (Haschemeyer and Rich, 1967) or electronic (Jordan and Pullman, 1968) reasons for any forbiddingly large energetic difference between these two forms; it may be assumed that the observed prevalence of the anti form is the result of such factors as efficiency of packing in crystals or solvation differences in solutions.

The present results are actually in accord with such views. Derivatives with an unsubstituted 5′-hydroxy group can undergo intramolecular hydrogen bonding only in the syn conformation; bonding of the type: 2′-hydroxyl → base is possible only in the anti conformation (Figure 2). Both types of bonding were detected in both uridine and adenosine derivatives in nonpolar solutions. In such solutions all possible hydrogen bonds are usually formed; transfer to a polar environment may then decrease the importance of these particular hydrogen bonds in favor of other ones in the solute–solvent system. Thus, our results are in no way contradictory to the prevalence of the anti conformation in water solutions.

The hydroxyl in position 2′ of the ribose moiety is influenced by two factors which make this group different from the 3′-hydroxyl. One is the inductive effect from the glycosidic 1′ carbon: this increases the hydroxyl's acidity (Fox *et al.*, 1953; Gin and Dekker, 1968, and references therein) and the rate of reactions such as methylation with diazomethane (Martin *et al.*, 1968; Gin and Dekker, 1968). The other 2′-hydroxyl modifying factor is its hydrogen bonding to the base. This bonding necessarily *lowers* the hydroxyl's acidity and reactivity, and therefore can be only of a secondary influence in the reactions mentioned. Nevertheless, there are other reactions, where both factors can act in the same direction. The higher acidity of the 2′-hydroxy group and its stabilization through hydrogen bonding are bound to increase the acylating ability of its esters compared to that of 3′-hydroxy derivatives. There is some chemical evidence for this. Michelson *et al.* (1956), Brown *et al.* (1956), and Fromageot *et al.* (1968) found that by partial acetylation to equilibrium the 3′,5′-diacetyl derivatives predominate. Also, adenosine derivatives were found (Neumann *et al.*, 1969) to undergo some intramolecular transacetylations; it was implied that the reaction could be a possible step in protein biosynthesis.

Our results, with the exception of unpublished material of Michelson (1961), give the first direct evidence for 2′-hydroxyl–base hydrogen bonding, which has been frequently evoked, and as early as 1953 (Fox *et al.*). They also show that the ribose–base hydrogen bonding indeed modifies the pairing abilities of base as envisaged by Ts'o (Ts'o *et al.*, 1966).

On the nucleotide or polynucleotide level there is another hydrogen-bond acceptor near the 2′-hydroxy group: phosphate. The interaction between the 2′-hydroxyl and phosphate (Brahms *et al.*, 1969, and references therein) is inaccessible to direct ir study in nonpolar solvents, but such a hydrogen bond must be at least comparable in strength to the bonding of the

TABLE II: Association Constants (l. mol^{-1}) of the Hydrogen-Bonded Complexes (1:1) at 20° in Carbon Tetrachloride Solutions.

Components	K_{assocn}	Reference
Methanol–*N*,*N*-dimethyl-formamide	6	Becker (1961)
Methanol–pyridine	3	Becker (1961)
Methanol–trimethyl phosphate	11	Gramstad (1961)
Phenol–1-methyl-2-pyrrolidone	163	Gramstad and Fuglevik (1965)
Phenol–δ-valerolactam	146	Gramstad and Fuglevik (1965)
Phenol–pyridine	60	Gramstad (1962)
Phenol–triethyl phosphate	239	Aksnes and Albriktsen (1968)

hydroxy group to the oxygen or nitrogen atoms of heterocycles. This is illustrated by the data from the literature collected in Table II.

In water solutions the situation is more complex. The 2′-hydroxy group is, in all the different helical structures, fully exposed to the water molecules, some of which are very strongly bound to polynucleotides (Falk, 1966). Under these conditions the hydrogen bonding observed in nonpolar solutions can be mediated by water molecules, the base, 2′-hydroxy group, and phosphate serving only as anchoring points, necessary for certain types of hydration or cation binding. But one parallel exists. Our results show that hydrogen bonding to the uracil moiety stabilizes the uracil–uracil association; Zmudzka *et al.* (1969), correspondingly, have found that the 2′-hydroxyl group stabilizes the presumably double-helical structures of uracil- and thymine-containing polynucleotides. A similar mechanism may be operative in both phenomena: the 2′-hydroxy group binds directly to the base or acts by fixing the water molecule bond to the base; and thus the uracil–uracil base pair is stabilized.

Acknowledgments

This work was made possible through the kind interest and support of Dr. G. L. Eichhorn. Further, I wish to thank Drs. D. M. Brown, R. W. Chambers, J. G. Moffatt, K. K. Ogilvie, and J. Zemlicka for samples of some of the compounds studied and to the referees for helpful comments.

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Nucleotide Distribution of *Escherichia coli* 16S Ribosomal Ribonucleic Acid*

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ABSTRACT: Chemical analyses have been performed on oligonucleotides arising from digestion of ^{32}P-labeled *Escherichia coli* 16S rRNA with pancreatic ribonuclease. The digestion products of the RNA were separated into individual oligonucleotides by two steps of column chromatography, first with DEAE-cellulose at neutral pH according to chain length, and second with Dowex 1-X2 at acidic pH according to nucleotide composition. The analyses indicated that several oligonucleotides existed in amounts of less than 1 mole, while the 5′-terminal sequence, pApApApUp, was present in *ca.* 1 mole in the RNA. These results suggest the internal heterogeneity in nucleotide sequence of *E. coli* 16S rRNA.

The molecular weight of the bacterial 16S rRNA has been estimated by physical and chemical methods, both giving 5.5×10^5. A 16S rRNA thus contains about 1500 nucleotides (Kurland, 1960; Green and Hall, 1961; Stanley and Bock, 1965a; Midgley, 1965; Nichols and Lane, 1967; Fellner *et al.*, 1970). The 16S rRNA preparations usually reveal physical homogeneity in the sedimentation or other physical analyses. On the other hand, DNA–rRNA hybridization experiments indicate that the 16S rRNA cistrons exist on bacterial chromosome in multiple (Yankofsky and Spiegelman, 1962, 1963; Attardi *et al.*, 1965; Oishi and Sueoka, 1965). It is then possible that the 16S rRNA, which appears to be homogeneous in terms of physical criteria, might be chemically heterogeneous. Several investigators have presented evidence for the heterogeneity of 16S rRNA in *Escherichia coli* (Aronson and Holowczyk, 1965; McIlreavy and Midgley 1967; Midgley and McIlreavy, 1967; Peacock and Dingman, 1968; Young, 1968; Maruyama and Mizuno, 1970). The recent sequence analysis by Fellner *et al.* (1970) also suggested the presence of occasional "point" heterogeneity in the 16S rRNA molecules.

It is the purpose of this paper to present further evidence for the chemical heterogeneity of *E. coli* 16S rRNA.

Experimental Section

A. Cells and Culture Conditions. Escherichia coli Q13, RNase I less mutant derived from K12 strain (Gesteland, 1966), was incubated at 37° in the phosphate-free Tris–salts–glucose medium (Mitsui *et al.*, 1963) containing 0.05% Casamino Acids (Difco) and 0.05% yeast extracts (Difco). In the exponentially growing phase, 0.1 mCi/ml of carrier-free [^{32}P]orthophosphate was added to the culture and the incubation was continued for about 3.5 generations (doubling time 70 min). Cells were harvested by centrifugation, washed with TM buffer,[1] and frozen at −20°.

B. Preparation of Ribosomes and rRNA. All operations described below were carried out at 4°. Crude extracts of the ^{32}P-labeled cells, which were prepared as described in the previous paper (Muto, 1968), were applied onto 5–20% linear sucrose gradient made in TM buffer (27 ml) and centrifuged at 20,000 rpm for 16 hr in an SW 25-1 rotor (Beckman-Spinco ultracentrifuge). About 1-ml fractions were collected from the bottom of the tube. A 0.02-ml portion was removed from each fraction, dipped on a filter paper of 1 cm^2, and dried. After measurements of radioactivity in a liquid scintillation spectrometer, the fractions containing 30S ribosomal subunits were combined, diluted threefold with TM buffer, and submitted to RNA preparation (Muto, 1968). The RNA prepared from 30S ribosomal subunits was further purified by centrifugation through 5–20% sucrose gradient at 25,000 rpm for 20 hr as described above. The ^{32}P-labeled 16S rRNA so prepared was collected together with about 5 mg of nonlabeled rRNA by ethanol precipitation and stored at −20° until use.

C. Pancreatic RNase Digestion. The ^{32}P-labeled 16S rRNA preparation was dissolved in 0.01 M Tris-HCl buffer (pH 7.5) containing 0.002 M EDTA. The RNA concentration was adjusted to 5 mg/ml with an addition of nonlabeled rRNA. About 10^7 cpm/mg of [^{32}P]RNA was digested with pancreatic RNase (RNase A; Worthington Biochemical Corp.; phosphate-free, RFA 8GD) using an enzyme to substrate ratio of 1:100 (w/w), at 37° for 13 to 18 hr in the presence of one drop of chloroform. The digests were diluted

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[1] Abbreviations used are: TM buffer, 0.01 M Tris-HCl–0.1 mM magnesium acetate (pH 7.5); BBOT, 2,5-bis[2-(5-*tert*-butybenzoxazolyl)]-thiophene (Packard).