[8] *Ibid.*, Definition 8.1.

[9] *Ibid.*, section 11.

[10] *Ibid.*, Theorem 10.2.

[11] Kodaira, K., L. Nirenberg, and D. C. Spencer, "On the existence of deformations of complex analytic structures," *Ann. of Math.*, **68**, 450–459 (1958).

[12] Atiyah, M. F., and F. Hirzebruch, "Riemann-Roch theorems for differentiable manifolds," *Bull. Amer. Math. Soc.*, **65**, 276–281 (1959).

[13] Enriques, F., *Le Superficie Algebriche* (Bologna, 1949).

---

*THE STRUCTURE OF THE THREE-STRANDED HELIX, POLY (A + 2U)**

BY H. TODD MILES

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES,
NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

Communicated by Norman Davidson, April 2, 1964

The interaction of polynucleotides to form helical structures results in characteristic changes in the infrared spectra[1–4] in aqueous (D_2O) solution. Although the origin of these spectral changes is complex and not at present understood in detail,[1, 5, 5a] it appears from all observations to date that when a carbonyl group in a nucleotide base is hydrogen-bonded to another polynucleotide chain in a helix, there is an increase in the vibrational frequency of that carbonyl group. We now propose this frequency shift as an empirical criterion of interbase hydrogen bonding in a helix, and apply the criterion in support of the structure proposed for poly (A + 2U) based on the hydrogen bonding scheme I. A stereochemically satisfactory three-dimensional model of the proposed structure has been constructed.

The essential conclusion which we derive from the infrared spectrum of the three-stranded helix (Fig. 1) is that the uracils in the two poly U strands are hydrogen-bonded to the adenine in the poly A strand by *different* oxygen atoms. This conclusion requires only that the 1691 cm^{-1} band and the 1657 cm^{-1} band of uridine involve the vibrations of different oxygen atoms and is independent of which assignment is made of the bands. A correct assignment is important, however, for the two-stranded helix, poly (A + U). From synthesis and spectroscopic observation of uridine-4-O^{18} we have obtained evidence that the two bands in question do have predominant contributions from the vibrations of different oxygens and that the higher frequency band has a major contribution from a C_2-O vibration and the 1657 cm^{-1} band from a C_4-O vibration.

Materials and Methods.—The infrared spectra of D_2O solutions were measured with a Beckman IR-7 spectrophotometer using matched CaF_2 cells of fixed path length.[1–4] The spectra were digitized at 1.25 cm^{-1} intervals with a Gerber X-Y reader and normalized to an extinction coefficient basis with a Honeywell-800 computer, as described in a recent communication.[3]

Uridine-4-O^{18} was prepared by nitrous acid deamination of cytidine[6] in 91 per cent D_2O^{18}. Because of isotope dilution by exchange between solvent and the nitrous acid oxygens the O^{18} content of the uridine was estimated to be ~82 per cent. Paper chromatography in two solvent systems (2-propanol-HCl and 1-butanol-

water-acetic acid) capable of resolving the two nucleosides showed that uridine was the sole product of the reaction and that no cytidine remained. Previous experiments had shown that neither nucleoside undergoes oxygen exchange with water in neutral solution on standing for two months. The spectra of cytidine, uridine, and uridine formed by deamination of cytidine in D_2O^{16} and in D_2O^{18} are shown in Figure 2.

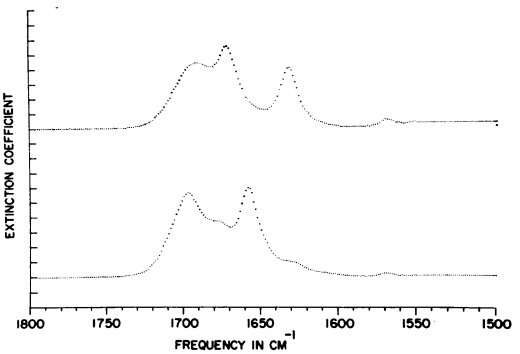


FIG. 1.—Upper curve, infrared spectrum of poly (A+U), 0.14 *N* Na^+, pD 7.3 ± 0.2. Lower curve, poly (A+2U), 0.14 *N* Na^+, pD 7.3 ± 0.2. Vertical index marks 100 units apart. Concentrations based on total polymer phosphate.

Three-dimensional models of the helices discussed here were constructed using models made by Cambridge Repetition Engineers (2 cm = 1 Å). We adopted the dimensions of the bases found by Hoogsteen[7] in the AT pair and used them to form the bonding schemes shown in I and II. Construction diagrams were prepared (to change the scale to 2 cm = 1 Å) and overlaid with clear plastic disks of 9-inch diameter to which grooved brass rods were screwed to preserve the glycosidic angles and C_1' positions indicated in I and II. These plates were attached to an axial rod at 6.8-cm (3.4 Å) intervals, and the ribose-phosphate chains attached by their 1′-carbons to the grooved brass rods. The plates can be rotated about the axis, and various sugar-phosphate conformations can be conveniently observed.

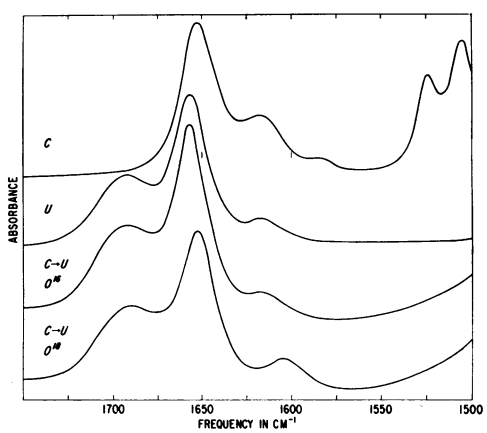
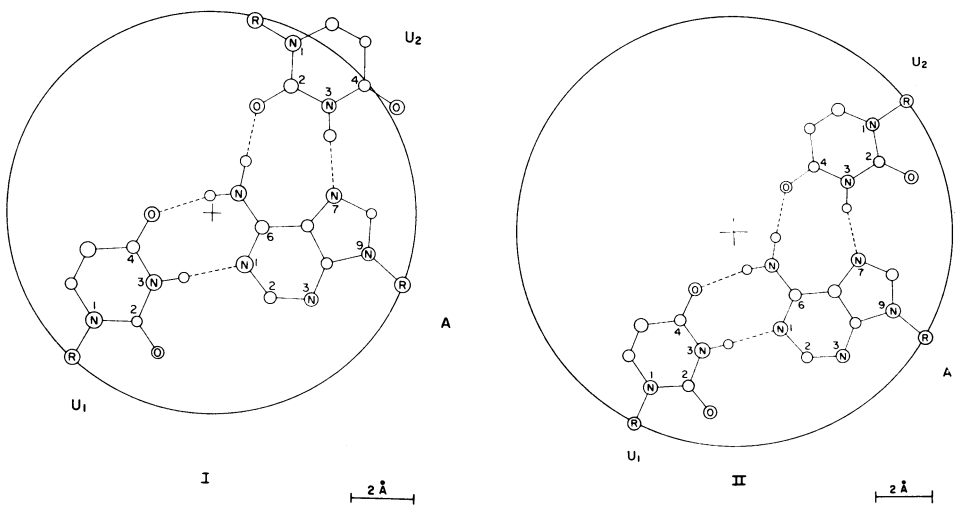


FIG. 2.—Infrared spectra in neutral D_2O solution of: C, cytidine; U, uridine; C → U (O^{16}) uridine formed by deamination of cytidine in D_2O^{16}; C → U (O^{18}) uridine formed by deamination of uridine in 90% D_2O^{18}. The rising base lines at lower frequencies in the latter two curves are attributable to contamination of the sample solutions with water.

Discussion.—Band assignment: While in general it is difficult to interpret the infrared spectra of large molecules, some of the vibrations in complex molecules may, to a first approximation, be considered to be primarily localized in a stretching motion between two of the atoms in the molecule. The practical application of this approximation had led to the recognition of characteristic group frequencies,[8, 9] the theoretical basis and limitations of which have been discussed by Herzberg.[10] We shall limit our attention to vibrations in the double-bond region, and particularly to carbonyl vibrations, for which the approximation of a diatomic vibration (strongly coupled in the case of conjugated unsaturation) may have sufficient validity to be structurally useful.



Our band assignments of nucleoside spectra have been based upon studies of alkylated model compounds,[1, 11, 12] upon the effect of deuterium exchange, and upon isotopic synthesis[13] with O^{18}. We have recently prepared inosine-6-O^{18}, inosinic acid-6-O^{18}, and guanosine-6-O^{18} by enzymic deamination in D_2O^{18}. These O^{18} compounds showed a decrease in frequency of 14 cm^{-1}, confirming the carbonyl assignment of the appropriate bands, but indicating strong coupling with other bonds in the ring.[14] Uridine-4-O^{18} (Fig. 2) showed a more complex isotope effect, with frequency decreases of both the 1657 cm^{-1} (-5 cm^{-1}) and the 1618 cm^{-1} bands (-12 cm^{-1}), but no frequency change of the 1691 cm^{-1} band. For the present purpose it suffices that the isotopic experiment supports the contribution of a C_4-O vibration to the 1657 cm^{-1} band, but not to the 1691 cm^{-1} band. A detailed interpretation of the two lower frequency bands[15] must await further work but is not essential to the structural conclusions reached here.

Frequency changes upon helix formation: The infrared spectra of polynucleotide helices which we have so far observed show an increase in the frequency of carbonyl bands upon helix formation if a carbonyl group is hydrogen-bonded to a base of a complementary polymer chain (summary, Table 1), but no increase if it is not so bonded. Thus, in poly (A + U) (Fig. 1, Table 1) we observe that the C_4 carbonyl, which is hydrogen-bonded in the Watson-Crick structure, has undergone a frequency increase of 15 cm^{-1}, while the C_2 carbonyl, which is not bonded, is unchanged in frequency.

Poly (A + 2U), on the other hand, shows a quite different spectral pattern (Fig. 1, Table 1). Here the high frequency band is at 1696 cm^{-1} instead of 1691 cm^{-1}, observed in poly (A + U). We interpret this band as a composite of a bonded C_2 carbonyl vibration (of higher frequency than the corresponding band in poly U) in the U_2 strand (scheme I) and an unbonded C_2 carbonyl band (unchanged from the uninteracted poly U frequency) in the U_1 strand. A second major difference in the poly (A + 2U) spectrum is the presence of a strong 1657 cm^{-1} band, absent in poly (A + U) but present in poly U. This band is assigned to the unbonded C_4 carbonyl of the U_2 strand in structure I. The band at $\sim$1677 cm^{-1} is common to both spectra and is attributed to the bonded C_4 carbonyl in the U_1 strand of I.

TABLE 1

CARBONYL ABSORPTION MAXIMA OF NUCLEOTIDES AND POLYNUCLEOTIDES IN D_2O SOLUTION[a]

Compound	Secondary structure	νmax in cm^{-1}	$\Delta\nu$[b]	Assignment	νmax in cm^{-1}	$\Delta\nu$	Assignment	Ref. to IR data
Uridylic acid	—		—	$C_2 = O$		—	$C_4 = O$	11
Poly U	Random coil	1692	—	$C_2 = O$ (U)[c]	1657	—	$C_4 = O$ (U)	1, 3
Poly (A + U)	Helical	1691	−1	$C_2 = O$ (U)	1672	+15	$C_4 = O$ (B)	1, 3
Poly (A + 2U)	Helical	1696	+4	$C_2 = O$ (B & U)[d]	1677	+20	$C_4 = O$ (B; U_1 strand)	1, 3
					1657	0	$C_4 = O$ (U; U_2 strand)	
Inosinic acid	—	1673	—	$C_6 = O$				1
Poly I	Random coil	1674	—	$C_6 = O$ (U)				1
Poly I	Helical	1685	+11	$C_6 = O$ (B)				2
Poly (I + C)		1695	+21	$C_6 = O$ (B)				1, 2
Poly (A + 2I)	Helical	1682	+8	$C_6 = O$ (B)				Fig. 3
Cytidylic acid	—	1652	—	$C_2 = O$ (U)				1, 11
Poly C	Random coil[e]	1656	—	$C_2 = O$ (U)				1
Poly (I + C)	Helical	1648	−8	$C_2 = O$ (U)				1
5′-GMP	Monomeric	1665	—	$C_6 = O$ (U)				4
5′-GMP	Helical	1680	+15	$C_6 = O$ (B)				4
3′-GMP	Monomeric	1666	—	$C_6 = O$ (U)				4
3′-GMP	Helical	1671	+5	$C_6 = O$ (B)				4
Poly G	Helical	1682	+17	$C_6 = O$ (B)				4
5′-GMP + poly C	Helical	1680	+15	$C_6 = O$ (B)				21

[a] Measured with a Beckman IR-7 spectrophotometer in fixed thickness CaF_2 cells. Frequencies estimated to be accurate to the nearest 1 cm^{-1}. Precision is somewhat less than this in some cases because of chemical variability.

[b] $\Delta\nu$ expressed as difference between the frequency of a band in the random coil homopolymer and that of the same band in the helix. In the case of the values for the guanylic acids the frequency of 5′-GMP is taken as the standard.

[c] The symbol (U) after the assignment indicates that the carbonyl oxygen is unbonded to the other helical strand; the symbol (B) indicates that it is bonded to the other helical strand. It is understood that in aqueous solution all "free" carbonyls are bonded to the solvent.

[d] This band is considered to result from the overlap of a $C_2 = O$ (U) band in the U_1 strand and a $C_2 = O$ (B) in the U_2 strand. The assignments for this helix are made in accordance with structure I.

[e] The term "random coil" is used for convenience. Poly C has a considerable amount of order in neutral solution.

These interpretations are consistent with the condition that the two uridine strands be bonded by different oxygen atoms, as well as with more detailed assignments of the uridine bands.

In the original paper[16] describing the formation of a three-stranded helix poly (A + 2U), a different bonding arrangement (II) for the third strand was suggested. Since scheme II involves bonding to the same carbonyl in both poly U strands, however, the arguments outlined above lead us to consider that this arrangement is much less compatible with the solution infrared spectra than is I. The possibility that there is bonding to the same carbonyl group in both strands of poly (A + 2U) but with different frequency shifts because of the different chemical environments of the two strands in I is made unlikely by consideration of the spectrum of poly (A + 2I) in Figure 2. This helix[17] in all probability has the structure III, since an arrangement analogous to I is not possible for inosine (in this case the greater distance of the inosine glycosidic carbon from the pyrimidine ring results in greater separation of the second ribose-phosphate chain of poly I from that of poly A than would exist in structure II). The spectrum of poly (A + 2I) in Figure 3 shows a single carbonyl band, however, which is both narrower and more symmet-

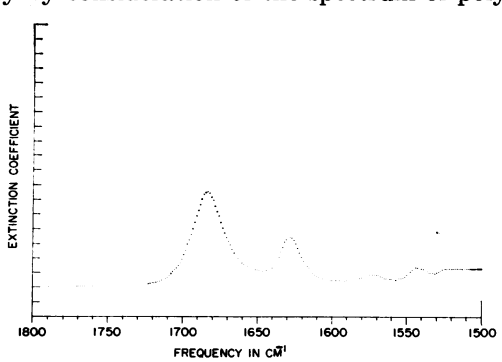
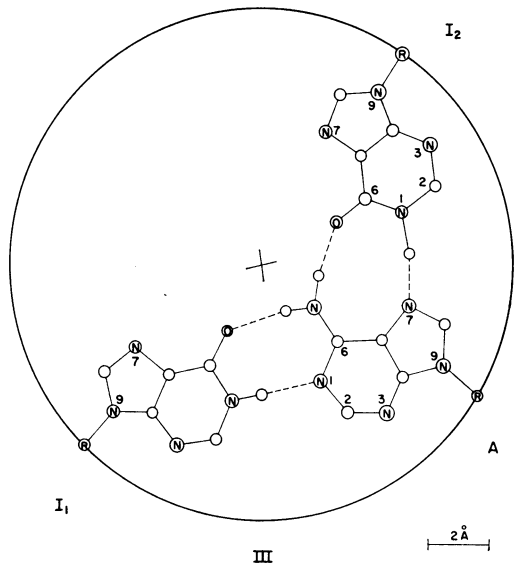


FIG. 3.—Infrared spectrum of poly (A+2I) in D_2O solution 0.14 N Na, 0.02 N Mg^{++}, pD 7.3 ± 0.2. Vertical index marks 100 units apart. Concentration based on total polymer phosphate.



III

rical than that of poly I. Thus, the difference in environment of the two strands in III does not result in detectably different frequency shifts of the same carbonyl group in different strands and would be unlikely to do so in II.

The bonding arrangement between strands A and U_2 in I has recently been observed in a mixed crystal[18] (adenosine-5-bromo-uridine), suggesting that there is nothing inherently improbable about such a bonding scheme.

We may draw a further, tentative conclusion from one of the noncarbonyl vibrations in the two poly A-poly U helices. The virtual disappearance of the adenosine ring band in the three-stranded helix (Fig. 1; band at ~1630) suggests that there is an additional point of interaction at the adenine ring, presumably a hydrogen bond, the only plausible position for such a bond being N-7, as in both I and II. When both the amino group and the N-1 position are bonded, as in poly (A + U), there is a decrease in the intensity of the adenosine band, though not nearly as great as in the three-stranded helix. An analogous case of a large reduction in intensity of the purine ring vibration,[4] in which the N-7 position is believed from X-ray evidence[19] to be hydrogen-bonded, is that of the 5′ and the 3′-GMP gels.

Stereochemical considerations: We have built a stereochemically satisfactory model of I, adopting Hoogsteen's dimensions for A from the AT pair since they are based on the only precise crystallographic analysis reported for an unprotonated 9-substituted adenine. It is quite possible that the base dimensions in the helix may depart from these values and that, in fact, helix formation may lead to minor deviations from "standard" dimensions, producing a better fit between the bases and more favorable orientations for the ribose-phosphate chains.[20]

Structure I has strands U_1 and U_2 antiparallel to strand A. Structure II has strand U_1 antiparallel to strands A and U_2, in both cases maintaining an *anti*-conformational relationship between the bases and the sugars. In addition to this difference in polarity of the chains, structure II places strands A and U_2 in closer proximity than I and may involve closer approach of the phosphates in the two

chains, though at the present time it does not appear to be possible to rule out either structure purely on stereochemical grounds. Our proposal of structure I is based on the infrared evidence presented above.

* Abbreviations, poly A, polyadenylic acid; poly U, polyuridylic acid; poly I, polyinosinic acid; poly G, polyguanylic acid; poly C, polycytidylic acid; poly (A + U), two-stranded helical product of poly A and poly U; poly (A + 2U), three-stranded helix; poly (I + C), helical product of poly I and poly C; poly (A + 2I), three-stranded helix formed between poly A and poly I; GMP, guanosine monophosphate.

[1] Miles, H. T., *Biochim. Biophys. Acta*, **30**, 324 (1958), and **45**, 196 (1960); these PROCEEDINGS, **47**, 791 (1961); *Nature*, **195**, 459 (1962).

[2] Sigler, P., D. R. Davies, and H. T. Miles, *J. Mol. Biol.*, **5**, 709 (1962).

[3] Miles, H. T., and J. Frazier, *Biochem. Biophys. Res. Commun.*, **14**, 21, 129 (1964).

[4] Miles, H. T., and J. Frazier, *Biochim. Biophys. Acta*, **79**, 216 (1964).

[5] Miles, H. T., *Nature*, **181**, 1814 (1959).

[5a] It is possible that a suggestion of Krimm [Krimm, S., *J. Chem. Phys.*, **23**, 1371 (1955)] regarding the difference in the spectra of α and β polypeptides may help to explain the frequency changes reported here. The angle formed by the C = O of the carbonyl with the O or N to which the bonding hydrogen is covalently attached may have an important influence on the carbonyl frequency. Thus, while a water molecule is free to assume any orientation with respect to the carbonyl group in the random coil form, the C = O . . . N angle in the helix is necessarily restricted to approximately 130°. This explanation would presuppose that the favored orientation of the water molecule involves an angle differing appreciably from 130°, a point on which clear experimental evidence appears to be lacking.

[6] Levene, P. A., and W. A. Jacobs, *Ber.*, **43**, 3150 (1910).

[7] Hoogsteen, K., *Acta Cryst.*, **12**, 822 (1959), and **16**, 907 (1963).

[8] Bellamy, L. J., *The Infrared Spectra of Complex Molecules* (Methuen & Co., Ltd., 2nd ed., 1958).

[9] Jones, R. N., and C. Sandorfy, in *Chemical Applications of Spectroscopy* (Interscience, 1956), chap. 4.

[10] Herzberg, G., *Infrared and Raman Spectra* (D. Van Nostrand Co., Inc., 1945), p. 194.

[11] Miles, H. T., *Biochim. Biophys. Acta*, **22**, 247 (1956), and **27**, 46 (1958).

[12] Miles, H. T., F. B. Howard, and J. Frazier, *Science*, **142**, 1458 (1963).

[13] For other examples of the use of O^{18} substitution as an aid in interpreting vibrational spectra, see, e.g., Pincas, S., D. Samuel, and M. Weiss-Broaday, *J. Chem. Soc.*, 2382, 3063 (1961); Becker E. D., H. Ziffer, and E. Charney, *Spectrochim. Acta*, **19**, 1891 (1963); Karabatsos, G. F., *J. Org Chem.*, **25**, 315 (1960).

[14] Howard, F. B., and H. T. Miles, *Biochem. Biophys. Res. Commun.*, **15**, 18 (1964).

[15] The two bands might, for example, be attributed to symmetric and antisymmetric modes of the $C_6 = C_5 - C_4 = O$ system. The relative intensities of the two bands would make it difficult simply to reverse the previous assignments.

[16] Felsenfeld, G., D. R. Davies, and A. Rich, *J. Am. Chem. Soc.*, **79**, 2023 (1957).

[17] Rich, A., *Nature*, **181**, 521 (1958).

[18] Haschemeyer, A. E. V., and H. M. Sobell, these PROCEEDINGS, **50**, 872 (1963).

[19] Gellert, M., M. N. Lipsett, and D. R. Davies, these PROCEEDINGS, **48**, 2013 (1962).

[20] A clear statement of the importance of small deviations from "standard" dimensions has been made by Sasisekharan, drawing examples primarily from the amino acids and peptides, *Collagen* (Interscience, 1962), p. 46.

[21] Howard, F. B., J. Frazier, and H. T. Miles, in preparation. Although the carbonyl frequency is the same in this case as in the 5′-GMP gel, the latter structure can be clearly excluded by the absence of the characteristic 1595 cm^{-1} ring vibration observed in the gel.[4]