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*THE ACCOMMODATION OF NONCOMPLEMENTARY BASES IN HELICAL POLYRIBONUCLEOTIDES AND DEOXYRIBONUCLEIC ACIDS**†

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It is now well established that deoxyribonucleic acid* is the molecular counterpart of the genetic apparatus. In this two-stranded helical polynucleotide the perfect crystalline geometry which normally prevails requires that adenine residues in one chain be hydrogen-bonded to thymine residues in the other; similarly, guanine residues can only be hydrogen-bonded to cytosine residues.[1–3] Thus, one chain is necessarily the complement of the other, and it appears likely that each chain serves as the template for the synthesis of its complement.[4–6]

Current views on the molecular basis of mutations hold that they are due to a variety of alterations in the nucleotide sequence within the two-stranded DNA helix. Presumably, many of these alterations arise from mistakes made during its replication. It appears from studies of fine structure genetics[7,8] that such mistakes could involve the substitution of noncomplementary bases, and also the addition or deletion of one or a sequence of nucleotides, during the synthesis of a progeny chain on its template. In view of these plausible mechanisms of mutation, we have examined the possibilities whereby noncomplementary base pairs can be accommodated within the two-stranded helical framework of DNA. We first investigated the effects of varying proportions of noncomplementary bases incorporated into pairs of complementary polyribonucleotides which are known to form two-stranded helices like DNA. The results of such experiments allowed us to exclude the notion that noncomplementary bases remain within the interior of the helix. Instead, they suggested that noncomplementary nucleotides of opposite chains rotate out of the helix in such a manner as to enable normal hydrogen-bonded base pairing of subsequent regions of nucleotides which are in register. This structural alternative was then explored by model building and found to be readily plausible.

Experimental Approach.—Polyadenylic and polyuridylic acids* are single stranded polyribonucleotides[9, 10] containing base residues equivalent to the complementary pair, adenine-thymine, present in DNA. Indeed, in neutral saline solution they form a 1 : 1 two-stranded helical complex which has essentially the same structure as DNA.[11–13] Under appropriate conditions, a second strand of poly U can be induced to wrap around this poly(A+U) helix, so that a three-stranded complex is formed in which the molar ratio of adenine to uracil is 1 : 2.[13]

Mixing curves: The interaction of polynucleotide strands to form helical complexes is accompanied by a marked decrease in ultraviolet absorbance.[10, 11, 13, 15] For long chains, this hypochromic change can be expected to reflect in a linear manner the number of nucleotides involved in the interaction. Thus, by titrating one species against another, it is possible to locate the mixture of maximum hypochromicity, and thereby to accurately determine the molar ratio of the reacting species in the complex being formed.[13]

In the present investigation, such mixing curves were used in order to study quantitative aspects of the interaction of poly A or poly U with a series of single stranded poly AU copolymers.[16] Complex formation here necessitates juxtaposition of the mismatched bases, adenine-adenine and uracil-uracil, at certain levels of the helix. The manner in which these pairs are structurally accommodated will influence the molar ratio at the point of maximum hypochromicity.

The underlying assumption behind these interaction experiments is, of course, that polynucleotide chains have the capacity to explore and find the partners which permit the complete fulfillment of their hydrogen bonding potentialities. This was previously shown for the interaction of poly A and poly U.[13, 17] The results to be described below also bear out this assumption. Even those single-stranded AU copolymers which exhibited a high degree of intramolecular hydrogen bonding (as evidenced by a large reversible hyperchromic change on heating) were found to interact optimally with homopolymer to form multistranded complexes.

Absorbance-temperature profiles: Helical macromolecules, being highly ordered, are found to denature or "melt" over relatively narrow temperature ranges.[18, 19] In the case of polynucleotides, the profile of this transition can be followed spectrophotometrically by measuring the increase in ultraviolet absorption (reversal of hypochromicity) as a function of temperature. In a given solvent, the temperature of the midpoint of this transition (T_m) will reflect the relative stability of the complex.[15] This property was assessed for the complexes formed between poly A or poly U and several poly AU copolymers in order to evaluate the effect of the mismatchings on the strength of the interaction.

Methods.—Polynucleotide samples: Samples of poly A and poly U, and samples of poly AU differing in their content of adenylic and uridylic residues were synthesized in this or other laboratories as previously described,[20] employing the reaction catalyzed by polynucleotide phosphorylase.[21]

Polymers were generally isolated from their polymerization reaction mixtures by precipitation with ethanol, and were purified by reprecipitation with ethanol from clarified neutral saline solution, followed by extensive dialysis against neutral saline containing versene or citrate. Stock solutions of polymers were stored at −20°C.

Base analysis of polymers: Duplicate determinations of the ratio of adenylic to uridylic residues in the copolymers were made according to a modification of a method of Markham and Smith.[22] About 0.5 mg of polynucleotide was hydrolyzed in an alkali resistant glass test tube in 0.4 *N* NaOH at 37°C for 18 hours. The hydrolysate was neutralized and divided into two parts for chromatography on Whatman No. 1 paper. Descending chromatograms were developed at 4°C with a solvent containing saturated aqueous $(NH_4)_2SO_4$ [79 vol], water [19 vol.], and isopropanol [2 vol.]. At this low temperature sharper spots were obtained, so

long as the chromatography paper was dry when the run was started. After thorough drying of the paper, the adenylate and uridylate spots and corresponding paper blanks were eluted quantitatively and their amounts determined spectrophotometrically.

Polymer concentration determination: Duplicate aliquots of stock solutions used for mixing curves were hydrolyzed in 0.4 *N* NaOH at 37°C for 18 hours. It was found necessary to incubate similar flasks containing 0.4 *N* NaOH in order to provide proper blanks. The hydrolysates were then diluted to a constant volume and final acid concentration of 0.1 *N* with HCl. Knowing the nucleotide composition and the extinction coefficients of the monomers at 259 mμ, the molar nucleotide residue concentration of the homopolymers and copolymers was calculated from the absorbance at 259 mμ.

Mixing curves: Polymer stock solutions were made up to the same nucleotide residue concentration. For two-stranded complex formation, the solvent used was either 0.15 *M* NaCl + 0.015 *M* sodium citrate, pH 7, or 0.15 *M* NaCl + 0.045 *M* sodium cacodylate, pH 6.9—the latter being preferred because of its ability to deter bacterial contamination. For each homopolymer-copolymer combination, a series of mixtures was made such that the total number of nucleotide residues was kept constant while the ratio of homopolymer to copolymer was varied. These mixtures were allowed to equilibrate at a constant temperature, and their absorbance at 259 mμ then determined, against a solvent blank, in a Beckman DU spectrophotometer adapted with a thermostated cell chamber. Additional mixtures, with polymer ratios clustered about the observed minimum, were then used to precisely locate the optimally interacting mixture. To detect any hypothetical three-stranded structure which might form, similar mixing curves were performed in solvent also containing 0.05 *M* $MgCl_2$.

TABLE 1

ANALYTICAL PROPERTIES OF SINGLE-STRANDED POLYRIBONUCLEOTIDES

Polymer	Mole % Adenylic Residues	$S^\circ_{20,w}$*	Approximate Molecular Weight
Poly A	100	9.0	600,000†
Poly U	0	4.3	150,000†
Poly AU "90"	90	3.0	50,000†
Poly AU "66"	66	1.4	8,000‡
Poly AU "53"	53	3.9	40,000‡
Poly AU "37"	37	2.9	25,000‡

* These mean sedimentation constants were determined in 0.15 *M* NaCl + 0.015 *M* sodium citrate, pH 7.
† Extrapolated from S° versus M.W. calibration curve for Poly A.
‡ Extrapolated from S° versus M.W. calibration curve for RNA.

Absorbance-temperature profiles: Absorbance measurements were made at 259 mμ on solutions contained in quartz stoppered cuvettes with a thermostated Beckman DU spectrophotometer controlled to ±0.3°C.

Sedimentation constants: Sedimentation velocity measurements were made at about 22°C in a Spinco Model E Ultracentrifuge using the ultraviolet absorption optical system. The mean $S^\circ_{20,w}$ was calculated from the moving boundaries traced with a Spinco analytrol.

Model building: Pauling–Corey space-filling nucleotide models,[23] (1″ = 2 Å) were used to explore possible configurations for the helical homopolymer-copolymer complexes.

Results.—Properties of polymers: In Table 1 are presented some analytical properties of the single-stranded polynucleotides used in the interaction studies. The compositional data are subject to an average deviation of ±1.5 per cent. Note that the identification number following each copolymer is derived from the mole per cent of adenylic residues that it contains. Approximate molecular weight values were derived from the sedimentation constants using either the calibration scale of S° versus molecular weight for RNA[24] or that for poly A.[9] It is recognized that these extrapolations may be subject to errors of as much as 50 per cent. Nevertheless, it is apparent that all the samples are of sufficiently high molecular weight so that hypochromicity can serve as a linearly proportional measure of complex formation.

Homopolymer-copolymer interactions: A priori, one might conceive of two possible ways in which noncomplementary base pairs could be accommodated into the DNA type of helical structure.

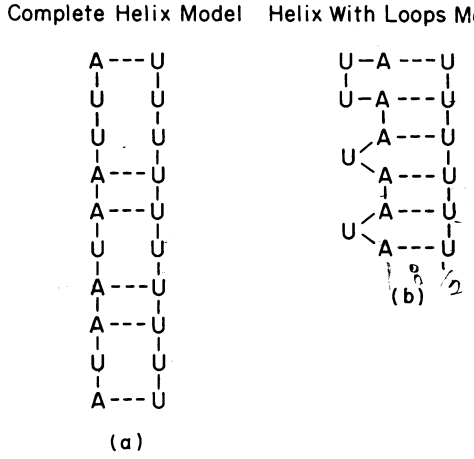

FIG. 1.—Structural alternatives for accommodating noncomplementary residues in DNA-like helices. (*a*) In any case of the Complete Helix Model the molar ratio of poly U:poly AU will always be 1:1 for the two-stranded complex. If a second strand of poly U is added, this ratio becomes 2:1. (*b*) In the Helix-With-Loops Model the molar ratio of poly U:poly AU varies with copolymer composition. In the example shown, this ratio is 6:10 for the two-stranded complex and 12:10 when a second strand of poly U is added.

The first possibility is that such nonmatching bases could be held *within* the helix either with or without hydrogen bonding. Conceivably this would involve occasional distortions of the helix to compensate for the steric variations involved in pyrimidine-pyrimidine and purine-purine pairings.

The alternative is that these noncomplementary nucleotide residues could in some manner rotate out of the helix to allow a continuity of complementary base pairs along its entire length.

These two possibilities are illustrated schematically for poly (AU + U) in Figure 1*a* and *b*. From Figure 1 it is clear that the overall composition of the two complexes will be different depending on the method by which nonmatching bases are accommodated. If these are retained within the helix (Fig. 1*a* "Complete Helix" model), the mixture of maximum hypochromicity in a mixing curve will always contain equimolar amounts of homopolymer and copolymer for the two-stranded complexes poly (AU + A) and poly (AU + U). In the alternative case, where the nonmatching residues in the copolymer do not reside within the helix (Fig. 1*b* "helix-with-loops" model), the homopolymer content of the complex will be *less* than 50 moles per cent. As is apparent from Figure 1 (*b*), the exact composition of such a complex can be calculated from the proportion of noncomplementary bases to be accommodated. If complex formation is complete, this scheme predicts that the mixing curve minimum will lie at the point where the

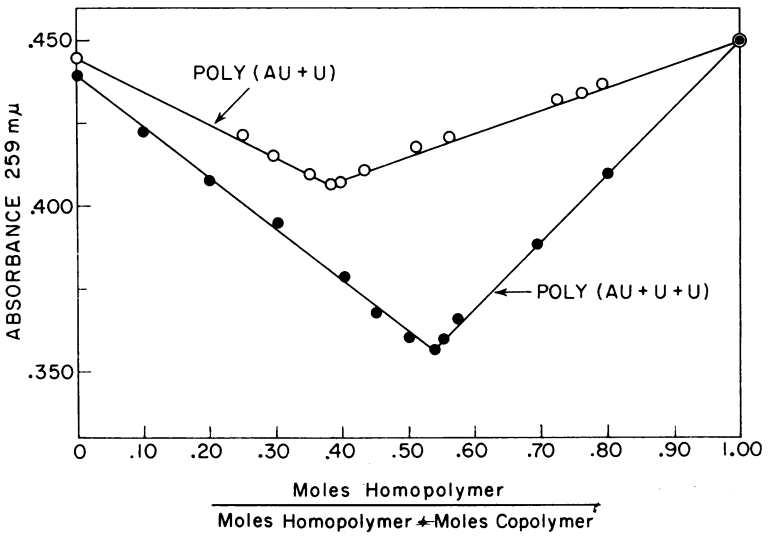

FIG. 2.—Mixing curves for poly AU "66" + poly U. *Open circle* points are for two-stranded complex formation at 21°C in 0.15 *M* NaCl + 0.045 *M* cacodylate, pH 6.9. *Filled circle* points are for three-stranded complex formation at 17°C in the same solvent + 0.05 *M* $MgCl_2$. The composition at the point of maximum hypochromicity did not change when the temperature was lowered to 4°C.

moles of homopolymer equals the moles of *complementary* residues in the copolymer.

Similar considerations can be used to analyze mixing curves involving the formation of the three-stranded complexes, poly (AU + U + U).

Mixing curves, such as those in Figure 2, were obtained on combinations of the copolymers and poly U or poly A. The composition of the various homopolymer-copolymer complexes were determined from the mixtures of maximum hypochromicity, and are compared in Table 2 with the theoretical values calculated for the two models described above. Two sets of the data included were derived from mixing curves published in another connection by Steiner.[25] It can be seen from Table 2 that in every case where two- or three-stranded complexes were found, their compositions agreed very closely with those expected for the model of the helix-with-loops. This is also evident from the examples in Figure 3 which show that *in every combination, maximum complex formation occurred at the point where*

TABLE 2

COMPOSITION OF HELICAL POLYRIBONUCLEOTIDE COMPLEXES

	Mole % of Homopolymer in Mixture of Maximum Hypochromicity					
	2-Stranded Complexes			3-Stranded Complexes		
		Theoretical			Theoretical	
Polymer Mixture	Experimentally Found	Helix With Loops	Complete Helix	Experimentally Found	Helix With Loops	Complete Helix
Poly A + Poly U	50	X	50	67	X	67
Poly AU "90" + Poly U	...	..	..	65 ± 1	64.5	67
Poly AU "66" + Poly U	38 ± 1	40	50	54 ± 1	57	67
Poly AU "53" + Poly U	34 ± 1	34.5	50	...	..	..
*Poly AU "62" + Poly U	...	..	..	57	55	67
*Poly AU "82" + Poly U	...	..	..	64	62	67
Poly AU "37" + Poly A	38 ± 3	39	50	X	X	X

* These sets of data were derived from the work of Steiner.[25]
Dots in place of a value indicate the data were not available, while an *X* indicates the value is not meaningful.

the moles of homopolymer either equals the moles of the complementary residue in the copolymer (two-stranded helix), *or equals twice that amount* (three-stranded helix).

These results clearly demonstrate that the noncomplementary residues of the copolymer strands do not remain within the helix. The only other way to explain our observations would be to assume significant compositional heterogeneity among the copolymer chains. In this event only a fraction of the total number of chains could participate in the interaction with the homopolymer. This possibility was evaluated and ruled out by the following considerations: (1) Chemical degradation of AU copolymers and isotopic studies of their enzymatic synthesis[20, 26] have shown

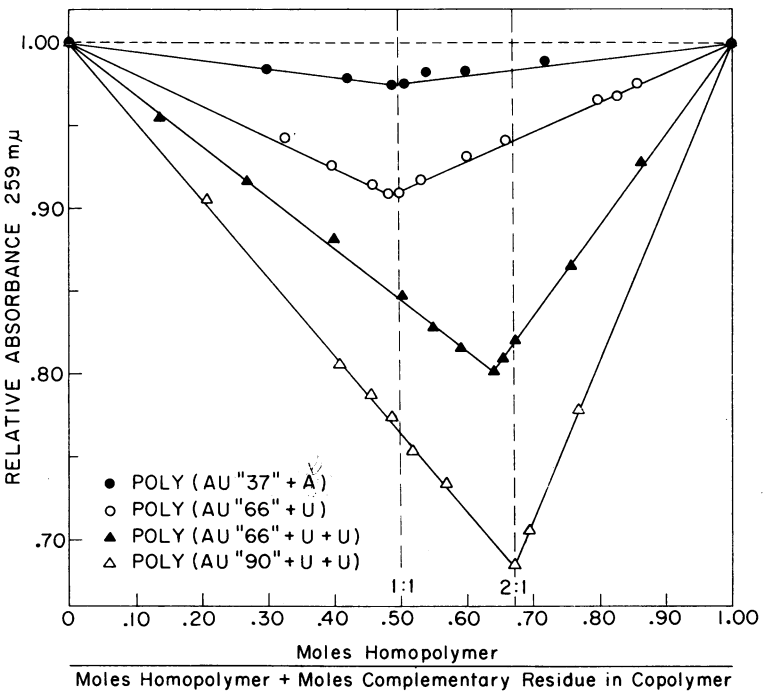

FIG. 3.—Mixing curves for the formation of homopolymer-copolymer polyribonucleotide complexes. Relative absorbance is the ratio of the absorbance of the mixture to that of the average of the unmixed constituents. It should be noted that the depth of these mixing curves will depend in part on the initial hypochromicity of the reacting species. The extreme shallowness of the (AU "37" + A) curve can be explained by the fact that both poly A and the AU copolymers are highly hypochromic, whereas poly U is not.

that the sequence of adenylic and uridylic residues is essentially random. Moreover, the chain lengths of our samples are sufficient to average out small compositional differences. (2) The self-consistency of all the interaction data argues strongly for a quantitative reaction. It is especially reflected by the equivalence of the amount of homopolymer required to form the poly (AU "66" + U) two-stranded complex, and the additional amount required to form the three-stranded complex. This rules out the possibility that the original copolymer solutions contained any stable two-stranded helical complexes which could not be titrated. (3) The fact that the hypochromicity increases in a *linear* manner as the copolymer

is titrated with homopolymer indicates that all the available copolymer molecules have an equal capacity for interaction. If some copolymer molecules had a preferential tendency to interact because of more favorable residue sequence or composition, the initial phase of the titration would result in a greater hypochromic change than that occurring when the titration was nearer to completion. In this event, the mixing curve would have a readily detectable concave character extending to the point of maximum hypochromicity.

Model building: Having concluded that the noncomplementary residues in the copolymer must loop out of the helix, we then proceeded to examine how this condition could be satisfied structurally. A poly (A + U) space-filling helix was built according to the DNA coördinates.[27] It was found that when a uridylic

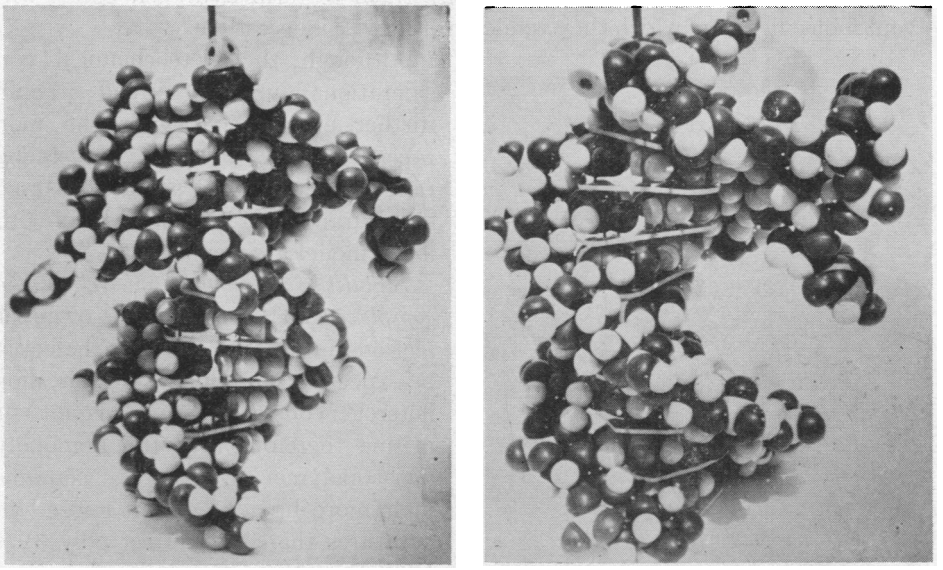

FIG. 4.—(*a*) A poly (A + U) helix containing a single nucleotide loop in each chain. This portrays the situation which might occur in DNA if cytosine and thymine are bases located in opposite chains at the same level, while all the other residues are in register. Note that the loops point away from the backbone and do not obstruct either groove.

FIG. 4.—(*b*) A poly (A + U) helix containing in the A-chain a looped region of two uridylic residues.

residue is incorporated into the A-chain, it can be readily looped out of its position within the helix, merely by rotation about its two adjacent phosphodiester bonds. This leaves the uracil base pointing radially out from the helix axis, and allows an adjacent nucleotide residue to move into the vacancy created without strain. (Fig. 4*a*). In addition, similar loops containing two, three or more nucleotides are also possible (Fig. 4*b*, *c*). Indeed, there appears to be no structural limitation on the number of nucleotide residues which can be incorporated into a loop. It should be noted, however, that whereas a loop of one residue is rigidly fixed in position except for the rotation about its phosphodiester bonds, larger loops become increasingly more flexible.

Exactly the same considerations are involved for any other noncomplementary base residues which might be incorporated into either chain. In all cases, the

regularity of the helix in the vicinity of the loop is unaffected, both the 3.4 Å separation and 36° translation of successive nucleotides being maintained. Furthermore, the loops do not interfere with either the "deep" or "shallow" groove, which explains why it is possible to add a second poly U chain to the poly (AU + U) complexes.

The only significant alteration in the helical backbone is that for each loop the separation of one pair of PO^- groups is reduced from 7 Å to approximately 6. However, it is apparent that other thermodynamic considerations such as the maximization of hydrogen bonding and the increased entropy brought about by loop formation tend to offset this small increase of charge repulsion. An additional factor favoring loop formation would seem to be that removing noncomplementary bases from the interior of the helix enables them to form hydrogen bonds with solvent molecules from which they would be otherwise inaccessible.

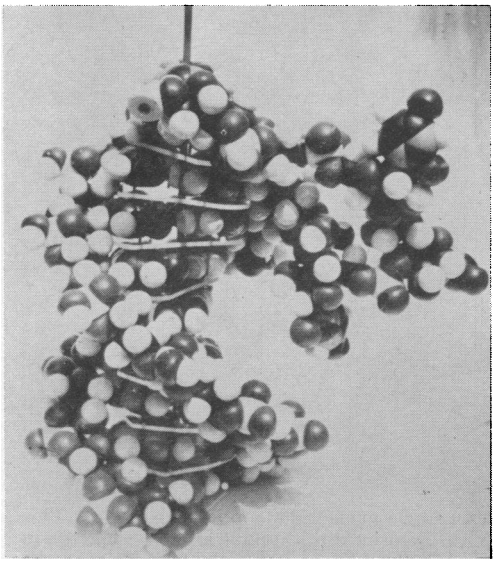

FIG. 4.—(*c*) A poly (A + U) helix containing in the A-chain a looped region of three uridylic residues.

Although the stereochemical considerations just described require further examination with the more precise models in use by crystallographers, it would appear that the broad structural features of the helix-with-loops model are correct.

Stability of homopolymer-copolymer complexes: The introduction of imperfections into a two-stranded helix can be expected to produce a weaker, more heterogeneous complex. It is not surprising therefore that the homopolymer-copolymer complexes examined melt more broadly and at lower temperatures than the perfect poly (A + U) helix. For example, in the saline-citrate solvent, whereas poly (A + U) melts almost entirely between 57 and 62°, poly (AU "53" + U) melts from 5 to 46° and has a T_m of about 24°.[28] The latter helix contains 31 per cent unpaired, looped out bases. Since its complete formation requires several hours at 2°, it would seem that a limit to the number of tolerable imperfections is being approached under these experimental conditions. In fact, for an AU copolymer containing 51 per cent adenylic residues, no interaction with poly U could be observed. In any case, the number of mismatchings which can be incorporated into a DNA-like helix is surprisingly large.

Discussion.—The helix-with-loops model adds a dimension of freedom to configurational considerations of helical polynucleotides. In the case of RNA, the model offers a means for satisfying the requirement for the short helical regions which have been recently suggested.[15] In addition, it involves the generation of a new type of tertiary structure which could have biological significance. A molecular structure for RNA incorporating these features will be described in a subsequent report.

There are several contexts in which the helix-with-loops model might be significant for DNA. In view of the similarity of essential structural features between the poly (A + U) helix and DNA, it seems likely that any base pair mismatchings arising during DNA synthesis would also be accommodated by loop formation. From the foregoing it is apparent that the looping mechanism does not require any significant alteration in the structural parameters of the poly (A + U) helix. It also does not result in significant crowding of the grooves, which in the case of DNA must remain unobstructed in order to contain protamine or histone.[29] The homopolymer-copolymer system does not provide an entirely analogous model to a DNA molecule with defective base pairs. Since one of the strands of the synthetic helix is a homopolymer, this strand need never form a loop in order to keep all nucleotides in register. Further, this helical complex is the resultant of an interaction between two *already existing* polynucleotide chains. In DNA, on the other hand, not only is there a specific sequence of residues, but it is believed that the helix is formed by one chain being synthesized in a linear fashion upon its complementary template chain.[4-6] Under these conditions, the accidental addition to the growing chain of a nucleotide which is not complementary to its partner in the template chain will lead to one of three obvious consequences. Any of these would appear to be a possible molecular counterpart for point mutations. These models showing how mutational events might be accommodated are represented schematically in Figure 5.

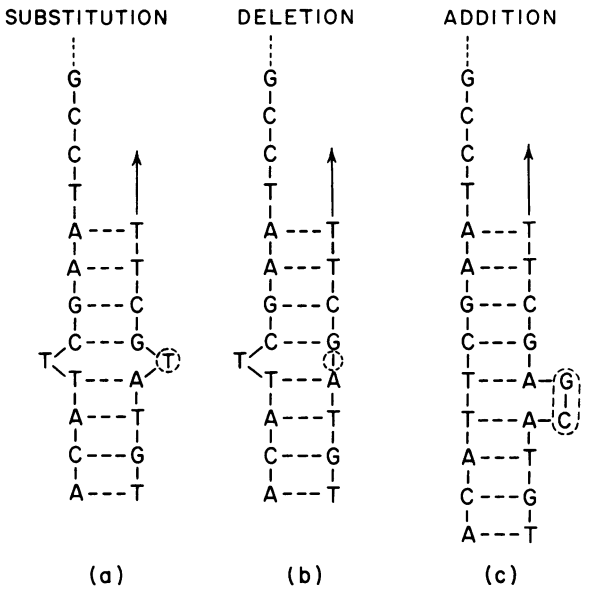

FIG. 5.—Hypothetical models for point mutations. In each case, the template chain is the one on the left. Note that while the templates are identical in each case, the sequence in the growing chains varies, depending on the kind of "mistake" made during replication. The "mistake" in the growing chain is indicated by a dotted circle.

In the first case, Figure 5*a* (compare with Fig. 4*a*), both the erroneous base and its noncomplementary partner have looped out of the growing helix, thus enabling the succeeding regions to pair normally. This type of accommodation merely involves the *substitution* of one residue for another, and results in no difference in chain length between the chains.

In Figure 5*b*, however, the erroneous base has paired with a succeeding base on the template, causing the intervening template residue(*s*) to loop out of the helix. Chain growth then continues, leading to a progeny chain which is shortened by one or more residues. Conceivably, such a *deletion* in the growing chain could also be caused by the presence of a pre-existing distortion in the template chain, rendering some of its residues inaccessible.

In the last case, Figure 5c, the errors made on the growing chain have been removed from the helix to enable succeeding chain growth to continue normally. This *addition* of residues to the growing chain results in a progeny chain longer than its template.

While it would seem that the *substitution* mechanism could produce reversible mutations (including those induced by base analogues in bacteriophage[7, 8]), the *addition* and *deletion* mechanisms could account for those mutational events which have been found to be irreversible.

It is worth noting that these models for point mutations can serve equally well to explain the incorporation into DNA of unnatural or unusual nucleotides incapable of forming satisfactory hydrogen bonds with any of the naturally occurring residues.

Finally, it is important to recognize that the helix-with-loops model may provide a basis for the molecular interpretation of such other genetic phenomena as recombination.[30]

Summary.—A quantitative study has been made of the helical complex formation between polyriboadenylic acid or polyuridylic acid and copolymers containing residues common to both members of this complementary pair. The interaction occurs in such a manner that the two-stranded helical complexes formed always contain equal moles of homopolymer residues and complementary residues in the copolymers. This indicates that the noncomplementary residues in the copolymers loop out of the helix. Model building shows that this can be done without altering the essential structural features of the helix, and that loops of one or more residues are feasible. While these loops do weaken the helix, it is apparent that the number of mismatchings which can be tolerated in a DNA-like helix is quite large.

The relevance of these findings to DNA is considered, and hypothetical schemes for the accommodation of point mutations by forming such loops are presented.

We should like to express our appreciation to Professor Paul Doty for encouraging and supporting this investigation. We are also indebted to Dr. E. Freese for stimulating discussions, to Dr. Leon Heppel, Dr. R. C. Warner, and Dr. A. Rich for the poly AU samples, and to Mr. Richard Blake for capable assistance. This work has been aided by grants from The American Heart Association, the United States Public Health Service, C-2170 and the National Science Foundation, G-7849.

* The following abbreviations have been used: RNA = ribonucleic acid; DNA = deoxyribonucleic acid; A = adenine; U = uracil; C = cytosine; G = guanine. The prefix *poly* is used to indicate a homo or copolymer of nucleotide(s) of the indicated base(s). Polynucleotide complexes are indicated by the prefix *poly* followed in parentheses by the summated homo or copolymer chain symbols.

† This paper is No. III in a series entitled *Polynucleotides*, of which the last was by J. R. Fresco, *J. Mol. Biol.*, 1, 103 (1959).

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[28] It is important to note that the homopolymer-copolymer complexes melt at temperatures low enough for the single-stranded copolymer chain (and the poly A chain) to reform a considerable number of hydrogen bonds within itself. Therefore, the experimentally determined profile of the complexes must be adjusted as follows in order to obtain a true measure of their stability:

where x = mole fraction of complex melted; t = temperature; a(t) = absorbance of the complex as a function of temperature; b(t) = absorbance of an equimolar amount of non-interacted constituent chains (calculated from their separate profiles); and c = absorbance of the completely formed complex [minimum value of a(t)];

$$a(t) = c[1 - x] + x[b(t)]$$

$$x = \frac{a(t) - c}{b(t) - c}$$

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