deed, it would appear that the specificity of adenine as a binding handle would be rather limited due to the large number of metabolically indispensable small molecules that contain an adenine moiety. Therefore, it is likely that the function of the adenine component in NAD^+, at least in one indispensable metabolic process, is something more than that of a passive binding handle.

At present this hypothetical function of the adenine component of NAD^+ is unknown. However, the present study suggests that it might involve the adsorption of an NAD^+ molecule in a stacked configuration to its binding site on an enzyme surface. During the reduction of this coenzyme molecule the function of its adenine moiety might be to act as a conduit for the transfer of an electron pair from the enzyme surface or from the substrate to the nicotinamide ring. The formation of a charge-transfer complex between the adenine and the nicotinamide rings of NAD^+ would facilitate such an electron pair transfer because it is expected that adenine would be the donor group[47] and nicotinamide would be the acceptor group[48] in such an intramolecular charge-transfer complex.

It is also possible that the conformation of NAD^+ bound to some enzymes is significantly different from that of its reduction product, NADH, bound to the same enzyme. For example, if the NAD^+ formed the postulated internally stacked complex, the NADH might assume an internally hydrogen bonded conformation. Alternatively, the NADH might be in an extended configuration such as that it assumes when bound to the dogfish enzyme lactate dehydrogenase.[46] Structural studies on NADH model compounds are presently being carried out so that conformational differences between NAD^+ and NADH can be more accurately assessed.

Acknowledgments. This work was supported by the National Institutes of Health (Grant No. GM18632) and by the Advanced Research Projects Agency, Office of the Secretary of Defense.

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Determination of Pyrimidine Nucleoside Syn, Anti Conformational Preference in Solution by Proton and Carbon-13 Nuclear Magnetic Resonance[1]

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Abstract: The glycosidic conformation of 11 pyrimidine nucleosides and one quinazoline nucleoside in solution has been investigated by ^1H and ^{13}C nmr spectroscopy. Proton chemical shift data as well as vicinal furanose coupling constants indicate that most of these nucleosides are preferentially anti. Bulky groups such as methyl at position 6 or a 5,6-fused benzene ring shift the torsional angle into the syn range. Measurements of the vicinal $^3J_{C_2-H_{1'}}$ about the glycosidic bond in cytidine and 6-methylcytidine confirm the conclusions based upon chemical shift data. Although the torsional angle may be altered somewhat, the relative proportion of syn and anti conformers appears to be about the same in DMSO as in water. Examination of 2′,3′-*O*-isopropylidene derivatives indicates that significant changes in the furanose conformation are less a determinant of glycosidic conformation than steric interaction between substituents on the base and ribose moieties.

An understanding of the conformational details of oligonucleotides and polynucleotides will undoubtedly be attained at least in part from thorough investigation of the conformational properties of the individual monomers. In pursuit of this goal, numerous investigations on nucleoside and nucleotide crystals and their solutions have been carried out. The solid state furanose conformation in nucleosides, nucleotides, and polynucleotides has been shown to be quite similar.[2] Nmr[3] studies of uridylic acid monomers[4] and polymers[4b] have been interpreted as indicating comparable furanose conformations in solution as well, at least for this particular example.

One important facet of nucleoside conformation is the relative position of the base and sugar moieties about the glycosidic bond, governed by the torsional angle, χ.[2a,5] X-Ray data,[2a,5,6] potential energy calculations,[7] and nmr studies[8] have shown that χ is dependent upon furanose ring puckering and that pyrimidine nucleosides generally exist in the anti

(1) Presented in part at the 15th Biophysical Society Meeting, New Orleans, La., Feb 1971, Abstracts No. WPM-M3; "Conformation of Biological Molecules and Polymers" (Proceedings of the Fifth Jerusalem Symposium), Israel Academy of Science and Humanities, Jerusalem, 1972, in press.

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(3) Abbreviations: nmr, nuclear magnetic resonance; pmr, proton magnetic resonance; cmr, carbon-13 nuclear magnetic resonance; CD, circular dichroism.

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range of χ, whereas purine nucleosides may be characterized by both anti and syn conformers. Bulky substituents on the base have the expected ability to influence χ. For example, 8-bromoguanosine and 8-bromoadenosine have been shown to be predominantly syn conformers by X-ray[9] and in solution by CD.[10,11]

Nmr techniques have been particularly useful in determinations of solution conformations of nucleosides,[12-15] mononucleotides,[4a,16,17] and oligonucleotides.[18-22] Determination of conformation about the glycosidic bond by pmr in the mononucleotide studies was facilitated by monitoring the differential electrostatic field effect of the 5′-attached phosphate anion upon the base protons.[16,17] This phosphate effect was manifested at H-8 of purine nucleotides and H-6 of pyrimidine nucleotides. Thus for these nucleotides in aqueous solution the preferred glycosidic conformation is anti. Perhaps one of the determinants of this conformational preference is the presence of the solvated phosphate group which is not readily accommodated in the syn form because of the possibility of close contact with the base.

In the case of the oligonucleotides, preference for the anti conformation for the individual nucleosides was deduced from the differential base proton chemical shifts as a result of both the 5′-phosphate effect and temperature perturbation of base stacking interactions.

The ability to discern conformational preference about the glycosidic bond in nucleosides is somewhat more difficult due to lack of the type of large intramolecular effects between bases as in oligonucleotides or between base and phosphate in mono- and oligonucleotides. For pyrimidine nucleotides, the deshielding influence of the ether oxygen on H-6 was used as an argument in favor of the anti conformation.[8,12] In addition, a measurable $^5J_{5-1'}$ in several pyrimidine nucleosides has been shown to exist only for anti conformers.[23]

The effect of anisotropic functional groups in the base moieties upon ribose proton chemical shifts has recently shown promise as a means of determining syn, anti preference in pyrimidine nucleosides.[24-27] We report here pmr studies expanding this approach to additional pyrimidine nucleoside systems in which large substituents on the base promote changes in the glycosidic conformation. For example, our chemical shift and coupling constant data show that 6-methyluridine, 6-methylcytidine, and 1-β-D-ribofuranosylquinazoline-2,4-dione are preferentially syn in dimethyl sulfoxide. The two methyl derivatives are also mainly syn in water. Measurement of $^3J_{C_2-N-C-H_{1'}}$ coupling constants in cytidine and 6-methylcytidine are consistent with glycosidic conformations in the anti and syn ranges, respectively.

Biological as well as structural significance is associated with consideration of nucleoside syn, anti conformational preference. Certain enzymes catalyzing polynucleotide synthesis, such as polynucleotide phosphorylase and RNA polymerase, will not function with purine and pyrimidine nucleoside di- or triphosphate substrates not having anti conformations.[28-30] Our interest in this topic was initially stimulated by the inability to achieve polymerization of 6-methylcytidine diphosphate with *E. coli* of *Micrococcus luteus* polynucleotide phosphorylase, in agreement with Kapuler and Reich.[29] In this communication we provide experimental verification of their contention that the syn glycosyl conformation is apparently the reason for the nonreactivity.

Experimental Section

Materials. The sources of nucleosides are listed in the legend to Figure 1. Deuterated solvents D_2O (99.8% *d*) and DMSO-d_6 (99.5% *d*) were from Diaprep., Inc., and ICN.

Instrumentation. Pmr spectra were taken on both Bruker HX-90 (90 MHz, ambient probe temperature, 28°) and Hitachi-Perkin-Elmer R20A (60 MHz, ambient probe temperature 34°) spectrometers. Chemical shifts were measured from TMS capillaries. The nucleosides were generally examined as 10% w/v solutions in D_2O. Concentration dependent shifts are not important for these nucleosides.[31] Spectra at elevated temperature (monitored by ethylene glycol splitting) were obtained with both spectrometers. Chemical shifts obtained from spectra at elevated temperature were corrected for bulk susceptibility changes by noting the change in resonance position between the external TMS capillary and DSS dissolved in D_2O. The natural abundance ^{13}C spectra were obtained on the Bruker HX-90 at 22.6 MHz operating in the Fourier transform mode in connection with a Nicolet 1074–Digital Equipment Corporation PDP-8/e computer package with 4K memory. Undecoupled spectra were obtained from 40% w/v solutions of the nucleosides in DMSO-d_6 in 10-mm tubes accummulating 30,000–40,000 transients. ^{13}C chemical shifts were obtained from noise decoupled spectra using hexafluorobenzene as external reference.

Results and Discussion

Evidence for changes in the population distribution of pyrimidine nucleosides having glycosidic conformations in the syn and anti ranges (as defined by the torsional angle, χ, according to Sundaralingam[2a]) may be obtained from various nmr data. First, we shall consider chemical shifts, particularly of the furanose protons.

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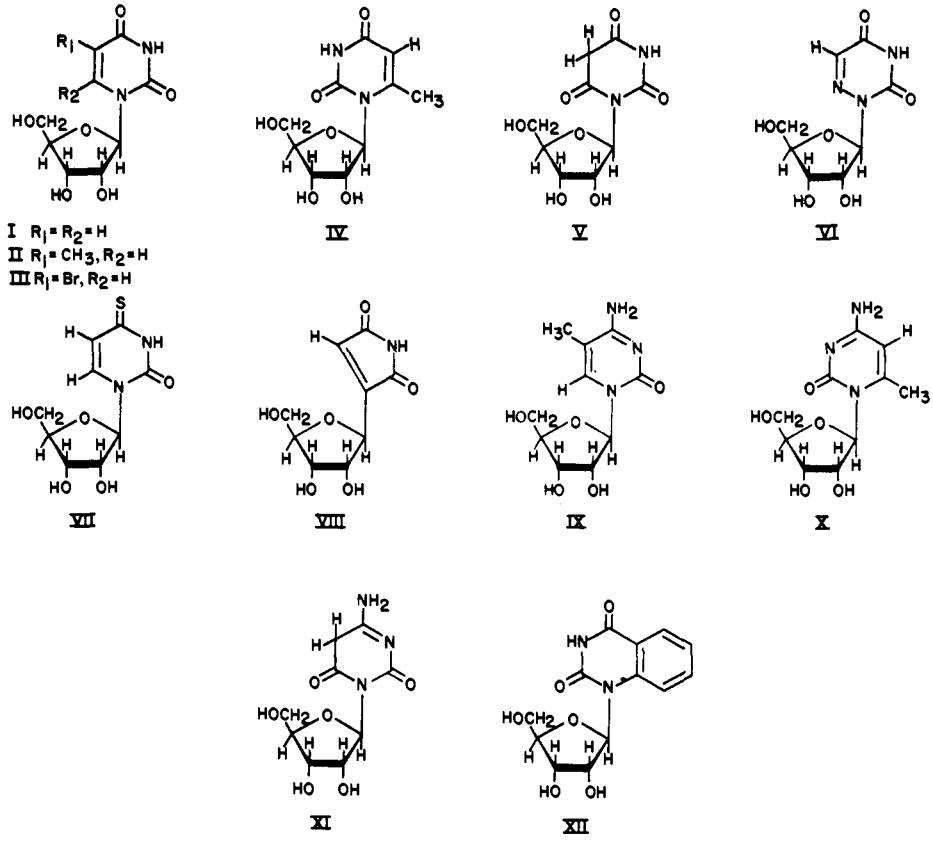

Figure 1. Nucleosides examined in the present study: (I) from Waldhof; (II) generously supplied by Dr. J. Fox, Sloan Kettering; (III) from ICN; (IV and X) M. W. Winkley and R. K. Robins, *J. Org. Chem.*, **33**, 2822 (1968); (V and XI) M. W. Winkley and R. K. Robins, *J. Chem. Soc. C*, 791 (1969); (VI) from Calbiochem; (VII) J. J. Fox, D. V. Praaz, I. Wempen, I. L. Doerr, L. Cheong, J. E. Knoll, M. L. Eidinoff, A. Bendich, and G. B. Brown, *J. Amer. Chem. Soc.*, **81**, 178 (1959); (VIII) K. R. Darnall, L. B. Townsend, and R. K. Robins, *Proc. Nat. Acad. Sci. U. S.*, **57**, 548 (1967); (IX) supplied by Cyclo Chemical Co.; (XII) M. G. Stout and R. K. Robins, *J. Org. Chem.*, **33**, 1219 (1968). The various nucleosides are drawn in the preferred glycosidic conformation as deduced in this work. The furanose ring is drawn planar, with the understanding that a dynamic equilibrium between the 2′-endo and 3′-endo puckered forms exists in solution.[3]

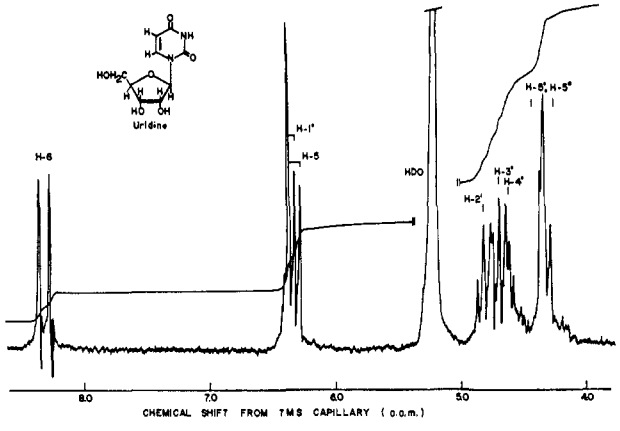

Figure 2. Pmr spectrum (90 MHz) of uridine, 10% w/v in D_2O, taken on a Bruker HX-90 spectrometer at 28°.

I. Chemical Shift Comparisons. Some of the data presented in this section have been communicated earlier.[2,4] In Figure 1 is presented the structural formulas of the nucleosides involved in the present study. Figure 2 contains the 90-MHz pmr spectrum of uridine (I) in D_2O. Spectral assignments were made by comparison with the literature.[1,3] The salient feature to be obtained from this spectrum is that the pseudo-triplet patterns for H-2′ and H-3′ plus the H-4′ multiplet are closely grouped at about δ 4.7 ppm. Substitution of a methyl group at position five of the uracil base has little effect on the chemical shifts of the ribose protons as may be seen at the top of Figure 3, containing the 60-MHz spectrum of 5-methyluridine (ribothymidine, II), a modified nucleoside found in most transfer RNAs. The H-2′,3′,4′ resonances remain clustered at $\delta \sim 4.7$ ppm. In the bottom portion of Figure 3 for 6-methyluridine (IV) it may be seen that a distinct change in the ribose proton chemical shifts has occurred. Protons H-2′ and H-3′ have moved to lower field, whereas H-1′, H-4′, and H-5′,5″ are shifted upfield. With reference to Table I, a direct comparison between I and IV serves to illustrate that in the latter, H-2′ and H-3′ are located downfield 0.46 and 0.19 ppm, respectively, while H-1′, H-4′ moved upfield 0.26 and 0.16 ppm. The exact shifts of H-2′,3′,4′ peaks in II cannot be determined at 60 MHz, but if one makes a crude comparison using the midpoint of the H-2′,3′,4′ cluster in the spectrum for II with the shifts for these protons in the spectrum for IV, H-2′ and H-3′ are shifted downfield 0.6 and 0.2 ppm, respectively, while H-4′ moves upfield 0.2 ppm.

The origin of these chemical shift changes is undoubtedly due in part to the magnetic anisotropy of the 2-keto group of the uracil base positioned over the ribose ring (Figure 4), provided the nucleoside is in the syn conformation as illustrated for IV. Additionally, electric dipolar effects may also be important, such that there is incipient bond polarization induced

Table I. Proton Chemical Shifts,[a] ppm, for Various Pyrimidine Nucleosides

Nucleoside	H-6 (or CH_3)	H-5 (or CH_3)	H-1′	H-2′	H-3′	H-4′	H-5′, 5′′	Syn, anti preference
Cytidine[b]	8.25	6.46	6.33	4.65	4.65	4.65	4.32	Anti
	8.20	6.47	6.32	4.64	4.64	4.64	4.30	
5-Methylcytidine[b] (IX)	8.18	2.44	6.40	4.71	4.71	4.71	4.40	Anti
6-Methylcytidine[b] (X)	2.85	6.31	6.15	5.30	4.86	4.48	4.31	Syn
	2.83	6.34	6.16	5.29	4.85	4.50	4.31	
6-Oxocytidine[c] (XI)			6.61	5.16	4.80	4.42	4.27, 4.13	Anti
			6.63	5.19	4.81	4.42	4.31, 4.15	
Uridine[c] (I)	8.34	6.33	6.36	4.81	4.69	4.62	4.42, 4.22	Anti
5-Methyluridine[b] (II)	8.15	2.35	6.36	4.69	4.69	4.69	4.34	Anti
	8.09	2.35	6.35	4.70	4.70	4.70	4.30	
6-Methyluridine[b] (IV)	2.86	6.19	6.10	5.27	4.85	4.46	4.30	Syn
	2.82	6.20	6.14	5.26	4.81	4.47	4.28	
6-Oxouridine[b] (V)			6.57	5.10	4.85	4.48	4.20	Anti
6-Azauridine[c] (VI)		8.05	6.56	5.03	4.82	4.56	4.31, 4.11	Anti
		8.06	6.54	5.02	4.78	4.55	4.29, 4.10	
5-Bromouridine[c] (III)	8.80		6.30	4.69	4.69	4.69	4.44, 4.28	Anti
	8.70		6.29	4.71	4.71	4.71	4.40, 4.26	
4-Thiouridine[b] (VII)	8.23	7.01	6.33	4.74	4.74	4.74	4.36	Anti
Showdomycin[c] (VIII)		7.14	5.23	4.60	4.60	4.60	4.24	Anti
		7.16	5.22	4.58	4.58	4.58	4.22	
1-β-D-Ribofuranosyl-quinazoline-2,4-dione[b,d] (XII)			6.55	4.93	4.54	4.22	4.10	Syn
			6.55	4.99	4.58	*e*	4.09	

[a] Measured from TMS capillary; 10% solution in D_2O. [b] 60-MHz spectra; top line 34°, bottom line 70°. [c] 90-MHz spectra; top line 28°, bottom line 70°. [d] 10% solution in DMSO-d_6. [e] Not resolved.

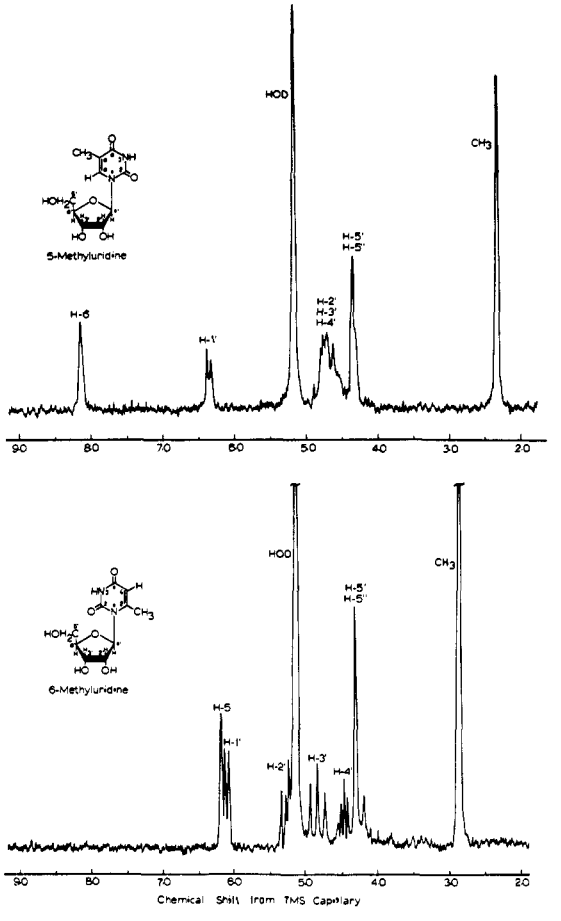

Figure 3. Pmr spectra (60 MHz) of 5-methyluridine and 6-methyluridine, conditions: 10% w/v in D_2O, taken on a Perkin-Elmer Hitachi R20A spectrometer at 34°.

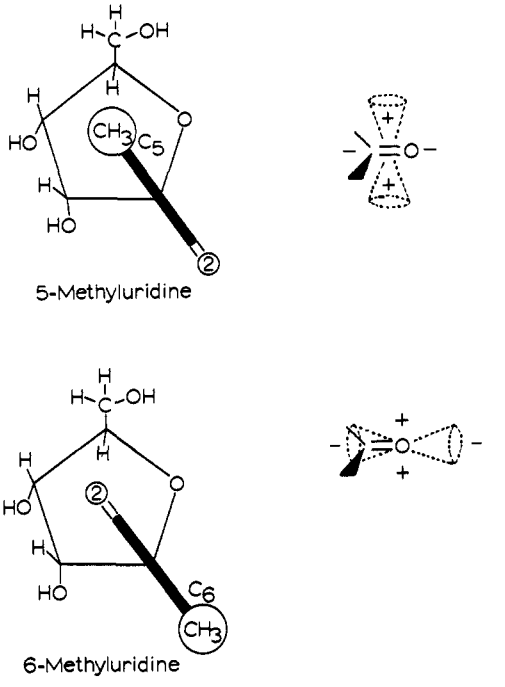

Figure 4. Diagrams of the orientation of methylated uracil with respect to the furanose ring, as seen by viewing from the top of the base. Two models of keto group anisotropy are shown at right.[33,34]

by the keto group resulting in altered charge density at the ribose hydrogens. The effect of a uracil 2-keto group on ribose proton shifts, namely H-1′, was first invoked by Prestegard and Chan.[8] Examination of space filling molecular models shows that contact between the 6-CH_3 and 5′-CH_2OH would prevent IV from assuming the normal anti conformation (as illustrated for II in Figure 4), and thus rotation of the base into the syn conformation would lead to a more stable situation. Miles, *et al.*,[32] have shown that, while 5-methylpyrimidine derivatives have normal positive Cotton effects, IV and 6-methylcytidine (X)

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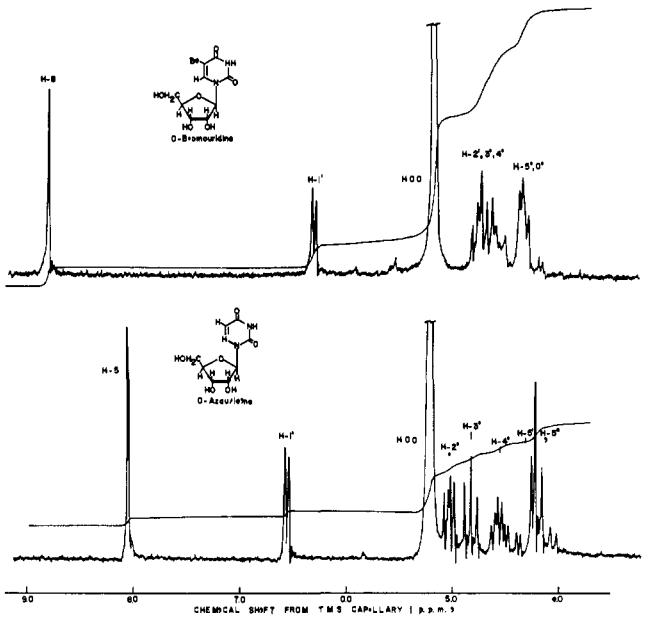

Figure 5. Pmr spectra (90 MHz) of 5-bromouridine and 6-azauridine, 10% w/v in D_2O, 28°.

display negative CD curves indicating a change in glycosidic conformation.

It can be noted in Table I that changes in ribose proton chemical shifts similar to those described above are found for the 5- and 6-methyl derivatives of cytidine. Furthermore, for 6-oxocytidine (XI) and 6-oxouridine (V), much the same effect is observed. Here either the 2- or 6-keto groups are expected to be located over the furanose ring. Dugas, *et al.*,[26] have reported on another pyrimidine nucleoside, 1-β-D-ribofuranosylcyanuric acid, which also contains a 6-keto function. The same sort of changes in the ribose proton shifts were found.

There are several differences between the 6-oxo- and 6-methylpyrimidines, namely H-1′, which is approximately 0.5 ppm to lower field in the 6-oxo derivatives. This may reflect the electron-withdrawing effect of an additional keto group in the base. It must be remembered that δ H-1′ is sensitive to the electron distribution of the base and thus will change according to the inductive and mesomeric nature of the substituents. Also, a somewhat different torsional angle may exist in the 6-methyl compounds. In Figure 4 a possible 2-keto orientation in 5- and 6-methyluridine is shown, with two current models of keto group anisotropy (top, ref 33; bottom, ref 34). The fact that in the 6-oxo compounds H-2′ is shifted downfield less than in the 6-methyl derivatives, but H-5′,5′′ is shifted further upfield, may denote a difference in χ between the 6-oxo and 6-methyl derivatives.

In the case of the quinazoline nucleosides, having a benzene ring fused 5,6 to the pyrimidine ring, we also observed deshielding at H-2′ and H-3′, for example, in 1-β-D-ribofuranosylquinazoline-2,4-dione (XII), indicating the syn conformation. Since most assuredly there exists in solution a distribution of conformers having torsion angles in the syn or anti ranges, we cannot specify χ from the shift data. Furthermore, since the exact nature of the carbonyl function anistropy is not precisely known, we can only suggest from the foregoing discussion that the significant alteration in the ribose proton chemical shifts observed when large groups are present at the 6 position of the base may most readily be interpreted as involving a change in preference of glycosidic conformation from the anti to the syn. A corollary which may be suggested is that in pyrimidine nucleosides where such changes in ribose proton shifts are not observed, the glycosidic preference is anti.

A recent report[25] describes ribose proton shifts for orotidine (6-carboxyuridine) which were interpreted in terms of a predominance of the syn conformer. It is of interest to note that IV has been shown to be syn in the crystalline state.[35]

Other uridine derivatives of interest include 5-bromouridine and 6-azauridine (Figure 5 and Table I). Examination of models shows that the bulky bromine atom can be readily accommodated at position five of uridine in the anti conformation. The cluster of resonances is found at about δ 4.7 ppm for H-2′,3′, and 4′ characteristic of pyrimidine nucleosides in the anti conformation. Wilson[36] has shown that in the solid state, 5-bromouridine is anti.

6-Azauridine is a nucleoside of considerable interest because of its chemotherapeutic activity.[37] It is obvious from comparisons of ribose proton shifts in Figure 5 and Table I for 6-azauridine with those of uridine that the nitrogen atom exerts a profound effect. The Δδ values are somewhat similar to those noted above for compounds where a keto group was located over the furanose ring. In 6-azauridine the anisotropy presumably comes from the lone pair of electrons in the sp^2 orbital of nitrogen at position 6. Similar deshielding of H-2′ and H-3′ is found in 8-azaadenosine compared with adenosine.[38] From this shift data, 6-azauridine in neutral aqueous media is considered to exist predominantly in the anti conformation. Hruska,[23] on the basis of a measurable $^5J_{5-1'}$ for 6-azauridine in alkaline aqueous solution, has reached the same conclusion. Saenger[39] has shown that 6-azauridine in the solid state is in the anti conformation.

Table I contains data for two additional nucleosides of biological interest, 4-thiouridine and showdomycin. 4-Thiouridine is found in many *E. coli* transfer RNAs.[40] Crystallographic data[41] have indicated that in the solid state 4-thiouridine exists as the syn conformer. However, the chemical shift data in Table I are interpreted such that 4-thiouridine in solution is anti. Similar conclusions have been reached by Hruska, *et al.*[42]

The *C*-glycosyl antibiotic showdomycin has been found to exist in the syn conformation in the solid

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Table II. Furanose Coupling Constants, J, for Pyrimidine Nucleosides[a]

	$J_{1'-2'}$	$J_{2'-3'}$	$J_{3'-4'}$	$J_{4'-5'}$	$J_{4'-5''}$	$J_{5'-5''}$
5-Methylcytidine	3.3					
6-Methylcytidine	3.7					
6-Oxocytidine[b]	3.6	6.0	6.0	3.0	6.2	*c*
Uridine[b]	4.6	5.3	5.0	2.5	3.9	13.2
5-Methyluridine	4.0					
6-Methyluridine[b]	3.3	6.2	6.6	*c*	6.0	*c*
6-Oxouridine	3.3					
6-Azauridine[b]	3.2	5.5	5.5	3.6	5.5	12.6
5-Bromouridine[b]	3.2	4.5	*c*	3.0	3.0	12.6
4-Thiouridine	3.6					
Showdomycin[d]	4.7[e]	4.8	5.0	3.2	4.5	12.7

[a] 10% solutions in D_2O. [b] Obtained from 90-MHz spectra. [c] Not resolved. [d] Obtained from 90-MHz spectra at 70°. [e] $J_{4-1'}$ = 1.4 Hz.

state.[43] The chemical shift data presented in Table I, however, are consistent with the anti form in solution. Two other *C*-glycosyl pyrimidine nucleosides have been shown by pmr to exist as anti conformers in aqueous solution, α and β-pseudouridine.[12,44]

II. Coupling Constant Data. Additional information regarding syn, anti preference in solution may be obtained by consideration of furanose conformational properties as monitored by the various vicinal H–C–C–H coupling constants and by examination of the dihedral relationship between C-2 of the base and H-1′ about the glycosidic bond as indicated by $^3J_{C_2-N-C-H_{1'}}$. The $^3J_{H-C-C-H}$ values obtained from 90-MHz spectra are listed for several nucleosides in Table II, including uridine, 5-bromouridine, 6-azauridine, 6-methyluridine, and 6-oxocytidine. Hruska, Smith, and coworkers have reported similar data for the first three compounds.[4a,13,15,45] Spectra for most of the other compounds given in Table II were obtained at 60 MHz and only the $J_{1'-2'}$ values were readily discernible.

The furanose conformational features in uridine have been explicitly delineated.[4a,13,15] $J_{1'-2'}$ and $J_{3'-4'}$ have values intermediate between 2′- and 3′-endo puckered forms and thus in solution it is thought that the best description of the uridine furanose ring conformation is a dynamic equilibrium between these two puckered extremes. In addition, the conformation aspects of uridine about the exocyclic $C_{4'}$–$C_{5'}$ bond have been described in terms of a population distribution between the classical staggered rotamers (Figure 6) gauche–gauche (gg), gauche–trans (gt) and trans–gauche (tg). The distribution is weighted in favor of the gg form, to the extent of 55–60%.[4a,46,47]

For nucleosides having a large percentage of gg rotomer, the $J_{4'-5'}$, $J_{4'-5''}$ coupling constants are relatively small as would be expected from the Karplus relationship between the vicinal coupling constants and the corresponding dihedral angles. Recently an assignment of the two 5′-protons has appeared,[47] based upon the differential change in chemical shifts in response to phosphate attachment at the 3′ position for the two examples: uridine, 3′-UMP, and pseudouridine, 3′-ψMP. Characteristically the 5′ proton displaying the greatest shift to lower field in response to the 3′-phosphate attachment is located at higher field and has a larger $J_{4'-5'}$ in the parent nucleoside. In Figure 6 this proton would be H-5″. Although the assignment of Remin and Shugar[47] probably holds for those nucleoside systems where the gg rotamer predominates, further examples, especially where the gg conformation is destabilized, should be reported, demonstrating general applicability.

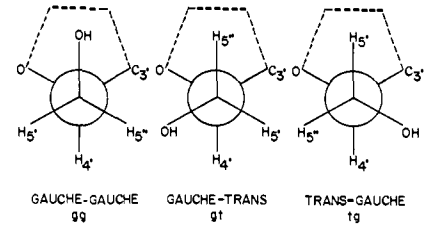

Figure 6. Newman projections of the three rotamers with respect to the $C_{4'}$–$C_{5'}$ bond.

It may be seen in Table II that substitution of uridine at position 5 with bromine results in a shift in the equilibrium population away from the 2′-endo conformation (lower $J_{1'-2'}$) and a higher population of gg rotamer (lower $H_{4'-5''}$). Hruska has also discussed the consequences of 5-halo substitution of pyrimidine nucleosides in greater detail,[46] concluding that the halogenation tends to shift the furanose puckering equilibrium toward 3′-endo concurrent with increasing the population of gg rotamers.

Examination of the furanose coupling constants for 6-azauridine, 6-methyluridine, and 6-oxocytidine in Table II reveals several interesting conformational features with reference to uridine (furanose coupling constants for cytidine are quite similar to those of uridine[15,46]). The decrease of $J_{1'-2'}$ coupled with an increase in $J_{3'-4'}$ may be interpreted as a shift in the dynamic puckering equilibrium away from 2′-endo. This shift in conformer population is also characterized by the increase in $J_{2'-3'}$ as the H-2′ and H-3′ become more nearly eclipsed.

Concomitant with the decrease of 2′-endo pucker is an increase in the gt and/or tg exocyclic rotamer population as is demonstrated by the increased $J_{4'-5'}$ and $J_{4'-5''}$ values, especially the latter. If one accepts the assignment of Remin and Shugar [47] for H-5′ and H-5″, the proton at highest field is H-5″. For the three nucleosides in question, this would mean that the preferred exocyclic conformation is gauche–

(43) Y. Tsukuda and H. Koyama, *J. Chem. Soc. B*, 1709 (1970).
(44) A. A. Grey, I. C. P. Smith, and F. E. Hruska, *J. Amer. Chem. Soc.*, **93**, 1765 (1971).
(45) F. E. Hruska in ref 27.
(46) F. E. Hruska, D. J. Wood, and J. G. Dalton, *J. Amer. Chem. Soc.*, in press.
(47) M. Remin and D. Shugar, *Biochem. Biophys. Res. Commun.*, **48**, 636 (1972).

Table III. ^{13}C Spectral Comparison of Cytosine, Cytidine, and 6-Methylcytidine

	Chemical shifts, ppm[a]									
	C-2	C-4	C-5	C-6	C-1′	C-2′	C-3′	C-4′	C-5′	CH_3
Cytosine	−38.4	−42.5	+34.6	−29.6						
Cytidine	−28.0	−38.0	+33.1	−14.4	+38.3	+50.4	+54.6	+43.3	+66.3	
6-Methylcytidine	−29.8	−38.7	+31.1	−27.6	+34.9	+55.3	+56.2	+41.5	+64.1	+106.5

	^{13}C–H coupling constants (Hz)[b]				
	$^3J_{C_2-H_6}$	$^3J_{C_2-H_{1'}}$	$^3J_{C_4-H_6}$	$^1J_{C_6-H_6}$	$^1J_{C_5-H_5}$
Cytosine[c]	9 ± 2		10 ± 2	170 ± 5	166 ± 5
Cytidine	9 ± 1	3 ± 1	9 ± 1	180 ± 2	185 ± 5[c]
6-Methylcytidine		6 ± 1			187 ± 5[c]

[a] Taken from noise decoupled FT spectra of 4000–8000 transients; probe temperature cooled to ~35°; shifts were measured from external hexafluorobenzene and converted to a benzene scale using the experimentally determined relationship $\delta_{C_6H_6} = \delta_{C_6F_6(ext)} - 9.9$ ppm. Samples were 30–40% w/v of solute in DMSO-d_6. The anion of cytosine was generated in DMSO by reaction with LiOH. [b] Taken from undecoupled FT spectra of 30,000–40,000 transients, sweepwidth 1.25 kHz, ambient probe temperature 30°. [c] Sweepwidth 5 kHz.

trans (Figure 6), although it is not apparent *a priori* why the gt should be favored over the tg. Mindful of the uncertainties which still exist in the assignment, especially since Ramin and Shugar did not present experimental verification for nucleosides where the gt or tg (or both) conformations are favored with respect to gg, the sum, $J_{4'-5'} + J_{4'-5''}$ (first suggested by Hruska[25]), will be utilized. Increases in this sum indicate relatively larger contributions of gt + tg rotamers, which is certainly the case for the three substituted nucleosides in question, going from 7.4 Hz in uridine to approximately 9 Hz.

The fact that in these nucleosides in question the population of furanose conformers has shifted from 2′-endo and that the gg rotamer is destabilized strongly suggests that repulsive interaction is occurring between the furanose substituents and the oxygen or nitrogen substituents in the aglycone lending further credence to the syn, anti preferences listed in Table I, namely that 6-azauridine and 6-oxocytidine are anti, whereas 6-methyluridine is syn.

Further information about the glycosidic conformation in unsubstituted *vs.* 6-methylated pyrimidine nucleosides has been obtained through examination of the vicinal ^{13}C–H coupling between C-2 and H-1′ in cytidine and 6-methylcytidine. Such an approach has recently been reported by Lemieux, *et al.*,[48a] for uridine and related compounds. These authors measured the various C–H coupling constants from the pmr spectra of nucleosides ^{13}C-enriched at C-2. We have studied the natural abundance cmr spectra of cytosine, cytidine, and 6-methylcytidine dissolved in DMSO-d_6.[48b] Although there are undoubtedly alterations in the glycosidic conformation between solvent water and DMSO, the data presented later on solvent perturbations indicate these changes are at the most small since similar ribose proton chemical shift changes are seen in DMSO as described above in D_2O. Pertinent chemical shifts and C–H couplings are listed in Table III. Assignments of the various ^{13}C resonances in cytidine and 6-methylcytidine were taken from the work of Jones, *et al.*,[49] except for a reversal of C-2′ and C-3′ as Mantsch and Smith have indicated.[50] The cytidine values are nearly identical with those of Jones, *et al.*, while those of 6-methylcytidine are within 1 ppm. The C-2 and C-4 resonances in cytosine anion were assigned by comparison with cytidine, using the parameters of Pugmire, *et al.*,[51] regarding the effects of nitrogen substitution on the ^{13}C shifts. C-5 and C-6 were readily assigned by comparison with cytidine, and from the one-bond C–H couplings (Table III).

In 6-methylcytidine the largest shift change occurs at C-6 (−13.2 ppm), as noted by Jones, *et al.*;[49] however, significant alterations also occur in the ribose carbon chemical shifts. C-2′ and C-3′ shift upfield respectively 4.9 and 1.2 ppm, whereas C-1′, C-4′, and C-5′ move downfield 3.4, 1.8, and 2.2 ppm. These carbon shift changes are in the opposite direction to the proton shifts for the respective directly bonded hydrogens (Table I). Taken together, these data suggest that C–H bond polarization, especially $C_{2'}$–$H_{2'}$, occurs in 6-methylcytidine due to an electric field effect of the 2-keto group of the base located over the ribose ring. Upfield shifts at C-2′ of slightly smaller magnitude (2–2.5 ppm) have been observed for 2,6-diketopyrimidine nucleosides such as 6-oxouridine and 1-β-D-ribofuranosylcyanuric acid compared with uridine.[49]

The vicinal $^3J_{C_2-H_6}$ of ~9 Hz listed in Table III for cytosine and cytidine is typical for these three-bond couplings. Lemieux[48a] reported similar values of $^3J_{C_2-H_6}$ for uracil and uridine. A splitting of the same magnitude is seen at C-4 which is presumably $^3J_{C_4-H_6}$. Two bond C–H couplings in this heterocycle were not resolved, but they must be small, 4–5 Hz or less, since in 6-methylcytidine with no $^3J_{C_4-H_6}$, C-4 is a singlet with a line width of ~4 Hz.

In cytidine the vicinal $^3J_{C_2-H_{1'}}$ is ~3 Hz, close to the value of 2.4 Hz reported by Lemieux[48a] for uridine. Based upon his graph relating the magnitude of the $^3J_{C-H}$ coupling to the dihedral angle between C-2 and various vicinal protons, the dihedral angle between C-2 and H-1′ is ±30–35°, corresponding to 3 Hz (Figure 7; the other unlikely possibility is ±130°, which brings the 2-keto and either H-2′ or O-1′ into intimate van der Waals contact). The torsional angle, χ, corresponding to $\phi_{C_2-H_{1'}}$ of +35° is −25°, whereas for $\phi = -35°$, χ is −95°. Both of these values are in the anti glycosidic range for $\chi_{C_6-O_{1'}}$.

For 6-methylcytidine the only coupling at C-2 is the vicinal interaction with H-1′ of ~6 Hz. Again with reference to the data of Lemieux,[48a] the dihedral angle

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(49) A. J. Jones, D. M. Grant, M. W. Winkley, and R. K. Robins, *J. Phys. Chem.*, 74, 2684 (1970).

(50) H. H. Mantsch and I. C. P. Smith, *Biochem. Biophys. Res. Commun.*, 46, 808 (1972).

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Table IV. Proton Chemical Shifts,[a] ppm, for Pyrimidine Nucleosides in DMSO-d_6[b]

Nucleoside	H-6 (or CH_3)	H-5 (or CH_3)	H-1′	H-2′	H-3′	H-4′	H-5′,5″
Uridine	8.27	6.03	6.18	4.40	4.40	4.26	3.98
	8.22	6.02	6.20	4.45	4.45	4.27	4.02
2′,3′-Isopropylidine-uridine	8.16	6.02	6.22	5.29	5.12	4.46	3.96
	8.13	6.00	6.23	5.29	5.13	4.47	3.98
5-Methyluridine	8.12	2.17	6.16	4.38	4.38	4.24	3.97
6-Methyluridine	2.67	5.97	5.87	5.00	4.51	4.16	3.97
2′,3′-Isopropylidine-6-methyluridine	2.68	6.10	5.98	5.63	5.17	4.37	3.92
5-Methylcytidine	8.09	2.25	6.19	4.35	4.35	4.26	4.02
6-Methylcytidine	2.67	6.02	5.91	5.03	4.54	4.17	3.96

[a] Measured from TMS capillary at 60 MHz; top line 34°, bottom line 70°. [b] 10% solutions.

corresponding to this coupling is about ±165° (Figure 7), in the syn range for χ of either 105 or 135°. Theoretically, ϕ could also be approximately ±15°, but this would place the 6-methyl group in close contact with the 5′-CH_2OH and other ribose substituents.

III. Solvent, Temperature, and Structural Perturbations. It was of interest to examine the effect of solvent environment and temperature on the glycosidic conformation as monitored by the relative chemical shift differences of the ribose protons. Table IV contains 60-MHz data for several nucleosides in DMSO-d_6. Here the data are similar to that observed in water (Table I). The ribose proton shifts are nearly identical for uridine and 5-methyluridine, but in 6-methyluridine (contrasted to uridine) H-2′ and H-3′ shift downfield approximately 0.60 and 0.11 ppm, respectively, while H-1′ and H-4′ move upfield 0.31 and 0.10 ppm. The same behavior is noticed for 5- and 6-methylcytidine. Thus it is probable that, though the torsional angle may be somewhat different between water and DMSO, overall glycosidic conformational preferences are the same in these milieus, at least for the examples given.

2′,3′-*O*-Isopropylideneuridine was examined as an example of a compound with a significantly altered furanose conformation. Since it was shown in section II that nucleosides with anisotropic groups located over the furanose ring, *e.g.*, syn 6-methylated nucleosides, have characteristically less 2′-endo pucker, then the converse situation may occur in derivatives such as 2′,3′-*O*-isopropylidenes or 2′,3′-cyclic phosphates with much less furanose puckering, that is, a shift away from the usual anti conformation.

2′,3′-*O*-Isopropylideneuridine in DMSO as listed in Table IV displays significant downfield shifts for H-2′, H-3′, and H-4′ compared with uridine, 0.89, 0.72, and 0.20 ppm, respectively. This behavior is contrasted to the 0.60 and 0.11 ppm low field shift of H-2′ and H-3′ and the 0.10 ppm upfield shift of H-4′ between uridine and 6-methyluridine. It is of interest to note that between 2′,3′-*O*-isopropylidene-6-methyluridine and 6-methyluridine H-2′, H-3′, and H-4′ shift downfield by 0.63, 0.66, and 0.21 ppm. It is thus tempting to ascribe most of the shift changes in the isopropylidene derivatives compared with the parent nucleosides to the drastic conformational restrictions imposed upon the furanose ring, with the attendant change in orientation of the various substituents. The torsional angle in 2′,3′-*O*-isopropylidene-6-methyluridine is undoubtedly in the syn range as has been demonstrated for the unblocked 6-methylated nucleosides, since a comparison between the two isopropylidene derivatives in Table IV shows H-2′ and H-3′ downfield 0.34 and 0.05 ppm, whereas H-1′ and H-4′ are upfield 0.24 and 0.09 ppm in the 6-methylated compound, these shift differences being very similar to the differences between uridine and 6-methyluridine.

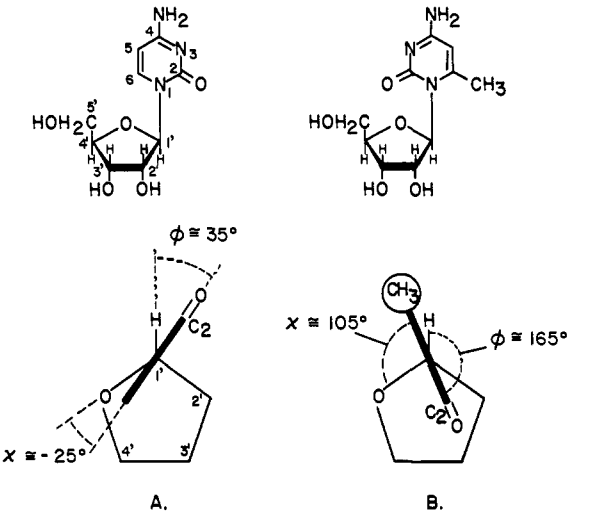

Figure 7. Projections looking down the base plane showing the dihedral angle, ϕ, between C-2 and H-1′ and the corresponding torsional angle, χ, between the C_6–N_1 and $C_{1'}$–$O_{1'}$ bonds in cytidine (A) and 6-methylcytidine (B).

Thus, it is apparent that the change in ribose conformation between free nucleoside and 2′,3′-*O*-isopropylidene derivatives has a minimal effect on syn, anti disposition, and that bulky substituents on the base are more significant determinants of the glycosidic conformation.

In general, the effect of temperature between 34 and 70° on both chemical shifts (Table I) and coupling constants is minimal, illustrating the relative conformational stability of these nucleosides. It is noted in Table I that the temperature-induced change in the H-6 chemical shift for 5-bromouridine is nearly double that for the other nucleosides. It is known[52] that 5-bromouridine associates in aqueous media so that the larger shift of H-6 to lower field may partially be due to break-up of aggregates.

Conclusions

Proton chemical shift data have shown that most of the pyrimidine nucleosides studied here prefer the anti

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glycosyl conformation in solution. However, bulky groups such as methyl at the 6 position shift the torsional angle into the syn range, as does a 5,6-fused benzene ring. Vicinal H–H and ^{13}C–H coupling constants are supportive of the assigned glycosyl preferences. The relative proportion of syn and anti conformers appears to be about the same in DMSO as in water, although the torsional angle may be altered. The population distribution of conformers is relatively unchanged by temperature alteration.

Acknowledgment. We thank Dr. George Kreishman for stimulating discussions and several ^{13}C spectra.

Synthesis of Phage Specific Deoxyribonucleic Acid Fragments. I. Synthesis of Four Undecanucleotides Complementary to a Mutated Region of the Coat Protein Cistron of fd Phage Deoxyribonucleic Acid

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Received December 4, 1972

Abstract: A general approach for the production of synthetic mutations in known sequences of circular single-stranded DNA by chemically prepared mutated oligonucleotides, used as primers, is proposed. In order to test this approach, synthesis of the four undecanucleotides dA-C-C-A-T-T-C-A-N-G-C (N = A, C, G, or T), corresponding to mutated minus strand fragments of a region of the coat protein cistron of fd phage DNA has been undertaken. In this sequence thymidylic acid in the sixth position replaces a cytidylic acid residue of the corresponding wild type sequence (N refers only to the degenerate position of an alanine codon). The octanucleotide sequence dA-C-C-A-T-T-C-A, common to all four undecanucleotides, was prepared in the protected form using protected dinucleotide blocks as intermediates for condensation reactions in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride. Individual reactions of the protected octanucleotide with one of the protected trinucleotides, dpbzA-ibuG-anC(Ac), dpanC-ibuG-anC(Ac), dpibuG-ibuG-anC(Ac), and dpT-ibuG-anC(Ac), yielded the four desired undecanucleotides. A new technique is described for the column chromatographic separation of trityl-containing nucleotide blocks or intermediates from compounds carrying N-protecting groups only: trityl-free compounds are eluted from DEAE-cellulose by using salt gradients in the presence of 20% methanol, whereas subsequent elution of the more lipophilic tritylated blocks or reaction products is achieved by salt gradients in the presence of 50% ethanol. Application of this technique is essential for the separation of protected tetra- and hexanucleotides from unutilized dinucleotides applied in excess and from the respective symmetric pyrophosphates. Unutilized blocks recovered by this method can also be reutilized for subsequent condensation steps without further purification.

Induction of mutations by chemical reagents or by irradiation in general is based on random processes in respect to the DNA or RNA regions involved, whereby the populations to be mutagenized usually become diminished by several orders of magnitudes. It seems therefore desirable to search for techniques, by which mutagenesis is achieved exclusively at small defined regions or even at distinct single base pairs without inactivation of the rest of the respective genoms.

In order to test the possibility of inducing specific mutations *in vitro* in circular single-stranded DNA by the use of synthetic mutated oligonucleotides acting as primers, synthesis of the four fd phage specific undecanucleotides dA-C-C-A-T-T-C-A-N-G-C (N = A, C, G, or T[1]) was undertaken. The selection of this sequence is based on the following considerations: from the amino acid sequence Ala-Trp-Met-Val, observed in the coat protein of the fd phage,[2] four possible undecanucleotide sequences of the corresponding mRNA and of the minus strand DNA can be deduced[3] (Chart I; the ambiguity in one position designated by N and M is due to the degeneracy of the alanine codons[3]). Conversion of the tryptophan codon UGG to the terminator codon UGA would be achieved by substitution of the cytidylic acid residue with a thymidylic acid residue in the sixth position of the complimentary undecadeoxynucleotide. Therefore one of the four oligomers synthesized should be

(1) The system of abbreviations is principally as has been suggested by the IUPAC–IUB commission published in *Eur. J. Biochem.*, **15**, 203 (1970). In this paper the prefix d (for deoxy) in all cases refers to the entire nucleoside residues of the oligonucleotide chains described; for clarity therefore brackets and hyphens usually following the prefix d are always omitted; thus brackets are only used to mark hydroxyl protecting groups. DCC, MS, and TPS refer to *N,N'*-dicyclohexylcarbodiimide, mesitylenesulfonyl chloride and 2,4,6-triisopropylbenzenesulfonyl chloride, respectively. One A_{260} unit is defined as the amount of nucleotide giving an absorbance of 1 at 260 nm when dissolved in 1 ml of solvent and measured in a 1-cm light path quartz cell. Different wavelengths are indicated by the respective subscripts.

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