to underestimate $\langle R_{ij}^{-1} \rangle$ between elements in loops that are smaller than average, more than it would overestimate $\langle R_{ij}^{-1} \rangle$ in loops larger than average, and thus would lower S_l/S_1. Thus, the effects of these two approximations will cancel to some extent.

The author is grateful to Professor Bruno Zimm for pointing out the semilogarithmic relationship (11), and for other helpful comments.

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PURINE BINDING TO DINUCLEOTIDES: EVIDENCE FOR BASE STACKING AND INSERTION

BY SUNNEY I. CHAN,* BENEDICT W. BANGERTER,† AND HEINRICH H. PETER

GATES AND CRELLIN LABORATORIES OF CHEMISTRY,‡ CALIFORNIA INSTITUTE OF TECHNOLOGY

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In an effort to understand the factors which may contribute to the stabilization of nucleic acids as well as the basic mechanism of the recognition process involved in the enzymatic replication of nucleic acids *in vitro*, we have embarked upon a systematic study of the interaction of biological bases and nucleosides with simple oligonucleotides by proton magnetic resonance. We wish to report here results which we have obtained on the interaction of purine with the following 3′ → 5′-dinucleotides: TpT, TpdU, and dUpT (T = thymidine, dU = 2′-deoxyuridine).

All the spectra reported in this communication were taken on a Varian A-60 NMR spectrometer with probe temperature at 30°C. The dinucleotides were dissolved in D_2O in the form of the ammonium salt.

The proton magnetic resonance spectrum of TpT in the regions of the thymine CH_3 and H_6 protons and the H_1' protons of the sugar moieties is shown in Figure 1*a*. For comparison, the same spectral regions for the thymidine nucleoside are also given (Fig. 1*b*). The protons of the two bases of TpT and the base protons in thymidine are, apparently, magnetically indistinguishable. The H_6 proton resonance in TpT is noticeably broader than that in thymidine, thereby suggesting that the two H_6 protons in TpT may not be exactly magnetically equivalent. The two H_1' protons of the dinucleotide are clearly not equivalent, and the resonance spectrum for these protons consists of a superposition of two 1:2:1 triplets. Evidently, the asymmetric attachment of the PO_4^- to the sugar moieties affects the magnetic environments at these protons differently. The chemical shift between the two H_1' protons is about 6 cps and is roughly equal to the spin-spin splittings

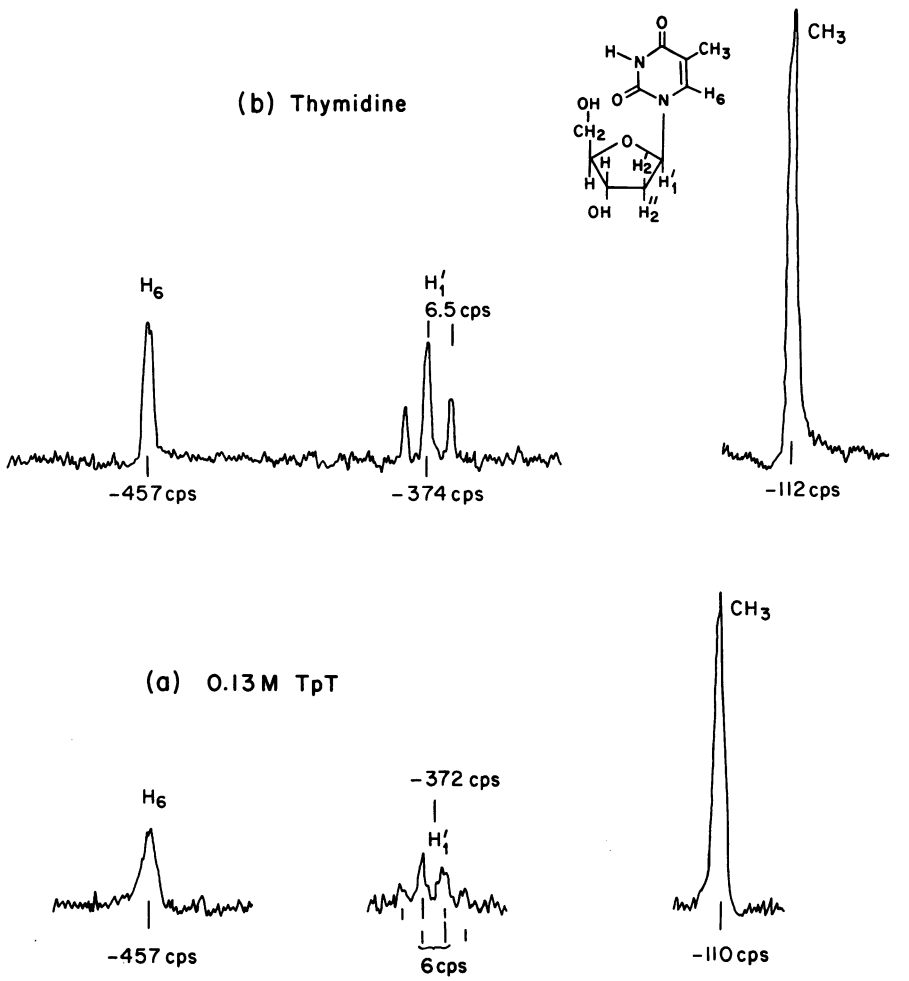

FIG. 1.—Proton NMR spectrum of (*a*) TpT, and (*b*) thymidine, in the regions of the CH_3, H_6, and H_1' protons.

arising from coupling to the (H_2', H_2'') sugar protons, yielding the observed 1:3:3:1 quartet.

Upon the addition of purine, the proton resonances for the thymine and H_1' protons in TpT are shifted to higher fields (Figs. 2*a* and *b*). The observed purine-induced shifts are not unexpected. These shifts, which arise from the ring current magnetic anisotropy of the purine base, have recently also been reported for a number of nucleosides: uridine, cytidine, and thymidine.[1] Not only do these results provide strong evidence for the binding of purine to these nucleosides, but, from the direction of the shifts and a comparison of the induced shifts for the base and sugar protons, it is also possible to conclude that the mode of interaction is that of vertical ring-stacking of the heterocyclic bases. However, for the TpT dinucleotide, the thymine CH_3 and H_6 protons are also split into two sets of resonances of *equal* intensity. (The CH_3 and H_6 resonances are already slightly split in the absence of purine due to mutual spin-spin coupling; the coupling constant is

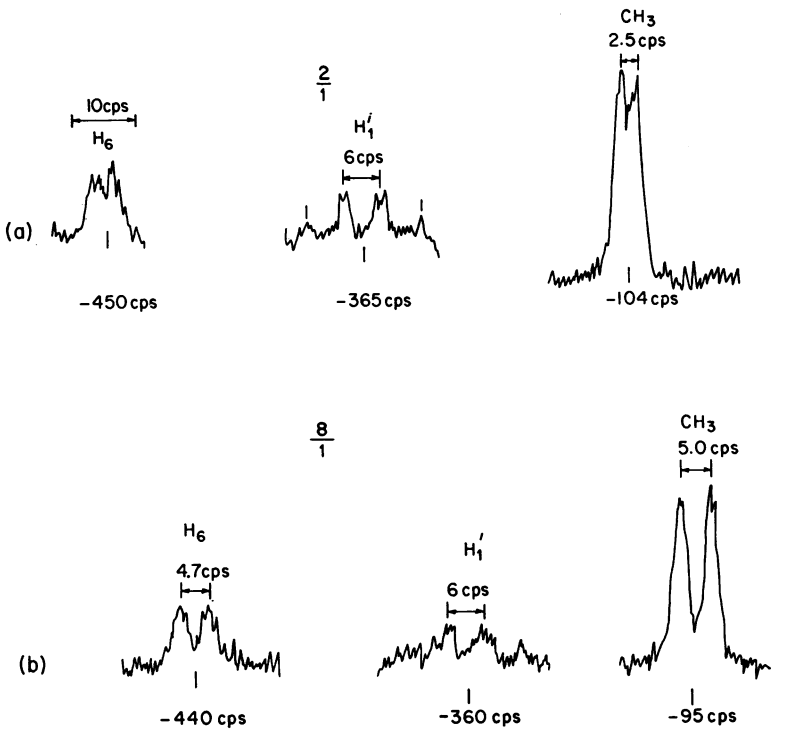

FIG. 2.—Effect of purine on the proton NMR spectrum of 0.13 *M* TpT. (*a*) Purine/dinucleotide = 2/1; (*b*) purine/dinucleotide = 8/1.

about 0.5 to 1.0 cps. With care, this spin-spin splitting can still be resolved after purine has been added to the dinucleotide solution. See Fig. 3.) This splitting is dependent upon the purine concentration (Fig. 3) and levels off at a maximum of 5 cps for both the CH_3 and H_6 base protons at the highest purine concentration studied (1.3 *M*). Such a purine-induced splitting is not evident for the H_1' protons. The stronger components of the H_1' quartet are somewhat broadened by the added purine. Perhaps the two sets of superimposed H_1' triplets are also moving relative to each other. However, the maximum relative shift appears to be of the order of 1 cps.

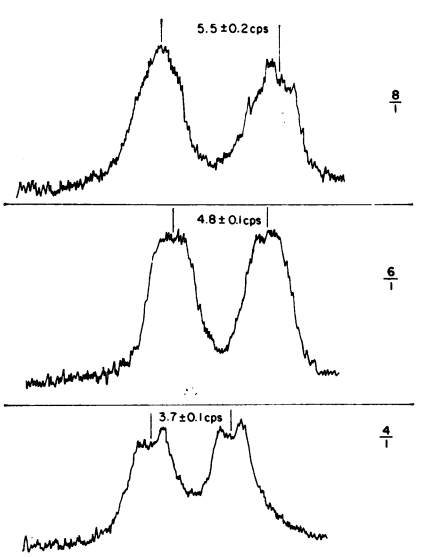

FIG. 3.—Effect of purine on the thymine CH_3 protons of 0.13 *M* TpT for purine/dinucleotide ratios of 4/1, 6/1, and 8/1.

Since a similar induced splitting is not observed for the thymine protons in thymidine and the two sets of resonances are of equal intensity over a wide range of purine/dinucleotide concentration ratios, it is possible to conclude that the addition of purine to TpT has resulted in the formation of a purine-dinucleotide complex in which the

TABLE 1

PURINE-INDUCED SHIFTS* FOR TpT

Purine/-dinucleotide†	CH_3		H_6		H_1'	
2/1	3.5	6.0	5.0	7.5	3.5	4.5
4/1	7.5	11.5	10.0	13.5	8.0	8.5
6/1	10.0	15.0	13.0	17.5	11.0	11.5
8/1	12.0	17.0	14.0	18.5	12.0	12.0
10/1	12.5	18.5	15.5	19.5	—	—

* Cps at 60 Mc for proton frequency.
† TpT concentration: 0.13 *M*.

two sets of thymine base protons of the TpT have become no longer magnetically equivalent as a result of a magnetic perturbation localized in the vicinity of the base protons. The magnetic nonequivalence arises presumably from the asymmetric phosphate attachment to the two deoxyriboses of the dinucleotide. The observed purine-induced shifts for TpT are summarized in Table 1.

There is ample evidence to indicate that purine will interact with the pyrimidine bases of the dinucleotide.[1, 2] However, there is, perhaps, little reason to expect an induced splitting of the otherwise nearly magnetically equivalent base protons of the two end thymine bases in TpT if the bases are "uncoiled" and purine molecules interact by stacking on both faces of the pyrimidine rings. Moreover, if the induced splitting can be accounted for in this manner, then it is difficult to understand why the H_1' proton resonances are not further split. To rationalize the observed results, we hypothesize that the two bases of the TpT dinucleotide are *either* already stacking and there is purine insertion between the thymine stacks in addition to external ring stacking, *or* the two bases are "uncoiled" initially and the purine molecule is interacting with the end bases in such a way as to bring them together to form a thymine-purine-thymine sandwichlike stack. On the basis of both recently published optical rotatory dispersion results on similar dinucleotides[3] and our own unpublished NMR results on the mixed purine-pyrimidine dinucleotides,[4] the latter interpretation would appear to be more probable. In either case, due to asymmetric attachment of the phosphate to the two deoxyribose moieties, the two bases of the dinucleotide can be in or brought into stereochemically nonequivalent configurations, so that the two sets of thymine protons experience slightly different magnetic anisotropy effects from the ring current of the incorporated purine base. This then leads to two sets of slightly different time-averaged resonances for the CH_3 and H_6 protons of the two thymine bases.

The above hypothesis may be tested by investigating the interaction of purine with dinucleotides containing different pyrimidine moieties. For this purpose, we have studied the effect of purine on dUpT and TpdU. The purine-induced shifts for the CH_3 and H_6 protons of the thymidine and those for the H_5 and H_6 protons of the uracil base are summarized in Table 2. From the table, it is clear that the limiting maximum purine-induced shifts are larger for those protons on the base of the 3′-nucleoside. This can be understood if the conformation of the sugar attachments to the phosphate in the complex is similar to that found in double helical DNA. To accommodate the inserting purine, obviously, some extension and untwisting of the phosphate-deoxyribose backbone must be made. In this conformation, the base of the 3′-nucleoside is more exposed so that the base

TABLE 2

PURINE-INDUCED SHIFTS* FOR TpdU AND dUpT

	TpdU (0.19 *M*)		dUpT (0.16 *M*)	
	Thymine Protons			
Purine/dinucleotide	CH_3	H_6	CH_3	H_6
1/1	6.5	7.0	4.5	4.0
2/1	10.5	10.0	6.5	7.0
4/1	15.0	15.5	10.5	11.5
6/1	19.5	20.0	14.0	15.0
8/1	21.5	22.5	15.5	17.0
10/1	23.0	23.5	17.0	18.5
	Uracil Protons			
	H_5	H_6	H_5	H_6
1/1	4.5	4.5	4.0	3.5
2/1	6.5	6.5	6.5	5.0
4/1	10.0	9.5	11.5	9.5
6/1	14.0	13.5	15.0	13.0
8/1	15.5	14.5	18.0	14.5
10/1	17.0	14.5	18.5	15.0

* Cps.

protons of the 3′-nucleoside experience, on the average, a larger ring current magnetic anisotropy from the inserting purine.

It is interesting to note that the maximum purine-induced shifts for the thymine base protons in TpdU and dUpT are larger than those for the corresponding base protons in TpT. We believe that the larger purine-induced shifts for the thymine base protons in TpdU and dUpT arise from preferential interaction of the purine bases with the thymine. There is evidence that purine stacks preferentially with thymine over uracil. It was recently reported,[1] for example, that the purine-induced shifts on the H_6 and H_1' protons of uridine are 60–70 per cent of the corresponding shifts for thymidine. It is also noteworthy that whereas the differential purine-induced shifts between the same thymine protons in TpdU and dUpT are comparable to the purine-induced splitting in TpT, the differential shifts for the H_5 and H_6 protons of the uracil base are quite small. This result probably suggests that the complex formation has not frozen out the relative rotation of the two ends of the dinucleotide about the bonds along the 5′—CH_2—O—P— linkage of the phosphate deoxyribose backbone. If the rate of this internal rotation is rapid compared with the reciprocal of the mean lifetime of a purine molecule in the complex, and if there is a preference for the intercalated purine to rotate with the thymine relative to the uracil base, the smaller differential shifts for the uracil base protons between TpdU and dUpT can readily be accounted for.

Further evidence for purine intercalation in a sandwichlike fashion between the bases of the dinucleotide is provided by the effect of the dinucleotide on the linewidths of the resonances of the purine protons. In the presence of the dinucleotide, the purine proton resonances are significantly broadened particularly at low purine/-dinucleotide concentration ratios where the fraction of incorporated to unbound purine is highest. The three observable proton resonances are not, however, equally broadened. For instance, the H_6 and H_8 resonances are affected to a considerably greater extent than the H_2 resonance. The effect is most pronounced for TpdU and least evident for dUpT, with TpT showing intermediate behavior. The purine proton resonances sharpen up with increasing purine/dinucleotide ratio presumably due to rapid exchange and averaging between bound and free purine. The dramatic change in the linewidths with varying purine concentration and the

TABLE 3

LINEWIDTHS* OF PURINE RESONANCES

Purine/-dinucleotide	TpdU			TpT			dUpT		
	H_6	H_2	H_8	H_6	H_2	H_8	H_6	H_2	H_8
1/1	~10	~3	~6	—	—	—	~1.5	~1.0	~2.0
2/1	6.0	2.4	3.4	4.0	2.2	2.4	1.6	1.4	1.2
4/1	4.0	1.6	2.4	2.7	1.7	1.9	1.4	1.0	1.2
6/1	3.2	1.5	2.0	2.4	1.4	1.6	1.2	1.0	1.0
8/1	2.6	1.3	1.6	1.9	1.1	1.5	1.1	0.8	0.8
0.125 *M* Purine									
	H_6	H_2	H_8						
	0.8	1.0	1.0						

* Full width at half-intensity in cps.

remarkable variation of this effect among the three dinucleotides TpT, dUpT, and TpdU, is depicted in Table 3 and Figure 4.

The observed line broadening does not appear to be attributable to slow exchange. The protons of purine molecules incorporated between the bases of the dinucleotide have slightly different chemical shifts from the protons of the unbound purine molecules in solution. Thus, if the exchange rate between bound and unbound purine is comparable to the chemical shift difference between the two species, line broadening is possible. However, if this were the case, it appears that a similar line broadening effect should also be noticeable for the base protons of the dinucleotide. This is not observed, even in the case of purine plus TpdU at the lowest purine/dinucleotide ratio, where the line broadening of the purine proton resonances is most pronounced. Furthermore, it would be difficult to rationalize on this basis the stereochemical specificity of the effect. The long lifetimes implied by this interpretation are also unreasonable.

We wish to propose that the purine protons experience a strong dipolar field when the purine is incorporated between the bases of the dinucleotide. The purine proton resonances can therefore be broadened by nuclear spin relaxation induced by fluctuations in these local magnetic fields. All the magnetic nuclei of the dinucleotide contribute to these dipolar fields; however, those arising from the 2′-, 3′-, 5′-, and 5″-ribose protons of the 3′-esterified nucleoside are expected to be most important. These protons are situated around the bend of the "U" on the inner side of the cage when the conformation of the dinucleotide corresponds to that for maximum interaction with the incorporated purine base. For these fluctuating local fields to be effective for specific line broadening of the purine proton resonances, the purine proton in question must be oriented favorably with respect to the ribose protons producing the local fields, and the stochastic process which is responsible for the fluctuations must be characterized by a correlation time which is of the order of 10^{-10} sec. It is not unreasonable that this characteristic time corresponds to the time scale of translational motion of the intercalated purine molecule relative to the dinucleotide and hence corresponds also to the mean lifetime of the purine molecule in the dinucleotide stack. Thus, if the mean lifetime of the intercalated purine is of the order of 10^{-10} sec, and if we can assume that in both the TpdU- and dUpT-purine complex, the inserted purine tends to rotate preferentially with the thymine base relative to the uracil end of the dinucleotide, then the observed specific line-broadening of the purine protons can be rationalized. In the TpdU-purine complex, where the purine would tend to stay in phase with the base of the 3′-nucleoside

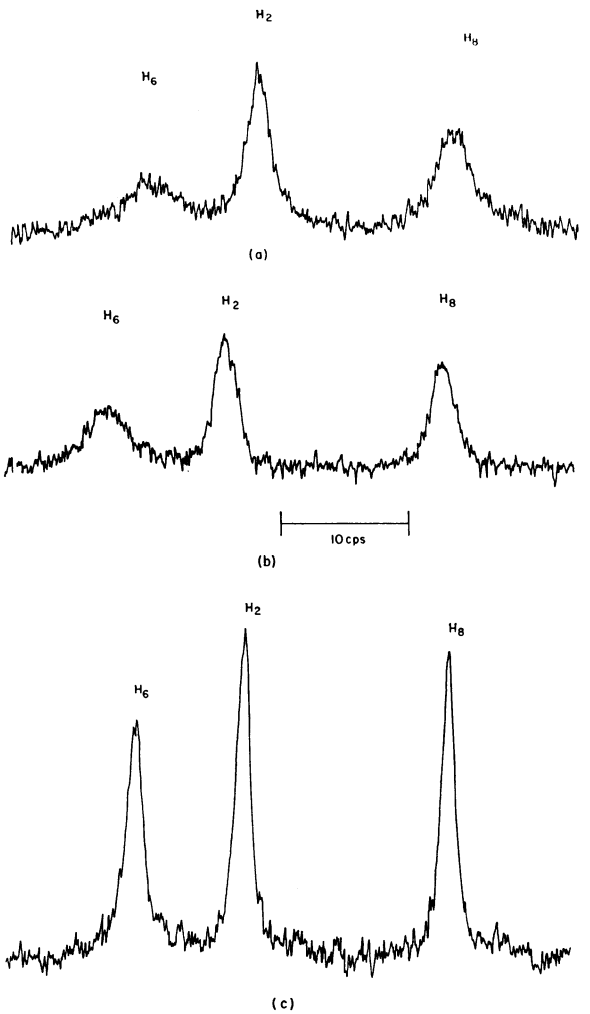

FIG. 4.—Effect of TpdU, TpT, and dUpT on the proton NMR spectrum of purine. Purine/dinucleotide = 2/1. (*a*) 0.19 *M* TpdU; (*b*) 0.13 *M* TpT; (*c*) 0.16 *M* dUpT.

on account of its stronger affinity for the thymine, the magnetic dipolar interaction between the purine proton and the ribose protons of the 3′-nucleoside is effectively modulated at a frequency of the order of the reciprocal of the mean lifetime of the purine incorporated, and hence the proton resonance for the appropriately oriented purine proton would be broadened. In the dUpT-purine complex, where the purine would rotate preferentially with the 5′-nucleoside, not only is the mean square field of the ribose protons significantly reduced due to the relative rotation of the two ends of the dinucleotide, but the magnetic dipolar interaction responsible for the purine line-broadening is modulated effectively by the rate of relative rotation of the two ends of the dinucleotide. The characteristic time associated with this internal rotation is expected to be shorter than the mean lifetime of the incorporated purine. The purine proton resonances are therefore expected to be sharp

for this system, as is observed. In the TpT plus purine system, there is no preferential interaction between the incorporated purine with either base of the dinucleotide. The linewidths of the purine resonances should therefore be intermediate between those for TpdU and dUpT. In fact, the widths of the H_6 and H_8 proton resonances are close to the mean of the widths for TpdU and dUpT. Similar line-broadening results have now been obtained for BdUpT, ApU, and UpA, which appear to be consistent with this model. (BdU = 5-bromo-2′-deoxyuridine, A = adenosine, and U = uridine.) For example, the purine proton resonances are sharp in UpA; but in BdUpT and ApU, they are so broad as to be unobservable for purine/dinucleotide ratios less than 2:1. On the basis of the larger self-association tendency of 5-bromouridine compared to uridine and thymidine,[2] purine is expected to interact more strongly with the 5-bromouracil base of BdUpT; it is also expected to interact preferentially with the adenosine base in UpA and ApU. Since the mean lifetime of the purine incorporated in these systems is expected to be longer than that in TpdU, dUpT, and TpT, the broadening of the purine proton resonances should be more pronounced, as observed.

The specificity of the line-broadening suggests that the orientation of the inserted purine molecule relative to the dinucleotide is not completely random. The larger line-broadening observed for H_6 and H_8 protons indicates that there may be some preference for the purine base to be oriented with these protons directed at the ribose protons of the 3′-nucleoside.

Similar studies on other dinucleotides (ApA, ApU, UpA, UpU, CpC, CpA, and ApC, where C = cytidine) have also been completed within our laboratories. These results will be published in detail elsewhere.

* Alfred P. Sloan fellow.

† National Science Foundation predoctoral fellow.

‡ Contribution no. 3321.

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*SPONTANEOUS ORIGIN OF AN INCIPIENT SPECIES IN THE DROSOPHILA PAULISTORUM COMPLEX**

BY THEODOSIUS DOBZHANSKY AND OLGA PAVLOVSKY

THE ROCKEFELLER UNIVERSITY

Communicated February 10, 1966

It has been questioned, by His Holiness Pius XII[1] among others, whether biology has really succeeded in making a species from another species. Fertile allopolyploids derived from hybrids between species have all the properties of new species. The clinching argument is that not only have new species been obtained