245. Nucleosides and Nucleotides. Part 13. Synthesis and Spectral Properties of 1-(3′-*O*-Phosphoryl-2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone-(3′-5′)-1-(2′-deoxy-β-D-ribofuranosyl)-2 (1*H*)-pyridone[1])

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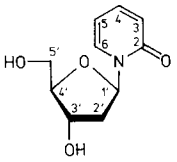
Summary

The dinucleoside phosphate $\Pi_d p\Pi_d$ (**4**) was synthesized from the monomers 1-(5′-*O*-monomethoxytrityl-2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone ((MeOTr)Π_d, **2**) and 1-(5′-*O*-phosphoryl-3′-*O*-acetyl-2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone (pΠ_d(Ac), **3**). Its 6.4% hyperchromicity and an analysis of the ^{1}H-NMR. spectra indicate that the conformation and the base-base interactions in **4** are similar to those in natural pyrimidine dinucleoside phosphates.

Introduction. – In an earlier paper on nucleosides and nucleotides we reported the synthesis of the deoxyriboside of 2(1*H*)-pyridone [2]. Condensation of this unnatural nucleoside, Π_d (**1**[2]) with natural deoxyribosides gave a variety of new oligonucleotides. These syntheses provided much valuable information on the stability of Π_d under the conditions of introduction and removal of protecting



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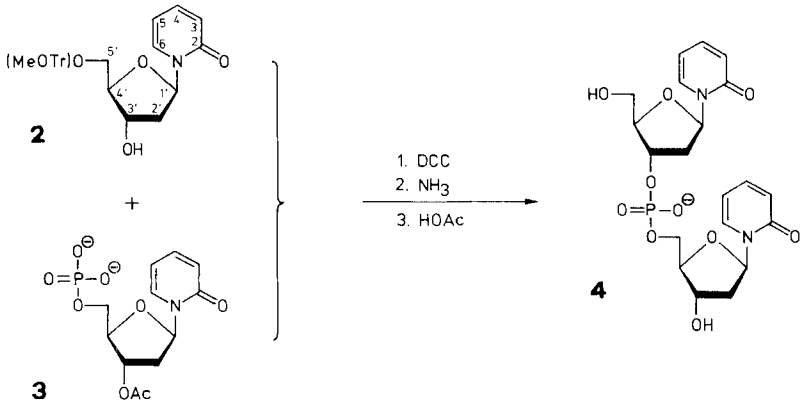
groups, phosphorylation and condensation [3–7]. In order to gain insight into the base stacking properties of Π_d we have now prepared the dinucleoside phosphate $\Pi_d p\Pi_d$ (**4**). The interaction between two pyrimidine bases is known to be smaller than that between pyrimidine and purine or between two purine bases [8] [9]. Nevertheless we decided to investigate the homodimer rather than a heterodimer, *e.g.* $\Pi_d pA_d$, for which – at least in the UV. spectra – it would be more difficult to separate the contributions of Π_d and A_d to any interactions.

[1]) Previous paper of this series: [1].

[2]) For abbreviations see [2].

Synthesis of $\Pi_d p\Pi_d$. – The dinucleoside phosphate $\Pi_d p\Pi_d$ (**4**) was obtained by condensation of (MeOTr)Π_d (**2**) [4] with pΠ_d(Ac) (**3**) [6] in the presence of dicyclohexylcarbodiimide (DCC). Subsequent removal of the protecting groups, whereby the lability of the 2(1*H*)-pyridone-containing nucleosides had to be taken into account [7], and chromatography on DEAE-Sephadex gave the desired product in 54% yield. Thin layer chromatography in two solvent systems revealed a slight impurity which could be neglected. Degradation of **4** with phosphodiesterase I gave equal amounts of Π_d and pΠ_d as expected. Similarly, phosphodiesterase II converted **4** into a 1:1 mixture of $\Pi_d p$ and Π_d, which again corroborated the structure of **4**. Both degradations proceeded to completion at rates similar to those observed for natural substrates[3]).



Hyperchromicity. – Interaction of the two bases in the dinucleoside phosphate **4** should lead to a smaller extinction coefficient for this compound than for a mixture of two corresponding monomers such as Π_d and pΠ_d. In other terms, any increase in absorbance (hyperchromicity) of a solution of $\Pi_d p\Pi_d$ observed upon cleavage of the internucleotide bond will indicate base-base interaction in the intact dinucleoside phosphate (*cf. e.g.* [11]).

Hyperchromicity was determined in two different ways:

a) A 30 µl aliquot of a stock solution of $\Pi_d p\Pi_d$ (**4**) was diluted with phosphate buffer of pH 7. Its extinction at 300 nm was 0.670 (see *Table 1*). Additional 30 µl aliquots were then subjected to enzymatic degradation with phosphodiesterase I or phosphodiesterase II. The extinction values of the resulting solutions containing equimolar amounts of monomer nucleosides and nucleotides were determined after appropriate dilution. The extinctions were 0.712 and 0.716, respectively;

b) To a 30 µl aliquot of the above-mentioned stock solution of $\Pi_d p\Pi_d$, phosphodiesterase I was added. The incubation mixture was rapidly diluted with phosphate buffer of pH 7. The extinction at 300 nm was 0.677. The extinction then in-

[3]) For a discussion concerning the cleavage of other unnatural dinucleoside phosphates with phosphodiesterases see [10].

Table 1. *Hyperchromicity measurements at 300 nm*

Degradation	Absorption of the dimer (D)	Absorption of the monomer mixture (M)	Hyperchromicity (H)	
Procedure 'a' with phosphodiesterase I	0.670	0.712	6.27%	average 6.4%
Procedure 'a' with phosphodiesterase II	0.670	0.716	6.87%	
Procedure 'b' with phosphodiesterase I	0.677	0.718	6.06%	

creased as the cleavage of the dinucleoside phosphate proceeded. After 6 days no further change in absorbance could be detected; the end value was 0.718.

From the above data, both experimental procedures lead to virtually the same results. The hyperchromicity H was then calculated using the equation [11]

$$H = \frac{M - D}{D} \cdot 100\%$$

where M and D represent respectively the absorptions of the monomer mixture and the intact dimer measured at the same concentration. The hyperchromicity in $\Pi_d p\Pi_d$ was 6.4% (see *Table 1*), which is comparable to the values obtained for $T_d pT_d$ [12] and similar natural dinucleoside phosphates [8].

Nuclear Magnetic Resonance. – Proton magnetic resonance spectroscopy is a very useful tool in nucleotide chemistry [13] [14]. It permits the determination of the conformation of the sugar moiety [15], the measurement of the torsion angle about the glycosidic bond (base *'syn'* or *'anti'*) [16], and monitoring of base-base interactions in dinucleoside phosphates [16]. Therefore, in addition to the hyperchromicity determination, ^{1}H-NMR. measurements were carried out with $\Pi_d p\Pi_d$ in order to gain further insight into its base stacking properties.

First, the concentration dependence of the chemical shifts of the base protons was determined in Π_d, $\Pi_d p$, $p\Pi_d$ and $\Pi_d p\Pi_d$. In all 4 compounds, the C(6) and C(4) protons – used as a probe and reference standard for base interactions, respectively, in the experiments described below – showed no concentration induced shifts greater than 0.01 ppm (or 0.88 Hz) in the range from 0.1 M to 0.0025 M. These findings, also observed with natural pyrimidine nucleoside derivatives [17], demonstrate that any *inter*molecular interactions do not express themselves in displacements of the C(6) and C(4) proton resonances.

Next, the chemical shifts of the C(6) and C(4) protons were determined for Π_d, $\Pi_d p$ and $p\Pi_d$ in D_2O at pD 5.8 (see *Table 2*), at which the nucleotides should mainly be present as the primary phosphates, thereby simplifying the comparison with the dimer **4** [18]. The chemical shift of the C(4) proton is constant in all 3 compounds. The C(6) proton, however, shows identical chemical shifts in Π_d and its 3'-phosphate, $\Pi_d p$, but it is displaced towards lower field by 0.13 ppm in the corresponding 5'-phosphate $p\Pi_d$. This is a clear indication that the orienta-

Table 2. *Chemical shifts of the C(6) and C(4) protons in D_2O, pD = 5.8*

	Π_d	$\Pi_d p$	$p\Pi_d$	$p\Pi_d$ at pD 7.6
C(6)–H	7.89	7.90	8.02	8.12
C(4)–H	7.61	7.60	7.60	7.60

The chemical shifts are given as δ values in ppm downfield from TMS. Dioxane served as internal standard (see experimental part).

tion of the base in 2(1*H*)-pyridone nucleosides is *anti* [19] as in most pyrimidine nucleosides [16] (corresponding values determined for T_d and pT_d are 0.103 ppm [19], 0.133 ppm [16], 0.09 ppm [18]). This was further corroborated when the spectrum of $p\Pi_d$ was measured at pD 7.6 at which the second dissociation of the phosphate is involved. While the C(4) proton resonance is not affected at all (see *Table 2*), the C(6) proton resonance is shifted further downfield by 0.1 ppm. Similar results were obtained by *Ts'o et al.* for pT_d, pU_d, pC_d and similar nucleotides [19].

The ^{1}H-NMR. spectrum of the dinucleoside phosphate $\Pi_d p\Pi_d$ (**4**) is shown in *Figure 1*. The two moieties of this compound can now be compared with the mononucleotides $\Pi_d p$ and $p\Pi_d$ which serve as models respectively for the 5′ and 3′ ends of the dimer **4**. The C(4) protons of the two moieties have almost identical chemical shifts (7.56 and 7.54 ppm, see *Fig. 2*), which corresponds nicely to what was found in the monomers $\Pi_d p$ and $p\Pi_d$ (see *Table 2*). It is not possible, however, to determine which of the two resonances belongs to which moiety of the dimer, nor can we explain why *both* C(4) protons are shifted by almost the same amount of 0.05 ppm to higher field in the dimer **4** as compared with the monomers.

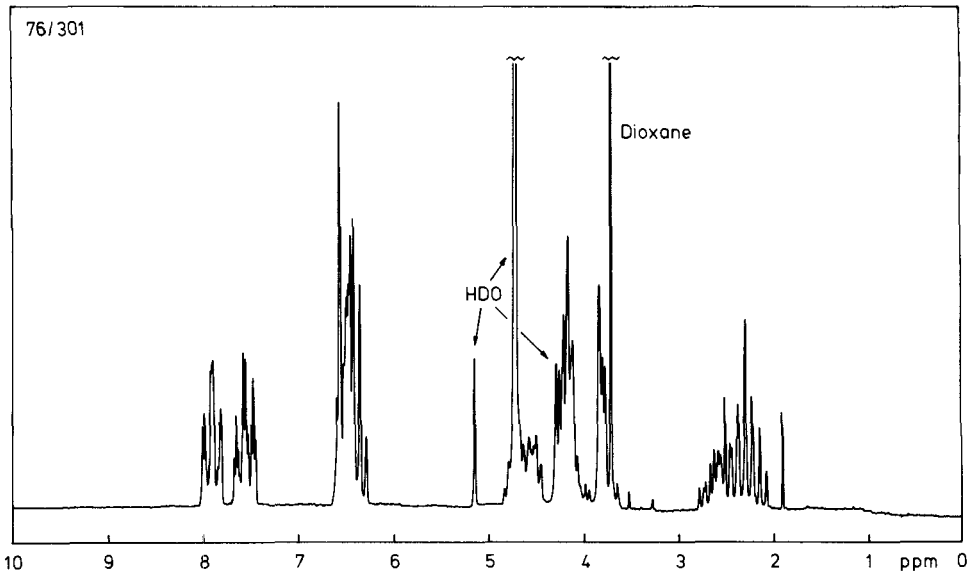


Fig. 1. *90 MHz ^{1}H-NMR. spectrum of $\Pi_d p\Pi_d$ (**4**) in D_2O*

The C(6) protons of **4** exhibit resonances at 7.92 and 7.83 ppm (see *Fig. 2*). Their assignment to the two moieties of the dinucleoside phosphate is possible in two ways: a) the 7.92 ppm signal may be attributed to the Π_dp part of the dimer; the 7.83 ppm resonance will then belong to the pΠ_d part - or b) the reverse assignment. From comparison of the resulting dimerization shifts with the values for T_dpT_d [16] [18] and UpU [16] (see *Table 3*) the assignment mode a) is probably the appropriate one, where the dimerization shifts for the Π_dp moiety of **4** is very small.

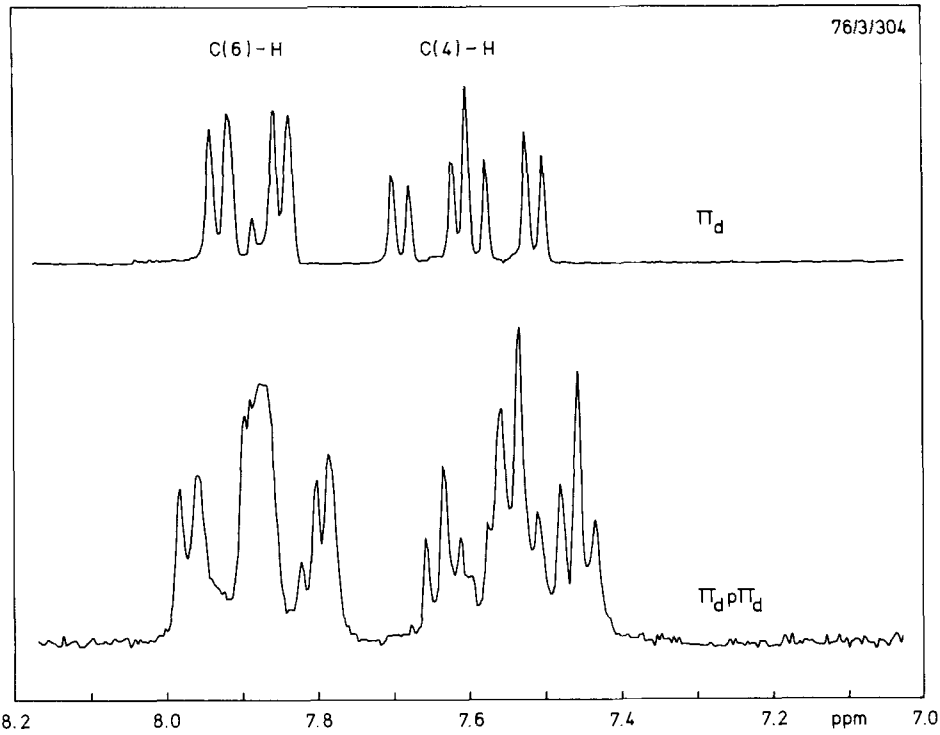


Fig. 2. *Sections of the 90 MHz ^{1}H-NMR. spectra of Π_d* (**1**) *and Π_dpΠ_d* (**4**) *showing the resonances of the C(4) and C(6) protons*

Table 3. *Dimerization shifts of the C(6) proton*

	δ of Np moiety of the dimer $-\delta$ of Np (monomer)	δ of pN moiety of the dimer $-\delta$ of pN (monomer)
In Π_dpΠ_d (**4**), assignment mode 'a'	+0.02 ppm	−0.19 ppm
In Π_dpΠ_d (**4**), assignment mode 'b'	−0.07 ppm	−0.10 ppm
In T_dpT_d [18]	+0.01 ppm	−0.06 ppm
[16]	+0.02 ppm	−0.08 ppm
In UpU [16]	+0.02 ppm	−0.10 ppm

Conclusion. – From the similarity of the hyperchromicity values and the dimerization shifts of the C(6) protons found for $\Pi_d p\Pi_d$ (**4**) with those of naturally occurring pyrimidine dinucleoside phosphates we conclude that the conformation of $\Pi_d p\Pi_d$ and the base-base interaction in this dimer are rather closely related to those in $T_d pT_d$, UpU and similar compounds. Thus, as one would expect, the conformation of such dimers is governed by the overall geometry of the base rather than by the presence or absence of its peripheral functional groups.

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Experimental Part

General. Solvent systems for paper and thin layer chromatography (TLC.) *(v/v)*, A: $CHCl_3$/methanol 1:1; B: 1-butanol/glacial acetic acid/water 5:2:3; C: 2-propanol/conc. NH_3-solution/water 7:1:2; D: CH_2Cl_2/methanol 9:1. For TLC. precoated silica gel plates F 254 *(E. Merck AG.)* were used. The spots were detected by observation under a UV. lamp, development with I_2 vapor, or spraying the plate with 10% perchloric acid and subsequent heating at 150–200°. *Whatman* 3MM paper served for paper chromatography (PC.); the descending technique was used.

1-(5′-*O*-Monomethoxytrityl-2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone ((MeOTr)Π_d, **2**) was prepared according to the procedure of *Gregor et al.* [4] and characterized by UV.- and IR. spectroscopy and by co-migration with a reference sample in the solvent systems A, B and D.

1-(5′-*O*-Phosphoryl-3′-*O*-acetyl-2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone ($p\Pi_d$(Ac), **3**) was prepared as described previously [6].

1. 1-(3′-*O*-Phosphoryl-2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone-(3′-5′)-1-(2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone ($\Pi_d p\Pi_d$, 4). – (MeOTr)Π_d (**2**, 242 mg, 0.5 mmol) and $p\Pi_d$(Ac) (**3**, pyridinium salt, 206 mg, 0.5 mmol) were dried 3 times by dissolving the mixture in 5 ml of abs. pyridine and evaporating the solvent. The residue was then dissolved in 7 ml of abs. pyridine and dicyclohexylcarbodiimide (DCC, 527 mg, 2.5 mmol) was added. The resulting clear yellow solution was stirred for 4 days at RT. in the dark. The reaction was stopped by addition of 7 ml of water while cooling with ice, and the mixture was stirred for another 24 h at RT. The dicyclohexylurea which had precipitated was removed by vacuum filtration and washed with 5 ml of 50% aqueous pyridine. The combined filtrate and washings were extracted 3 times with ether, which was then washed once with water. The aqueous layers were pooled and, after the addition of some pyridine, concentrated by evaporation to about 3 ml. The mixture was then treated with 3 ml of pyridine and 30 ml of conc. ammonia for 24 h at RT. The volume was reduced to 3 ml by evaporation and 12 ml of acetic acid were added [7]. After 4 h at RT. the acetic acid was removed under high vacuum. Residual acetic acid was repeatedly coevaporated with ethanol/water 2:1. The residue was distributed between 3×30 ml portions of water and 3×30 ml portions of ether. The aqueous layers were pooled, concentrated i.V. and applied to a DEAE-Sephadex A 25 column (100× 1 cm, HCO_3^--form). The elution was effected with a linear gradient of 4 l of 0–0.03M aqueous NH_4HCO_3-solution; fractions of 23 ml each were collected at a flow rate of 1.5 ml/min. A Uvicord I analyser of *LKB* was used to monitor the elution of the substances. Corresponding fractions were pooled and concentrated by evaporation *i.V.* Residual NH_4HCO_3 was removed by co-evaporation with several portions of methanol/water 9:1. Evaporation of the solution to complete dryness caused decomposition, so was strictly avoided. Fractions 75–93 (0.013–0.016M NH_4HCO_3) contained 3100 OD_{300} units of $\Pi_d p\Pi_d$ (**4**) (54%) and fractions 160–174 (0.028–0.03M NH_4HCO_3) contained 785 OD_{300} units of $p\Pi_d$ and $\Pi_d 5'pp5'\Pi_d$ (26% of the starting **3**). The aqueous solution of $\Pi_d p\Pi_d$ (**4**) was lyophilized to give a product containing only a very slight amount of a contaminant. Rf values: solvent B: 0.32 (TLC.); C: 0.56 (TLC.), 0.60 (PC.). – UV. (water): 300 and 226 nm ($\varepsilon_{300}/\varepsilon_{226} = 1.12$). – ^{1}H-NMR. ($D_2O$): see *Figure 1*.

2. Enzymatic degradation of $\Pi_d p\Pi_d$ with phosphodiesterases. – *Enzyme solutions.* 100 EU of phosphodiesterase I, EC 3.1.4.1 (Venom phosphodiesterase I, *Crotalus adamanteus, Worthington Biochem.* Corp.) were dissolved in 2 ml of 0.33M tris buffer, pH 9.1.

13 EU of phosphodiesterase II, EC 3.1.4.18 (bovine spleen, *P-L Biochemical* Inc.) were dissolved in 2 ml of 0.2M NH_4OAc buffer, pH 5.7.

Substrate solution. A stock solution of $\Pi_d p\Pi_d$ (**4**) was prepared by dissolving a certain amount of the ammonium salt in 0.5 ml water. UV. measurements indicated an approximate concentration of 16.3mM (calculated using an ε-value of 12,000, neglecting any hypochromicity). The exact concentration of this solution is irrelevant to the degradation experiments described below, where only absorption ratios are determined.

Assay with phosphodiesterase I. 30 µl of substrate stock solution and 100 µl of enzyme solution were incubated together for 5 h at 37°. A 10 µl aliquot of the incubation mixture was developed on a TLC. plate in system C to check for complete degradation of the substrate. For the determination of the ratio of the degradation products, 60 µl of the incubation mixture were applied as a spot on *Whatman* 3MM paper and the chromatogram developed in system C. After drying the paper, the UV.-absorbing spots were cut out, dissected to small pieces and eluted for 10 h with 2 ml of water. The eluates were filtered through cotton wool into volumetric flasks. The paper was then washed with water (3 × 1 ml) to give a total volume of 5 ml. At equal distances from the origin as the spots containing substance, blanks of about the same size were cut from the paper and treated as above. The UV. absorption of the solutions containing nucleosides or nucleotides were then determined at 300 nm in 1 cm cells against the corresponding blanks. Ratio found: $E(\Pi_d)/E(p\Pi_d) = 0.272:0.270 = 1.00$.

Assay with phosphodiesterase II. 30 µl of substrate solution and 50 µl of enzyme solution were incubated together for 5 h at 37°. The ratio of the degradation products was determined as described above. Ratio found: $E(\Pi_d p)/E(\Pi_d) = 0.480:0.484 = 0.99$.

3. Hyperchromicity of $\Pi_d p\Pi_d$ (4). – The degradation of $\Pi_d p\Pi_d$ (**4**) for the hyperchromicity measurements was effected in two ways.

a) 30 µl of a $\Pi_d p\Pi_d$ stock solution (*ca.* 9.3mM) were incubated with 100 µl of phosphodiesterase I solution for 3 h at 37°. The mixture was then transferred to a volumetric flask, made up to 5 ml with 0.1M phosphate buffer, pH 7.0, and the extinction measured at 300 nm in a 1 cm cell against 0.1M phosphate buffer[4]). $E(300) = 0.712$.

A second degradation was carried out in an analogous manner, but using 50 µl of phosphodiesterase II solution instead of the 100 µl of phosphodiesterase I. The extinction measured after the degradation was $E(300) = 0.716$.

The absorption of the intact dimer $\Pi_d p\Pi_d$ (**4**) was determined by measuring 30 µl of the above-mentioned substrate stock solution after dilution to 5 ml with phosphate buffer in a 1 cm cell against the appropriate blank. $E(300) = 0.670$.

b) 30 µl of the substrate stock solution and 100 µl of phosphodiesterase I solution were together made up to 5 ml with 0.1M phosphate buffer, pH 7.0. The UV. absorption was measured immediately at 300 nm in a 1 cm cell: $E(300) = 0.677$. The solution was then held at RT. and the change of the absorption was followed. After 6 days no further change was observed and the maximal extinction was reached: $E(300) = 0.718$.

The hyperchromicity was calculated from these measurements as described in the theoretical part.

4. ^{1}H-NMR. measurements. – The ^{1}H-*FT*-NMR. measurements were determined on a *Bruker* WH 90 spectrometer in the spectral laboratory of the Institute (we acknowledge *K. Aegerter*'s help). A spectral width of 1200 Hz was used and 8k FID's were collected and *Fourier* transformed to give 4k real spectra. Maximum resolution: 0.294 Hz/point. Dioxane was used as internal standard; chemical shifts were converted to the TMS scale using $\delta_{dioxane} = 3.70$ ppm.

The sodium salts of $p\Pi_d$, $\Pi_d p$ and $\Pi_d p\Pi_d$ were used for the measurements; they were obtained by passing aqueous solutions of the corresponding ammonium salts through a Dowex-50 column (Na^+-form). The substances were then isolated by lyophilization.

Apparent pH values in D_2O were determined with a *Metrohm* pH meter and converted to pD values using the equation pD = pH (meter reading) + 0.4 [20].

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[4]) Using phosphate buffer containing the appropriate amounts of enzyme gave exactly the same results.

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246. ^{13}C-NMR. Spectra of 4-Substituted Quinuclidines. Polar Effects, Part V.

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Summary

^{13}C-NMR. spectra of 37 4-substituted quinuclidinium perchlorates, 15 4-substituted quinuclidines and the corresponding 1-methylquinuclidinium iodides have been measured. The chemical shifts δ for all compounds lie in the expected range. No correlation is found between δ and the inductive substituent constant σ_I^q of the substituent. Abnormal shift differences between quinuclidines bearing a nucleofugal group and the corresponding protonated or *N*-methylated quinuclidinum salt are observed for the bridgehead carbon C(4). These differences are ascribed to incipient fragmentation, *i.e.* C,C-hyperconjugation in the ground state.