Conformational Analysis of 2- and 3-Phosphoglyceric Acids by ^1H and ^{13}C Nuclear Magnetic Resonance Spectroscopy

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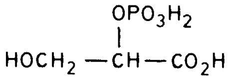
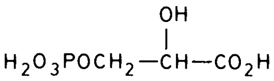
The staggered rotamer populations about the C(2)–C(3) and C–O (phosphate) bonds of 2- and 3-phosphoglyceric acids (2PPG and 3PPG) have been estimated as a function of pH from ^1H–^1H, ^1H–^{31}P, ^{13}C–^1H, and ^{13}C–^{31}P vicinal coupling constants. For 2PPG in the pH range 5.0—10.8, between four and five conformations of the molecule may be significantly populated($\geqslant 10$ %). For 3PPG at low pH that conformer with the planar *trans*-arrangement of the C(2)–H and O–P bonds predominates and is possibly stabilised by intramolecular hydrogen bonding. At higher pH (9.8) there is an increasingly important contribution from that conformer with the C(2)–CO_2^- and O–PO_3^{2-} bonds in a planar *trans*-arrangement, and this conformer may be stabilised by a combination of intramolecular hydrogen bonding and the electrostatic interaction.

THE substances 2-phosphoglyceric acid (2PPG) (I) and 3-phosphoglyceric acid (3PPG) (II) are important intermediates in the enzyme-catalysed process of glycolysis. In glycolysis a sugar (*e.g.* D-glucose) is converted to lactate *via* 3PPG and 2PPG, with a high proportion of the overall free-energy decrease being conserved as the phosphate bond energy of adenosine triphosphate (ATP). Our interest lies in the reversible conversion of 3PPG to 2PPG which is catalysed by the enzymes phosphoglyceromutase. The equilibrium constant for this reaction has been measured by Britton and his co-workers[1] and found to be in the region 8.6—11.7 (pH 5.4—8.1) in favour of 3PPG. An anomalous temperature dependence of the equilibrium constant was tentatively ascribed[1] to more extensive internal hydrogen bonding in 2PPG relative to 3PPG, limiting internal mobility in the former. Since n.m.r. spectroscopy is known to be a powerful tool in the elucidation of molecular conformations we have undertaken a study of 2PPG and 3PPG using this technique. In the absence of enzyme the rate for equilibration of 2PPG and 3PPG is slow on the n.m.r. time scale at all pH values employed in this study.



$$HOCH_2{-}\overset{OPO_3H_2}{\overset{|}{C}}H{-}CO_2H$$

(I)

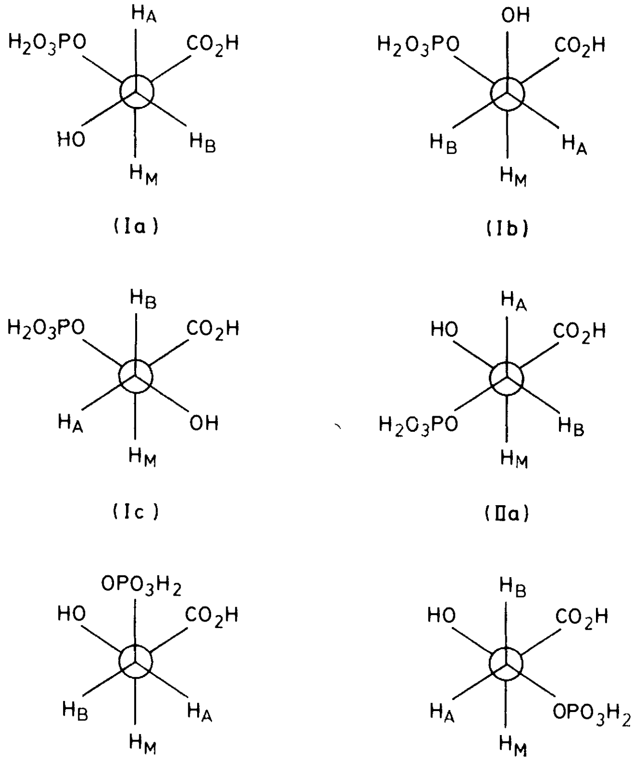


$$H_2O_3POCH_2{-}\overset{OH}{\overset{|}{C}}H{-}CO_2H$$

(II)

The Conformations about the C(2)–C(3) *Bond*.—Feeney and his co-workers[2] have detailed a method for estimating rotamer populations about the C_α–C_β bond in amino-acids by measurement of the vicinal ^1H–^1H and ^{13}C–^1H coupling constants. This method is readily applicable to 2PPG and 3PPG to investigate conformations about the C(2)–C(3) bond. The idealised staggered rotamers whose populations (P_i) are to be estimated are shown in Figure 1, and the conformation-dependent coupling constants are H_A–H_M, H_B–H_M, C(1)–H_A, and C(1)–H_B. Since the rotamer populations will depend upon the ionisation state of the phosphate and carboxy-groups, the analyses of the ^1H n.m.r. spectra were performed on samples at pH 5.0 and 10.8 for 2PPG and pH 0.6, 1.0, 2.8, 4.7, and 9.8 for 3PPG.

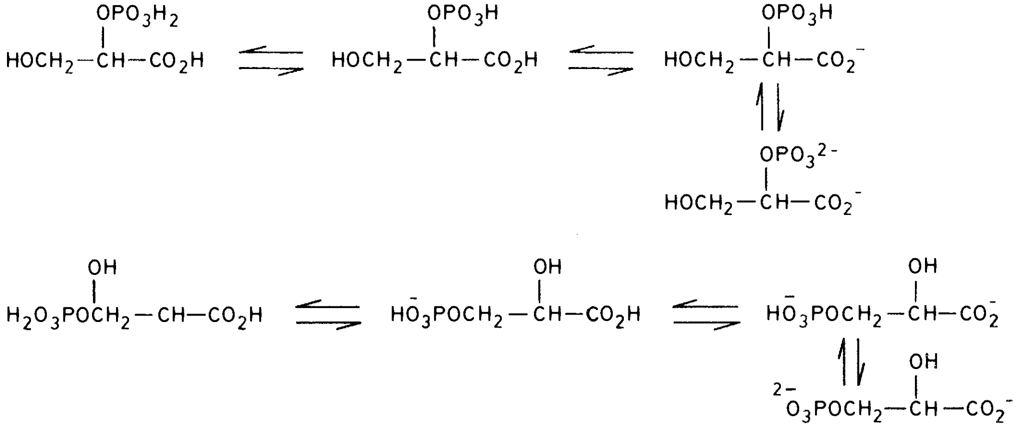
The species involved in the dissociation equilibria are illustrated in the Scheme (the pK_i are taken from the literature[3]).



(Ia) (Ib) (Ic) (IIa) (IIb) (IIc)

FIGURE 1 Staggered rotamers about C(2)—C(3) available to D—(+)—2PPG and D—(−)—3PPG

Typically (see Figure 2) in the cases most suitable for analysis, the ^1H spectra of 2PPG and 3PPG were second order showing 24 resonances characteristic of the ABM part of an ABMX spectrum (the X nucleus being ^{31}P). The splittings due to the ^{31}P nucleus were readily identified from the 12-line ^{1}H spectra (at 100 MHz) employing ^{31}P decoupling (at 40.5 MHz). ^{1}H-(^{31}P) Spectra of 2PPG at pH < 5 were deceptively simple[4] yielding less than 12 lines and were not suitable for full analysis of the inter-proton couplings. The coupling constants obtained by analysis (see Experimental section) of the ^{1}H spectra are collected in Table 1 together with the ^{1}H chemical shifts. Of the three groups of ^{1}H resonances, that of the methine proton (H_M) is to highest frequency (lowest field) while of the methylene group resonances those due to H_A have been arbitrarily assigned to lower frequency than H_B in most cases. For 3PPG at pH < 2.8, H_B has been assigned to lower frequency than H_A. This seems reasonable since the H_A–H_B chemical shift separation is a minimum at pH 2.8 (Table 1) and this proposed cross-over in chemical shifts avoids a switch in the relative magnitudes of J_{AM} and J_{BM} (and subsequently in P_{IIa} and P_{IIc}) and in J_{AP} and J_{BP} in the pH region 1.0—2.8. In addition the consequences of the cross-over are reasonable on chemical grounds (see Discussion).


SCHEME $pK_1 \simeq 2$, $pK_2 = 3.6$, $pK_3 = 7.0$ for 2PPG; $pK_1 \simeq 2$, $pK_2 = 3.6$, $pK_3 = 6.8$

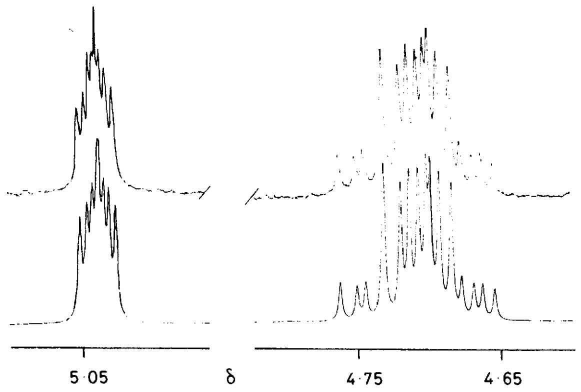


FIGURE 2 360 MHz ^{1}H Spectrum of 3PPG at pH 0.6: (a) experimental spectrum, (b) calculated spectrum

Because of rapid rotation about the C(2)–C(3) bond for both 2PPG and 3PPG, the vicinal couplings in Table 1 are values averaged over the three rotamers. In order to further analyse the vicinal couplings in terms of the rotamer populations,[2] values for the *trans*-vicinal coupling [$^{3}J_{AM}$ in rotamer (Ia), Figure 1] and the *gauche* [$^{3}J_{AM}$ in rotamer (Ib), Figure 1] must be obtained from model systems wherein the pattern of substituents about the C–C bond is as similar as possible to 2PPG and 3PPG. Such a model is 3′,5′-cyclic thymidine monophosphate (III) from which values for the *trans* ($^{3}J_{HH^t}$) and *gauche* $^{3}J_{HH^g}$) vicinal couplings between the protons at C(4′) and C(5′) have been measured[5] to be 10.1 and 2.1 Hz, respectively.

TABLE 1

^{1}H–^{1}H and ^{1}H–^{31}P coupling constants[a] and chemical shifts[b] for 2PPG and 3PPG

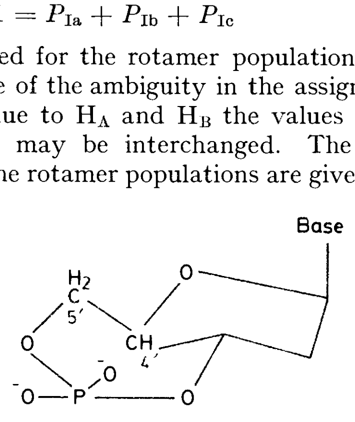
	2PPG				3PPG				
pH	1.0	2.8	5.0	10.8	0.6	1.0	2.8	4.7	9.8
$^{3}J_{AM}$	$\|7.0_3\|$[b]	$\|7.4_8\|$[b]	5.0_0	5.4_4	4.1_6	4.3_0	5.4_9	5.8_7	6.1_5
$^{3}J_{BM}$			3.0_7	2.9_4	3.0_6	3.1_2	2.3_8	2.6_1	2.7_5
$^{2}J_{AB}$	-12.4_7		-12.1_3	-11.7_4	-10.9_0	-10.9_6	-10.9_6	-10.7_9	-11.0_6
$^{3}J_{MP}$	9.4_8	9.2_8	8.8_7	8.6_0					
$^{3}J_{AP}$					6.4_7	6.4_9	6.2_2	5.6_4	5.9_4
$^{3}J_{BP}$					5.1_7	5.0_3	5.0_2	5.2_5	5.9_1
$^{4}J_{MP}$					1.7_7	1.5_9	1.3_6	1.2_3	0.5_7
δ_A	4.47_6	4.42_6	4.36_6	4.30_5	4.72_7	4.67_4	4.59_4	4.47_4	4.38_9
δ_B	4.45_6	4.42_6	4.40_9	4.40_7	4.72_7	4.67_4	4.59_4	4.47_4	4.38_9
δ_M	5.24_7	5.13_3	5.00_4	4.96_4	5.04_2	5.01_2	4.89_3	4.69_5	4.69_6

[a] Coupling constants in Hz; chemical shifts in p.p.m. to high frequency of external Me_4Si, from analysis of 360-MHz ^{1}H spectra; labelling of protons as in Figure 1. [b] Only the sum $^{3}J_{AM} + ^{3}J_{BM}$ can be calculated from these deceptively simple ^{1}H spectra.

The equations relating the rotamer populations to the vicinal couplings have been given by Feeney *et al.*,[2] and applied to 2PPG are of the form (1); these equations

$$\begin{aligned} J_{AM} &= P_{Ia}J_{HH}^{t} + P_{Ib}J_{HH}^{g} + P_{Ic}J_{HH}^{g} \\ J_{BM} &= P_{Ia}J_{HH}^{g} + P_{Ib}J_{HH}^{g} + P_{Ic}J_{HH}^{t} \\ 1 &= P_{Ia} + P_{Ib} + P_{Ic} \qquad (1) \end{aligned}$$

may be solved for the rotamer populations P_i. However, because of the ambiguity in the assignment of the resonances due to H_A and H_B the values obtained for P_{Ia} and P_{Ic} may be interchanged. The values calculated for the rotamer populations are given in Table 2.



(III)

The uncertainty is removed by consideration[2] of the vicinal ^{13}C–^{1}H couplings at C(1). This procedure may be understood if we consider the two solutions (2) and (3)

$$P_{Ia} = l,\ P_{Ib} = m,\ P_{Ic} = n \qquad (2)$$

$$P_{Ia} = n,\ P_{Ib} = m,\ P_{Ic} = l \qquad (3)$$

$$J_{C(1)H_A} + J_{C(1)H_B} = (2l + m + n)J_{CH}^{g} + (m + n)J_{CH}^{t} \qquad (4)$$

$$J_{C(1)H_A} + J_{C(1)H_B} = (l + m + 2n)J_{CH}^{g} + (l + m)J_{CH}^{t} \qquad (5)$$

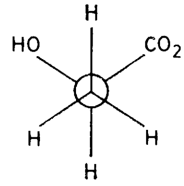
for the P_i. If solution (2) is true then (Figure 1), then equation (4) holds, or if solution (3) is true, equation (5), where J_{CH}^{t} and J_{CH}^{g} are values for the *trans*- and *gauche*-vicinal ^{13}C–^{1}H couplings obtained from a model system, for which we have selected lactic acid (IV).

TABLE 3

^{13}C–^{1}H and ^{13}C–^{31}P coupling constants[a] and ^{13}C chemical shifts[a] for 2PPG and 3PPG

	2PPG				3PPG			
pH	1.0	2.8	5.0	10.8	1.0	2.8	4.7	9.8
$^2J_{C(1)M}$			-3.7_0				-3.8_6	
$^3J_{C(1)A}$			0.2_0 [b]				1.0 [c]	
$^3J_{C(1)B}$			6.0_0 [b]				4.8_7 [c]	
$J_{C(1)P}$	4.4_0	4.6_4	4.15	6.5_9	0.0_0	0.0_0	0.0_0	0.0_0
$J_{C(2)P}$	-5.1_3 [d]	-5.1_3 [d]	-5.8_6 [d]	-4.8_8 [d]	8.0_6	7.8_1	8.0_6	7.0_8
$J_{C(3)P}$	5.1_3	5.1_3	5.1_3	2.4_4	-4.8_8 [d]	-4.8_9 [d]	-5.1_3 [d]	-4.6_4 [d]
$\delta_{C(1)}$	173.8_1	175.0_9	177.4_5	178.9_2	175.4_9	176.4_9	178.5_9	179.5_5
$\delta_{C(2)}$	75.9_3	76.2_3	77.9_5	77.0_8	70.9_2	71.6_5	73.0_4	73.8_0
$\delta_{C(3)}$	63.4_8	63.8_4	64.5_8	65.6_0	67.9_9	67.8_5	68.5_9	67.5_6

[a] Coupling constants in Hz; chemical shifts in p.p.m. to high frequency of external Me_4Si from analysis of 90.5 MHz ^{13}C spectra [except for A, B, M, P couplings to C(1) at pH 5.0, this ^{13}C spectrum at 50.3 MHz]. [b,c] The C(1)A and C(1)B couplings may be interchanged. [d] These two-bond couplings are assumed negative.



(IV)

TABLE 2

Rotamer populations[a] for 2PPG (P_{Ii}) and 3PPG (P_{IIi})

pH	1.0	2.8	5.0	10.8	Bond
P_{Ia}			0.36	0.42	
P_{Ib}			0.52	0.47	C(2)—C(3)
P_{Ic}			0.12	0.11	
P_{Id}	0.27	0.27	0.25	0.24	O—C(2)
P_{Ie}	0.27	0.27	0.29	0.60	
P_{If}	0.46	0.46	0.46	0.16	

pH	0.6	1.0	2.8	4.7	9.8	Bond
P_{IIa}	0.26	0.28	0.42	0.47	0.51	
P_{IIb}	0.62	0.59	0.54	0.47	0.41	C(2)—C(3)
P_{IIc}	0.12	0.13	0.04	0.06	0.08	
P_{IId}	0.73	0.74	0.75	0.77	0.73	
P_{IIe}	0.16	0.16	0.15	0.12	0.14	O—C(3)
P_{IIf}	0.11	0.10	0.10	0.11	0.13	

[a] Estimated error on the rotamer populations is ±10%.

The single resonance ^{13}C spectrum of the carboxy-carbon of (IV) yielded $^2J_{CH}^{g} + J_{CH}^{t} = 11.1$ Hz. The coupling constants at C(1) taken from the analysis * of the single-resonance ^{13}C spectra of 2PPG and 3PPG are given in Table 3. Using the rotamer populations (Table 2) for 2PPG at pH 5.0 together with the data in Table 3 in equation (6), which together with the data from lactic acid ($2J_{CH}^{g} + J_{CH}^{t} = 11.1$ Hz), yields J_{CH}^{g} −11.3 and J_{CH}^{t} 33.0 Hz. Interchanging the values for P_{Ia} and P_{Ic} (Table 2) in equation (2), with the lactic

$$6.20 = 1.36J_{CH}^{g} + 0.64J_{CH}^{t} \qquad (6)$$

acid data yields J_{CH}^{g} 5.6 and J_{CH}^{t} −0.06 Hz. A similar treatment for 3PPG at pH 4.7 yields J_{CH}^{g} 0.0 and J_{CH}^{t} 11.1 Hz for the populations as shown in Table 2,

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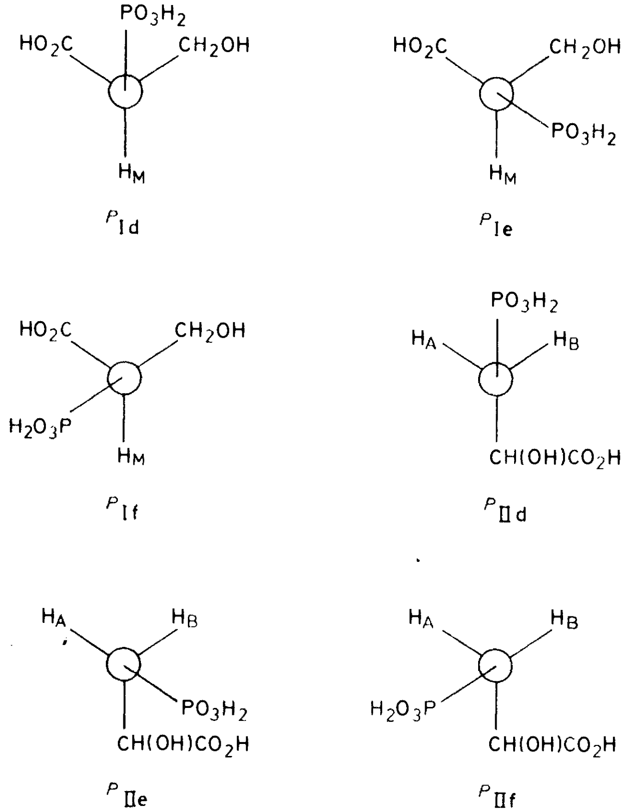
* In the analysis, calculated $^{13}C(1)$ band shapes are independent of the assignment between $^3J_{C(1)A}$ and $^3J_{C(1)B}$. However this does not affect the subsequent analysis since only the sum $J_{C(1)A} + J_{C(1)B}$ is used.

or J_{CH^g} 5.6 and J_{CH^t} 0.0 Hz on interchanging the values for P_{IIa} and P_{IIc}.

For α-amino-acids Feeney and his co-workers[2] have estimated J_{CH^g} 1.0 ± 1.0 and J_{CH^t} 10.6 ± 2.0 Hz, and adopt the criterion that $J_{\mathrm{CH}^t} > J_{\mathrm{CH}^g}$. Clearly the first solution for 3PPG, with the populations as given in Table 2, is the correct one. For 2PPG the situation is not so clear-cut, for while the first solution gives $J_{\mathrm{CH}^t} > J_{\mathrm{CH}^g}$, the magnitudes of the couplings are too large. However, values for J_{CH^g} and J_{CH^t} obtained from this analysis for 2PPG are very sensitive to small changes in the populations $P_{\mathrm{I}i}$. For example, if the populations shown in Table 2 were changed slightly to P_{Ia} 0.41, P_{Ib} 0.47, P_{Ic} 0.12 then *via* equation (2) we obtain J_{CH^g} −1.5 and J_{CH^t} 14.0 Hz, or on interchanging the values for P_{Ia} and P_{Ic}, J_{CH^g} 5.6 and J_{CH^t} −0.1 Hz. Thus the solution with $P_{\mathrm{Ia}} > P_{\mathrm{Ic}}$ is more reasonable, and the populations are as given in Table 2 as is the assignment of $\mathrm{H_A}$ to lower frequency of $\mathrm{H_B}$ (Table 1).



P_{Id} P_{Ie} P_{If} P_{IId} P_{IIe} P_{IIf}

FIGURE 3 Staggered rotamers about O–C(2) and O–C(3) available to D-(+)-2PPG and D-(−)-3PPG, respectively

The Conformations about the C–OPO$_3$H$_2$ *Bond*.—Sufficient coupling constant data ($^3J_{\mathrm{HP}}$, $^3J_{\mathrm{CP}}$) are available from the ^{1}H and ^{13}C spectra of 2PPG and 3PPG to perform an analysis of the conformation about the C–OPO$_3$H$_2$ bond in the manner described above for the C(2)–C(3) bond. Here the rotamers to be considered are $P_{\mathrm{Id-f}}$ for 2PPG and $P_{\mathrm{IId-f}}$ for 3PPG [see Figure 3 for projections along the O ⟶ C(2) and O ⟶ C(3) bonds respectively].

The analysis for the rotamer populations about the O–C bonds follows that for the C(2)–C(3) bond, with the advantage that for 2PPG the question of the ambiguity in the assignment between $\mathrm{H_A}$ and $\mathrm{H_B}$ does not arise, and the assignment has already been made for 3PPG. The relevant equations for 2PPG are of the form (7).

$$^3J_{\mathrm{C(3)P}} = P_{\mathrm{Id}}J_{\mathrm{CP}^g} + P_{\mathrm{Ie}}J_{\mathrm{CP}^g} + P_{\mathrm{If}}J_{\mathrm{CP}^t}$$
$$^3J_{\mathrm{H_MP}} = P_{\mathrm{Id}}J_{\mathrm{HP}^t} + P_{\mathrm{Ie}}J_{\mathrm{HP}^g} + P_{\mathrm{If}}J_{\mathrm{HP}^g}$$
$$1 = P_{\mathrm{Id}} + P_{\mathrm{Ie}} + P_{\mathrm{If}} \quad (7)$$

Clearly values for the *gauche*- and *trans*-^{13}C–^{31}P and *gauche*- and *trans*-^{1}H–^{31}P couplings are required. In extensive studies on the conformational analysis of the amino-acids phosphoserine and phosphothreonine in aqueous solutions, Ziessow and his co-workers[6] have employed the values J_{CP^g} 1.0 and J_{CP^t} 10.0 Hz (after Mantsch and Smith[7]) and J_{HP^g} 2.5 and J_{HP^t} 27.9 Hz (after Donaldson and Hall[8]). We use these same values, which together with experimental vicinal ^{1}H–^{31}P and ^{13}C–^{31}P couplings (Tables 1 and 3) in equations (3) yield the rotamer populations $P_{\mathrm{Id-f}}$ shown in Table 2. The experimental values for the vicinal couplings ^{13}C(1)–^{31}P were not employed in the rotamer population analysis since the values for J_{CP^g} and J_{CP^t} used above related to *sp*^{3} hybridised carbons and the possibility exists that different values for the *gauche*- and *trans*-couplings would be appropriate to the *sp*^{2} hybridised C(1). However a regression analysis based upon the derived populations $P_{\mathrm{Id-f}}$ (Table 2) and values for $^3J_{\mathrm{C(1)P}}$ (Table 3) yielded $J_{\mathrm{C(1)P}^g}$ 2.7 and $J_{\mathrm{C(1)P}^t}$ 9.1 Hz (correlation coefficient 0.96). In spite of the cumulative errors involved in this estimation these values seem entirely reasonable.

In analysing for rotamer populations about the O–C(3) bond of 3PPG it is possible to use equations analogous to (3) but based upon $^3J_{\mathrm{H_AP}}$, $^3J_{\mathrm{H_BP}}$, *or* $^3J_{\mathrm{H_AP}}$, $^3J_{\mathrm{C(3)P}}$, *or* $^3J_{\mathrm{H_BP}}$, $^3J_{\mathrm{C(3)P}}$. The solutions for $P_{\mathrm{IId-f}}$ of 3PPG shown in Table 2 were obtained from the vicinal ^{31}P couplings to $\mathrm{H_A}$ and $\mathrm{H_B}$. Populations $P_{\mathrm{IId-f}}$ obtained using one ^{1}H–^{31}P coupling and the ^{13}C(3)–^{31}P coupling were very similar to those given in Table 2 and did not differ by more than 0.04 in any instance.

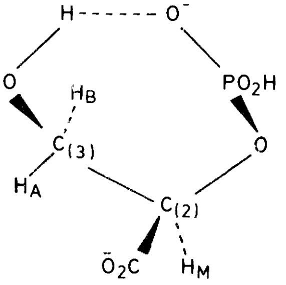
DISCUSSION

The rotamer populations in 2PPG and 3PPG may be influenced by several factors. First the combination of steric, dipolar, and electrostatic repulsion, this last being more important at higher pH values. This might be expected to destabilise C(2)–C(3) rotamers (Ib) (2PPG) and (IIb) (3PPG) since here the polar groups form a *syn*-arrangement. However, other factors must influence the rotamer populations since (Ib) and (IIb) contribute significantly (Table 2) at all pH values. An important factor to consider is intramolecular hydrogen bonding affecting rotamer populations, for which several schemes are possible. It is possible to form a five-membered hydrogen-bonded ring between the hydroxy-proton and the phosphate oxygen (directly bonded to carbon) which would stabilise rotamers (Ia and b) for 2PPG and (IIa and b) for 3PPG. At low pH (below *ca.* 3.6) a six-membered-ring hydrogen bond between the carboxy-proton and hydroxy-oxygen (for 2PPG) or the phosphate oxygen at C(3) (for 3PPG) would stabilise (Ib, c) and (IIb, c) respectively. At higher pH values for 2PPG, a six-membered hydrogen-bonded ring is also possible between a carboxylate oxygen and hydroxy-proton in rotamers (Ib and c).

The five- and six-membered hydrogen-bonding schemes would affect the conformations about the C(2)–C(3) bond directly. Additionally it is possible to form a seven-membered-ring hydrogen bond between the hydroxy-proton and a terminal phosphate oxygen which would favour rotamers (Ia and b) (2PPG) and (IIa and b) (3PPG) when in combination with suitable rotamers about the C–O (phosphate) bond.

Inspection of molecular models indicates that the five-, six-, and seven-membered hydrogen bonds are all reasonable with the observation that with the seven-membered ring the grouping –O–H · · · O– (phosphate) is almost linear which could make this a particularly favourable arrangement. Such seven-membered hydrogen-bonded ring structures have been postulated in the solution conformation of ethanolamine phosphate [9] and phosphoserine [6] at high pH.

Overall Conformation of 2PPG.—In view of the possibility of rationalising all rotamer populations about the individual C(2)–C(3) and C–O bonds of 2PPG and 3PPG in terms of steric, dipolar, or electrostatic repulsion and/or various combinations of the intramolecular hydrogen bonding schemes, it is desirable to look at the overall conformation of the molecule. However for 2PPG there are no experimental data to correlate the rotamer populations about C(2)–C(3) with those about C(2)–O. For example rotamers (Ib and d) are significantly populated at pH 5.0 (52 and 25%, respectively). The rotamer combination of (Ib and d) at pH 5.0 allows the formation of a seven-membered hydrogen-bonded ring.



(Ib and d) at pH 5.0

This combination might contribute as much as 25% to the overall conformation at this pH if all rotamer (Id) is associated with (Id) and *not* with (Ie and f). But this combination could contribute 0% if all rotamer (Id) were distributed between (Ie and f). On this basis it is only possible to consider *probable* overall conformations which are simply the product of the individual rotamer populations about the C(2)–C(3) and C(2)–O bonds. The probabilities for these overall conformations are shown in Table 4. At pH 5.0 there are five combinations with a probability of ⩾10% and at pH 10.8 there are four such combinations. However as pointed out above, there is no experimental evidence to correlate the overall conformation of 2PPG and we will not speculate further on this.

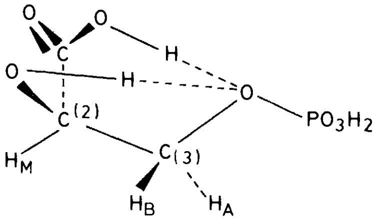
Overall Conformation of 3PPG.—The probabilities for the various rotamer combinations at pH 0.6 and 9.8 are shown in Table 4. These indicate that two combinations [(IIa, b) and (IIb, d)] predominate. At pH 0.6 the probability of combination (IIb, d) is 45%, and this may be stabilised by the six-membered hydrogen-bonded ring involving the carboxyl proton and the five-membered ring involving the hydroxy-proton.

TABLE 4

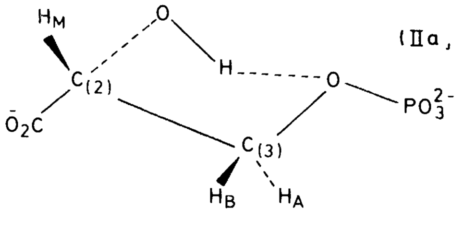
Probable contributions of the rotamer combinations to the overall conformation of 2PPG and 3PPG

Rotamer combination	2PPG pH 0.5	pH 10.8
(Ia, d)	0.09	0.10
(Ia, c)	0.10_4	0.25
(Ia, f)	0.17	0.07
(Ib, d)	0.13	0.11
(Ib, e)	0.15	0.28
(Ib, f)	0.24	0.08
(Ic, d)	0.03	0.03
(Ic, e)	0.03	0.07
(Ic, f)	0.06	0.02
	3PPG pH 0.6	pH 9.8
(IIa, d)	0.19	0.37
(IIa, e)	0.04	0.07
(IIa, f)	0.03	0.07
(IIb, d)	0.45	0.30
(IIb, e)	0.10	0.06
(IIb, f)	0.07	0.05
(IIc, d)	0.09	0.06
(IIc, e)	0.02	0.01
(IIc, f)	0.01	0.01

At higher pH, the contribution of (IIb, d) probably decreases (to 30%) while (IIa, d) becomes more significant (37%). In this conformation the ionised carboxylate and phosphate groups will have a minimum electrostatic interaction while the possible five-membered hydrogen-bonded ring is retained. For the case of 3PPG there is one piece of experimental evidence to support a significant contribution from rotamer (IIb, d) at low



(IIb, d) at pH 0.6



(IIa, d) at pH 9.8

pH, which decreases at high pH. This is the observation of a four-bond coupling (Table 1) $^4J_{\mathrm{H_MP}}$ which has the value 1.7_7 Hz at pH 0.6, decreasing to 0.5_7 Hz at pH 9.8. The rotamer combination (IIb, d) has $\mathrm{H_M}$ and P related by a planar *W* path and it is generally considered [6,10] that values for $^4J_{\mathrm{HP}}$ (*ca.* 2 Hz) are a maximum for the *trans*-anticoplanar arrangement of the four bonds involved. As the contribution of (IIb, d) decreases, then the magnitude of $^4J_{\mathrm{H_MP}}$ should decrease, as is observed.

Conclusions.—The analysis for the rotamer populations about the C(2)–C(3) and C(2)–O bonds of 2PPG at pH 5.0 and 10.8 indicates that 4 or 5 possible conformations for the molecule may be significantly populated ($\geqslant 10\%$). However in the absence of experimental evidence to correlate the rotamer populations about the individual bonds it is not possible to speculate on the various factors which may influence the conformational stability.

In the case of 3PPG there is evidence ($^4J_{\mathrm{H_MP}}$) to correlate the rotamers about the C(2)–C(3) and C(3)–O bonds and two conformations [(IIa, d) and (IIb, d)] predominate in the pH range 0.6–9.8.

The information derived here on the conformational preferences about the C(2)–C(3) and C–$\mathrm{OPO_3}$ bonds for 2PPG and 3PPG is not sufficient to substantiate the earlier proposal [1] that internal mobility in 2PPG is more restricted than in 3PPG. While a smaller number of combination rotamers are significantly populated ($\geqslant 10\%$) for 3PPG than for 2PPG, no information is available on other bond rotations within the molecule [*e.g.* C(1)–C(2); C–OH *etc.*] and therefore it is not possible to even speculate about entropy differences between 2PPG and 3PPG arising from restrictions on internal mobility. A more promising approach to the problem would appear to be the study of any conformational changes occurring on binding 2PPG and 3PPG to the enzyme phosphoglyceromutase, through the use of ^{1}H spin-echo n.m.r.

EXPERIMENTAL

2PPG (Boehringer) and 3PPG (Sigma) were dissolved in $\mathrm{D_2O}$ (99.8%; Goss Scientific Instruments) to give 0.5M solutions. The pH of the samples was adjusted by the addition of DCl or NaOD solutions. The pH values were measured using a Corning-Eel model 7 meter. The pH values quoted in the text are the meter reading and are uncorrected.

^{1}H N.m.r. spectra (5 mm o.d. sample tubes) were obtained in the continuous-wave mode at 100 and 220 MHz (Varian HA-100 and HR-220 instruments) and employing the pulse-Fourier-transform technique for spectra at 270 and 360 MHz (Bruker WH-270 and WH-360 instruments). ^{13}C N.m.r. spectra (10 mm o.d. sample tubes) were obtained in the pulse-Fourier-transform mode at 50.2 and 90.5 MHz (Bruker WP-200 and WH-360 instruments).

For many spectra, particularly both ^{1}H and ^{13}C with pH > 4, the resonances were quite broad and in these cases better resolution was obtained after treatment of the sample with a chelating resin (Chelex-100; Bio-Rad Laboratories) according to the procedure of Irving and Lapidot [11] to remove trace paramagnetic impurities. In those ^{1}H and ^{13}C spectra, obtained with the WP-200 and WH-360 instruments the convolution-difference technique [12] was employed to improve the resolution of closely spaced lines. In addition all ^{1}H spectra obtained by the pulse-Fourier-transform method employed the pre-irradiation solvent (*H*OD) suppression technique.[13] ^{1}H–(^{31}P) Experiments were performed with the HA-100 instrument using a doubly tuned probe; the ^{31}P frequency (40.5 MHz) was generated with a Schomandl ND 100-M synthesiser.

Spectral analyses for coupling constants and chemical shifts were made *via* iterative computer simulation of the spectra using the LAOCOON III program [14] with the ICL 1900 series computer at Queen Mary College and a more compact version of the program,* ITERCAL VIB, with a Bruker BNC-12 computer. Analyses of ^{1}H spectra run at 100, 220, 270, and 360 MHz were in excellent agreement; however, only the data from analysis of the 360-MHz ^{1}H spectra are included in the text.

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