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α- and β-Glycopyranosyl Phosphates and 1,2-Phosphates. Assignments of Conformations in Solution by ^{13}C and ^{1}H NMR†

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ABSTRACT: The ^{1}H and ^{13}C NMR parameters of the anomeric pairs of aldopyranosyl phosphates and their rigid 1,2-phosphate derivatives are reported. The derivatives of D-glucose, D-galactose, and D-mannose exist in the $^{4}C_{1}$ conformation while the L-fuco derivatives are in the $^{1}C_{4}$ conformation. As judged by ^{31}P–^{1}H and ^{31}P–^{13}C coupling constants, all of the α anomers of the aldopyranosyl phosphates have the phosphate moiety predominantly trans to C(2) while in the β anomers other rotamers make significant contributions. This relationship remains the same for the biologically important nucleoside diphosphate sugars (UDPGlc, UDPGal, GDPMan, and GDPFuc). From the pH dependence of ^{13}C chemical shifts, observed in 0.5 M solutions, the pK'_{a2} of the α anomers is 6.1 while the pK'_{a2} of the β anomers is 0.6–0.8 pH unit lower. In the 1,2-phosphates, the chair conformation of the parent aldose is retained while an envelope conformation is formed by the cyclic phosphate. In the α anomers, the plane is formed between C(2), C(1), O(1), and P while O(2) is above the plane. In the β anomers, O(1) is out of the plane formed by the other atoms. The β anomers have phosphorus coupled to C(3) with coupling constants of 10.8–11.7 Hz, approximately 2 Hz greater than the maximum reported for trans coupling (Lapper, R. D., & Smith, I. C. P. (1973) *J. Am. Chem. Soc. 95*, 2880).

Glycosyl phosphates are precursors or components of nucleoside diphosphate sugars, glycoproteins, glycolipids, and complex carbohydrates. Their involvement in biological processes has made the elucidation of their structures, reactivities, and conformations in aqueous solution relevant to studies of structure–function relationships in many biochemical systems.

Few studies of glycosyl phosphate conformations have been reported. Sarma et al. (1973) and Lee & Sarma (1976) have examined the conformation of several glycosyl phosphates and nucleoside diphosphate sugars by ^{1}H NMR and have shown that in the most abundant conformer the phosphate group is positioned trans to C(2) of the pyranosyl ring or bisects the angle between H(1) and C(2). Bundle et al. (1973) examined α- and β-2-acetamido-2-deoxy-D-glycopyranosyl phosphates and polymers derived from them by ^{13}C NMR and concluded that the phosphorus atom was trans to C(2) in all cases. It appears that, in these systems, both homonuclear and heteronuclear vicinal couplings follow a Karplus type of angular dependence, which allows certain conclusions to be drawn with respect to conformation although the exact relationships of coupling to dihedral angle have not been established.

The present study was undertaken to examine the effects of configurational changes, anomeric form, and pH on the NMR parameters of the commonly occurring α-glycosyl phosphates of D-glucose, D-mannose, D-galactose, and L-fucose. In addition, the less common β anomers and the 1,2-phos-

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phates, in which the number of available conformations is limited, have been examined. The data are interpreted in terms of conformational preferences in aqueous solution and of the effects of structural or environmental factors on NMR parameters. Preliminary accounts of this study have been presented elsewhere (Nunez, 1977; Nunez et al., 1977).

Materials and Methods

The α-D-gluco- and -galactopyranosyl phosphates were purchased from Sigma. α-D-Mannopyranosyl phosphate was synthesized by the method of MacDonald (1962). The α- and β-fucopyranosyl phosphates and the rest of the β anomers were synthesized by an adaptation of the methods of Prihar & Behrman (1973) and Kochetkov (Shibaev et al., 1974, 1976). Details of the procedure will be published elsewhere (O'Connor & Barker, submitted for publication). The 1,2-phosphates were prepared by the method of Piras (1963).

The ^{13}C NMR spectra were obtained using a Bruker WP-60 spectrometer equipped for Fourier transform operation, using quadrature detection and operating at 15.08 MHz with a sweep width of 3000 Hz and 4096 data points. The resonances are reported in parts per million relative to tetramethylsilane with the instrument standarized to give the literature value for C(1) of β-D-glucopyranose of 97.4 ppm (Walker et al., 1976). Chemical shifts were assigned in comparison to shifts in model compounds and in some cases on the presence of coupling to phosphorus.

^1H NMR spectra were obtained on a Bruker WP-180 spectrometer equipped for Fourier transform operation using an 1800 Hz sweep width and 16K data points. Chemical shifts are reported in parts per million downfield from sodium 3-(trimethylsilyl)-1-propanesulfonate. Where possible, chemical shifts of ^1H were assigned by first-order analysis. In complex spectra, chemical shifts and coupling constants were obtained by comparison of the experimental data with the theoretical spectra generated by the ITRCAL program available from the Nicolet Computer Co., Madison, WI. This program, which is a modification of LAOCN3 (Castellano & Bothner-By, 1964) for a 12K Nicolet 1080 computer system, allows the calculation of NMR spectra first by entering reasonable estimates of the chemical shifts and coupling constants for the nuclei in question and then by entering the actual frequency of the lines in the experimental spectra. By changing either the chemical shifts or the coupling constants or both, iteration to a best fit of the theoretical with the real spectrum can be obtained.

pH measurements were made on a Corning pH meter and determinations in D_2O were corrected using the equation pH = pD − 0.4 (Lumry et al., 1951). Ionization constants of the sugar phosphates were determined from plots of log $[(\delta_{HB} - \delta)/(\delta - \delta_B)]$ vs. pH, where δ_{HB} is the chemical shift of the protonated species, δ_B is the chemical shift of the unprotonated species, and δ is the shift at the experimental pH value (James, 1975).

Results and Discussion

The ^1H and ^{13}C NMR parameters of the glycopyranosyl phosphates are listed in Tables I, II, and III. Where no data are given, the spectra were too complex or resolution was insufficient to allow an assignment.

Assignment of ^1H Parameters. Figure 1A is the proton spectrum of α-L-fucopyranosyl phosphate and Figure 1B is the spectrum of α-L-fucopyranosyl 1,2-phosphate. These spectra illustrate that a unique fit of the experimental data can be achieved by evaluating coupling patterns on the basis of the expected $^1C_4$ conformation (Coxon, 1972). In all the proton spectra, H(1) produces the furthest downfield resonance. As shown in Figure 1B, the derivatization of C(2) by the 1,2-phosphate ring shifts H(2) downfield by 0.6 ppm with small changes in the rest of the spectrum. In spectra that are too complex to be resolved by first-order analysis, computer fitting of the experimental spectrum was employed. Figure 2A is the ^1H spectrum of β-D-galactopyranosyl phosphate at pH 4. Figure 2B is the theoretical spectrum generated from estimates of chemical shifts and coupling constants, followed by iteration to obtain the best fit. Assignments obtained in this manner give chemical shifts accurate to ±0.005 ppm and coupling constants accurate to ±0.1 Hz.

Table I: ^1H NMR Chemical Shifts[a] for Glycopyranosyl Phosphates and 1,2-Pyranosyl Phosphates

compd	pH	H(1)	H(2)	H(3)	H(4)	H(5)	H(6)	H(6′)
Glycopyranosyl Phosphates								
α-L-Fuc	4.1	5.435	3.721	3.932	3.841	4.270	1.200	
	8.8	5.414	3.693	3.913	3.803	4.264	1.194	
β-L-Fuc	4.4	4.865	3.548	3.716	3.784	3.843	1.282	
	7.5	4.845	3.526	3.700	3.752	3.827	1.262	
α-D-Gal	4.0	5.521	3.810	3.905	4.017	4.177	3.754	3.717
	8.0	5.479	3.740	3.902	3.982	4.182	3.725	3.702
β-D-Gal	4.4	4.903	3.594	3.745	3.966	3.799	3.845	3.787
	8.8	4.859	3.559	3.713	3.922	3.730	3.810	3.730
α-D-Glu	4.0	5.484	3.541	3.754	3.449	3.839	3.783	3.840
	8.0	5.442	3.465	3.775	3.378	3.920	3.872	3.725
β-D-Glu	4.2	4.925	3.337	3.547	3.337	3.525	3.934	3.707
	8.3	4.888	3.298	3.531	3.322	3.520	3.908	3.662
α-D-Man	4.0	5.404	3.959	3.896	3.659	3.810	3.878	3.762
	8.0	5.315	3.945	3.935	3.579	3.879	3.887	3.704
β-D-Man	4.5	5.159	4.021	3.715	3.551	3.442	3.938	3.715
	7.8	5.095	4.020	3.708	3.531	3.436	3.938	3.701
1,2-Pyranosyl Phosphates								
α-L-Fuc	8.0	5.800	4.343	4.06	3.826	4.166	1.26	
β-L-Fuc	8.0	4.936					1.32	
α-D-Gal	8.0	5.866	4.392	4.073	4.017	4.069	3.834	3.800
β-D-Gal	8.0	4.958						
α-D-Glu	8.0	5.779	4.309	3.950	3.492	3.805	3.821	3.764
β-D-Glu	8.0	5.037	3.900	3.729	3.523	3.680	3.940	3.796
β-D-Man	8.0	5.541	4.635	3.834	3.749	3.438	3.835	3.801

[a] The chemical shifts are expressed in parts per million from internal sodium 3-(trimethylsilyl)-1-propanesulfonate and are reported with four significant figures for the purpose of computer simulating spectra.

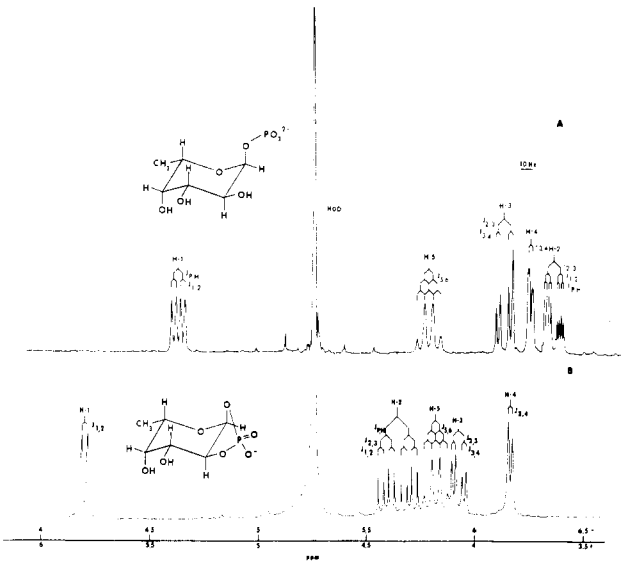

FIGURE 1: The 180-MHz Fourier transform ^1H NMR spectra of 0.1 M solutions of (A) α-L-fucopyranosyl phosphate (sodium salt) and (B) the 1,2-phosphate (sodium salt) in D_2O, pD 8.0, at 40 °C. Resonances are reported in parts per million from internal sodium 3-(trimethylsilyl)-1-propanesulfonate.

Table II: ^1H-^1H and ^{31}P-^1H Coupling Constants[a] for Glycopyranosyl Phosphates and 1,2-Pyranosyl Phosphates

compd	pH	J_{1-2}	J_{2-3}	J_{3-4}	J_{4-5}	J_{5-6}	$J_{5-6'}$	$J_{6-6'}$	J_{P-1}	J_{P-2}
Glycopyranosyl Phosphates										
α-L-Fuc	4.1	3.7	10	3.2	1.0	6.7			7.4	2.6
	8.8	3.7	10.2	3.3	0.9	6.8			7.0	1.8
β-L-Fuc	4.4	7.7	9.8	3.3	1.1	6.6			7.7	0
	7.5	7.5	9.3	3.3	1.1	6.6			7.5	0
α-D-Gal	4.0	3.3	10.2	3.1	1.0	7.0	6.0	−12.0	7.0	3.3
	8.0	3.3	9.9	3.3	1.1	6.2	6.0	−12.0	8.2	1.4
β-D-Gal	4.4	7.7	10	3.3	0.8	2.0	2.2	−11.2	7.7	0
	8.8	7.5	9.2	3.3	0.8	2.0	2.2	−11.7	7.5	0
α-D-Glu	4.0	3.5	9.9	9.7	9.8	4.9	2	−12.6	7	2.6
	8.0	3.4	9.7	9.4	9.1	2.5	5.7	−12.4	7.5	1.8
β-D-Glu	4.2	7.8	8.8	8.8	8.9	2.2	6.3	−12.3	7.8	0
	8.3	7.7	8.8	8.8	8.9	2.2	6.7	−12.1	7.7	0
α-D-Man	4.0	2.1	3.3	9.7	9.8	1.2	6.0	−12.3	7.7	0
	8.0	1.8	3.5	9.7	9.7	1.6	6.6	−12.3	8.8	0
β-D-Man	4.4	0.8	3.1	9.4	9.4	2.2	6.4	−12.2	8.4	0
	7.8	0.8	2.8	9.1	9.1	1.5	5.3	−11.4	8.8	0
1,2-Pyranosyl Phosphates										
α-L-Fuc	8.0	4.8	8.6	3.0	0.8	6.5			0	19.2
β-L-Fuc	8.0	4.4[b]				6.5			3.3[b]	
α-D-Gal	8.0	4.9	8.4	3.4	0.1	5.0	2.0	−12.2	0	19.2
β-D-Gal	8.0	4.7							1.2	
α-D-Glu	8.0	4.9	7.5	8.8	9.0	2.0	5.5	−12	2.2	17.2
β-D-Glu	8.0	7.5	10.2	6.6	8.1	2.0	4.6	−11.2	0	1
β-D-Man	8.0	2.1	2.3	9.3	9.5	1.6	6.6	−12.2	25.1	0

[a] The coupling constants are accurate to ±0.1 Hz. [b] These assignments may be reversed.

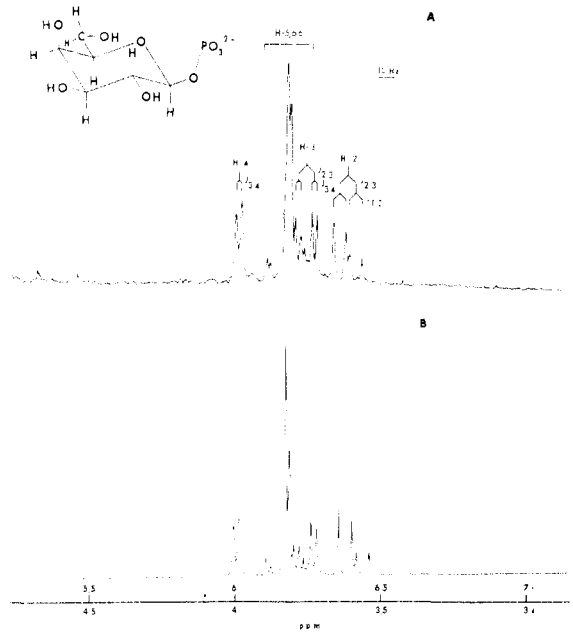

FIGURE 2: (A) The 180-MHz Fourier transform ^1H NMR spectrum of a 0.1 M solution of β-D-galactopyranosyl phosphate in D_2O, pD 4.4, at 40 °C. Resonances are reported in parts per million from internal sodium 3-(trimethylsilyl)-1-propanesulfonate. (B) The spectrum generated using the ITRCAL program and the chemical shifts and coupling constants listed in Tables I and II. The peaks corresponding to H(1) are not shown.

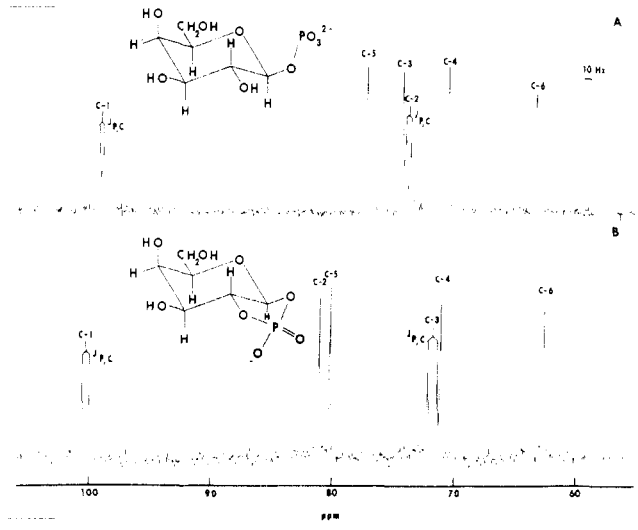

FIGURE 3: The 15.08-MHz Fourier transform ^{13}C NMR spectra of (A) β-D-galactopyranosyl phosphate (sodium salt, 0.5 M in water, pH 8.0) and (B) the 1,2-phosphate derivative (sodium salt, 0.5 M in water at pH 8). The spectra (1024 transients) are proton decoupled and are reported in parts per million downfield from external Me_4Si.

Assignment of ^{13}C Parameters. In all of the noncyclic glycosyl phosphates C(1) and C(2) are coupled to P. In some of the 1,2-phosphates C(3) is also coupled and appears as a doublet. These patterns of coupling are shown in the ^{13}C spectrum of β-D-galactopyranosyl phosphate at pH 8 (Figure 3), in which C(1) is the furthest downfield and is coupled to P with $^2J_{P-C(1)}$ = 2.9 Hz. The C(2) resonance shows $^3J_{P-C(2)}$ = 6.6 Hz. In this, as in all of the spectra, C(6) produces the resonance furthest upfield. In Figure 3B, the spectrum of the 1,2-phosphate is shown. Here, C(1) is shifted slightly downfield, whereas C(2) is shifted dramatically downfield (7.2 ppm) but shows no coupling to P. A large downfield shift of the derivatized carbon is commonly observed on formation of a phosphate ester. The absence of coupling, which is unexpected, is discussed below. In the 1,2-phosphate, C(3) is shifted upfield and is coupled to P and can be assigned on this basis.

The remaining resonances, due to C(4) and C(5), cannot be assigned with certainty. Assignments given in Table III were made on the basis that the small shifts that occur when the glycosyl phosphate is formed from the parent aldose or when the 1,2-phosphate is formed from the glycosyl phosphate fit a common pattern for all of the compounds examined. An excellent correlation of these trends is apparent in the data presented in Table IV. For most compounds C(3) as well as C(4) and C(5) is assigned on the basis of the pattern of chemical-shift changes that occur on substitution. Although the data in Table IV demonstrate that these patterns of change are highly reproducible, these assignments in Table III should

Table III: ^{13}C Chemical Shifts[a] and ^{31}P-^{13}C Coupling Constants[b] of Glycopyranosyl Phosphates, 1,2-Pyranosyl Phosphates, and Pyranosyl Phosphate Moieties of Nucleoside Diphosphate Sugars

compd	pH	chemical shift (ppm)						coupling constants (Hz)		
		C(1)	C(2)	C(3)	C(4)	C(5)	C(6)	P–C(1)	P–C(2)	P–C(3)
Glycopyranosyl Phosphates										
α-L-Fuc	3.5	96.5	69.6	70.9	73.3	69.0	16.7	6.6	8.8	0
	8.7	95.2	70.4	71.4	73.5	68.3	17.0	5.1	6.6	0
β-L-Fuc	4.0	99.4	72.8	74.1	72.8	72.8	17.0	3.7	*c*	0
	7.4	98.9	73.6	74.4	72.9	72.6	17.1	4.4	4.4	0
α-D-Gal	4.5	96.5	69.7	70.7	70.7	73.1	62.7	6.2	8.8	0
	7.9	95.3	70.5	71.1	71.0	72.6	62.9	6.2	7.1	0
β-D-Gal	4.0	99.5	73.0	73.9	70.3	77.2	62.9	5.9	7.3	0
	8.1	99.0	73.6	74.1	70.7	77.1	63.0	2.9	6.6	0
α-D-Glu	4.4	96.3	72.9	74.3	70.9	74.1	61.9	7.1	8.0	0
	8.2	95.0	73.7	74.7	71.2	73.5	62.2	5.3	5.3	0
β-D-Glu	4.1	98.9	75.3	76.9	71.2	77.9	62.4	5.1	8.1	0
	7.7	98.6	76.0	77.2	71.5	77.8	62.8	3.7	6.6	0
α-D-Man	4.5	97.3	72.1	71.6	68.1	75.0	62.4	6.2	8.8	0
	8.5	96.5	72.6	71.7	68.6	74.5	62.8	4.4	6.2	0
β-D-Man	4.2	96.7	72.6	74.2	68.2	78.2	62.7	3.7	5.9	0
	7.9	96.3	72.9	74.3	68.3	78.0	62.8	3.0	5.1	0
1,2-Pyranosyl Phosphates										
α-L-Fuc	7.7	98.7	78.7	72.2	72.2	70.6	17	8.8	*d*	0
β-L-Fuc	7.7	100.4	80.8	71.7	74.5	76.1	16.5	8.8	0	11
α-D-Gal	7.7	98.7	79	72.1	69.6	74.7	62.5	8.8	2.7	0
β-D-Gal	7.7	100.5	80.9	71.5	70.9	80.0	62.2	8.8	0	11.7
α-D-Glu	7.7	98.3	80.4	75.4	69.2	75.1	61.8	8	*d*	0
β-D-Glu	7.7	99.6	81.7	74.4	71.9	80.4	61.8	9.5	0	11
β-D-Man	7.7	97.7	79.6	72.6	67.3	76.3	61.9	0	2.2	10.8
Pyranosyl Phosphate Moiety of Nucleoside Diphosphate Sugars										
GDP β-L-Fuc	7	99.8	72.9	74.1	72.9	72.9	16.9	3.8		0
UDP α-D-Gal	7	96.8	69.3			73.0	62.1	4.4	8	0
UDP α-D-Glu	7	97.0	73.0		70.9		62.1	6.2	8	0
GDP α-D-Man	7	97.2	71.7		67.9		62.2	5.8		0

[a] Chemical shifts are downfield from Me_4Si and are accurate to ±0.1 ppm. [b] J_{P-C} are ±0.7 Hz. [c] The overlapping chemical shifts of C(2), C(4), and C(5) did not allow assignment of the coupling constant. [d] $J_{P-C} < 0.7$ Hz.

Table IV: Change in ^{13}C Chemical Shift upon Substitution[a]

	compd	pyranose to pyranosyl phosphate at pH 7						pyranosyl phosphate to 1,2-pyranosyl phosphate at pH 7					
		C(1)	C(2)	C(3)	C(4)	C(5)	C(6)	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)
pH 4	α-L-Fuc	−2.7	+0.2	+0.1	+0.2	−1.2	+0.5	−2.2	−9.1	−1.3	+1.1	−1.6	−0.3
	α-D-Gal	−2.7	+0.3	+0.1	+0.2	−1.1	+0.1	−2.2	−9.3	−1.4	+1.1	−1.6	+0.2
	α-D-Glu	−2.7	+0.3	+0.2	+0.5	−1.1	+0.4	−2.0	−7.5	−1.1	+1.7	−1.0	+0.1
	α-D-Man	−1.8	+0.2	+0.3	+0.4	−1.1	+0.2						
	β-L-Fuc	−1.6	+0.6	+0.5	+0.3	−0.5	+0.2	−1.0	−8.0	+2.4	−1.7	−3.3	+0.5
	β-D-Gal	−1.5	+0.6	+0.5	+0.1	−0.6	−0.3	−1.0	−7.9	+2.4	−0.6	−2.8	+0.7
	β-D-Glu	−1.5	+0.6	+0.6	+0.1	−0.5	+0.1	−0.7	−6.4	+2.5	−0.7	−2.5	+0.6
	β-D-Man	−1.5	+0.2	+0.6	+0.1	−0.6	−0.1	−1.0	−7.1	+1.6	+0.9	+1.9	+0.6
pH 8	α-L-Fuc	−1.4	−0.6	−0.4	0	−0.5	+0.2	−3.5	−8.3	−0.8	+1.3	−2.3	0
	α-D-Gal	−1.5	−0.5	−0.3	−0.1	−0.6	−0.1	−3.5	−8.5	−1.0	+1.4	−2.1	+0.4
	α-D-Glu	−1.4	−0.5	−0.2	+0.2	−0.5	+0.1	−3.3	−6.7	−0.7	+2.0	−1.6	+0.4
	α-D-Man	−1.0	−0.3	+0.2	−0.1	−0.6	−0.2						
	β-L-Fuc	−1.1	−0.2	+0.2	+0.2	−0.3	+0.1	−1.5	−7.2	+2.7	−1.6	−3.5	+0.8
	β-D-Gal	−1.0	0	+0.3	−0.3	−0.5	−0.4	−1.5	−7.3	+2.6	−0.2	−2.9	+0.8
	β-D-Glu	−1.2	−0.1	+0.3	−0.2	−0.4	−0.3	−1.0	−5.7	+2.8	−0.4	−2.6	+0.1
	β-D-Man	−1.1	−0.1	+0.5	+0.1	−0.4	−0.2	−1.4	−6.8	+1.7	+1.0	+1.7	+0.9

[a] Negative sign means downfield, positive means upfield from the parent compound. Chemical shifts are accurate to ±0.1 ppm.

be regarded as tentative. Firm assignments must await preparation of appropriate isotopically enriched compounds.

Conformational Assignments. Values of ^3J coupling constants between ring protons [H(1)–H(2), H(2)–H(3), H(3)–H(4), and H(4)–H(5)] are similar to those reported for the parent aldopyranoses (Coxon, 1972), indicating that, in the D-glycopyranosyl phosphates, the $^4C_1$ conformation is retained and that the $^1C_4$ conformation is retained in the L-fucose derivatives (Table II).

All of the α anomers of the glycopyranosyl phosphates have $^3J_{P-H(1)}$ values between 7.0 and 8.8 Hz, indicating a similar spatial relationship between the phosphorus atom and H(1). Similarly all of the α anomers have $^3J_{P-C(2)}$ values between 5.3 and 8.8 Hz, indicating a similar spatial relationship between P and C(2). The three most stable rotamers about the C(1)–O(1) bond are shown in Figure 4. Each rotamer is expected to display a unique combination of coupling constants between P and H(1) (White & Verkade, 1970; Kainosho et al., 1969) and between P and C(2) (Lapper et al., 1973; Lapper & Smith, 1973; Bundle et al., 1973). The experimental data clearly support the assignment of rotamer III with C(2) trans to P as the most abundant form present in all cases. As pointed out by Lee & Sarma (1976), the value of $^3J_{P-H(1)}$ can vary from 0 Hz at 90° to 24 Hz at 180°, and the observed

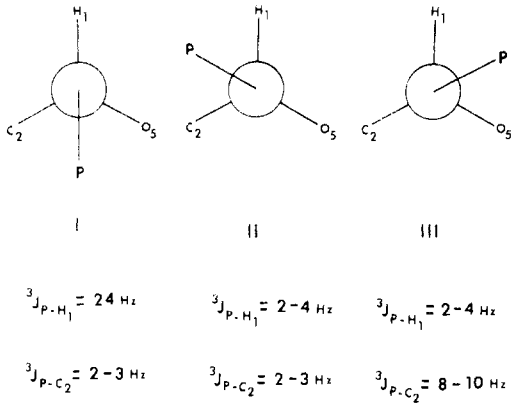

FIGURE 4: Newman projections showing rotamers around the C–O bond of glycosyl phosphates and associated values of $^3J_{P\text{-}C(2)}$ and $^3J_{P\text{-}H(1)}$.

value of 8 Hz does not allow selection between rotamers II and III. The assignment of a large population of rotamer III can be made, however, on the basis of the observed $^3J_{P\text{-}C(2)}$ = 8 Hz since this approaches the maximum value previously observed (Bundle et al., 1973; Lapper et al., 1973; Lapper & Smith, 1973). This conformational assignment is also supported by the observation of $^4J_{P\text{-}H(2)} \sim 2$ Hz for all of the α-pyranosyl phosphates having H(2) axial. Coupling through four bonds depends on the presence of a trans-antiplanar arrangement of the five atoms involved (Hall & Malcolm 1968, 1972). No $^4J_{P\text{-}H(2)}$ coupling is observed in α-D-mannopyranosyl phosphate in which H(2) is equatorial.

In the case of α-D-glucopyranosyl phosphate at pH 8.2 the value of $^3J_{P\text{-}C(2)}$ does not change over the temperature range 10–90 °C, indicating that this conformational preference is probably due to space-filling characteristics rather than hydrogen bonding or some other specific intramolecular interaction which would be disrupted at the higher temperature.

In the β-pyranosyl phosphates it is possible that appreciable amounts of rotamers other than that in which P is trans to C(2) are present. This conclusion is supported by the observation of $^3J_{P\text{-}C(2)}$ values ranging from 4.4 to 8.1 Hz combined with $^3J_{P\text{-}H(1)}$ values ranging from 7.5 to 8.8 Hz. The most probable rotamers in this case are the mirror images of rotamer II and III in Figure 4, and the observed values of $^3J_{P\text{-}C(2)}$ are the weighted averages of the contributions from the two rotamers. The values of $^3J_{P\text{-}H(1)}$ are not affected, since, in both rotamers II and III, P is gauche to H(1). Alternatively, it is possible that the smaller values of $^3J_{P\text{-}C(2)}$ are due to the presence of a slightly longer C(1)–O(1) bond in the β anomers as has been observed in the β-glycosides (Marchessault et al., 1977). If this is the case, however, the value of $^3J_{P\text{-}H(1)}$ should also be decreased. It is not, indicating that the observed decrease in $^3J_{P\text{-}C(2)}$ is most probably due to the presence of alternate rotamers.

The mole fractions of glycosyl phosphates that exist in the various rotamers can be calculated using values for both $^3J_{P\text{-}C(2)}$ and $^3J_{P\text{-}H(1)}$ since a unique solution can be obtained to equations relating proportions of rotamers to observed coupling constants. The appropriate equations are

$$^3J_{P\text{-}C(2)} = (P_I + P_{II})J_g + P_{III}J_t$$
$$^3J_{P\text{-}H(1)} = (P_{II} + P_{III})J_g + P_IJ_t$$

where P_I, P_{II}, and P_{III} are the mole fractions of rotamers I, II, and III and J_g and J_t are the coupling constants for gauche and trans conformations, respectively. We have assumed J_t = 22.9 Hz and J_g = 2.1 Hz for P–H(1) couplings and J_t = 14 Hz and J_g = 2.0 Hz for P–C(2) coupling. The mole fraction of rotamer I is computed from $^3J_{P\text{-}H(1)}$, that of rotamer III from $^3J_{P\text{-}C(2)}$, and that of rotamer II is obtained by difference.

Table V: Mole Fractions of Rotamers about the C(1)–O(1) Bond of Glycopyranosyl Phosphates[a]

compd	pH	I[b]	II[c]	III[c]
α-L-Fuc	4.1	25	19	56
	8.8	24	38	38
β-L-Fuc	4.4	27	*d*	*d*
	7.5	26	56	18
α-D-Gal	4.0	24	20	56
	8.0	25	33	42
β-D-Gal	4.4	27	29	44
	8.8	26	36	38
α-D-Glu	4.0	24	20	50
	8.0	25	48	27
β-D-Glu	4.2	27	22	51
	8.3	27	35	38
α-D-Man	4.0	28	16	56
	8.0	31	34	35
β-D-Man	4.4	32	36	32
	7.8	32	42	26

[a] See text for calculation. [b] These values are calculated from $^3J_{P\text{-}H}$ and are accurate to ±1%. [c] These values are calculated from $^3J_{P\text{-}C}$ and are accurate to ±7%. [d] Unable to calculate due to the overlapping resonance of C(2), C(4), and C(5).

The mole fractions of rotamers I, II, and III calculated in this fashion are listed in Table V. The accuracy of these calculations depends on the values assigned the trans and gauche coupling constants. Even if extreme values for $^3J_{P\text{-}H(1)}$ are assigned, however, it appears that rotamer I is present in significant amounts. In this rotamer the phosphate group lies below the ring bisecting the angle formed between C(2), C(1), and O(5). This result is surprising considering the bulk of the phosphate group, but it would be necessary to assume a value of $^3J_{P\text{-}H(1)}$ = 8 Hz in the gauche conformation to eliminate a significant contribution from rotamer I. Such a high value of J_g is unlikely in view of the present study and the reports of Lapper et al. (1973), Lapper & Smith (1973), and Lee & Sarma (1976) that indicate values of approximately 2 Hz.

These calculations also indicate that rotamer III is present in larger amounts in the α anomers than in the β anomers, except in the case of glucose. In addition, the amount of rotamer II is increased at higher pH values, possibly due to the repulsion of the dianion by the π orbitals of the ring oxygen. Assignment of values for $^3J_{P\text{-}C(2)}$ ranging from 8 to 14 Hz significantly alters the calculated contribution of rotamer III. Most observations of $^3J_{P\text{-}C}$(trans) fall in a small range around 8 Hz. In the present study we have observed a value of $^3J_{P\text{-}C} \sim 12$ Hz between P and C(3) in the β-glycopyranosyl 1,2-phosphates (Table III). In our view, the dihedral angle between P and C(3) in these compounds is approximately 160°, so that the 180° trans conformation may have $^3J_{P\text{-}C}$ = 14 Hz. When this value is used, rotamer III appears to contribute approximately 50% to the population. When $^3J_{P\text{-}C(2)}$ = 8 Hz is used, rotamer III would appear to be the sole contributor. This cannot be the case since the $^3J_{P\text{-}H(1)}$ values indicate that rotamer I contributes approximately 25% to the population. Thus the value of $^3J_{P\text{-}C}$ for a dihedral angle of 180° must be greater than 8 Hz (Lapper et al., 1973; Lapper & Smith, 1973); in fact, $^3J_{P\text{-}C}$ must be greater than 10.5 Hz to allow a real solution to the above equations if the contribution by rotamer I calculated from $^3J_{P\text{-}H(1)}$ is accepted.

It is possible that, in rotamer III, there is repulsion between the phosphate group and the ring oxygen which increases the

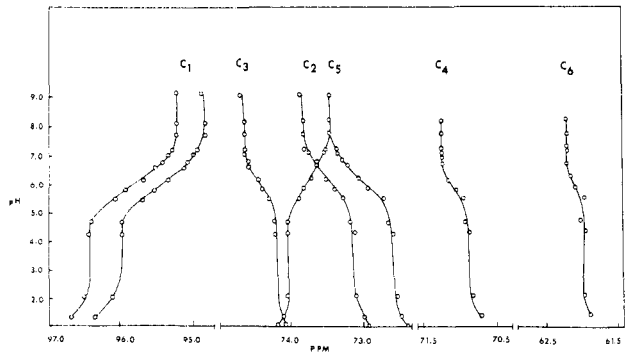

FIGURE 5: The effect of ionization of the phosphate group on the ^{13}C chemical shifts of α-D-glucopyranosyl phosphate in water. C(1) and C(2) are coupled to phosphorus, giving rise to two lines in each case.

Table VI: Ionization Constants of the Glycopyranosyl Phosphates[a]

compd	pK'_{a2}
α-L-Fuc	6.0
β-L-Fuc	5.4
α-D-Gal	6.2
β-D-Gal	5.4
α-D-Glu	6.1
β-D-Glu	5.3
α-D-Man	6.1
β-D-Man	5.3

[a] Computed from log $[(\delta_{HB} - \delta)/(\delta - \delta_B)]$ vs. pH. See text for further explanation.

C(1)–O–P bond angle and decreases the magnitude of $^3J_{P-C(2)}$. If this is the case a greater proportion of rotamer III is present than is predicted from the measured values of $^3J_{P-C(2)}$ discussed above. Additional evidence for the predominance of rotamer III at lower pH values is the increase in $^4J_{P-H(2)}$ from ~1.6 to ~3.0 Hz as the pH is decreased from 7.0 to 4.0 for α-L-fuco, α-D-gluco, and galacto isomers. The increased long-range coupling is probably due to an increased proportion of the extended trans-antiplanar arrangement between the coupled atoms as the pH is reduced.

Effects of Ionization. That the ^{13}C chemical shifts and coupling constants of the pyranosyl phosphates are sensitive to the state of ionization of the phosphate group is shown by the data of Table III. On the other hand, ^1H chemical shifts and couplings are unaffected. In all cases examined, the resonances of C(1) and C(5) move upfield while those of the remaining carbons move downfield as the pH is adjusted from 4 to 8. A typical titration is shown in Figure 5 for the carbons of α-D-glucopyranosyl phosphate and the values for pK'_{a2} of the glycosyl phosphates are listed in Table VI. These values are lower than others have reported (Behrman, 1974; Ashby et al., 1955; Cori et al. 1937), which may be due to the high concentration (0.5 M) used for the NMR titration experiment (Ashby et al., 1954, 1955).

The chemical-shift changes accompanying ionization of the phosphate group of the glycopyranosyl phosphates with formation of the dianion are difficult to rationalize. The phosphate dianion is probably less strongly electron withdrawing than the monoanion. Thus, it is reasonable to expect greater shielding, and upfield shifts, of C(1) and perhaps C(5) if the effect is readily transmitted through the C(1)–O–C(5) bond. These effects would be much smaller for protons since the chemical shifts of the ring protons range over 1.8 ppm while chemical shifts for ring carbons range over 40 ppm. Thus, a similar effect on protons would be barely discernible.

The phosphate dianion moiety is probably larger than the monoanion (Cozzone & Jardetzky, 1976) and may influence, through space, the chemical shifts of atoms close to it, increasing shielding and causing an upfield shift of the reasonances of the affected atoms [C(1) and C(5)]. This effect at C(5) should be greater in the α anomers than in the β anomers due to the closer proximity of the phosphate to C(5) in the former. This is the case in the gluco, manno, and fuco isomers in which the effect of ionization on the chemical shift at C(5) is two to four times greater in the α than in the β anomer. The pattern of ^{13}C chemical-shift changes at C(2), C(3), C(4), and C(6) accompanying ionization might reflect a relatively small change in the geometry of the pyranosyl ring. If the C–C bonds in the ring of the dianion are slightly longer (0.02 Å) than in the monoanion, chemical shifts might move downfield. The upfield movement of chemical shifts for C(1) and C(5) could then be explained on the basis of a strong inductive or through-space effect of the dianion relative to the monoanion.

The effect of pH on the ^{13}C chemical shifts in ethyl phosphate was also examined. As in the glycosyl phosphates the C(1) resonance shifts upfield from 63.4 to 61.7 ppm as the pH is raised from 4.0 to 8.4. Simultaneously, the resonance of C(2) moves downfield from 17.2 to 17.4 ppm. Chemical shifts and coupling of the methylene and methyl protons are affected only slightly. It appears that in this case, as with the pyranosyl phosphates, more than one effect is involved in producing the observed shifts.

1,2-Phosphates. Introduction of a fused-ring system in the cyclic 1,2-phosphates restricts conformational possibilities and permits some conclusions to be drawn concerning the values of coupling constants between H–P and C–P in these systems.

As shown in Table II and Figure 1 the conformation of the pyranosyl ring is not greatly affected by introduction of the 1,2-phosphate ring system in the α series. Although the H(1) and H(2) resonances are shifted downfield, $^3J_{H-H}$ couplings between ring hydrogens generally are only slightly altered. The α-1,2-phosphates of glucose, galactose, and fucose show an increase in $^3J_{H(1)-H(2)}$ from 3.5 ± 0.2 to approximately 4.8 Hz, and coupling between H(2) and H(3) is decreased from 9.9 ± 0.2 to values ranging from 7.5 to 8.6 Hz. These changes probably reflect a slight flattening of the pyranosyl ring with a decrease in the angles between H(1) and H(2) and between H(2) and H(3).

In the β anomers, the gluco and manno isomers show small changes in $^3J_{H-H}$ couplings, which are consistent with the flattening of the pyranosyl ring to accommodate the cyclic phosphate. However, both the galacto and fuco isomers are significantly affected by introduction of the 1,2-phosphate ring system. For both derivatives, $^3J_{H(1)-H(2)}$ changes from 7.5 Hz to approximately 4.5 Hz as the cyclic phosphate is formed (Table II). This change probably reflects a decrease in the H(1)–H(2) dihedral angle from approximately 160 to 140° (Coxon, 1972) and might indicate the pyranosyl ring has adopted a skew conformation. Unfortunately, the 180-MHz ^1H NMR spectra of these compounds do not permit the assignment of coupling constants between the other vicinal pairs of hydrogens, so that this possibility cannot be evaluated.

The conformation of the five-membered 1,2-phosphate ring can be deduced from consideration of the coupling of the P to C and H in the pyranosyl ring (Tables II and III). Three-bond coupling between ^{31}P and ^1H is known to show angular dependence with $^3J_{P-H}$ values ranging from approximately 0 Hz at 90° to 24 Hz at 180° (White & Verkade, 1970; Kainosho et al., 1969). In the α-1,2-phosphates of glucose, galactose, and fucose $^3J_{P-H(1)}$ = 0, 0, and 2.2 Hz, respectively. Values of $^3J_{P-H(2)}$ are 19.2, 19.2, and 17.2 Hz

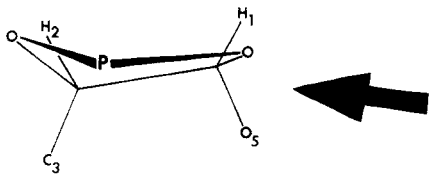

FIGURE 6: The proposed conformation of the cyclic 1,2-phosphate derivatives of the glycosyl phosphates. C(1), C(2), O(1), and P are approximately planar and O(2) is above the plane of the ring.

in the same series. These values indicate that the five-membered cyclic phosphate ring exists in a conformation similar to that proposed for methyl ethylene phosphate by Steitz & Lipscomb (1965) on the basis of X-ray diffraction data, that is, with dihedral angles of 11° between the C(2)–O(2)–P and the P–O(1)–C(1) plane normals and 2° between P–O(1)–C(1) and O(1)–C(1)–C(2) plane normals (Figure 6). The structure is close to that of an envelope with O(2) above the plane of the ring. In this conformer the dihedral angle between P and H(1) is approximately 90° and that between P and H(2) is approximately 160°, requiring $^3J_{P-H(1)} \simeq 0$ Hz and $^3J_{P-H(2)} \simeq 20$ Hz, in good agreement with the observed values.

The β-1,2-phosphates of glucose, galactose, and fucose have $^3J_{P-H(1)}$ values of 0, 1.2, and 3.3 (or 4.4) Hz, respectively. These couplings require a P–H(1) dihedral angle close to 90° as is present in a slightly deformed envelope with O(1) out of the plane of the other four atoms. The same dihedral angles can be achieved in several other conformations; however, $^3J_{P-H(2)}$ is not discernible in the spectra of these derivatives so that the assignment cannot be evaluated. The β-manno isomer has $^3J_{P-H(1)} = 25.2$ Hz, a value that requires a P–H(2) dihedral angle close to 180°. A suitable conformation is achieved with an envelope having O(1) below the plane of the other four atoms.

The interpretation of P–C couplings in the cyclic phosphate esters is complicated by the simultaneous coupling of carbons in the five-membered ring through two bonds and through three bonds to phosphorus. For example, C(1) is coupled to P through O(1), and $^2J_{P-C(1)}$ is expected to be approximately 5 ± 2 Hz; C(1) is also coupled to P through C(2) and O(2), and $^3J_{P-C(1)}$ is expected to vary between 0 and 12 ± 2 Hz depending on the dihedral angle. Although the signs of the $^2J_{P-C}$ and $^3J_{P-C}$ are not known, it is reasonable to assume that they will be of opposite sign since alternation for each additional bond is known to occur in other systems (Coxon, 1972). On this basis couplings between P and C(1) or C(2) will reflect the algebraic sum of the two- and three-bonded coupling constants.

The two-bonded coupling constants between carbon and phosphorus in glycosyl phosphate monoanions range from 6.2 to 7.1 in the α series and from 3.7 to 5.9 in the β series. These values may not be applicable to the 1,2-phosphate where the POC angle is probably reduced from 119 to 112° (Steitz & Lipscomb 1965). It is difficult to assign values to the relationships between dihedral angle and coupling constant for the $^3J_{P-C}$ case. Maximum values in the range of 10 Hz have been reported previously for $^3J_{P-C}$ where the coupled atoms are probably trans to the intermediate C–O bond (Lapper et al., 1973; Lapper & Smith, 1973; Bundle et al., 1973). In this study we have observed $^3J_{P-C}$ values of 10.8 and 11.7 Hz in β-1,2-phosphates between P and C(3). Although the dihedral angle in these cases may approach 180°, it is probably at least 10–15° lower. On this basis, $^3J_{P-C}$ values may range up to 14 Hz and be in the range of 12 Hz for dihedral angles close to 0 or 160°. When these ranges of coupling constants are used to evaluate P–C coupling in the 1,2-phosphates, an approximate fit to the experimental data can be obtained, which generally supports the conformations proposed on the basis of P–H coupling constants.

In the α-1,2-phosphates $J_{P-C(1)}$ varies from 8.0 to 8.8 Hz. These values can be explained if $^2J_{P-C(1)} \sim -5$ Hz and $^3J_{P-C(1)} \sim +12$ Hz, the latter value being associated with a dihedral angle of 15° for P vs. C(1) around the O(2)–C(2) bond. The sum of these two couplings is +7 Hz (observed values are 8.0–8.8 Hz).

When a similar analysis is applied to P–C(2) coupling in the α-1,2-phosphates, it becomes difficult to reconcile the P–H coupling constants with the P–C data. The observed values range from less than 1 to 2.7 Hz, which require that three-bond coupling almost exactly match two-bond coupling in magnitude and be of opposite sign. Thus, $^3J_{P-C(2)}$ should have a value between +3 and +7 Hz, and the associated dihedral angles should be between 60 and 30°. The proposed conformer has a dihedral angle close to 0° and would be expected to have $^3J_{P-C(2)} \simeq 12$ Hz. Since $^2J_{P-C}$ values are generally much smaller than 12 Hz, the residual coupling should be substantial (4–8 Hz) in contrast to the observed value.

The β-1,2-phosphates of glucose, galactose, and fucose have $J_{P-C(1)}$ values of 9.5, 8.8, and 8.8 Hz and $J_{P-C(2)}$ values of 0 Hz. The proposed conformation with C(1), C(2), O(2), and P approximately in a plane predicts $J_{P-C(1)}$ and $J_{P-C(2)}$ couplings of approximately 7 and 0 Hz, respectively, in good agreement with those observed.

It appears that P–C coupling constants in the α-1,2-phosphate ring system are unusual with respect to those observed in acyclic systems. Further studies will be required to determine whether coupling constants between P and C within the five-membered ring are of value as conformational probes. The results reported do demonstrate, however, that coupling between P and C(3), H(1), or H(2) are valuable in establishing conformations of the five-membered cyclic phosphate esters.

Nucleoside Diphosphate Sugars. The chemical shifts and coupling of atoms in the pyranosyl ring of the nucleoside diphosphate sugars are essentially the same as those in the corresponding pyranosyl phosphates (Tables I–III), indicating that in these compounds rotamer III is favored also. Furthermore, C(1) does not appear to be coupled to the phosphorus atom of the 5′-ribosyl phosphate group, indicating that the four intervening bonds may not lie in the same plane. This interpretation is made with caution, however, since the maximum value expected of $^4J_{P-C}$ would be small.

The presence of the second negative charge on the pyrophosphate group does not significantly affect the chemical shifts of atoms in the pyranosyl ring unlike the situation which occurs when a second negative charge is introduced into a pyranosyl phosphate. Thus, the NMR parameters of the pyranosyl moiety of nucleoside diphosphate sugars at pH 8 are essentially identical to those of α-pyranosyl phosphates at pH 4.0. The chemical shifts of the latter are strongly affected by the titration of the phosphate monoanion as discussed above. The nucleoside diphosphate sugars reported here (except for GDP fucose) were examined by Sarma et al. (Lee & Sarma, 1976) by ^{1}H NMR. The conclusions they drew are confirmed by this study and extended by the conclusions made possible by ^{13}C NMR analysis.

In summary, we have presented evidence for the following. (a) The α- and β-hexopyranosyl phosphates have conformations in aqueous solutions similar to those of the parent aldopyranoses. (b) Chemical shifts of all ring carbons are

sensitive to the ionic state of the phosphate, whereas the chemical shifts of ring hydrogens are relatively unaffected. (c) The formation of a 1,2-phosphate ring generally has only minor effects on the conformation of the pyranosyl ring. (d) Coupling between P and C(2) suggests that, in α-glycopyranosyl phosphates, P is trans to C(2) in the predominant conformers. In the β anomers substantial amounts of other rotamers are present. (e) P–C(1) and P–C(2) coupling constants are decreased as the monoanion is converted to the dianion in both α- and β-pyranosyl phosphates. (f) P–H(1), P–H(2), and P–C(3) coupling constants are compatible with a conformation of the 1,2-phosphate ring in which O(2) is out of the plane of the other atoms. (g) Observed P–C(1) and P–C(2) couplings probably represent the algebraic sum of coupling through two and three bonds. The magnitude of the observed couplings in the cyclic systems cannot always be explained on the basis of values observed in acyclic systems. (h) In nucleoside diphospho sugars, conformations of the pyranosyl phosphate moiety are essentially identical with those in the pyranosyl phosphates at pH 4.0.

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