Neu, N. C., and Heppel, L. A. (1964), *J. Biol. Chem. 239*, 2927.
Ogur, M., and Small, J. D. (1960), *J. Biol. Chem. 235*, PC 60.
Rabinowitz, J. C., and Pricer, W. E., Jr. (1962), *J. Biol. Chem. 237*, 2898.
Rammler, D. H., and Rabinowitz, J. (1962), *Anal. Biochem. 4*, 116.
Schmidt, G. (1968), *Methods Enzymol. 12*, 230.
Steinschneider, A. (1971), *Biochemistry 10*, 173.
Tener, G. M. (1961), *J. Amer. Chem. Soc. 83*, 162.
Whitfeld, P. R. (1954), *Biochem. J. 58*, 390.
Whitfeld, P. R., and Markham, R. (1953), *Nature (London) 171*, 1151.

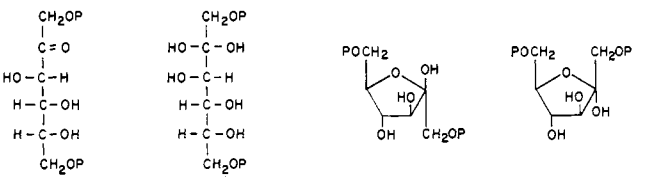
An Examination of D-Fructose 1,6-Diphosphate and Related Sugar Phosphates by Fourier Transform ^{31}P Nuclear Magnetic Resonance Spectroscopy*

Gary R. Gray

ABSTRACT: D-Fructose 1,6-diphosphate (FDP) and stereochemical analogs of its α- and β-furanose forms have been examined by ^{31}P nuclear magnetic resonance spectroscopy. The 1- and 6-phosphorus resonances of 2,5-anhydro-D-mannitol 1,6-diphosphate and methyl β-D-fructofuranoside 1,6-diphosphate, analogs of the β-furanose form of FDP, have very similar chemical shifts. Conversely, the 1-phosphorus resonances of 2,5-anhydro-D-glucitol 1,6-diphosphate and methyl α-D-fructofuranoside 1,6-diphosphate, analogs of the α-furanose form of FDP, are shifted downfield relative to the 6-phosphorus resonances. The ^{31}P nmr spectrum of FDP contains resonances ascribable to the 1- and 6-phosphate groups of both furanose forms, the β-furanose form being predominant (~90%). The assignment of these phosphorus resonances was confirmed with specifically deuterated reference compounds. In the ^{31}P nmr spectrum of D-fructose-6,6-d_2 1,6-diphosphate, the 1-phosphorus resonances of the α- and β-furanose forms remained as triplets, but the 6-phosphorus resonances collapsed to "singlets" since phosphorus–hydrogen coupling was lost. New evidence is presented confirming earlier work which demonstrated that the acyclic free keto and hydrated keto forms of FDP were not present in solution to an appreciable extent. These studies demonstrate that the four forms of FDP are present in the following percentages: β-furanose (~90), α-furanose (~10), keto (<1.7), and hydrated keto (<0.1).

Since in many reactions in which sugar phosphates participate the free aldehydo or free keto form is the actual substrate (Gracy and Noltmann, 1968; Dyson and Noltmann, 1968; Trentham *et al.*, 1969; Rutter, 1961), it is important to know the exact proportion of the reactive form in the tautomeric equilibrium. Those enzymes with substrates which can exist in multiple tautomeric forms in solution may be able to bind each but may be able to utilize only one in the catalytic reaction. Where only a single form is utilized, the other forms may either be converted to the reactive form by the enzyme (Dyson and Noltmann, 1968) or they may function as inhibitors, especially if the reactive form is present in a very low proportion.

Fructose 1,6-diphosphate aldolase, a good example of this kind of enzyme, catalyzes the conversion of D-fructose 1,6-diphosphate (FDP)[1] to 1,3-dihydroxy-2-propanone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (G-3-P). Each of these substrates may exist in solution in multiple forms. Solutions of G-3-P have been shown (Trentham *et al.*, 1969; Swenson and Barker, 1971) to contain a mixture of the aldehydo form (4%) and the hydrated aldehydo (gem-diol) form (96%); and DHAP exists as a mixture of the free keto (55%, 25°) and hydrated keto (45%) forms (Gray and Barker, 1970; Reynolds *et al.*, 1971). D-Fructose 1,6-diphosphate would be expected to exist in solution as a mixture of the keto **1**, hydrated keto **2**, β-furanose **3**, and α-furanose **4**



1 2 3 4

forms, but a considerable controversy has arisen regarding the relative proportion of each. In an examination of aqueous solutions by ultraviolet spectroscopy, McGilvery (1965) concluded that Na_2FDP existed predominately in form **1**, but Gray and Barker (1970), in a study of FDP and various acyclic 2-ketose phosphates in D_2O by ^{1}H nuclear magnetic resonance (nmr) spectroscopy and infrared spectroscopy, found that the keto **1** and hydrated keto **2** forms of FDP were not present in solution to an appreciable extent (<2%). In another study of 2-ketoses and 2-ketose phosphates by ultraviolet and circular

* From the Department of Biochemistry and the Laboratory of Chemical Biodynamics, Lawrence Radiation Laboratory, University of California, Berkeley, California 94720. *Received July 21, 1971.* Supported by USPHS Postdoctoral Fellowship No. 1 F02 AI42452-01 and by the U. S. Atomic Energy Commission. Address inquiries to the Department of Biochemistry, University of California, Berkeley, Calif. 94720.

[1] Abbreviations used are: FDP, D-fructose 1,6-diphosphate; DHAP, 1,3-dihydroxy-2-propanone phosphate; G-3-P, D-glyceraldehyde 3-phosphate.

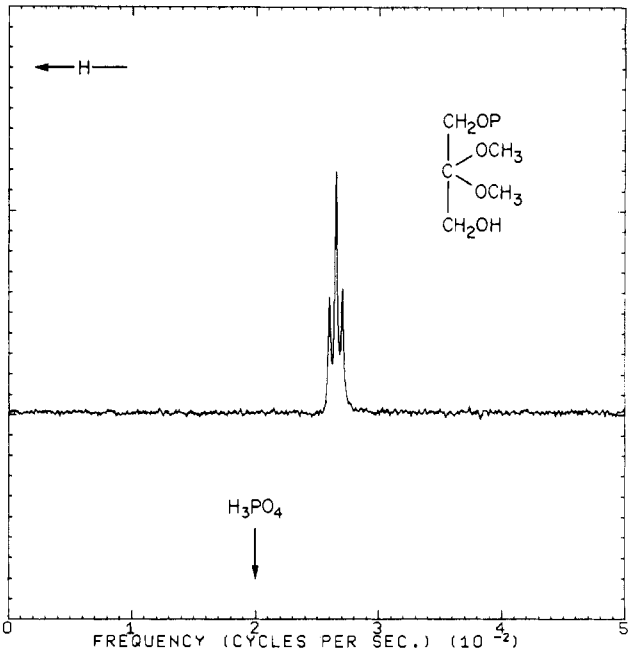


FIGURE 1: ^{31}P nuclear magnetic resonance spectrum of 1,3-dihydroxy-2-propanone phosphate dimethyl acetal (pH 6.5).

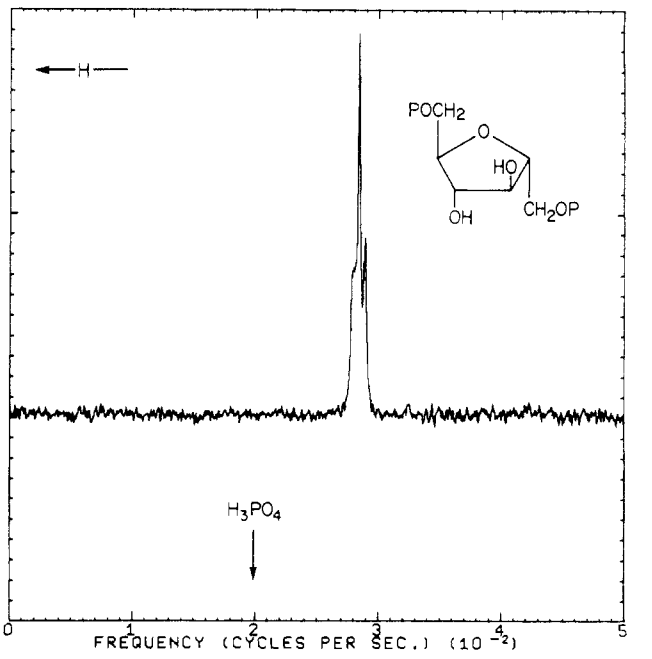
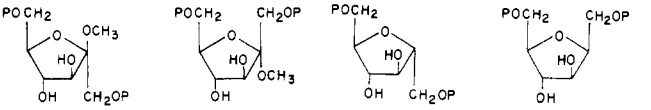


FIGURE 3: ^{31}P nuclear magnetic resonance spectrum of 2,5-anhydro-D-mannitol 1,6-diphosphate (**7**) (pH 7.0).

dichroic spectroscopy, Avigad *et al.*, (1970) concluded that solutions of FDP contain about 25% of the keto form.

This paper summarizes pertinent data regarding the structure of FDP in solution and will present evidence obtained from a study of FDP and related analogs by ^{31}P nmr spectroscopy which demonstrates that the β-furanose form **3** predominates in solution. The ability to distinguish between the two furanose forms of FDP in solution is based on the assignment of phosphorus resonances of structural analogs, and for this purpose, methyl β-D-fructofuranoside 1,6-diphosphate (**5**), methyl α-D-fructofuranoside 1,6-diphosphate (**6**), 2,5-anhydro-D-mannitol 1,6-diphosphate (**7**), and 2,5-anhydro-D-glucitol 1,6-diphosphate (**8**) have been examined. In addition to these analogs of the furanose forms of FDP, DHAP, and its dimethyl acetal, analogs of the acyclic forms of FDP have also been examined by ^{31}P nmr spectroscopy.



5 6 7 8

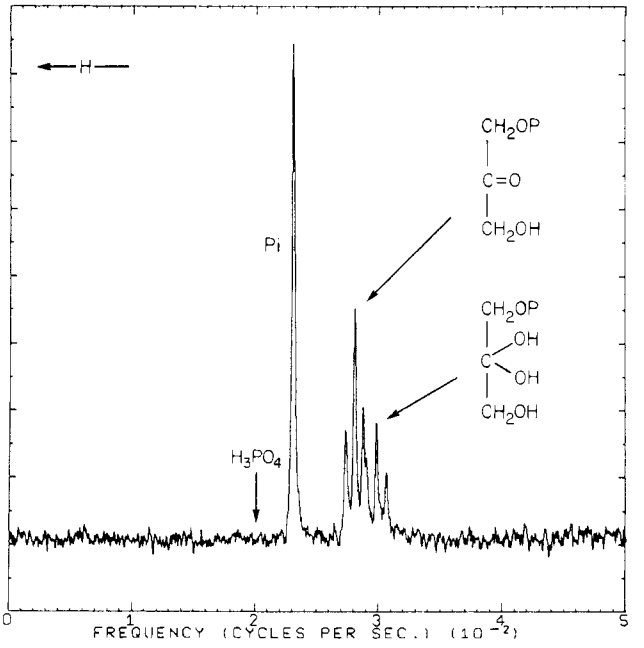


FIGURE 2: ^{31}P nuclear magnetic resonance spectrum of 1,3-dihydroxy-2-propanone phosphate (pH 6.5).

Results

A study of DHAP and its dimethyl acetal by ^{31}P nmr spectroscopy provides evidence for the presence of both the keto and hydrated keto forms in solution. In the ^{31}P nmr spectrum of the dimethyl acetal of DHAP (Figure 1), the phosphorus resonance occurs as a triplet, a result of coupling to the two hydrogen atoms at C-1 (J = 5.4 Hz). Hydrolysis of the acetal (Ballou and Fischer, 1956) produces an equilibrium mixture of two forms of DHAP in which the phosphate groups have different chemical shifts (Figure 2). The assignment of the phosphorus resonances of the two forms can be made by ^{1}H nmr spectroscopy (Gray and Barker, 1970) which indicates a 3:2 mixture of the keto and hydrated keto forms at 30°. In the ^{31}P nmr spectrum of DHAP, therefore, the phosphorus atom of the hydrated keto form occurs at a lower field. The reason for the chemical shift difference between the phosphorus resonances of the keto and hydrated keto forms is not known. An examination of DHAP by ^{13}C nmr spectroscopy (G. R. Gray and F. W. Dahlquist, 1971, unpublished data) has also demonstrated the presence of the keto and hydrated keto forms in a 3:2 ratio.

In the ^{31}P nmr spectrum of 2,5-anhydro-D-mannitol 1,6-diphosphate (7, Figure 3), a single triplet (J = 5.0 Hz) is ob-

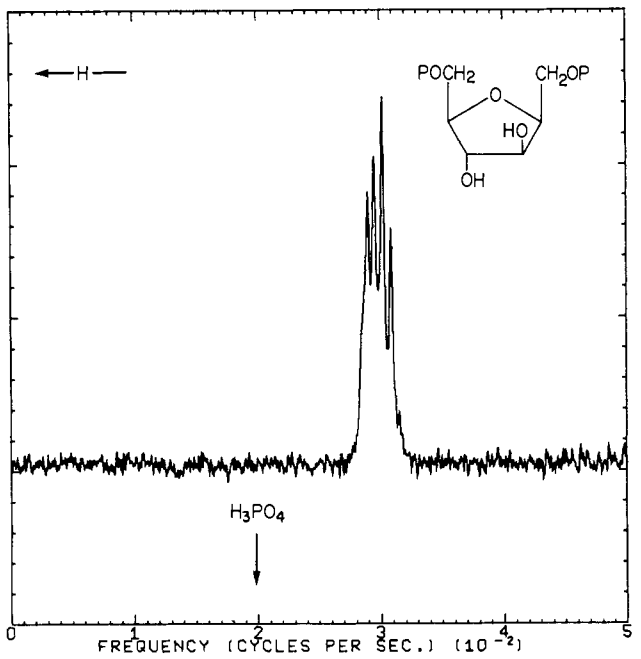


FIGURE 4: ^{31}P nuclear magnetic resonance spectrum of 2,5-anhydro-D-glucitol 1,6-diphosphate (**8**) (pH 7.6).

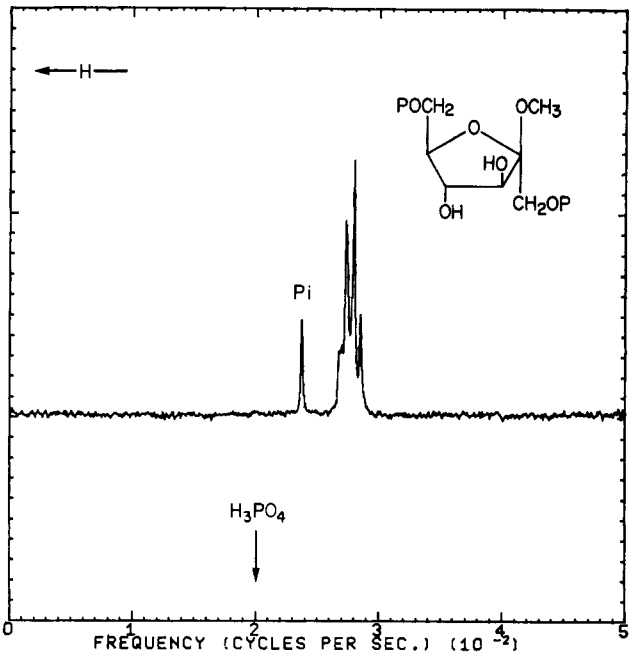


FIGURE 5: ^{31}P nuclear magnetic resonance spectrum of methyl β-D-fructofuranoside 1,6-diphosphate (**5**) (pH 7.0).

served because the two phosphorus atoms are stereochemically identical. This identity can be seen by rotation of the molecule about an axis bisecting the ring oxygen atom and the C-3,-C-4 bond. The spectrum of 2,5-anhydro-D-glucitol 1,6-diphosphate (**8**, Figure 4), however, is more complex. In this derivative the phosphorus atoms are stereochemically nonequivalent. One of the phosphorus resonances has the same chemical shift and apparently the same coupling constant as the phosphorus nuclei of **7**, but the other resonance is shifted approximately 0.5 ppm to lower field. In addition, the phosphorus resonance at lower field has a larger coupling constant to the methylene hydrogens (J = 6.8 Hz) than observed for the phosphorus nuclei of **7** (J = 5.0 Hz). From these results, the resonance at lower field in the spectrum of **8** can be assigned to the C-1 phosphate group, which differs from the phosphate group at C-6 only in that it is cis to the neighboring hydroxyl group.

Similar results were obtained by comparing the spectra of methyl β-D-fructofuranoside 1,6-diphosphate (**5**) and methyl α-D-fructofuranoside 1,6-diphosphate (**6**). The ^{31}P nmr spectrum of **5** (Figure 5) contains two overlapping triplets separated by 0.26 ppm. The triplet at higher field is poorly resolved and has a small coupling constant (J = 4.9 Hz) while the triplet at lower field has a slightly larger coupling constant (J = 5.5 Hz). The assignment of these resonances can be made by a comparison with the spectrum of **7**. Methyl β-D-fructofuranoside 1,6-diphosphate (**5**) differs from **7** only by the introduction of the methoxyl group at C-2, which results in a downfield shift of the 1-phosphorus resonance. The spectrum of **6** (Figure 6) contains two well-resolved phosphorus resonances. The resonance at higher field has a small coupling constant and a chemical shift identical with the resonance at higher field in the spectrum of **5** at the same pH. The other phosphorus resonance in the spectrum of **6** (assigned to the 1-phosphate) has a large coupling constant (J = 7.1 Hz) and is shifted 0.94 ppm downfield from the 6-phosphorus resonance.

The ^{31}P nmr spectrum of D-fructose 1,6-diphosphate was obtained at pH values of 7.45, 6.15, and 5.00. The spectrum at pH 7.45 was identical in appearance with the spectrum at pH 6.15, although at pH 7.45 the phosphorus resonances were shifted downfield as expected (Moedritzer, 1967). The ^{31}P nmr spectrum at pH 6.15 (Figure 7) contains resonances ascribable to both the β-furanose **3** and α-furanose **4** forms. A comparison of the ^{31}P nmr spectra of FDP and the other analogs (Table I and Figures 3–6) demonstrates conclusively that the β-furanose form **3** predominates in solution (~90%). The weak triplet at lower field can be assigned to the 1-phosphate of the α-furanose form **4**, both because of its chemical shift and J_{PH} value (Table I), and the two intense overlapping trip-

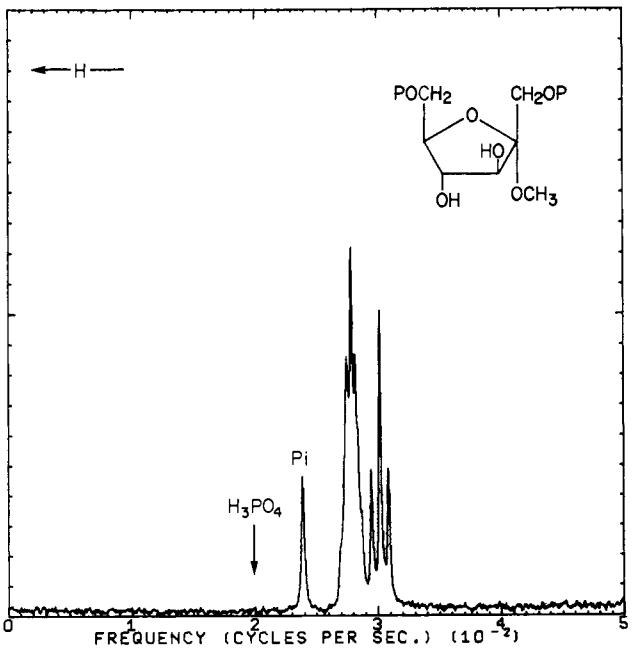


FIGURE 6: ^{31}P nuclear magnetic resonance spectrum of methyl α-D-fructofuranoside 1,6-diphosphate (**6**) (pH 7.0).

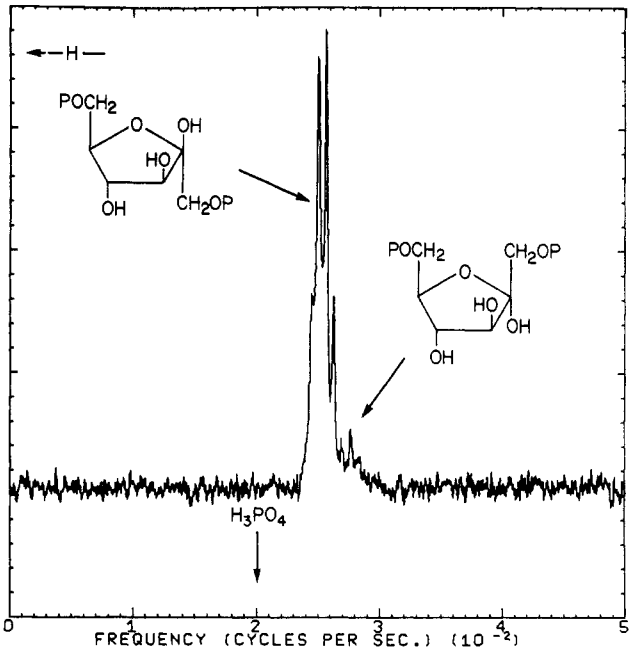


FIGURE 7: ^{31}P nuclear magnetic resonance spectrum of D-fructose 1,6-diphosphate (pH 6.15).

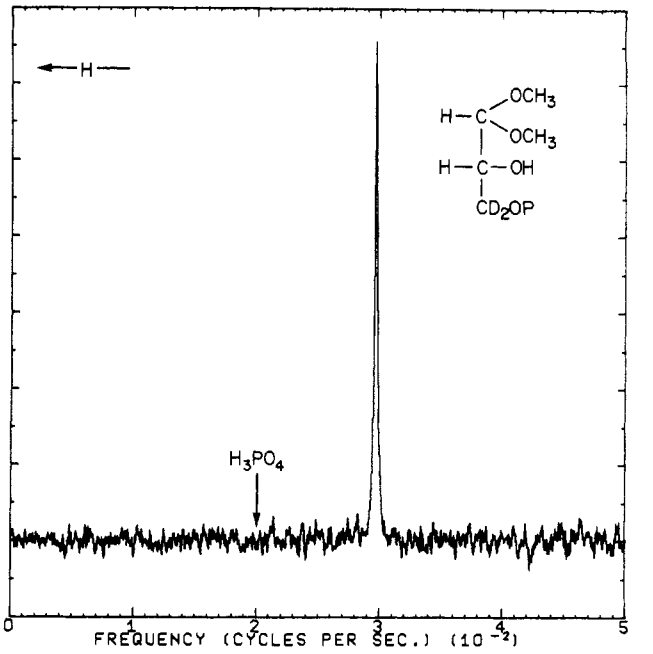
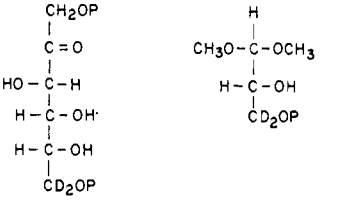


FIGURE 8: ^{31}P nuclear magnetic resonance spectrum of D-glyceraldehyde-3,3-d_2 3-phosphate dimethyl acetal dicyclohexylammonium salt (**10**).

lets can be assigned to the 1- and 6-phosphates of the β-furanose form **3**. The 6-phosphorus resonance of the α-furanose form **4** is obscured by the intense resonances of the β-furanose form. At pH 5.0, the ^{31}P nmr spectrum of FDP did not contain a weak triplet shifted to lower field, indicating either the absence of the α-furanose form, or coincidence of the resonances of the α- and β-furanose forms at this pH.

The conclusion that FDP exists primarily in the β-furanose form **3** in solution is based on the assignment of the chemical shifts and J_{PH} values of the phosphorus resonances of the two cyclic forms. In order to demonstrate that these assignments were correct, D-fructose-6,6-d_2 1,6-diphosphate (**9**) has been synthesized (Gray and Barker, 1971). Since the 6-phosphorus resonances of the two furanose forms of FDP have identical chemical shifts, they yield little information regarding the proportion of the two forms in solution. Replacement of the C-6 hydrogens with deuterium, therefore, simplifies the ^{31}P nmr spectrum by collapsing the 6-phosphorus resonances of the two forms from triplets essentially to singlets, since the deuterium–phosphorus coupling is not resolved. This effect can be seen by examining the ^{31}P nmr spectrum of the dimethyl acetal of D-glyceraldehyde-3,3-d_2 3-phosphate (**10**, Figure 8),



9 10

an intermediate in the synthesis of **9**. This spectrum demonstrates that complete replacement of the C-3 hydrogens with deuterium was achieved, since the phosphorus resonance of the undeuterated analog is a triplet. The spectrum also demonstrates that no migration of the phosphate from the 3 to the 2 position occurred (Ballou and Fischer, 1955), since the phosphorus resonance in the latter would be a doublet. The ^{31}P nmr spectrum of D-fructose-6,6-d_2 1,6-diphosphate (Figure 9) confirms the assignments of the phosphorus resonances in the spectrum of FDP. The 6-phosphorus resonances of both furanose forms of **9** occur as a singlet at the high-field portion of the spectrum as expected. Overlapping this singlet is a triplet due to the 1-phosphate of the β-furanose form, and a minor triplet (partially unresolved) is present at lower field, assigned to the 1-phosphate of the α-furanose form. These resonances have the same chemical shifts and coupling constants as the corresponding resonances in the spectrum of FDP (Table I).

TABLE I: Chemical Shift Differences and ^{31}P–^{1}H Coupling Constants of Phosphorus Resonances in the ^{31}P Nmr Spectra of FDP and Related Diphosphates.

1,6-Diphosphate Derivative	J_{PH} (Hz) 1-Phosphorus	6-Phosphorus	$(P_1 - P_6)$, ppm
2,5-Anhydro-D-mannitol	5.0	5.0	0.0
2,5-Anhydro-D-glucitol	6.8	5.1	0.49
Methyl β-D-fructofuranoside	5.5	4.9	0.26
Methyl α-D-fructofuranoside	7.1	*a*	0.94
β-D-Fructofuranose	5.4	5.1	0.26
α-D-Fructofuranose	~7.1	*a*	~1.06
β-D-Fructofuranose-6,6-d_2	5.8	0[b]	0.26
α-D-Fructofuranose-6,6-d_2	~7.1	*a*	0.98

[a] Could not be measured; obscured by other resonances. [b] Phosphorus–deuterium coupling not resolved.

Discussion

These studies and those of Gray and Barker (1970) clearly establish the structure of D-fructose 1,6-diphosphate in solution. Both proton magnetic resonance spectroscopy and measurement of the carbonyl stretching frequency in the infrared indicate that less than 1.7% of the keto form **1** is present, and that the proportion of the hydrated keto form is probably less than 0.1%. An examination of FDP and stereochemically related analogs of its α- and β-furanose forms by ^{31}P nmr spectroscopy has demonstrated that both the β-furanose form (**3**, ~90%) and α-furanose (**4**, ~10%) form are present.

These findings are in disagreement with those based on an examination of FDP by ultraviolet spectroscopy (McGilvery, 1965; Avigad *et al.*, 1970). The conclusion that FDP exists predominately or to a large extent in the keto form **1** is based on the two erroneous assumptions that the observed molar absorption intensity in the ultraviolet can be assigned only to the n $\rightarrow \pi^*$ transition of the carbonyl group, and that the extinction coefficient of 1,3-dihydroxy-2-propanone (ϵ_{270} 19.2) represents the molar absorption intensity of the carbonyl group of 2-ketose phosphates in solution. Regarding the first assumption, it has been shown that the observed absorption intensity varies considerably between different preparations of the compounds examined, and can be enhanced enormously by small amounts of impurities (Gray and Barker, 1970). For example, the tetracyclohexylammonium salt of FDP has been reported to have ϵ_{280} = 1.06 (Hartman and Barker, 1965), and the trisodium salt has been reported to have ϵ_{278} = 2.7 (Gray and Barker, 1970), ϵ_{280} = 5.0 (Avigad *et al.*, 1970), and ϵ_{293} = 9.8 (McGilvery, 1965). The tetracyclohexylammonium salt is probably the purest since it is easy to crystallize, and it has the lowest ϵ_{max}, suggesting that higher values are due to contaminants. The observed absorption in the ultraviolet, therefore, cannot be assigned unequivocally to the n $\rightarrow \pi^*$ transition of the carbonyl group. Regarding the second assumption, the authors failed to recognize that a carbonyl group can exist in a gem-diol or hydrated form in solution, and that consequently the observed ϵ_{max}, even if due only to the n $\rightarrow \pi^*$ transition of the carbonyl group, reflects only the amount of *free* carbonyl present. For example, using the data of Avigad *et al.*, D-glyceraldehyde (ϵ_{275} 15) would be assumed to exist predominately (~75%) in the free aldehydo form, and DHAP (ϵ_{260} 25) would exist entirely in the free keto form. Measurements in the infrared, however, have shown that solutions of glyceraldehyde contain only 4% of the aldehydo form (Swenson and Barker, 1971), and several independent techniques, ^{1}H nmr, ir spectroscopy (Gray and Barker, 1970), and stopped-flow fast reaction kinetics (Reynolds *et al.*, 1971), have shown that 55% of the keto form of DHAP is present in solutions at 25°.

Thus, it is clear that ultraviolet spectroscopy does not yield reliable data for quantitation of the amount of a free carbonyl form in solution. The agreement between the techniques of infrared spectroscopy, ^{1}H nmr spectroscopy, ^{31}P nmr spectroscopy, ^{13}C nmr spectroscopy, and stopped-flow fast reaction kinetics as to the equilibrium composition of DHAP in solution clearly indicates that any of these methods can be reliably applied, where appropriate, to an examination of carbonyl forms in solution.

On the basis of these arguments, it is clear that FDP does not exist to an appreciate extent in the free keto form **1** as claimed by McGilvery (1965) and Avigad *et al.* (1970). The presence of the hydrated keto form **2** can also be ruled out as demonstrated by Gray and Barker (1970). Therefore, FDP must exist predominately in furanose form **3** or **4** in solution. This conclusion is further substantiated by the fact that the carbonyl oxygen of FDP is much more resistant to exchange with solvent than the carbonyl oxygen of DHAP (Model *et al.*, 1968), and the fact that the 1-phosphate group of FDP is not released as inorganic phosphate within 20 min in 1 N sodium hydroxide at room temperature, conditions which readily degrade acyclic 2-ketose phosphates (Meyerhof and Lohmann, 1934).

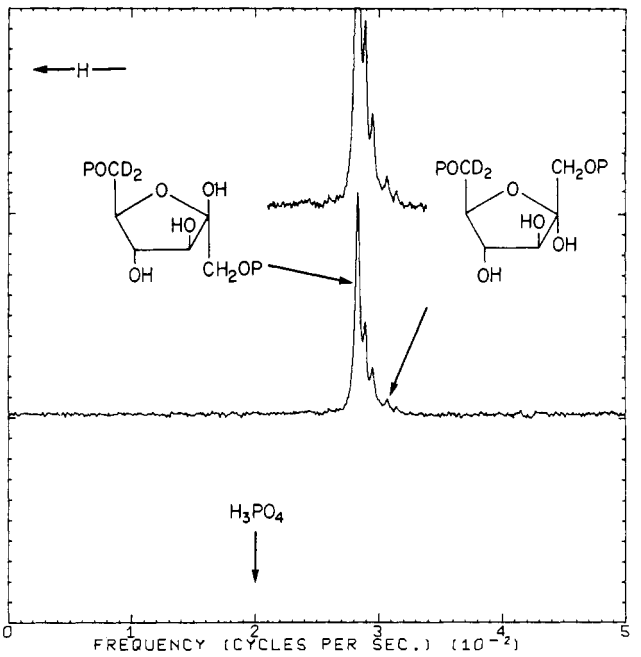


FIGURE 9: ^{31}P nuclear magnetic resonance spectrum of D-fructose-6,6-d_2 1,6-diphosphate (**9**) (pH 7.0).

In an initial examination of FDP by ^{31}P nmr spectroscopy, Gray and Barker (1970) concluded that the β-furanose form **3** predominated in solution. This conclusion was based on a comparison of the ^{31}P nmr spectra of FDP, methyl β-D-fructofuranoside 1,6-diphosphate (**5**), and methyl α-D-fructofuranoside 1,6-diphosphate (**6**). The spectra, however, were obtained at a high temperature (50°) and at an alkaline pH, conditions which degrade FDP. Indeed, the ^{31}P nmr spectrum of FDP did contain a resonance line due to inorganic phosphate, indicating some decomposition of the sample during the experiment.

To demonstrate conclusively the presence of one or both furanose forms of FDP in solution, these ^{31}P nmr studies have been extended in a way (1) to ensure that no decomposition of the samples occurred during the experiment; (2) to obtain spectra under the same conditions of temperature and pH; (3) to verify the interpretation of a downfield shift resulting from deshielding of the 1-phosphate group by the cis C-3 hydroxyl in analogs of the α-furanose form of FDP; and (4) to assign unequivocally the phosphorus resonances in the spectrum of FDP.

It is evident from the above data that ^{31}P nmr spectroscopy is capable of distinguishing between the phosphate groups of structural analogs of the furanose forms of FDP. The diphosphates **5** and **7** are analogs of the β-furanose form of FDP in which a methoxyl group and hydrogen atom, respectively, replace the anomeric hydroxyl group at C-2, and the diphosphates **6** and **8** are analogs of the α-furanose form of FDP in which a methoxyl group and hydrogen atom, re-

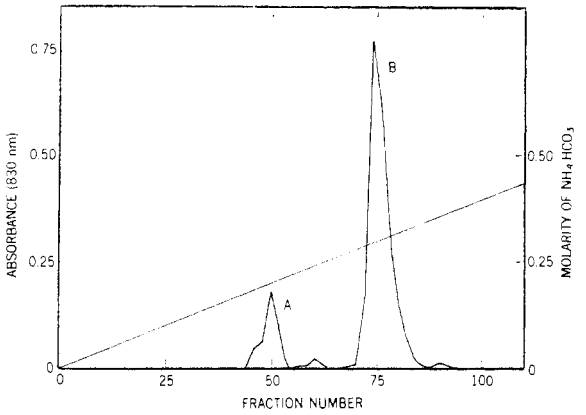


FIGURE 10: Purification of 2,5-anhydro-D-glucitol 1,6-diphosphate (**8**) by chromatography on Sephadex DEAE A-25. Peak A is inorganic phosphate and peak B is **8**.

spectively, replace the hydroxyl group at C-2. The relative orientations of OH and CH_2OP groups in these analogs are identical with those in the respective furanose analogs of FDP, and these analogs therefore serve as excellent models for examining the stereochemical dependence of the phosphorus chemical shifts. The data may be summarized by comparing the proton–phosphorus coupling constants and chemical shift differences between the phosphorus resonances of each of the analogs (Table I). The 6-phosphorus resonances had essentially the same chemical shifts and coupling constants and could not be used to differentiate between the various ring forms. This was expected, since the C-6 phosphate groups of all the analogs are stereochemically equivalent with respect to substitution at C-3, C-4, and C-5. The 1-phosphorus resonances, however, have very different chemical shifts and ^{31}P–^{1}H coupling constants depending on the configuration at carbons-2 and -3. In the β series (C-2 CH_2OP trans to C-3 OH) the 1- and 6-phosphorus resonances have very similar chemical shifts and ^{31}P–^{1}H coupling constants, but in the α series (C-2 CH_2OP cis to C-3 OH) the 1-phosphorus resonance has a larger ^{31}P–^{1}H coupling and is shifted downfield relative to the 6-phosphorus resonance. Therefore, a cis arrangement of OH and CH_2OP groups on a five-membered ring leads to greater deshielding of the phosphorus nucleus than does a trans arrangement. A precise explanation of the mechanism of this shift, however, is difficult since several factors determine ^{31}P chemical shifts (Van Wazer and Letcher, 1967). The chemical shifts of the phosphorus resonances vary with pH (Moedritzer, 1967; Ho *et al.*, 1969), but the difference between the 1- and 6-phosphorus resonances remains fairly constant from pH 5 to 8.

Since the keto form of FDP is present in solution in a very low proportion and is an obligatory intermediate in the reaction catalyzed by fructose 1,6-diphosphate aldolase, it will be necessary to examine more carefully the interaction of the furanose forms with the enzyme in order to determine whether they serve as substrates by conversion to the keto form on the enzyme's surface, or whether they are bound to the enzyme in a catalytically inactive configuration and act as inhibitors. Studies have shown that analogs of the keto and furanose forms of FDP are bound about equally well and a hypothetical binding site has been formulated to account for these facts (Hartman and Barker, 1965), but the precise geometry of the binding has not yet been determined. These analogs should be explored further in order to determine if they are bound in a common geometry with respect to the catalytically functional residues of the active site.

Experimental Section

^{31}P Nmr Spectra. The ^{31}P nmr spectrometer was operated at 24.287 MHz and the magnetic field was locked on an external water reference signal. Spectra were obtained by the Fourier transform technique (Ernst and Anderson, 1966) using a 90° pulse of 40-μsec duration at a repetitive rate of 5 sec. The free induction decay signal was digitized in 2048 sample channels equidistant in time (1 msec/channel), collected in a computer based data acquisition system, and subsequently Fourier transformed. Spectra presented in this paper were obtained at a pulsing frequency 200 cps lower than the resonance frequency of 85% H_3PO_4 (24,287,204 cps); therefore, the standard 85% H_3PO_4 reference signal is offset 200 cps downfield from zero frequency, as indicated by an arrow on the spectra. Some samples contained inorganic phosphate, and its resonance frequency is also labeled on the spectra.

All measurements were made at a probe temperature of 31°, and all samples were converted to sodium salts which were dissolved in 0.005 M EDTA to prevent line broadening in the spectra due to paramagnetic ion contamination. Chemical shifts were not affected by addition of EDTA. Chemical shifts and coupling constants were calculated from computer printouts of signal intensity *vs.* frequency, and the proportions of tautomeric forms observed in the spectra of FDP and DHAP were estimated from these data.

Phosphate Esters. The crystalline trisodium salt of D-fructose 1,6-diphosphate was obtained from Wessex Biochemicals Limited, Bournemouth, England, and was assayed as previously described (Gray and Barker, 1970). 1,3-Dihydroxy-2-propanone phosphate dimethyl acetal was a gift from Dr. C. E. Ballou, and 1,3-dihydroxy-2-propanone phosphate was prepared by hydrolysis of the dimethyl acetal as described by Ballou and Fischer (1956). Proton magnetic resonance spectroscopy indicated that complete hydrolysis was achieved. Methyl β-D-fructofuranoside 1,6-diphosphate (**5**) and methyl α-D-fructofuranoside 1,6-diphosphate (**6**) were the same samples previously used (Gray and Barker, 1970), and were purified as described below for 2,5-anhydro-D-glucitol 1,6-diphosphate. 2,5-Anhydro-D-mannitol 1,6-diphosphate (**7**) and 2,5-anhydro-D-glucitol 1,6-diphosphate (**8**), obtained from Dr. Robert Barker, were purified by chromatography on Sephadex DEAE A-25. The purification of **8** is representative. The tetracyclohexylammonium salt of **8** (100 mg) was applied in water to a 2 × 16 cm column of Sephadex DEAE A-25 (bicarbonate form). The column was eluted with a 1-l. linear gradient of 0.0–0.5 M ammonium bicarbonate (Figure 10). Fractions (8 ml) were collected at a flow rate of 40 ml/hr, and were assayed for total phosphate and inorganic phosphate as described by Bartlett (1959b). Inorganic phosphate (peak A) eluted with 0.2 M ammonium bicarbonate and diphosphates (peak B) eluted with 0.3 M ammonium bicarbonate. Fractions containing **8** were combined and were repeatedly evaporated *in vacuo* from water to remove ammonium bicarbonate. The pure diphosphates were converted to their sodium salts, titrated to pH 7.0, and lyophilized.

D-Fructose 6,6-d_2 1,6-diphosphate (**9**) was prepared as described by Gray and Barker (1971) and was purified by column chromatography on Sephadex DEAE A-25 as described above, except that a 0.0–1.0 M ammonium formate gradient was used (Bartlett, 1959a) to avoid the alkaline pH of ammonium bicarbonate. Inorganic phosphate eluted with 0.19 M ammonium

formate and FDP eluted with 0.34 M ammonium formate. Fractions containing D-fructose-6,6-d_2 1,6-diphosphate were combined, acidified with Dowex 50 (H^+), and extracted six times with three volume portions of ether to remove formic acid. The sample, which still contained some formic acid, was adjusted to pH 7.0 and lyophilized.

Acknowledgments

The author expresses his gratitude to Dr. Melvin P. Klein, Laboratory of Chemical Biodynamics, University of California, Berkeley, for his interest in this work and for use of the ^{31}P nmr spectrometer, and his special gratitude to Dr. Irving Salmeen for demonstration of the use of the spectrometer system, and for helpful discussions on Fourier transform nmr spectroscopy and the optimal conditions for obtaining ^{31}P nmr spectra. I also thank Dr. Robert Barker and Dr. C. E. Ballou for supplying some of the samples used in this work.

References

Avigad, G., Englard, S., and Listowsky, I. (1970), *Carbohyd. Res. 14*, 365.
Ballou, C. E., and Fischer, H. O. L. (1955), *J. Amer. Chem. Soc. 77*, 3329.
Ballou, C. E., and Fischer, H. O. L. (1956), *J. Amer. Chem. Soc. 78*, 1659.
Bartlett, G. R. (1959a), *J. Biol. Chem. 234*, 459.
Bartlett, G. R. (1959b), *J. Biol. Chem. 234*, 466.
Dyson, J. E. D., and Noltmann, E. A. (1968), *J. Biol. Chem. 243*, 1401.
Ernst, R. R., and Anderson, W. A. (1966), *Rev. Sci. Instrum. 37*, 93.
Gracy, R. W., and Noltmann, E. A. (1968), *J. Biol. Chem. 243*, 5410.
Gray, G. R., and Barker, R. (1970), *Biochemistry 9*, 2454.
Gray, G. R., and Barker, R. (1971), *Carbohyd. Res.* (in press).
Hartman, F. C., and Barker, R. (1965), *Biochemistry 4*, 1068.
Ho, C., Magnuson, J. A., Wilson, J. B., Magnuson, N. S., and Kurland, R. J. (1969), *Biochemistry 8*, 2074.
McGilvery, R. W. (1965), *Biochemistry 4*, 1924.
Meyerhoff, O., and Lohmann, K. (1934), *Biochem. Z. 275*, 89.
Model, P., Ponticorvo, L., and Rittenberg, D. (1968), *Biochemistry 7*, 1339.
Moedritzer, K. (1967), *Inorg. Chem. 6*, 936.
Reynolds, S. J., Yates, D. W., and Pogson, C. I. (1971), *Biochem. J. 122*, 285.
Rutter, W. J. (1961), *Enzymes 5*, 341.
Swenson, C. A., and Barker, R. (1971), *Biochemistry 10*, 3151.
Trentham, D. R., McMurray, C. H., and Pogson, C. I. (1969), *Biochem. J. 114*, 19.
Van Wazer, J. R., and Letcher, J. H. (1967), Topics in Phosphorus Chemistry, Vol. 5, New York, N. Y., Interscience Publishers, p 169.

Isolation of Glycopeptides from Low- and High-Density Platelet Plasma Membranes*

A. J. Barber and G. A. Jamieson†

ABSTRACT: Glycopeptides have been isolated from both the low- (*d* 1.090) and high- (*d* 1.120) density membranes isolated by the glycerol-lysis technique. Three size classes of glycopeptide were obtained on treatment with trypsin which were identical with those obtained by proteolytic digestion using intact platelets. However, a chondromucopeptide obtained from intact platelets by trypsin treatment was not obtained using isolated membranes suggesting that it is a product of the platelet "release reaction." Brief digestion of intact platelets with chymotrypsin, which does not induce the release reaction, did not yield the chondromucopeptide and in this case the isolated *macroglycopeptide* was larger than that obtained by tryptic treatment. When intact platelets were subjected to catalytic iodination (^{125}I) with lactoperoxidase the incorporation of radioactivity, and its distribution in sodium dodecyl sulfate–polyacrylamide gels, were identical in both the high- and low-density vesicles. The amount of residual sialic acid in both types of vesicle was also equal following limited treatment with neuraminidase. These results show that both types of membrane vesicle are derived from the outer surface of the platelet and may reflect areas of anatomical specialization on the platelet surface, as previously suggested from electron microscopy.

A *macroglycopeptide* is released from intact platelets by brief proteolytic digestion (Pepper and Jamieson, 1970). This macroglycopeptide is unique in having a molecular weight (120,000) considerably larger than that of the glycopeptides isolated from other cell surfaces (Winzler *et al.*, 1967; Winzler, 1969; Buck *et al.*, 1971) and it may be related to the thrombocyte specific antigen (Hanna and Nelken, 1971). It contains both N- and O-glycosidic linkages, has galactose as its major

* From the Blood Research Laboratory, The American National Red Cross, Bethesda, Maryland 20014. *Received June 28, 1971.* This work was supported, in part, by USPHS Grants GM-13057 and AI-09017 and is Contribution No. 230 from the Blood Research Laboratory of the American National Red Cross.

† To whom correspondence should be addressed.