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Studies on the Substrates of D-Fructose 1,6-Diphosphate Aldolase in Solution*

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ABSTRACT: Ketose mono- and diphosphates have been examined by infrared, ultraviolet, and nuclear magnetic resonance spectroscopy in deuterium oxide solutions. Those which have a hydroxyl group at C-5 (D-fructose 1,6-diphosphate) or C-6 (D-*glycero*-D-*altro*-octulose 1,8-diphosphate) are in the furanose and pyranose ring forms, respectively. Ketose phosphates which cannot exist in ring forms are in the free keto and hydrated keto forms in solution. The proportion of the keto form at 25° for the phosphates examined is given in parentheses in the following list: 5,6-dideoxy-D-*threo*-hexulose 1-phosphate (96%), D-*erythro*-pentulose 1,5-diphosphate (84%), 1,5-dihydroxy-2-pentanone 1,5-diphosphate (84%), 1,3-dihydroxy-2-propanone phosphate (55%), D-fructose 1,6-diphosphate (<1.7%), D-*glycero*-D-*altro*-octulose 1,8-diphosphate (none). The K_m values of the ketose diphosphates for rabbit muscle aldolase relate to the distance between the phosphate groups in the predominant form in solution.

D-Fructose 1,6-diphosphate ($FruP_2$), the substrate of aldolase, would be expected to exist in aqueous solution as a mixture of forms **1, 2, 3, 4,** or **5.** It has usually been assumed (Rutter, 1961) that it exists predominately in a furanose form (3 or 4), since in the monosaccharides, ring forms are usually more stable than acyclic forms. However, no direct evidence has been obtained to demonstrate that this is the case. From an examination of ultraviolet spectra, McGilvery (1965) concluded that $FruP_2$ dianion exists predominately as the free keto form **1,** and the tetraanion as the enediol form **5.** The established importance of acyclic forms of $FruP_2$ and other phosphorylated ketoses as intermediates in enzymatic reactions makes their relative abundance in solution an important consideration in describing the mechanism of enzyme action (Mehler and Cusic, 1967; Koshland and Neet, 1968; Knowles and Pon, 1968).

The acyclic ketose phosphates, 1,3-dihydroxy-2-propanone phosphate (**6**), 5,6-dideoxy-D-*threo*-hexulose 1-phosphate (**7**), D-*erythro*-pentulose 1,5-diphosphate (**8**), and 1,5-dihydroxy-2-pentanone 1,5-diphosphate (**9**), and the potentially cyclic $FruP_2$, and D-*glycero*-D-*altro*-octulose 1,8-diphosphate (**10**) have been examined.

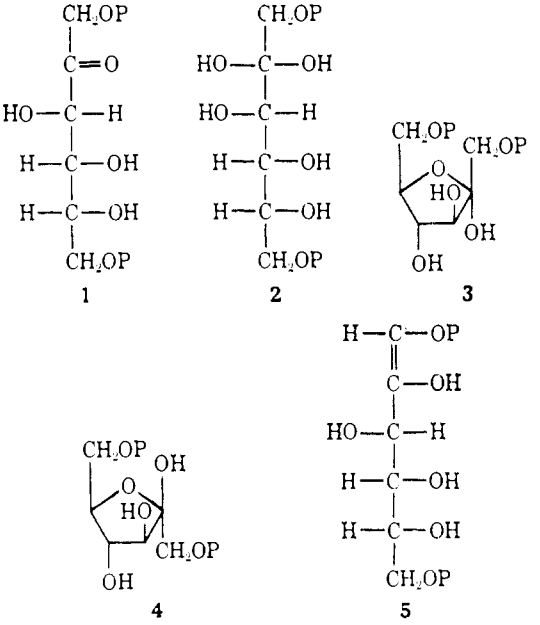

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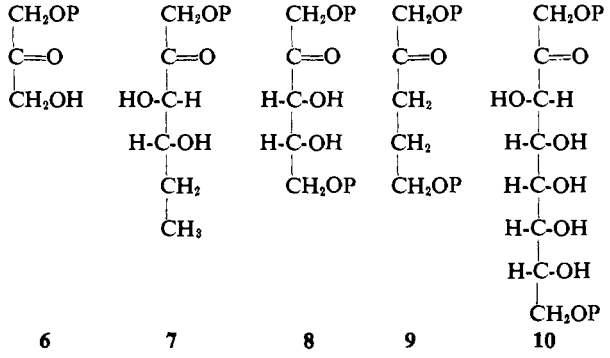
TABLE I: Infrared and Ultraviolet Absorption Maxima and Percentage of Keto Form in Solutions of the Sodium Salts of Ketose Phosphates in Water at pH 7 or D_2O at pD 7.

Phosphate	Infrared			Ultraviolet Wavelength		Nuclear Magnetic Resonance
	Frequency (cm^{-1})	ϵ	% Keto ϵ_0 211 at 25°	mμ	ϵ	% Keto
5,6-Dideoxy-D-*threo*-hexulose 1-phosphate	1730	203	96	279	59.7	91, 28°
D-*erythro*-Pentulose 1,5-diphosphate	1731	177	84	282	85.4	
1,5-Dihydroxy-2-pentanone 1,5-diphosphate	1725	177	84	267	37.5	84, 28°
1,3-Dihydroxy-2-propanone phosphate	1735	115	55	268	15.4	63, 37°
D-Fructose 1,6-diphosphate	1730	3.5	1.7	278	2.7	<3, 37°
D-*glycero*-D-*altro*-Octulose 1,8-diphosphate		0	0			

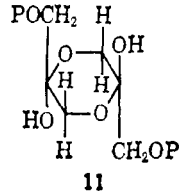


6 **7** **8** **9** **10**

Results and Discussion

Infrared Spectroscopy. Infrared spectra obtained using deuterium oxide solutions of the sodium salts of various ketose phosphates are given in Figure 1. The molar extinction coefficients at the frequency of maximum absorption are given in Table I.

Differences in molar extinction coefficients could be due to inherent differences in the force constants of the carbonyl bonds. However, the ϵ_{max} for **6** (115) differs from ϵ_{max} for **7** (203) by a factor of approximately 2. This is too large a difference to attribute to differences in bond force constants since infrared absorption intensities of groups in closely related environments are usually quite insensitive to substitution (Jones, *et al.*, 1952). It is more probable that about 43% of **6** exists as the hydrate or as a dimeric hemiacetal (**11**) in aqueous solution. To estimate the proportions of hydrate or hemiacetal, the value for the molar extinction coefficient of the free carbonyl in aqueous solution was calculated to be 211 from the ϵ_{max} 177 of **9**, and the estimate that the compound is 84% in the keto form by nuclear magnetic resonance spectroscopy. The proportion of keto form calculated on this basis for the compounds examined is given in Table I.



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Ultraviolet Spectroscopy. Values for wavelength of maximum absorption and molar extinction coefficients of aqueous solutions are given in Table I. All of the ketose phosphates examined absorb in the region 268–288 mμ. Although $FruP_2$

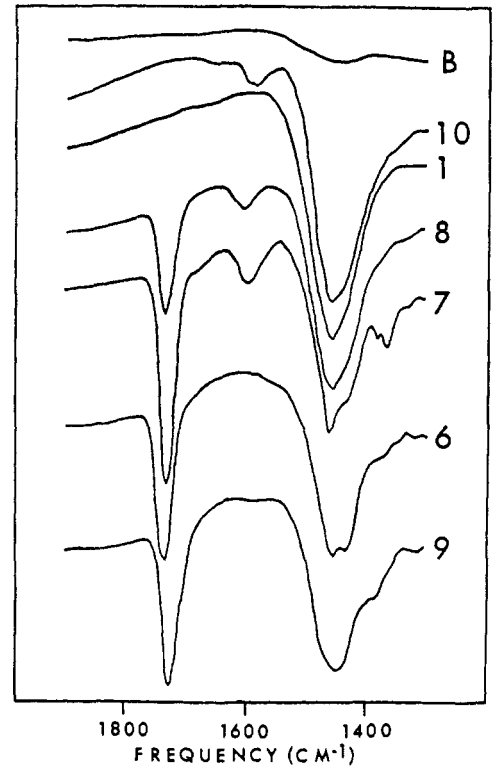


FIGURE 1: Infrared spectra in the 1300–1900 cm^{-1} region, obtained using deuterium oxide solutions of D-*glycero*-D-*altro*-octulose 1,8-diphosphate (**10**), D-fructose 1,6-diphosphate (**1**), D-*erythro*-pentulose 1,5-diphosphate (**8**), 5,6-dideoxy-D-*threo*-hexulose 1-phosphate (**7**), 1,3-dihydroxy-2-propanone phosphate (**6**), and 1,5-dihydroxy-2-pentanone 1,5-diphosphate (**9**) at pH 7, 25°.

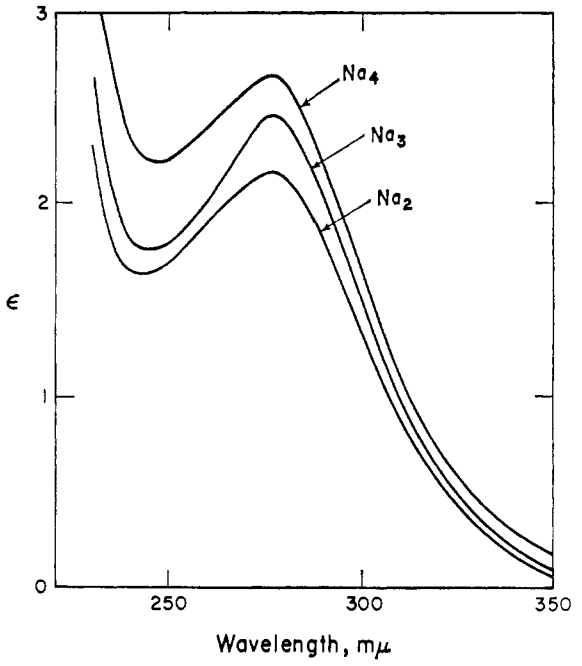


FIGURE 2: The ultraviolet absorption spectra of D-fructose 1,6-diphosphate. Na_3, commercial trisodium salt; Na_2, dianion formed by titration of the commercial salt with hydrochloric acid; Na_4, tetraanion formed by titration of the sodium salt with NaOH.

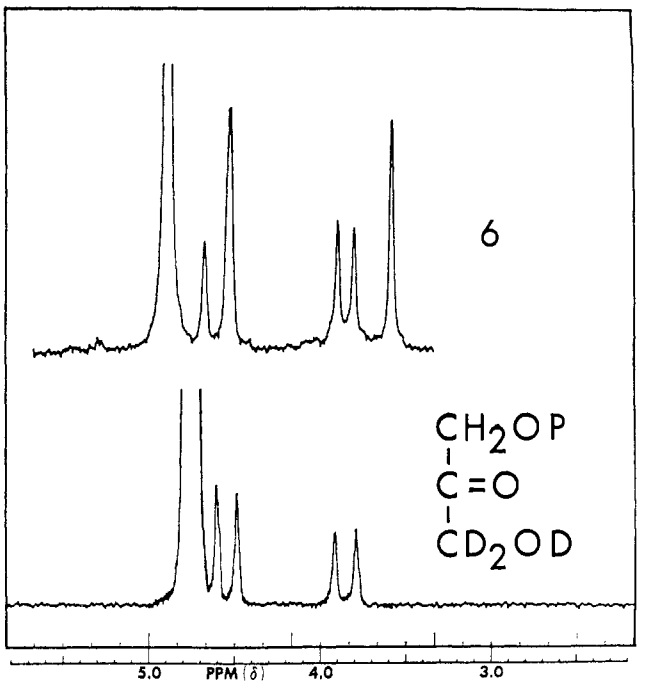


FIGURE 3: 60-MHz proton magnetic resonance spectra of 1,3-dihydroxy-2-propanone phosphate (**6**) in deuterium oxide at pH 4.5, 10°, and 3,3'-dideuterio-1,3-dihydroxy-2-propanone phosphate in deuterium oxide at pH 7, 37°. Me_4Si external standard.

has the lowest ϵ_{max} (2.72) and **6** has the next lowest (15.4) in agreement with the infrared data, the other values do not parallel the extinction coefficients of the 1730-cm^{-1} band. In addition, $FruP_2$ showed a much lower ϵ_{max} than reported by McGilvery (1965) for the dianion (9.8 at 293 mμ) and a much higher value than reported by Hartman and Barker (1965) for the tetracyclohexylammonium salt (1.06 at 280 mμ). These differences suggest that the ultraviolet absorption spectra may be due to an impurity and may depend upon the method of preparation; this was shown to be the case. For example, when a solution of the $FruP_2$ trisodium salt (2.7 at 287 mμ) used in this study was brought to pH 4.5 (disodium salt) with Dowex 50W-X8(H^+), the absorption maximum shifted to 273 mμ and the molar extinction coefficient increased to 5.53. Titration with base back to the trisodium salt (pH 6.5) caused a further increase in the molar extinction coefficient to 7.05 without a change in the wavelength of maximum absorption. However, when the trisodium salt was converted to the disodium salt by the addition of hydrochloric acid rather than Dowex 50(H^+) the wavelength of maximum absorption was unaffected and the molar extinction coefficient fell to 2.16. Titration back to the trisodium salt regenerated the starting spectrum (Figure 2). Washings from the resin had no absorption in this region.

In another experiment, $FruP_2$ trisodium salt was dried under high vacuum at 100°. The loss of 8.1 waters of crystallization was accompanied by the destruction of 7.1% of the $FruP_2$, assayed with aldolase (Blostein and Rutter, 1963). The slightly colored residue had ϵ_{max} 1160 at 288 mμ, very close to the band observed by McGilvery (1965) for his preparation of $FruP_2$.

The addition of 1 molar equiv of $MgCl_2$ to a solution of $FruP_2$ trisodium salt enhances the absorbance only slightly (~3%) and does not change the position of maximum absorption.

Although there are significant differences between the ultraviolet spectra of the preparations we used and those used by McGilvery (1965), we found that our preparation had an optical rotatory dispersion qualitatively and quantitatively resembling those published by McGilvery.

Clearly, $FruP_2$ is very sensitive to strong cation-exchange resins and heat, and the different ultraviolet spectra observed for different preparations are probably due to contamination. Because of this, the ultraviolet data in Table I cannot be interpreted entirely in terms of the amount of carbonyl present since a strong acid resin was used at some stage in the preparation of each of the compounds.

Nuclear Magnetic Resonance Spectroscopy. Nuclear magnetic resonance spectroscopy has been used to distinguish between free and hydrated carbonyls in solution (Lombardi and Sogo, 1960; Hine and Houston, 1965; Hooper, 1967). The inductive effect as well as the diamagnetic anisotropy of a carbonyl group leads to deshielding of hydrogen nuclei attached to the carbonyl carbon atom or on atoms adjacent to it. However, deshielding is significantly reduced in the hydrate or acetal and the hydrogens at C-1 and C-3 of a ketose phosphate should absorb at lower field in the keto form than in the hydrated keto form or hemiacetal.

Each of the acyclic ketose phosphates examined was found to exist predominately in the free keto form in solution. The nuclear magnetic resonance spectra all had a doublet in the region τ 5.2–5.4 which was assigned to the C-1 hydrogens of the free keto form split by the phosphorus of the phosphate group (Crutchfield *et al.*, 1962).

In the spectrum of **6** (Figure 3), the doublet due to

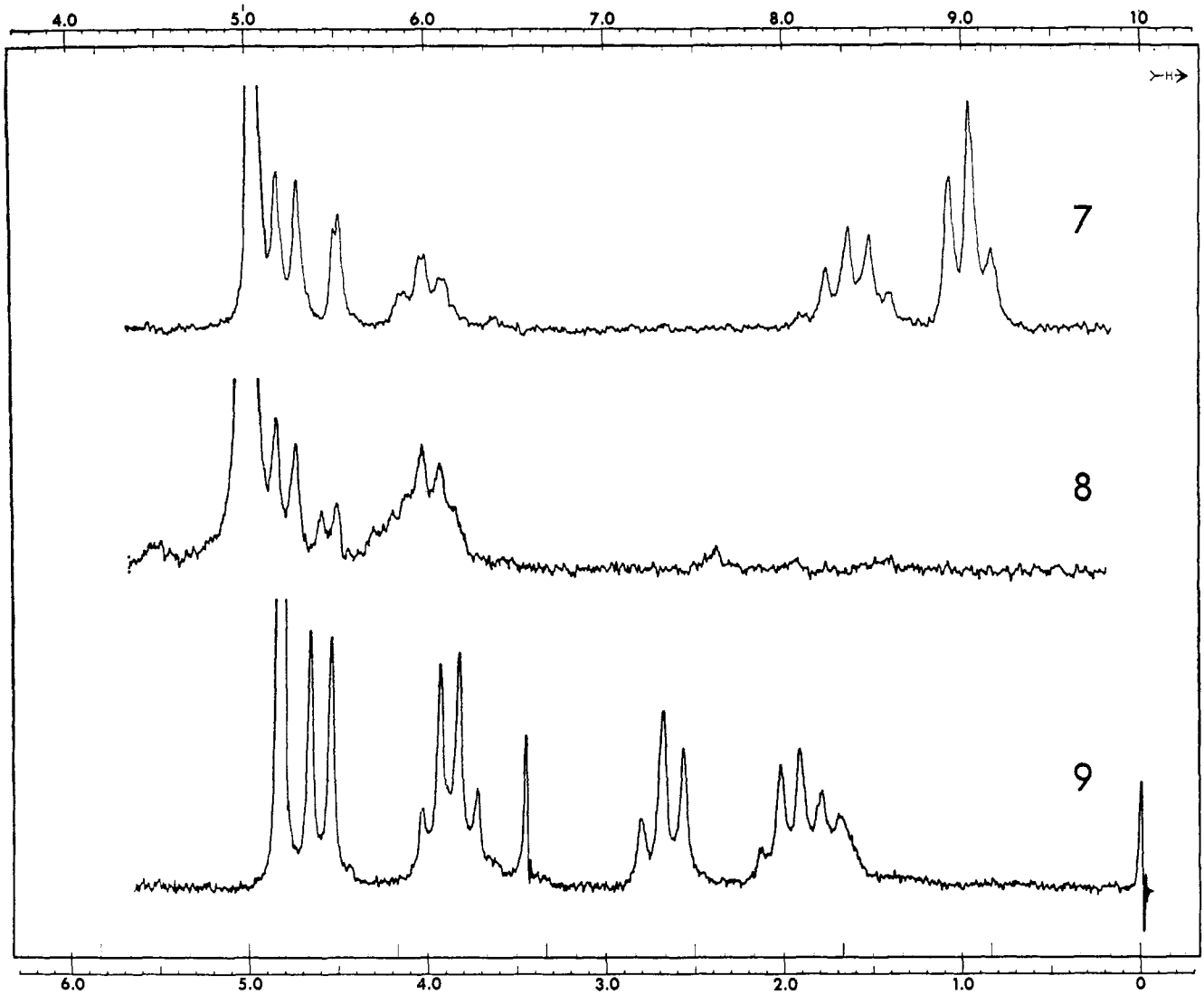


FIGURE 4: 60-MHz proton magnetic resonance spectra of 5,6-dideoxy-D-*threo*-hexulose 1-phosphate (**7**), D-*erythro*-pentulose 1,5-diphosphate (**8**), and 1,5-dihydroxy-2-pentanone 1,5-diphosphate (**9**) in dueterium oxide at pH 7, 28°. Me_4Si external standard.

the C-1 hydrogens of the free keto form is not well resolved. At pH 4.5, the higher field portion of the doublet is superimposed on the singlet for the C-3 hydrogens of the keto form. This was shown to be the case by replacing the C-3 hydrogens with deuterium. Replacement was accomplished enzymatically using aldolase (Rose, 1958) followed by triosephosphate isomerase (Reider and Rose, 1959) after removal of the aldolase. In addition, the spectrum contains a doublet at τ 6.15 and a singlet at 6.42 assigned to the C-1 and C-3 hydrogens of the hydrated keto form. The fact that the C-3 hydrogens occur as a singlet makes the presence of the dimeric hemiacetal form **11** unlikely (Gardiner, 1966). If this form is present, the chemical shifts of the axial and equatorial hydrogens must be the same since geminal coupling is absent. In addition, absorptions due to the hydrate would have to be coincident with those of the dimer or with those of the *keto* form; *or* the amount of hydrate would have to be small enough to escape detection by nuclear magnetic resonance. The presence of absorptions ascribable to hydrated forms in **7** and **9** makes these possibilities seem unlikely, and it is most probable that only monomeric *keto* and hydrate forms of **6** exist in aqueous solution. The proportion of keto form estimated by integration is given in Table I.

In the spectra of ketoses **7**, **8**, and **9** (Figure 4), the doublet due to the C-1 hydrogens of the keto form is not obscured by other resonances, and is easily integrated. For example, in the spectrum of **9**, the doublet at τ 5.37 contains 1.68 hydrogens and the multiplet at 6.08 contains 2.33 hydrogens. It appears that the C-1 hydrogens of the hydrated form are superimposed on the multiplet due to the C-5-methylene protons at 6.08. Therefore, 84% of the keto form is present. The percentages of keto forms in solutions of the other acyclic phosphates were estimated using the same technique (Table I).

The nuclear magnetic resonance spectrum of $FruP_2$ (Figure 5) has no doublet at τ 5.22 characteristic of the C-1 hydrogens of the keto form. If present at all, the keto form must constitute less than 3% of the $FruP_2$ in solution. In the model compounds examined, the equilibrium between keto and

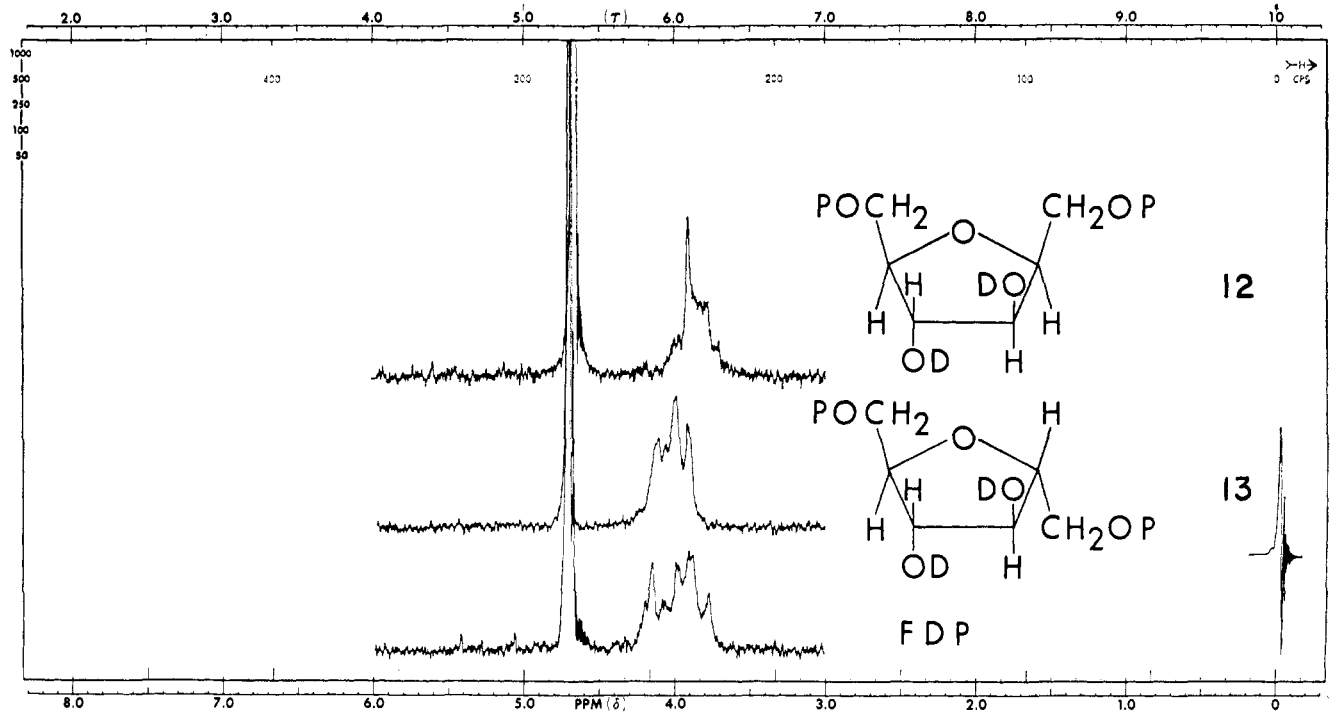


FIGURE 5: 60-MHz proton magnetic resonance spectra of D-fructose 1,6-diphosphate, 2,5-anhydro-D-glucitol 1,6-diphosphate (**12**), and 2,5-anhydro-D-mannitol 1,6-diphosphate (**13**) in deuterium oxide at pH 7, 37°. Me_4Si external standard.

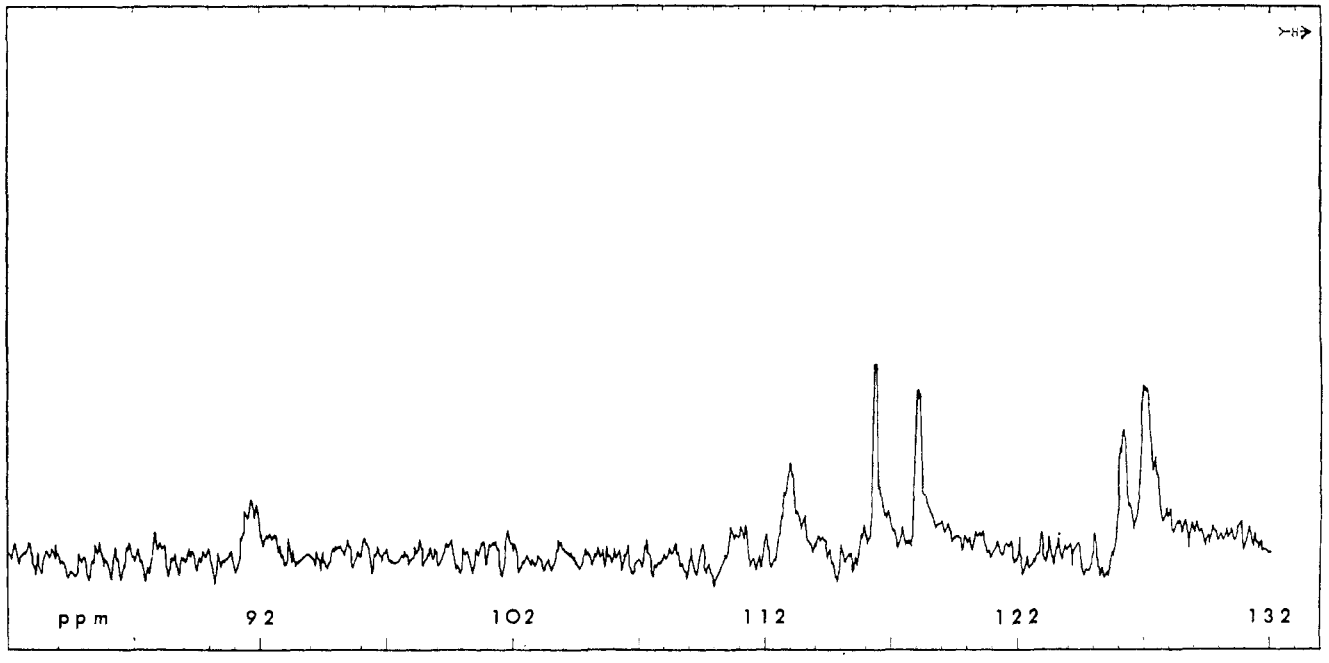


FIGURE 6: Proton noise decoupled 25.15-MHz ^{13}C nuclear magnetic resonance spectrum of trisodium D-fructose 1,6-diphosphate in water at 32°. Average of 100 scans. Chemical shift expressed in parts per million from carbon disulfide external standard. Courtesy of Dr. L. F. Johnson, Varian Associates.

hydrate favors the keto form so that by analogy, the hydrate form of $FruP_2$ cannot be a major component in solution. The spectrum does, however, resemble the spectra of 2,5-anhydro-D-glucitol 1,6-diphosphate (Figure 5, **12**) and 2,5-anhydro-D-mannitol 1,6-diphosphate (Figure 5, **13**), analogs of the α- and β-furanose forms of $FruP_2$, respectively, in which a hydrogen replaces the hydroxyl at C-2.

To determine if one or both furanose forms of $FruP_2$ is present in solution, the 25.15 MHz ^{13}C nuclear magnetic resonance with proton noise decoupling (Weigert *et al.*,

1968), 220 MHz proton magnetic resonance, and 40.5 MHz ^{31}P nuclear magnetic resonance with proton noise decoupling (Johnson and Tate, 1969) spectra were obtained. The proton noise decoupled ^{13}C nuclear magnetic resonance spectrum should give a single sharp absorbance for each carbon atom in a magnetically distinct environment. Thus, if a single form of $FruP_2$ is present in solution, six lines should be observed; if two forms are present, twelve lines should be observed. The areas under each set of six lines should represent the proportion of the form giving rise to the absorptions. However, the ^{13}C nuclear magnetic resonance spectrum of $FruP_2$ is complicated, probably by carbon-13–phosphorus-31 spin coupling causing splitting of the signals from C-1 and C-6. Nevertheless, the spectrum indicates that one form predominates in solution since only four sharp absorbances are present (Figure 6) due to signals from C-2, C-3, C-4, and C-5. If more than one form were present in solution, four sharp absorbances would be expected for each form. The 220 MHz proton magnetic resonance spectrum was too complex to allow an evaluation of forms present in solution. In Figure 7 the proton noise decoupled ^{31}P spectrum of $FruP_2$ is compared to those of methyl β-D-fructofuranoside 1,6-diphosphate (**14**) and a mixture of methyl α- and β-D-fructofuranoside 1,6-diphosphates enriched in the α form (**15**). These glycosides are close structural analogs of the α- and β-furanoses **3** and **4**. Both the 1- and the 6-phosphate groups of the β-furanoside have the same chemical shift (−4.2 ppm) while those of the α-furanoside have different chemical shifts. The phosphate groups in the β compound are *trans* to the neighboring hydroxyl groups and are in similar chemical environments. In **15** the 6-phosphate group is similarly disposed but the 1-phosphate is *cis* to the 3-hydroxyl group which would be expected to produce a downfield shift. On this basis the signal at −5.0 ppm is assigned to the 1-phosphate group and that at −4.3 ppm to the 6-phosphate group of **15**. The spectrum of $FruP_2$ shows two strong signals at −2.1 and −1.7 ppm and two weak signals at −2.9 and −0.44 ppm. If the signal at −0.44 ppm is disregarded, the spectrum can be accounted for approximately by assuming that there are two pairs of signals, a strong pair at −1.7 and −2.1 ppm superimposed on a weak pair at −1.8 and −2.9 ppm, the strong lines being due to the β-furanose form (the major component) and the weak pair to the α-furanose form (the minor component). It is possible that all of the weak signals are due to impurities produced in the sample by exposure to the high temperature of the spectrometer (50°) and the slightly basic pH. The broad lines in the spectrum of $FruP_2$ in water may be due to the occurrence of rapid equilibration among tautomers or conformers (Model *et al.*, 1968).

Structure of D-*Fructose 1,6-Diphosphate.* Neither the nuclear magnetic resonance nor the infrared spectrum of $FruP_2$ indicates the presence of much of the free keto form (**1**) in solution. The data from the acyclic phosphates also indicates that the hydrated keto form of $FruP_2$ (**2**) does not exist in solution to an appreciable extent, since those with a secondary hydroxyl at C-3 (**7**, **8**) tend to have a high proportion of the free keto form present in solution. Therefore, we conclude that $FruP_2$ must exist predominately in a furanose form in solution.

This conclusion is supported by studies on the rate of exchange of ^{18}O from 2-^{18}O-labeled $FruP_2$ and **6** into $H_2{}^{16}O$ (Model *et al.*, 1968). The oxygen atom of a free carbonyl group exchanges with the oxygen atom of water *via* reversible hydrate formation, and the rate of this exchange depends partly upon the concentration of free carbonyl in solution. At 25°, the rate of exchange of ^{18}O from **6**

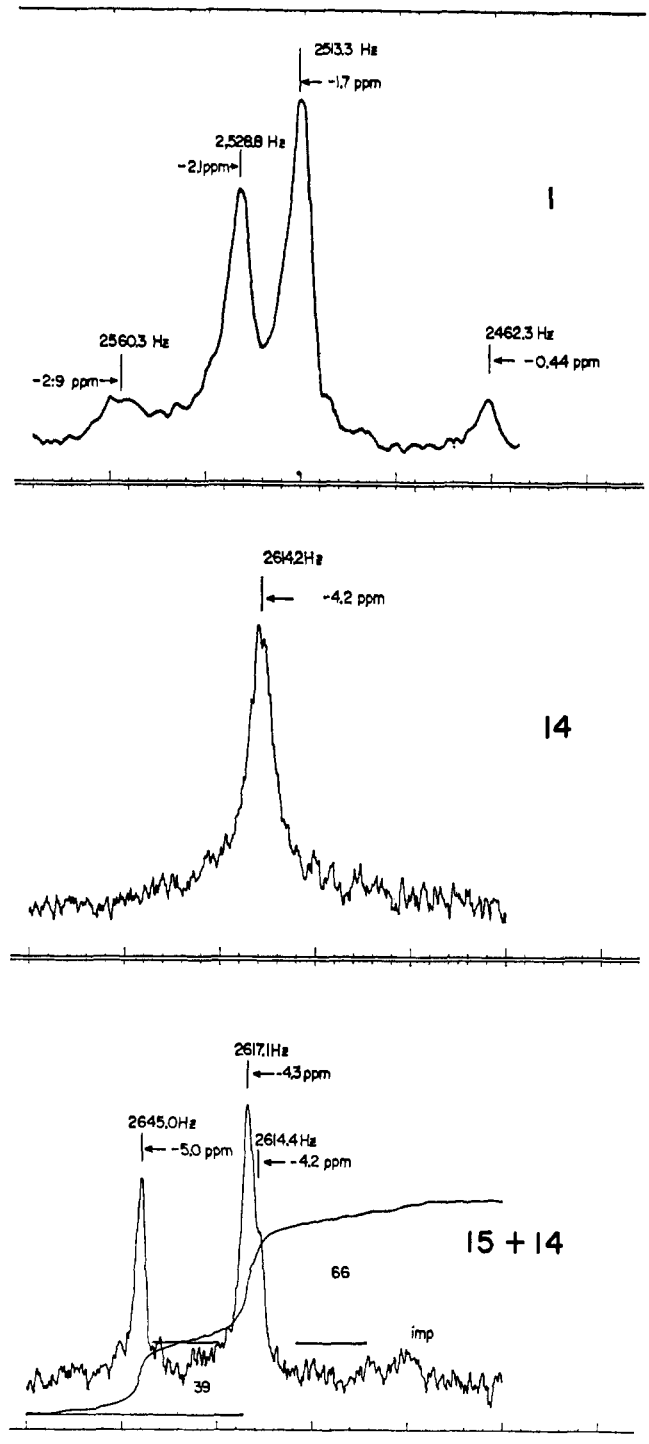
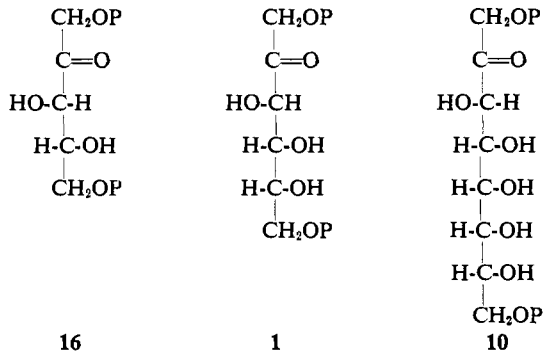


FIGURE 7: Proton noise decoupled 40.5-MHz ^{31}P nuclear magnetic resonance spectra of trisodium D-fructose 1,6-diphosphate (**1**) at 50° (average of 255 scans), methyl β-D-fructofuranoside 1,6-diphosphate (**14**) at 40°, pH 9, and a 3:1 mixture of methyl α-D-fructofuranoside 1,6-diphosphate (**15**) and methyl β-D-fructofuranoside 1,6-diphosphate (**14**) at 40°, pH 9. Chemical shifts expressed in parts per million downfield from 85% H_3PO_4. Courtesy of Dr. L. F. Johnson, Varian Associates.

into $H_2{}^{16}O$ is at least 30 times the rate of exchange of ^{18}O from $FruP_6$ into $H_2{}^{16}O$. Thus, the carbonyl of $FruP_2$ is much more resistant to exchange with water than is the carbonyl of **6**, which would be expected if $FruP_2$ existed predominately in a furanose form in solution.

These findings have a bearing on the mechanism of the aldolase reaction. Hartman and Barker (1965) measured the enzyme–inhibitor dissociation constants (K_I) of diphosphates in the series ethylene glycol diphosphate.....1,8-octanediol diphosphate and observed that 1,5-pentanediol diphosphate and 1,6-hexanediol diphosphate were bound most strongly to aldolase with diphosphates of shorter and longer chain lengths binding less strongly. Because these alkanediol diphosphates would be expected to exist in solution in a fully extended form, these authors concluded that aldolase serves as a fairly rigid template on which the inhibitor complex is formed.

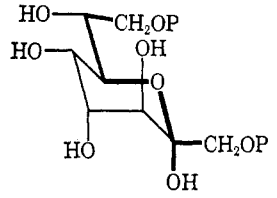
However, Mehler and Cusic (1967) have observed that



16 **1** **10**

D-*threo*-pentulose 1,5-diphosphate (**16**), $FruP_2$ (**1**), and **10** bind equally well to the active site of aldolase in spite of the fact that their phosphate groups, which are responsible for binding, are separated by carbon chains of varying lengths. However, the compounds are cleaved at significantly different rates. They state that these three substrates are identical at the first four carbon atoms, and differ only in the length of the sugar chain, which determines the separation of the phosphate groups. They conclude that the location of the *weak* binding site for phosphate (Ginsberg and Mehler, 1966) is not fixed, but is determined by the substrate, and that the induced-fit concept of Koshland (1963) therefore applies to aldolase.

However, the free keto form of **10** is not observed in solution (Table I); rather the pyranose form will be favored (**17**). In this form the phosphates are separated by 5 carbon atoms and an ether–oxygen linkage (dark lines) and would bind to aldolase about as well as does $FruP_2$. Similarly, the pentulose phosphates (**8** and **16**), which cannot exist in cyclic forms, fit tightly into the active site. The rates of cleavage of the three types of ketose should depend upon the positioning of their reaction sites relative to the reactive groups of the enzyme and should differ significantly.



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In agreement with Mehler and Cusic (1967) we find that $FruP_2$ and **10** have similar K_m values (2×10^{-5} M) but are cleaved at different rates (V_{max} = 10 and 0.14 μmoles min^{-1} mg^{-1}, respectively). **8** is bound equally well (K_I = 1×10^{-5} M) but is cleaved very slowly (V_{max} <0.05 μmole min^{-1} mg^{-1}). Mehler and Cusic found that **16** was cleaved at a much greater rate. We have not examined this compound but would expect it to exist in solution principally as the *keto* form. The difference in rate between the *erythro* (**8**) and *threo* isomers (**16**) must depend upon the position of the 3-hydroxyl group and clearly indicates the sensitivity of V_{max} to changes in the geometry of the substrate. Differences in rate between **1, 10,** and **16**, which all have the same configuration at carbons 1 through 4 in the *keto* form, might be due to differences in the proportion of *keto* form available to the enzyme. If this is the case, **16** is cleaved at a much slower rate than might be expected—perhaps because of steric or inductive effects of the 5-phosphate group. Possibly the rate differences reflect the ease with which the enzyme can convert the form bound to the acyclic intermediate required for cleavage. In this case the position of substituents at carbons 2, 3, and 4 in the predominant form in solution would be expected to determine the rate of the reaction. The values of K_m would then reflect the binding of the pyranose form of **10**, the furanose form of $FruP_2$, and the *keto* forms of **8** and **16** and reflect the separation of the phosphate groups in these forms. This interpretation of the similarity in binding is in agreement with the finding of Hartman and Barker (1965) that diphosphates are bound most strongly to aldolase if they are separated by 5 or 6 carbon atoms and seems to indicate that aldolase has a fairly rigid binding site and that it is not an "induced-fit" enzyme, at least in the binding step.

Experimental Section

General. Proton magnetic resonance spectra were obtained on a Varian A-60 spectrometer. Me_4Si was used as an external reference for samples dissolved in deuterium oxide.

Infrared spectra were obtained on a Perkin-Elmer Model 521 grating infrared spectrometer at 25 ± 2°. Samples were dissolved in deuterium oxide and placed in a 0.05-mm calcium fluoride cell previously balanced against a variable thickness cell containing solvent so that a relatively straight base line was obtained in the region 1600–1900 cm^{-1}.

Ultraviolet spectra were obtained at 25 ± 2° on a Cary Model 15 spectrometer in quartz cells with a 1-cm light path. Optical rotatory dispersion spectra were obtained at 27° on a Cary Model CD 60 spectropolarimeter.

Phosphate esters used in this study were examined for purity by chromatography on Whatman 40 paper in one of two solvent systems. Acid sensitive phosphates were chromatographed in ethanol–ammonia–water, 6:2:2 (solvent 1). Base sensitive phosphates were chromatographed in butanol–acetic acid–water, 7:2:5 (solvent 2). The chromatograms were dried and developed by the procedure of Hanes and Isherwood (1949).

D-*Fructose 1,6-Diphosphate.* The crystalline heptahydrate of Na_3FruP_2 was purchased from Wessex Biochemicals Limited, Bournemouth, England, and used without further

purification. Chromatography in solvent 1 and solvent 2 revealed only one compact spot. The material had $[\alpha]_D^{25}$ $+1° \pm 0.3$ (c 9.5, H_2O) and showed a maximum rotation of $+0.35°$ M^{-1} cm^{-1} at 300 mμ and a minimum of $-1.90°$ M^{-1} cm^{-1} at 215 mμ with a shoulder at 246 mμ. When assayed using aldolase according to Blostein and Rutter (1963), a molecular weight of 567 ± 5 was obtained. Titration of the acid liberated by treatment with Dowex 50 (H^+) to pH 9.0 gave a molecular weight of 571 ± 5. Both of these values are in close agreement with the salt being a nonahydrate (mol wt 568). Drying at 100° for 70 hr resulted in the loss of 8.1 molecules of water based on mol wt 568. The dried $FruP_2$ had ϵ_{max} 1160 at 288 mμ. The aldolase assay showed that 7.1% of the $FruP_2$ was destroyed by this treatment.

D-*erythro-Pentulose 1,5-Diphosphate* (**8**). The dibarium salt of D-*erythro*-pentulose 1,5-diphosphate was purchased from Sigma Chemical Co., and converted into the sodium salt before use. Chromatography in solvent 2 revealed a compact blue spot and a trace of inorganic phosphate.

5,6-Dideoxy-D-*threo-hexulose 1-Phosphate* (**7**). 5,6-Dideoxy-D-*threo*-hexulose 1-phosphate (**7**) was prepared by a modification of the procedure of Lehninger and Sice (1955). Na_3-$FruP_2$ (2 g) was dissolved in 45 ml of water adjusted to pH 7.5 with 1 N sodium hydroxide. Aldolase (5 mg, specific activity 10 μmoles/min per mg of protein) and triose phosphate isomerase (4 mg) were added. After 15 min, a solution of 2.70 ml of propionaldehyde (37.5 mmoles) in 35 ml of water was added. The reaction was complete in 5.5 hr as estimated by the formation of base-labile phosphate (1 N sodium hydroxide, 20 min). The pH was adjusted to 5.6 with Dowex 50 (H^+) and the solution was concentrated *in vacuo* at 50°. The residue was dissolved in 20 ml of water, and 1 g of cyclohexylammonium sulfate, 2 ml of cyclohexylamine, and 200 ml of 1-butanol were added. Vigorous shaking produced a homogeneous solution which was stored at −10° overnight. After filtration to remove inorganic salts, 200 ml of ethyl ether was added, and the solution was stored at 4°, resulting in the deposition of 1.83 g of crystalline material. Chromatography in solvent 2 revealed a major component, R_F 0.54, and traces of $FruP_2$ (R_F 0.22) and inorganic phosphate, R_F 0.50. Neither contaminant interferes with the spectrophotometric analysis of the compound. When assayed with aldolase in the system of Blostein and Rutter (1963), it gave 1 molar equiv of **6** per mole of base-labile phosphate. The compound has a nuclear magnetic resonance spectrum compatible with the assigned structure (Figure 4).

1,5-Dihydroxy-2-pentanone 1,5-Diphosphate (**9**) *and 1,3-Dihydroxy-2-propanone Phosphate* (**6**). 1,5-Dihydroxy-2-pentanone 1,5-diphosphate (**9**) and 1,3-dihydroxy-2-propanone phosphate (**6**) were prepared essentially according to published procedures (Hartman and Barker, 1965; Ballou and Fischer, 1956) except that phosphorylation was accomplished with cyanoethylphosphoric acid–dicyclohexylcarbodiimide (Tener, 1961) rather than diphenyl chlorophosphate. The preparation of 1,3-dihydroxy-2-propanone phosphate is representative.

Acetyl-1,3-dihydroxy-2-propanone dimethyl ketal (4 mmoles) and 8 ml of a 1 M solution of cyanoethylphosphoric acid in pyridine and water were combined and evaporated *in vacuo* to remove the water. The residue was dissolved in 30 ml of anhydrous pyridine and 4 g of dicyclohexylcarbodiimide was added. After remaining at room temperature for 20 hr, water (5 ml) was added and the reaction mixture was concentrated *in vacuo* to remove solvent. Barium hydroxide (16 mmoles) was added in 100 ml of hot water; the reaction was refluxed for 1.5 hr, cooled, and filtered. A concentrated solution of cyclohexylammonium sulfate in water was added to the filtrate until the precipitation of barium sulfate was complete, and, after filtration, the filtrate was concentrated *in vacuo* at 50°. The residue was extracted with absolute ethanol and the extract was concentrated to a crystalline mass which was filtered and washed thoroughly with acetone to yield 2.4 mmoles of pure dicyclohexylammonium 1,3-dihydroxy-2-propanone phosphate dimethyl ketal. Conversion into the free ketone was accomplished as described by Ballou and Fischer (1956).

Methyl α- *and* β-D-*Fructofuranoside 1,6-Diphosphate* (**15**) *and* **14**). These glycosides were prepared from the free acid form of D-fructose 1,6-diphosphate rather than from its calcium salt as described by Schlubach and Bartels (1939) and were isolated by fractional crystallization of their brucine salts. The composition of the α:β mixture was determined by dephosphorylating with alkaline phosphatase and separating the resulting α- and β-methyl fructofuranosides by gas chromatography of the silyl derivatives.

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Purification and Properties of Nucleoside Triphosphate–Adenosine Monophosphate Transphosphorylase from Beef Heart Mitochondria*

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ABSTRACT: A simple method for preparing mitochondrial nucleoside triphosphate–adenosine monophosphate transphosphorylase, purified about 40-fold, is described. The enzyme seems to be fairly stable under different conditions, but optimum activity occurs at pH 8.5. Ethylenediaminetetraacetic acid, glutathione, cysteamine, and high inorganic phosphate concentrations have no effect on its activity. The molecular weight of the enzyme is estimated to be about 52,000, and an ultraviolet spectrum is given. The reversible incorporation of [γ-^{32}P]guanosine triphosphate and [^{14}C]guanosine triphosphate shows that the enzyme forms a nucleotide–enzyme complex and not a phosphorylated enzyme intermediate. K_M and K_i values for different substrates show that the enzyme is nonspecific toward nucleoside triphosphates. However, there is a specificity for adenosine monophosphate. The substrate specificity, expressed in V_{max}, is determined by assay and by calculation from kinetic studies. In the direction of adenosine monophosphate phosphorylation all substrates tested inhibit competitively. Adenosine diphosphate and guanosine diphosphate are noncompetitive inhibitors in the reverse reaction. The enzyme is stimulated by metal ions, optimally by 4.0×10^{-3} magnesium. The nucleoside triphosphate–adenosine monophosphate transphosphorylase seems to follow a sequential type of reaction mechanism in both directions. Two active sites of the enzyme (for nucleotide alone and for nucleotide magnesium complex) are proposed.

A large number of nucleotides in their different phosphorylated stages were shown to occur in mitochondria (Siekevitz and Potter, 1955; Heldt, 1966). Only the adenosine nucleotides seem to be immediate products of the oxidative phosphorylation. Most of the other nucleotides are derived from these by coupled phosphorylation at the substrate level (Slater and Holton, 1953; Sanadi *et al.*, 1954, 1955; Heldt *et al.*, 1964; Rossi and Gibson, 1964; Lardy *et al.*, 1965). One of the most important steps in nucleotide phosphorylation is carried out by the nucleoside phosphate kinases (Strominger *et al.*, 1954; Lieberman *et al.*, 1954; Herbert *et al.*, 1955; Gibson *et al.*, 1956).

In mitochondria, over 90% of the endogenous AMP is phosphorylated by these enzymes (Slater and Holton, 1953). The adenosine-specific adenylate kinase (EC 2.7.4.3) can be substituted by the nonspecific nucleoside triphosphate–adenosine monophosphate transphosphorylase (EC 2.6.4.d) which uses other nucleoside triphosphates as substrates for the phosphorylation of AMP. It was described and partially purified by Gibson *et al.* (1956), Strominger *et al.* (1954, 1959), Heppel *et al.* (1959), and Chiga *et al.* (1961), from cell homogenates. Heldt and Schwalbach (1967) showed that the enzyme is located in mitochondria.

In this report the purification, some properties, and the kinetics of the mitochondrial NTP–AMP transphosphorylase are described. The enzyme is compared with the mitochondrial adenylate kinase and nucleoside triphosphate–nucleoside diphosphate kinase (EC 2.7.4.6). All three enzymes seem to be most important in the regulation of the mitochondrial adenosine nucleotide level.

Experimental Procedure

Reagents. Nucleotides, substrates, and most enzymes used were obtained from C. F. Boehringer & Soehne, GmbH, Mannheim, Germany. The hexokinase, type III (EC 2.7.1.1), was a preparation from Sigma Chemical Co., St. Louis, Mo.; the NTP–NDP kinase used occurred in a sufficient amount as an impurity in this same preparation. [γ-^{32}P]GTP was prepared by Dr. H.-W. Heldt, Department of Physical Biochemistry, University of Munich, and was generously donated for these experiments.

Protein Determination. The protein content in the suspension of mitochondria and in the enzyme extracts was deter-

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