Fructose 1,6-Diphosphate. Acidic Dissociation Constants, Chelation with Magnesium, and Optical Rotatory Dispersion*

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ABSTRACT: Fructose 1,6-diphosphoric acid has pK_3' and pK_4' values separated by about 0.8. The values change little from 25 to 40° but vary sharply with both ionic strength and [Na$^+$]. Association constants with Mg^{2+} are near 500 for the tetrabasic anion and 130 for the tribasic anion, determined by titrimetry at ionic strengths near 0.08. The compound has at least two Cotton effects in its optical rotatory dispersion, which change with ionization or chelation with metals. These observations, together with the absorption spectra, support the conclusion that the ions exist mostly in the open chain form, with a ketone structure probable for the dibasic anion and an enediol structure possible for the tetrabasic anion and the chelates. Treatments of the titration data are described that permit the use of standard least-squares programs for digital computers in calculating the 1:1 association constants for cations with dibasic acids, using titration data obtained with nearly equivalent concentrations of metal and ligand. Sample calculations with the constants obtained show that the behavior of fructose 1,6-diphosphatase with variations in pH and [Mg^{2+}] cannot be explained on the basis of simple metal–substrate chelation.

The activity of fructose 1,6-diphosphatase varies in a complex fashion with alterations in the concentrations of H$^+$ and Mg^{2+} (McGilvery, 1964). Interpretation of this phenomenon requires estimates of the acidic dissociation constants and the formation constants for the magnesium chelate of fructose 1,6-diphosphate (FDP).[1] Kiessling (1934) reported values of 1.52 and 6.31 for pK'. These are evidently averages of the first and last pairs, because even a crude titration shows a pronounced inflection near pH 4 after addition of 2 equiv of base. This paper reports more nearly correct values for pK_3' and pK_4' and gives some information from measurements of optical rotatory dispersion and absorption spectra bearing on the structure of FDP.

Experimental Procedures

Preparation of FDP. The procedure used hinges on a complete charge of Dowex 1 with FDP, thus eliminating contaminants with fewer ionizable groups. All steps were conducted at 0 to 4°. Commercial monobarium FDP was precipitated twice with EtOH (McGilvery, 1953), dissolved in water, and converted to the acid by passage over Dowex 50-X8-H$^+$.[2] The solution was adjusted to pH 2.5 with NaOH, diluted to 0.2 M FDP, and immediately passed through a column of Dowex 1-X8-formate, 100–200 mesh, having an exchange capacity of 1.7 equiv/mole of FDP. The column was washed with 1.6 bed volumes of 0.007 M formic acid and 2 bed volumes of water, the effluent containing all of the P$_i$ in the original preparation. Perchloric acid (1 M) was passed into the column, and effluent fractions containing FDP, estimated with anthrone reagent (Mokrasch, 1954), were quickly brought to pH 3.0 with solid BaCO$_3$. Monobarium FDP was precipitated with EtOH from the combined fractions and reprecipitated twice; yield: 55–60% by weight from the original commercial material. Hydrolysis of FDP occurred during the elution with HClO$_4$, and the final product had 5–6 mmoles of P$_i$/mole of FDP. Analytical chromatography of the least pure preparation on Dowex 1-formate (L. M. Hall, unpublished procedure) showed trace quantities of other anthrone-reacting material spread over the fractions in which hexose monophosphates appear. The total maximum absorbancy of these fractions after heating with anthrone was less than 0.007 of that from the FDP fractions. Analysis by Dr. L. M. Hall of a similar preparation gave a barium content corresponding to BaH$_2$FDP·3H$_2$O.

The stock compound described above was refractionated for this study, with 0.003 M HClO$_4$ used for rinsing the column after charging wih FDP. The final product contained, per mole of FDP: 6.0 mmoles of

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[1] The abbreviation FDP is used to designate D-fructose 1,6-diphosphate. This abbreviation is combined to show specific ions and salts of the compound. Thus, MgHFDP$^-$ is the magnesium chelate of the tribasic anion, H$_2$FDP^{2-} is the dibasic anion, FDP^{4-} is the tetrabasic anion, and so on. G6P represents glucose 6-phosphate.

[2] The ionic charge used is listed last with each form of Dowex. Unless specified otherwise, each kind of Dowex was recycled once and fines were discarded before use.

P_i measured by a modified Fiske–Subbarow method; <0.03 mmole of glucose 6-phosphate (G6P) measured with glucose-6-phosphate dehydrogenase; 0.84 mmole of F6P measured with phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. Equivalent weight by the titrations reported here was 99.47% of the theoretical for the trihydrate without correction for the known content of P_i, and there were no detectable monobasic ions.

Modified Fiske–Subbarow Method. We have had increasing difficulty with the classical method, including a loss of the biphasic kinetics of color development necessary for good reproducibility and crystallization of the color reagent in the reaction mixture. No trouble has yet appeared with this modified procedure: to a 3-ml sample add, with mixing after each addition, 1.5 ml of 20 μM $CuSO_4$ in 1 M H_2SO_4, 1.0 ml of 15 mg of ammonium molybdate tetrahydrate/ml, and 0.5 ml of 1.2 mg of 1-amino-2-naphthol-4-sulfonic acid/ml (Eastman) in 72 mg of $NaHSO_3$/ml, read at 700 mμ after 20 min at 25°. Deviation from Beer's law is approximately 3% over the range to 1.5 μmole of P_i, but replicates check within ±0.001 absorbance (±1.7 nanomole), permitting accurate standardization.

Measurement of pH. The pH of samples was calculated from the potential between Corning No. 476022 glass and No. 476002 calomel electrodes, measured with a Beckman Model GS pH meter which was selected for stability through the courtesy of Phipps and Bird, Inc., and was used in an area shielded with grounded aluminum foil. The efficiency of the electrodes changed less than 0.003, and the potential of a phosphate standard less than 0.5 mv over a period of 1 month. Primary standards were NBS 0.05 *m* phthalate, 0.05 *m* phosphate, and 0.01 *m* borax solutions (Bates, 1962). Secondary standards, prepared from Mallinckrodt reagents, gave the same potential within 0.05 mv. The Mallinckrodt Na_2HPO_4 and KH_2PO_4 gave identical quadruplicate P_i analyses per nominal mole.

The over-all efficiency of the electrodes was 0.9973 between the phthalate and Borax standards, 0.9924 between the phosphate and phthalate, and 1.0033 between the phosphate and Borax, all at 25°. These efficiencies were confirmed at different ranges on the Beckman potentiometer and with the Corning pH meter. Similar values were obtained at other temperatures.

The pH of the test samples was calculated according to the NBS convention (Bates, 1962) using the appropriate electrode efficiencies. The electrodes were equilibrated in phosphate buffer at the proper temperature for 12 hr in advance to minimize temperature hysteresis of the calomel electrode. Temperatures were determined with a 0–100° thermometer graduated at 0.1°, which gave readings of 0.15° for the ice point and 32.50° for the transition point of $Na_2SO_4 \cdot 10H_2O$.

Titration of FDP. A solution of 1 mmole of monobarium FDP was quantitatively passed through a 13 × 30 mm column of Dowex 50-X8, which had been recycled with NaOH and HCl until the effluent had negligible absorbance above 240 mμ and finally charged with HCl to a negative flame test for Na^+. The effluent was collected directly in a titration vessel, and 2 mmoles of NaOH was added immediately after the sample passed through the column to diminish hydrolysis during the rinses of the column. Water rinse was continued until the total volume was 250 ml. Titrations and electrode standardizations were made with magnetic stirring under washed nitrogen in completely jacketed vessels, using Scientific Industries Co. Model 200 Ultra-burets, which are the mercury-displacement type of 7-ml capacity readable to 0.0001 ml. By cementing the ground joints, calibrating at 0.5-ml intervals, and temperature control to ±0.1°, a precision of ±0.0003 ml was attained. Measurements of pH were made with the solution at rest and with the buret tip lifted from the solution. Otherwise, the sample |1 M NaOH:Hg:steel electrode represented by the buret created a large spurious potential.

Titrations were made with 50-μmole increments of 1 M NaOH or HCl, standardized to 2 parts in 10,000 against potassium hydrogen phthalate. After a preliminary complete titration of H_4FDP, later titrations were made only of Na_2H_2FDP. At 25 and 30° the samples were titrated with NaOH, followed by titration with HCl. NaCl (4.5 mmoles) was added, titration with NaOH repeated, and the sample returned to the starting point with a single addition of HCl. The titration and return were repeated after each of two further additions of 12.5 mmoles of NaCl. At 35° it was necessary to eliminate the first titration with HCl and titrate with HCl after the final increment of NaCl, so as to decrease the number and duration of exposures to the more extreme pH values. At 40°, the procedure was further shortened by alternate titrations with NaOH and HCl after additions of NaCl. Even so, the appearance of P_i during the titrations, which was 0.4 μmole at 25°, increased to 4.2 μmoles at 40°. The disadvantage in reverse titration with HCl arose from incomplete reversal of the drift in electrode response that occurred during the previous exposure above pH 9, but this was accepted as less of an error than that resulting from more extensive hydrolysis of the sample.

To estimate p*K'* values at varying concentrations of FDP, solutions were made by adding 6.75, 7.50, and 8.25 mmoles, respectively, of NaOH to 2.5-mmole batches of H_4FDP, obtained as above and diluted to ~0.025 M. The pH was measured after successive dilutions with water.

Solutions containing 1 mmole of FDP, 15 mmoles of NaCl, and 0.502, 1.046, or 2.092 mmoles of $MgCl_2$ were titrated with NaOH for estimation of the formation constants for the chelates. The $MgCl_2$ was standardized to 1 part in 500 with NaOH and trisodium EDTA.

Optical Rotatory Dispersion and Absorption Spectra. Measurements of optical rotatory dispersion were made with a 5-cm cell in a Durrum–Jasco recording spectropolarimeter[3] at a scan of 4 mμ min^{-1}. In all cases re-

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[3] Available through the courtesy of Dr. Paul Schatz of the Department of Chemistry.

ported here the final concentration of FDP was 0.01 M, and NaCl was added as required to give an ionic strength of 0.1. Varying amounts of NaOH, $MgCl_2$, or $CaCl_2$ were used. A solution containing 5 mM $MnCl_2$ developed a fine haze, which was removed by centrifugation before measurement. No detectable pellet formed, but higher concentrations of $MnCl_2$ gave gross precipitates and were not used. Measurements were also made with higher concentrations of Na_4FDP than those reported here, but with no qualitative differences. For comparison, measurements were made of 0.01 M D-fructose and 0.01 M L-sorbitol, with and without 0.02 M $MgCl_2$. Absorption spectra were run in the Cary Model 14 at a scan speed of 1 mμ sec^{-1}, using 0.05 M solutions of Na_2H_2FDP, Na_3HFDP, Na_4FDP, and Na_4FDP containing $MgCl_2$.

Calculations

The Apparent Acidic Dissociation Constants. Hand calculation of pK_3' and pK_4' gave values separated by about 0.8 and sufficiently close to 7 to be able to neglect the concentrations of H^+ and OH^- in the stoichiometry without introduction of significant error. It was also evident that the precision of the data warranted complete treatment. Hence, the moles of titrant were corrected for the known P_i in the sample, using the data of Bates (1962, 1964). An exact end point was calculated from the first estimates of the constant. The equation for the titrations was cast in the form:

$$\frac{\mathrm{H}^2 f}{K_3'(2-f)} - \frac{\mathrm{H}(1-f)}{(2-f)} - K_4' = 0 \qquad (1)$$

in which $\mathrm{H} = [\mathrm{H}^+]$, and f = moles of NaOH added/mole of Na_2H_2FDP. This equation can be treated as $y = ax + b$, and solved by linear least squares. Two solutions are possible, in which a,b are $1/K_3', K_4'$ or $K_3', K_3'K_4'$.

The data were programmed for the Burroughs 5000 computer,[4] and both solutions were calculated for: (1) the 28–31 points on each titration with $0.24 < f < 1.78$, (2) the 12–14 points with $0.33 < f < 1.00$, and (3) the 10–14 points with $1.00 < f < 1.67$.

Estimates of pK_3' and pK_4' at varying concentrations of FDP (see Experimental Procedures) were made by hand calculation from the three points obtained.

The Formation Constants for the Magnesium Chelates. It is desirable to have a more rigorous solution for the 1:1 chelate formation constants than the approximations hitherto used. If the titration in the presence of Mg^{2+} is completely described by the expressions for K_3', K_4', the formation constant for $MgFDP^{2-}$, and the formation constant for $MgHFDP^-$, neglecting $[H^+]$ and $[OH^-]$, then the seven simultaneous equations and eight unknowns (p. 107 in Chaberek and Martell, 1959) can be reduced without further assumptions or approximations to the form

$$AK_\alpha^2 + BK_\beta^2 + CK_\alpha K_\beta + DK_\beta + EK_\alpha = 0 \qquad (2)$$

in which

$$K_\alpha = \frac{[\mathrm{MgFDP}^{-2}]}{[\mathrm{Mg}^{2+}][\mathrm{FDP}^{4-}]}$$

$$K_\beta = \frac{[\mathrm{MgHFDP}^{-}]}{[\mathrm{Mg}^{2+}][\mathrm{HFDP}^{3-}]}$$

$$A = (2-f)\left\{M\left[\frac{2\mathrm{H}^2}{K_3'K_4'} + \frac{\mathrm{H}}{K_4'}\right] - G\left[\frac{f\mathrm{H}^2}{K_3'K_4'} - (1-f)\frac{\mathrm{H}}{K_4'} - (2-f)\right]\right\}$$

$$B = (1-f)\left[\frac{\mathrm{H}}{K_4'}\right]^2\left\{M\left[\frac{\mathrm{H}^2}{K_3'K_4'} - 1\right] - G\left[\frac{f\mathrm{H}^2}{K_3'K_4'} - (1-f)\frac{\mathrm{H}}{K_4'} - (2-f)\right]\right\}$$

$$C = \frac{\mathrm{H}}{K_4'}\left\{M\left[(4-3f)\frac{\mathrm{H}^2}{K_3'K_4'} + (1-f)\frac{\mathrm{H}}{K_4'} - (2-f)\right] - G(3-2f)\left[\frac{f\mathrm{H}^2}{K_3'K_4'} - (1-f)\frac{\mathrm{H}}{K_4'} - (2-f)\right]\right\}$$

$$D = -\frac{\mathrm{H}}{K_4'}\left[\frac{\mathrm{H}^2}{K_3'K_4'} - 1\right]\left[\frac{f\mathrm{H}^2}{K_3'K_4'} - (1-f)\frac{\mathrm{H}}{K_4'} - (2-f)\right]$$

$$E = -\left[\frac{2\mathrm{H}^2}{K_3'K_4'} + \frac{\mathrm{H}}{K_4'}\right]\left[\frac{f\mathrm{H}^2}{K_3'K_4'} - (1-f)\frac{\mathrm{H}}{K_4'} - (2-f)\right]$$

In these terms, f and H are as in eq 1, M is the total concentration of magnesium, and G is the total concentration of FDP.

An iterative least-squares program for the computer was adapted for eq 2, using 17 points from each of the three titrations. Relative differences were minimized by two of the five possible methods: (1) with term E as the dependent variable, and (2) with term D as the dependent variable. A first approximation was made by both methods from rough estimates of the ionic strength and the resultant estimated values for K_3' and K_4'. A better estimate of ionic strength was then possible in arriving at the second approximation.

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[4] Mrs. Frances Ferguson, statistician of the Computer Science Center, kindly made the programs and gave valuable advice.

TABLE I: Apparent Dissociation Constants of Fructose 1,6-Diphosphate.[a]

Temp. (°C)	Titrant	[FDP]	[Na$^+$]	μ	pK_3'	pK_4'	$\frac{1}{2}$ [pK_3' + pK_4']	Observed pH
25	NaOH	0.00395	0.0118	0.0237	6.220	7.056	6.638	6.636
	HCl	0.00392	0.0157	0.0274	6.157	7.042	6.600	6.599
	NaOH	0.00389	0.0369	0.049	6.052	6.873	6.462	6.460
	NaOH	0.00383	0.091	0.103	5.893	6.671	6.282	6.281
	NaOH	0.00377	0.144	0.155	5.826	6.559	6.192	6.190
	[b]	0.00143	0.0043	0.0086	6.324	7.190	6.757	6.753
	[b]	0.00282	0.0085	0.0169	6.261	7.101	6.681	6.678
	[b]	0.00420	0.0126	0.0252	6.223	7.055	6.639	6.630
	[b]	0.00555	0.0167	0.0333	6.193	6.997	6.595	6.593
	[b]	0.0082	0.0246	0.0492	6.140	6.929	6.534	6.529
	[b]	0.0122	0.0366	0.073	6.092	6.854	6.473	6.469
	[b]	0.0161	0.0483	0.097	6.055	6.799	6.427	6.422
	[b]	0.0238	0.071	0.143	5.994	6.724	6.359	6.355
30	NaOH	0.00395	0.0118	0.0237	6.269	7.104	6.686	6.688
	HCl	0.00392	0.0157	0.0274	6.194	7.091	6.642	6.639
	NaOH	0.00388	0.0368	0.0485	6.075	6.906	6.490	6.490
	NaOH	0.00383	0.090	0.102	5.910	6.695	6.302	6.303
	NaOH	0.00377	0.143	0.154	5.810	6.584	6.197	6.196
35	NaOH	0.00395	0.0118	0.0237	6.272	7.100	6.686	6.687
	NaOH	0.00388	0.0369	0.0485	6.073	6.913	6.493	6.493
	NaOH	0.00383	0.091	0.103	5.905	6.704	6.304	6.305
	HCl	0.00380	0.0142	0.153	5.801	6.594	6.197	6.197
40	NaOH	0.00395	0.0118	0.0237	6.286	7.127	6.706	6.707
	HCl	0.00392	0.0333	0.0450	6.092	6.959	6.526	6.526
	NaOH	0.00389	0.085	0.096	5.935	6.745	6.340	6.341
	HCl	0.00385	0.0136	0.148	5.836	6.619	6.228	6.227

[a] All of the listed data are for the point in the titrations when fructose 1,6-diphosphate is present as the trisodium salt. [b] Values with no titrant listed were obtained by dilution of three solutions of fixed composition. See the text for details.

Results

Apparent Acidic Dissociation Constants. The results obtained from calculations with $0.24 < f < 1.78$ are listed in Table I, in which [total FDP], [Na$^+$], and the ionic strength are given for the midpoints of the titrations. The listed pK' values are negative logarithms of the mean constants obtained by the two methods of calculation, although the difference was in no case great enough to change pK' more than 0.001. The calculated pH at the midpoint and the value interpolated between the two closest points on the titration curve are also given. The values at 25° with no titrant listed are those obtained by dilution of more concentrated FDP solutions, and the greater error from these three-point calculations is shown by the greater discrepancy between the calculated and observed midpoint pH. Standard errors are meaningless owing to the nature of eq 1, but some guide to the validity of the expression is provided by the correlation coefficients for the titrations, which varied from a low of 0.99986 to a high of 0.999991.

The results of calculations over a limited range of j are not shown. In general, titration below the midpoint gave lower values for pK_3' and higher values for pK_4' than did titration above the midpoint. The worst scatter was obtained from the third titration listed at 40°, in which the respective values from below the midpoint, the complete range, and above the midpoint were 5.936, 5.935, 5.951 for pK_3', and 6.755, 6.745, 6.730 for pK_4'.

Association Constants for the Magnesium Chelates. Results by the two methods of calculation from the titrimetric data are shown in Table II. The change in pH at the midpoint resulting from the additions of $MgCl_2$ was 0.107, 0.188, and 0.311, respectively, for the three concentrations listed. It may be seen from the table that relatively small errors in the estimation of the acidic dissociation constants will thus cause large changes in the apparent association constants for the chelates.

Optical Rotatory Dispersion of FDP and the Magnesium Chelate. Figure 1 shows the rotatory dispersion of a solution containing Na_2H_2FDP with 0.2 equiv of NaOH added to diminish hydrolysis during measure-

TABLE II: Apparent Association Constants for Mg^{2+} and Fructose 1,6-Diphosphate.[a]

Approximation	Total [FDP]	Total [Mg]	$[Na^+]$	μ	pK_3'	pK_4'	Method 1 K_α	Method 1 K_β	Method 2 K_α	Method 2 K_β
1	0.00395	0.00207	0.071	0.079	5.959	6.760	549	146	553	138
	0.00370	0.00387	0.070	0.072	5.978	6.787	612	155	644	165
	0.00331	0.00693	0.066	0.072	5.975	6.784	587	132	596	134
2	0.00395	0.00207	0.071	0.080	5.949	6.743	448	132	457	129
	0.00370	0.00387	0.070	0.077	5.956	6.754	479	134	506	143
	0.00331	0.00693	0 066	0.071	5.974	6.775	569	131	577	134

[a] Values obtained by titrimetry at 25°. Concentrations at the midpoint of the titrations are listed. The estimated values for ionic strength and acidic dissociation constants used in the first approximation were corrected on the basis of the resultant chelate association constants to obtain the values used in the second approximation. The methods of calculation are described in the text. $K_\alpha' = [MgFDP^{2-}]/[Mg^{2+}][FDP^{-4}]$. $K_\beta' = [MgHFDP^-]/[Mg^{2+}][HFDP^{3-}]$.

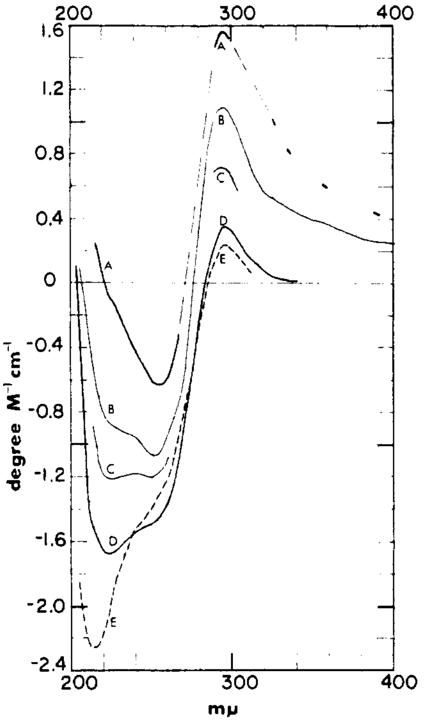

FIGURE 1: Optical rotatory dispersion of D-fructose 1,6-diphosphate and the magnesium chelate. (A) 0.01 M Na_4FDP + 0.02 M $MgCl_2$ (the heavy lines are the measured sections, and the light broken line is only to indicate continuity); (B) 0.01 M Na_4FDP + 0.01 M $MgCl_2$; (C) 0.01 M Na_4FDP + 0.004 M $MgCl_2$; (D) 0.01 M Na_4FDP; (E) 0.01 M Na_2H_2FDP + 0.002 M NaOH. The rotations are given in degrees/M/cm to be comparable to absorbancy measurements.

ment (curve E), Na_4FDP (curve D), and Na_4FDP with increasing amounts of $MgCl_2$ (curves C, B, and A). The last solutions ought to contain 24, 50, and 72%, respectively, of the FDP as $MgFDP^{2-}$. The rotatory dispersion was unexpectedly complex. The shoulder near 255 mμ with Na_4FDP was partially lost in Na_2H_2FDP, whereas it became the prominent feature of the magnesium chelate, owing to the loss of the trough at 223 mμ. Solutions with intermediate amounts of NaOH added gave curves intermediate between curves D and E. Above 400 mμ plain curves were obtained. At the concentration used Na_4FDP had too little rotation compared to the noise level to be significant in the visible region. In an experiment not shown, 0.08 M excess NaOH was added to 0.01 M Na_4FDP, and scattered points were quickly measured by hand drive. The points fitted a plain curve resembling those obtained with fructose or sorbitol.

For comparison, the measurements shown in Figure 2 were made with the manganese chelate, and with sorbitol and fructose. The Mn chelate had a rotatory dispersion similar to that of the Mg chelate, except for an additional small peak at 470 mμ. Sorbitol and fructose gave only plain curves to the limit of the instrument (205 mμ with 5 cm of aqueous solution). Addition of $MgCl_2$ to sorbitol or fructose gave slightly more negative rotations, but no change in the qualitative nature of the curves.

Replacement of $MgCl_2$ with $CaCl_2$ gave the same qualitative result, but of a smaller magnitude consistent with the smaller association constants expected from analogy with other phosphate compounds (Smith and Alberty, 1956).

Ultraviolet Absorption Spectra. The spectra are shown in Figure 3. Absorptions continued to rise at lower wavelengths than those illustrated, and a continued rise was measured with more dilute solutions to the limit of the spectrophotometer near 190 mμ. The value of these results near the vacuum ultraviolet is clouded by the discovery that the neutralized effluent from the same batch of Dowex 50 used in preparing the FDP solutions, but with $BaCl_2$ used as a sample, had an absorption beginning at 214 mμ and rising sharply at shorter wavelengths. This absorption was considerably less than that of the FDP solutions, and not detectable

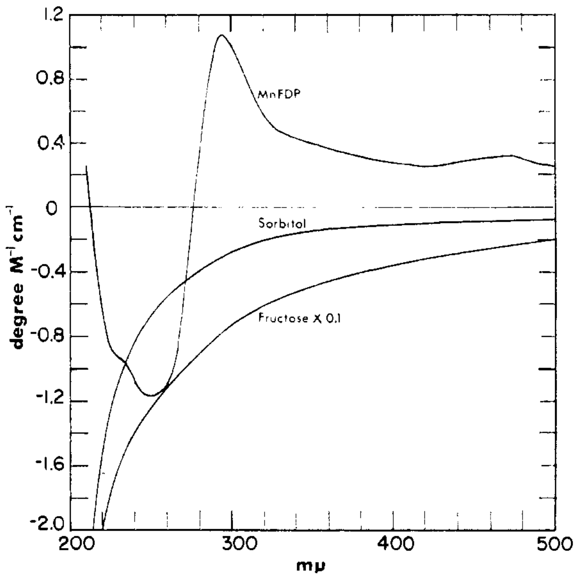

FIGURE 2: Optical rotatory dispersion of the manganous chelate of D-fructose 1,6-diphosphate, of L-sorbitol, and of D-fructose. The hexose derivatives were all 0.01 M. The FDP solution contained 0.0049 M $MnCl_2$. The rotations of the fructose solution were ten times the values plotted.

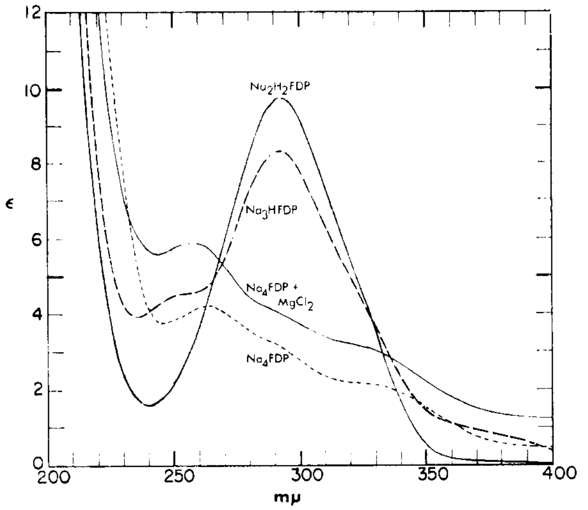

FIGURE 3: Absorption spectra of the ionic species of D-fructose 1,6-diphosphate and the magnesium chelate. All solutions were 0.05 M to FDP; 0.042 M $MgCl_2$ was present in the case indicated.

above 215 mμ, thus not affecting the results of Figure 3. However, since Dowex columns were used at all stages of preparation of the FDP, the extent of this spurious response at lower wavelengths is unknown.

Good quantitative measurements of absorption spectra of the Ca and Mn chelates could not be made because of the formation of precipitates at the concentrations needed for adequate absorbance. However, the liquid phase from a mixture 0.05 M to Na_4FDP and 0.025 M to $MnCl_2$ had a detectable broad absorption in the visible region with double maxima ($A = 0.070$ with 1 cm lightpath) near 454 and 472 mμ, corresponding to the region of the peak in the rotatory dispersion curve.

Discussion

The Equilibrium Constants. It ought to be remembered that the constants reported here are, like the pH scale, pragmatic values, useful to the degree that they can aid quantitative treatment of related phenomena dependent upon the same parameters.[5] The measurements were deliberately made in the presence of varying amounts of Na^+, despite the unknown extent of association of FDP with this ion, because solutions containing varying amounts are most likely to be used in biochemical experimentation. It is hoped sufficient information is given here to permit useful extrapolations for other conditions. For example, plots of pK_3' and pK_4' against μ at 25° give two curves, depending upon whether μ was varied by addition of NaCl or by changing the concentration of FDP. A plot of pK_4' against $[Na^+]$ gives a single smooth curve, but not of pK_3'. Extrapolation of all of these plots to zero concentration gives approximate values of 6.425 for pK_3 and 7.276 for pK_4.

In addition to these qualifications, there is the inherent error of the determinations themselves. With the methods used, the error in the acidic dissociation constants increases at each temperature with increasing μ for the titrations and decreases for the values obtained by dilution. The error certainly increases with temperature, owing to the hydrolysis of FDP. Experience with prediction of pH with the values makes it likely that the error in the pK' values at 25° is generally less than ±0.005. However, it may be as high as ±0.02 at 40°.

The values for the chelate association constants are considerably more shaky, owing to their small magnitude and the resultant small differences in titration with and without the metal present. No really satisfactory experimental method has been found for the case of dibasic acids with small differences in pK'. The practice of adding large excesses of the metal ion has the advantage of increasing the changes in pH and simplifying the calculations, but the overwhelming disadvantages of necessitating a very dubious extrapolation of the acidic dissociation constants to fit the new conditions, as is brought out by the data reported here, of limiting the possible range of titration because of precipitation of metallic hydroxides, and of increasing the error caused by any formation of 2:1 chelates. The values reported here probably are of interest primarily in establishing the range of concentrations of ionic species

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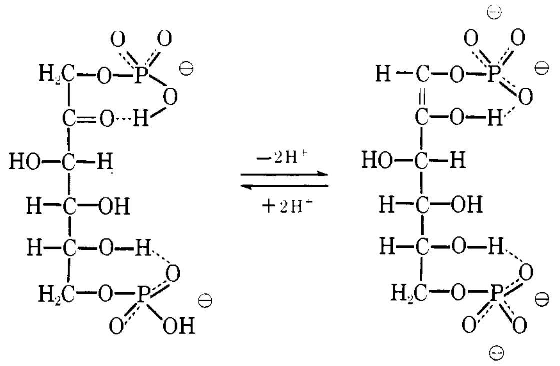
[5] Bates (1964) has an excellent discussion of the problems in applying strict theory to the pH scale and related measurements.

to be expected in a given circumstance. For example, a solution at 25° and pH 7.0 containing 0.001 M each of FDP and $MgCl_2$ with sodium salts to bring the ionic strength to 0.077 would be expected to contain 48% of the total FDP as FDP^{4-}, 28% as $HFDP^{3-}$, 2.5% as H_2FDP^{2-}, 19% as $MgFDP^{2-}$, and 3.0% as $MgHFDP^{-}$. If the pH was raised so as to completely ionize FDP, the concentration of the chelate would still only represent 26% of the total FDP. It is apparent that the behavior of fructose 1,6-diphosphatase with variations in $[H^+]$ and $[Mg^{2+}]$ cannot be explained only on the basis of the formation of the Mg chelates of the substrate, because the corresponding variation in enzymatic activity is severalfold greater than the resultant changes in chelate concentration. The involvement of the protein as a primary ligand for the metal is more likely. Parenthetically, it may be noted that qualitative reasoning concerning other enzymes activated by divalent metallic ions may be unsatisfactory. Even with association constants an order of magnitude larger than those reported here, the concentrations of free substrate and metal ion are not negligible when the total concentrations of each are near 0.001 M.

The Structure of FDP. It is clear from the two Cotton effects and the absorption spectra that a large fraction, perhaps most, of the ionic species of FDP and its chelates does not exist in the furanose form. An exact statement of the quantity from these measurements alone is not possible because of the lack of values for aqueous solutions of related compounds with fixed ketone or furanose structures. It is also clear that FDP^{4-} and $MgFDP^{2-}$ contain yet a different structure. Stabilization of the open chain form may occur through hydrogen bonding. The spectrum of H_2FDP^{2-} is consistent with a ketone structure, and the shift of the peak absorption to a lower wavelength and the development of a more complex spectrum with FDP^{4-} may be a result of formation of a substituted 1,2-enediol. This would also explain the instability of FDP^{4-} manifested by development of a yellow color in solutions standing a few hours at room temperature. Examination of molecular models[6] shows that structures of these types are possible



The loss of the Cotton effect in more alkaline solutions might be a result of ionization of the enediol. Although it is possible to make tetrahedral configurations of four oxygen atoms with the enediol structure, speculation about the structure of the Mg chelate is not warranted with the information at hand.

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[6] E. Leybold's Nachfolger kindly made and donated tetrahedral models for P.