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Proportion of Keto and Aldehydo Forms in Solutions of Sugars and Sugar Phosphates*

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ABSTRACT: A number of aldoses and ketoses have been examined in deuterium oxide and aqueous solution by infrared, ultraviolet, and circular dichroic spectroscopy. Only infrared spectroscopy in deuterium oxide is a reliable index of the proportion of the free carbonyl tautomer present in solution. Ultraviolet spectra are due principally to impurities and circular dichroism spectra cannot be interpreted quantitatively. The percentages of keto or aldehydo forms in the sugars tested are D-fructose, <0.4; D-fructose 1-phosphate, <1.0; D-fructose 6-phosphate, 2.5; D-glucose 6-phosphate, <0.4; D-glyceraldehyde, 4.4; DL-glyceraldehyde 3-phosphate, 4.4.

Reducing sugars, *i.e.*, carbohydrates having a potentially free aldehydo or keto function, are capable of existing in aqueous solution in several tautomeric forms and as a hydrate. A stable enol form has been suggested also for D-fructose 1,6-diphosphate (McGilvery, 1965). The proportions of these forms are important to considerations of the mode of action of enzymes that use the reducing sugars as substrates and to the chemistry of the substances in water. The phosphorylated monosaccharides are of particular importance. Gray and Barker (1970) have presented nuclear magnetic resonance and infrared evidence that D-fructose 1,6-diphosphate exists in deuterium oxide solution in a furanose form, probably β, and that less than 2% is in the free keto form. Similarly, D-*glycero*-D-*altro*-octulose 1,8-diphosphate exists entirely in ring form(s). Several acyclic ketose phosphates were examined also and found to exist predominantly in the keto rather than the hydrated (gem diol) form, thus making improbable the presence of acyclic structures in the case of D-fructose 1,6-diphosphate.

Avigad *et al.* (1970) examined a number of ketoses, ketose phosphates, and substituted ketoses using ultraviolet and circular dichroic spectroscopy. These authors concluded that aqueous solutions of D-fructose 1-phosphate, D-fructose 6-phosphate, and D-fructose 1,6-diphosphate may contain up to 20% of the acyclic forms; presumably these are the keto form. They did not consider the possibility of hydrated forms which would not be detected by these methods. This difference between the two studies is sufficiently large to warrant further examination of the ketoses in solution.

The experimental difficulty in these studies lies in finding an analytical method that allows the determination of relatively small amounts of free keto forms. Chemical methods can all be suspected of perturbing the system and the methods of choice are physical methods. In the nuclear magnetic resonance spectra of the ketoses the signals due to the keto forms can only be identified in the simplest cases, *e.g.*, 1,3-dihydroxy-2-propanone phosphate (Gray and Barker, 1970). Ultraviolet absorptions in the 260- to 280-nm region cannot be assigned unequivocally to be carbonyl function and can be enhanced enormously by small amounts of impurities, *e.g.*, D-fructose 1,6-diphosphate (Gray and Barker, 1970). Circular dichroic spectra may be more reliable because they depend on a chromophore associated with an asymmetric center but impurities that might be present in reducing sugar preparations, such as osazones, could contribute to ellipticities. Infrared spectroscopy in deuterium oxide solution allows observation of the carbonyl absorption and only impurities with a carbonyl group interfere with the analysis. Large molar extinction coefficients for this region as compared to the ultraviolet ($n-\pi^*$) lead to greater sensitivity for the detection of carbonyl groups in the infrared. If the molar extinction coefficient of a suitable model of known per cent keto form can be obtained the per cent keto form for similar compounds can be estimated readily as shown by Gray and Barker (1970) in their study of several acyclic ketose phosphates. The infrared method was shown to agree well with the nuclear magnetic resonance analyses when discrimination between keto and hydrated keto forms is possible by this latter technique.

Proton magnetic resonance spectroscopy can be used to advantage in examining aldoses for the presence of aldehyde forms; *e.g.*, Trentham *et al.* (1969) have examined D-glyceraldehyde 3-phosphate and Horton and Wander (1971) have studied a number of acetylated *aldehydo*-aldoses.

Materials and Methods

Infrared spectra were obtained on a Perkin-Elmer Model 521 grating spectrometer at 25 ± 2°. Samples dissolved in deuterium oxide solution were placed in a 0.05-mm calcium

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TABLE I: Ultraviolet, Infrared, and Circular Dichroism Parameters for Several Sugars and Sugar Phosphates and the Percentage of Keto Form Present in Solution.

Compound	Ultraviolet		Circular Dichroism		Infrared		% Keto	
	λ_{max} nm ± 1 (0.05 M)	ϵ	λ_{max} nm ± 2 (0.15–0.50 M)	$[\theta]$	$\bar{\nu}_{max}$ cm^{-1} ± 1 (0.3 M)	ϵ	*a*	*b*
Fru	281.5	0.84	275	+29.5	None		0	0
Fru[c]	277	0.38	272	+30.4				
Fru-1-P	274	5.30	279	+29	None		0	0
Fru-6-P	286	6.20	275	+182	1724	11.5	5.4	2.5
Fru-1,6-P_2[d]	278	2.7			1730	<3.5	<1.7	<0.8
Glu-6-P	None		272	+0.17	None		0	0
Gly	273	63	291	−46	1729	20.4	9.8	4.4
Gly[c]	273	5.0	291	−47	1729	20.5	9.8	4.4
DL-Gly-3-P	None				1708	20.5	9.8[e]	4.4
1,3-Dihydroxy-2-propanone-P[d]	268	15.4			1735	115	55	25
							63[f]	
Acetone[g]	265	17.3			1696	460		100

[a] Calculated from the infrared absorbance at 1700–1735 cm^{-1} assuming ϵ = 211 for the carbonyl group in deuterium oxide (Gray and Barker, 1970). [b] Calculated from the infrared absorbance at 1700–1735 cm^{-1} assuming ϵ = 460 (acetone) for the carbonyl group in deuterium oxide. Samples passed over a small column containing dry charcoal–Celite (1:1, v/v). [d] Data from Gray and Barker (1970). [e] A shoulder at 1680 cm^{-1} that accounted for 25% of the total absorbance in the carbonyl region was subtracted. [f] Calculated from the proton magnetic resonance spectrum, Gray and Barker (1970). [g] The proton magnetic resonance spectrum shows only one strong signal indicating the very low concentration of hydrated form.

fluoride cell that had been balanced over the region 1600–1900 cm^{-1} against a similar cell containing deuterium oxide. Solutions were nominally 0.3 M and spectra were obtained with a spectral slit of 1.5 cm^{-1}.

Ultraviolet spectra were obtained at 25 ± 2° on a Cary Model 17 spectrometer using quartz cells with a 1-cm light path. Circular dichroism spectra were obtained on a Cary Model 60 with a 6001 CD attachment using a 1-cm path-length cell.

Sugar phosphates purchased from Boehringer und Söhne, GmbH, Mannheim, Germany, were shown to be pure within the limits of thin-layer chromatography using microcrystalline cellulose plates and methanol–ammonia–water (7:2:1, v/v) or isobutyric acid–ammonia–water (66:1:33, v/v) as developers. Phosphate esters were detected with acidic molybdate (Hanes and Isherwood, 1949) and reducing sugars using alkaline silver nitrate.

Fructose, purchased from Pfanstiehl, Waukegan, Ill., gave a single spot on paper chromatography in ethyl acetate–pyridine–water (10:4:3, v/v). Glyceraldehyde was purchased from Sigma Chemical Co., St. Louis, Mo., and gave a single spot on chromatography in the same system.

All solutions had pH values between 6.5 and 7.5.

Results

The wavelengths of the ultraviolet, infrared, and circular dichroic maxima and the corresponding extinction coefficients for the compounds tested are given in Table I and in Figure 1 are shown their infrared spectra over the region 1300–1900 cm^{-1}.

Discussion

The data in Table I demonstrate that there is no quantitative correspondence between adsorptions in the ultraviolet or infrared and the observation of a circular dichroism spectrum. An absorption in the infrared region between 1650 and 1750 cm^{-1} does show that a free keto or aldehydo form is present in solution or that there is a contaminating carboxylic acid or ester present. The absence of an absorption in this region, however, demonstrates the absence of free keto or aldehydo forms. On this basis we can conclude that D-fructose, D-fructose 1-phosphate, D-fructose 1,6-diphosphate, and D-glucose 6-phosphate have no, or only trace amounts of, free keto or aldehydo forms in solution. The infrared method would detect less than 1% of these forms. Since it was previously shown (Gray and Barker, 1970) that the keto forms of ketose phosphates usually predominate over the hydrated keto forms it is improbable that acyclic structures are important in solutions of these compounds.

The proportion of hydrated keto form present in deuterium oxide solutions can be estimated in certain cases from proton magnetic resonance spectra. Protons adjacent to the carbonyl function absorb at lower field in the free keto form than in the hydrated form (Gray and Barker, 1970). Similar observations have been made by others for the aldehydo and hydrated (aldehydrol) forms of aldoses (Wolfrom *et al.*, 1965; Horton *et al.*, 1968; Trentham *et al.*, 1969; Horton and Wander, 1971). The ultraviolet absorption decrease following mixing of simple aldehydes with water or deuterium oxide proved satisfactory (Pocker and Meany, 1965; Fridovich, 1966; Naylor and Fridovich, 1968) for estimation of the relative proportions

of aldehyde and hydrate. These techniques cannot always be used. The proton magnetic resonance spectra of monosaccharide phosphates are complex and small proportions of aldehydo forms may be observed only with time averaging. Ultraviolet spectra are not a sufficiently specific indicator of the carbonyl structure and small proportions of degraded materials can cause large increases in absorption. This was shown to be the case with D-fructose 1,6-diphosphate where mild treatment with resins resulted in significant changes in the 270- to 290-nm region (Gray and Barker, 1970). The infrared method is specific and the approximate proportion of keto form can be estimated from the extinction coefficient observed for a series of ketose phosphates (200–211) (Gray and Barker, 1970) and acetone (460). For D-fructose 1-phosphate, D-glucose 1-phosphate, and D-fructose 1,6-diphosphate the appropriate standard is 1,5-dihydroxy-2-pentanose 1,5-diphosphate, while acetone is a proper standard for D-fructose 6-phosphate, D-glyceraldehyde, and D-glyceraldehyde 3-phosphate. Substitution of a phosphate group α to the carbonyl group decreases the carbonyl absorption intensity by a factor of two whereas substitution at more distant sites has little effect. It should also be noted that the carbonyl absorption intensity is relatively insensitive to the changes in mechanical coupling (frequency) such as would occur in a homologous series (Jones and Sandorfy, 1956).

The discrepancies between the methods are well illustrated by experiments with D-glyceraldehyde. As obtained from the supplier it is an orange-brown syrup and in deuterium oxide has $\epsilon_{273\ nm} = 63$ and $\epsilon_{1729\ cm^{-1}} = 20.4$ corresponding to 4.4% aldehydo form. Treatment with charcoal results in a decrease in $\epsilon^{M}_{273\ nm}$ to 5 but has no effect on $\epsilon_{1729\ cm^{-1}}$. The circular dichroism spectrum is not altered by this treatment, indicating that in this case the principal ultraviolet absorbing substance is not asymmetric.

This was also the case for D-fructose where charcoal treatment reduced only the ultraviolet spectrum; the circular dichroism spectrum and the optical rotatory dispersion curves were unaltered.

The circular dichroism data presented in Table I agree with those presented by Avigad *et al.* (1970) for the same sugars except for the magnitude of the molar ellipticity, $[\theta]$, for D-fructose 6-phosphate, D-glucose 6-phosphate, and D-glyceraldehyde. The ultraviolet absorption data show less agreement, with differences in λ_{max} for D-fructose, D-fructose 1-phosphate, D-fructose 6-phosphate, D-glyceraldehyde, and 1,3-dihydroxy-2-propanone phosphate, and large differences in ϵ for D-fructose 1,6-diphosphate, D-glucose 6-phosphate, D-glyceraldehyde, and 1,3-dihydroxy-2-propanone phosphate. The fact that charcoal treatment reduces ultraviolet absorption but not the carbonyl content of D-glyceraldehyde nor the rotatory power and ellipticity of D-glyceraldehyde and D-fructose indicates that the infrared and circular dichroism values may both be indicating the presence of free carbonyl groups. The ellipticity value cannot be easily interpreted in terms of percent carbonyl, however, since it is sensitive to the configuration and substitution at asymmetric centers close to the carbonyl. The observation of a circular dichroism spectrum then may serve as a useful qualitative test for keto forms. If the infrared data for D-fructose 6-phosphate are taken as accurately indicating the proportion of keto form present in solution then the circular dichroism data for D-fructose and D-fructose 1,6-diphosphate indicate very low proportions of keto form present in solutions of the latter compounds.

Our observation that DL-glyceraldehyde 3-phosphate has approximately 4.4% of the aldehydo form at 25° agrees well with the observation of Trentham *et al.* that DL-glyceraldehyde 3-phosphate has approximately 4% of the aldehydo form by proton magnetic resonance analysis. These authors also found that D-glyceraldehyde 3-phosphate is 3.3% in the form that serves as a substrate for D-glyceraldehyde 3-phosphate dehydrogenase; *i.e.*, the aldehydo form.

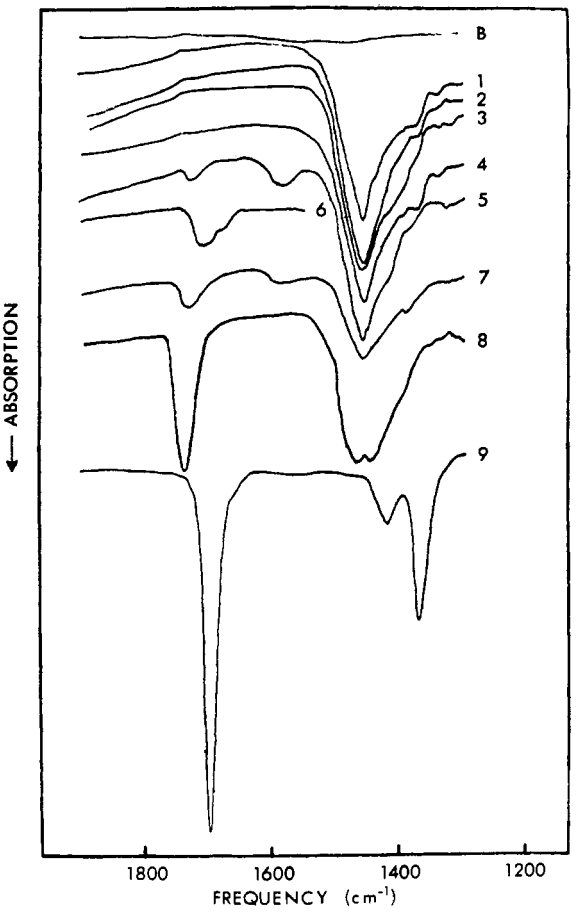

FIGURE 1: Infrared spectra in the 1300- to 1900-cm^{-1} region measured in 0.05-mm CaF_2 cells at approximately 0.3 M in D_2O solutions: Base line (B), D-fructose (1), D-fructose 1-phosphate (2), D-glucose 1-phosphate (3), D-fructose 1,6-diphosphate (4), D-fructose 1-phosphate (5), DL-glyceraldehyde 3-phosphate (6), D-glyceraldehyde (7), 1,3-dihydroxy-2-propanone phosphate (8), acetone (9).

The data presented here clearly demonstrate that ultraviolet spectroscopy is not a reliable method for estimating the proportion of carbonyl forms of sugars in aqueous solution. The percentages of keto form we obtained are in most cases lower than those obtained by ultraviolet spectroscopy (Avigad *et al.* 1970; McGilvery, 1965). Our results, which we feel are based on appropriate models, are not likely to be in error by more than a factor of two. Given this factor we still cannot reconcile our results with the literature values for the fructose phosphates which are higher by more than an order of magnitude. Furthermore, we have demonstrated for D-fructose 1,6-diphosphate and D-glyceraldehyde that highly absorbing impurities invalidate the ultraviolet method. Thus we are convinced that the infrared region with its higher molar extinction coefficients for carbonyl groups (200–500 *vs.* <30 for ultraviolet region) gives more consistent results and is the method of choice.

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Preferential Uptake of D-Glucose by Isolated Human Erythrocyte Membranes*

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ABSTRACT: A sensitive method for the measurement of the stereospecific uptake of D-glucose by isolated human erythrocyte membranes has been developed. The method is based on the difference in uptake of L-[^{14}C]glucose and D-[^{3}H]glucose as measured by the retention of radioactivity by the membrane preparation. Detection of the preferential D-glucose uptake activity was found to be dependent on the presence of $(NH_4)_2SO_4$ and the uptake increased with increasing concentrations of this neutral salt. This effect of $(NH_4)_2SO_4$ could not be attributed to either ammonium or sulfate ions *per se* or to a restoration of D-glucose transport activity. Properties of the D-glucose uptake activity have been studied. The uptake was reversible and did not involve any chemical alteration of D-glucose. Changes of pH (5.7–8.8) and fragmentation of the erythrocyte membrane did not greatly affect the uptake; all D-glucose uptake activity was lost upon incubation at 50° or by boiling the membrane preparation for 5–10 min. The apparent dissociation constant for the D-glucose–membrane complex was 2.9×10^{-5} to 4.6×10^{-5} M at 0° and increased as the temperature was increased. From the temperature dependence, it was calculated that the apparent enthalpy of dissociation of the complex was 3.3 kcal/mol. In the presence of a finite concentration of substrate, the maximum capacity for D-glucose uptake varied from 394.5 to 519.1 pmoles per mg of membrane protein and was independent of temperature (0–37°). The D-glucose-uptake activity was shown to be closely associated with glucose transport in erythrocytes since it exhibited the following properties characteristic of this carrier-mediated transport system. (a) The uptake was specific for the D isomer of glucose. (b) Phloretin, a competitive inhibitor of erythrocyte monosaccharide transport, inhibited the uptake. (c) The uptake activity was inhibited by monosaccharides known to be competitive inhibitors of D-glucose transport in human erythrocytes; the degree of inhibition paralleled the apparent affinities of the monosaccharides for the glucose transport system. (d) Saturation of D-glucose uptake occurred with increasing concentrations of D-glucose. (e) The uptake activity was inhibited by sulfhydryl reagents previously reported to inhibit D-glucose transport. Evidence suggesting that the uptake activity represents the stereospecific binding of D-glucose to the erythrocyte membrane rather than the transport of the sugar into a vesicle lumen is discussed.

Of all the mammalian transport systems, that of monosaccharide transport in the erythrocyte has been studied the most extensively (LeFevre, 1954, 1961a; Wilbrandt and Rosenberg, 1961; Stein, 1967). The kinetic data accumulated on this process have generally been interpreted to indicate that the translocation of monosaccharides across the cell membrane does not occur by simple diffusion but involves an obligatory reversible association of the sugar with a membrane component during transport. However, the reactions essential to this carrier-mediated transport of monosaccharides have not been identified.

All models of carrier-mediated transport assume the first step to be the specific binding of the permeant to an active site on the extracellular surface of the cell membrane (Stein, 1967; Pardee, 1968). Within recent years several permeant-binding proteins associated with bacterial membrane transport systems have been isolated and characterized (Pardee, 1968; Heppel, 1967, 1969; Roseman, 1969). However, parallel studies in mammalian cells have not been reported. Several attempts to identify the glucose-binding component of erythrocyte membranes have been described. LeFevre *et al.* (1964) have demonstrated that phospholipids extracted from the

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