Ring Opening and Closing Rates for Thiosugars

C. E. Grimshaw,[1] R. L. Whistler,[2] and W. W. Cleland*[1]

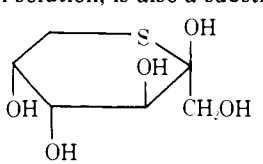
Contribution from the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706, and the Department of Biochemistry, Purdue University, Lafayette, Indiana 47907. *Received August 25, 1978*

Abstract: Reaction of 4,4′-dipyridyl disulfide (4PDS) with thiols requires the RS^- form, and shows a Brønsted β_{nuc} value of 0.34. The second-order rate constants for the process are 6.6×10^4, 4.6×10^6, and 6.9×10^7 M^{-1} s^{-1} for reaction of mercaptoethanol (pK = 9.5) with the neutral, mono-, and diprotonated forms of 4PDS. With 2,2′-dipyridyl disulfide (2PDS) the corresponding rate constants are 5.6×10^4, 1.5×10^8, and 1.7×10^{10} M^{-1} s^{-1}. The increased reactivity of the protonated forms of 4PDS and 2PDS permits the dipyridyl disulfides to be used as reagents for thiols over the pH range −1 to 9. Ring opening and closing rates and the proportion of the sugar with a free SH group have been measured for several thiosugars by reaction with 4PDS, 2PDS, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Ring opening and closing are base catalyzed from pH 6 to 10, and at lower pH values a pH-independent rate is seen. For most thiosugars, base-catalyzed ring opening shows k_B = 40–150 M^{-1} s^{-1}, but the value for 6-thio-D-fructose is 17 200 M^{-1} s^{-1}. The pH-independent rate for 6-thio-D-fructose is also 180 times that for 5-thio-D-glucose. Mutarotation of 5-thio-α-D-glucopyranose ($[\alpha]^{25}_D$ +215°) to the equilibrium mixture (80% α and 20% β; $[\alpha]^{25}_D$ +188°) is base catalyzed at high pH, acid catalyzed below pH 1, and pH independent between pH 1 and 3, with the base-catalyzed and pH-independent rates being 520 times faster than and roughly equal to, respectively, the corresponding ring-opening rates. Base-catalyzed mutarotation is postulated to proceed through intermediates in which the sulfur has a negative charge and forms an induced dipolar bond with the carbonyl carbon of the aldehyde. Rotation of the aldehyde group and ring closure produce mutarotation, while ring opening, as measured by reaction with disulfide reagent, requires movement of the sulfur away from the aldehyde so it can react. 6-Thio-β-D-fructopyranose is converted to the β-furanose form at a rate sufficient for it to be a substrate for fructokinase (which is specific for the β-furanose) with a V_{max} the same as that of D-fructose and a K_m of 6–10 mM, although at high enzyme levels ring opening becomes rate limiting. The disulfide of 6-thio-D-fructose and 4-mercaptopyridine (which should exist 82% as the β-furanose) has a V_{max} 16% that of D-fructose and a K_m of 51 μM, the lowest known for this enzyme; the calculated K_m values for the β-furanose forms of this disulfide and D-fructose are identical. By assuming the K_m of the β-furanose of 6-thio-D-fructose also to be the same as that of D-fructose, and the relative amounts of the anomeric forms with a free SH group to be the same as with D-fructose, 6-thio-D-fructose is calculated to contain 0.6% β-furanose, 0.11% α-furanose, and 0.02% acyclic form. At pH 7 the ring-opening rate for the β-furanose is 0.19 s^{-1}, while the ring-closing rates to β-furanose and β-pyranose are 6 and 9 s^{-1}. Equilibration between the furanoses of 6-thio-D-fructose, and presumably also of D-fructose, is thus rapid (estimated half-time 1.2–3.5 s at 25 °C).

Fructokinase catalyzes the reaction

$$\text{D-fructose} + \text{MgATP} \rightleftharpoons \text{MgADP} + \text{D-fructose 1-phosphate} \quad (1)$$

and has been shown to be specific for the β-furanose anomer of D-fructose.[3] 6-Thio-D-fructose, which exists largely as the β-pyranose form in solution, is also a substrate for fructokinase,[4] suggesting that the β-pyranose is in equilibrium with a small amount of the enzymatically active β-furanose anomer, and that the thiopyranose ring can open at a rate sufficient to permit substrate activity. We have confirmed this by using the reaction of 4,4′-dipyridyl disulfide (4PDS) with free thiols[5] to study the ring-opening rate for 6-thio-β-D-fructopyranose. In addition, we have investigated the reaction of 4PDS and 2,2′-dipyridyl disulfide (2PDS) with thiols as a function of pH and the pK of the thiol. These results permit the calculation of the apparent ring-closing rate and thus the proportion of thiosugar existing with a free thiol group at equilibrium (which can be verified by direct observation).



6-thio-β-D-fructopyranose

We have also examined the ring-opening and ring-closing rates for several other thiosugars by this method. In particular, comparison of the ring-opening and mutarotation rates for 5-thio-D-glucose leads to some interesting conclusions concerning the mechanisms of both processes.

Materials and Methods

6-Thio-D-fructose,[4] 5-thio-D-fructose,[6] 5-thio-D-xylose,[7] 4-thio-D-arabinose,[8] 4-thio-D-ribose,[9] 4-thio-D-xylose,[10] and 1,6-dithiodulcitol[11] were prepared by published procedures and 5-thio-D-glucose was purchased from Pfanstiehl or Sigma. 4,4′-Dipyridyl disulfide (4PDS) was purchased from Aldrich. 2,2′-Dipyridyl disulfide (2PDS), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and all other biochemicals were purchased from Sigma. 6-Thio-D-fructofuranose-4-mercaptopyridine disulfide was prepared by reacting 10 μmol of 4PDS with 9.5 μmol of 6-thio-D-fructose in a volume of 10 mL at pH 7.0 until there was no further change in absorbance at 324 nm. Excess 4PDS was not removed since it did not inhibit fructokinase.

Reactions of thiols with 4PDS, 2PDS, and DTNB were conducted in quartz cuvettes of 1-cm path length with a buffer concentration of 30 mM and were followed spectrophotometrically at 324, 343, and 412 nm, respectively. Reactions were monitored using a Beckman DU monochrometer with deuterium lamp, a Gilford optical density converter, and a 10-mV recorder with adjustable zero and multispeed drive. The cell compartment was thermostated at 25 °C. The rates were calculated using the literature values for the extinction coefficient and dissociation constant for 4-thiopyridone (ϵ_{mM}^{324nm} 19.8 at pH 7.2,[5] pK = 8.83[12]), 2-thiopyridone (ϵ_{mM}^{343nm} 7.06 at pH 7.2,[5] pK = 9.97,[12] and 5-thio(2-nitrobenzoic acid) dianion (ϵ_{mM}^{412nm} 13.6 at pH 7.0[13]). Under conditions where the disulfide showed substantial absorbance at the normal wavelengths, reactions were followed at 340, 380, and 475 nm, with 4PDS, 2PDS, and DTNB, respectively, using the extinction coefficients calculated from end-point assays of a stock solution of mercaptoethanol. Buffers used were glycylglycine (pH 8.5 and 9), HEPES[14] or TES (pH 7.5 and 8), PIPES or MOPS (pH 7), MES (pH 6), acetate (pH 4 and 5), citrate (pH 3), phosphate or glycine–HCl (pH 2), and aqueous solutions of HCl (pH 0–2) and H_2SO_4 (pH < 0[15]). A buffer composed of equimolar HEPES, glycylglycine, CHES, and CAPS was used for the 6-thio-D-fructose and 5-thio-D-glucose reactions with both 4PDS and DTNB at pH 7.5 and above. Solutions of 4PDS were calibrated using the extinction coefficient ϵ_{mM}^{247nm} 16.3 at pH 7.2.[5] Thiol, 2PDS, and DTNB solutions were calibrated from end-point assays with excess 4PDS, mercaptoethanol, and mercaptoethanol, respectively. Concentrated solutions of DTNB (100 mM) were prepared by dissolving the mixed Na^+/K^+ salt, recrystallized from methanol, in water. Distilled, deionized water

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was used in all solutions. The pH was measured using a Radiometer Model 26 pH meter with a GK2321C glass electrode standardized at pH 4, 7, and 10, with buffers purchased from Beckman, and at pH 1.10 with 0.100 M HCl.[16]

The concentrations of sugar and (in parentheses) of 4PDS, 2PDS, or DTNB, used in initial velocity experiments for determination of ring-opening rates and half-saturation constants, follow: 6-thio-D-fructose, 30–50 μM (0.05–1.5 mM); 5-thio-D-fructose, 30 μM (3–120 μM); 5-thio-D-xylose and 5-thio-D-glucose, 300 μM, except 6.7–40 mM 5-thio-D-glucose used below pH 3 (0.2–1.8 mM, except 6 and 36 mM 4PDS and DTNB, respectively, at pH <3 and 7.7, respectively); 4-thio-D-xylose, 20 μM (2–15 μM); 4-thio-D-arabinose, 300 μM (2–15 μM); 4-thio-D-ribose, 250 μM (2–15 μM). General acid catalysis of ring opening and mutarotation for 5-thio-D-glucose was measured at constant ionic strength (0.5 M) over the range 0.1–1.0 M total buffer concentration using either a 1:1 or 4:1 acid:salt mixture of formic, acetic, or cacodylic acids, or of imidazole. The pH varied less than 0.1 unit over this concentration range. Ionic strength was maintained when necessary with KCl. 4-Thiosugars were preincubated in dilute solution at the desired pH and temperature to ensure equilibration of all anomeric forms, and 4PDS was added last to start the reaction. In all other cases, thiol or thiosugar was added last to initiate the reaction.

Values for percent free SH group were calculated by extrapolation to zero [4PDS] of the burst observed upon addition of 4PDS to a solution of thiosugar. Similar experiments with 5-thio-D-glucose showed an impurity at 0.05% levels which could be removed by pretreatment with a trace of 4PDS. Pretreated 5-thio-D-glucose solutions were used for all kinetic experiments, excluding mutarotation measurements. For measurement of the relative rates of thiosugar reaction with 4PDS and the 4-thiosugar-4-mercaptopyridine disulfide, a 20-fold excess of sugar (200–300 μM) over 4PDS (10–15 μM) was employed.

Mutarotations of 5-thio-D-glucose were measured using a 20-mg/mL solution in 30 mM buffer at the desired pH with a Perkin-Elmer Model 141 spectropolarimeter. Reactions were conducted in a 1.0-dm water-jacketed cell maintained at 25 °C. Mutarotation rates were calculated from least-squares fits to the equation

$$(\alpha_0 - \alpha_t) = (\alpha_0 - \alpha_\infty)(1 - e^{-k_{mut}t}) \quad (2)$$

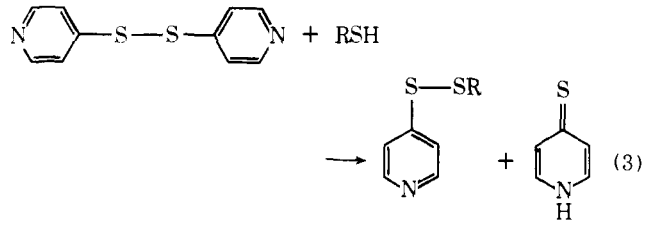
where α_0, α_t, and α_∞ are the observed optical rotations at zero time, t, and equilibrium, respectively. Crystalline 5-thio-D-glucose is the α anomer with a specific rotation $[\alpha]^{25}_D$ +215°.[17] At anomeric equilibrium the specific rotation is $[\alpha]^{25}_D$ +188°, and, since 270-MHz ^{1}H NMR studies show that the equilibrium mixture is 80% α and 20% β anomer, the calculated specific rotation of the β anomer is $[\alpha]^{25}_D \approx +80°$.

Fructokinase was purified according to the procedure of Raushel and Cleland.[18] Enzymatic assays were run in 1.0-cm cuvettes containing 50 mM buffer, 167 μM NADH, 1 mM phosphoenolpyruvate, 4 mM ATP, 100 mM KCl, 5 mM $MgCl_2$, 167 μM dithiothreitol (except when 6-thio-D-fructose-4-mercaptopyridine disulfide was the substrate), 30 units pyruvate kinase, and 50 units lactate dehydrogenase in 3.0-mL volume. Enzyme was added to start the reaction and the disappearance of NADH at 340 nm (ϵ_{mM}^{340nm} 6.30[19]) used to calculate the initial velocity. Blanks containing no enzyme were run in all cases and used to correct the observed initial velocities. For the phosphorylation of 6-thio-D-fructose-4-mercaptopyridine disulfide, coupling enzymes were added first to remove traces of ADP, fructokinase was added next, followed by 67 μM 4PDS to react with dithiothreitol present in the enzyme solution, and finally substrate was added to start the reaction.

For statistical analysis, data were fitted to the equations in the text by the least-squares method assuming equal variances for the velocities,[20] and using the Fortran programs of Cleland.[21] Spectral titrations of 4PDS and 2PDS were performed on a Cary 118 spectrophotometer equipped with a repetitive scan accessory.

Results and Discussion

Reaction of 4PDS and 2PDS with Thiols. The reaction of free thiols with 4PDS by disulfide interchange to generate 4-thiopyridone can be followed at 324 nm (eq 3). The comparable reaction with 2PDS can be followed at 343 nm. The use of 4PDS as a thiol reagent has several advantages over the use of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[22] The product 4-thiopyridone has an extinction coefficient at neutral pH which is 1.5-fold higher than that of the 5-thio(2-nitrobenzoic acid) dianion (19.6 vs. 13.6 × 10^4 M^{-1} cm^{-1}). In addition, the effective pH range for the 4PDS reaction (pH 1–9) is broader than that for DTNB (pH 5–9). The kinetics of the reaction of thiols with DTNB have recently been studied,[23,24] and, in order to determine the chemical basis for the difference in pH behavior of the two compounds, and to obtain rate constants for use in the following calculations, we have carried out a similar study of the reaction of thiols with 4PDS and 2PDS.



(3)

Table I. Rate Constants for Reaction of 4,4′-Dipyridyl Disulfide with Thiols at pH 7, 25 °C

thiol	pK	k_{obsd}, M^{-1} s^{-1}	$k \times 10^{-4}$, M^{-1} s^{-1}
mercaptoethylamine	8.4[a]	2300 ± 200	6.0 ± 0.5
L-cysteine	8.6[a]	1880 ± 70	7.7 ± 0.3
5-thio-D-sorbitol	9.25	690 ± 20	12.3 ± 0.4
1,6-dithiodulcitol	9.33, 10.3	650 ± 40	14.0 ± 0.9
1-thioglycerol	9.44	510 ± 20	14.1 ± 0.6
mercaptoethanol	9.5[b]	410 ± 30	13 ± 1
3-mercaptopropionic acid	10.3	145 ± 3	29.0 ± 0.6
2-mercaptosuccinic acid	10.6	87 ± 5	33 ± 2

[a] Reference 81. [b] Reference 23.

The second-order reaction of acyclic monothiols with 4PDS was assumed to follow the rate law

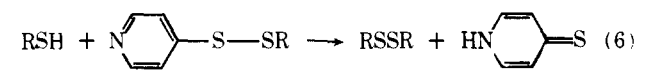
$$\frac{d[4TP]}{dt} = k[RS^-][4PDS] \quad (4)$$

where 4TP is 4-thiopyridone, and we assume that the contribution of the uncharged thiol to the observed rate is negligible. The observed rate will then be

$$\frac{d[4TP]}{dt} = k_{obsd}[RSH]_{total}[4PDS] = \frac{k}{(1 + [H^+]/K)}[RSH]_{total}[4PDS] \quad (5)$$

where $[RSH]_{total} = [RS^-] + [RSH]$ and K is the acid dissociation constant for the thiol. The apparent second-order rate constant, k_{obsd}, was obtained directly from the slope of $v_i/[RSH]_{total}$ plotted vs. [4PDS]. The values of k_{obsd} for 4PDS are listed along with the thiol pK values in Table I. The values calculated for k are also shown in Table I, and when log k is plotted vs. thiol pK, a linear Brønsted relationship[25] is seen, from the slope of which $\beta_{nuc} = 0.34 \pm 0.02$. Thus the reactivities of this set of organic thiolate anions toward 4PDS are proportional to their basicities. Similar values have been found for the reaction of thiolate anions with DTNB.[26]

Under conditions of excess thiol, a second, slower phase is observed corresponding to the reaction with the mixed disulfide of 4-mercaptopyridine (eq 6). The observed ratios of slow to



(6)

fast phase rate at pH 8 for mercaptoethanol (0.074), 1-thioglycerol (0.069), and 3-mercaptopropionic acid (0.019) are similar to the values seen with DTNB,[23] and show the effect of a negative charge from the mercaptopropionate in slowing

down the reaction with the mixed disulfide. Similar data were obtained at pH 8 for 4-thio-D-xylose (0.016), 4-thio-D-ribose (0.0056), and 4-thio-D-arabinose (0.0032). The steric bulk of a pyranose ring with all equatorial substituents has roughly the same effect as one negative charge, but axial hydroxyls on the ring (or an axial sulfur) clearly hinder attack on the disulfide.

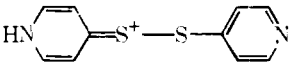
Explanation of the pH–Rate Profile for Reaction of 4PDS and 2PDS with Mercaptoethanol. Figure 1 is a plot of log k_{obsd} vs. pH for reaction of mercaptoethanol with 4PDS and 2PDS. 4PDS has two ionizable pyridine groups, and spectral titration over the pH range 1–8 showed two different isosbestic points, indicating a three-component mixture of neutral, mono-, and diprotonated species. Plots of absorbance vs. pH for the high pH (261 nm) and low pH (250 nm) isosbestic points gave simple titration curves with pK values from least-squares fits of 3.59 ± 0.02 and 5.055 ± 0.025. The rate data were fitted to the equation

$$k_{obsd} = (ax_1 + bx_2 + cx_3)/(1 + [H^+]/K) \qquad (7)$$

where x_1, x_2, and x_3 are the fraction of neutral, mono-, and diprotonated 4PDS present at a given pH (calculated from the pK values above), a, b, and c are the respective k values for the three forms, and K is the acid dissociation constant of mercaptoethanol. The resulting k values for neutral, mono-, and diprotonated 4PDS were 6.6 ± 0.7 × 10^4, 4.6 ± 0.5 × 10^6, and 6.9 ± 0.4 × 10^7 M^{-1} s^{-1}. Thus the first protonation causes a 70-fold increase in the rate, and the second one a 15-fold change.

A similar spectral titration of 2PDS over the pH range −1 to 7 gave pK values of 0.35 ± 0.02 (for the isosbestic point at 290 nm) and 2.37 ± 0.01 (at 320 nm). The rate data above pH 1 were fitted to eq 7 to yield k values of 5.3 ± 0.8 × 10^4, 1.5 ± 0.2 × 10^8, and 1.7 ± 0.3 × 10^{10} M^{-1} s^{-1}, for the various protonation states, corresponding to a 2800-fold increase and a 110-fold increase in rate upon mono- and diprotonation, respectively. The rate below the pK of 0.35 appears to be due to reaction of the thiol form of mercaptoethanol with diprotonated 2PDS with k_{RSH} = 25 M^{-1} s^{-1}. The k value for DTNB and mercaptoethanol (6.6 ± 0.9 × 10^4 M^{-1} s^{-1}) is comparable to that for neutral 4PDS and 2PDS.[29]

Advantages of 4PDS and 2PDS as Thiol Reagents. The increased rates that accompany protonation of 4PDS and 2PDS are explainable on the basis of the increasing positive charge on the sulfurs due to resonance forms such as i. Protonation



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of both rings not only enhances the electrophilic nature of the sulfurs, but also provides a better leaving group in the protonated thiopyridone. The contribution of such resonance forms is indicated by the spectra, since neutral 4PDS has a λ_{max} of 247 nm, while the doubly protonated species has a λ_{max} of 280 nm and 4-thiopyridone has a λ_{max} of 324 nm. Similarly, neutral 2PDS, diprotonated 2PDS, and 2-thiopyridone have λ_{max} values of 281, 297, and 343 nm, respectively.

The reasons why 4PDS and 2PDS are such good thiol reagents are thus both kinetic and thermodynamic. First, the expected drop in rate with drop in pH that results from the decreasing level of thiolate is largely offset by the enhanced reactivity of the disulfide when the pyridine rings become protonated. Figure 1 shows that 2PDS is better in this regard at pH <2.8 while 4PDS is the more reactive at higher pH. Second, release of a thiol which exists almost entirely as the tautomeric thiopyridone makes the reaction essentially irreversible.[30] Thus the pH range over which the reaction can be monitored is much larger than that for DTNB. The practical

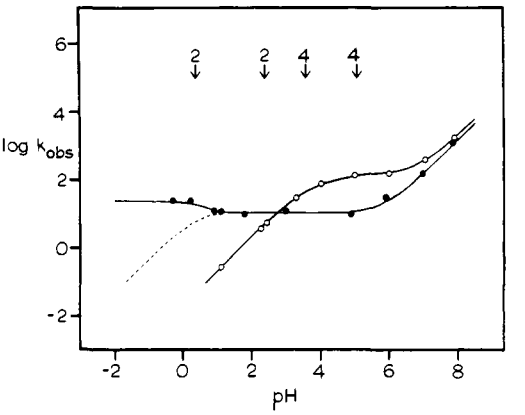
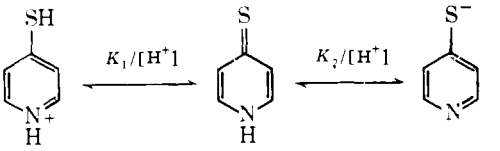


Figure 1. pH profile of rate of reaction of 2PDS (closed circles) and 4PDS (open circles) with mercaptoethanol in 30 mM buffer at 25 °C. The pK values indicated are those of 2PDS (2→) and 4PDS (4→). The curves are fits to eq 7; except for 2PDS it is also observed that free thiol reacts with diprotonated 2PDS with $k_{RSH} \simeq 25$ M^{-1} s^{-1}. The dotted curve shows the expected rate if k_{RSH} were zero.

limits are set by base-catalyzed decomposition (pH >10) on the high-pH side and protonation of the respective thiopyridone at low pH, since the protonated pyridine thiol does not have a spectrum appreciably different from the starting disulfide.



(8)

For 4PDS pK_2 = 8.83 and pK_1 = 1.43,[12] while for DTNB the analogous limits are set by base-catalyzed decomposition (pH >10) and the pK = 4.8 for protonation of the 5-thio(2-nitrobenzoic acid) dianion.[31,32] For 2PDS the range is even larger with pK_2 = 9.97 and pK_1 = −1.07.[12]

Reaction of Disulfides with Thiosugars. If the disulfide reacts only with free thiols, the following kinetic scheme can be used to describe the reaction between 4PDS, 2PDS, or DTNB and a thiosugar such as 5-thio-D-glucose:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow{k_3[C]} \text{products} \qquad (9)$$

where A is the thiopyranose, B is the total of acyclic and furanose forms, C is the disulfide, and k_2 and k_3 are apparent rate constants and do not refer to a particular species. Since the level of B is low, we can assume a steady state and

$$v_i = \frac{k_1[A][C]}{(k_2/k_3 + [C])} \qquad (10)$$

Thus when initial velocities are determined at a fixed level of 5-thio-D-glucose and at varying levels of 4PDS, 2PDS, or DTNB, $[A]/v_i$ can be plotted vs. $1/[C]$ to give a straight line with vertical intercept of $1/k_1$ and a horizontal intercept of $-(k_3/k_2)$ (for statistical analysis the data were fitted to eq 11, where $V = k_1$, $K = k_2/k_3$, and $v = v_i/[A]$[33]).

$$v = \frac{V[C]}{K + [C]} \qquad (11)$$

Similar experiments were performed with 6-thio-D-fructose, 5-thio-D-fructose, 5-thio-D-xylose, 4-thio-D-xylose, 4-thio-D-ribose, and 4-thio-D-arabinose. The temperature variation of k_1 for 5-thio-D-xylose and 6-thio-D-fructose at pH 7 corresponded to activation energies of 22 and 18 kcal/mol, respectively.

Table II. Comparison of pH-Independent and Base-Catalyzed Ring Opening and Mutarotation Rates[h]

thiosugar or sugar	ring-opening rate $k_0 \times 10^6$, s^{-1}	ring-opening rate $k_B \times 10^{-2}$, $M^{-1}\,s^{-1}$	mutarotation rate $k_0 \times 10^6$, s^{-1}	mutarotation rate $k_B \times 10^{-2}$, $M^{-1}\,s^{-1}$	percent anomers with a free SH: calcd	percent anomers with a free SH: obsd[e]
5-thio-α-D-glucopyranose	0.16	0.64	0.18	330.	0.0012[d]	<0.01
5-thio-α-D-xylopyranose	0.55	0.48	<12[a,g]	290.[a]	0.003	<0.01
5-thio-α-D-ribopyranose			<7[b,g]	400.[b]		
4-thio-D-arabinofuranose	2.5	0.42			0.12	0.11
4-thio-D-ribofuranose	4.5	0.78			0.47	0.29, 0.44
4-thio-D-xylofuranose	26.	0.94			3.5	2.0
5-thio-D-fructose	11.	1.5			0.06	
6-thio-β-D-fructopyranose	29.	172.			0.66,[d] 0.73[f]	0.42
D-glucose			390.[c]	4.8[c]		
D-fructose			3400.[c]	880.[c]		

[a] Calculated from the data in ref 44. [b] Calculated from the data in ref 45. [c] Calculated[83] from the data in ref 82. [d] From least-squares fit of experimental 1/[percent free thiol] vs. pH to a simple titration curve. [e] From burst observed upon addition of 4PDS. [f] From comparison of rates of reaction with infinite [4PDS] and of phosphorylation with infinite [fructokinase]. See Discussion. [g] See footnote 43. [h] Values calculated for 25 °C.

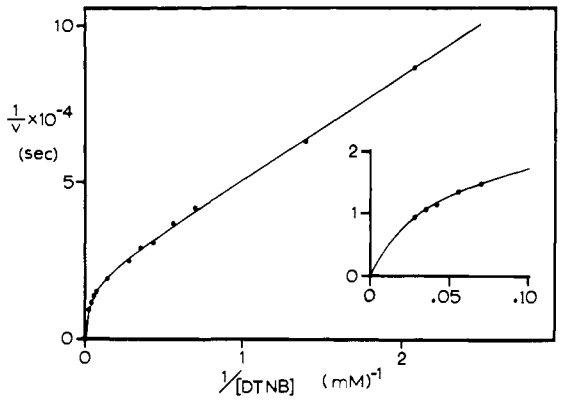


Figure 2. Double reciprocal plot for the reaction of 0.333 mM 5-thio-D-glucose with DTNB at pH 7.7 (100 mM TES), 25 °C and 0.250 M ionic strength. The curve is a fit to eq 13. When the data were fitted to an equation in which the line did not go through the origin, the vertical intercept was not significantly different from zero.

However, when high concentrations of DTNB at pH 7.7 or of 4PDS at pH 2.6 were used, the plot of [5-thio-D-glucose]/v_i vs. 1/[DTNB] or 1/[4PDS] was concave down and appeared to go through the origin (Figure 2), suggesting a direct reaction between the thiosugar, or an intermediate in equilibrium with it (see below), and the disulfide. Thus we must add the step

$$A \xrightarrow{k_3'[C]} \text{products} \qquad (12)$$

to eq 9 and the term k_3'[A][C] to eq 10. The resulting equation has the form

$$v = \frac{V[C]}{(K + [C])} + k_3'[C] \qquad (13)$$

where v, K, V, and [C] have the same meaning as above. From fits to eq 13, the value of k_3' at pH 2.6 with 4PDS (1.9×10^{-5} $M^{-1}\,s^{-1}$) is 1400-fold higher than would be expected from the value of k_3' found for DTNB at pH 7.7 (1.7×10^{-3} $M^{-1}\,s^{-1}$), assuming that 4PDS and DTNB react with an ionized form of the thiosugar so that k_3' decreases an order of magnitude per pH unit, and that the rate is the same for DTNB and neutral 4PDS. Since the reaction of diprotonated 4PDS with a thiolate is 1100-fold faster than the reaction of neutral 4PDS, it seems likely that the pH variation of k_3' values will be the same as that of k_3 values already described above for 4PDS. This assumption has been used in making the corrections discussed below.

Except at low pH 4PDS is not soluble enough to permit direct determination of k_3' values, and thus one has only the linear asymptote region of the plot of [thiosugar]/v_i vs. 1/[4PDS] to work with. In order to determine the true values of k_1 and k_2/k_3, the following equations must be applied:

$$k_1 = c^2V \qquad (14)$$

$$k_2/k_3 = cK \qquad (15)$$

where K and V are obtained from fits of the asymptote region to eq 11 and $c = 1 - (k_3'K/V)$. Thus for all data where a linear [thiosugar]/v_i vs. 1/[4PDS] plot was observed, k_1 and k_2/k_3 were obtained from eq 14 and 15 using k_3' values calculated by assuming that the pH variation of k_3' paralleled that of k_3. The correction factor c, which depends on the relative size of k_3' and k_1k_3/k_2, ranged from insignificant for 6-thio-D-fructose and the thiofuranoses to 0.87 for 5-thio-D-glucose.[34]

Proportion of Thiosugar with Free Thiol Groups. The pK for those thiosugars with a primary thiol can be approximated by that for mercaptoethanol of 9.5, since the rates shown in Table I for 1-thioglycerol and dithiodulcitol show that longer chain polyhydroxy thiols have essentially the same reactivity as mercaptoethanol. Values of k_3 which should apply for reaction of 4PDS and 2PDS with thiosugars having primary thiols can thus be calculated for any desired pH from eq 7 (using the coefficients and pK for mercaptoethanol).

For 5-thio-D-glucose, 5-thio-D-fructose, and the 4-thio-D-aldofuranoses, which contain secondary thiols with hydroxyl groups on both adjacent carbons, the pK can be approximated by the value of 9.25 for 5-thio-D-sorbitol prepared by sodium borohydride reduction of 5-thio-D-glucose.[35] For these thiosugars, the pK used in eq 7 was thus 9.25, and in addition the coefficients in eq 7 were corrected according to the measured Brønsted relationship (i.e, multiplied by 0.82), since the rates for the secondary thiol anions, 2-mercaptosuccinic acid and 5-thio-D-sorbitol (Table I), fall on the same line with the primary thiols.

With k_3 known, it now becomes possible to calculate from each experimental value of k_2/k_3 the value of k_2, the ratio k_1/k_2, and thus the proportion of the thiosugar in the acyclic and other forms which have a free sulfhydryl group. These calculated values are shown in Table II. Where the proportion of free SH-containing forms is sufficient to be determined directly from the burst upon addition of 4PDS, the agreement with the calculated values is good and supports the validity of the assumptions involved in the calculations. The highest percentages of free SH-containing forms are seen for those thiosugars which can exist in stabilized oxygen containing ring forms; thus while D-xylose and D-fructose contain >99% py-

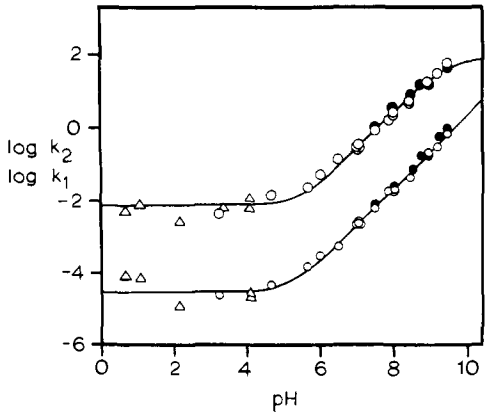


Figure 3. pH profile of ring opening (k_1, lower curve) and ring closing (k_2, upper curve) rates for 6-thio-D-fructose measured by reaction with 4PDS (open circles), DTNB (closed circles), and 2PDS (triangles) in 30 mM buffer at 25 °C. Curves are fits to eq 16 of the 4PDS data (below pH 8.5 for k_2), except that k_2 is assumed to level off with a pK value of 9.45 (this value is from a fit of all k_2/k_1 values to a simple titration curve).

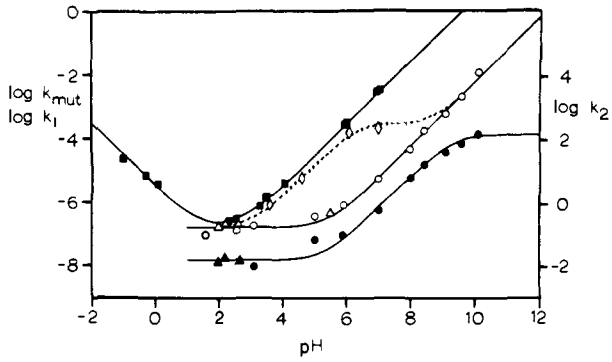


Figure 4. pH profile of ring opening (k_1, open symbols) and ring closing (k_2, closed symbols) rates measured by reaction with 4PDS (circles) and 2PDS (triangles), and of mutarotation rates (k_{mut}, closed squares) measured by polarimetry, for 5-thio-D-glucose in 30 mM buffer at 25 °C. The bottom two curves are fits to eq 16 (data below pH 8.5 for k_2) except that k_2 is assumed to level off with a pK of 9.30 (this value is from a fit of all k_2/k_1 values to a simple titration curve). The mutarotation rates are fitted to eq 16 with an additional $k_A[H^+]$ term. The dotted curve (diamonds) is the ring-opening rate (k_1) for 1.0 M general acid concentration at the pK of the catalyzing acid, 25 °C, and 0.50 M ionic strength, calculated from experimental results with formate, acetate, cacodylate, and imidazole buffers.

ranose[37] and 25% furanose,[38] respectively, 4-thio-D-xylose shows 3% pyranose, and 6-thio-D-fructose, 0.66% furanose. This method would appear to be the best way to estimate the amount of free SH-containing forms for those thiosugars where direct measurement is not practical.

pH Variation of Ring-Opening and -Closing Rates for Thiosugars. Ring-opening (k_1) and ring-closing (k_2) rates are plotted vs. pH for 6-thio-D-fructose in Figure 3 and 5-thio-D-glucose in Figure 4. It is clear that in the pH range 6–10 both reactions appear to be base catalyzed, but that at low pH the reactions become pH independent. No acid catalysis is observed for 5-thio-D-glucose down to pH 1.5 and for 6-thio-D-fructose down to pH 0.7.[39] The data were fitted to eq 16 where k_0 and k_B are the pH-independent and base-catalyzed rate constants, respectively, and the results are shown in Table II.

$$k = k_0 + k_B[OH^-] \qquad (16)$$

For 6-thio-D-fructose, the k_B values for ring opening determined with 4PDS (1.7×10^4 M^{-1} s^{-1}) and DTNB (1.9×10^4 M^{-1} s^{-1}) are in good agreement, showing that the rate of the ring-opening reaction is independent of the nature of the disulfide reagent used to measure it. The limited data for the other thiosugars in Table II show that above pH 7 ring opening and closing are base catalyzed in these cases also.

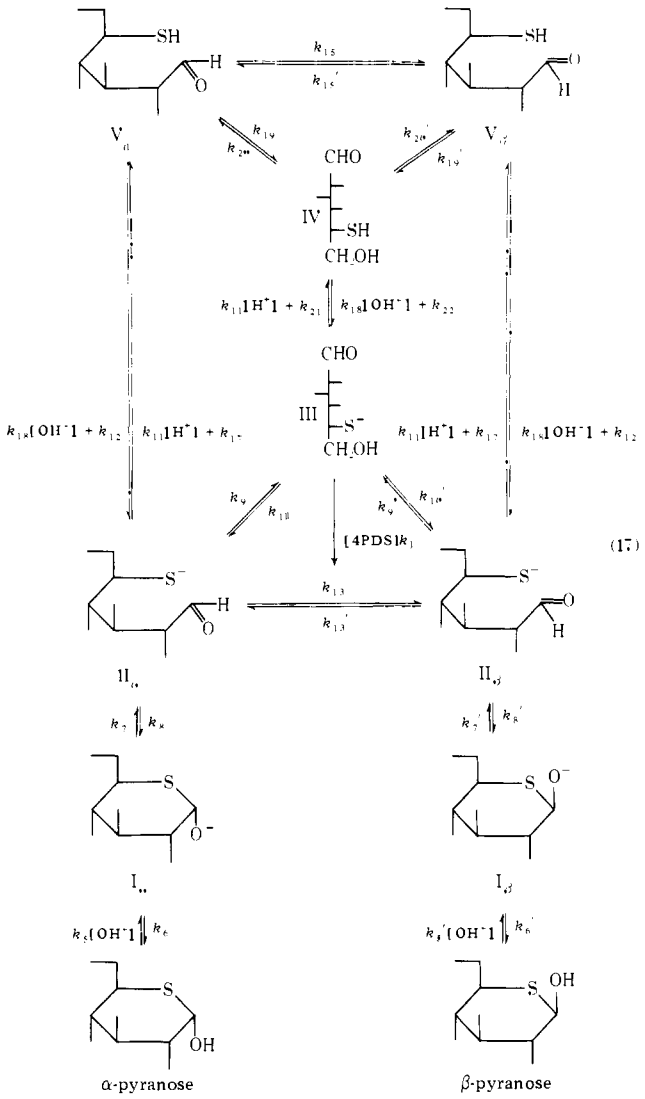
The equilibrium constant (k_1/k_2) between free thiol and thiopyranose forms should be constant below pH 8 and increase at high pH values as the ionized thiol appears in equilibrium with the protonated species. This change must be reflected in k_1 and k_2, and one might expect that k_1 would continue to increase by a factor of 10 per pH unit, but that k_2 would level off with a pK equal to that of the free thiol group, if ring opening required ionization of the anomeric hydroxyl and ring closing involved the thiolate species. Figures 3 and 4 show this to be the case. Least-squares fits of the ratio k_2/k_1 (1/percent free thiol species) to a simple titration curve provided pK values of 9.30 ± 0.05 and 9.45 ± 0.09 for 5-thio-D-glucose and 6-thio-D-fructose, respectively, confirming the earlier pK assignments. The limited data for the other thiosugars in Table II show that above pH 7 ring opening and closing are base catalyzed in these cases also.

Mechanism of Ring Opening and Mutarotation of Thiosugars. Mutarotation of thiosugars with the sulfur in the ring is reported to be base, but not acid, catalyzed owing to the low basicity of the sulfur compared to the oxygen in normal sugars.[40a] However, it is clear from our results with 5-thio-D-glucose (Figure 4) that mutarotation is not only base catalyzed, but is also acid catalyzed below pH 1, and that a pH-independent reaction occurs for pH 1–3.[41] The values for k_0 and k_B from analysis according to eq 16 are listed in Table II for the ring-opening and mutarotation rates observed in this study and for literature values for 5-thio-D-xylose and 5-thio-D-ribose. Values of k_0 and k_B for mutarotation of the corresponding oxygen-containing sugars are also included in Table II.

Base-catalyzed mutarotation of 5-thio-D-glucose and 5-thio-D-xylose is about 520 times faster than base-catalyzed ring opening, suggesting the existence of intermediates[42] which can undergo mutarotation, but not reaction with 4PDS. In contrast, pH-independent mutarotation of 5-thio-D-glucose at low pH is roughly equal to ring opening, suggesting that ring opening must accompany mutarotation under these conditions.[43] further, neither the pH-independent ring opening nor mutarotation shows general base catalysis,[46] in contrast to the pH-independent mutarotation of normal sugars, which is subject to pronounced general acid and general base catalysis.[40a] The pH-independent reactions of thiosugars thus involve a different mechanism from that of sugars, presumably one involving specific base catalysis by OH^- and specific acid catalysis by H^+. These results can be rationalized by the following scheme, which is shown for 5-thio-D-glucose, but appears to apply to all of the thiosugars studied.

In eq 17 base-catalyzed mutarotation proceeds by interconversion of II_α and II_β without ring opening, but at low pH II_α and II_β are converted to V_α and V_β, and ring opening is a necessary part of the mutarotation process. Ring opening itself proceeds via direct conversion of II_α and II_β to III at high pH, but at low pH II_α is protonated to V_α, and II_β to V_β, which in turn become IV and then III. As we will show later, the proximity of the SH group in V_α and V_β to the carbonyl carbon displaces the thiol pK from the value of 9.25 in IV to a value of about 7.7, and thus $k_{17} < k_{21}$ and $k_{12} > k_{22}$. The following discussion will show how to estimate most of the rate constants in eq 17, and, in particular, how to estimate for II_α and II_β, the spontaneous ring opening rates (k_9 and k_9'), the equilibrium constant for conversion of III to II_α and II_β, and the rates of interconversion between II_α and II_β (k_{13} and k_{13}').

In considering the ring-opening process, one must remember that mutarotation occurs rapidly relative to ring opening, ex-



(17)

cept below pH 3, and thus that II_α and II_β will be in equilibrium with each other, and, as will be shown, with I_α and I_β, and the α- and β-pyranoses as well. Further, ring-opening experiments at all pH values were carried out using mutarotated solutions of 5-thio-D-glucose. In the following discussion, $\bar{k}_5$–$\bar{k}_9$ are the weighted averages of k_5 and k_5', k_6 and k_6', etc., for the equilibrium mixture of α and β forms of free thiosugar, I, and II. Subsequent discussion will consider which rate constants differ sufficiently to generate the equilibrium α/β ratio of 4 for the free thiosugar, and whether the II_α/II_β ratio will also be 4, or closer to unity.

Ring Opening. The first step in eq 17 should proceed at the diffusion-controlled limit, and, assuming that $\bar{k}_5$ has a value of 10^{10} M^{-1} s^{-1},[47] $\bar{k}_5[OH^-]$ will always greatly exceed the observed rates of ring opening and mutarotation, and this step will be at equilibrium (below pH 2 this step may be water catalyzed, but the rate will still be 10^5 times the ring-opening rate). The value of $\bar{k}_6$ will be about 5×10^8 s^{-1}, taking the pK of the anomeric hydroxyl to be 12.7.[48] The second step will also be rapid, but have a low equilibrium constant, and, although we cannot yet calculate a value for $\bar{k}_7/\bar{k}_8$, we can proceed to estimate the ring-opening rate for II by considering the average equilibrium constant ($\bar{K}_{11}[OH^-] = \bar{k}_5\bar{k}_7[OH^-]/\bar{k}_6\bar{k}_8$) for formation of II_α and II_β from thiopyranose.

The rate constants k_{11} (4×10^{10} M^{-1} s^{-1} [51]) and k_{18} (10^9 M^{-1} s^{-1} [51]) represent the diffusion-controlled reactions between thiolate and hydronium, and thiol and hydroxide ion, respectively. k_{12} (7.1×10^2 s^{-1}), k_{17} (560 s^{-1}), k_{21} (1.8×10^4 s^{-1}), and k_{22} (22 s^{-1}) are the water-catalyzed rate constants and can be calculated from the pK_V = 7.7 (see below) and the above values of k_{11} and k_{18}. Since ring-opening rates are extrapolated to infinite 4PDS, 2PDS, or DTNB concentration and III is the reactive species, the back reactions involving k_{10} and k_{10}', and the conversion of III to IV, need not be considered.[52] If we assume that $\bar{k}_{13} \gg \bar{k}_9$ (because base-catalyzed mutarotation is faster than ring opening), and $\bar{k}_8 \gg \bar{k}_{13}$ (we will prove this shortly), the ring-opening rate can be written as

$$k_1 = \bar{K}_{11}[OH^-](\bar{k}_9 + \text{app } k_{11}) \qquad (18)$$

where app k_{11} is the net rate constant for conversion of II_α and II_β to III via intermediates V_α, V_β, and IV.

While the complete expression for app k_{11} is complicated, below pH 8 it will equal $k_{11}[H^+] + k_{17}$, since V_α will be converted to IV (or to V_β) much faster than it returns to II_α.[53] The subsequent conversion of IV to III, although slow, will proceed exclusively to III, rather than back to II_α or II_β. This is a result of the difference in pK values for IV and V_α or V_β, since the equilibrium constant for formation of V_α and V_β from IV will be 30-fold smaller than the value of 0.04 determined below for the formation of II_α and II_β from III. Thus eq 18 can be written

$$k_1 = \bar{K}_{11}[OH^-](\bar{k}_9 + k_{17} + k_{11}[H^+]) \qquad (19)$$

It can be seen from eq 19 that the ring-opening rate (k_1) will become pH independent below the pH where $k_{11}[H^+] = \bar{k}_9 + k_{17}$. If $k_{11} = 4 \times 10^{10}$ M^{-1} s^{-1}, then $\bar{k}_9 = 1.6 \times 10^5$ since the experimental transition for 5-thio-D-glucose occurs at pH 5.40 and the contribution of k_{17} (560 s^{-1}) is negligible. As noted above, this is the ring-opening rate for the equilibrium mixture of II_α and II_β.

Equation 17 also predicts that the ring-opening rate for 5-thio-D-glucose will again appear to be base catalyzed below the pH value where $k_{11}[H^+]$ becomes equal to $\bar{k}_8$, with the slow step now being conversion of I to II ($\bar{k}_7$) followed by rapid reaction to V and subsequently III as discussed above. However, at pH values as low as 1.5, k_1 is still within a factor of 2 of the pH-independent rate, and thus the pH where $k_{11}[H^+] = \bar{k}_8$ is at least this low.[54] Since $k_{11}[H^+]$ at pH 1.5 is 1.3×10^9 s^{-1}, $\bar{k}_8$ is greater than or equal to this value.

For base-catalyzed ring opening, one needs to consider the expression for app k_{11} at high pH. The maximum value for app k_{11} under these conditions will be $k_{11}[H^+] + k_{17}$, and, since k_{17} is much less than $\bar{k}_9$, and $k_{11}[H^+]$ must also be smaller than $\bar{k}_9$ above the point where the reaction becomes base catalyzed, we have

$$k_B = \bar{K}_{11}\bar{k}_9 \qquad (20)$$

$\bar{K}_{11}$ can now be determined as 4.0×10^{-4} M^{-1} from the experimental k_B value in Table II and the $\bar{k}_9$ value calculated above. We can also calculate a value for $\bar{k}_7/\bar{k}_8$ of 2.0×10^{-5}, since $\bar{K}_{11}[OH^-]$, which is the equilibrium constant for formation of II from pyranose, is the product of $\bar{k}_7/\bar{k}_8[OH^-]$ and the dissociation constant of the anomeric hydroxyl (pK = 12.7[49]) divided by $K_w = 10^{-14}$. A minimum value of $\bar{k}_7$ can now be estimated as 2.6×10^4 s^{-1} using $\bar{k}_8 \geq 1.3 \times 10^9$ s^{-1}. Note how unfavorable this conversion from I to II really is, with I being five orders of magnitude more stable than II.

In addition one can estimate the value for $\bar{k}_{10}/\bar{k}_9$ by considering the following equation, which applies to an equilibrium mixture of α and β forms of the pyranose and II:

$$\text{pyranose} \overset{K_1}{\rightleftharpoons} \text{IV} \overset{K_2}{\rightleftharpoons} \text{III} \overset{K_3}{\rightleftharpoons} \text{II} \qquad (21)$$

K_1 is the proportion of free carbonyl form present at equilibrium, which can be estimated by multiplying the proportion of 5-thio-D-glucose with a free SH group from Table II

Table III. Analysis of Rate and Equilibrium Constants in Equation 17 for Thiosugars and Sugars[p]

compd	K_1	$K_2/[OH^-]$	K_3	transition pH[j]	$\bar{k}_7/\bar{k}_8$[m]	$\bar{k}_9 + k_{17}$, s^{-1}	$\bar{k}_{13}$, s^{-1}
5-thio-D-glucose	1.7×10^{-7} [a]	5.6×10^4 [g]	0.042	5.40	2.0×10^{-5}	1.6×10^5	$2.2\text{-}3.6 \times 10^7$
5-thio-D-xylose	1.2×10^{-6} [d,o]	3.2×10^4 [h]	0.047	6.06	3.6×10^{-5}	3.5×10^4	(10^6)[n]
6-thio-D-fructose	2×10^{-4} [c]	3.2×10^4 [h]	0.011	5.23	5.6×10^{-4}	2.4×10^5	
5-thio-D-fructose	6.5×10^{-6} [b]	5.6×10^4 [g]	0.080	6.86	7.3×10^{-4}	5.3×10^3	
4-thio-D-arabinose	4.5×10^{-7} [b]	5.6×10^4 [g]	0.27	6.77	3.4×10^{-4}	6.3×10^3	
4-thio-D-xylose	8.2×10^{-6} [b]	5.6×10^4 [g]	0.14	7.43	3.2×10^{-3}	1.4×10^3	
4-thio-D-ribose	3.4×10^{-6} [b]	5.6×10^4 [g]	0.063	6.76	6.0×10^{-4}	6.6×10^3	
D-glucose	2.6×10^{-5} [e]	1.0[i]	0.041[q]		2.1×10^{-8} [q]	6×10^8 [k]	$2.2\text{-}3.6 \times 10^7$ [l]
D-fructose	8.4×10^{-3} [f]	1.0[i]	0.023[q]		1.9×10^{-6} [q]	6×10^8 [k]	

[a] See text. [b] Free SH containing forms (see Table III) multiplied by the ratio of free carbonyl[56] to total pyranose for the normal sugar.[37,38] [c] See eq 37. [d] Free SH containing forms (see Table III) multiplied by the ratio of free carbonyl[56] to total furanose for the normal sugar.[37] [e] Reference 84. [f] Reference 56. [g] $pK = 9.25$. [h] $pK = 9.50$. [i] pK assumed to be 14. [j] pH below which ring opening is pH independent. [k] Rate of protonation by water, assuming $pK = 12.5$ and diffusion-controlled proton transfers. [l] Assumed to be the same as for 5-thio-D-glucose. [m] The pK of the anomeric hydroxyl is assumed to be 12.7 for 5-thio-D-glucose and the other thioaldoses, and 12.4 for 6-thio-D-fructose and the other thioketoses.[48] [n] Calculated from data in Table II.[43] [o] Calculations made assuming 0.5% total furanose in D-xylose.[37] [p] Rate constants with bars (k) are weighted averages for the α and β forms. Values calculated at 25 °C. [q] Calculated[83] from data in ref 82.

(0.0012%) by the ratio of free carbonyl (0.0024%[55]) to the sum of β-furanose (0.11%[57]) and hydrate (0.06%[60]) present in D-glucose.[63] Thus $K_1 = 1.7 \times 10^{-7}$.[64] K_2 is for ionization of the free SH group ($pK = 9.25$), and is thus $5.6 \times 10^4[OH^-]$. K_3 represents the desired equilibrium constant for formation of the closed forms II_α and II_β from the open thiolate III, and is given by

$$\bar{K}_{II}[OH^-] = K_1K_2K_3[OH^-] \quad (22)$$

Thus, $K_3 = 0.042$ from the values of K_1, K_2, and $\bar{K}_{II}$ calculated above.

Similar calculations can be made for 6-thio-D-fructose and 5-thio-D-xylose and the results are shown in Table III. Note that, despite a 350-fold range in k_B values (Table II), K_3 and $\bar{k}_9$ are fairly constant (Table III), varying only 5.5- and 6.5-fold, respectively, over the three thiopyranoses. These data also suggest that there is nothing unique about 6-thio-D-fructose from the kinetic point of view; it is the greater thermodynamic stability of II relative to the thiopyranose ring which leads to the unusually high ring-opening rate for 6-thio-D-fructose relative to the other thiosugars.

The results of calculations made in the same manner for the thiofuranoses 5-thio-D-fructose, 4-thio-D-arabinose, 4-thio-D-xylose, and 4-thio-D-ribose are also shown in Table III. Although the $\bar{k}_9$ values are, on the average, 25-fold lower than those for the thiopyranoses, they are internally consistent, varying only fivefold over the four examples.[65]

Mutarotation. For analysis of the mutarotation rates, II_α and II_β can no longer be considered to be in equilibrium, since it is their interconversion which constitutes the actual mutarotation reaction. (These experiments were all conducted with freshly dissloved 5-thio-α-D-glucopyranose.) The k_{mut} measured is the sum of the rate constants for conversion of α-pyranose to β-pyranose, and for the reverse process. It is clear from an examination of eq 17, and from the values derived above for the various rate constants involved, that II_α or II_β return to I_α and I_β much faster than they are converted to III, IV, or V, and, as will be shown, much faster than they are directly interconverted via k_{13} and k_{13}'. One can thus formulate k_{mut} as (k_9, k_9', and k_{17} have again been ignored as they are much less than k_{13} and k_{13}')

$$k_{mut} = \frac{k_5[OH^-]k_7}{k_6k_8}(k_{13} + k_{11}[H^+]f_\beta) + \frac{k_5'[OH^-]k_7'}{k_6'k_8'}(k_{13}' + k_{11}[H^+](1 - f_\beta)) \quad (23)$$

In eq 23, the first term represents conversion of α-pyranose to β-pyranose, and the second term the reverse reaction. f_β is the proportion of II which will be formed as II_β when III, IV, or V return to either II_α or II_β, and will probably be close to the amount found in the equilibrium mixture of II_α and II_β. At high pH II_α is converted directly to II_β by the step with rate constant k_{13}, and mutarotation is base catalyzed. At low pH, however, $k_{11}[H^+]f_\beta$ becomes larger than k_{13}, and the rate will become pH independent. Equation 17 does not allow for the acid-catalyzed mutarotation seen at still lower pH (see below), which presumably results from protonation of the thiopyranose and subsequent ring opening to a protonated form of V.

At the transition pH between base-catalyzed and pH-independent mutarotation (pH 2.75 for 5-thio-D-glucose) eq 23 predicts that

$$k_{13} = \frac{k_{11}[H^+](f_\beta + R(1 - f_\beta))}{(1 + Rk_{13}'/k_{13})} \quad (24)$$

where

$$R = \frac{k_5'k_7'k_6k_8}{k_6'k_8'k_5k_7} \quad (25)$$

Since Rk_{13}'/k_{13} simply equals the equilibrium ratio of α-pyranose to β-pyranose, which is 4.0 for 5-thio-D-glucose, the denominator of eq 24 is 5.0. If one assumes that f_β is the proportion of II in the β form at equilibrium, then

$$R = 4.0f_\beta/(1 - f_\beta) \quad (26)$$

and eq 24 can be written

$$k_{13} = k_{11}[H^+]f_\beta \quad (27)$$

In addition

$$k_{13}' = k_{13}(1 - f_\beta)/f_\beta \quad (28)$$

In order to proceed further, a value must be assigned to f_β. If one assumes, by analogy with D-glucose,[66] that the equilibrium α/β ratio of 4 is due entirely to a difference in ionization constant for the two thiopyranoses, then $f_\beta = 0.5$ and $k_{13} = k_{13}' = 3.6 \times 10^7\ s^{-1}$. However, if the equilibrium[II]/[pyranose] ratio is the same for the α and β forms, then $f_\beta = 0.2$ and k_{13} is $1.4 \times 10^7\ s^{-1}$, while k_{13}' is $5.7 \times 10^7\ s^{-1}$. In either case, these values for k_{13} and k_{13}' are roughly two orders of magnitude greater than $\bar{k}_9$, but smaller than $\bar{k}_8$ by about the same factor.

It is interesting to note that eq 17 requires the near equivalence of the pH-independent ring-opening rate (k_1) and the pH-independent k_{mut} value at low pH, as experimentally observed. To demonstrate this, the pH-independent ring-opening

rate can be expressed more completely than was done in eq 19 as

$$k_1 = \frac{0.8k_5[OH^-]k_7k_{11}[H^+]}{k_6k_8} + \frac{0.2k_5'[OH^-]k_7'k_{11}[H^+]}{k_6'k_8'} \quad (29)$$

where the first term represents ring opening of II_α, and the second term, of II_β. By employing eq 25 and 26, this can be written

$$k_1 = \frac{k_5[OH^-]k_7k_{11}[H^+](0.8)}{k_6k_8(1-f_\beta)} \quad (30)$$

while eq 23 at low pH becomes

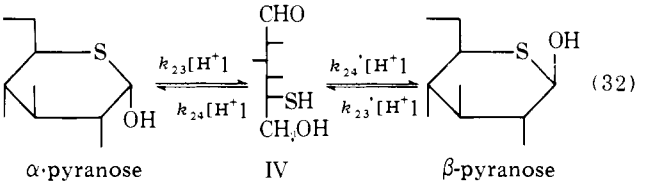
$$k_{mut} = \frac{k_5[OH^-]k_7k_{11}[H^+](5f_\beta)}{k_6k_8} \quad (31)$$

If $f_\beta = 0.2$, k_1 and k_{mut} will be equal (they are also equal if f_β is 0.8, which is very unlikely), while if $f_\beta = 0.5$, $k_{mut} = 1.56k_1$. The near equivalence of the actual values in Table II for k_1 and k_{mut} suggests that f_β is closer to 0.2 than 0.5, and lends credence to eq 17.

General Acid Catalysis of Ring Opening and pK of Intermediate V. Equation 17 predicts that protonation of II_α and II_β should be catalyzed by diffusion-controlled reaction with weaker acids than hydronium ion, provided that $pK_V > 0$. Buffer catalysis of ring opening for 5-thio-D-glucose is in fact observed for acids of pK 3.7–7.0, and comparison of the catalytic constants obtained in buffers composed of 50 or 80% general acid confirms that it is this species, and not the general base, which is responsible for the catalysis. The dotted curve in Figure 4 shows k_1 values calculated from the experimental data for a general acid concentration of 1.0 M at the pK of the catalyzing acid.[68]

For a simple proton transfer reaction in water which is diffusion controlled in the thermodynamically favored direction, the Brønsted plot of the logarithm of the catalytic constant (k_{HA}) vs. the pK of the catalyzing acid is expected to follow an Eigen curve with a break from a slope of zero when the pK of the general acid is far below that of the acceptor, to a slope of minus one when the pK considerably exceeds that of the acceptor.[51] The observed ring-opening rate at 1.0 M general acid, which is the product of the catalytic constant and the equilibrium concentration of II (which varies by a factor of 10 per pH unit), should thus follow a curve such as the dotted curve in Figure 4, and a least-squares fit of the data on the dotted curve gives an apparent pK of 6.23 ± 0.08 for the break in the profile. This is equivalent to a fit of the actual Eigen curve, since these k_1 values are calculated at the pK of the general acid catalyst. The catalytic constants (k_{HA}) for formic acid ($2.2 \pm 0.2 \times 10^7$ M^{-1} s^{-1}), acetic acid ($2.5 \pm 0.3 \times 10^7$ M^{-1} s^{-1}), cacodylic acid ($2.2 \pm 0.2 \times 10^7$ M^{-1} s^{-1}), and imidazole ($3.5 \pm 1.5 \times 10^6$ M^{-1} s^{-1}) are roughly two orders of magnitude slower than the predicted diffusion-controlled rate, but, when allowance is made for steric factors, are consistent with values obtained for the reaction of mercaptoethanolate ion with neutral acid species (~10^8 M^{-1} s^{-1} for acetic acid, for example[69]). Thus, there is apparently a barrier to thiolate protonation by general acids, which is not present for the hydronium ion (7.5×10^{10} M^{-1} s^{-1} for H^+ and HS^- and ~9×10^{10} M^{-1} s^{-1} for H^+ and $HOCH_2CH_2S^-$ [51]), and which accounts for the two to three orders of magnitude difference in the diffusion-controlled rate for the two cases. A similar but smaller barrier exists for the reaction of hydroxide ion with a thiol (~10^9 M^{-1} s^{-1} for OH^- and mercaptoethanol[51]). As a result of these effects, the Eigen curve for neutral acid catalyzed protonation of mercaptoethanolate ion shows a break which is fairly sharp, but which is displaced about 1.5 pH units below the actual thiol pK value.[51,69] Assuming a similar situation with form V of 5-thio-D-glucose, $pK_V = 7.7$, which is 1.5 pH units less than the pK of form IV.

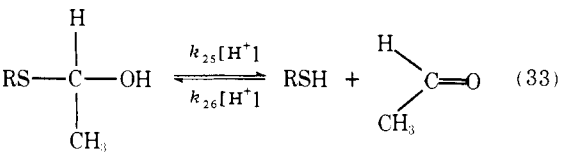
Comparison of Thiosugar Ring Opening and Closing with Acyclic Thiohemiacetal Breakdown and Formation. The reaction of thiol addition to the carbonyl group to yield the corresponding thiohemiacetal or thiohemiketal has been thoroughly studied in both the forward and reverse directions.[47,49,70] For basic thiols (pK > 7), thiohemiacetal formation from acetaldehyde proceeds by specific-base-catalyzed addition of the thiol anion and general-acid-catalyzed addition of the uncharged thiol. No general acid catalysis is observed for the addition of the basic thiol anion, and the contribution of the uncatalyzed reaction involving the free thiol and the carbonyl is negligible. These results predict that thiosugar ring opening and mutarotation should also be acid catalyzed, and this is in fact seen for k_{mut} in Figure 4.[71] Thus, the following reaction must be added to eq 17:



(32)

α-pyranose IV β-pyranose

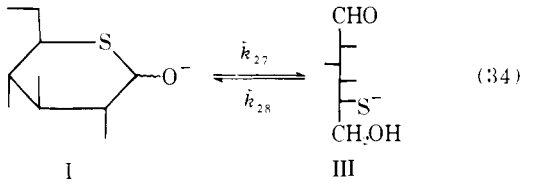
Equation 32 can be analyzed by setting the acid-catalyzed mutarotation rate equal to the pH-independent rate at the experimental transition pH 1.24. To account for the equilibrium α/β ratio of 4, we can assume as the two extreme cases $\bar{k}_{24}/k_{24}' = 4$, for which $\bar{k}_{23}[H^+] = 1.8 \times 10^{-7}$ s^{-1} and thus $\bar{k}_{23} = 3.1 \times 10^{-6}$ M^{-1} s^{-1}; or $k_{23}'/\bar{k}_{23} = 4$, in which case $1.56 \cdot \bar{k}_{23}[H^+] = 1.8 \times 10^{-7}$ s^{-1}, hence $\bar{k}_{23} = 2.0 \times 10^{-6}$ M^{-1} s^{-1}. From the value of K_1 for 5-thio-D-glucose and $\bar{k}_{23}$ above, one can estimate a range for $\bar{k}_{24}$ of 12–18 M^{-1} s^{-1}. These values can be compared with the acyclic rate constants for addition of 2-methoxyethanethiol (pK = 9.5) to acetaldehyde (eq 33),



(33)

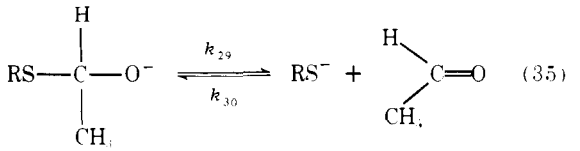
where R = $-CH_2CH_2OCH_3$, $k_{25} = 0.28$ M^{-1} s^{-1}, and $k_{26} = 8.8$ M^{-2} s^{-1}.[70] Thus for thiols of similar pK, the cyclic thiohemiacetal is five orders of magnitude less reactive than the acyclic compound toward acid-catalyzed breakdown. The ratio of the unimolecular ($\bar{k}_{24}$) and bimolecular (k_{26}) rate constants for such a reaction has been used as a measure of the local concentration or "approximation" effect of combining both functional groups on the same molecule. For acid-catalyzed thiohemiacetal formation the ratio is only 2 M, indicating a small effect. Acid catalysis should also contribute to ring opening for 5-thio-D-glucose below the pH value where $\bar{k}_{23}[H^+]$ becomes equal to the pH-independent rate. Using the above values for $\bar{k}_{23}$, the calculated transition pH value is in the range 1.10–1.29.

In order to make a similar comparison of the specific-base-catalyzed reactions 34 and 35, the rate constants of eq 34 must be expressed in terms of eq 17. Since $\bar{k}_8 \gg \bar{k}_9$, the values of $\bar{k}_{27}$ and $\bar{k}_{28}$ for 5-thio-D-glucose are given by $\bar{k}_7\bar{k}_9/\bar{k}_8$ (3.2 s^{-1}) and $\bar{k}_{10}$ (6.6×10^3 s^{-1}), respectively, since, as shown above, II is in rapid equilibrium with I. The corresponding acyclic rate constants k_{29} (2.3×10^7 s^{-1}) and k_{30} (4.7×10^5 M^{-1} s^{-1}) for R = $-CH_2CH_2OCH_3$[70] show the same trend as in the acid-catalyzed reaction. In both cases, the equilibrium constant for cyclic thiohemiacetal formation is five orders of magnitude greater than that for the bimolecular acyclic re-



(34)

I III



(35)

action, with the major contribution to this difference arising from the decomposition rates ($\bar{k}_{23}$ vs. k_{25} and $\bar{k}_{27}$ vs. k_{29}). While part of the difference here may be caused by the different reactivities of the thiosugar and acetaldehyde carbonyl groups, this represents a small fraction of the total effect, since the equilibrium constant for hydration of the former is only 13 times that for the latter. Most of the remaining factor of roughly 10^4 can be accounted for, however, by considering the equilibrium [pyranose]/[hydrate] ratio of 2.1×10^3 for the normal aldohexose, D-glucose. Thus the factors which influence the relative position of the equilibrium for the cyclic vs. the acyclic reaction appear to be similar in the case of either hemiacetal (hydrate) or thiohemiacetal formation.

The rate-determining step in the base-catalyzed breakdown of thiohemiacetals of acetaldehyde has been shown to be expulsion of the thiolate anion from the ionized tetrahedral intermediate for basic thiols.[47] This step is preceded by a diffusion-controlled proton-transfer step (which becomes rate limiting for more acidic thiols) and followed by diffusion-limited separation of the products. For the thiosugars, however, carbon–sulfur bond scission occurs in a rapid equilibrium step followed by a slower (10^3–10^5 s^{-1}) separation of the thiol and carbonyl groups. Partitioning of intermediate II (eq 17) through V and subsequently to III accounts for the pH-independent reactions observed for both ring opening and mutarotation, respectively. Once carbon–sulfur bond scission occurs, the decomposition of acyclic thiohemiacetals (eq 35) partitions completely toward products and as a result displays only acid and base catalysis, with no pH-independent rate.

Structure of Intermediate II. The mechanism presented for thiosugar mutarotation (eq 17) contains the minimum number of cyclic but not covalently bonded intermediates (II_α and II_β) required to explain the data. A single-intermediate model cannot account for the break in k_{mut} to a pH-independent rate since k_{13} and k_{13}' are necessarily infinite in such a model, and eq 24 shows that the transition pH would be infinitely negative. Electrostatic energy calculations based on simple ion–dipole interactions show that the lowest energy configuration for II has the thiolate ion located directly behind the carbonyl and nearly collinear with the carbon–oxygen double bond ($\theta = 180°$ in Figure 5). Immediately following ring opening, the angle θ should be very close to the tetrahedral angle of 109°.[72] A simple calculation of the electrostatic energy for an intermediate with a van der Waals contact distance $r = 3.4$ Å (Figure 5) shows that for $\theta = 180°$ the stabilization is $10.3/\epsilon$ kcal/mol, relative to the separated species, while the 109° complex is $4.7/\epsilon$ kcal/mol less stable (ϵ is the local dielectric constant). Thus the steps labeled k_{13} and k_{13}' may represent interconversion of II_α and II_β (for which $\theta = \pm 109°$) through a more stable intermediate with $\theta = 180°$, with the rate constants being actually determined by the conversion of this intermediate to II_β or II_α. A value for the stabilization of the noncovalently-bonded intermediate II relative to III of 2.0 kcal/mol can be estimated from the experimentally determined pK difference, $pK_{IV} - pK_V = 1.5$ units, and thus $\epsilon \simeq 5.0$.

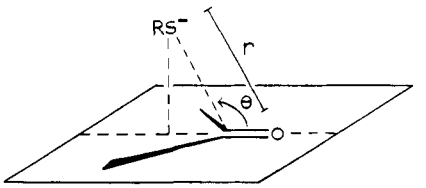


Figure 5. Model for thiolate interaction with the carbonyl group in thiosugars.

Mutarotation and Ring Opening of Oxygen-Containing Sugars. As noted earlier, the mutarotation of sugars in the neutral pH range is water and buffer catalyzed and clearly goes by a different mechanism than that in eq 17. However, the base-catalyzed mutarotations may well proceed by a mechanism similar to that in eq 17, although the pH-independent reaction resulting from such a mechanism will be too slow relative to the water-catalyzed rate to be observed. To test this hypothesis an analysis similar to that made above for thiosugars has been made for D-glucose and D-fructose, and the data are also shown in Table III. It is necessary to assume a pK for the hydroxyl which becomes the ring oxygen and we have chosen 14.[73] The value of K_3 is not sensitive to this choice, but, if we pick 15 instead, $K_2/[OH^-]$, $\bar{K}_{II}$, $\bar{k}_7/\bar{k}_8$, k_{12}, and k_{22} become smaller, and k_{17} and k_{21} larger, by a factor of 10. We have chosen to calculate average values for all anomers, and thus have used k_B values for mutarotation directly in the calculations.

If we assume that $\bar{k}_{13}$ has the same value for 5-thio-D-glucose and D-glucose and that the pK in II is lowered by 1.5 pH units, as appears to be the case for 5-thio-D-glucose, then we can calculate that k_{17} is at least 6.3×10^8 s^{-1}, since proton transfer to hydroxide ($k_{18}[OH^-]$) in the other direction should have a rate of 2×10^{10} M^{-1} s^{-1}.[51] This is a minimum value for $\bar{k}_9 + k_{17}$, and increases proportionately if the drop in pK corresponding to stabilization of the negative charge in II_α is taken to be less than 1.5 units. Since this value is a factor of 18–29 greater than $\bar{k}_{13}$, the calculation indicates that base-catalyzed mutarotation of oxygen-containing sugars may proceed through the acyclic form 94–97% of the time. This is an approximate calculation based on several assumptions, and the mutarotation process for normal sugars may proceed to a greater or lesser extent through the acyclic form analogous to III, depending on the actual values of $\bar{k}_9$, $\bar{k}_{13}$, and pK_V. The question can only be answered by measuring the ring-opening rates for sugars, for which suitable methods do not now exist.

Mechanism of Direct Reaction of Thiosugars with Disulfide Reagents. As noted above, an ionized form of 5-thio-D-glucose appears to react directly with high levels of 4PDS or DTNB. The amounts of the two possible candidates for this reaction, I and II, present at pH 7.7 are approximately 1×10^{-5} and 3×10^{-10}, respectively, relative to the concentration of intact thiopyranose. From the value of k_3' at this pH one can calculate that, if II is the reactive species, the bimolecular rate constant for reaction of II with disulfide reagent must be 100 times that for reaction of the free thiolate (5.4×10^4 M^{-1} s^{-1} for RS^- of pK = 9.25 and DTNB, for example), which seems highly unlikely since pK_V is 1.5 units lower than pK_{IV}. However, if one invokes reaction with intermediate I, which is present at 10^5 higher concentration, the rate constant for reaction with DTNB need be only 170 M^{-1} s^{-1}. Although the analogous rate constants calculated for reaction of DTNB with intermediate II for 4-thio-D-xylose (5.3×10^4 M^{-1} s^{-1}), 5-thio-D-fructose (1.2×10^5 M^{-1} s^{-1}), and 6-thio-D-fructose (1.5×10^5 M^{-1} s^{-1}) are comparable to that of the free thiolate, they are still three to nine times the expected value when one takes into account the decreased pK of the sulfur anion in II.

Table IV. Kinetic Constants for Substrates of Fructokinase

substrate	pH	V_{max}[a]	K_m, mM[b]	V_{max}/K_m[b,c]
D-fructose	7.0	(1.00)	0.17 ± 0.03 (0.036)	0.21 ± 0.03 (1.00)
	8.0	0.99 ± 0.03	0.23 ± 0.02 (0.048)	0.154 ± 0.014 (0.74)
6-thio-D-fructose	7.0	1.2 ± 0.6	10 ± 5	0.004 ± 0.002
	8.0	0.64 ± 0.02	6.2 ± 0.2	0.0037 ± 0.0002
6-thio-D-fructose-4-mercaptopyridine disulfide	7.0	0.159 ± 0.004	0.051 ± 0.005 (0.042)	0.11 ± 0.01 (0.14)

[a] Relative to D-fructose at pH 7.0 (50 mM PIPES) and 25 °C. [b] Values in parentheses are calculated for the β-furanose forms, assuming 21% to be present for fructose,[38] and 82% for 6-thio-D-fructose-4-mercaptopyridine disulfide (the remainder being 15% α-furanose and 3% acyclic; these values are calculated on the assumption that the ratios among the possible anomers are the same as for fructose). [c] Relative to the value for the β-furanose form of D-fructose at pH 7.0 (50 mM PIPES) and 25 °C.

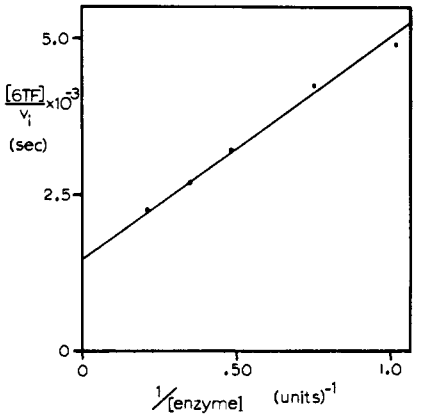
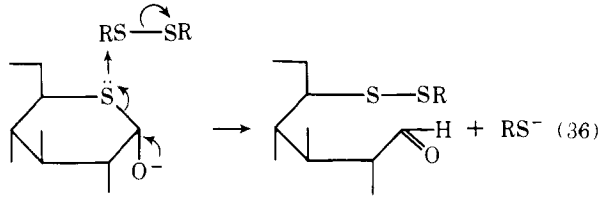


Figure 6. Double reciprocal plot of the rate of phosphorylation of 0.41 mM 6-thio-D-fructose by 4.0 mM MgATP in the presence of fructokinase at pH 7 (50 mM PIPES) and 25 °C, as a function of varying fructokinase concentration. Units are micromoles ADP produced per minute per milligram of protein.

Thus, while the reaction of II may occur, the reaction of I, which is present in much higher concentration, is probably the major factor responsible for the curved reciprocal plots which go through the origin.

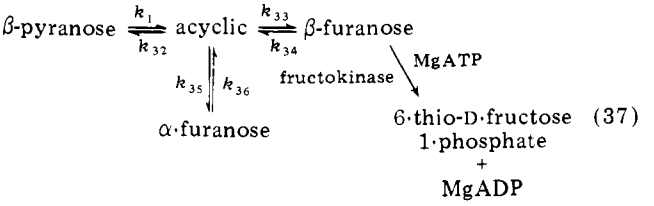
Reaction of hydroxide ion with DTNB to produce 5-thio(2-nitrobenzoic acid) dianion and a sulfenate anion is relatively slow (0.3 M^{-1} s^{-1} [31]), so it seems unlikely that it is the oxygen of I which reacts. A possible mechanism involving the sulfur atom is shown in eq 36, where the disulfide acts as



(36)

a "soft" electrophile to induce carbon-sulfur bond breakage. There is precedent for such reactions in the iodine-assisted hydrolysis of thiophosphates[74] and metal ion catalyzed cleavage of thioamides to yield the metal sulfide.[75] A similar mechanism may be responsible for the rapid oxidation of 4-thio-D-ribose by iodine at room temperature.[76]

Interconversion Rates among 6-Thio-D-fructose Anomers. The work described so far allows calculation of ring-opening rates and of average rates of ring closing for all forms with a free SH group, as well as the proportion of the sugar with a free SH group. By assuming that anomeric equilibria among the forms with a free SH group are the same as for the analogous sugar, one can then tell how much of each is present, but one cannot calculate rates of interconversion. For 6-thio-D-fructose, however, we can do more than this, since the rate of phosphorylation by fructokinase at infinite enzyme measures the net rate of conversion from β-pyranose to β-furanose. The experimental results can be described in terms of the following mechanism:



(37)

The ring-opening rate for the β-pyranose, k_1, is the same as that in mechanism 9, and can be derived from the 4PDS reaction. As fructokinase is increased to a saturating level, the level of β-furanose drops to zero and thus the step involving k_{34} can be ignored. However, the extrapolated rate at infinite enzyme is not given by k_1 alone, but also depends on the partitioning of the acyclic form between β-furanose and β-pyranose. Under these conditions the net rate constant for the conversion of β-pyranose to β-furanose is given by

$$\text{app } k_1 = \frac{k_1 k_{33}}{(k_{32} + k_{33})} \tag{38}$$

Figure 6 shows the results of an enzyme saturation experiment in which [6-thio-D-fructose]/v_i is plotted vs. 1/[enzyme] at a constant level of 6-thio-D-fructose. The extrapolated value of v_i/[6-thio-D-fructose] at saturating fructokinase was 6.7 ± 0.2 × 10^{-4} s^{-1} at pH 7 and 1.29 ± 0.38 × 10^{-4} s^{-1} at pH 6. At pH 7 with $k_1 = 1.7 \times 10^{-3}$ s^{-1} and app $k_1 = 6.7 \times 10^{-4}$ s^{-1}, we have $k_{32}/k_{33} = 1.54 \pm 0.07$. At pH 6, $k_{32}/k_{33} = 1.0 \pm 0.3$.

Up to this point no assumptions have been made concerning the anomeric composition of 6-thio-D-fructose. If we assume that the Michaelis constants are the same for the β-furanose anomers of D-frucose and 6-thio-D-fructose,[77] we can calculate the fraction of this anomer present at equilibrium from a comparison of the K_m values in Table IV. The reported equilibrium composition of D-fructose[38,56] is β-pyranose (75%), β-furanose (21%), α-furanose (4%), acyclic (0.8%[55]). Thus at pH 7 the fraction of 6-thio-D-fructose in the β-furanose form is 0.21 × 0.17/10 = 0.004. At pH 8 the value is 0.008. Adopting 0.006 as an average value, we get the relationship

$$[\beta\text{-furanose}]/[\beta\text{-pyranose}] = k_1 k_{33}/k_{32} k_{34} = 0.006 \tag{39}$$

Substituting the value of k_{32}/k_{33} into eq 39, $k_1/k_{34} = 0.009$, and, since k_1 is known, $k_{34} = 0.19$ s^{-1}. If we further assume that the relative amounts of β-furanose, α-furanose, and acyclic forms at equilibrium are the same for D-fructose and 6-thio-

D-fructose, we can estimate the proportion of these forms present as 0.11% α-furanose and 0.02% acyclic. We can then calculate $k_{32} = 9\ s^{-1}$ and $k_{33} = 6\ s^{-1}$, but only the ratio $k_{35}/k_{36} = 6$ (although k_{35} and k_{36} are unlikely to differ much in magnitude from k_{33} and k_{34}). We now have for pH 7

$$\underset{(99.3\%)}{\beta\text{-pyranose}} \underset{9\ s^{-1}}{\overset{1.7 \times 10^{-3}\ s^{-1}}{\rightleftharpoons}} \underset{(0.02\%)}{\text{acyclic}} \underset{0.19\ s^{-1}}{\overset{6\ s^{-1}}{\rightleftharpoons}} \underset{(0.6\%)}{\beta\text{-furanose}}$$

$$\Updownarrow \qquad (40)$$

$$\underset{(0.11\%)}{\alpha\text{-furanose}}$$

The low level of furanose forms present at equilibrium explains why only β-pyranose was detected by NMR[4] and also why no mutarotation is observed for 6-thio-D-fructose.[79] The agreement with the estimated amount of free SH forms in Table II is excellent.

The above analysis is consistent with mutarotation studies in which sucrose was rapidly hydrolyzed at 4 °C by the addition of a large excess of invertase to generate β-D-fructofuranose.[59] Under these conditions, a very fast equilibration of furanose forms was observed, followed by a slower rate of furanose–pyranose interconversion. If the ring-closing and -opening rates are 6 and 1 s^{-1} for the α-furanose in eq 40, the two furanoses would interconvert with a half-time of 1.2 s at 25 °C, or 8.2 s at 4 °C, assuming an activation energy[40b] of 15 kcal/mol. If values of 1.2 and 0.2 s^{-1} are used, the half-times would be 3.5 and 24 s at the two temperatures. The rates calculated here for ring opening and closing of the β-furanose can also be compared with the values recently determined for the furanoses of D-galactose by Wertz and Anderson[80] at pH 4.3 and 6.2. Correction of these data to pH 7 gives ring-opening rates for the α- and β-furanoses of 0.088 and 0.074 s^{-1}, and ring closing rates of 8.6 and 15 s^{-1}. These rates are very close to those shown in eq 36 for 6-thio-D-fructose.

Substrate Specificity of Fructokinase. Table IV lists the kinetic constants for the phosphorylation of D-fructose, 6-thio-D-fructose, and 6-thio-D-fructose-4-mercaptopyridine disulfide by MgATP in the presence of normal low levels of fructokinase. The results reported in Table IV and Figure 6 confirm the conclusions of Raushel and Cleland[3,18] that substrates with six-membered rings are not phosphorylated, that active substrates must contain a β-D-furanose structure, and that the substituents attached to C-5 of the furanose ring have relatively little influence on the kinetics. Substitution of a mercaptomethyl group for a hydroxymethyl group does not change the V_{max} appreciably, and probably has little effect on the Michaelis constant. The Michaelis constant for 6-thio-D-fructose-4-mercaptopyridine disulfide (which cannot exist as a pyranose and thus should contain 82% β-furanose, if the ratio between the furanoses and the acyclic form is the same as for fructose) is the same as that of the β-furanose anomer of D-fructose, and is in fact the lowest K_m value of any substrate known for the enzyme, although V_{max} and V_{max}/K_m are both sixfold less than the values for D-fructose.

Acknowledgments. This work was supported by grants to W.W.C. from the National Science Foundation (BMS 75-16134) and the National Institutes of Health (GM 18938), and to R.L.W. from the National Institutes of Health (AM 18482). We wish to thank Dr. Laurens Anderson and Dr. William P. Jencks for many helpful discussions.

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(29) These results agree fairly well with previously determined pK values for 4PDS of 4.11 and 5.00, and for 2PDS of 2.45 (pK_1 was not measured).[28] The similarity of the rate enhancements observed for reaction with mercaptoethanol upon protonation of 4PDS (70-fold) and 2PDS (2.8×10^3-fold) and the values (70- and 1.4–4.0 $\times 10^3$-fold, respectively[28]) found using L-cysteine indicates that the enhancement is independent of the nature of the attacking thiol.
(30) The equilibrium [thiopyridone]/[pyridinethiol] ratio for the neutral species as estimated spectrophotometrically is 4.9×10^4 for 2PDS and 3.5×10^4 for 4PDS.[12]
(31) J. P. Danehy, V. J. Elia, and C. J. Lavelle, *J. Org. Chem.*, **36**, 1003 (1971); J. P. Danehy and K. N. Parameswaran, *ibid.*, **33**, 568 (1968).
(32) All three reagents are about equally susceptible to base-catalyzed decomposition with a rate constant of 0.3–0.8 $M^{-1}\ s^{-1}$.[31] The main disadvantage of the dipyridyl disulfide reagents is their poor solubility at neutral pH, a problem not encountered with DTNB. However, solubility increases markedly upon protonation of the pyridine rings. At low pH solutions of 2PDS and 4PDS show substantial background absorbance at the thiopyridone λ_{max} values, but this problem can be avoided by monitoring the reaction at slightly longer wavelength where the contribution due to the diprotonated species is decreased. The advantage of enhanced reactivity over a wide pH range, combined with their greater sensitivity, makes the PDS reagents the ones of choice for study of thiosugars and related thiol chemistry.
(33) For the reaction of 6-thio-D-fructose and 5-thio-D-glucose with 4PDS and DTNB at high pH where the rates are extremely fast, assays were conducted under pseudo-first-order conditions with disulfide in large excess and the time course was analyzed in terms of a pseudo-first-order rate equation of the form $v_i = k'$[thiosugar], where $k' = k$[4PDS].
(34) Thiosugar data with 2PDS were corrected in a similar fashion using k_3' values calculated by assuming that $k_{3(4PDS)}/k_{3(2PDS)} = k_3'_{(4PDS)}/k_3'_{(2PDS)}$.
(35) Literature data[36] show that primary aliphatic thiols have pK values of about 10.6, with secondary and tertiary thiols having slightly higher values of 10.9 and 11.3, respectively. Addition of one hydroxyl group on the adjacent carbon has an inductive effect, decreasing the pK 1.45 units for a tertiary

and 1.10 units for a primary thiol (with secondary thiols presumably showing an intermediate value). The measured pK of 9.25 for 5-thio-D-sorbitol shows that addition of a second hydroxyl adjacent to the thiol gives an overall decrease of 1.65 units, so that the inductive effect of the second hydroxyl is somewhat less than that of the first.
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(39) We have looked for acid-catalyzed ring opening of 5-thio-D-glucose and 6-thio-D-fructose. Unfortunately, under conditions where acid catalysis should be observable (pH <1.1 to 1.3 for 5-thio-D-glucose and pH <−1.0 to −1.2 for 6-thio-D-fructose), accurate measurement of the ring-opening rates becomes impractical owing to experimental difficulties with decreased sensitivity and significant background color production due to breakdown of 2PDS.
(40) (a) H. S. Isbell and W. Pigman, *Adv. Carbohydr. Chem.*, **24**, 13 (1969); (b) W. Pigman and H. S. Isbell, *ibid.*, **23**, 11 (1968).
(41) The mutarotation data were fitted to eq 16 with a $k_A[H^+]$ term added. The calculated values for k_B and k_A are not sufficient to account for the observed rates at pH 2.3 and 2.5. Thus between the pH values of 1.24 and 2.75 where acid- and base-catalyzed mutarotation, respectively, become equal to k_0, the main contribution must be due to a pH-independent reaction.
(42) These intermediates correspond roughly to the "pseudoacyclic" intermediates proposed by H. S. Isbell et al., *Carbohydr. Res.*, **9**, 163 (1969), to explain the transformations of normal oxygen containing sugars in alkaline solution.
(43) The values of k_0 and k_B for 5-thio-D-xylose[44] and 5-thio-D-ribose[45] are calculated from mutarotation rates reported at pH 4.4 and 6.6. While the k_B value is well determined, k_0 is estimated from the increase of the observed k_{mut} value at pH 4.4 over that expected on the basis of the value at pH 6.6. Since these factors were only 1.7–2.5, it is possible (and by comparison with the value we have observed for 5-thio-D-glucose, very likely) that the k_0 values are, in fact, considerably smaller than those calculated here.
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(53) k_{19} is certainly as great as k_9, whose value exceeds 10^5 s^{-1} (see above), while k_{12} is only 710 s^{-1}, and $k_{18}[OH^-]$ is only 10^3 s^{-1} at pH 8.
(54) Acid-catalyzed ring opening of 5-thio-D-glucose should become important below pH 1.1–1.3 (see above). Thus while eq 17 predicts that k_1 will again appear to be base catalyzed below the pH value where $k_{11}[H^+] = k_8$, this break in the pH profile should be unobservable owing to the contribution of the acid-catalyzed pathway.
(55) Value measured at 20 °C by CD and corrected to 25 °C.[56]
(56) L. D. Hayward and S. J. Angyal, *Carbohydr. Res.*, **53**, 13 (1977).
(57) Value measured at 43 °C (0.14%)[58] by ^{13}C NMR and corrected to 25 °C using the temperature variation of β-furanose concentration in D-fructose.[59]
(58) C. Williams and A. Allerhand, *Carbohydr. Res.*, **56**, 173 (1977).
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(64) This value can be compared with the previous estimate of 5×10^{-5} for 5-thio-D-glucose by the CD method.[56]
(65) Water-catalyzed protonation ($k_{17} = 10^3$ s^{-1}) may represent 15–71% of the total base-catalyzed rate $(k_9 + k_{17})K_{II}$ if $pK_{IV} - pK_V$ for the analogous thiofuranose intermediates is only 1.5 units, as in 5-thio-D-glucose. The contribution of k_{17} decreases as the pK difference increases.
(66) The reported ionization constants[67] for the anomeric hydroxyl group of α-(pK = 12.17) and β-D-glucose (12.47) are sufficiently different at 25 °C to account for the observed equilibrium α/β ratio of 0.61, assuming a mechanism for base-catalyzed mutarotation which involves interconversion of the two ionized pyranoses through one or more intermediates. If this is the case for 5-thio-D-glucose ($pK_\alpha - pK_\beta \simeq$ log 4.0), f_β may still be 0.2 if $(k_7'/k_8')/(k_7/k_8) = 0.25$ (eq 25 and 26). Unfortunately, measurement of the ionization constants for 5-thio-α- and 5-thio-β-D-glucose is not feasible owing to the 100-fold greater base-catalyzed mutarotation rate (Table II) and the less favorable equilibrium (only 20% change from the pure α-D-anomer vs. 62% change from pure α-D-glucose).
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(78) After prolonged exposure to silylating conditions, 6-thio-D-fructose is reported in ref 79 to consist of two components in a ratio of 2:1 as analyzed by GLC. If this second component is the α-pyranose of 6-thio-D-fructose, the results shown in eq 39 and 40 are not affected, except that k_1 and k_{32} now are rate constants referring to the combined pyranoses. Since these authors could observe no mutarotation of 6-thio-D-fructose it seems unlikely that any second component is actually present in more than the concentrations shown in eq 40 for the furanose anomers. A more plausible explanation is that the second component is an artifact of the analytical procedure.
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