

Anomerization Rates and Enzyme Specificity for Biologically Important Sugars and Sugar Phosphates

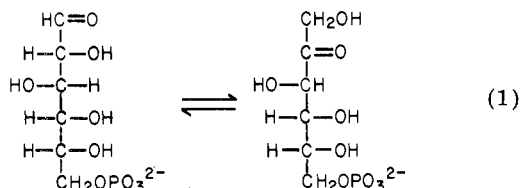
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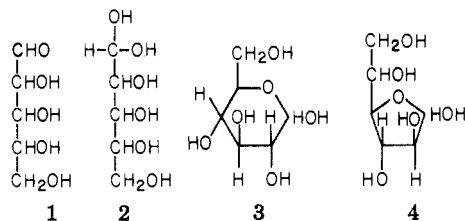
Enzymes, particularly those catalyzing steps for the various cellular pathways, have long been known to possess a very high degree of specificity with regard to their substrates. That is, only a single chemical compound is transformed at significant rates. Modifications or isomers of this compound are disallowed (rates of less than 1%) unless the structural change is minor.

A number of enzymes catalyze transformations of sugars and their derivatives. These also are remarkably specific, usually discriminating between the various stereochemical isomers of the carbohydrate very effectively. Phosphoglucose isomerase, for example, catalyzes the isomerization of D-glucose 6-phosphate to D-fructose 6-phosphate (eq 1) and does not accept as



substrate any of the other hexose isomers.

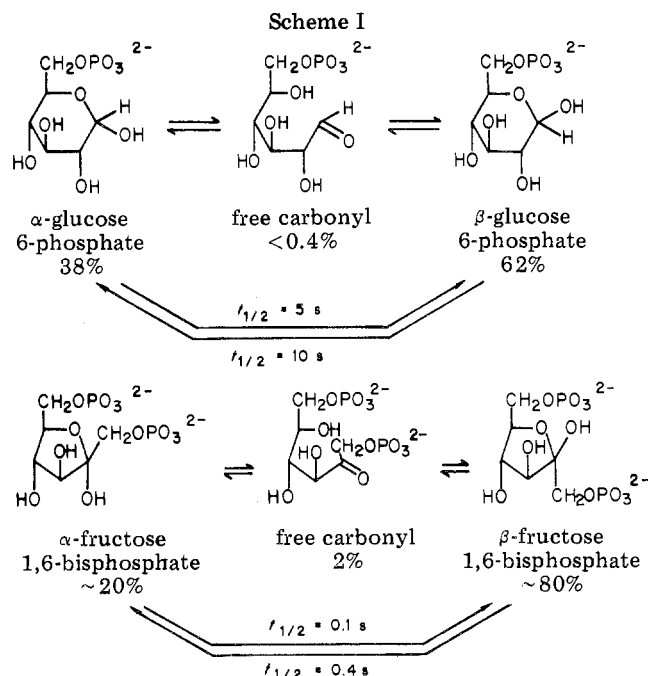
These various sugars may exist in a number of forms in solution. Initiating what may well be the longest running investigation of a fairly narrowly defined field, Dubrunfaut in 1846 pointed out the changes which occur in the effect of glucose on polarized light. Investigations of this complex field continue actively today. It is known that glucose, for example, may exist in the acyclic free carbonyl form (1), the cyclic pyranose



(3) and furanose (4) forms, and the hydrated carbonyl form (2). The particular forms which predominate for

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a particular sugar must be determined, as they are a complex function of subtle differences in structure between sugars.

The ring forms may themselves exist as two isomers resulting from formation of an additional asymmetric center. These are termed α and β and are referred to as anomers. The alteration of the rotation of plane-polarized light, subsequently termed mutarotation (for change of rotation), is caused in the representative case of glucose by conversion of one of the pyranose anomers (the normally crystallized α anomer) into an equilibrium mixture of both α - and β -pyranose forms.

In the cases of five- and six-carbon sugars and their mono- and diphosphorylated derivatives, the cyclic structures are the principal form. Also, pyranose forms, if structurally possible, constitute the largest component, often to the exclusion of furanose forms. The experimentally observed structures for the substrates of greatest immediate interest, D-glucose 6-phosphate (G6P) and D-fructose 1,6-bisphosphate (FDP), are shown in Scheme I. The equilibrium in solution generally favors one anomer, with the free carbonyl form constituting a very small percentage.¹⁻⁶ Addi-

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Table I
Half-Lives^a of Anomerization of Sugars upon
Phosphorylation

Sugar	Half-life of $\alpha \rightarrow \beta^b$	Half-life of $\beta \rightarrow \alpha^b$	Ref
Glucose	1740	3500	1
Glucose-6-P	5	10	14,15
Fructose-6-P	0.4	1.6	16
Fructose-1,6-P ₂	0.1	0.4	6

^a At ca. 25 °C and neutral pH. ^b In seconds.

tionally, fructose 6-phosphate (F6P) is 80–90% β F6P, 10–20% α -F6P, and <2% free carbonyl.^{3-5,7}

There are several methods for determining the state of equilibrium in solution for these and other ketoses. Determination of optical rotation or rates of oxidation was prominent in early work. More recently infrared and ¹H and ¹³C NMR studies have been very informative.⁷ The anomeric forms interconvert readily via the free carbonyl,^{1,8} this process being termed anomerization (the terms mutarotation and 1-epimerization also occur in the literature). The rates and mechanisms of this interconversion have been studied for many years with use principally of polarimetric methods, but infrared spectroscopy, solubility, polarographic methods, and others have also found application. The mechanism is believed to involve general acid-base catalysis but, despite many attempts, it remains uncertain whether catalysis is concerted or stepwise.^{1,9}

This Account deals with two areas related to these equilibria: (1) current studies on rates of anomerization of biologically important ketose and aldose phosphates; and (2) more extensively, enzyme selectivity in catalyzing reactions of the different anomeric and acyclic species, considering both mechanistic and regulatory implications.

In Table I are listed half-lives for anomerization of a number of compounds of interest. The rates of nonenzymatic anomerization determined for various sugars vary by factors of less than 10 as the number of carbon atoms or the stereochemistry is changed.¹ Points of special interest in Table I are the increases in rate which occur on phosphorylation and with ring contraction to furanose from pyranose (compare glucose-6-P with fructose-6-P). These data have become available only recently because the rapid rates of these anomerizations and the unavailability of pure anomeric forms made earlier methods insufficient. Rapid kinetics techniques making use of enzyme selectivity, as discussed below, and NMR line-broadening studies and the use of other NMR techniques⁷ to determine equilibrium values have been combined to obtain this information.

The rate enhancement observed in proceeding from the six-membered pyranose ring in glucose 6-phosphate to the five-membered furanose ring in fructose 6-phosphate confirms previous observations.¹ More interesting, perhaps, is the increased rate resulting from monophosphorylation. Anomerization is known to be subject to general acid-base catalysis,⁹ and Bailey et

al.¹⁰ have observed catalysis of glucose anomerization by inorganic phosphate. They have found the dianionic HPO₄²⁻ species to be ca. 25-fold more effective than the more fully protonated H₂PO₄⁻ species. The fully ionized PO₄³⁻ is ca. 10-fold more effective than HPO₄²⁻. The pH-rate profile for glucose 6-phosphate anomerization reveals that the dianionic species is responsible for the enhanced anomerization rate of glucose 6-phosphate over glucose.¹¹ These results strongly implicate general-base catalysis rather than a bifunctional¹² or tautomeric¹³ catalytic effect.

A single intramolecular phosphoryl group appears to provide the maximum catalytic effect. This is shown by the relatively small rate increase observed in comparing fructose 6-phosphate to fructose 1,6-bisphosphate and by the facts that fructose 1,6-bisphosphate anomerization is not catalyzed by high concentration of the bisphosphate⁶ and that glucose 6-phosphate¹¹ anomerization is not catalyzed by inorganic phosphate.

The majority of previous investigations of enzyme-catalyzed reactions of sugars and sugar phosphates have neglected that difficult question of whether one or both of the possible cyclic forms or the more commonly drawn acyclic form (with a free carbonyl group) is the true substrate. Considerable progress has recently been made in determination of the forms of the substrates that are actually utilized by enzymes that catalyze reactions of various sugars and more particularly their mono- and bisphosphates.

The earliest demonstration of an enzymatic anomeric specificity was by Keilin and Hartree,¹⁷ who showed in 1952 that glucose oxidase is specific for the β -anomer of D-glucose. Glucose dehydrogenase and galactose dehydrogenase have been shown to act only upon the β anomers of their substrates.^{18,19} These reactions are easily studied since pure anomeric forms with slow anomerization rates are available. Likewise the determination of anomeric specificity for glycosidases presents no problem since these enzymes catalyze reactions of nonanomerizing structures.

The pioneering work of Salas and his collaborators¹⁴ initiated efforts to determine the anomeric specificity of enzymes that act upon phosphorylated sugar substrates. This is a much more difficult problem due to the unavailability of pure anomers and the rapidity of their anomerization. By determination of previously unknown anomeric ratios by ¹³C NMR and by utilization of rapid kinetic techniques, these early results have been extended and now a rather complete picture has emerged.

Anomeric Preferences Observed and Their Significance

An overview of this picture is presented in Scheme II. The main features of glycolysis-gluconeogenesis

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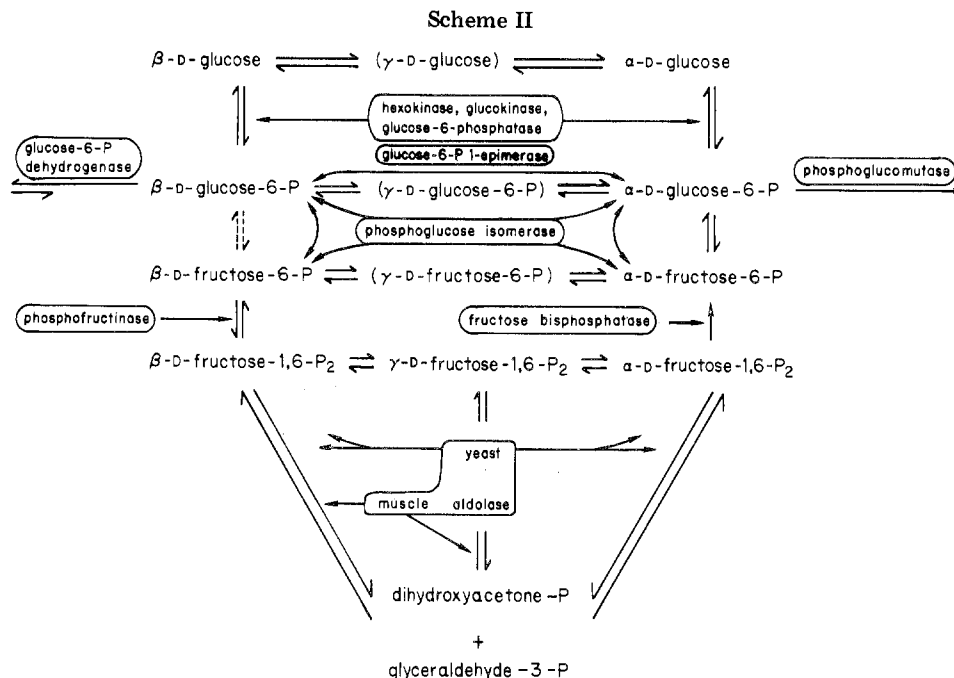
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may be summarized as follows. More extensive descriptions are available.²⁰⁻²²

(1) Hexokinase and glucose-6-phosphatase are non-specific, catalyzing reactions of both anomers.^{15,23}

(2) (a) Phosphoglucumutase that produces α -glucose 1-phosphate utilizes α -glucose 6-phosphate.²⁴ (b) Glucose-6-phosphate dehydrogenase requires the β anomer.¹⁴ (c) Phosphoglucose isomerase (PGI) prefers α -G6P and α -F6P by a factor of at least 20 over the β anomers.^{2,14,25} Not shown in this scheme are the anomeric specificities of the D-xylose, L-arabinose, phosphomannose, and glucosamine-6-phosphate isomerases²⁶⁻²⁸ which are specific for the α , β , β , and α substrate anomers, respectively.

(3) Phosphofruktinase (PFK) is specific for β -F6P.^{16,29-31}

(4) Fructose-1,6-diphosphatase appears to be specific for α -FDP. It has been shown to prefer α - to β - by a factor of 5- to 10-fold,³² but its action on the β anomer now appears to be due to a contaminating nonspecific phosphatase.³³

(5) Muscle aldolase uses as substrate both β -FDP and acyclic FDP,^{34,35} while yeast aldolase is nonspecific.³⁴

(6) Muscle aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase are specific for the free carbonyl (unhydrated) forms of the triose phosphates, though these are not anomeric forms.^{36,37}

(7) (a) Mutarotase catalyzes anomerization of D-glucose. (b) Phosphoglucose isomerase possesses anomerase activity toward glucose 6-phosphate, fructose 6-phosphate,^{2,8,25} and mannose 6-phosphate.²⁷ (c) Glucose-6-phosphate 1-epimerase catalyzes the anomerization of glucose 6-phosphate.³⁸ (d) Yeast aldolase anomerizes its substrate fructose 1,6-bisphosphate.³⁴

The question may be raised as to whether these observed specificities have significance beyond a random selection due to their fit to their respective substrate binding sites. Two possible alternatives suggest themselves: reasons related to the mechanism of the catalyzed reaction and/or reasons related to regulatory selection.

Arguments Related to Enzyme Mechanism

Clearly the postulated enzyme reaction mechanism must deal with the anomeric specificity (or lack of it) in the reaction. Minimally, the geometry of the substrate or reaction intermediates requires catalytic groups to have precise loci. The use of a particular substrate form becomes mechanistically significant if the other form(s) could not be used equally well by the enzyme because of the mechanism of the transformation. That is, the mechanism itself rather than simply the location of catalytic residues imposes a stereochemical requirement which is not satisfied equally well by all substrate forms.

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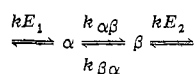
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anomeric forms the rate of anomerization will be an important rate-determining step as the rate of the second or depleting step becomes more rapid than the reverse anomerization step in the steady state:



Thus when $kE_2 > \beta k_{\beta\alpha}$ there will be a lowering of the concentration of β relative to α , and the steady state attained will be a nonequilibrium state in the anomerization step. The result will be a slowing of the E_2 step. This will be most severely felt in a transition between steady states when, due to slow anomerization, there will be a longer transient time and an underutilization of E_2 compared to the situation where anomeric equilibrium obtains or that in which the two enzymes are coupled through a common anomeric form.

The spontaneous rate of anomerization of the sugar phosphates is rapid particularly for F6P and FDP. These are, of course, first-order rates and thus the amount of substrate anomerized is directly proportional to the amount of substrate free to anomerize. Thus, at very low substrate levels the amount of substrate anomerized may be small. Calculations indicate that catalysis of anomerization of G6P may be required to account for the high rate of yeast fermentation.¹⁴

Based upon such considerations Wurster and Hess proposed and then discovered³⁸ an enzyme in yeast, the sole function of which is apparently the anomerization of G6P. In higher organisms this special G6P anomerase function may be performed by PGI which has much broader specificity, being very active with F6P and mannose-6-P as well. Moreover, Gernert and Keston⁴² have recently shown that the widely distributed enzyme mutarotase shows anomerase activity toward G6P at pH 5.5 and have suggested that it may play such a role in tissues of high glycolytic rate such as retina. Furthermore the anomerase activity of PGI is not simply a necessary side reaction of isomerase activity since it has been shown that phosphomannose isomerase does not manifest anomerase activity. It can be noted that phosphomannose isomerase is predicted to produce β -F6P, thus coupling directly to PFK and making an F6P anomerase activity unnecessary as opposed to the case of PGI. All of the above observations suggest a requirement for G6P anomerase activity, and the presence of this activity has been demonstrated.

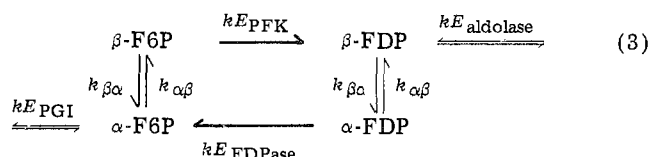
At the FDP level it is interesting that yeast, which utilizes the gluconeogenic pathway, possesses FDP anomerase activity through its FDP aldolase whereas muscle, involved primarily with glycolytic flux, does not. This suggests that liver because of its gluconeogenic activity may require an FDP anomerase if liver aldolase is also specific for a single anomer.

(3) The PFK-FDPase pair constitutes one of the main regulatory points of glycolysis-gluconeogenesis. Regulation is necessary to avoid the breakdown of adenosine triphosphate that results from an uncontrolled "futile cycle" in which PFK uses adenosine triphosphate to phosphorylate F6P and FDPase hydrolyzes the FDP back to F6P. The two enzymes are well known to have reciprocal activator-inhibitor re-

lationship such that effectors which inhibit one enzyme activate the other, and vice versa. The direction of the pathway is thus controlled by the concentrations of these effectors.

It may be that this regulatory control is further facilitated by the circumstance that the two steps do not have common substrates but prefer opposite anomers of F6P and FDP. That is, α -F6P produced by FDPase cannot be immediately utilized by PFK, and the β -FDP produced by PFK is not acted upon by FDPase.

In recent investigations Clark et al.⁴³ have shown that considerable cycling between F6P and FDP occurs in bumblebee flight muscle but that under conditions of increased glycolytic flux the amount of cycling decreases. For conditions of increased flux, it is reasonable to postulate more rapid turnover of substrate pools and possibly decreased concentrations. Anomerization rates that would lead to the incorrect anomer would not compete as effectively, and a smaller percent of the total flux would anomerize. In the example given in eq 3 for



muscle glycolysis, $kE_{\text{aldolase}} > \beta\text{FDP} \times k_{\beta\alpha}$; for liver gluconeogenesis, $kE_{\text{PGI}} > \alpha\text{FDP} \times k_{\alpha\beta}$. One may attempt to answer the two questions which are thus raised: Are the anomerization rates $\alpha\text{F6P} \rightarrow \beta\text{F6P}$ and $\beta\text{FDP} \rightarrow \alpha\text{FDP}$ sufficient to be non-rate-limiting in glycolysis and gluconeogenesis respectively or slow enough to prevent spontaneous cycling under conditions of high flux?

Fructose 1,6-bisphosphate and fructose 6-phosphate are present in liver at levels of 8–15 nmol/g and 20–70 nmol/g,^{44–46} respectively, the lower values of F6P occurring during high gluconeogenic flux. Rate constants for $\alpha\text{F6P} \rightarrow \beta\text{F6P}$ and $\beta\text{FDP} \rightarrow \alpha\text{FDP}$ of 1.6 s⁻¹ and 8.1 s⁻¹ (25 °C, pH 7.6 and 7.2, respectively) have been determined, and one may arbitrarily multiply them by three to obtain an approximate rate at 37 °C. One may then estimate ca. 43–54 nmol s⁻¹ g⁻¹ α -FDP formation (assuming 80% to 100% βFDP at 10 nmol/g) and ca. 21–104 nmol s⁻¹ g⁻¹ βF6P (assuming 20–100% αF6P in 20 nmol/g) or ca. 63–312 nmol s⁻¹ g⁻¹ βF6P (at 60 nmol/g). Overall glycolytic flux of 3–4 and 12–25 nmol s⁻¹ g⁻¹ for rat liver and brain, respectively, have been measured, and gluconeogenic rates up to 20 nmol s⁻¹ g⁻¹ have been measured for rat liver.^{44–46}

The following conclusions may be drawn from these data. (1) $\alpha\text{F6P} \rightarrow \beta\text{F6P}$ rates appear to be sufficiently rapid to be non-rate-limiting in glycolysis. In gluconeogenesis this rate is seemingly rapid enough to allow considerable anomerization, making cycling by PFK possible. However, we note a decrease in F6P concentration (3-fold) in high gluconeogenesis conditions which would possibly serve to reduce cycling from the normal state. (2) $\beta\text{FDP} \rightarrow \alpha\text{FDP}$ rates are within a

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factor of two of gluconeogenic flux and may require nonspecific aldolase or FDP anomerase activity. It is also possible that this step could limit the amount of cycling occurring in glycolysis if no anomerase activity were functioning. (3) These calculations may be altered by the effect of pH on anomerization rates, the availability of substrates for anomerization (i.e., whether they are protein-bound or free to anomerize), and whether the above values are maximal. These effects would probably serve to decrease spontaneous anomerization in relation to flux rates and make the anomerization steps more critical.

Thus the anomerization rates *in vitro* are not more than an order of magnitude faster than rates of metabolic flux. *In vivo* rates may be considerably slower. The similar rates, the pattern of coupled and uncoupled specificities, and the observed anomerase activities all suggest a role for these specificities in the partitioning and possibly rates of utilization of these anomeric substrate pools. The possibility arises that anomerase activities may be required and that these steps are potential regulatory points in addition to those already known. Whether this latter possibility is true awaits determination of anomerase activity levels and studies

investigating possible regulators of these activities.

Conclusion

The determination of the anomeric specificities of many of the enzymes of sugar metabolism allows the development of an overview of the reasons for and of the effects of these specificities. Enzyme reaction mechanisms must take into account these specificities, which may account for the anomeric preference observed. These mechanistic considerations or, additionally, possible regulatory needs may have given rise to enzymic development of these specificities. The specificities are well coupled after the rate-determining $F6P \rightleftharpoons FDP$ step in both glycolysis and gluconeogenesis but are uncoupled prior to this step. These uncoupled steps may act to inhibit cycling of F6P and FDP by imposition of an anomerization step between the enzymes. At the same time there is evidence from rate comparisons and observations of anomerase activities that anomerase activities acting on these steps may be required for maximal flux rates.

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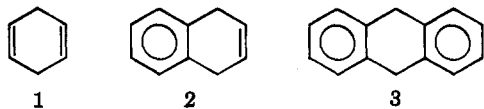
The Conformational Analysis of 1,4-Cyclohexadienes: 1,4-Dihydrobenzenes, 1,4-Dihydronaphthalenes, and 9,10-Dihydroanthracenes

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As evidenced by coverage in classic tests on stereochemistry,¹ much of our information concerning the conformational analysis of carbocyclic rings has traditionally been based on (and in some cases prejudiced by) the vast amount of data available for the cyclohexane ring system. In recent years, largely due to the continued development of NMR spectroscopic techniques, investigators such as Anet, Roberts, and others have provided reports concerned with a variety of conformational types.² As it turns out, the 1,4-cyclohexadiene ring system must be dealt with in three classes of compounds, 1,4-dihydrobenzenes (1), 1,4-



dihydronaphthalenes (2), and 9,10-dihydroanthracenes

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(3), and each of these systems has caused considerable controversy concerning some aspect of its stereochemistry. It would appear that most of these difficulties have now been resolved, and it is the purpose of this Account to convey an accurate review of our current understanding of these systems, as well as to provide a perspective concerning the emergence of a reasonable pattern of conformational behavior in spite of our efforts to hinder this development through preconceived notions and theoretical predictions.

The basic stereochemical concerns in these systems can be categorized in four general divisions. (1) *The shape of the 1,4-cyclohexadiene ring*: this includes the possibility of a planar ring or a boat-shaped conformation. (2) *The values of the homoallylic coupling constants*: the geometrically dependent long-range, five-bond coupling across the 1,4-cyclohexadiene ring. (3) *The behavior of substituents*: this includes the effect of substituents on the conformational preference of the ring, as well as the effect of the ring on rotational populations of the substituents. (4) *Dynamic processes*:

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