Deuterium Isotope Effects in the Fermentation of Hexoses to Ethanol by *Saccharomyces cerevisiae*. II. A Steady-State Kinetic Analysis of the Isotopic Composition of the Methyl Group of Ethanol in an Isotopic Mirror Fermentation Experiment*

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ABSTRACT: A steady-state kinetic analysis of deuterium isotope effects on glycolysis in the alcohol fermentation of ordinary and deuteriohexoses in H_2O and D_2O has been made. The computer calculations have led to a consistent set of relative rates of reaction and primary and secondary deuterium isotope effects that reproduce the isotopic composition of the product ethanol. The deduced rate constants and isotope effects are restricted to a remarkably small range, and the isotopic composition of the ethanols require exchange with the solvent at two stages: (1) exchange accompanying hydrogen transfer between C_2 and C_1 during isomerization of hexose 6-phosphate, and (2) introduction of hydrogen from the medium by enzymatic enolization of pyruvate.

Symbol Table

μ = fraction of PMI exchangeable hydrogens actually exchanged.
γ = fraction of PGI exchangeable hydrogens actually exchanged.
η = fraction of unlabeled hexose species at steady state.
σ = fraction of singly labeled hexose species at steady state.
δ = fraction of doubly labeled hexose species at steady state.
k_0^{HH} = input rate (as mole fraction) of $PEPH_2$.
k_0^{HD} = input rate (as mole fraction) of PEPHD.
k_0^{DD} = input rate (as mole fraction) of $PEPD_2$.
k_p^{H} = rate of adding a proton to $PEPH_2$.
k_p^{D} = rate of adding a deuteron to $PEPH_2$.
k_{-p}^{H} = rate of removing a proton from $PYRH_3$.
k_{-p}^{D} = rate of removing a deuteron from $PYRH_3$.
ϵ = secondary isotope effect on pyruvate exchange (>1).
k_{dec} = rate of pyruvate decarboxylation.
t_g = fraction of protons transferred by PGI rather than exchanged with medium.
t_m = fraction of protons transferred by PMI rather than exchanged with medium.
k_g = rate constant for conversion of glucose-6-P to fructose-6-P *via* PGI.
k_{-g} = rate constant for conversion of fructose-6-P into glucose-6-P *via* PGI.
k_m = rate constant for conversion of mannose-6-P into fructose-6-P *via* PMI.
k_{-m} = rate constant for conversion of fructose-6-P into mannose-6-P *via* PMI.
For definition of $k_{-g}^{H'}$, $k_{-g}^{D'}$, $k_{-m}^{H'}$, and $k_{-m}^{D'}$, see text.
k_0^{G} = input rate of glucose-6-P to hexose phosphate isomerization.
k_0^{M} = input rate of mannose-6-P to hexose phosphate isomerization.
k_t = rate constant for disappearance of fructose-6-P in hexose-6-P isomerization.
k_c = rate constant for aldolase forward reaction.
k_{-c} = rate constant for aldolase back reaction.
k_d = rate constant for conversion of GAP into 1,3-diphosphoglyceraldehyde.
k_i = first-order rate constant for conversion of DHAP into GAP.
k_{-i} = first-order rate constant for conversion of GAP into DHAP.
F^{HH} = fructose-6-P with two H on C_1.
F^{HD} = fructose-6-P with H in the PMI-exchangeable position and D in the PGI-exchangeable position.
F^{DH} = fructose-6-P with H in the PGI-exchangeable position and D in the PMI exchangeable position.
F^{DD} = fructose-6-P with two D on C_1.
G = glucose-6-phosphate, M = mannose-6-phosphate } Figure 3 has four species of each.
See paper I (Saur *et al.*, 1968), footnote 1, for additional symbol definitions.

Part I of this series (Saur *et al.*, 1968) presents the results of isotopic mirror experiments in the study of hexose fermentation by *Saccharomyces cerevisiae*, and discusses the isotopic composition of the product ethanol in a qualitative way in terms of the Embden–Meyerhof–Parnas scheme as it is presently understood.

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While considerations of this sort led us rather firmly to conclusions about the relative importance of alternative hydrogen-exchange mechanisms and to some idea about the rate sequences involved, it appeared desirable to undertake a quantitative treatment of the results.

The Embden–Meyerhof–Parnas pathway has been sufficiently well studied both *in vitro* and *in vivo* by a variety of methods to suggest that a more rigorous analysis might succeed. Our reasons for adopting a steady-state analysis rather than the more laborious method of computer simulation by numerical integration were twofold. First, there is ample experimental evidence that during fermentation by *S. cerevisiae* steady-state conditions are reached very rapidly and are maintained thereafter throughout the process (Holzer and Freytag-Hilf, 1959). Moreover, preliminary calculations using numerical integration of the rate equations showed that the concentrations would, in fact, build up to their steady-state values in a time very short relative to the over-all fermentation time.

The present paper is concerned with the isotopic composition of the methyl group, the analysis of the isotopic composition of the methylene group being deferred for separate consideration.

As will be seen in detail below, the applicable steady-state equations were sometimes sufficiently simple to be handled with a desk calculator.[1] Sometimes, however, the number of simultaneous equations was larger than could be easily solved by hand. Moreover, there were usually more unknowns than equations, so that several of the former had to be treated as parameters and each of them swept through its permissible range, each solution then being checked for its compatibility with the experimental results. All this is most conveniently done by computer. One might expect, as we did initially, that the apparently large number of variables would make it possible to fit the experimental results to several models, leaving the choice among them to biochemical plausibility. As it turned out, however, the experimental results, particularly the matching of those from corresponding isotopic mirrors, were remarkably restrictive. We were invariably led to a single model in which each of the relevant rates fell within a well-defined range. Fortunately the numerical values arrived at do not require violation of any accepted chemical ideas about primary and secondary isotope effects or of biochemical principles. They are also fully consistent with the large body of evidence amassed by previous workers and offer considerable further support for and amplification of the conclusions based on the qualitative considerations in part I (Saur *et al.*, 1968) of this series.

The isotopic composition of the methyl group of ethanol produced in the four fermentation experiments is given in Table III of the preceding paper (Saur *et al.*, 1968). The over-all scheme leading to introduction of hydrogen or deuterium from the solvent into the methyl group is shown in Figure 1, in which the reactions leading to exchange at the hexose-6-P level and the pyruvate level, respectively, are isolated. We will consider, in order, (a) isotopic exchange limited to the hexose-6-P level, (b) hydrogen exchange by pyruvate, and (c) pyruvate exchange superimposed on exchange during hexose-6-P isomerization.

A. Exchange Limited to the Hexose-6-P Level. It is well known from both *in vitro* experiments and yeast fermentation studies that isotopic exchange does occur during hexose-6-P isomerization, its extent depending upon the substrate and other experimental conditions (Rose and O'Connell, 1961; Rose, 1962). The question that arises is whether this exchange is in itself sufficient to account for the isotopic compositions reported in the preceding paper (Saur *et al.*, 1968) in Table III.

Hydrogen exchange with the medium during hexose-6-P isomerization directly affects only the protons at C_1 and C_2 of the starting sugar. Hydrogen exchange at C_6, which must also occur to account for the results of our experiment with glucose-6-d_2, can only take place at the hexose-6-P level *via* recombination of the two isomerized triose phosphates on aldolase and subsequent dephosphorylation (by phosphofructokinase) to fructose-6-P in which C_1 and C_6 have been interchanged.

The crucial characteristic of exchange during enzymatic hexose-6-P isomerization is stereospecificity: of the two equivalent hydrogen atoms at C_1 of fructose-6-P, PGI[2] can exchange only that one derived from C_2 of glucose or C_1 of mannose. Similarly, PMI can exchange only the one derived from C_2 of mannose or C_1 of glucose (Topper, 1957). This strict stereospecificity should also govern the exchange of the hydrogen atoms originally present at C_6 in the hexose. It is easy to see that one of these two atoms is potentially exchangeable by PGI and the other by PMI, provided they retain their stereochemical identity throughout the entire reaction sequence. The only step during which racemization could occur would be dephosphorylation at C_1 by phosphofructokinase, but only if this took place with cleavage of the C–O bond. However, phosphokinases as a rule operate by P–O bond cleavage, and thus leave the stereochemical nature of C_1 intact (Dixon and Webb, 1964).

The fact that exchange by PGI and exchange by PMI are two distinct processes which must, barring secondary isotope effects, be uncorrelated allows us to apply a statistical criterion which, though mathematically almost trivial, is quite powerful.

Let μ be the fraction of PMI-exchangeable hydrogen atoms which are actually exchanged and γ be the corresponding fraction of PGI-exchangeable hydrogen atoms actually exchanged at C_1 and C_6 of the hexose-6-P. $1 - \mu$ and $1 - \gamma$ will then be the respective fractions of unexchanged hydrogens. If the reasonable assump-

[1] The analysis of Benjamin and Collins (1956), using the area theorem of J. Z. Hearon, may also be applied to our data. Although this method does not require the assumption of a steady state, it offers no advantage over the steady-state treatment.

[2] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PGI, phosphoglucose isomerase; PEP, phosphoenolpyruvate; PMI, phosphomannose isomerase; PK, pyruvate kinase; $PEPD_2$, phosphoenolpyruvate with two deuterons on C_3; PEPDH, phosphoenolpyruvate with one deuteron and one proton on C_3; $PEPH_2$, phosphoenolpyruvate with two protons on C_3.

TABLE I: Required Relative Input Rates of Isotopically Labeled Phosphoenolpyruvate into PK Step.[a]

Rate Constants	ϵ	Glucose-6-d_2 in H_2O	Deuterio-D-mannose in H_2O	D-Mannose in D_2O	D-Glucose in D_2O
k_0^{HH}	1.000	0.50	0.161 ± 0.008	0.505 ± 0.016[b]	0.728 ± 0.020
	1.125	0.50	0.158 ± 0.007	0.505 ± 0.016[b]	0.589 ± 0.022
k_0^{HD}	1.000	0.0	0.333 ± 0.012	0.331 ± 0.006	0.153 ± 0.011
	1.125	0.0	0.339 ± 0.011	0.370 ± 0.007	0.192 ± 0.012
k_0^{DD}	1.000	0.50	0.505 ± 0.016[b]	0.164 ± 0.016	0.119 ± 0.015
	1.125	0.50	0.505 ± 0.016[b]	0.126 ± 0.023	0.219 ± 0.029

[a] For definitions of K_0's, see text. [b] Our model requires that these values be at least 0.50 since C_6 provides half of the input.

tion is made that the rate of PGI labeling is unaffected by the H/D ratio in the PMI position and *vice versa*, simple statistical considerations require that the fractional distribution of labels[1] is as listed in eq 1

$$\eta = (1 - \mu)(1 - \gamma)$$
$$\sigma = (1 - \mu)\gamma + (1 - \gamma)\mu \quad (1)$$
$$\delta = \mu\gamma$$

where η, σ, and δ represent, respectively, unlabeled, singly labeled, and doubly labeled species. The values μ and γ will then be the two roots of the quadratic equation

$$x^2 = (\sigma + 2\delta)x + \delta = 0 \quad (2)$$

For real roots of μ and γ, we must have

$$(\sigma + 2\delta)^2 \geq 4\delta \quad (3)$$

Introduction of experimental values for σ and δ from Table I yields no real solutions for either of the two mannose experiments or for the glucose–D_2O experiment. The glucose-6-d_2 values can be fit, but only with either $\gamma = 0$ or $\mu = 0$, *i.e.*, either no PMI or no PGI exchange, a result clearly inconsistent with the appearance of doubly exchanged species in the other experiments.

The criterion just employed would not apply if PMI and PGI exchange were in fact interdependent, by virtue of a secondary isotope effect on hexose-6-P isomerization. The experimental distribution of variously labeled species is so different from that required by eq 2 as to require unreasonably large secondary isotope effects. Moreover, the observed ratio of singly to doubly labeled species is too low for an agreement with uncorrelated stereospecific labeling in *both* mannose mirror experiments, requiring that the second exchanged atom be introduced more easily than the first, both when the first atom is H and when it is D. It is difficult to reconcile such a secondary isotope effect with established chemical principles. It seems hardly more reasonable to postulate a positive correlation of this type between PGI and PMI activity. Clearly, hexose-6-P isomerization cannot alone account for the experimental results, even if both hexose-6-P isomerases are present and C_1–C_6 interconversion is allowed.

B. Labeling by Pyruvate Exchange. The only remaining step in the Embden–Meyerhof–Parnas scheme in which isotopic exchange leading to methyl labeling can occur is the interconversion of phosphoenolpyruvate and pyruvate by PK. The detailed scheme for this exchange is shown in Figure 2. Although our model requires that enolization of pyruvate take place while pyruvate is bound to the enzyme as shown by Rose (1960), it does not depend upon whether pyruvate released by the enzyme can recomplex for further exchange or whether the discharge from the enzyme is essentially

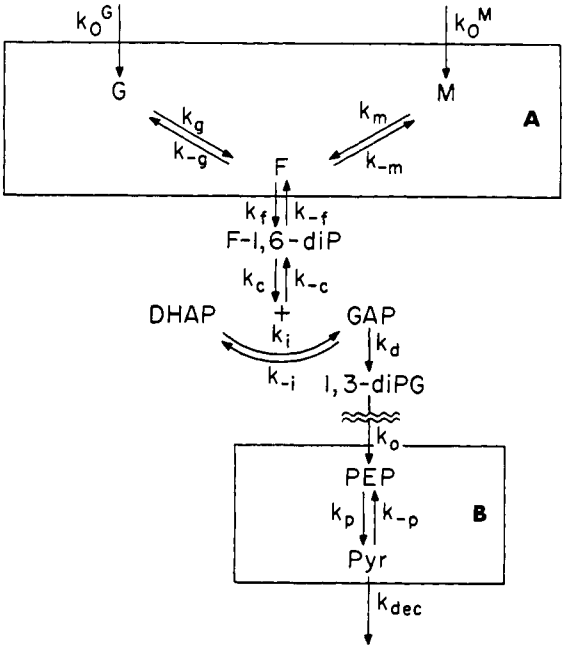

FIGURE 1: The general scheme of enzymatic steps in glycolysis leading to the labeling of methyl groups in ethanol fermentation. Box A shows the reactions that lead to isotopic exchange at C_1 mediated by hexose-6-P isomerases. Box B shows the conversion of PEP into pyruvate by pyruvate kinase at which there will be isotopic exchange at carbon atoms corresponding to C_1 and C_6 of the original hexose.

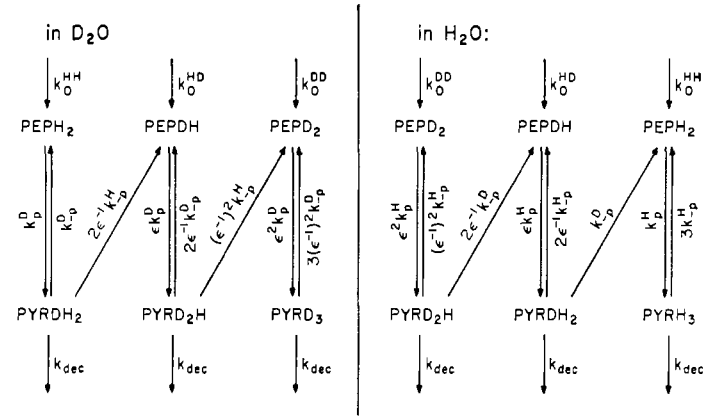

FIGURE 2: A detail of the course of isotopic exchange with the medium at the pyruvate kinase step. The exchange rates, k_p and k_{-p}, are normalized for the secondary isotope effect, ϵ, and statistically weighted to account for the possible multiplicity in back reactions. k_{dec} is the decarboxylation rate.

irreversible, as appears from the work of Kerson *et al.* (1967).

The reaction schemes in H_2O and D_2O have been shown separately in order to keep the isotopic nature of the various species clear. k_0^{HH}, k_0^{HD}, and k_0^{DD} represent the input rates of the phosphoenolpyruvate species ($PEPH_2$, PEPDH, $PEPD_2$) carrying H or D previously introduced from the solvent by hexose-6-P isomerization. k_p^H and k_{-p}^H are the rate constants for adding and removing, respectively, a proton from a carbon atom bearing two H atoms. The corresponding rate constants for addition or removal of a deuterium atom are k_p^D and K_{-p}^D. The presence of other deuterium atoms on the reacting carbon atom is taken into account in two ways: (a) the statistical factors governing number of exchangeable H or D atoms are introduced explicitly, and (b) the possibility of a secondary isotope effect is included by introducing a factor ϵ for each D atom present in the PEP entering the reaction, and assuming each D atom exerts its isotope effect independently. The secondary isotope effect on the reverse reaction is assumed to be ϵ^{-1}, as will be explained below. The rate constant for decarboxylation of pyruvate, k_{dec}, is assumed to be isotope independent. The steady-state condition requires that the total input ($k_0^{HH} + k_0^{HD} + k_0^{DD}$), which is normalized to unity, be equal to the output rate $k_{dec}\Sigma[Pyr]$. Since the pyruvate concentrations are also normalized to unity, $k_{dec} = 1$. The further requirement that the concentrations of the various species remain constant leads in each of the two mirrors to a simple set of three simultaneous equations. For H substrate in D_2O, they are

$$k_0^{HH} = (1 + 2\epsilon^{-1}k_{-p}^H)[PyrDH_2] \quad (4)$$

$$k_0^{DH} + 2\epsilon^{-1}k_{-p}^H[PyrDH_2] = (1 + (\epsilon^{-1})^2k_{-p}^H)[PyrD_2H] \quad (5)$$

$$k_0^{DD} + (\epsilon^{-1})^2k_{-p}^H[PyrD_2H] = [PyrD_3] \quad (6)$$

For D substrate in H_2O, they are correspondingly

$$k_0^{DD} = (1 + 2\epsilon^{-1}k_{-p}^D)[PyrD_2H] \quad (7)$$

$$k_0^{HD} + 2\epsilon^{-1}k_{-p}^D)[PyrD_2H] = (1 + k_{-p}^D)[PyrDH_2] \quad (8)$$

$$k_0^{HH} + k_{-p}^D[PyrDH_2] = [PyrH_3] \quad (9)$$

Consider first the experiments with glucose-6-d_2 in H_2O. Since we are not allowing any C_1–C_6 interconversion, all the hydrogens from C_1 and C_2 enter PEP as H, and all those from C_6 as D, *i.e.*, $k_0^{HH} = k_0^{DD} = 0.50$ and $k_0^{HD} = 0$. At steady state, the isotopic composition of the pyruvate leaving the reaction must be identical with that of the methyl group in the product ethanol. Substituting this experimental fraction of CD_2H into eq 7 as [$PyrD_2H$] along with its experimental error yields eq 10 but does not allow us to evaluate separately

$$(2\epsilon^{-1}k_{-p}^D) = 0.214 \pm 0.030 \quad (10)$$

k_{-p}^D and ϵ^{-1}. Equation 7 can, of course, also be used to solve for the input of $PEPD_2$ in the deuteriomannose experiment using the same value of $(2\epsilon^{-1}k_{-p}^D)$, because pyruvate exchange should be the same for both glucose and mannose input. Since the output of the unexchanged $PYRD_2H$ is the same as in the glucose-6-d_2 experiment, k_0^{DD} necessarily turns out to be the same. The clear implication is that only the hydrogen atoms at C_6 survive exchange at the hexose-6-P level, while those originating from C_1 are fed into the PK step as either PEPDH or $PEPH_2$. However, in order to arrive at values of k_0^{HH} and k_0^{HD} by solving eq 8 and 9, some value must be assigned to ϵ, the secondary isotope effect.

It is well established that in reactions involving a transition from the tetrahedral to the trigonal configuration of a carbon atom bearing an H atom, replacement of the latter by D reduces the rate by about 10–15%. The deuterium isotope effect on addition to double bonds is inverse, increasing the rate to about the same extent (Halevi, 1963). ϵ will therefore be larger than unity, but probably no larger than 1.15. Equations 8 and 9 were solved with two test values of ϵ, 1.000 and 1.125, representing a zero secondary isotope effect and a characteristic one. The corresponding values for k_0^{HH}, k_0^{HD}, and k_0^{DD} in the deuteriomannose experiment are shown in third column of Table I. The test values of ϵ evidently make no essential difference in this experiment.

The mannose–D_2O experiment can now be easily accommodated if it is assumed, in analogy to its mirror, that C_6 enters the pyruvate exchange only as unex-

TABLE II: Relative Output of Fructose-6-P Species Labeled at C_1 from Hexose-6-P Isomerization.[a]

Fructose Species	Deuterio-D-mannose in H_2O	D-Mannose in D_2O	D-Glucose in D_2O
F^{HH}	0.316 ± 0.014	0.010 ± 0.032	0.178 ± 0.044
$F^{HD} + F^{DH}$	0.678 ± 0.022	0.740 ± 0.014	0.384 ± 0.024
F^{DD}	0.010 ± 0.032	0.252 ± 0.047	0.438 ± 0.058

[a] Converted from Table II (see text).

changed $PEPH_2$, and that the amount of C_1 entering as unexchanged phosphoenolpyruvate cannot exceed the slight excess over 50% found in the deuteriomannose experiment. Introducing this value of k_0^{HH} and the experimental value for $[PyrDH_2]$ into eq 4 yields

$$(2\epsilon^{-1}k_{-p}^{H}) = 1.805 \pm 0.097 \qquad (11)$$

Taking the ratio of this result and the corresponding value from eq 10 gives the primary isotope effect on pyruvate enolization to be $k_{-p}^{H}/k_{-p}^{D} = 8.4$ (with limits of 6.9 and 10.2). This value is independent of any assumption about the secondary isotope effect. This apparently large effect is, in fact, characteristic of primary deuterium isotope effects observed in enolization of a variety of ketones (Melander, 1960). Simon and Medina (1968) have also observed exchange at the pyruvate kinase step by means of tracer experiments using tritium and ^{14}C-labeled glucose (Simon *et al.*, 1968; Schmidt *et al.*, 1968).

The accompanying values of k_0^{HD} and k_0^{DD} for the mannose–D_2O experiment consistent with eq 5 and 6 are shown in the fourth column of Table I. These are somewhat more sensitive to the choice of ϵ because the exchange proceeds much farther in D_2O than in the corresponding mirror.

The input rates of the various PEP species for the glucose–D_2O experiment are now determined using eq 4-6 and exchange parameters determined from the mannose–D_2O data. These rates are listed in column five of Table I. The larger k_0^{HH} value than in the corresponding mannose experiment requires that a good proportion of the hydrogen atoms originally present at C_1 survive hexose-6-P isomerization. Just how much $PEPH_2$ must enter the PK step now depends strongly upon the value assumed for ϵ, but a choice cannot be made until the hexose-6-P isomerization is considered in the section C.

C. Hexose-6-P Isomerization Superimposed on Pyruvate Exchange. The sets of k_0^{HH}, k_0^{HD}, and k_0^{DD} that appear as required input to the PK step in Table I also represent the relative rates at which the correspondingly labeled triose phosphates emerge from the isomerization–phosphorylation–dealdolization sequence. The distribution of isotopic species is, of course, determined by hexose-6-P isomerization, which is stereospecific, so the relation expressed by eq 3 can be applied to the proportions of singly and doubly exchanged species (k_0^{HD} and k_0^{DD} in the D_2O experiments, k_0^{HD} and k_0^{HH} in the H_2O experiments). This criterion cannot be applied to the glucose-6-d_2 experiment since the fraction 0.5 listed as k_0^{HH} is derived from C_1 and C_2 of the glucose-6-d_2, which were nondeuterated to begin with. Moreover, k_0^{DD} and k_0^{HD} were fixed at 0.5 and 0.0, respectively, because our present model does not allow any exchange of C_6 hydrogens prior to the PK step. If secondary isotope effects on the hexose isomerization can be neglected, the other three experiments should obey eq 3. Both sets of values for each of the mannose mirrors do so. However, of the two sets listed for the glucose–D_2O experiment, the one required as input to pyruvate exchange without a secondary isotope effect ($\epsilon = 1.0$) does not obey the criterion. It can be easily determined that ϵ must be at least 1.1 for the required hexose-6-P output to fit eq 3. The preferred value, $\epsilon = 1.125$, is, as already noted, a characteristic one and lies at the midpoint between the minimum value permitted by the data and a 15% secondary isotope effect, which chemical considerations place near the upper limit of plausibility.

For a detailed consideration of the hexose-6-P isomerization scheme it is convenient to subtract the unexchanged 50% derived from C_6 and to renormalize the remaining 50% from C_1 to unity. As explained above, only those values obtained with $\epsilon = 1.125$ in the PK step need be considered. The renormalized figures which represent the output of fructose-6-P variously labeled at C_1 are listed in Table II. The isomerization scheme is outlined in Figure 3. G and M represent glucose-6-P and mannose-6-P in which the isotopic labeling in the 1 or 2 position is marked. In the four fructose-6-P species, F, the first superscript refers to the atom exchangeable by PMI, the second to that exchangeable by PGI. t_g is the fraction of protons or deuterons transferred between C_1 and C_2 of glucose-6-P, rather than exchanged with the medium. Following Rose, we assume this fraction to be the same in both directions (Rose, 1962). t_m is the corresponding quantity for transfer in PMI. k_0^{G} is the rate of glucose-6-P input and k_0^{M} is the rate of mannose-6-P input. We arbitrarily set either k_0^{G} or k_0^{M} to unity, according to which aldohexose is being fed; the other will necessarily be zero. Since the over-all fermentation rate for the four experiments was about the same, we use the same input (unity) in each case. The quantities k_g and k_{-g} are the forward and backward rate constants for PGI, and k_m and k_{-m} the corresponding PMI rate constants. These rate constants are superscripted according to the isotopic nature of the atom removed, to allow for an isotope effect on isomerization rate. In *in vitro* experiments on the glucose-6-P to fructose-6-P isomerization, Rose and O'Connell (1961) have found

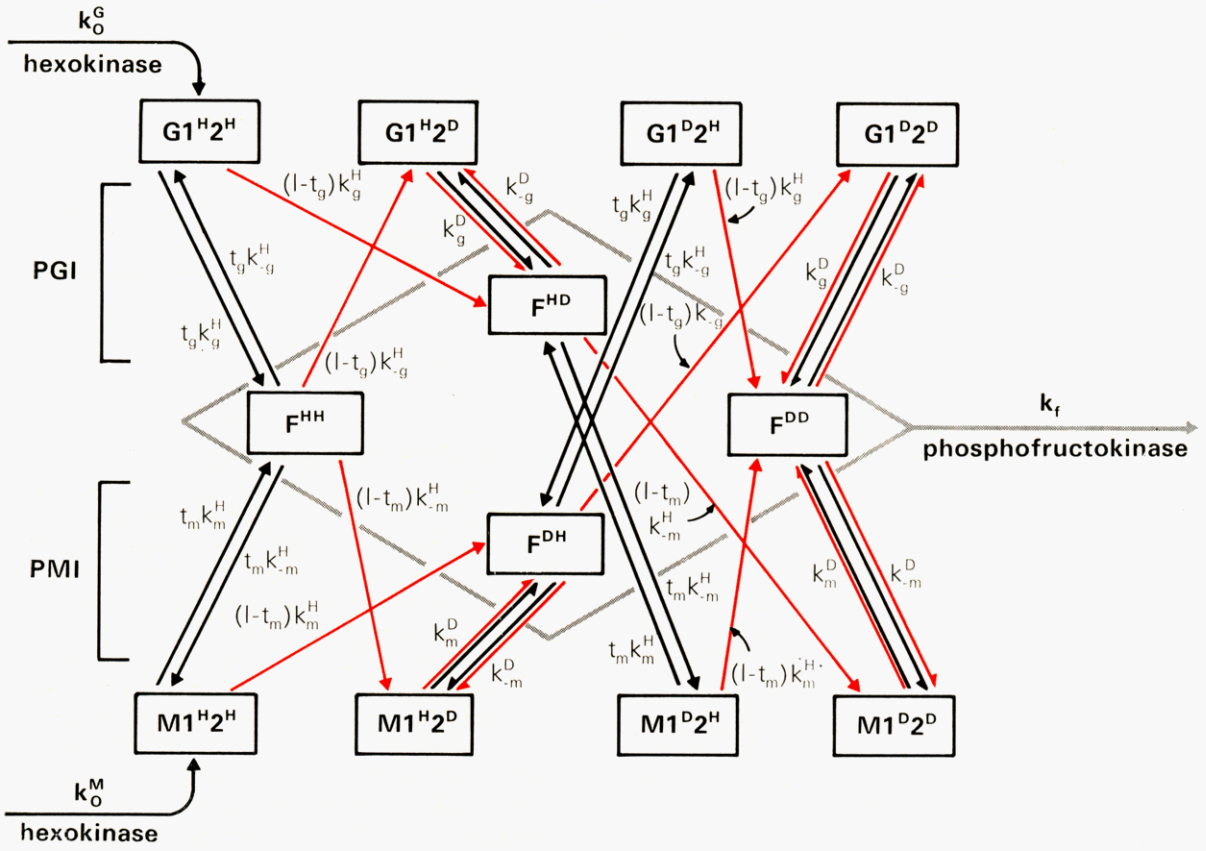

FIGURE 3: The scheme of isotopic exchange with the medium during isomerization of hexose-6-P; M and m, mannose-6-P; t_g and t_m, transfer fractions; k, isomerization rate constants; H and D, protium and deuterium. Black reactions are those in which there is only transfer of hydrogen. The red arrows indicate exchange reactions. Black and red together show reactions in which transfer or exchange leads to the same product.

that deuterium in the exchangeable position slows the rate by a factor of 1.86 in both directions, and we may reasonably assume a similar effect in the PMI isomerization. Saturation of the isomerase would reduce this factor, the limit being 1.00 for a completely saturated enzyme. We are not assuming a substrate isotope effect on t_g and t_m since according to the mechanism proposed by Rose (1962), t_g represents the probability of a label being transferred from the enzyme back to the substrate rather than being lost to the medium. This competition for the label should not depend upon mass of the label, though it may well be subject to a solvent isotope effect for reasons similar to the temperature dependence of t_g found by Rose (1962). Finally, k_f is the rate constant for removal of fructose-6-P from the hexose-6-P isomerase system *via* phosphofructokinase. Since we are expressing the concentrations of fructose-6-P species as mole fractions (their sum consequently being unity), and since steady-state kinetics require that

$$k_0 = ki\Sigma[\text{fructose-6-P}] \qquad (12)$$

it follows that k_f must be unity. This convention converts k_f, k_{-g}, and k_{-m} from first-order rate constants into zero-order rate constants. Our treatment uses k_0 equal to unity. If we had set k_0 at its physiological value of 0.25 μmole/g (see Lynen, 1963, and Betz and Chance, 1965) and expressed [fructose-6-P] in the same units, the magnitude and dimensions of k_f would change accordingly, but the quantities derived from the calculations would change only by a scale factor and the conclusions would be identical.

For each system (D_2O and H_2O), the steady-state treatment requires setting up a steady-state equation for each of the 12 species shown in Figure 4. These equations are obtained by assuming that the rate of appearance of each species equals its rate of disappearance. The resulting system of 12 equations can be reduced by substitution to the following three simultaneous equations (see Appendix A).

For glucose in D_2O, these are

$$t_g^{D_2O} = (1 + k_{-g}^{H'} + k_{-m}^{H'})[F^{HH}] \qquad (13)$$

$$k_{-m}^{H'}[F^{HH}] = (k_{-g}^{H'} + 1)[F^{DH}] \qquad (14)$$

$$k_{-g}^{H'}[F^{DH}] + k_{-m}^{H'}[F^{HD}] = [F^{DD}] \qquad (15)$$

For mannose in D_2O, they are

$$t_m^{D_2O} = (1 + k_{-m}^{H'} + k_{-g}^{H'})[F^{HH}] \qquad (16)$$

$$k_{-g}^{H'}[F^{HH}] = (k_{-m}^{H'} + 1)[F^{HD}] \qquad (17)$$

$$k_{-m}^{H'}[F^{HD}] = k_{-g}^{H'}[F^{DH}] = [F^{DD}] \qquad (18)$$

For deuteriomannose in H_2O, they are

$$t_m^{H_2O} = (1 + k_{-g}^{D'} + k_{-m}^{D'})[F^{DD}] \qquad (19)$$

$$k_{-g}^{D'}[F^{DD}] = (k_{-m}^{D'} + 1)[F^{DH}] \qquad (20)$$

$$k_{-m}^{D'}[F^{DH}] + k_{-g}^{D'}[F^{HD}] = [F^{HH}] \qquad (21)$$

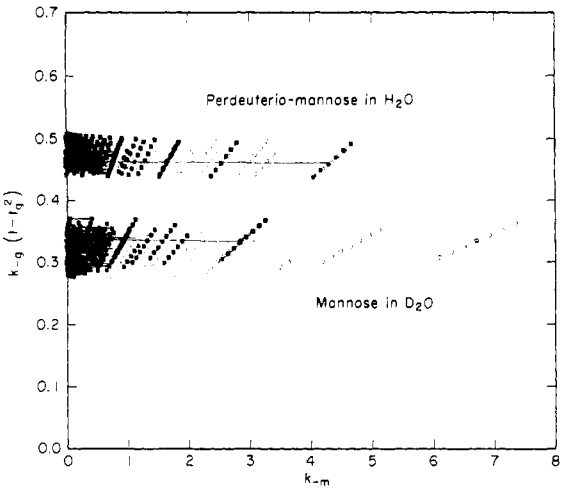

FIGURE 4: The values plotted above represent points for k_{-m} and $k_{-g}(1 - t_g^2)$ corresponding to experimental concentrations for the four fructose-6-P species. One point was calculated for each increment of $[F^{HD}] = i(0.1)[[F^{HD}] + [F^{DH}]]$ with $i = 0, 1, 2 \ldots, 10$. The multiplicity of points indicates the experimental uncertainty in the fructose concentrations. Filled squares, solutions where $0 < t_m \leqslant 0.05$; Open circles, solutions for $0.05 < t_m \leqslant 0.10$. The connected line links the values found for varying $[F^{HD}]/[F^{DH}]$ when $[F^{DD}]$ and $[F^{HH}]$ were set at the midvalue of their experimental range (Table III, columns 1 and 2 of Saur *et al.*, 1968) and the stipulation was made that $[F^{DD}] + [F^{DH}] + [F^{HD}] + [F^{HH}] = 1$.

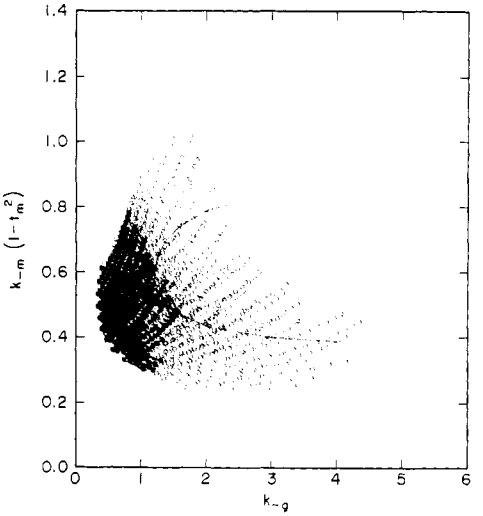

FIGURE 5: Points for k_g and $k_m(1 - t_m^2)$ corresponding to experimental concentrations for the four fructose species in the fermentation of ordinary glucose in D_2O. When the fructose concentrations are swept over their experimental range, a family of parabolas are obtained, giving the "blur" of the figure. Hence, the range of k_{-g} *vs.* $k_{-m}(1 - t_m^2)$ which could give the observed experimental range is indicated in the figure. The filled squares are solutions for $0.73 \leqslant t_g < 0.80$, the open circles for $0.80 < t_g \leqslant 0.90$. The connected line is obtained by varying $[F^{HD}]/[F^{DH}]$ when $[F^{HH}]$ and $[F^{DD}]$ are set at the midvalue of their experimental range (Table III, column 3 of Saur *et al.*, 1968).

In these equations, the fructose species are as identified in Figure 3; the primed rate constants are: $k_{-g}^{H} = k_{-g}^{H}[1 - (t_g^{D_2O})^2]$, $k_{-m}^{H'} = k_{-m}^{H}[1 - (t_m^{D_2O})^2]$, $k_{-g}^{D'} = k_{-g}^{D}[1 - (t_g^{H_2O})^2]$, and $k_{-m}^{D'} = k_{-m}^{D}[1 - (t_m^{H_2O})^2]$.

Each of the above sets of three simultaneous equations can be used to determine directly two rate constants and a transfer ratio when the concentrations of the four fructose species are given. Equations 13–15 give $t_g^{D_2O}$, k_{-g}^{H}, and $k_{-m}^{H'}$; eq 16–18 give $t_m^{D_2O}$, k_{-m}^{H}, and $k_{-g}^{H'}$; eq 19–21 give $t_m^{H_2O}$, k_{-m}^{D}, and $k_{-g}^{D'}$. Several points should be noted. (a) Equations 16–18 can be obtained from eq 13–15 by reflection in the aldohexose mirror, *i.e.*, interchanging subscripts g and m, and taking into account the stereospecificity by also interchanging F^{HD} and F^{DH}. Equation 15 is identical with eq 18 because it is symmetric to this reflection. (b) Equations 19–21 are obtained from eq 16–18 by reflection in the isotopic mirror, *i.e.*, interchanging H and D throughout, wherever they appear. (c) The concentrations of the aldohexoses and the rate constants leading from them do not appear. (d) The various k^D values disappear when the substrate does not initially contain deuterium, as does k^H when it is initially fully deuterated. (e) k_{-g} and k_{-m} always appear multiplied by a factor containing the corresponding transfer fraction, t_g or t_m. Since t_g appears separately when glucose is fermented and t_m when mannose is fermented, t_g and k_{-g} can be individually evaluated in glucose experiments, k_m' remaining as a product of k_m and $1 - t_m^2$ that cannot be further reduced unless information based on other experiments is used. Similarly, mannose experiments yield t_m and k_{-m} separately, but retain $k_{-g}(1 - t_g^2)$ as a product. (f) The solution of the three simultaneous equations for these three unknowns is complicated by the fact that although $[F^{HH}]$ and $[F^{DD}]$ can be derived from experiment, only the sum of $[F^{HD}]$ and $[F^{DH}]$ is known (see Table II). The entire range of the latter two concentrations, from pure F^{HD} to pure F^{DH}, consistent with their sum being equal to $(1 - [F^{HH}] - [F^{DD}])$, had, therefore, to be scanned necessitating a computer solution. In each case $[F^{HH}]$ and $[F^{DD}]$ were also swept over the range defined by their experimental errors.

The results are most easily discussed on the basis of a graphical representation. Therefore, the punched-card output from the CDC 3600, in the form of matched values of t_g, k_{-g}, and $k_{-m}(1 - t_m^2)$ or t_m, k_{-m}, and $k_g(1 - t_g^2)$, corresponding to a given set of fructose concentrations, was subsequently fed into an IBM 1620 with an on-line CALCOMP plotter. Figure 4 comprises a superposition of two such plots of $k_{-g}(1 - t_g^2)$ *vs.* k_{-m}, one for each of the two mannose mirrors. Regions corresponding to different ranges of t_m are indicated by differently shaped symbols, the density of the calculated points being sufficiently large to map out regions in which matching constants fit the data in Table II within the stated experimental error. Figure 5 is the corresponding plot of $k_{-m}(1 - t_m^2)$ *vs.* k_g for the glucose–D_2O case. In each plot the connected line represents fits of the data in which all the fructose concentrations are assigned the midvalue of their experimental range.

Consider first Figure 4. For both mannose mirrors, k_{-m} is unrestricted, an increase in k_{-m} being compensated by a corresponding increase in t_m. However, there is independent evidence that t_m must be very small, Simon and Medina (1966 and 1968) having found $t_m^{H_2O}$

to be about 0.05. While, for reasons that will be discussed below in connection with glucose, $t_m^{D_2O}$ may be somewhat larger, we would hardly expect it to exceed 0.10. This restriction limits k_{-m}^{D} to less than about 5 and k_{-m}^{H} to be no larger than approximately 8. We go on to note that, regardless of k_{-m}^{H}, $k_{-g}^{H}[1 - (t_g^{D_2O})^2]$ has a most probable value of 0.35, and, independent of k_{-m}^{D}, $k_{-g}^{D}[1 - (t_g^{H_2O})^2]$ has a most probable value 0.47. From *in vitro* experiments of Rose and O'Connell (1961) we know that for unsaturated PGI, k_{-g}^{H} is larger than k_{-g}^{D} by a factor of 1.86. It follows that if this enzyme is unsaturated *in vivo* when mannose is being fermented, $[1 - (t_g^{H_2O})^2]/[1 - (t_g^{D_2O})^2]$ is equal to $1.86 \times (0.47/0.35)$ or 2.5. Simon and Medina (1966) have found $t_g^{H_2O}$ to be 0.55–0.58, so that $t_g^{D_2O} \cong 0.86$. This is a maximum value, since if the enzyme is saturated and enzyme complex formation becomes rate determining, the isotope effect should vanish. In this case, since $k_{-g}^{D} = k_{-g}^{H}$, we find, retracing the calculation, a minimum value of $t_g^{H_2O} \cong 0.69$. The fact that $t_g^{D_2O} > t_g^{H_2O}$ is not surprising. Rose has shown that t_g increases as the temperature is lowered, and D_2O is universally considered to be a "cooler" solvent than H_2O by virtue of its being more highly structured (Némethy and Scheraga, 1962). Finally for the mannose experiments, we can evaluate k_{-g}^{D} to be about 0.7 and k_{-g}^{H} to be between 0.7 and 1.3 depending upon the degree of unsaturation of the PGI.

We can now turn to Figure 5. The shape of this graph is very different, evidently because proton transfer on PGI is so much higher than on PMI. The most striking feature is that no solutions at all were obtained for $t_g^{D_2O} < 0.73$. Increasing $t_g^{D_2O}$ toward unity allows k_{-g}^{H} to increase without bound. However, the mannose experiments restrict the accessible region to well below 0.90, as has just been shown. The two areas distinguished in the graph correspond to $t_g^{H_2O}$ 0.73–0.80 and 0.80–0.90. We see that k_{-g}^{H} is centered at about 1.3, within about a factor of two. $k_{-m}[1 - (t_m^{D_2O})^2]$, which is virtually identical with k_{-m} since $t_m^{D_2O}$ is so low, is about 0.5, again within a factor of two or so.

It is rather surprising that in a yeast fermenting glucose, the PMI activity is so close to that of the PGI activity. It is no less remarkable that the PGI activity in a yeast fermenting mannose is nearly the same as in a yeast fermenting glucose. The complementary evidence with regard to PMI activity in the two cases is less clear-cut, but it cannot be much more than ten times as great in a mannose-fermenting yeast than in a glucose-fermenting yeast, and the data are compatible with PMI activity being very much more closely similar in the two cases.

Summary

In this research, we have considered the isotopic makeup of the methyl group of ethanol produced by *S. cerevisiae* by fermentation of glucose and mannose in D_2O, and from glucose-6-d_2 and deuteriomannose in H_2O.

The Embden–Myerhof–Parnas glycolytic pathway requires that one of the methyl hydrogen atoms be derived from the medium at the conversion of phosphenolpyruvate to pyruvate. There are two steps in the scheme where the incorporation of additional hydrogen (or deuterium) from the medium can be reasonably assumed to occur: (a) exchange accompanying hydrogen transfer between C_2 and C_1 during isomerization of the hexose 6-phosphate; (b) exchange by enolization of pyruvate, presumably while this is still bound to pyruvate kinase.

A steady-state analysis of isotopic exchange during these steps was carried out in order to ascertain whether a consistent set of relative rates could be found that would reproduce the isotopic composition of the ethanol derived from fermentation under the four different experimental conditions. It was found that neither a nor b alone can account for all the isotopic exchange. Partial exchange during step a followed by further exchange during step b could be made to fit the experimental results, and here too the data were remarkably restrictive with regard to the values which could be assigned to the relative rates.

It is gratifying that the relative rates which had to be assumed in order to match the experimental results are concordant with the results of a large number of *in vivo* and *in vitro* studies of glycolysis, and in particular with those dealing with the isotopic exchange associated with stereospecific hydrogen transfer during hexose 6-phosphate isomerization (Rose and O'Connell, 1961; Rose, 1962; Topper, 1957; Simon and Medina, 1966, 1968). With regard to pyruvate exchange, the data compel the adoption of a primary and a secondary isotope effect. The direction and magnitude of each of these effects is characteristic of the corresponding isotope effect on enolization in nonbiological systems (Halevi, 1963; Melander, 1960).

In addition to establishing the fact that isotopic exchange occurs during enzymatically catalyzed conversion of PEP into pyruvate, a finding that has since been confirmed *in vitro* (H. L. Crespi, W. K. Saur, and J. J. Katz, 1968, unpublished data), this research also casts some light on the relative activities of the hexose phosphate isomerases. Specifically, it shows that the activity of PMI during glucose fermentation, or of PGI during mannose fermentation, is similar in magnitude to that of the isomerase actually required for fermentation.

Probably the most significant result of the present study is the fact that isotopic mirror experiments (Katz and Crespi, 1966; Katz *et al.*, 1966) are not limited to qualitative comparisons, but can be rendered quantitative, provided the details of the biochemical sequence under study are well enough understood. How valuable this tool will be in the investigation of biochemical systems more complicated or less well established than the Embden–Meyerhof–Parnas glycolysis pathway remains for future work.

Appendix A

Steady-State Treatment of Hexose-6-P Exchange. We will derive the equations for the D_2O experiments. Their isotopic mirrors can be handled in the same way.

For simplicity of notation, t_g and t_m will be used, instead of the more cumbersome $t_g^{D_2O}$ and $t_m^{D_2O}$; $k_f = 1$. Other symbols are as defined in the text.

When the steady-state condition (time derivative equals zero) is applied to the four glucose concentrations, we obtain

$$k_0^G + t_g k_{-g}^H[F^{HH}] = \{t_g k_b^H + (1 - t_g)k_g^H\}[G1^H2^H] = k_g^H[G1^H2^H] \quad (A1)$$

$$(1 - t_g)k_{-g}^H[F^{HH}] + k_{-g}^D[F^{HD}] = k_g^D[G1^H2^D] \quad (A2)$$

$$t_g k_{-g}^H[F^{DH}] = k_g^H[G1^D2^H] \quad (A3)$$

$$(1 - t_g)k_{-g}^H[F^{DH}] + k_{-g}^D[F^{DD}] = k_g^D[G1^D2^D] \quad (A4)$$

From the four mannose concentrations, we get

$$k_0^M + t_m k_{-m}^H[F^{HH}] = k_m^H[M1^H2^H] \quad (A5)$$

$$(1 - t_m)k_{-m}^H[F^{HH}] + k_{-m}^D[F^{DH}] = k_{-m}^D[M1^H2^D] \quad (A6)$$

$$t_m k_{-m}^H[F^{HD}] = k_{-m}^H[M1^D2^H] \quad (A7)$$

$$(1 - t_m)k_{-m}^H[F^{HD}] + k_{-m}^D[F^{DD}] = k_m D[M1^D2^D] \quad (A8)$$

From the concentrations of the four fructoses

$$t_g k_g^H[G1^H2^H] + t_m k_m^H[M1^H2^H] = (1 + k_{-g}^H + k_{-m}^H)[F^{HH}] \quad (A9)$$

$$(1 - t_g)k_g^H[G1^H2^H] + t_m k_m^H[M1^D2^H] + k_g^D[G1^H2^D] = (1 + k_{-g}^D + k_{-m}^H)[F^{HD}] \quad (A10)$$

$$(1 - t_m)k_m^H[M1^H2^H] + t_g k_g^H[G1^D2^H] + k_m^D[M1^H2^D] = (1 + k_{-m}^D + k_{-g}^H)[F^{DH}] \quad (A11)$$

$$(1 - t_g)k_g[G1^D2^H] + (1 - t_m)k_m[M1^D2^H] + k_g^D[G1^D2^D] + k_m^D[M1^D2^D] = (1 + k_{-g}^D + k_{-m}^D)[F^{DD}] \quad (A12)$$

We now substitute eq A1–A8 into eq A9–A12, eliminating explicit reference to glucose and mannose concentrations.

Substituting eq A1 and A5 into eq A9 and rearranging, we get

$$t_g k_0^G + t_m k_0^M - (1 - t_g^2)k_{-g}^H[F^{HH}] - (1 - t_m^2)k_{-m}^H[F^{HH}] = [F^{HH}] \quad (A13)$$

From eq A1, A2, A7, and A10, we get

$$(1 - t_g)k_0^G + (1 - t_g^2)k_{-g}^H[F^{HH}] - (1 - t_m^2)k_{-m}^H[F^{HD}] = [F^{HD}] \quad (A14)$$

From eq A3, A5, A6, and A11

$$(1 - t_m)k_0^M + (1 - t_m^2)k_{-m}^H[F^{HH}] - (1 - t_g^2)k_{-g}^H[F^{DH}] = [F^{DH}] \quad (A15)$$

Finally, from eq A3, A4, A7, A8, and A12, we obtain

$$(1 - t_g^2)k_{-g}^H[F^{DH}] + (1 - t_m^2)k_{-m}^H[F^{HD}] = [F^{DD}] \quad (A16)$$

Equations A13–A16 give a system of four equations, any three of which are independent. The three we choose for analysis of a given experiment become purely a matter of convenience.

When glucose is being fed, we recall that $k_0^G = 1$; $k_0^M = 0$. We also set $(1 - t_m^2)k_{-m}^H = k_{-m}^{H'}$ and $(1 - t_g^2)k_{-g}^H = k_{-g}^{H'}$, refer t_g specifically to solvent D_2O, and condense eq A13, A15, and A16 to

$$t_g^{D_2O} - k_{-g}^{H'}[F^{HH}] - k_{-m}^{H'}[F^{HH}] = [F^{HH}] \quad (A17)$$

$$- k_{-g}^{H'}[F^{DH}] + k_{-m}^{H'}[F^{HH}] = [F^{DH}] \quad (A18)$$

$$- k_{-g}^{H'}[F^{DH}] + k_{-m}^{H'}[F^{HD}] = [F^{DD}] \quad (A19)$$

These three equations are rearranged to give eq 13–15 of the text.

For mannose in D_2O, we recall that $k_0^G = 0$; $k_0^M = 1$. Using the same definitions for $k_{-m}^{H'}$ and $k_{-g}^{H'}$, we select eq A13, A14, and A16 to obtain eq 16, 17, and 18 of the text. When these last equations are reflected in the isotopic mirror by interchanging H and D throughout, we obtain eq 19, 20, and 21 of the text for the deuteriomannose–H_2O experiment.

Each set of three equations can be expressed in matrix form, AX = C, as follows. For glucose in D_2O

$$\begin{pmatrix} 1 - [F^{HH}] & -[F^{HH}] \\ 0 - [F^{DH}] & [F^{HH}] \\ 0 & [F^{DH}] \quad [F^{HD}] \end{pmatrix} \begin{pmatrix} t_g^{D_2O} \\ k_{-g}^{H'} \\ k_{-m}^{H'} \end{pmatrix} = \begin{pmatrix} [F^{HH}] \\ [F^{D.I}] \\ (F^{DD}] \end{pmatrix}$$

For mannose in D_2O

$$\begin{pmatrix} 1 - [F^{HH}] & -(F^{HH}] \\ 0 - [F^{HD}] & [F^{HH}] \\ 0 & [F^{HD}] \quad [F^{DH}] \end{pmatrix} \begin{pmatrix} t_m^{D_2O} \\ k_{-m}^{H'} \\ k_{-g}^{H'} \end{pmatrix} = \begin{pmatrix} [F^{HH}] \\ [F^{HD}] \\ [F^{DD}] \end{pmatrix}$$

For deuteriomannose in H_2O

$$\begin{pmatrix} 1 & -[F^{DD}] & -[F^{DD}] \\ 0 & -[F^{DH}] & [F^{DD}] \\ 0 & [F^{DH}] & [F^{HD}] \end{pmatrix} \begin{pmatrix} t_m^{H_2O} \\ k_{-m}^{D'} \\ k_{-g}^{D'} \end{pmatrix} = \begin{pmatrix} [F^{DD}] \\ [F^{DH}] \\ [F^{HH}] \end{pmatrix}$$

For each experiment, the values for entries on the coefficient matrix, A, and the constant vector, C, are introduced and the matrix equation is solved for the unknown vector, X.

As explained in the text, this procedure was carried out by computer, the input parameters ($[F^{HH}]$, $[F^{HD}]$, $[F^{DH}]$, and $[F^{DD}]$) being varied by small increments over their experimental ranges. A program was written in FORTRAN IV for the ANL CDC3600 computer, incorporating a standard ANL matrix inversion subroutine. Nested do-loops were used to vary $[F^{HH}]$ and $[F^{DD}]$ over their experimental ranges, obtaining ($[F^{DH}] + [F^{HD}]$) by difference from 1. A check was made to see that ($[F^{DH}] + [F^{HD}]$) fell within its experimental range. Subsequently, a nested do-loop was used to vary $[F^{DH}]/[F^{HD}]$ by setting $[F^{DH}] = k([F^{DH}] + [F^{HD}])$ and varying k from 0 to 1 in increments of 0.05. These values for $[F^{HH}]$, $[F^{HD}]$, $[F^{DH}]$, and $[F^{DD}]$ were then used to solve the unknown vector, X. Solutions which were physically meaningless (negative rate constants, t_g or t_m outside the range 0–1) were automatically rejected. In the glucose experiment $t_g^{D_2O}$ and $k_{-g}^{H'}$ were then used to obtain k_{-g}^{H}, and in mannose experiments t_m and k_{-m}^{1} were used to obtain k_{-m}. The resulting solutions for each experiment were outputted on punched cards for a plot of the solutions using an IBM 1620 computer with an on-line CALCOMP plotter. The plots produced in this manner thus showed the ranges for t_g, k_{-g}, and k_{-m}^{1} or t_m, k_{-m}, and k_{-g}^{1} consistent with the experimental error in the fructose 6-phosphate concentrations.

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