Deuterium Isotope Effects in the Fermentation of Hexoses to Ethanol by *Saccharomyces cerevisiae*. I. Hydrogen Exchange in the Glycolytic Pathway*

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ABSTRACT: Deuterium isotope effects have been used to study the fermentation of hexoses to ethanol. Ordinary D-glucose and D-mannose have been fermented by *Saccharomyces cerevisiae* in D_2O and fully deuterated mannose and glucose-6-d_2 have been fermented in H_2O. The product ethanol has the general composition $CH_xD_{3-x}CH_yD_{2-y}OH$, and a quantitative analysis of the mixture of partially deuterated ethanols can be accomplished by proton magnetic resonance and mass spectroscopy. From the isotopic composition of the ethanols, we conclude the following. (1) Exchange with the medium of the methylene group of the product ethanol occurs at the triose phosphate level during the sequence triose phosphate isomerase–aldolase–glyceraldehyde diphosphate dehydrogenase. These three steps are listed in probable order of decreasing rate, the final step occurring under conditions in which the hydrolysis of the acyl–enzyme complex by inorganic phosphate is rate determining. (2) The incorporation of H or D from the medium into the methyl group in the ethanol requires the presence of both phosphoglucose and phosphomannose isomerase during fermentation of either mannose or glucose. It also requires further exchange by pyruvate kinase. The alternative to pyruvate exchange, C_1–C_6 interconversion by adolase–triose phosphate isomerase, is much more difficult to reconcile with the data, although it cannot be rigorously excluded at this point.

Deuterium isotope studies have provided much useful information about the kinetics and mechanisms of enzyme–substrate interactions (see Thomson, 1963, for a comprehensive review). For the most part, such studies have been confined to *in vitro* systems where single, isolated enzyme systems can be observed. The applications of deuterium isotope studies in living organisms have by contrast been far more limited. It is the purpose of this and the following paper (Saur *et al.*, 1968b) to show that the deuterium isotope effects in even a fairly complicated reaction sequence in a living cell can be unraveled and, indeed, be given a quantitative interpretation. Such an interpretation, it will be shown, provides new information about the biosynthetic pathway itself, and in addition serves to reveal detailed information about the participation of the solvent in the reactions under study.

The biochemical sequence we have undertaken to study is the Embden–Meyerhoff–Parnas glycolytic pathway for the conversion of hexoses to ethanol by the yeast *Saccharomyces cerevisiae*. The glycolysis and alcoholic fermentation of hexoses are among the best characterized biochemical reaction sequences. The 12 enzymatic steps leading from hexose to ethanol have been studied extensively (Mahler and Cordes, 1966), not only in isolated enzyme systems, but also in many living systems; this circumstance greatly facilitates interpretation of deuterium experiments. The final product, ethanol, is relatively simple chemically speaking, and the development of new analytical techniques, which are referred to below, makes it practical to determine not just the over-all isotopic composition of the product, but the concentrations of all the isotopic species present in the product ethanol. Finally, *S. cerevisiae* is a very suitable organism for these studies, because it converts up to 90% of the supplied hexose by one well-known sequence of enzyme steps without appreciable side reactions. A steady state is reached very soon after fermentation begins and is maintained over most of the course of the conversion. Steady-state kinetics (Saur *et al.*, 1968) thus are readily applied to an analysis of the experimental data.

A number of recent developments have made important contributions to the practical aspects of our studies. The first is the ability to grow many kinds of organisms in very high concentrations of D_2O in essentially fully deuterated form (Katz and Crespi, 1966). Fully deuterated algae (DaBoll *et al.*, 1962) are a convenient source of fully deuterated glucose and mannose (Blake *et al.*, 1961). The deuterated hexoses obtained by biosynthesis in D_2O make it possible to obtain supplemental data which make an important contribution to the assignment of deuterium isotope effects to particular steps in the reaction sequence. The ability to grow *S. cerevisiae* in either H_2O or D_2O and to use either ordinary

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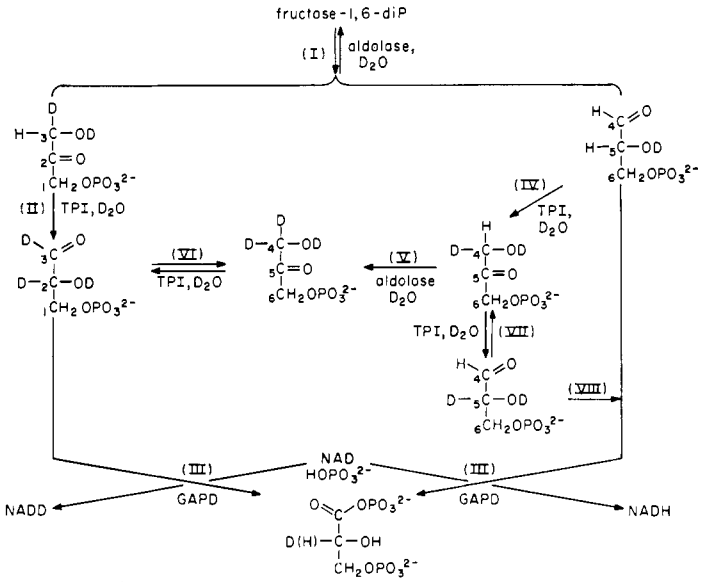

FIGURE 1: Scheme showing the possible mechanisms of deuterium labeling of aldo- and ketotriose phosphates in D_2O during the reactions catalyzed by aldolase and triose phosphate isomerase.

or deuterated hexose as the substrate makes it possible to carry out what we designate as an isotope mirror experiment (Katz and Crespi, 1966; Katz *et al.*, 1966) and so to maximize the information that can be obtained from deuterium isotope effects.

The Embden–Meyerhoff–Parnas scheme for the glycolytic conversion of hexoses into alcohol requires that the final product, ethanol, contain four of the six carbon atoms originally present in the hexose. The methyl group of the ethanol originates from C_1 and C_6, and the methylene group of the ethanol arises from C_2 and C_5 of the hexose. In the first study designed to determine the extent to which deuterium is incorporated into alcohol by fermentation of ordinary glucose in D_2O, Reitz (1936) showed that deuterium is indeed introduced to a large extent into the fermentation intermediates. Limited by the analytical techniques and the knowledge of the glycolytic pathway available at that time, Reitz predicted and found the product to be one compound with the formula CH_2DCD_2OD. In the light of more recent advances in the biochemistry of the Embden–Meyerhoff–Parnas pathway, however, a much more complex mixture of isotope hybrid ethanols, with the general formula $CH_xD_{3-x}CH_yD_{2-y}OD$, is to be expected. We have developed analytical procedures based on proton magnetic and mass spectroscopy (Sauer *et al.*, 1968a) that allow the quantitative determination of all of the isotopic species of ethanol produced in isotope mirror experiments. This paper presents a qualitative interpretation of an isotope mirror experiment on the glycolysis of mannose, and a description of deuterium isotope effects on the fermentation of glucose in D_2O and glucose-6-d_2 in H_2O. The accompanying paper (Saur *et al.*, 1968b) provides a quantitative account for the isotopic composition of the methyl group of the product ethanol.

Experimental Procedure

Materials. Reagent grade dextrose (Baker) was used as received. D-Mannose (NBC) was clarified with charcoal before use. To prepare hexose containing OD, 100-g batches of mannose or glucose were exchanged four times with 200 ml of 99.8% D_2O. Deuteriomannose was prepared in this laboratory (Blake *et al.*, 1961) or purchased from Merck Sharp and Dohme (Montreal). These preparations have hydrogen at all exchangeable positions, so no equilibration with H_2O was necessary. D-Glucose-6-d_2 was kindly provided by Dr. Charles Sweeley, University of Pittsburgh. Yeast extract was obtained from Difco. *S. cerevisiae*, ATCC 7754, was used in growth and fermentation experiments.

Cultivation of S. cerevisiae (ATCC 7754). The nutrient medium contained the following salts (in grams per liter): NH_4Cl, 1.20; KH_2PO_4, 0.30; K_2HPO_4, 0.04; $CaCl_2$, 0.05; $MgSO_4$, 0.05; NaCl, 0.10; and $FeSO_4 \cdot 7\ H_2O$, 0.010. Minor elements (in parts per million) were: B, 0.05; Mn, 0.25; Zn, 0.05; Cu, 0.02; and Mo, 0.05. The vitamin mixture contained the following (mg/100 ml): biotin, 0.2; nicotinic acid, 2.0; thiamin, 1.0; *p*-aminobenzoic acid, 1.0; panthothenic acid, 0.5; pyridoxamine hydrochloride, 5.0; riboflavin, 1.0; and vitamin B_{12}, 0.5.

The yeast cultures for the D_2O fermentation experiments were grown in 5- and 10-l. batches with pH control and vigorous aeration in an H_2O–nutrient medium containing 3% ordinary glucose as carbon source, 0.02–0.04% yeast extract, and 20 ml of vitamin mixture. After 20 hr the yeast was harvested by centrifugation in a yield of approximately 2 g/l. of medium (dry weight). The packed cells were washed four times with the D_2O–salt solution (over 99% exchangeable hydrogen is thereby exchanged). The cultivation of the yeast used for the H_2O–fermentation experiments was done in a 600-ml batch with 3% deuteriomannose as carbon source in H_2O–salt solution, yeast extract and vitamins being used in the same relative amounts as described above.

Fermentation Experiments in D_2O and H_2O. For the fermentation experiments, the washed yeast cells were resuspended in the fermentation medium to roughly one-third of the original volume. The medium thus contained approximately 6 g/l. of yeast (dry weight), 10% hexose, and added vitamins. The fermentations were carried out in 1-l. batches and were complete in 3–4 days. The yeast was then separated by centrifugation, and the solution was distilled slowly through a Widmer column, a fraction being collected from 78 to 95°. This portion was redistilled slowly through a Vigreux column, and this time 90% of the total volume could be collected between 78 and 80°, the boiling range for the ethanol–water azeotrope. The last of the water was removed by distillation over Molecular Sieves A4 (Linde). The final yield of pure ethanol, based on the invested sugar, was between 55 and 60%.

The final deuterium content of the D_2O medium after removal of the ethanol was determined by near-infrared spectroscopy (Crespi and Katz, 1961) and

found to be 97.8 ± 0.6%, indicative of only slight isotopic dilution during the course of the fermentation.

Analytical Methods. A detailed description for the qualitative and quantitative analysis of mixtures of partially deuterated ethanols by nuclear magnetic resonance and mass spectroscopy has been given elsewhere (Saur *et al.*, 1968a).

Results and Discussion

In order to interpret the results obtained from our isotopic fermentation studies, it seems useful at this point to discuss the Embden–Meyerhoff–Parnas scheme in terms of possible exchanges of carbon-bound hydrogen with the hydrogen of the medium. We will follow the treatment of Mahler and Cordes (1966).

Interconversion of Hexose 6-Phosphates to Fructose 6-Phosphate. In this step, cleavage and formation of carbon–hydrogen bonds at positions C_1 and C_2 in glucose and mannose 6-phosphates occurs, and thus exchange with the solvent is possible at these two positions. Two different enzymes are involved in the isomerization of glucose-6-P and mannose-6-P: PGI[1] (D-glucose 6-phosphate ketol isomerase, EC 5.3.1.9) and PMI (D-mannose 6-phosphate ketol isomerase, EC 5.3.1.8). Some pertinent literature exists on hydrogen exchange during the isomerization. In experiments carried out with purified enzymes in D_2O, Topper (1957) showed that isomerization of both glucose-6-P and mannose-6-P is highly stereospecific. Complete deuteration at C_1 of fructose-6-P and D_2O can therefore be expected only if this intermediate is isomerized at least once by both hexose isomerases before it enters the following step of fermentation.[2] Studying yeast phosphoglucose isomerase *in vitro*, Rose and O'Connell (1961) found approximately 33% of the protons were transferred intermolecularly from C_1 of fructose-6-P to C_2 of glucose-6-P. The deuterium isotope effect, k_H/k_D, for the isomerization in both directions was found to be 1.86. Simon and Medina (1966) confirmed this *in vitro* exchange-to-transfer ratio, and also investigated the corresponding hydrogen transfer by phosphomannose isomerase. Here they found only 5% of the hydrogen being transferred intermolecularly from C_2 of mannose-6-P to C_1 of fructose-6-P. Fermentation of glucose-2-T by yeast resulted in a product with 18–20% of the label transferred to the methyl group of ethanol, whereas the ethanol from fermentation of mannose-2-T retained only 2.8–3% of the label. Particularly relevant to our problem is the conclusion of these workers that isomerization with PGI has a lower exchange-to-transfer ratio than does PMI. Phosphorylation of fructose-6-P to fructose-1,6-diP by phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) involves no cleavage of C–H bonds and therefore no possibility for incorporation of deuterium.

Conversion of Fructose-1,6-diP into Triose Phosphate and Interconversion of Aldo- and Ketotriose Phosphate. The subsequent cleavage of fructose-1,6-diP by aldolase (fructose 1,6-diphosphate–D-glyceraldehyde 3-phosphate lyase, EC 4.1.2.13) into the two triose fragments dihydroxyacetone-P and glyceraldehyde-3-P takes place with stereospecific incorporation of deuterium at C_3 of dihydroxyacetone-P (Rose and Rieder, 1958; Rutter, 1964). The enzyme complementary in stereospecificity to aldolase, triose-P isomerase (D-glyceraldehyde 3-phosphate–ketol isomerase, EC 5.3.1.1), catalyzes the isomerization of the two metabolic fragments, thus labelling the remaining proton at C_3 of dihydroxyacetone-P (Bloom and Topper, 1956; Rieder and Rose, 1959). During the course of these reactions there is no exchange with the medium of the hydrogen atoms at C_1 of DHAP.

Conversion of Glyceraldehyde-3-P into D-3-Phosphoglycerate. Glyceraldehyde-3-P then undergoes oxidation by NAD, catalyzed by glyceraldehyde-3-P dehydrogenase (D-glyceraldehyde 3-phosphate–NAD oxidoreductase, EC 1.2.1.12), losing the proton at C_1 to NAD. The incorporation of deuterium from the solvent during these three reactions is summarized in Figure 1. Of all the deuterium introduced into the fermentative pathway during these last three steps, that bound by NAD is particularly important, because in the reduction of acetaldehyde to ethanol by NADH, catalyzed by alcohol dehydrogenase (alcohol–NAD oxidoreductase, EC 1.1.1.1), the isotopic composition of the NADH determines the isotopic composition of the methylene group of the product ethanol. This transfer of a hydrogen atom, *via* NAD, from the aldehydic carbon atom of glyceraldehyde-3-P to the methylene group of the product ethanol is stereospecific and takes place without exchange with the medium (Vennesland and Westheimer, 1954). Moreover, it is stoichiometric, so that the final H/D ratio in the methylene group is determined by the deuterium content of the glyceraldehyde-3-P at the time when it undergoes oxidation to 1,3-diP-glycerate.

Conversion of 1,3-DiP-glycerate into Phosphoenolpyruvate and Then into Pyruvate. Transformation of 1,3-diP-glycerate to 2-P-glycerate should not involve C–H exchange. During conversion of 2-P-glycerate into phosphoenolpyruvate, similarly, no exchange of carbon-bound protons is expected to take place. There is, however, such a possibility for exchange in the pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40) reaction.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: α, probability of diluent H uptake in pyruvate exchange; β, isotopic dilution of solvent (hence α/β = isotope effect); GAPD, glyceraldehyde phosphate dehydrogenase; H_G, proton on C_1 of fructose-6-P not exchangeable by PGI; H_M, proton on C_1 of fructose-6-P not exchangeable by PMI; PEP, phosphoenolpyruvate; $PEPD_2$, 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; PGI, phosphoglucose isomerase; PK, pyruvate kinase; PMI, phosphomannose isomerase; $PYRD_3$, pyruvate with three deuterons on the methyl group; $PYRD_2H$, pyruvate with two deuterons and one proton on the methyl group; $PYRDH_2$, pyruvate with one deuteron and two protons on the methyl group; $PYRH_3$, pyruvate with three protons on the methyl group; TPI, triose phosphate isomerase.

[2] This statement is rigorously correct only if no fructose 6-phosphate is re-formed by back reaction from deuterated pyruvate or other exchange intermediates.

TABLE I: Composition of Ethanol Produced by Fermentation of Hexoses in D_2O and of Deuterated Hexoses in H_2O by *S. cerevisiae*.

	D-Glucose in D_2O		D-Mannose in D_2O			Deuterio-D-mannose in H_2O		D-Glucose-6-d_2 in H_2O	
Compound	Expt	Mol %	Expt	Mol %	Compound	Expt	Mol %	Expt	Mol %
CD_3CD_2OD	G1	33.3	M1	37.5	CH_3CH_2OH	MD1	17.5	GD1	50.0
	G2	33.0	M2	37.5		MD2	17.4		
CHD_2CD_2OD	G1	28.8	M1	30.6	CH_2DCH_2OH	MD1	33.6	GD1	8.7
	G2	29.0	M2	30.9		MD2	33.6		
CH_2DCD_2OD	G1	23.2	M1	17.0	CHD_2CH_2OH	MD1	36.1	GD1	41.2
	G2	23.3	M2	16.9		MD2	35.8		
CH_3CD_2OD	G1	1.8[a]	M1	1.3[a]	CD_3CH_2OH	MD1			
	G2	1.9[a]	M2	1.2[a]		MD2			
CD_3CHDOD	G1	4.7	M1	5.7	CH_3CHDOH	MD1	2.5		
	G2	4.8	M2	5.7		MD2	2.3		
CHD_2CHDOD	G1	4.8	M1	5.3	$CH_2DCHDOH$	MD1	4.2		
	G2	4.7	M2	5.2		MD2	5.1		
$CH_2DCHDOD$	G1	3.4	M1	2.6	CHD_2CHDOH	MD1	6.1[b]		
	G2	3.3	M2	2.7		MD2	5.9[b]		

[a] The concentration of CH_3CD_2OD is much higher than would be expected (about 0.4%) from random incorporation of H from the solvent introduced by isotopic dilution during the fermentation. It is consistent with the large solvent isotope effect on the enzymatic formation of pyruvate from phosphoenolpyruvate, as was found by Simon and Palm, (1966). [b] These values are based on mass spectral data and include any CD_3CH_2OH present. The latter is assumed to be formed by isotopic dilution of the solvent during the experiment, and, since the solvent isotope effect referred to above would tend to inhibit its formation, was assumed to be in negligible concentration.

The three protons of the methyl group of the pyruvate, one of which is necessarily derived from the solvent, are chemically identical. In D_2O, significant equilibration during this reaction would therefore lead to an amount of deuterium exceeding 1 atom/molecule of pyruvate.

Conversion of Pyruvate into Acetaldehyde. Acetaldehyde is produced by decarboxylation of pyruvate with thiamine pyrophosphate, catalyzed by pyruvate decarboxylase (2-oxo acid carboxylase, EC 4.1.1.1). According to recent studies upon the mechanism of this reaction (Schellenberger *et al.*, 1966), the proton introduced at the carbonyl carbon must originate from the solvent and can therefore only be deuterium in a D_2O experiment.

Summary of C–H Exchange Possibilities. The isotopic composition of the product ethanol is determined as follows. One hydrogen atom of the methyl group will be derived from the medium; the other two will reflect the isotopic composition of the phosphoenolpyruvate. These two hydrogen atoms are derived from either C_1 or C_6 of the fructose-6-P produced by hexose-6-P isomerase. If recondensation of the triose phosphates to the hexose-6-P pool does not take place to any significant extent, C_1 or C_5 will remain isotopically distinct. In this case both hydrogen atoms from C_6 will appear in PEP, as will the hydrogen atom originally on C_1 of the aldohexose. Whether or not the hydrogen originally on C_2 of the aldohexose will also appear in the phosphoenolpyruvate depends upon the extent of intramolecular transfer by the appropriate hexose-6-P isomerase. To the extent that the triose–hexose recondensation does occur, C_1 and C_6 will be interconverted, so that one of the hydrogen atoms of C_6 could also be exchanged with the medium. It should be stressed, however, that this enzymatic scheme can lead to incorporation of at most one H or D atom from the solvent into phosphenolpyruvate, the other one being necessarily retained from C_1 or C_6 of the hexose because of the stereoselectivity of the hexose-6-P isomerase reaction. Initial formation of a phosphoenolpyruvate molecule containing more than one H or D atom from the solvent would only be possible by the simultaneous presence of adequate amounts of both phosphoglucose isomerase and phosphomannose isomerase during fermentation. If such a possibility is excluded, the presence of ethanol with a fully exchanged methyl group, which is in fact observed, necessarily requires random isotopic exchange after the formation of the phosphoenolpyruvate.

One proton of the methylene group in the ethanol is directly introduced from the medium in the conversion from pyruvate to acetaldehyde. The second one undergoes exchange in the triose-P isomerization step before

TABLE II: Isotopic Composition of the Methylene Group.[a]

Experiment	CH_2 (mol %)	CHD (mol %)	CH_2 (mol %)
D-Glucose in D_2O		11.2 ± 1.0	88.8 ± 1.0
D-Mannose in D_2O		12.0 ± 1.0	88.0 ± 1.0
Deuterio-D-mannose in H_2O	88.6 ± 3.0	11.4 ± 3.0	

[a] The values are taken from Table I and corrected for isotopic dilution of the solvent as follows: since the solvent is isotopically diluted by about 2% at the end of fermentation, an average dilution of 1% was used to correct for statistical incorporation of the diluent isotope into species that would have remained labeled in the pure solvent.

TABLE III: Isotopic Composition of the Methyl Group.

	CH_3 (mol %)		CH_2D (mol %)		CHD_2 (mol %)		CD_3 (mol %)	
Experiment	Exptl[a]	Cor[b]	Exptl[a]	Cor[b]	Exptl[a]	Cor[b]	Exptl[a]	Cor[b]
D-Glucose-6-d_2 in H_2O	50.0	50.0 ± 1.5	8.7	8.8 ± 0.5	41.2	41.2 ± 1.0		
Deuterio-D-mannose in H_2O	19.8	20.3 ± 0.2	38.3	38.2 ± 0.5	41.9	41.5 ± 0.3		
D-Mannose in D_2O	1.2		19.6	18.0 ± 0.1	36.0	34.6 ± 0.2	43.2	47.3 ± 0.2
D-Glucose in D_2O	1.8		26.6	25.8 ± 0.1	33.7	32.6 ± 0.2	37.9	41.6 ± 0.2

[a] From Table I. [b] The addition of one H or D atom is assumed to be subject to a solvent isotope effect. All others were taken to be statistically incorporated (see footnotes to Tables I and II). The isotopic fractionation factor was adjusted in order to account for all the CH_3 product in both D_2O experiments on this basis. For details, see Appendix A. The errors represent the maximum deviation between two experimentally obtained values for each compound.

being transferred by NADH to the product ethanol. The product of a hexose fermentation in D_2O proceeding along the Embden–Meyerhof–Parnas pathway should therefore be a mixture of isotope hybrid ethanols with the sum formula $CH_xD_{3-x}CH_yD_{2-y}$ with $0 < x < 3$ and $0 < y < 2$. The results of such fermentation experiments as well as their isotopic mirrors are summarized in Tables I–III.

Isotopic Composition of the Methylene Group of Ethanol. It can be seen in Table II that the isotopic composition of the methylene group is very nearly the same in the mannose and the glucose experiments. This is expected, of course, because the reactions involved are essentially the same. However, there are also no significant differences observed between the results of the two mirror experiments, *i.e.*, the fermentation of mannose in D_2O, and of deuteriomannose in H_2O.

Consider first the experiments with unlabeled substrate (ordinary mannose) in D_2O. As noted above, one hydrogen (or deuterium) atom is derived from the solvent, so that the remaining 0.88–0.89 atom of fraction D (Table II) must reflect the amount of NADD in the total reduced NAD. Of this, one-half is abstracted from the aldehydic carbon atom (C_3 in the hexose), derived from the dihydroxyacetone-P by triose phosphate isomerization. The stereospecificity of the aldolase reaction ensures that this half of the total reduced NAD is deuterated. The remaining 38–39% deuterium represents abstraction of deuterium, rather than hydrogen, from the initially formed glyceraldehyde-P, *i.e.*, at the C_4 position of the hexose. A consideration of the sequence determining the fate of the C_4–C_6 moiety (Figure 1) reveals that complete deuteration of the reducing NAD could only occur if the oxidation step (III) were very much slower than both triose-P isomerization (II, IV, VI, and VII) and labeling by aldolase (V), regardless of the relative rates of these latter two steps. The fact that the reduced NAD from this source is about 76% NADD suggests that oxidation is, in fact, the slowest step, but that either aldolase labeling or isomerization occurs at a comparable rate, *i.e.*, only three or four times as fast.

It is possible to make an estimate of the relative rates of triose-P isomerization and labeling by aldolase if we also consider the mirror experiment. Here, in complete analogy with the preceding argument, we conclude that the reduced NAD from the C_4–C_6 moiety is again 76% NADH, the same precentage as that of NADD in the reverse, D_2O, experiment. The absence of an over-all isotope effect requires that oxidation of glyceraldehyde-3-P, which we were led to conclude is rate determining, is itself isotope insensitive.

According to recent studies on the mechanism of glyceraldehyde-3-P dehydrogenase action, the cleavage of the NAD-oxidized acyl-enzyme complex requires inorganic phosphate, 1,3-diP-glycerate being directly formed as a product. Under conditions in which the

concentration of P_i determines the over-all rate of fermentation, the actual transfer of H or D from the substrate to NAD will not be rate limiting, and therefore the over-all oxidation of glyceraldehyde-3-P to 1,3-diP-glycerate will not be subject to a deuterium isotope effect. Our isotopic mirror experiment was carried out under steady-state conditions in which, according to Holzer and Freytag-Hilf (1959), the P_i concentration determines the over-all fermentation rate. Our results, which preclude an isotope effect on the oxidation step, are thus consistent with their findings. If we were now to assume that the isomerization is nearly comparable in rate with oxidation, and recall that the former is subject *in vitro* to a large deuterium isotope effect (k_H/k_D = 2.2) (Rieder and Rose, 1959), it is difficult to escape the conclusion that the steady-state concentrations of the variously deuterated trioses would differ in the two systems. The absence of a significant difference in methylene labeling between the two isotopic mirrors speaks against this assumption. If, however, the isomerization is rapid, it may be considered to be an equilibrium. Assuming an equal deuterium effect on the forward and reverse rate constants, in analogy to hexose-P isomerization (Rose, 1962), this equilibrium will not be affected by different deuterium substitution. The rate of aldolase labeling (V, Figure 1) has also been shown to be insensitive to isotopic substitution (Rose and Rieder, 1958). It is thus reasonable to conclude from the similar results in the two mirror experiments that the latter reaction is the one nearly comparable in rate to glyceraldehyde-P oxidation, the triose-P isomerization being a rapidly attained equilibrium.

Isotopic Composition of the Methyl Group. Table III shows the isotopic composition of the methyl group in the alcohol product from the four experiments: mannose and glucose fermentation in D_2O, deuteriomannose and glucose-6-d_2 fermentation in H_2O.

Probably the most striking feature of Table III is the presence of completely exchanged methyl groups in all experiments. As noted above, exchange of the C_1 hydrogen of the glucose cannot be affected by phosphoglucose isomerase, nor can that of mannose be exchanged by phosphomannose isomerase. The complete exchange can be effected at the hexose phosphate level only if both isomerases are present, whatever the nature of the sugar being fermented. Alternatively, or in addition, random exchange of the methyl group can occur in the pyruvate kinase reaction. Both of these possibilities are *a priori* open to objection. On the one hand, it is generally assumed that enzyme levels are regulated by the concentrations of substrates available to the organism. In these terms it seems unreasonable that a yeast fermenting glucose would be adequately supplied with phosphomannose isomerase and *vice versa*. On the other hand, the pyruvate kinase equilibrium is known to be strongly shifted in the direction of pyruvate, the back reaction taking place very much slower than the forward reaction (Kerson *et al.*, 1967). Consequently, extensive enzymatic hydrogen exchange of pyruvate with the solvent would not be expected. Be this as it may, these are the only two reactions in the Embden–Meyerhof–Parnas scheme that allow for complete isotopic exchange of the hydrogens derived from C_1 and C_2 of the aldohexose. The hydrogens from C_6 can, of course, also be exchanged at the pyruvate level. They cannot be exchanged at the hexose level unless reversal of the aldolase–triose-P isomerase step is sufficiently rapid to interconvert C_1 and C_6. This postulate is not attractive either because such an interconversion occurs to only a very minor extent even in photosynthesis by *Chlorella* (Dorrer *et al.*, 1966), where conditions are presumably less forcing of the forward reaction than in yeast fermentation.

Glucose-6-d_2 in H_2O must yield at least 50% CH_3 from the hydrogen at C_1 and C_2. Consequently the amount of CH_3 derived from C_6 has to be negligible in this experiment since the observed total CH_3 is, within experimental error, 50%. The 41% of the unexchanged CHD_2 necessarily comes from C_6. The percentage of CHD_2 in the deuteriomannose–H_2O experiment is also 41%. We know that phosphomannose isomerase converts mannose-6-P into fructose-6-P with virtually complete isotopic exchange of one hydrogen atom (Simon and Medina, 1966) so that practically no CHD_2 can be derived from the C_1 of deuteriomannose. If exchange of the D atoms takes place at the hexose level, after C_1–C_6 interconversion, the 41% CHD_2 represents that fraction of C_6 that either did not undergo realdolization, or, having done so, was exchanged by neither of the hexose phosphate isomerases. The fact that this proportion is identical in the two cases could then be explained by requiring either identical hexose-P isomerase levels in the two experiments or a fortuitous cancellation of several factors. Alternatively, if C_1–C_6 interconversion does not occur, the 41% of CD_2H in the glucose-6-d_2 experiment is that fraction which survives random exchange at the pyruvate level, the same amount necessarily remaining in the deuteriomannose experiment as well.[3]

Let us first examine the consequences of the assumption that there is no C_1–C_6 interconversion and that exchange of the C_6 hydrogen atoms occurs only in the pyruvate kinase step. Assigning 8.7% of CH_2D to this source in the deuteriomannose experiment requires the methyl group from C_1 to have the composition: 20.3% CH_3, 29.5% CH_2D. In both of these species, one deuterium atom must have been exchanged by phosphomannose isomerase. If we do not concede that isomerization by phosphoglucose isomerase also takes place, all of the CH_3 must be formed from CH_2D during pyruvate exchange. The rate required for this is much too large to be consistent with that calculated from the glucose-6-d_2 experiment. The experimental results discussed so far thus require, quite apart from pyruvate exchange, also considerable phosphoglucose isomerase activity in a yeast cell fermenting mannose.

Turning now to the mannose mirror experiment (ordinary mannose in D_2O) we are struck by the large increase in the proportion of completely exchanged

[3] The percentage of the completely exchanged CH_3 derived from C_6 after random exchange by pyruvate would then be estimated from the pyruvate steady state (see part II) to be 0.5%, *i.e.*, less than can be detected by our analytical methods.

species, CD_3, and the corresponding decrease in the unexchanged one, CH_2D. Qualitatively this is readily explained in terms of an increase in the rate of pyruvate exchange. Nonenzymatic enolizations are generally subject to very large isotope effects (Melander, 1960). There is no reason not to expect a similarly large effect in an enzymatic enolization as well, provided the rate-determining step is proton abstraction (Rose *et al.*, 1965), as it should be, since the enzyme is very far from being saturated by pyruvate (Holzer and Freytag-Hilf, 1959; Kerson *et al.*, 1967). However, since here, too, the fructose-6-P would initially be formed with virtually complete exchange of one proton with the solvent, then, unless allowance is made for substantial phosphoglucose isomerase activity, the subsequent pyruvate exchange could not generate a sufficiently large proportion of fully exchanged product.

The results of the glucose–D_2O experiments are very similar to those of the mannose–D_2O fermentation except for a small increase in the unexchanged CH_2D, largely at the expense of the completely exchanged species CD_3. This is reasonable, when we recall that, contrary to phosphomannose isomerase, the phosphoglucose isomerase transfers approximately 50% of the label between C_1 and C_2 per isomerization pass (Simon and Medina, 1966). More transfer favors the unexchanged species in the fructose-6-P and (if the rate of pyruvate exchange is the same as in the corresponding mannose experiment) would yield a less exchanged product. Once again, pyruvate exchange alone cannot account for the proportions of the various species, and substantial phosphomannose isomerase activity must be postulated.

Although the scheme just outlined is preferred to the alternative mechanism involving C_1–C_6 interconversion instead of pyruvate exchange, the latter cannot be rejected on the basis of qualitative or semiquantitative arguments.[4] It is, however, much more difficult to reconcile with the experimental results: the identical amounts of CHD_2 in the glucose-6-d_2 and the deuteriomannose experiments could be rationalized in terms of C_1–C_6 interconversion *via* the aldolase–triose-P isomerase system and an equal amount of exchange by phosphomannose isomerase and by phosphoglucose isomerase in both cases. The large increase in the extent of exchange in the mirror experiment, however, would require a very large isotope effect on either aldolization of the two triose phosphates, C_1–C_2 hydrogen transfer, or both. The former reaction is known to occur on the native enzyme without an isotope effect (Rose *et al.*, 1965); the isotope effect on the latter reaction was found to be about 1.9 (Rose, 1962), far too small to account for the difference between our mirror experiments.

Appendix A

Correction for Isotope Dilution. Consider the fermentation of glucose or mannose in D_2O. The distribution in pure D_2O of the product methyl groups of ethanol is the same as in pyruvate and also the same as the PEP species from which it is derived by addition of a deuteron.

Schematically, we shall specify concentrations of this *ideal* distribution in parentheses and reserve brackets for actual concentrations. Hence

$$\begin{aligned} (PYRD_3) &= (PEPD_2) \\ (PYRD_2H) &= (PEPDH) \\ (PYRDH_2) &= (PEPH_2) \\ (PYRD_3) &= 0 \end{aligned} \tag{A1}$$

The isotopic dilution that gradually takes place during fermentation necessarily distorts this distribution. Without serious error, we can regard the isotopic composition of the solvent as effectively constant, assigning it a roughly average atom fraction of H, $\beta = 0.01$, instead of letting it vary linearly, as it does, from $\beta \cong 0.003$ to 0.022.

The uptake of a proton (or deuteron) by PEP is subject to a large isotope effect (Simon and Palm, 1966) so that the probability of picking up a diluent H atom is much larger than 0.01. Let us denote this probability by α, α/β representing the isotope effect.

The actual pyruvate concentrations will be

$$\begin{aligned} [PYRD_3] &= [PEPD_2](1 - \alpha) \\ [PYRD_2H] &= [PEPDH](1 - \alpha) + [PEPD_2]\alpha \\ [PYRDH_2] &= [PEPH_2](1 - \alpha) + [PEPDH]\alpha \\ [PYRH_3] &= [PEPH_2]\alpha \end{aligned} \tag{A2}$$

The actual concentrations of PEP would correspond with their ideal values only if there were no dilution, which is not the case. Since the conclusions of our study on the mechanism of exchange in the steps preceding pyruvate exchange cannot justifiably be fed into a correction procedure used to obtain values on which these conclusions are to be based, we had no recourse but to assume that dilution of these two exchanged atoms is statistical

$$\begin{aligned} [PEPD_2] &= (1 - 2\beta - \beta^2)(PEPD_2) \\ [PEPDH] &= (1 - \beta)(PEPDH) + 2\beta(PEPD_2) \\ [PEPH_2] &= (PEPH_2) + \beta(PEPDH) + \beta^2(PEPD_2) \end{aligned} \tag{A3}$$

Introducing eq A3 into A2 and recalling eq A1, leads to

$$\begin{aligned} [PYRD_3] &= (1 - \alpha)(1 - 2\beta - \beta^2)(PYRD_3) \\ [PYRD_2H] &= (1 - \alpha)\{(1 - \beta)(PYRD_2H) + \\ &\quad 2\beta(PYRD_3)\} + \alpha(1 - 2\beta - \beta^2)(PYRD_3) \\ [PYRDH_2] &= (1 - \alpha)\{(PYRDH_2) + \beta(PYRD_2H) + \\ &\quad \beta^2(PYRD_3)\} + \alpha\{(1 - \beta)(PYRD_2H) + 2\beta(PYRD_3)\} \\ [PYRH_3] &= \alpha\{(PYRDH_2) + \beta(PYRD_2H) + \beta^2(PYRD_3)\} \end{aligned} \tag{A4}$$

The assumed value of β and the experimental concentrations can then be used to solve for α and hence the corrected concentrations. It is easily verified that a value of $\alpha = 0.083$ is the correct one to use for both

[4] See, however, part II (following paper).

the glucose– and mannose–D_2O experiments, corresponding to an isotope effect of about eight on the pyruvate kinase step.

In the deuteriomannose–H_2O experiment, α should be about 0.01/8.3, or effectively zero, and the only correction remaining is that on the initial, presumably statistical, exchange (eq A3). In the glucose-6-d_2–H_2O experiment, the dilution is so slight as to render any correction unnecessary.

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