

BBA Report

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MAGNITUDE OF THE EQUILIBRIUM ISOTOPE EFFECT ON CARBON-TRITIUM BOND SYNTHESIS

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Summary

The exchange of tritium from ^3HOH into the methyl group of pyruvate catalyzed by 6-phospho-2-keto-3-deoxygluconate aldolase (6-phospho-2-keto-3-deoxy-D-gluconate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.14) of *Pseudomonas putida* shows an equilibrium isotope effect of 0.78. From this value and the deuterium effect on the fumarase equilibrium (Thomson, J.F. (1960) *Arch. Biochem. Biophys.* 90, 1), one can calculate by use of the relative fractionation factors of Hartshorn and Shiner (Hartshorn, S.R. and Shiner, Jr., V.J. (1972) *J. Am. Chem. Soc.* 94, 9002), fractionation factors for transfer of deuterium or tritium from water to a number of organic molecules of interest.

The effect of deuterium or tritium substitution on the equilibrium constant for a reaction becomes of considerable practical importance in interpreting isotope effects on enzyme-catalyzed reactions, since the ratio of the kinetic isotope effects in the two directions equals the equilibrium isotope effect. With malate dehydrogenase, malic enzyme, and glutamate dehydrogenase, Schimerlik et al. [1] originally reported equilibrium fractionation factors for deuterium transfer from NADH or NADPH to L-malate of 1.32 and 1.22, and from NADPH to L-glutamate of 1.18. More careful determinations of the fractionation factors from NADH to L-malate, isopropanol, and cyclohexanol, using appropriate enzymes, gives identical values of 1.18 (Cook, P. and Cleland, W.W., unpublished results). A theoretical basis for these values is provided by the work of Hartshorn and Shiner [2], who showed that deuterium or tritium will become enriched in the molecule with the stiffest C-H bond. The stiffness of any C-H bond is determined largely by the other atoms bonded to this carbon, with only small differences resulting from more remote structural differences. For the molecules of interest to biochemists,

TABLE I

EQUILIBRIUM ISOTOPE EFFECT IN PYRUVATE-³H₂O EXCHANGE CATALYZED BY 6-PHOSPHO-2-KETO-3-DEOXYGLUCONATE ALDOLASE

400 mg sodium pyruvate (Calbiochem) were incubated with 630 units of the aldolase at 35°C in 1 ml tritiated water ($5.41 \cdot 10^5$ dpm/ μ atom H). At the times indicated, 0.2-ml samples were treated with 20 μ l of 1% sodium dodecyl sulfate to inactivate the enzyme and then lyophilized in distillation tubes. The residue, dissolved in 0.2 ml water, was treated with 1 ml ethanol to induce crystallization. The crystalline sodium pyruvate was isolated by centrifugation and dried in vacuo. A portion of the product, dissolved in water, was chromatographed on a 2.5 \times 30-cm bed of Sephadex G-10 with 100 μ M EDTA. Pyruvate, located by scintillation counting in an ethanol/toluene cocktail, was pooled and lyophilized. Specific activities were determined by scintillation counting and assay with NADH and lactate dehydrogenase [4]. The specific activity of the ³H₂O was determined on aliquots of the incubation mixture which were lyophilized in distillation tubes by diluting and counting the recovered water.

Incubation time (h)	Pyruvate specific activity (dpm/ μ mol)	Tritium incorporation (equiv./mol)	Fractionation factor
2	$12.75 \cdot 10^5$	2.356	0.785
6	$12.77 \cdot 10^5$	2.360	0.787
24	$12.56 \cdot 10^5$	2.322	0.774

TABLE II

FRACTIONATION FACTORS FOR ²H AND ³H BETWEEN WATER AND VARIOUS MOLECULES

Values calculated from the experimental value determined in this work, and by Thomson [3] using the factors of Hartshorn and Shiner [2]. Tritium effects assumed to be the 1.442 power of deuterium ones.

	² H	³ H	Structure
H ₂ O \rightarrow [3- ³ H]pyruvate	0.84	0.78 [†]	C-CH ₂ [³ H]
H ₂ O \rightarrow [3- ³ H]malate	0.93 ^{††}	0.90	C-CH[³ H]-C
H ₂ O \rightarrow [1- ³ H]dihydroxyacetone-3-P	1.00	1.00	C-CH[³ H]OH
H ₂ O \rightarrow [2- ³ H]glyceraldehyde-3-P	1.10	1.15	C-C[³ H]OH-C
H ₂ O \rightarrow [1- ³ H]glyceraldehyde-3-P	1.18	1.27	C-C[³ H](OH) ₂ ^{†††}

[†]This work.

^{††}Experimental value from Thomson [3].

^{†††}Glyceraldehyde-3-P is 97% hydrated in water at neutral pH [6].

these predictions can be summarized by saying that replacement, on the carbon holding the C-H bond of interest, of H by C, N, or O will result in fractionation factors of about 1.10, 1.15, and 1.18 in favor of the new molecule*. The experimental values discussed above agree reasonably well with these predictions.

A number of biological reactions involve solvent water as a reactant, and thus it becomes necessary to know the fractionation factors between water and various organic molecules. The only accurate value in the literature is the value of 0.93 for deuterium between water and the CH₂ group of malate determined by Thomson [3] with fumarase. From the rules of Hartshorn and Shiner [2], an even lower value is expected for water and the CH₃ group of pyruvate. Comparing carbon 3 of pyruvate and malate, H replaces C, so the predicted fractionation factor of H₂O \rightarrow pyruvate is 0.93/1.10, i.e. 0.85 for deuterium. Since the isotope effects for tritium are always the 1.442 power of the deuterium effects, the fractionation factor for tritium in going from water to pyruvate should be (0.85)^{1.442}, i.e. 0.79.

We have measured this fractionation factor by using the pyruvate-tritiated

*Hartshorn and Shiner [2] did not include oxygen-containing compounds in their calculations, but Dr. Shiner in a private communication has indicated that oxygen should have a very similar effect to fluorine, for which the calculated factor is 1.18. As noted above, this value has now been experimentally confirmed for three secondary alcohols relative to NADH.

water exchange [4] catalyzed by 6-phospho-2-keto-3-deoxygluconate aldolase of *Pseudomonas putida* [5]. Sodium pyruvate was incubated in tritiated water containing sufficient enzyme to reach 99% of theoretical equilibration in 1.4 h, and crystalline sodium pyruvate was then reisolated after 2, 6 and 24 h. The data in Table I indicate that the exchange reaction had reached equilibrium by 2 h, and yield an average fractionation factor of 0.78 ± 0.01 , in excellent agreement with the value calculated above.

With this result and the one from the fumarase equilibrium, one can calculate fractionation factors for various structures as shown in Table II. The third and fourth entries in this table are experimentally measurable with triose-phosphate isomerase, and the measured values with tritium (Albery, W.J. and Knowles, J.R., private communication) are 1.03 ± 0.04 and 1.0 ± 0.2 , which agree reasonably well with these predictions. Biochemists who use deuterium and tritium as tracers should be aware of these fractionation factors and correct their experimental results for them where appropriate.

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