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Hydrogen isotope effects and atom tunnelling

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The abnormally high mass-dependent isotope effects in liquid-phase hydrogen (deuterium) atom transfer reactions, which are customarily regarded as quantum effects, are actually the products of two classical effects, namely, kinetic and thermodynamic ones. The former is determined by the rate constants for atom transfer and the latter is caused by nonbonded (or noncovalent) isotope effects in the solvation of protiated and deuterated reacting molecules. This product can mimic the large isotope effects that are usually attributed to tunnelling. In enzymatic reactions, tunnelling is of particular interest; its existence characterizes an enzyme as a rigid molecular machine in which the residence time of reactants on the reaction coordinate exceeds the waiting time for the tunnelling event. The magnitude of isotope effect becomes a characteristic parameter of the internal dynamics of the enzyme catalytic site.
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I. Introduction

Three hydrogen isotopes differ by the mass (this gives rise to mass-dependent isotope effect), magnetic moment (this is the source of magnetic isotope effect) and the volume (this is responsible for nuclear volume isotope effect). The last-mentioned effect is expected to be most pronounced exactly for hydrogen, as the successive addition of neutrons to a proton increases the nuclear volume twofold (in deuterium) and threefold (in tritium). The physics of this effect is that the increase in the volume generates more plumpy distribution of the nuclear charge; as a result, the Coulomb interaction of electrons with the distributed charge differs from the interaction that would have been observed if the nuclear charge had been concentrated at one point. However, no nuclear volume effect was found for hydrogen (although it was detected even for mercury isotopes [1]), because it is camouflaged by the much more pronounced mass-dependent effect. The magnetic isotope effect for hydrogen is known but is less pronounced than for other element isotopes.[2, 3]

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The mass-dependent H/D and H/T isotope effects are most important for chemistry and biochemistry; over the last 50 years they have been measured in quite a number of reactions.[4] The numerical values of these effects differ considerably: some values are normal, being up to several units, while some other are anomalous, comprising several tens of units. The former are attributed, without any hesitation, to the classical over-barrier transfer of the atoms, while the latter are ascribed with equal confidence to tunnelling, *i.e.*, under-barrier penetration of the atom as a wave.

The purpose of this study is to provide a comparative analysis of normal and anomalous isotope effects. This analysis has unexpectedly shown that anomalous effects, which are commonly considered to be of quantum nature, are actually superpositions (products) of two classical effects and just mimic the tunnelling. In enzymatic reactions, tunnelling is possible only for rigid molecular machines in which the retention time of the reactants on the reaction coordinate exceeds the waiting time for a tunnelling event.

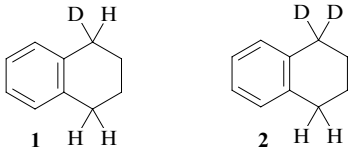
II. Anomalous H/D effects

It was believed that tunnelling is significant only in enzymatic reactions in which modulation of the energy barrier height and shape, *i.e.*, adjustment of the barrier for tunnelling, is provided by molecular motions of the protein fragments of the catalytic site. Abnormally high isotope effects were found by Klinman and co-workers [5–7] in the oxidation reactions of arachidonic acids and 1,4-*cis*,*trans*-diene lipids catalyzed by the iron-containing enzyme lipoxygenase. These isotope effects reached 80 (or more). This was an intriguing phenomenon: it seemed improbable that quantum effects can be significant under mild conditions (in liquids, at room or elevated temperature). These effects are known to be detectable only under exotic conditions (at extremely low temperature when all over-barrier processes are ruled out) and to be inconceivable and unachievable within the framework of classical chemistry. Only for this reason, the abnormally high isotope effects were attributed to quantum tunnelling.[5] Elegant mechanisms and physical models based on modulation of the height and width of energy barriers by molecular motions have been proposed to interpret these effects.† Later, a discouraging fact was discovered: the same anomalous isotope effects also exist in conventional (rather than enzymatic) reactions. The accuracy and reliability of their determination are beyond doubt; however, there are some enigmatic contradictions and discrepancies, which are discussed below.

Extensive investigations of the H/D effects were performed by Porter and co-workers.[10–12] The rates of chain oxidation of the isotopic substrates LH_2 and LD_2 (ethyl linolenates, 11,11-H_2-Lin and 11,11-D_2-Lin) differ by almost an order of magnitude: the rate constants are related as $k^H/k^D = 9.3 \pm 1.1$. In experiments on co-oxidation of the same substrates with dioxygen, the isotope effect (IE) was 12.8 ± 0.6.[12] The intramolecular isotope effect in the oxidation of 11,11-D,H-Lin proved to be different, amounting to 8.9.[12] However, all these values are at variance with the isotope effect ($k^H/k^D = 6 \pm 1$) measured by EPR in the direct reaction of peroxy radicals with the same substrates;[13] that effect was in line with the isotope effect observed in the reaction of peroxy radicals with α-deuterated cumene (IE = 5.5).[14] In the reactions of $Cl^•$, $Br^•$, $Me_3CO^•$ and $Me^•$ with toluene, ethylbenzene and cumene, the isotope effects are also moderate, ranging from 2.0 to 5.5.[14]

The isotope effect of linolenic acid oxidation in which hydrogen atom transfer to the peroxy radical is the rate-limiting step (as in the oxidation of linoleic acid) was almost three times higher ($k^H/k^D = 36 \pm 3.6$),[12] and this fact is also contradictory and unexplainable. Porter and co-workers [15] measured the H/D isotope effects for the oxidation of tetralins **1** and **2**.

Structures 1, 2



The effects refer to the reactions of peroxy radicals with these molecules and amount to 13.0 and 15.9 for compounds **1** and **2**, respectively.[15]

An abnormally high isotope effect (23 ± 2.3) was found for co-oxidation of 11,11-H_2-Lin and 11,11-D_2-Lin using α-tocopherol as an antioxidant.[11] The unexpected doubling of the isotope effect in the presence of α-tocopherol indicates unambiguously that the measured values do not characterize the elementary hydrogen transfer reactions, but are caused by a combination of isotope effects in consecutive or parallel processes. It is also improper to attribute these effects to tunnelling, which would not depend on the presence of α-tocoferol.

Anomalous isotope effects were found not only in reactions of peroxy radicals. For the processes of hydrogen atom transfer from cyclohexane, *p*-xylene, benzaldehyde, benzhydrol, fluorene, toluene and their deuterated 'twins' to the phthalimide nitroxide radical (PINO) in acetic acid, the isotope effects are in the range of 17 – 28.[16] However, the isotope effects for reactions of HBr_2 with the same compounds are usual, namely, 5.3 – 8.2.[17] For the reaction of PINO with ethylbenzene and its fully deuterated 'twins' in acetic acid, $k^H/k^D = 21.6$;[18] meanwhile, the same reaction with pure ethylbenzene (without acetic acid) has $k^H/k^D = 8.7$.[19] Even a lower value ($k^H/k^D = 3.8$) was found for the same reaction by Ishii *et al.*[20] The peroxy radical detaches an H(D) atom from α- and β-naphthols with isotope effects of 5.4 and 6.7, respectively, while the related $Me_3CO^•$ radical eliminates H(D) from phenol with IE = 4.8.[21]

For the reaction of PINO with benzhydrol in acetic acid, IE = 11.8, but in MeCN, this value is 10.6.[17,19] The reactions of hydrogen atoms with propan-2-ol-H_8 and propan-2-ol-D_8 involve no anomalous effects (IE = 8.2).[22] Remarkably, the IEs for reactions of PINO with C_6H_{14} and ethylbenzene are 24 and 21.6, respectively, but the IE value for the reaction with fluorene is twice lower (13.3).[17] Ueda *et al.*[19] found that IE values for the reaction of PINO with benzhydrol are 10.7 (25 °C) and 7.2 (35 °C);

† A summary of these mechanisms and models is given in excellent papers.[8,9] The models actually simulated the barriers with properties corresponding to tunnelling and, hence, they were always consistent with experiments.

however, IE = 19.6 was also reported for the same reaction.[23]

Special mention should be made of the publication of Wu *et al.*[24] devoted to the kinetics of hydrogen and deuterium atom transfer to the nitroxide radical from the corresponding hydroxylamines

$$R_2NO^{\bullet} + R'_2NOH(D) \longrightarrow R_2NOH(D) + R'_2NO^{\bullet} \quad (1)$$

These exchange reactions are thermally neutral, being characterized by isotope effects of 13 – 23 dependent on the solvent (MeCN, CH_2Cl_2, CCl_4). In the case of the reaction involving $(CF_3)_2$ NO$^{\bullet}$ and toluene, which is highly endothermic, the isotope effect falls in the same range ($k^H/k^D = 15$).[25] However, for the same reaction carried out in an inert solvent (hexane), the IE is an order of magnitude lower ($k^H/k^D = 1.5 – 1.9$) and no signs of tunnelling are present.[24]

The isotope effects of many reactions are solvent-dependent; this fact implies that the solvation of reactants is also isotope-dependent. Indeed, the IE for the reactions of 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) with its hydroxylamines in acetonitrile varies in the following way:[16]

T/K	278	308	318
IE	11 ± 1	17 ± 2	21 ± 6

This type of temperature dependence indicates unambiguously that the major contribution to the IE is made by solvation of hydroxylamines. Moreover, these reactions obey the Marcus rule, which is inconsistent with tunnelling.[24, 26]

III. Isotope effects of tritium

The tritium isotope effects were studied by Berezin and Antonovskii[27] for elimination of hydrogen (tritium) atoms from hydrocarbons and phenols by a methyl radical. For n-heptane

$$\frac{k^H}{k^T} = 0.2 \exp\frac{3450}{RT} \quad (2)$$

the difference between the activation energies ($E_T - E_H$) for the T and H atoms is nearly equal to the difference between the zero-point energies. For toluene, cyclopentane, cyclohexane and cycloheptane, the measured ($E_T - E_H$) values are 2200, 3500, 2700 and 3400 cal mol^{-1}, respectively; they also nearly coincide with the zero-point energy differences. The absolute k^H/k^T values are normal and imply no tunnelling.

The isotope effect for hydrogen (tritium) atom transfer to the methyl radical from the hydroxyl groups of 2,6-di-*tert*-butylphenols is

$$\frac{k^H}{k^T} = 0.02 \exp\frac{5800}{RT} \quad (3)$$

In this case, the ($E_T - E_H$) difference is much greater than the zero-point energy difference, although the absolute values of k^H/k^T are normal, showing no signs of tunnelling. It is known that the phenol hydroxyl group can occupy two positions relative to the aromatic ring — axial and equatorial ones. The ($E_T - E_H$) value is not the true difference between the zero-point energies, because it results from superposition of two effects involved in the reaction of the methyl radical with the OH groups in the axial and equatorial positions. In order to derive the pure isotope effect of the reactions of the methyl radical with hydroxyl groups, it is necessary to take into account the equilibrium between the axial and equatorial groups, which is also isotope-dependent and includes an isotope effect;[27] its characteristics are unknown as yet.

IV. Kinetics of reactions of solvated molecules

The total rate constant for a typical hydrogen atom transfer reaction

$$R^{\bullet} + MH \longrightarrow RH + M^{\bullet} \quad (4)$$

is the additive sum

$$k_{\Sigma}^H[MH]_{\Sigma} = k_f^H[MH]_f + k_s^H[MH]_s \quad (5)$$

in which the first addend refers to the reaction of free (non-solvated) molecules, MH_f, and the second one refers to the reaction of solvated molecules, MH_s; k_f^H and k_s^H are the corresponding rate constants. A similar equation holds for deuterated MD molecules

$$k_{\Sigma}^D[MD]_{\Sigma} = k_f^D[MD]_f + k_s^D[MD]_s \quad (6)$$

Here the total concentrations are given by the equations

$$[MH]_{\Sigma} = [MH]_f + [MH]_s \quad (7)$$

$$[MD]_{\Sigma} = [MD]_f + [MD]_s \quad (8)$$

The experimentally measurable isotope effect

$$IE = \frac{k_{\Sigma}^H}{k_{\Sigma}^D} \quad (9)$$

is a function of single rate constants k_f^H, k_f^D, k_s^H and k_s^D and concentrations of free and solvated MH and MD molecules. Using the equations for solvation equilibria

$$MH_f + S \overset{K^H}{\rightleftharpoons} MH_s \quad (10)$$

$$MD_f + S \overset{K^D}{\rightleftharpoons} MD_s$$

one can easily derive expressions for k_{Σ}^H and k_{Σ}^D

$$k_{\Sigma}^H = \frac{k_f^H}{1 + K^H[S]} \quad (11)$$

$$k_{\Sigma}^D = \frac{k_f^D}{1 + K^D[S]} \quad (12)$$

where [S] is the concentration of solvating molecules S. Since the equilibrium constants $K^H[S] \gg 1$ and $K^D[S] \gg 1$, the equation for the isotope effect has the form

$$\frac{k_{\Sigma}^H}{k_{\Sigma}^D} = \frac{k_f^H}{k_f^D}\frac{K^D}{K^H} \quad (13)$$

It is clear that the measurable isotope effect is the product of two effects, the kinetic (k_f^H/k_f^D) and thermodynamic (K^D/K^H) ones. Since $K^D > K^H$ and $K^D/K^H > 1$

(this follows from nonbonded isotope effects, see below), the measurable effect $k_{\Sigma}^{H}/k_{\Sigma}^{D}$ can far exceed the kinetic effect k^{H}/k^{D}.

Consideration of isotope effects of solvation accounts for the huge quantitative diversity and the solvent dependence of the measurable effects. Apart from the hydrogen-bonded associates, isotope sensitivity can be inherent in π-complexes and any other donor – acceptor complexes. Equation (13) was derived for a single H/D substitution; if the reaction involves n atoms (for example, for the CD_2 group, n is 2), Eqn (13) has the form

$$\frac{k_{\Sigma}^{H}}{k_{\Sigma}^{D}} = \frac{k_{f}^{H}}{k_{f}^{D}} \left(\frac{K^{D}}{K^{H}}\right)^{n} \qquad (14)$$

V. Nonbonded isotope effects

For participating in a reaction, a molecule should, most often, get free from the solvation shell, *i.e.*, the intermolecular bonds with the solvating molecules should break. The energies of these bonds are different for protiated and deuterated molecules; this difference constitutes what is called the intermolecular, or nonbonded isotope effect.[28] It is detected in a variety of processes including chromatographic ones.

Encapsulation of protiated and deuterated *p*-xylene and *p*-xylene-D_n (n = 3, 4, 6 or 8) molecules into calixarenes to give a host – guest structure is accompanied by an isotope effect:[29] the deuterated molecules always bind more strongly to the host molecule. The ratio of the equilibrium constants of binding, K^{D}/K^{H}, amounts to 1.2 – 1.3. A similar isotope effect is observed in binding of 4,4′-diacetoxy(biphenyls) (with CH_3 and CD_3 substituents), 4,4′-diacetoxybis(cyclohexanes) (also with CH_3 and CD_3 substituents) and $X-(CH_2)_8-X$, where $X = OC(O)CH_3$ or $OC(O)CD_3$.[30] The isotope effect, *i.e.*, the K^{D}/K^{H} ratio, amounts to 1.12 – 1.73 and depends on temperature: it increases with decreasing temperature.

Nonbonded isotope effects are reliably detected in the separation of protiated and deuterated molecules by reverse phase chromatography.[31, 32] This regime includes the equilibrium transfer of solute molecules between the mobile phase (usually water or water – alcohol hydrophilic medium) and the hydrophobic stationary phase. The distributions of protiated and deuterated molecules between these phases are different: the deuterated molecules bind more strongly to the polar molecules of the mobile phase; therefore, the peak for these molecules appears earlier in the chromatograms. In other words, deuterated molecules are held more strongly by the hydrophilic phase than their protiated 'twins'.

The chromatographic isotope effect of H- and D-molecules is defined as the K^{H}/K^{D} ratio of the retention factors for these molecules; the retention factor K is determined by the reduced difference between the retention times of a component in the chromatogram

$$K = \frac{t - t_0}{t_0} \qquad (15)$$

where t and t_0 are the retention times measured on the chromatographic scale.[31, 32]

The question of what interactions are responsible for the isotope-selective binding of molecules can be answered by analyzing the dependences of isotope effects of the retention of protiated and deuterated molecules on the number of deuterium atoms (n) in the molecule, which are shown in Figs 1 – 3. The linear dependences for various molecules (aromatic and aliphatic) reliably demonstrate that the major contribution to the nonbonded isotope effects is made by atomic potentials, that is, by local atom – atom interactions (such as hydrogen bonds) rather than by macroscopic properties (dielectric constant, optical polarizability and so on).

The nonbonded interactions responsible for the separation of H/D isotope pairs can be reliably detected in the NMR spectra of paramagnetic molecules.[33 – 35] They are accompanied by partial transfer of the unpaired electron from the paramagnetic molecule or ion to the solvating

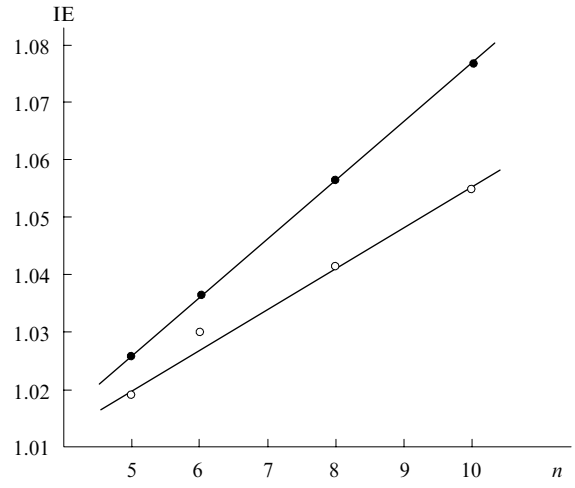


Figure 1. Isotope effects in the chromatographic separation of H/D isotope pairs.[32]
n = 5, nitrobenzene; n = 6, benzene; n = 8, toluene; n = 10, anthracene. The empty and black circles refer to different stationary phases.

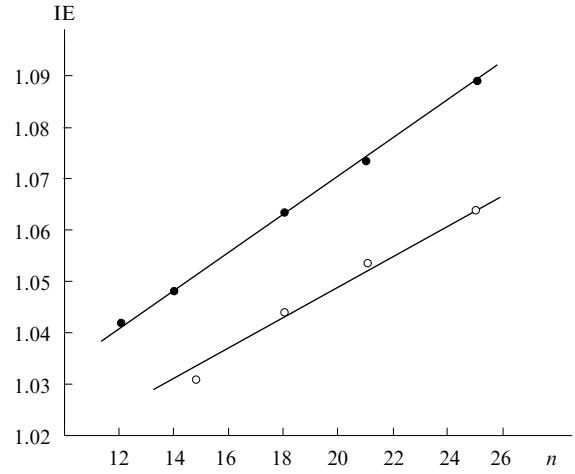


Figure 2. Isotope effects in the chromatographic separation of H/D isotope pairs.[32]
n = 12, cyclohexane; n = 14, hexane; n = 18, octane; n = 21, decanol; n = 25, dodecanol. The empty and black circles refer to different stationary phases.

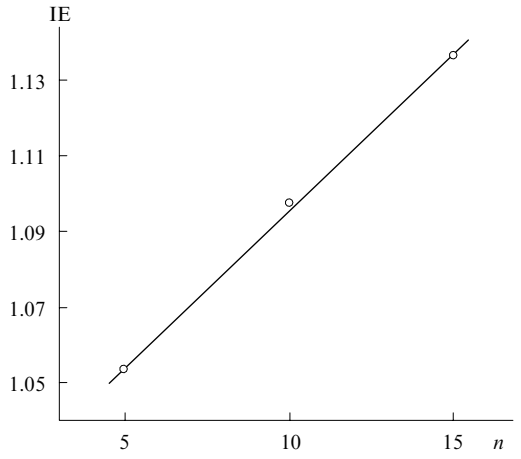


Figure 3. Isotope effects in the chromatographic separation of H/D isotope pairs.[31]
n = 5, 10, 15, diethylaniline.

ligand molecule. The appearance of the electron spin on the ligand nuclei induces paramagnetic shifts and broadening of NMR lines. Strictly speaking, this means that nonbonded interactions are partly bonded in the sense that upon contact of molecules in the complex, their electron shells are partly shared.[36]

Theoretical calculations confirm stronger binding for deuterated molecules. For example, olanzapine-D_3 binds to the silanol groups on the silica gel surface more strongly than the protium 'twin' by 0.12 kcal mol^{-1}; des-methyl-olanzapine-D_3 also binds more strongly (by 0.19 kcal mol^{-1}) than the protium form.[37] This energy difference corresponds to the K^H/K^D isotope effects of 1.22 and 1.38, respectively, which is in quantitative agreement with the chromatographic isotope effects. It also follows from these calculations that the K^H/K^D ratio of the retention factors is identical to the ratio of equilibrium constants for isotopic molecules.

The nonbonded isotope effects provide the chromatographic separation of tritium-labelled molecules and molecules labelled by isotopes of other, heavier elements (carbon, oxygen, nitrogen). For example, the isotope effects in the chromatographic separation of deuterium- and tritium-labelled 2-(*N*,*N*-dipropylamino)tetrahydronaphth-8-ols are 1.037 (for H_8/D_8) and 1.029 (for D_8/T_8).[31] A general trend is that the isotope effects of solvation with aromatic molecules are much greater than those for aliphatic molecules; this also attests to localized nonbonded interactions. The nonbonded isotope effects are found in the fractionation of the isotope 'twins' or triads of heavy nuclei such as $^{15}N/^{14}N$, $^{14}C/^{13}C/^{12}C$ or $^{18}O/^{17}O/^{16}O$.[28, 38, 39]

VI. Enzymatic reactions

The discovery of giant isotope effects in enzymatic reactions created the illusion that tunnelling predominates in this case. The anomalous H/D effects appear mysterious, as they are observed under mild conditions in which tunnelling seems implausible. An anomalous isotope effect ($k^H/k^D \approx 80$) was found in the hydrogen (deuterium) atom transfer during the oxidation of linoleic acid (LA) with soybean lypoxygenase SLO-1;[40] the observation of this effect was a significant event in chemistry. Later, this isotope anomaly was demonstrated[41] in the same reaction with SLO-1 (isotope effect 48 ± 5) and with the human enzyme 15-HLO (human 15-lypoxigenase, isotope effect 47 ± 7). Not only the absolute magnitudes but also the behaviour of the effects proved to be anomalous. First, the effects depended substantially on the concentration of the substrate (linoleic acid) being oxidized: a 2 – 3-fold increase in the LA concentration entailed an increase in the isotope effect from 19 (for LA concentration of 5 μmol litre^{-1}) to 46 (for LA concentration of 19 μmol litre^{-1}). Second, the presence of a foreign substance — oleic acid — had a pronounced influence on the isotope effect of LA oxidation: in the case of SLO-1, the presence of oleic acid decreased the isotope effect by 20% – 30%. Meanwhile, in the case of 15-HLO, the effect increased by an equal value of 20% – 30%.[41] Neither of these findings has yet been explained.

Moreover, the isotope effects for both enzymes are highly temperature-dependent, the dependences being different at different concentrations of the substrate being oxidized. When H_2O present in the reaction system is replaced by the isotopic analogue, D_2O, the isotope effect decreases by 10% – 40%.[41] Yet another remarkable feature is that the isotope effects depend on the enzyme mutant forms: modification of the catalytic site is accompanied, due to mutagenesis, by a change in both the enzyme catalytic activity and isotope effect.[42] Anomalous isotope effects with the same unusual behaviour were also observed[43] for the oxidation of arachidonic acid (AA) with the SLO-1 enzyme. The most significant result is shown in Fig. 4. It can be seen that at low oxygen pressure, the oxidation rates for the protiated and deuterated AA molecules are equal, *i.e.*, no isotope effect is present. No doubt that under these conditions, the rate-limiting step is the addition of oxygen to the AA-derived alkyl radicals, which are generated in the primary reaction of the FeO active site of the enzyme with the C – H (or C – D) bond. At high concentrations of oxygen, the primary hydrogen (deuterium) atom transfer reaction becomes the rate-limiting step, and particularly this

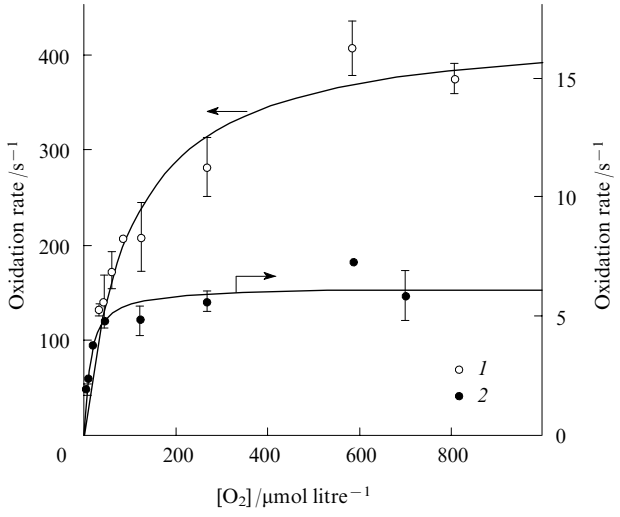


Figure 4. Initial rates of oxidation of arachidonic acid (*1*) and its deuterated form (*2*) *vs.* concentration of oxygen.[43]

step is isotope-dependent (the isotope effect is ~60). The same magnitude and the same oxygen concentration dependence of the effect were observed in the oxidation of linoleic acid with the SLO-1 enzyme.[44]

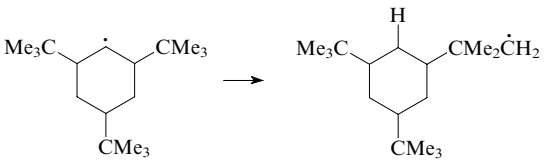
Glickman and Klinman[44] convincingly demonstrated that the primary reaction in the catalytic site is the hydrogen (or deuterium) atom transfer from the substrate (LA or AA) to the FeO active site; the appearance of oxygen and its addition to the alkyl radical is the secondary process. The isotope effect (the value of ~60 for this effect seems most trustworthy) appears particularly in this reaction and is clearly indicative of tunnelling.

VII. Tunnelling in enzymatic reactions

The tunnelling in enzymatic reactions is of special interest: the presence (or, conversely, the absence) of tunnelling contributes to the understanding of the enzyme physics. It is commonly considered that the phenomenon of catalysis (including enzyme catalysis) is a consequence of lowering of the energy barrier. However, when lowered, the barrier becomes wider and less steeply sloping. Both factors suppress the contribution of tunnelling; therefore, the experimental observation of tunnelling seems paradoxical.

The physical mechanisms of tunnelling are discussed in many books and papers (see, for example, Refs 4, 45–47). Tunnelling is a quantum event; it follows from quantum mechanics in which every object is both a particle and a wave. When a hydrogen atom is unable to jump over the energy barrier, it 'pretends' to be a wave and penetrates through the barrier (in the same way as a photon penetrates through glass). This is a rare event; for this to happen, a long waiting time is required. For this reason, tunnelling is observed only in crystals and in amorphous solids at very low temperatures where molecular motions are suppressed and the energy barriers of reactions and the positions of reactants are rigidly fixed in time and space. For instance, a huge H/D isotope effect (13 000 at 123 K) was observed in the intramolecular hydrogen transfer (Scheme 1).[47]

Scheme 1



Even a greater effect (28 000 at 77 K) was observed in the reaction of methyl radicals with acetonitrile.[48]

It has been noted above that mechanisms and models based on modulation of the height and width of energy barriers by molecular motions have been proposed for interpreting the anomalous H/D effects.[8,9] However, one should recall that the energy barrier of a reaction is determined by strong Coulomb potentials in the A—H···B triatomic system and the H atom is presumed to migrate between the heavy atoms A and B. The barrier size and profile are formed by local Coulomb interactions in this system. The molecular motions (including the motions of the protein fragments of the enzyme catalytic site) generate fluctuations of the barrier and force the reacting system to climb along the barrier energy profile. At some point of the profile attained, the reacting system should stop and stay for a sufficiently long period for a tunnelling event to happen. In the simplest case of a parabolic barrier, the equation for the rate constant for tunnelling has the form[49–51]

$$k_{\text{tun}} = v_0 \exp\left[\frac{-\pi d(mE_a/2T)^{1/2}}{\hbar}\right] \quad (16)$$

where v_0 is the vibration frequency of the tunnelling atom; m is the tunnelling mass; d and E_a are the width and the height at that point of the potential barrier where the tunnelling atom is located; $\hbar$ is the Planck constant; T is temperature.

The unusual enigmatic behaviour of tunnelling in the enzymatic transport of H and D is explained in terms of physical models in which the major role belongs to the barrier modulation dynamics.[52] However, tunnelling requires relatively long waiting time

$$\tau = (k_{\text{tun}})^{-1} \quad (17)$$

This is the key condition, which implies that the settled lifetime of the system at the achieved point of the barrier profile should be at least τ. This condition can be provided only by a rigid structure of the catalytic site. It is fulfilled only in rigid molecular machines in which the retention time of the reactants exceeds the waiting time for the tunnelling event. The enzyme as a molecular machine captures the substrate according to the host–guest pattern, confines the reactants in the catalytic site and holds them for a rather long time to provide for the tunnelling. In 'soft' enzyme molecular machines, the reactant contact time is short and the contribution of tunnelling is low. In other words, the contribution of tunnelling is controlled by the rigidity of the catalytic site; the rigidity depends on temperature, the presence of plasticizers and the mutant form of the site. Different allosteric forms of enzymes have different rigidity; this accounts for the experimentally observed diversity of H/D isotope effects and their behaviours (see the previous section). Moreover, if tunnelling is controlled by the rigidity of the catalytic site, the magnitude of the isotope effect can characterize the enzyme and its rigidity as a molecular machine.

One should distinguish the notions of enzyme power and rigidity. The enzyme power can be measured quantitatively and is determined as the force at which the enzyme, as a molecular motor, transforms a molecule. For this purpose, an optico-mechanical technology has been created, the key unit of which is an optical trap, or optical tweezers. The trap is a ~1 μm polystyrene or quartz bead. On exposure to a focused laser beam, the bead is drawn into the focus with a force depending on the light intensity. Hence, the trap attached to the end of a molecule pulls the molecule, and the molecule is distorted. The optical trap is actually an analogue of an atomic force microscope. This technique is used to study molecular motors (ATP synthases, kinesin, myosin, immunoglobulin proteins and so on) and their functioning.[53–56]

Thus RNA polymerase draws the DNA double strand inside and pulls it past, simultaneously generating an RNA thread, with a force of 25 pN. This is considered to be the most powerful known molecular motor: it is 5–6 times more powerful than kinesin. It is assumed that most energy of this motor is spent for unwinding the DNA strands.[54]

Another protein motor is λ-exonuclease — a component of a bacterial virus. Unlike RNA polymerase, which is powered by ATP, λ-exonuclease operates on the DNA energy: while pulling the DNA double strand through itself, it 'eats up' one of the strands. It is a weaker motor than RNA polymerase and develops force of only 5 pN.[56]

The notion of enzyme rigidity characterizes its relaxation properties: slow molecular rearrangements of protein fragments in the catalytic site imply long lifetimes for fixed states of a rigid enzyme, while fast relaxation and high mobility of protein fragments correspond to the notion of a soft enzyme. Certainly, these definitions lack quantitative rigour; perhaps, particularly the presence (or, conversely, the absence) of the tunnelling effect could be regarded as a criterion for enzyme rigidity.

It is clear that tunnelling is not a necessary feature of any enzyme. Northrop [57] used elegant and physically perfect high-pressure technique to measure the H, D and ^{13}C isotope effects and the activation volumes for hydrogen atom transfer catalyzed by yeast alcohol dehydrogenase and concluded that there is no tunnelling in this reaction. The oxidation of isotopic H/D/T forms of benzyl alcohol with liver alcohol dehydrogenase is accompanied by normal isotope effects, $k^H/k^D = 3.8$ and $k^D/k^T = 1.9$,[58] and no tunnelling is detected. In the biomimetic tyrosine redox system, no tunnelling is present either ($k^H/k^D = 3.0$).[50] Evidently, dehydrogenases and oxidoreductases, unlike lipoxygenases, are soft molecular machines providing no rigid conditions necessary for tunnelling.

Dihydrofolate reductases,[59, 60] which reduce dihydrofolate to tetrahydrofolate using nicotinamide adenine dinucleotide as the reducing agent, should also be classified as soft molecular machines. Both reductases, chromosomal and R-plasmid ones, used in the study of Sen *et al.*[60] reduced H- and T-dihydrofolates with a normal k^H/k^T isotope effect of 6.1 – 6.7. No signs of tunnelling were found in these enzymes.

VIII. Conclusion

The abnormally great H/D isotope effects cannot serve as reliable evidence for the tunnelling mechanism of hydrogen atom transfer. For reactions occurring under mild conditions, nonbonded isotope effects, *i.e.*, the difference between the solvations of protiated and deuterated reactants, should be taken into account. Then the measurable total isotope effect is the product of purely kinetic isotope effect in the atom transfer and the thermodynamic effect of solvation, which is isotope-depended *via* nonbonded interactions (such as hydrogen bonds). This product can reach large values, thus mimicking the tunnelling.

The isotope effects of solvation can also be involved in enzymatic reactions, as the isotope substitution in substrate molecules changes their affinity for the catalytic site and gives rise to the isotope effect of host – guest substrate binding. However, as convincingly shown by experiments, the major contribution to the isotope effects is made by tunnelling. The physical source of tunnelling is probably the rigid fixation and compression of reactants in the catalytic site of the enzyme as a molecular machine. This compression is possible only in rigid molecular machines (such as lipoxygenases), in which the retention time of the reactants on the reaction coordinate at a specified point of the energy barrier profile is longer than the waiting time for a tunnelling event at this point.

Recently, an elegant idea of confinement of isotope molecules has been proposed:[61] model calculations for the H_2, D_2 and T_2 molecules demonstrated that confining is accompanied by a decrease in the interatomic distances and considerable increase in the gap between the zero-point energies and isotope effects. The authors deem that compression of molecules in a catalytic site can produce similar effects. However, this compression (especially for large molecules rather than H_2, D_2 or T_2) does not mean compression of C – H, C – D or C – T chemical bonds; this elegant idea does not seem useful for the understanding of isotope effects in enzymatic catalysis.

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