Chang-Hwei Chen

# Deuterium Oxide and Deuteration in Biosciences

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# Preface

Water in tissues and cells intervenes with cellular components. The interactions of water affect the structures, dynamics, and functions of biomolecules, including proteins, nucleic acids, and lipids. Biomolecular folding, stability, and binding are mediated by water through hydrogen bonds and hydrophobic interaction. Water also participates in catalytic functions of enzymes, and plays crucial roles in protein secondary and tertiary structures, nucleic acid helix formation, and lipid bilayer membranes.

Deuterium is a naturally occurring, stable, and non-radioactive isotope of hydrogen. There are distinct differences in physical properties between deuterium oxide ($D_2O$) and regular water ($H_2O$). Investigations of deuterium oxide and deuteration is a promising component in various areas of sciences, including physics, chemistry, biochemistry, biomedical, pharmacology, and biotechnology.

There are distinct differences between deuterium oxide (heavy water) and hydrogen oxide (regular water) as the solvent for biological systems. Changes in the aqueous $H_2O$ environment with $D_2O$ affect the interaction, stability, and functions of biomolecules, including the strengths of hydrogen bond, hydrophobic interaction, and carbon-hydrogen bond that serve as the driving forces for various biomolecular processes.

There are many advances that have been made in the past decades toward the understanding of functions, interactions, and implications of deuterium oxide and deuteration. Studies of deuterium oxide and deuteration effects on the interactions, stability, and functions of biomolecules offer valuable applications to health-related issues, including diseases, vaccines, and drugs. Deuterium oxide and deuteration also mediate pharmacokinetic process involving adsorption, distribution, metabolism, excretion, and toxicity.

An organized, concise overview of deuterium oxide and deuteration in biosciences is needed for readers who are exposed to this important subject, particularly for students and researchers in the areas of biochemistry, biophysics, biomedical sciences, and pharmacology, who are interested in gaining knowledge on the background and implications of deuterium oxide and deuteration.

This book addresses deuterium oxide and deuteration in broad scope with emphasis on biochemical, biomedical, and pharmacological aspects. Individual subjects covered include distinct characteristics of heavy water versus regular water, deuterium bonding versus hydrogen bonding, hydrophobic interaction in deuterium oxide versus hydrogen oxide, deuterium oxide and deuteration effects on biomolecules, effects of deuterium oxide and deuteration on biomembranes, biochemical effects of deuterium oxide and deuteration, physical methods for investigating deuterium oxide and deuteration effects, living cells grown in deuterium oxide and deuteration, deuterium oxide and deuteration effects on health issues, deuterium oxide effects on thermostability of vaccines, and deuterium oxide and deuteration effects on pharmacology. A bibliography is provided in each chapter for readers who wish to pursue topics in more detail.

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# Part I
# Roles of Regular and Heavy Water in Biosciences

# Chapter 1
# Overview: Background and Applications

The structure of liquid water and its interaction with biological molecules is a very active area of experimental and theoretical research. Water is essential for life by not only serving as a solvent but also contributing to an active role in biological structures and functions. Accordingly, water should not be treated as an inert environment alone but rather as an integral component of biomolecular systems. Researching water advances our understanding of the chemistry and physics of biological systems.

When dissolve in water, solute disrupts the local ordering of solvent molecules at the interface between a hydrated ion and the surrounding solvent. As an integral and active component of biomolecular systems, water actively participates in molecular recognition by mediating the interactions between biomolecular binding partners, including molecular interfaces, biological assembly, conformational changes, and biochemical reactivities.

Changes in the water environment affect the structure and function of biomolecules. Water participates in the catalytic function of proteins and nucleic acids, is involved in the protein folding through the hydrophobic interaction, mediates biomolecular binding through the hydrogen bond in complex formation, and also plays a critical role in the formation of lipid bilayer membranes.

Deuterium is a naturally occurring stable, nonradioactive isotope of hydrogen. Hydrogen consists of one electron and one proton with an atomic mass of 1.0, while deuterium has one neutron in addition to one electron and one proton with an atomic mass of 2.0. Deuterium occurs at a natural abundance of about 0.0015%. Regular water ($H_2O$) is the source of producing enriched heavy water (deuterium oxide; $D_2O$).

Physical properties of deuterium oxide differ to some extent from those of regular water. As solvent, there are fundamental differences between $D_2O$ and $H_2O$. Investigations of deuterium oxide is a promising component in various areas of sciences, including chemistry, biochemistry, biotechnology, and pharmacology. Changes in the aqueous $H_2O$ environment with $D_2O$ affect the structure and stability of biomolecules (proteins, lipids, and nucleic acid) due to solvent effects as well as

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deuteration effects. Deuterium solvent isotope effects are those due to the special properties of $D_2O$ as the solvent, while deuterium substitution effects (deuteration) are resulted from deuterium replacing hydrogen in biological molecules.

Deuterium oxide is also a promising component in research related to human health.

$D_2O$ is able to activate or inhibit biological systems and thus influences the metabolism and functions of tissues and organs. $D_2O$ also has numerous therapeutic applications to health issues, such as hypertension, cancer, cardiac, and aging. Moreover, vaccines such as polio, influenza, and yellow fever undergo changes upon exposure to heat. The addition of deuterium oxide prompts the stabilization of viral vaccines.

Deuteration also affects biomolecules, such as the structures and functions of proteins and nucleic acids, and the interactions of lipids, lipid phases, and lipid peroxidation. Moreover, deuteration can also alter the pharmacological profile of a drug compound by producing an effect on its pharmacokinetics. The incorporation of deuterium into pharmacologically active agents offers potential benefits, such as improvements in efficacy, tolerability, or safety.

## 1.1 Role of Water in Biological Systems

As indicated above, water is an essential participant in the stability, structure, dynamics, and function of biomolecules. The interactions of biomolecules with water affect their structures, functions, and dynamics. Furthermore, water in tissues and cells is confined by intervening cellular components and is subject to structural effects which are not present in its bulk counterpart. Thus, the biological functions of water detail not only the stabilization effect on proteins, nucleic acids, and lipids but also involve the direct role that water molecules play in biochemical processes, such as enzyme kinetics, protein synthesis, and nucleic acid-drug interaction. Water molecules within the complex nucleic acid structures also assist in drug binding and catalytic reactions. Proper protein folding and helix structure of nucleic acids are driven by intermolecular interactions between biomolecules and water.

### *1.1.1 Integral Component of Biomolecules*

The role of water as a solvent helps cells transport and utilize substances like oxygen or nutrients. Thus, water is an essential constituent of the protoplasm of living cells, which is directly involved in countless biochemical reactions like photosynthesis and respiration. Without water, cells could not move waste and by-products, take in nutrients, and perform intracellular transportation.

The behavior of water molecules at hydrophilic sites is different from that at hydrophobic sites. This dissimilar behavior promotes the anisotropy of the

hydration shell of biomolecules. Such anisotropy of the hydration shell is essential for the enzyme function. Hydration water generally has slower correlation times than water in bulk. The chemically complex surfaces of protein molecules alter the structure of the surrounding layer of hydrating water molecules.

Specific water-mediated interactions in protein complexes have been incorporated into potential energy functions for protein folding. Studies of bacteriorhodopsin revealed the importance of water molecules for proton transport and biological function and how protein conformational changes reposition or reorient internal water molecules, thereby guiding proton transport.

### *1.1.2 Interface Water*

As pointed out above, the structure and function of biomolecules are strongly influenced by their hydration shells. Experimental and theoretical evidences revealed that solvation water is not a passive spectator in biomolecular processes. There is significant hydrogen bond dynamics for the function of proteins and for molecular recognition. Bound water is a major component of biological membranes and is required for the structural stability of lipid bilayers.

Membrane-water interface underlines the important role of membrane hydration properties. The understanding of structural changes of lipid bilayers, as a result of different properties of the environment outside or inside the membrane, provides a foundation for better insights into the structure-function relationships of biological membranes.

Infrared absorption is sensitive to hydrogen bonding and vibrational motion of water, as well as membrane proteins and lipids. Spectroscopic studies of fluorescence emission of membranes as a function of hydration revealed that increasing rigidity or decreasing polarity of the membrane-aqueous interface occurs with removal of water. Molecular dynamics simulations have led to insight into fluctuations of water structure and hydrated biomolecular interfaces.

## 1.2 Distinct Characteristics of Deuterium Oxide ($D_2O$)

Physicochemical properties of $D_2O$ differ to some extent from those of $H_2O$. The molar mass for deuterium oxide (heavy water) is 20.028 versus 18.016 for hydrogen oxide (regular water). The dielectric constant at 25 °C is 78.06 for $D_2O$ and 78.39 for $H_2O$. The viscosity of $D_2O$ at room temperature has a value of 1.23 times the viscosity of $H_2O$.

Moreover, the mobility of ions dissolved in $D_2O$ is smaller than in $H_2O$. Consequently, the conductivity of ions in $D_2O$ is smaller than in $H_2O$. For instance, the ratio of conductivity in $D_2O$ and $H_2O$ is 0.82 for KCl, 0.83 in NaCl, and

0.82 in $CH_3COOK$. Furthermore, acids become weaker in heavy water than in regular water. The viscosity of liquid $D_2O$ is 1.23 times that of $H_2O$.

### *1.2.1 Hydrogen Bonding: $D_2O$ Versus $H_2O$*

A hydrogen bond is formed between a covalently bonded hydrogen atom on the donor group (such as H-O or H-N<) and a pair of nonbonding electrons on an acceptor group (like :O=C- or :N=), where the strength of a donor depends on its electronegativity. In biomolecules, O and N have appropriate electronegativities to serve as donors. Hydrogen bonds can be intermolecular or intramolecular. Intermolecular hydrogen bonding is responsible for the high boiling point of water.

Intramolecular hydrogen bonding plays an important role in the structures of proteins and nucleic acids. Many amino acid side chains carry groups that are either hydrogen bond donors or acceptors. Alpha-helix and beta-sheet are two important secondary structures of proteins. In the alpha-helix, hydrogen bonds are within a single chain (NH--O). In the beta-sheet, hydrogen bonds are between chains that run side by side. While in the base pairing in the helix structure of nucleic acid, hydrogen bonds are between base pairs (N-H--O) or N---H-N<), such as guanine-cytosine base pair.

### *1.2.2 Hydrophobic Interaction: $D_2O$ Versus $H_2O$*

The interactions of nonpolar substance or group with water molecular framework cause a perturbation in the hydrogen-bond network of water due to hydrophobic effect. This alters the ordering of the surrounding water structure, leading to a loss of randomness and a decrease in the entropy of the system. Molecular dynamics studies of the structure of water in the hydration shells of small hydrophobic solutes showed that a subset of water molecules in the first hydration shell of a nonpolar solute have a significantly enhanced tetrahedrality and a slightly larger number of hydrogen bonds, supporting the proposed hydrophobic effect.

Hydrophobic interaction is an important factor that makes a major contribution to the stability of biomolecules. Hydrophobic interaction has been extensively investigated within the context of solvent-induced effects that contribute to the driving force for biomolecular processes. Like hydrogen bonding, an understanding of hydrophobic interaction is important to the structure and stability of biomolecules in water medium.

When the polypeptide chain folds into a globular structure, the hydrophobic residues are buried within the folded proteins. In addition to hydrogen bonding, hydrophobic interaction also contributes to driving important biological processes, like the double-helix formation of nucleic acids and a polar, hydrophilic head region connected to the hydrophobic hydrocarbon tail portion in lipids.

According to the calculated structural parameters, more structural order is present in $D_2O$ than in $H_2O$ at a given temperature. The free energy of hydrophobic interaction in $D_2O$ was found to be – 1.76 kJ/mol, for each contact between two methylene groups, compared with −1.40 kJ/mol in $H_2O$. Hence, hydrophobic interaction is stronger in $D_2O$ than in $H_2O$ medium.

### *1.2.3 Zero-Point Energy: $D_2O$ Versus $H_2O$*

Molecules possess residual vibrational energy at 0 °K. Zero-point energy is the lowest possible energy that a quantum mechanical system may have. This finite, minimum amount of kinetic energy is present in all matter even at 0 °K. The relative energies of hydrogen (H) and deuterium (D) bonds are due to the differences in zero-point vibrational energy.

Because of a greater atomic mass of *deuterium*, a deuterium-carbon bond has a lower vibrational frequency, leading to a deuterium-carbon bond having a lower zero-point energy than a corresponding hydrogen-carbon bond. Such lower zero-point energy translates to a higher activation energy for C-D bond cleavage and a slower rate constant than C-H bond.

Moreover, the zero-point vibrational energy of the D-bond is also lower than that of the H-bond in the neutral dimer and trimer of water. The total zero-point vibration energy of the ($H_2O$--H--$OH_2$) is higher when a D occupies the bridge position ($D_2O$--D--$OD_2$). Theoretical studies revealed that more structural order presents in $D_2O$ than in $H_2O$ at a given temperature, which is consistent with a comparative infrared study of hydrogen bonding in water and deuterium oxide.

## 1.3 $D_2O$ Effects on Biochemical Reactions

The differences in physicochemical properties between $D_2O$ and $H_2O$ as indicated above significantly contribute to $D_2O$ effects on the thermodynamics of biochemical reactions. While the differences in zero-point potential energy and vibration frequency between deuterium and hydrogen contribute significantly to $D_2O$ effects on the kinetics of biochemical reactions.

### *1.3.1 Thermodynamic Effects*

Elucidation of the stabilization of proteins by deuterium oxide is an active research area. For example, in tubulin, the protein loses its ability to form microtubules rapidly when stored in solution, while $D_2O$ stabilizes tubulin against such thermal inactivation. Calorimetric studies showed that the temperatures for tubulin unfolding are

58.6 °C and 62.17 °C in $D_2O$, compared to 55.4 °C and 59.35 °C in $H_2O$. In addition, tubulin is inactivated after 40 hours at 4 °C in $H_2O$, while in $D_2O$, no activity was lost after 54 hours.

Moreover, $D_2O$ effects on helix formation were demonstrated in the comparative studies of the thermodynamics of double-helix formation by (dG-dC)3 in $H_2O$ and $D_2O$. The average of the enthalpy was found to be −59.6 kcal/mol of helix in $H_2O$ versus −65.8 kcal/mol of helix in $D_2O$, which reveals that $D_2O$ solvent exhibits a stabilization effect on (dG-dC)3 double-helix formation. Moreover, polypeptide (Pro-Pro-Gly)10, which forms a triple-helical structure in $H_2O$ solution, increases its melting temperature from 24.5 °C in $H_2O$ to 40 °C in $D_2O$.

### *1.3.2 Kinetics Effects*

Deuterium kinetic isotope effect is the change in the reaction rate of a chemical reaction when one of the atoms in the reactants is replaced by deuterium. Kinetics reaction rate change is a quantum mechanical effect, which primarily results from deuterium having lower vibrational frequencies as compared to hydrogen. This implies a greater energetic input needed for deuterium to reach the reaction transition state and consequently a slower reaction rate.

The C-D bond has a lower zero-point energy and a lower vibrational frequency than a corresponding C-H bond. This translates to a higher activation energy for C-D bond cleavage and a slower reaction rate. Hence, the deuterium isotope effect has the potential to affect the biological metabolism pathways involving C-H bond scission.

The study of kinetic isotope effects can help the elucidation of the mechanism of a biochemical reaction. For instance, cytochrome P450s are major enzymes that catalyze oxidation reactions involving the cleavage of C-H bond. The presence of a significant primary deuterium kinetic isotope effect is evident in cytochrome P450 reactions, where hydrogen abstraction is at least partially rate-limiting.

## 1.4 Preparations of Deuterated Biomolecules

Deuterated biomolecules are largely produced in bacterial systems, especially *Escherichia coli* (*E. coli*) grown in $D_2O$ medium. Yeast, moss, and animal cells are also grown in a medium containing $D_2O$ to produce deuterated biomolecules. Studies of the growth of different organisms in various concentrations of $D_2O$ revealed that the growth is increasingly inhibited in higher concentrations as compared to lower concentrations of $D_2O$.

There seem to be certain species-specific limits to their tolerance to heavy water. Some organisms are unable to grow in heavy water, while others have no difficulties. Single-celled organisms can often be grown in an environment that is fully

deuterated. Lower organisms have been reported to survive in 30% $D_2O$. Mice and dogs tolerate long-term replacement of 10–15% of body $H_2O$ with $D_2O$. However, toxicity is observed with exposure to above 25% $D_2O$.

## 1.5 Physical Methods in Studying $D_2O$ and Deuteration Effects

Spectroscopic methods that are applied to investigate deuterium oxide and deuteration effects on the structures and functions of biomolecules include nuclear magnetic resonance, infrared, ultraviolet, and fluorescence spectroscopies. Deuterium nuclear magnetic resonance, neutron scattering, and Fourier transfer infrared spectroscopies are also applied to study deuteration effects on biomolecules, while non-spectroscopic methods include differential scanning calorimetry, circular dichroism, and molecular dynamic simulation.

## 1.6 Deuterium Oxide Solvent Effects on Biomolecules

Deuterium solvent effect is referred to those due to the special properties of $D_2O$ as the solvent. $D_2O$ favors the burial of nonpolar surfaces and van der Waals packing in the biomolecule cores. While in $H_2O$, macromolecules would present more water-exposed surfaces, in particular at the macromolecular surface.

A broad variety of morphological and physiological changes have been observed in deuterium-treated cells and organisms. $D_2O$-induced changes in biological processes include cell division and energy metabolism. The mechanisms underlying the effects of deuterium oxide are likely to provide insight into the fundamental significance of hydrogen bonds in biological functions. Moreover, the hydrophobic effect is known to be stronger in $D_2O$ than in $H_2O$.

$D_2O$ affects biomolecules, including proteins, lipids, and nucleic acids. The stability of a protein is vital for its biological function, and proper folding is partially driven by intermolecular interactions between protein and water. Investigations of the effect of $D_2O$ on the structural stability of bovine serum albumin revealed that $D_2O$ enhances its conformational stability and reduces its monomer loss.

Studies of the effects of deuterium oxide on the stability of collagen peptides revealed that the transition temperatures of the protonated peptide (Pro-Pro-Gly)(10)-OH are increased from 25.4 °C in $H_2O$ to 28.7 °C in $D_2O$. Moreover, the phase-transition properties of lipid vesicles prepared from 1,2-distearoyl-, 1,2-dipalmitoyl-, and 1,2-di-myristoyl-L-3-glyceryl-phosphatidylcholines were compared in $D_2O$ and $H_2O$, which showed that their phase-transition temperatures are 0.2–0.4 °C higher in $D_2O$ than in $H_2O$.

## 1.7 Deuteration Effects on Biomolecules

Deuteration effects are resulted as deuterium (D) replaces hydrogen (H) in biological molecules. Lower organisms such as bacteria, algae, yeast, and molds can be grown in 100% $D_2O$, resulting in the stable deuteration of their cellular components. The higher mass of deuterium affects the physicochemical properties of biomolecules.

For example, comparative investigations of normal and deuterated phycocyanins isolated from blue-green algae revealed that deuterated proteins are less resistant to the denaturant urea than are normal proteins. The value of the thermal denaturation temperature in deuterated protein is about 5°C lower than that in normal protein. The magnitudes of the enthalpy of denaturation in deuterated protein are 18–36% lower than in normal protein.

In addition, deuterium was found to slow the rates of hydrogen transfer reactions. Depending on the site of deuteration, deuterium can be exchanged into drug compounds or into reagents that are useful for synthesizing drug molecules. Incorporation of deuterium may significantly alter the metabolic profile of a drug molecule.

## 1.8 Deuterium Oxide and Deuteration Effects on Human Health

As indicated above, the effects of deuterium oxide on living systems are generally categorized into two areas: (a) deuterium solvent effect based on the properties of $D_2O$ molecule, particularly its effects on biological molecules and the structure of water, and (b) deuterium isotope substitution effect, which results from the replacement of H with D in biological molecules.

Deuterium oxide is a promising component in the areas of biotechnology, chemistry, and medicine. Deuterium oxide and deuteration effects on human health include diseases, vaccination, and pharmacology.

### *1.8.1 $D_2O$ Effects on Diseases*

Deuterium oxide has therapeutic implications, which can be applied as a therapeutic agent against disease conditions, such as hypertension, cancers, cardiac disease, and aging. For instance, a minimum dose of 10% deuterium oxide was found to prevent the development of hypertension, elevated aortic calcium uptake, and renal vascular changes in spontaneously hypertensive rats. Deuterium-depleted water was shown to be an anticancer agent with potential clinical applications. Moreover, a tracer dose of deuterium oxide can be applied to determine the total body water by the deuterium nuclear magnetic resonance method.

### 1.8.2 Deuteration Effects on Health-Related Issues

Deuteration effects on health-related issues include autoxidation, oxidative damage, and deuterated medicine. Autoxidation of polyunsaturated fatty acids is initiated by the abstraction of bis-allylic hydrogen atoms, resulting in a chain reaction that generates toxic products associated with various health disorders. Replacement of bis-allylic hydrogens in polyunsaturated fatty acids with deuterium atoms inhibits their peroxidation, leading to cell protection against oxidative stress.

Oxidative damage resulting from increased lipid peroxidation is considered an important factor in the development of Alzheimer's disease. Administration of deuterated polyunsaturated fatty acids represents a promising strategy to reduce rates of lipid peroxidation, which can be beneficial in improving Huntington's disease condition.

### 1.8.3 $D_2O$ Effects on Pharmacokinetics

A significant number of studies have appeared in the literature over the years reporting the deuterium isotope effect on enzyme-catalyzed reactions, including cytochrome P450s, monoamine oxidase, alcohol dehydrogenase, and aldehyde oxidase. These enzymes are responsible for drug metabolism. Due to the kinetic isotope effect, the replacement of hydrogen with deuterium has the potential to slow down their metabolic pathways.

Accordingly, deuterium oxide has been utilized to improve the pharmacokinetic properties of drugs. Deuterium substitution can potentially affect the overall pharmacological profile of a drug compound. The application of deuterium medicinal chemistry to drug compounds with well-understood therapeutic utility can potentially provide a risk-reduced approach to creating new drugs that address important medical needs.

### 1.8.4 Thermal Stabilization of Vaccines by $D_2O$

Live attenuated vaccines, such as polio, influenza, yellow fever, measle, and mump vaccines, are made up of proteins, nucleic acids, lipids, and carbohydrates. These vaccines undergo changes when expose to heat. Therefore, the thermostability of vaccine in solution is an important consideration.

Enhancement of thermostability of vaccines can also lead to the improvement of vaccine effectiveness. Deuterium oxide has been known to stabilize attenuated viral RNA against thermal degradation. Accordingly, thermal stabilization effect of deuterium oxide has been studied on vaccines, including oral poliovirus and influenza virus vaccines.

### *1.8.5 Deuteration Effects on Pharmacology*

One critical area of pharmacologic research is to improve the effective therapeutics of drugs. Research has been carried out to prolong the residence time of the drug to achieve greater efficacy and to alter the rate of metabolism to afford greater tolerability. Another critical area of pharmacologic research is the studies of the reactions between drugs and living systems, including the body's responses to drugs and interactions between the drug and the human body.

Certain drugs may be favorably influenced when deuterium is substituted for protium, which could result in improved safety, tolerability, or efficacy. To slow down the metabolic process and improve the half-life of the drugs, incorporation of deuterium in the drug (referred to as deuterated drugs) has been carried out, which revealed that deuterated versions of existing drugs can exhibit improved pharmacokinetic or toxicological properties, due to the stronger deuterium-carbon bond that modifies their metabolism. An increased interest in deuterium substituted medicines is reflected by a greater number of patent filings. Their early clinical results have been encouraging.

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# Chapter 2
# Characteristics of Deuterium Oxide Versus Hydrogen Oxide

Water ($H_2O$) exhibits unique properties that distinguish it from other liquids. High heat capacity and high dielectric constant make water an excellent solvent for a wide variety of organic and inorganic compounds. Although water molecule is neutrally charged, its electrons are asymmetrically distributed, making the molecule polar. Furthermore, the oxygen in $H_2O$ draws electrons away from the hydrogen, leaving hydrogen with a net positive charge, while the excess electron density creates a weak negative charge on the oxygen atom. As a result, water has unique properties that form hydrogen bonds between its molecules.

Water is composed of 70% of mammalian cells, including the human body. As the solvent, $H_2O$ plays an essential role in chemical and biological processes from molecules, cells to tissues in living organisms. Moreover, water is confined by intervening cellular components and is subject to structural effects that are not present in its bulk counterpart. Consequently, water in biological systems is not just a solvent but also a partner in the formation of the structures of biomolecules, including proteins, nucleic acids, and lipids.

## 2.1 Role of Water in Biological Systems

Water molecules are as essential as amino acids for proton transport and biological function. For example, protein-bound internal water molecules are essential features of the structure and function of microbial rhodopsin. The importance of water molecules for proton transport has been revealed through this protein, where protein-bound water acts as proton conductors and even proton storage sites.

As an integral and active component of biomolecular systems, water has both dynamic and structural roles. Focusing on water also sheds light on the function of biological molecules and their assembly. Water is also directly involved in countless biochemical reactions, such as respiration, photosynthesis, and cell transport.

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Without water, cells cannot move waste and by-products, take in nutrients, or perform intracellular transport and functioning.

Two extremely important biological molecular interactions involving water as the solvent of life are hydrogen bonding and hydrophobic interaction. Hydrogen bonding is an electrostatic attraction force that involves a partially positively charged hydrogen atom covalently bound to a nearby partially negatively charged atom. Hydrogen bonds are essential to maintaining the structure and stability of biomolecules, including proteins, nucleic acids, and membrane lipids.

While hydrophobic interaction describes the relations between water and nonpolar molecules or groups, it is also critical to maintaining the structure, function, and assembly of biomolecules. A better understanding of the hydrophobic hydration of biomolecules may yield insights into biomolecular structures and functions and intracellular assembly. Comparable to hydrogen bonding, hydrophobic interactions also play a critical role in protein folding, nucleic acid helical formation, and lipid bilayer membranes.

## 2.2 Deuterium Oxide

Deuterium is a naturally occurring, stable, nonradioactive isotope of hydrogen. Hydrogen consists of one electron and one proton with an atomic mass of 1.0, while deuterium has a single electron, but its nucleus contains one neutron and one proton with an atomic mass of 2.0. Deuterium oxide (heavy water; $D_2O$) occurs naturally in much smaller quantities than hydrogen oxide (regular water; $H_2O$). In nature, the abundance of deuterium is 1 per about 7000 hydrogen atoms.

The replacement of regular water with deuterium oxide or hydrogen with deuterium in biomolecules affects hydrogen bonding and hydrophobic interaction. A broad variety of morphological and physiological changes have been observed in deuterium-treated cells and organisms, including changes in fundamental processes, such as cell division or energy metabolism. Deuterium solvent effects exhibit unique physicochemical properties and distinctive kinetic effects. In addition, deuteration involving deuterium isotope substitution compounds, such as deuterated drugs, is of significant biotechnological potential.

Some organisms have no difficulties growing in deuterium oxide medium, while some are unable to grow. Single-celled organisms can often be grown in an environment that is fully deuterated. Lower organisms, such as fish, have been reported to survive in at least 30% $D_2O$. Mice and dogs tolerate long-term replacement of at least 10–15% of body fluid hydrogen with deuterium. However, toxicity is observed with acute or chronic exposure to above 25% of $D_2O$. Humans can tolerate moderate exposure to deuterium in body fluids, and $D_2O$ is excreted by humans via the urine with a half-life of about 10 days.

**Table 2.1** Comparisons of physical properties of $D_2O$ with $H_2O$

| Physical characteristics | $D_2O$ | $H_2O$ |
|---|---|---|
| Radioactivity | No | No |
| Molar mass | 20.028 | 18.016 |
| Dielectric constant (at 25 °C) | 78.06 | 78.39 |
| Zero-point vibrational energy (kcal/mol) | 9.69 | 13.25 |
| Density (g/cm$^{-3}$) | 1.1044 | 1.0 |
| Viscosity (cp at 30 °C) | 0.969 | 0.91 |
| Bond length (×10$^8$ cm) | 0.9575 | 0.9572 |
| Bond angle | 104.474 | 104.523 |
| Melting point (°C) | 3.82 | 0 |
| Boiling point (°C) | 101.42 | 100 |
| pD and pH at 25 °C | 7.44 | 7.0 |

## 2.3 Physical Properties of $D_2O$ Versus $H_2O$

Water has attracted the most scientific attention among other liquids due to its anomalous properties, such as negative volume of melting, density maximum at 4 °C, high melting and boiling points, high dielectric constant, and high mobility transport for $H^+$ and $OH^-$ ions.

Comparisons of physical properties of deuterium oxide with hydrogen oxide are listed in Table 2.1, including radioactivity, molar mass, dielectric constant, zero-point vibrational energy, density, viscosity, bond length, bond angle, melting point, boiling point, and values of pD and pH. In deuterium oxide, the O-D bond length is shorter than O-H, and the D-O-D angle is lesser than H-O-H.

As shown in the table, the boiling point for heavy water is 101.4 °C versus 100.0 °C for regular water. The melting point for heavy water is 3.8 °C versus 4.0 °C for regular water. The viscosity of heavy water at 25 °C is 1.23 times as great as that of regular water. The boiling point of $D_2O$ is 101.42 °C versus 100.0 °C for regular water.

Moreover, the value of the dielectric constant is 78.39 for regular water and 78.06 for deuterium oxide at room temperature, and the viscosity of $D_2O$ has a value of 1.23 times that of $H_2O$. The mobility of ions dissolved in $D_2O$ is smaller than in $H_2O$, leading to the smaller conductivity of ions in $D_2O$ than in $H_2O$.

## 2.4 Zero-Point Potential Energy of $D_2O$ Versus $H_2O$

Temperature is a measure of the intensity of random molecular motion. Even as the temperature is reduced to absolute zero, molecular motion never vanishes. There is a finite, minimum amount of kinetic energy in all matter even at O$^o$K. This residual

vibrational energy that a molecule possesses is called the zero-point energy, which is the lowest possible energy that a quantum mechanical system may have.

The heavier mass of deuterium lowers the frequency and hence is associated with lower zero-point vibrational energy. Table 2.1 shows that zero-point vibrational energy is 9.69 kcal/mol for deuterium oxide versus 13.25 kcal/mol for hydrogen oxide. Because of this difference, the lower energy of a hydrogen (H) bond in HOH··OH is compared to a deuterium (D) bond in DOD··OD. Raising the temperature invoking thermal vibration tends to preferentially stabilize H bond over D bond.

## 2.5 Deuterium Bond Versus Hydrogen Bond

The equilibrium properties of systems containing hydrogen or deuterium bonds are dependent on the vibrational motion of the nuclei, which reflects the shape, as well as the depth of the potential energy surface. Differences in the stabilities of H- and D-bonded species are related to differences in the zero-point vibration energy. A lower zero-point vibrational energy for deuterium oxide than hydrogen oxide generally leads to weaker deuterium bonding than hydrogen bonding.

The formation of hydrogen bond and deuterium bond is illustrated in Fig. 2.1. A more detailed description of deuterium bond versus hydrogen bond is discussed in Chap. 3.

## 2.6 C-D Bond Versus C-H Bond

The heavier atom has a lower frequency of vibration than the light atom, which leads to the heavier atom having a lower frequency of vibration and lower zero-point energy for the lighter atom. Accordingly, due to the greater atomic mass of deuterium, the deuterium-carbon (C-D) bond has a lower vibrational frequency than the hydrogen-carbon bond (C-H). Since C-D bond has a lower zero-point energy than the corresponding C-H bond, the C-D bond vibrates less than C-H bond, leading to stronger gravitational attraction between them.

Figure 2.2 illustrates the comparisons of C-D bond with C-H bond. The lower zero-point energy leads to the higher activation energy for C-D bond cleavage. As a result, the bond dissociation energy for C-D is greater than that of C-H.

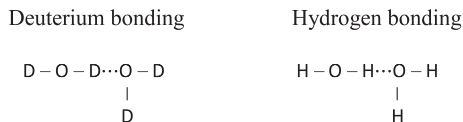


**Fig. 2.1** Hydrogen bonding versus deuterium bonding

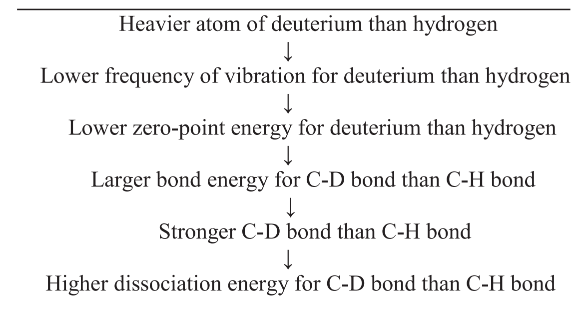


**Fig. 2.2** Comparisons of C-D bond with C-H bond

## 2.7 pD of $D_2O$ Versus pH of $H_2O$

Since deuterium (D) is heavier than hydrogen (H), D-OD vibration is weaker than H-OH vibration. Thus, D-O bond is more stable than H-O bond. Hence, $D_2O$ is a weaker acid than $H_2O$. At 25 °C, the pH of $H_2O$ is 7.0, and the pD of $D_2O$ is 7.4 (Table 2.1), as shown in Equations (2.1) and (2.2):

$$\mathrm{pH} = -\log\left[\mathrm{H}^+\right] = 7.0 \ \text{in}\, \mathrm{H_2O} \tag{2.1}$$

$$\mathrm{pD} = -\log\left[\mathrm{D}^+\right] = 7.4 \ \text{in}\, \mathrm{D_2O} \tag{2.2}$$

Equation (2.2) minus Eq. (2.1) leads to

$$-\log\left[\mathrm{D}^+\right] + \log\left[\mathrm{H}^+\right] = \log\left(\left[\mathrm{H}^+\right]/\left[\mathrm{D}^+\right]\right) = 0.40 \tag{2.3}$$

Therefore,

$$\left[\mathrm{H}^+\right]/\left[\mathrm{D}^+\right] = 2.51 \tag{2.4}$$

Equation (2.4) shows that $H_2O$ is more acidic than $D_2O$, implying that H-O bond is weaker than

D-O bond.

## 2.8 Dissociation Constants of Acids in $D_2O$ Versus $H_2O$

Differences in dissociation constants of acids in $H_2O$ and $D_2O$ solvents are a consequence of distinction in zero-point energy between proton and deuterium when attached to a water molecule. Such differences are associated primarily with changes in hydrogen bonding strength of acid in the solvent. The dissociation constant of acid in $H_2O$ solvent can be represented as follows:

**Table 2.2** Bonding characteristics in $D_2O$ versus $H_2O$

| Physical property | Deuterium oxide | Hydrogen oxide |
|---|---|---|
| Zero-point energy | Lower | Higher |
| Activation energy | Higher | Lower |
| Deuterium/hydrogen bond | Stronger | Weaker |
| O-D/O-H bond | Stronger | Weaker |
| C-D/C-H bond | Stronger | Weaker |
| Rate of D-C H-C cleavage | Slower | Faster |
| Acid dissociation constant | Weaker | Stronger |

$$HA(\text{in } H_2O) = H^+(\text{in } H_2O) + A^-(\text{in } H_2O) \tag{2.5}$$

$$K_{H2O} = [H^+]_{H2O}[A^-]_{H2O}/[HA]_{H2O} \tag{2.6}$$

$$pK_{H2O} = -\log K_{H2O} \tag{2.7}$$

while the dissociation of acid in $D_2O$ solvent is represented as

$$HA(\text{in } D2O) = H^+(\text{in } D_2O) + A^-(\text{in } D_2O) \tag{2.8}$$

$$K_{D2O} = [H^+]_{D2O}[A^-]_{D2O}/[HA]_{D2O} \tag{2.9}$$

$$pK_{D2O} = -\log K_{D2O} \tag{2.10}$$

Equations (2.10)–(2.7) give

$$\Delta pK = pK_{D2O} - pK_{H2O} \tag{2.11}$$

$$= \log(K_{H2O}/K_{D2O}) \tag{2.12}$$

Since acid dissociation constant in $D_2O$ solvent is weaker than that in $H_2O$, ΔpK therefore is positive.

Table 2.2 lists bonding characteristics in $D_2O$ versus $H_2O$ medium, including acid dissociation constant.

## 2.9 $D_2O$ Versus $H_2O$ as the Solvent in Biological System

The structure of liquid water and its interaction with biomolecules is a very active research area. Studying how water interacts with biomolecules reveals water motion toward functional sites of biomolecules. The behavior of water molecules at

hydrophilic sites is different from that at hydrophobic sites. This dissimilar behavior promotes the anisotropy of the hydration shell of proteins. Such anisotropy of the hydration shell is essential for the enzyme function.

Changes in the aqueous environment can also affect the stability and function of biomolecules. Investigations of $D_2O$ as the solvent can also provide insight into the role of solvation and hydrogen bonding in biomolecules. Accordingly, changes in the aqueous environment by replacing $H_2O$ with $D_2O$ as the solvent can have a large impact on the stability and function of biomolecules.

Moreover, the interaction of solvent with a polypeptide chain is a primary factor in maintaining protein folding and stability. For instance, substitution of $H_2O$ with $D_2O$ affects the formation of the immunoglobulin module of human cardiac titin. Water also participates in the catalytic function of proteins. Molecular dynamics simulations revealed a specific water-protein coupling as the cause of its dynamical heterogeneity.

Furthermore, water plays a critical role in double-helical structure of nucleic acids and bilayer structure of membrane lipids. Water also participates in the catalytic function of proteins and nucleic acids, as well as mediates binding through the hydrogen bond in complex formation. Hence, the replacement of $H_2O$ with $D_2O$ as the solvent affects the structures, functions, and activities of biomolecules, including conformation stability, molecular interaction, and membrane transport as discussed below.

### *2.9.1 Structure Stability*

The stability of a protein is vital for its biological function, and proper folding is driven by intermolecular interactions between protein and water. In the studies of how water interacts with biomolecules differing from bulk water, molecular dynamics simulations provided evidence for a specific water-protein coupling as the cause of the observed dynamical heterogeneity.

In turn, the complex surfaces of protein molecules alter the structure of the surrounding layer of hydrating water molecules.

Specific water-mediated interactions in protein complexes have been incorporated into potential energy functions for protein folding. The stability of proteins is significantly affected by replacing regular water with deuterium oxide. Deuterium oxide is known to have strong stabilization effects on the assembly dynamics of several proteins including tubulin, lysozyme, and bovine serum albumin. $D_2O$ was also found to stabilize proteins against denaturants. For example, ovalbumin undergoes urea denaturation more slowly in $D_2O$ than in $H_2O$. Moreover, in many physical measurements, $H_2O$ is replaced by $D_2O$, because $H_2O$ interferes with the measured signal.

The increase of protein stability in $D_2O$ was interpreted by the observation that $D_2O$ is a poorer solvent for nonpolar amino acids than $H_2O$, which implies that the hydrophobic effect is larger in $D_2O$. Nevertheless, thermodynamic analysis of the

*Drosophila* signal transduction protein revealed that the stabilizing effect of $D_2O$ compared with $H_2O$ is enthalpic and has a small to insignificant effect on the entropy and the heat capacity of unfolding.

Moreover, ribonuclease dissolved in $D_2O$ was found to be more stably held in the alpha-helix form than that in water. Studies of double-helix formation by (dG-dC)3 also revealed differences in the enthalpy of formation: -59.6 and -65.8 kcal/mol of helix in $H_2O$ and $D_2O$, respectively.

In lipid bilayer studies, fluorescence measurements on 1,2-dimyristoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine membranes revealed significantly slower dynamics and lower hydration of the headgroup region for a bilayer hydrated with $D_2O$ than $H_2O$.

### *2.9.2 Molecular Interaction*

Water actively participates in molecular recognition by mediating the interactions between binding partners. For instance, $D_2O$ was found to enhance the initial rate and final extents of polymerization of tubulin. The yields of polymerization are increased with the increasing $D_2O$ concentration. In addition, deuterium oxide is also known to promote the assembly of tubulin into microtubules and to inhibit mitosis. Replacing 50% or more of $H_2O$ with $D_2O$ promotes microtubule polymerization and stabilizes microtubules. The observed enhancement of the polymerization of tubulin by $D_2O$ could be the result of strengthening hydrophobic interactions of the tubulin molecules. The mechanism responsible for the ability of $D_2O$ to stabilize microtubule dynamics may also involve the substitution of deuterium bonds for hydrogen bonds.

### *2.9.3 Membrane Transport*

Water molecules are essential for proton transport and biological function. In the transport of sucrose across membranes, the protonmotive force that stimulates translocation activity is a function of the pH at the inner face of the membrane and is stimulated by the transmembrane pH gradient and the transmembrane electrical potential. Such translocation activity of proton-transfer reaction is retarded in $D_2O$ medium, indicating that $D_2O$ can affect membranous transport processes by solvent and kinetic isotope effects.

Moreover, the voltage-activated $H^+$ selective conductance of rat alveolar epithelial cells was investigated to examine the effects of substituting $D_2O$ for $H_2O$ on the conductance. $D^+$ was found to permeate proton channels, but with a conductance about half that of $H^+$. Such reduction in conductance in $D_2O$ is more than that which could be accounted for by the lower mobility of $D^+$ than $H^+$, suggesting that $D^+$ interacts specifically with the channel during permeation.

**Table 2.3** Effects of $D_2O$ and deuteration on biomolecules

| ***$D_2O$ as the solvent*** | ***Effects*** |
|---|---|
| Structure stability | Stabilization of proteins against denaturants and the assembly dynamics |
| | Larger hydrophobic effect in $D_2O$ |
| | More stable alpha-helix form of RNA in $D_2O$ |
| | Slower dynamics and lower hydration of lipid |
| | Bilayer headgroup region with $D_2O$ |
| Molecular interaction | $D_2O$ enhances the initial rate and final extents of polymerization of tubulin |
| | The ability of $D_2O$ to stabilize microtubule |
| Membrane transport | Translocation activity of proton-transfer reaction is retarded in $D_2O$ |
| ***Deuteration on biomolecules*** | |
| Kinetics of deuteration | A slower reaction rate occurs with deuteration |
| | A substantial kinetic isotope effect on deprotonation and re-protonation reactions |
| Metabolics of deuteration | Potential benefits result from deuteration of pharmacologically active agents |
| | Improving exposure profiles and decreasing toxic metabolites |

Furthermore, as a major component of biological membranes, bound water is required for the structural stability of the lipid bilayer and is also involved in membrane fusion and mobility of proteins and lipids. Changes in the rigidity or the polarity of the membrane-aqueous interface may occur with the replacement of $H_2O$ with $D_2O$ as the solvent.

The above-discussed effects of $D_2O$ versus $H_2O$ as the solvent on structural stability, molecular interaction, and membrane transport of biomolecules are briefly summarized in Table 2.3.

## 2.10 Deuteration of Biomolecules

Deuteration involves a whole or part of hydrogen atoms being replaced with deuterium atoms. In the case of a deuterated drug, one or more hydrogen atoms are replaced with deuterium atoms. Deuteration of a drug can potentially affect its pharmacokinetics, leading to a lower rate of metabolism and a longer half-life than the non-deuterated drug, when the drug is metabolized by pathways involving C-H bond scission.

### *2.10.1 Kinetics of Deuteration*

Carbon-deuterium (C-D) bond has a lower vibrational frequency and a lower zero-point energy than a corresponding carbon-hydrogen (C-H bond). Due to the greater atomic mass of deuterium, the cleavage of C-D bond requires greater energy than C-H bond. A greater energetic input is needed for the heavier atom (D) to reach the transition state, so that a slower reaction rate occurs with deuterium isotope. The kinetic isotope effect is referred to the change in the reaction rate of a chemical reaction when one of the atoms in the reactants is replaced with its isotopes.

When hydrogen is replaced with deuterium, kinetic isotope effect is represented by the ratio of rate constants for the reactions involving the lighter H atom ($k_H$) and the heavy D atom ($k_D$):

$$\text{Kinetic isotope effect} = k_H / k_D \tag{2.13}$$

Equation (2.13) is referred to as the primary deuterium isotope effect, which is the ratio of the rate of C-H versus C-D bond cleavage. For example, investigations of kinetic isotope effects of carboxylic groups of aspartic and glutamic acid residues were carried out by monitoring their C=O stretching vibrations in $H_2O$ and in $D_2O$, which revealed a substantial kinetic isotope effect (>2) in its deprotonation and re-protonation reactions.

### *2.10.2 Metabolism of Deuteration*

Studies of kinetic isotope effects can help not only the elucidation of the reaction mechanism of biochemical reactions but also the exploitation of drug development to improve the unfavorable pharmacokinetics involving metabolically vulnerable C-H bonds. The incorporation of deuterium into pharmacologically active agents offers potential benefits, such as improving exposure profiles and decreasing the production of toxic metabolites.

Besides drug development, deuteration can also be applied to alter the metabolism of a compound allowing its use as a mechanistic probe. In summary, the above-discussed deuteration effects on kinetics and metabolisms of biomolecules are also briefly listed in Table 2.3.

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# Part II
# Molecular Interactions of Deuterium Versus Hydrogen Oxide

# Chapter 3
# Deuterium Bonding Versus Hydrogen Bonding

Intermolecular forces that mediate interactions between molecules include hydrogen bonding, hydrophobic interaction, dipole-dipole interaction, and van der Waals forces. Hydrogen bonding is an electrostatic attraction force that involves a partially positively charged hydrogen atom covalently bound to a nearby partially negatively charged atom, such as oxygen, nitrogen, or fluorine that carries a lone pair of electrons. Polar molecules that are capable of forming hydrogen bonds have hydrogen atoms that are covalently bonded to highly electronegative elements (O, N, F). For example, the hydroxyl group of water-soluble $CH_3$-OH can form hydrogen bond with water.

In contrast, other intermolecular force, such as hydrophobic interaction, describes the interactions between water and nonpolar molecules or groups. The low solubility of hydrophobic solutes or groups in water makes nonpolar substances to clump up together in water medium, so as to allow the nonpolar molecules to have minimal contact with water. While dipole-dipole interactions are associated with the fluctuating polarizations of nearby particles, which occur when the partial charges formed within one molecule are attracted to an opposite partial charge in a nearby molecule. Van der Waals forces are relatively weak intermolecular forces that consist of attraction and repulsions between atoms and molecules.

Hydrogen bonds are extremely important in biology. They are essential to the structure and stability of globular proteins. Many of the amino acid side chains carry groups that are either hydrogen bond donors or acceptors, such as the hydroxyl of serine or threonine, the amino group and carbonyl oxygen of asparagine or glutamine, or the ring nitrogen in histidine.

While in nucleic acids, hydrogen bond is formed between the positive hydrogen end of a polar N-H bond and a pair of electrons on either a nitrogen or a carbonyl oxygen, such as between adenosine and thymine bases, or between cytosine and guanine bases. Moreover, H, N, and O atoms in major bases and HO- and PO3- groups in the backbone act as hydrogen bond donors or acceptors. Hydrogen

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bonding between complementary base pairs in a DNA double helix provides the essential stability for the structure of nucleic acid.

Hydration contributes to structural expressions of molecules in a phospholipid membrane environment. Hydrogen bonding also contributes to the maintenance of the phase structure of membrane lipids. For example, monoolein, which is composed of a hydrocarbon chain attached to a glycerol backbone by an ester bond. In phosphatidylethanolamine**,** hydrogen bonds are formed between the $NH_3^+$ group and either the $PO_2^-$ or the CO group.

## 3.1 Hydrogen Bond Donors and Acceptors

Table 3.1 presents atoms or groups in biomolecules that serve as hydrogen bond donors or acceptors, including O, H, OH, SH, N, S, SH, $NH_2$, NH, $CONH_2$, $^+NH$, $^+NH_3$, $^+NH_2$, $COO^-$, $HO^-$, or $PO_3^-$, as shown in the table.

## 3.2 Vibrational Energy: $D_2O$ Versus $H_2O$

As mentioned above, deuterium and hydrogen bonds differ in zero-point vibrational energy. Figure 3.1 illustrates the plot of vibrational energy versus interatomic distance for deuterium bond versus hydrogen bond. The heavier mass atom (D) has a lower zero-point vibration energy than the lighter one (H). As a result, the energy required to break the deuterium bond is larger than that to break the hydrogen bond.

## 3.3 Bonding Energies of Deuterium Versus Hydrogen

The equilibrium properties of systems containing hydrogen bond are dependent on the vibrational motion of the nuclei, which is related to the shape and the depth of its potential energy. Investigations of the change in the conformational stability for the mutation of a hydrogen- bonded residue to one incapable of hydrogen bonding revealed a stabilization of 1.0 kcal/mol per hydrogen bond.

As shown in Fig. 3.1, the relative stabilities of hydrogen – and deuterium – bonded molecules are related to differences in their zero-point vibrational energies. The replacement of H by D lowers the intermolecular zero-point vibrational energy, leading to a larger energy required to break the deuterium bond than the hydrogen bond. A lower energy of a H bond as compared to a D bond is also observed in H-O-H··O-H system.

**Table 3.1** Biomolecular atoms or groups serving as hydrogen bond donors or acceptors

| Biomolecule | Atoms or groups as hydrogen bond donors or acceptors |
|---|---|
| Protein | |
| Aliphatic amino acids | |
| Serine, threonine, tyrosine | HO |
| Cysteine | SH |
| Tryptophan | N |
| Methionine | S |
| Cysteine | SH |
| Histidine | $^{+}NH$ |
| Lysine | $^{+}NH_3$ |
| Arginine | $NH_2$, NH, $^{+}NH_2$ |
| Aspartic acid | $COO^-$ |
| Glutamic acid | $COO^-$ |
| Asparagine | $CONH_2$ |
| Glutamine | $CONH_2$ |
| Aromatic amino acid | |
| Phenylalanine | O, OH, $NH_2$ |
| Nucleic acids | |
| Ribonucleic acid (RNA) | |
| Backbone | O, $HO^-$ or $PO_3^-$ |
| Major bases | |
| Adenine (A) | H, N |
| Guanine (G) | H, N |
| Cytosine (C) | N, O |
| Uracil (U) | H, O |
| Deoxyribonucleic acid (DNA) | |
| Backbone | O, H, or $PO_3^-$ |
| Major Bases | |
| Adenine | H, N |
| Guanine | H, N |
| Cytosine | N, O |
| Thymine | H, O |
| Lipids | |
| Head group | O or $COO^-$ |

## 3.4 Hydrogen Bonding in Water Solvent

Based on the convention that “like dissolves like,” polar molecules are soluble in water since water is a polar solvent. The presence of hydrogen bonding between molecules of a substance implies that the substance is soluble in water. Alternatively, molecules that can hydrogen bond with water have a higher solubility in water. Hydrogen bonding is critical in determining the structure of water. Liquid water can

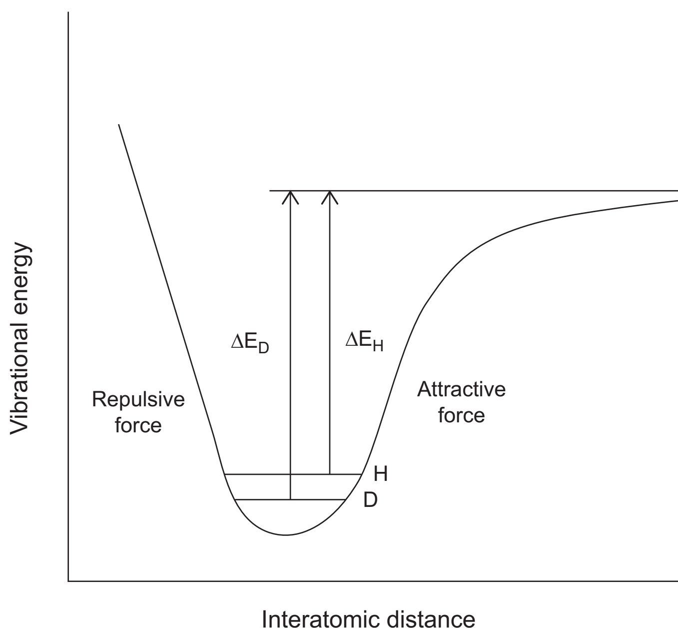


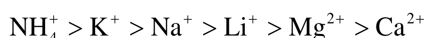

**Fig. 3.1** Vibrational energy and interatomic distance of deuterium versus hydrogen

be viewed as an instantaneously changing random hydrogen-bonded network, which consists of differently coordinated hydrogen-bonded molecules with their distinct solvation shells.

The formation of deuterium bonding in $D_2O$ versus hydrogen bonding in $H_2O$ is illustrated in Fig. 3.2. Extensive molecular dynamics simulation revealed that the average number of hydrogen bonds correlates well with various order parameters as well as the temperature of maximum densities.

Changes in water structure between the ions and the surrounding water molecules can affect biomolecule – water interactions. For instance, the presence of cations and anions affects the solubility of proteins in water. The ability of ions to salt-out or salt-in proteins affect the secondary and tertiary structural stability of proteins. The Hofmeister series classifies anions and cations in their order of ability to salt out or salt in proteins, according to

$$F^- > Cl^- > Br^- > NO_3^- > ClO_4^- > SCN^-$$



$$NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+}$$

The variation in solvation energy between the ions and the surrounding water molecules seems to underlie the mechanism of the Hofmeister series.

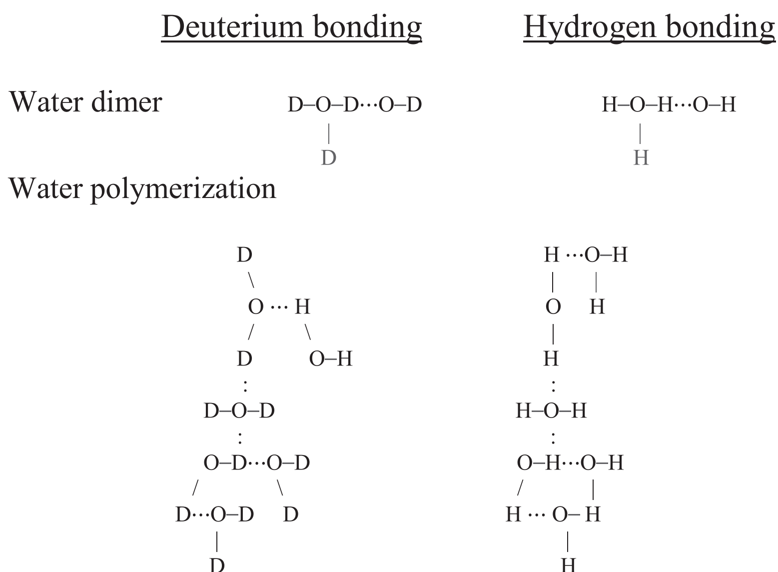



**Fig. 3.2** Deuterium bonding versus hydrogen bonding

## 3.5 The Structure of Water

The structure of water has been the subject of extended research for many decades. Mainly, three kinds of models of the structure of water have been proposed to address hydrogen bonding in water, which are the continuum model, the mixture model, and the interstitial model. They are briefly described below.

### *3.5.1 Continuum Model*

The continuum model of water structure proposes that water is a hydrogen – bonded network with a connectivity different from that of ice crystal, the connectivity is assumed to be in some way random, and almost completely hydrogen bonded water molecules are present.

The model proposes that there is no bond breaking, although the water bond bending and deformations occur, and assumes that all water molecules continue to be hydrogen bonded to four neighboring water molecules. The intermolecular links can be bent and stretched to produce irregular networks that varies with temperature and pressure. However, liquid water is composed of a single component although distortion of hydrogen bond may occur, rather than broken hydrogen bond as temperature or pressure changes.

**Table 3.2** Models of the structure of water

| Structure of water | Proposal |
|---|---|
| Continuum model | Water molecules are hydrogen-bonded, and some of them are distorted rather than broken |
| Mixture model | Mixture of a small number of distinguishable species of water molecules |
| Interstitial model | Water molecule forms hydrogen-bonded framework in which non-hydrogen-bonded reside |

### *3.5.2 Mixture Model*

In the mixture model, water consists of differently hydrogen-bonded species with zero, one, or both hydrogens engaged in hydrogen bonding network. The model is consistent with the claims based on the spectroscopic evidence that water is a mixture of two components. Moreover, the observed pressure and temperature dependence of water structure and thermodynamic properties are also consistent with the choice of this water model.

### *3.5.3 Interstitial Model*

The interstitial model of water proposes that water consists of hexagonal lattice with vacant lattice and interstitial water molecules. Disorder is introduced by the displacement of molecules from a bonded icelike framework onto interstitial sites. The model proposes that there are water molecules present in the cavities of ice-like lattice.

In summary, a comparison of the above three models of the structure of water is presented in Table 3.2. The mixture model of water structure is further elaborated below.

## 3.6 Mixture Model of Water Structure

Among these three models, the mixture model has most been studied from experimental and theoretical points of view. The following flickering cluster model is the most extensively investigated mixture model of water structure. The mixture model of water is supported by virtual single-particle energy distributions in water in comparison with other liquids.

### 3.6.1 Flickering Cluster Model

Frank and Wen's flickering cluster model proposes that hydrogen bonding keeps on holding water molecules, leading to the formation of a cluster of water. Flickering clusters consist of hydrogen – bonded molecules, in which the cooperative nature of clusters form and relax, where the hydrogen-bonded icelike clusters of $H_2O$ molecules are in equilibrium with non-hydrogen-bonded liquid.

A cluster is an unsteady state that keeps on forming and deforming. The thermodynamic parameters of liquid water derived based on the flickering cluster model revealed that the average cluster size is in the ranges from 91 to 25 $H_2O$ molecules over the temperature range from 0° to 70 °C.

The flickering cluster model is applicable to liquid deuterium oxide. In $D_2O$, the intermolecular vibrational frequencies are changed in accord with the theoretical ratios for isotopic substitution. The energy of the deuterium bond is 0.24 kcal/mole higher than that of the hydrogen bond. The calculated structural parameters indicate that more structural order exists in $D_2O$ than in $H_2O$ at a given temperature.

### 3.6.2 Virtual Single-Particle Energy Distributions

The mixture model of water structure is supported by virtual single – particle energy distributions of $H_2O$ comparing with methanol, ethanol, carbon disulfide, mercury, and benzene. Virtual single – particle energy distribution was determined by the energy moments calculated from constant-volume heat capacity at a constant volume. In contrast to methanol, ethanol, carbon disulfide, mercury, and benzene which exhibit a single-peaked distribution, a bimodal distribution was found for water.

The distinctive characteristic of the virtual single-particle energy distribution in $H_2O$ is the appearance of a lower-energy peak in the neighborhood of 1.0 kcal/mol and a higher-energy peak around 3.1 kcal/mol. These two energy peaks in water could represent two distinguishable states of structure. At a fixed volume, the ratio of the higher- to the lower-energy peak increases as temperature increases, and at a constant temperature, the ratio increases as volume decreases.

Moreover, virtual single – particle energy distributions of $D_2O$ also revealed a bimodal distribution. The lower and higher energy peaks also represent two distinguishable states of structure, implying that the structure of $D_2O$ also consists of mixed H-bonded species.

### *3.6.3 Thermodynamics Aspects of Water Structure*

#### 3.6.3.1 Entropy Factor

Studies of the nature of deviations found for nonpolar solutes in water lead to the concept that water forms frozen patches or microscopic icebergs around solute molecules. The extent of the iceberg increases with the size of the solute molecule. Specifically, icebergs are formed in the nonpolar parts of the molecules of polar substances, such as amines dissolved in water. These findings are in agreement with the observation that the increasing insolubility of large nonpolar molecules is an entropy effect.

In proteins, structural analysis demonstrated that water molecules are commonly found in the internal cavities of proteins. Quantification of enthalpic and entropic contributions of water molecules in five different proteins was investigated by a statistical mechanical method. The results revealed that water molecules in protein cavities contain charged residues, which is subject to entropy changes that contribute > 2.0 kcal/mol to the free energy.

#### 3.6.3.2 Entropy – Enthalpy Compensation

Another thermodynamic consideration is the entropy – enthalpy compensation resulting from a consequence of the properties of $H_2O$ as a solvent, regardless of the solutes and the solute processes studied. The existence of a specific linear relationship between the entropy and the enthalpy changes has been proposed for a variety of processes of small solutes in water solution, such as the solvation of ions and nonelectrolytes.

The compensation temperature lies in a relatively narrow range from about 250 to 315 °K for biochemical processes. A similar relationship is present in a variety of protein reactions, suggesting that liquid water plays a direct role in many protein processes and may be a common participant in the physiological function of biomolecules.

## 3.7 Deuterium Effects on Hydrogen Bond in Biomolecules

Protein stability is critical for its biological function. The intermolecular interaction of solvent with a polypeptide chain is one of the primary factors controlling protein folding and stability. Substitution of $H_2O$ with $D_2O$ as the solvent affects the structural stability of protein. Comparative studies of protein stability in $D_2O$ and $H_2O$ could provide insight into the role of hydration and hydrogen bonding in protein folding. Hydrogen bond in proteins not only mirrors the motion of hydrogen but also forms water lattice surrounding protein molecules.

The proposal that water lattice is perturbed in its entropic configuration by the motion of hydrogen bond was tested by changing from $H_2O$ to $D_2O$ as the surrounding medium, which results in a substantial solvent isotope effect. This finding demonstrates the influence of the environment on the individual hydrogen bond. Typical deuterium oxide effects related to hydrogen bonding are shown in Table 3.3.

Furthermore, the substitution of $D_2O$ for $H_2O$ was also found to increase the transition temperature and decrease the enthalpy of protein unfolding. Nevertheless, the total stability of proteins appears to be unchanged, as a result of entropic compensation for the decrease in enthalpy, which is also applied to changes in hydration of proteins in $D_2O$.

### 3.7.1 *Proteins*

Elucidation of the stabilization of proteins and peptides by deuterium oxide was investigated, such as protein tubulin and peptide (Pro-Pro-Gly)10. The rate of decrease of tryptophan fluorescence of tubulin was found to be significantly lower in $D_2O$ than in $H_2O$, suggesting stabilization of the secondary structure of tubulin in $D_2O$. This finding was consistent with the results obtained from differential

**Table 3.3** Typical effects of deuterium oxide and deuteration related to the strength of hydrogen bond

| In deuterium oxide medium | Biological processes affected by the relative strength of hydrogen bond |
|---|---|
| Protein | Stabilization of the secondary structure of tubulin |
| | Increasing the melting temperature of polypeptide |
| | Enhancement of hydrophobic interaction, resulting increasing hydrogen bond |
| Nucleic acid | Stabilization of attenuated viral RNA against thermal degradation |
| | Increasing the stability of the folded RNA |
| | Enhancing the flexibility of RNA structure of polio vaccine. |
| | Increasing the stability of nucleic acids in living systems |
| Lipid | Lowering the cubic to inverted hexagonal phase transition temperature |
| | Reduction of the surface occupied area of monoolein at the interface |
| | Increasing the O-H stretching signal of the alkanol – DPPC complex |
| | Slowing the cleavage of O-D bond in lipid peroxidation kinetics |
| Deuteration of biomolecules | Biological processes affected by deuteration |
| Protein | Lectin-carbohydrate interactions that provide hydrogen bond network information |
| Lipid | Location of cholesterol in the two outer layers and the lipid subclasses in the central layer |
| Nucleic acid | Difference in the transition temperature for deuterated ribonuclease |

scanning calorimetric experiments. The temperatures for tubulin unfolding are higher in $D_2O$ than $H_2O$ (58.6 °C and 62.17 °C as compared to 55.4 °C and 59.35 °C).

Collagen – like polypeptide (Pro-Pro-Gly)10 forms a triple-helical, polyproline structure in $H_2O$ solution. Studies of its circular dichroism spectra demonstrated an increase of the melting temperature in the presence of increasing amounts of $D_2O$, implying an enhancement of the enthalpy of unfolding in $D_2O$ versus $H_2O$. Moreover, the folding dynamics of poly(glutamic acid) was also investigated by circular dichroism. Its difference in $D_2O$ and $H_2O$ was assigned to different hydrogen bond energies in these solvents.

Moreover, the denaturant – dependent relaxation kinetics of folding and unfolding have been measured with a protonated (amide) backbone and a deuterated backbone in $H_2O$ and $D_2O$. The folding reaction was found to be significantly influenced by the nature of the bulk solvent. In $D_2O$, the stability of each state in the folding pathway is enhanced to a degree which is proportional to the extent of the exposure of nonpolar protein groups to the solvent. The solvent isotope effect arises from enhanced hydrophobic interactions which, in turn, results from an increase of hydrogen bond in $D_2O$.

### *3.7.2 Nucleic Acids*

Thermal stability of DNA structure is associated with the properties of water molecules around DNA. RNA base pairs also have variable stability depending on their degree of solvation. These findings have biological implications for nucleic acid structure and RNA – protein complexes. Molecular dynamics simulations of RNA base pairs revealed that water competes for base – base hydrogen bonds.

There is a considerable increase in the stability of the folded RNA in $D_2O$ medium. $D_2O$ has been known to stabilize viral RNA against thermal degradation. A higher melting temperature in $D_2O$ is compared with $H_2O$. Studies of the hydration dynamics of RNA showed that $D_2O$ strengthens the hydrogen bond network in the solvent.

The substitution of deuterium for hydrogen will affect the strength of a hydrogen bond.

$D_2O$ was found to considerably increase the stability of intramolecular hydrogen bond and the flexibility of RNA structures of polio vaccine. By strengthening the hydrogen bonding network in the solvent and lengthening life time of inter residue water, $D_2O$ can act as a thermal stabilizer for polio vaccine.

### *3.7.3 Lipids*

Investigation of the hydration of carbonyl moieties in dimyristoylphosphatidylcholine (DMPC) bilayer revealed that excitation of the OD stretching mode of heavy water produces a time dependent change of the absorption band of

the phospholipid carbonyl groups. Intermolecular vibrational coupling also affects the entire C=O band. Moreover, sn-1 carbonyl has a higher propensity to form hydrogen bonds with water in comparison to sn-2 carbonyl.

Studies of water – DMPC interaction also suggested that the primary hydration site of the lipid is the phosphate moiety, where up to 18 water molecules are restricted at the polar head group and about 9 water molecules strongly interact with the phospholipid head group.

Substitution of $D_2O$ for $H_2O$ was also found to lower the cubic to inverted hexagonal phase transition temperature of lipid. The structural analysis of the cubic phase indicated that $D_2O$ reduces the surface occupied area of monoolein at the interface by 12% in comparison with $H_2O$, which probably is due to the difference in the strength of hydrogen bond.

When a part of lipid – bound water is replaced by alcohols, a new infrared broad band of O-H stretching appears, which represents alcohol molecules bound to the phosphate moiety of lipid. As the alcohol concentration increases, alcohols interact with the phosphate moiety and replace the bound water, indicating that alcohols interact with lipid membranes at the phosphate moiety of the hydrophilic head, weaken the membrane-water interaction, and destabilize membranes.

Hydrogen bonding of alkanols to dipalmitoyl-phosphatidylcholine (DPPC) was also investigated by Fourier transform infrared spectroscopy in $D_2O$. Alkanols were found to form hydrogen bonds with the phosphate moiety of DPPC and release the lipid – bound deuterated water, as evidenced by increases in the bound O-H stretching signal of the alkanol – lipid complex.

Furthermore, low- density lipoprotein peroxidation plays a major role in many physiological and pathophysiological processes. The mechanisms involve free radical production that occurs via decomposition of hydroperoxides. The step of rate – limiting reaction includes the cleavage of O-H bond of hydroperoxide. In $D_2O$, cleavage of O-D bond is slower than cleavage of O-H bond in $H_2O$.

## 3.8 Deuteration Effects on Hydrogen Bonding

Deuteration effects on hydrogen bonding of biomolecules were less reported than the above $D_2O$ solvent effects. Such deuteration effects are briefly described below.

### *3.8.1 Proteins*

Carbohydrate – binding proteins, from pathogenic bacteria and fungi and in complex with deuterated L-fucose, were studied by neutron diffraction. A detailed structural analysis was able to provide information on the hydrogen bond network, the role of water molecules, and the extent of interactions between fucose and the

aromatic amino acids in the binding site. No large differences were observed between H and D forms, implying that deuteration did not seem to affect the native state of the protein.

### 3.8.2 Lipids

Either protiated lipids or selectively deuterated lipids were applied to obtain information on the locations of various lipid classes, including hydrogen bond with the ester group. A model was proposed to suggest that cholesterol is located in the two outer layers, and the two other lipid subclasses are predominantly located in the central layer.

### 3.8.3 Nucleic Acids

A deuterated helical macromolecule is expected to have a different stability from a hydrogen – containing one. Such altered stability could result in a change in the transition temperature between a helix and a random coil. The effect of deuterium – hydrogen substitution was investigated using poly- γ-benzyl-L-glutamate. The observed decrease in the transition temperature upon denaturation has implications for the stability of helical protein and nucleic acids in living systems.

Ribonuclease is folded in a compact form and held together by covalent bonds and hydrogen bonds. In poly-γ-benzyl-L-glutamate, the transition temperature of deuterated ribonuclease in $D_2O$ is different from that of hydrogen – containing ribonuclease in $H_2O$. The analysis of pH dependence of reversible denaturation can provide a means of examining hydrogen bonds among ionizable side-chain groups.

The above- discussed deuteration studies of hydrogen bonding in biomolecules are also presented in Table 3.3.

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# Chapter 4
# Hydrophobic Interaction in $D_2O$ Versus $H_2O$

In their interpretation of the thermodynamic properties of aqueous solution of hydrophobic substances, Frank and Evans first attempted to provide a detailed theory of the hydrophobic effect. They described water molecules rearranging into a microscopic iceberg around a nonpolar molecule and discussed the entropic effect of this iceberg formation. The simple hydrophobic substances hydrocarbons and nonpolar organic substances.

Later, in analyzing the forces that stabilize the native structure of proteins, Kauzmann suggested that hydrophobic interaction is probably among the most important factors in the stabilization of folded configurations in native proteins. Due to amino acids containing nonpolar and polar groups, hydrophobic bonding in proteins arise as a consequence of the *interaction* of their hydrophobic amino acids with the polar solvent water.

In biomolecules, hydrophobic interaction describes the relations between water and nonpolar molecules or groups. A better understanding of the hydrophobic hydration of biomolecules can yield insights into biomolecule structure dynamics and functions and intracellular assembly. Hydrophobic interactions play important role in protein folding, nucleic acid helical structure, and lipid bilayer membranes.

Nucleic acids have significant polar and nonpolar parts which interact with water solvent, where there is a delicate balance between hydrophilic and hydrophobic interactions. The contributions of hydrophobic interaction are critical in driving important processes like the double-helix formation of DNA.

Membrane phospholipid consists of two long fatty acid hydrocarbon chains esterified to a glycerol backbone with a phosphate group. It is energetically favorable for lipid hydrocarbon chains to minimize their surface area of contact with water. Hydrophobic interaction occurs as hydrocarbon regions of lipids interact with hydrocarbons, causing a decrease in entropy.

Hydrophobic interaction is affected by the replacement of regular water (hydrogen oxide) with deuterium oxide as the solvent. The free energy of hydrophobic interaction between hydrocarbon chains in deuterium oxide at 25 °C was found to

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be −1.76 kJ/mol for each contact between methylene groups, as compared to −1.40 kJ/mol in $H_2O$, which implies that hydrophobic interaction is stronger in $D_2O$ than in $H_2O$.

## 4.1 Hydrophobic Effect

As described in the previous chapter, water is a highly structured liquid with hydrogen bonds linking individual molecules to each other. The tetrahedral structure of ice suggests a locally tetrahedral arrangement of molecules in liquid water. The presence of a hydrophobic solute strengthens water hydrogen bonding around it. As a result, water displays extensive structural ordering resembling that in clathrates. This arrangement is disrupted by the solute dissolved in water, depending on the polarity of the solute.

The low solubility of hydrophobic solutes in water makes nonpolar substances to clump up together in water medium. This allows the nonpolar molecules to have minimal contact with water. Hydrophobic effect has been given to interpret the thermodynamic properties of hydrophobic substances in aqueous solutions and to analyze the force that stabilizes the secondary structure of proteins.

The idea of iceberg formation is followed to explain the hydrophobic effect. Since direct experimental measurements of the mutual interactions of liquid water molecules and their associated kinetic and transport processes are inaccessible, hypothetical models need to be considered to account for the results of observations made upon water.

### *4.1.1 Iceberg Model*

The water molecules at the surface of the cavity created by a nonpolar solute are capable of rearranging themselves in order to regenerate the broken hydrogen bonds. In doing so, they create a higher degree of local order that exists in pure liquid water and form transient microscopic icebergs arising from strengthened water hydrogen bonding.

This ordering results in the rearrangement of a small number of water molecules near the nonpolar solutes, leading to forming a kind of cage consisting of the molecules of water. Such ordered structure is similar to the structure of a clathrate and therefore is referred to as iceberg.

#### 4.1.1.1 Proposal

Iceberg concept can be regarded as a tendency of water molecules in the solvation shell of hydrophobic solutes to possess structural features that exhibit some similarities to the behavior of water molecules in the solid phase. Such icebergs are also formed at the nonpolar parts of the molecules of polar substances, such as alcohols and amines that are dissolved in water. In contrast, depending on their sizes and charges, ions may cause a breaking down of water structure nearest them. Structural influences can also modify the distribution of ions in an electrolyte solution.

#### 4.1.1.2 Research Supports

Investigations of infrared of oxygen-deuterium stretching mode of HDO water near hydrophobic solutes, such as methane, ethane, krypton, and xenon, supported the presence of strengthened water hydrogen bonding near these hydrophobic solutes, which match those in ice and clathrates. In turn, water molecules involved in the enhanced hydrogen bonding display extensive structural ordering resembling that in clathrates.

Molecular dynamics simulations also confirmed that water molecules in the vicinity of methane form stronger tetrahedrally oriented hydrogen bonds than those in bulk water, where their mobility is restricted. These findings confirm the classical view of hydrophobic hydration.

Moreover, molecular dynamics studies of the structure of water in the hydration shells of small hydrophobic solutes showed that a subset of water molecules in the first hydration shell of a nonpolar solute has a significantly enhanced tetrahedrality as well as a slightly larger number of hydrogen bonds. This finding is consistent with the experimentally observed negative excess entropy and enhanced heat capacity of hydrophobic solutions at room temperature.

Furthermore, computer stimulation investigations of the structural properties of solvation water of hydrophobic molecules at 250–295 °K revealed that, in the solvation layer of hydrophobic molecules, ordered aggregates consisting of water molecules are formed and become more profound with the decrease in temperature. Such existence of the ordered aggregates around the hydrophobic solutes also complies with the concept of the above-described iceberg model.

### *4.1.2 Thermodynamics Aspects*

The idea of iceberg formation can provide an explanation to the entropy and the enthalpy of solvation of nonpolar solutes in water. From the thermodynamic point of view, the hydrophobic effect manifests itself by the occurrence of significant unfavorable free energy of mixing of the hydrophobic substance with water. The extent of this effect is determined by the entropic contribution. Loss of entropy is

associated with the structural changes when hydrophobic solutes are present in liquid water.

On the other hand, enthalpy-entropy compensation proposes a linear correlation between enthalpy and entropy changes in chemical processes, where closely related structures are involved. In ligand binding, a ligand modification results in a change in the enthalpic contribution to binding, which is offset by a similar change in the binding entropy.

#### 4.1.2.1 Entropy Effect

As nonpolar solute dissolves in water, neighboring solvent molecules loosen the forces to produce a solvent reaction. The increasing insolubility of nonpolar molecules in water is an entropy effect, which leads to the idea that water forms frozen patches or microscopic icebergs around nonpolar molecules. The extents of the entropy effect and the iceberg increase depend on the size of the nonpolar solute molecule. Nevertheless, the entropy of hydration of ion depends on not only its size but also its charge. Ions may cause a breaking down of water structure nearest them, thus causing an entropy increase.

#### 4.1.2.2 Entropy-Enthalpy Compensation

The entropy-enthalpy compensation pertains to structural changes induced in the solvent. When the icelike structure of water breaks down, water still keeps its tendency to form crystal-like clusters. These clusters are unstable and flickering. With temperature increase the probability of forming these flickering clusters decreases.

A large number of thermodynamic data, including the free energy, enthalpy, and entropy changes, were analyzed for the protein denaturation. Enthalpy-entropy compensation was found in protein unfolding, indicating that the change in enthalpy is essentially compensated by a corresponding change in entropy, which results in a smaller net free energy change. This was proposed to result from the water molecule reorganization.

As a result of ligand binding, localized change in a protein encounters the conformational fluctuation of the rest of the protein. Both the entropy and the enthalpy associated with the change consist of contributions from fluctuations in the atoms surrounding the binding site. The enthalpy-entropy compensation reflects the flexibility of the surrounding structures. Its fluctuations contribute to the entropy, and the conformational change contributes to the enthalpy.

A comparative study of the stability of several proteins in $H_2O$ and $D_2O$ showed that the substitution of $D_2O$ for $H_2O$ leads to an increase in the transition temperature and a decrease in the enthalpy of unfolding. The stability of the proteins, however, appears to be largely unchanged as a result of entropic compensation for the

decrease in enthalpy. This enthalpy-entropy compensation is attributed to changes in the hydration of proteins in $D_2O$ as compared to $H_2O$. The changes in the enthalpy of unfolding and in the protein-ligand interactions, due to water isotopic substitution, can be rationalized by changes in hydration of the buried nonpolar groups.

### *4.1.3 Structure Maker and Breaker*

As discussed above, water structure can be perturbed by solute molecules, resulting in altering the hydrogen bonding network of water. A nonpolar molecule that promotes water structure is characterized as water structure maker, while polar molecules that demote water structure is considered as a water structure breaker. For example, methane and urea are considered as water structure maker and breaker, respectively.

Such classification has relevance to the interactions between ligands and biomolecules. Ligands may alter protein conformation through effects on water structure. While, lysine, an α-amino acid that consists of a protonated -$NH_3^+$, an α-carboxylic acid group, and a side chain $CH_2)_4NH_2$, is considered as a water structural breaker.

Investigations of the native DNA molecule conformation revealed that the solvent structure is an important factor in determining the interactions between organic compounds and the native DNA molecule. Water structure maker and breaker can also affect the ordered water at the membrane-aqueous interface. Studies of phospholipid properties suggested that lysine can produce an effect on the lipid-water interface that controls its stability.

Investigations of the effect of perturbing the interfacial water structure on the glycoprotein arrangement in the bilayer revealed a possible new arrangement of membrane-bound glycoprotein. Moreover, water structure maker was also found to broaden the transition and change the phase profile of phosphatidylcholine, and a water structure breaker causes a shift in the transition temperature.

## 4.2 Hydrophobicity in Biomolecules

Hydrophobic interaction describes the interactions of nonpolar molecules or groups with water solvent. Many biomolecules are characterized by surfaces that contain extended nonpolar regions. Hydrophobicity of biomolecules refers to the removal of biomolecular surfaces from contacting water framework, in which understanding is critical to gain insights into intracellular assembly and biochemical processes, such as protein folding, nucleic acid double helix, and lipid bilayer structure. Accordingly, hydrophobicity in proteins, nucleic acids, and lipids are discussed below.

### *4.2.1 Proteins*

Amino acids that make up proteins have the same basic structure but differ in the side chain (R-group). The side chain is an assembly of charge, polarity, and hydrophobicity. These properties impact the structure and conformation of proteins. Figure 4.1 shows the structure of amino acid and lists nine amino acids each containing a hydrophobic side chain.

The primary thermodynamic driving force for the formation of globular structure is hydrophobic interactions, which keep nonpolar groups from contacting water framework. Thus, globular proteins fold by minimizing exposure of their nonpolar surface to water, while simultaneously providing hydrogen bonding interactions for buried backbone groups.

### *4.2.2 Nucleic Acids*

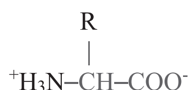
Nucleic acids are polynucleotides. Each nucleotide consists of a nitrogen-containing aromatic base, which is attached to a pentose sugar that is connected to a phosphate group. Four nitrogen-containing bases of nucleic acids are adenine (A), guanine (G), cytosine (C), and thymine (T) in deoxyribonucleic acid (DNA) and adenine (A), guanine (G), cytosine (C), and uracil (U) in ribonucleic acid (RNA).

Table 4.1 below lists the interaction forces involving nucleic acid bases. In secondary structure interaction between bases, two strands of DNA are held together by hydrogen bonds, while stacking interactions are stabilized by hydrophobic interactions and van der Waals forces.

### *4.2.3 Lipids*

Most lipids present in biological membranes are composed of amphiphilic phospholipids. The lipid bilayer is made of two layers of amphiphilic lipid molecules that have a hydrophilic phosphate head and a hydrophobic tail consisting of two fatty acid chains. Figure 4.2 shows the structure of an amphiphilic phospholipid, where

**Fig. 4.1** Hydrophobic side chains of amino acids

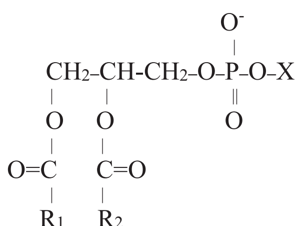



Amino acids containing a hydrophobic R group: alaine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, and glycine

**Table 4.1** Interaction forces involving nucleic acid bases

| Forces involved | Nucleic acid bases |
|---|---|
| Hydrophobic interaction and van der Waals force | DNA: adenine, cytosine, quinine, and thymine |
| | RNA: adenine, cytosine, quinine, and uracil |
| Hydrogen bond | Sugar: deoxyribose (DNA) ribose (RNA) |
| | Phosphate group: DNA and RNA |

**Fig. 4.2** Amphiphilic lipid molecules




where $R_1$ and $R_2$ represent long hydrocarbon chains, and X may be zwitterionic or positively charged.

$R_1$ and $R_2$ are hydrocarbon chains and X is a charge group which may be positive or zwitterionic.

When $R_1$ and $R_2$ are saturated long hydrocarbon chains, the lipid bilayer exhibits hydrophobic cores in an ordered configuration. In contrast, when $R_1$ and $R_2$ contain unsaturated hydrocarbon chains, the hydrophobic core may be in a fluid state.

Comparative thermodynamic studies were conducted on the interactions of aqueous dispersions of dipalmitoyl-phosphatidylcholine bilayer vesicles with hydrophobic species. Cyclohexanol, a strong hydrophobic species, lowers the temperature of the lipid main phase transition from the gel to the liquid-crystalline phase but increases the enthalpy of the transition. The enthalpy change is positive, and the free energy change is negative, implying a characteristic of solute-solute hydrophobic interaction.

## 4.3 Deuterium Oxide Effects on Hydrophobic Interaction

The presence of strengthened water hydrogen bonds near hydrophobic solutes, such as methane, ethane, krypton, or xenon, was found to match those in ice and clathrates, where water molecules involved in the enhanced hydrogen bonds display extensive structural ordering resembling that in clathrates. Molecular dynamics simulations confirmed that water molecules in the vicinity of methane form stronger and more tetrahedrally oriented hydrogen bonds than those in bulk water, implying the classical view of hydrophobic hydration.

Comparative studies of the kinetics of the reaction between decylamine and 4-nitrophenyl decanoate with that of the corresponding reaction without hydrophobic side chains (the reaction between ethylamine and 4-nitrophenyl acetate) were also carried out to delineate the extent to which hydrophobic interaction stabilizes the transition state of the reaction.

Based on the flickering cluster concept, investigations of the energy of the hydrogen bond revealed that the deuterium bond is 0.24 kcal/mole higher than for $H_2O$. The calculated structural parameters also indicate that more structural order of proteins exists in $D_2O$ than in $H_2O$ at a given temperature. The stronger hydrophobic interaction in $D_2O$ than in $H_2O$ was also demonstrated in the comparative studies of hydrophobic species in both media.

The free energy of hydrophobic interaction between hydrocarbon chains in deuterium oxide at 25 °C was also investigated in micellar solutions of a series of hexadecyl-trimethylammonium carboxylates. Examinations of the effects of hydrocarbon chain length on the binding a counter ion (carboxylate ion) to the micelle revealed that the free energy of hydrophobic interaction in $D_2O$ is −1.76 kJ/mol for each contact between methylene groups, as compared to −1.40 kJ/mol in $H_2O$.

### *4.3.1 Proteins*

Studies of internal dynamics of proteins, including ribonuclease T1, superoxide dismutase, beta-lactoglobulin, liver alcohol dehydrogenase, and apo- and Cd-azurin, showed that $D_2O$ significantly increases the rigidity of the protein native structure. The structure tightening effect of deuteration was assigned to the solvent effect, rather than to stronger intramolecular deuterium bonding. Moreover, such structure tightening effect by $D_2O$ is generally amplified at higher temperatures, which supports the hydrophobic nature of the underlying interaction.

The conformational stability presented by the free energy change has also been measured for 72 aliphatic side-chain mutants from four proteins, in which a larger side chain is replaced by a smaller side chain. The results revealed that proteins gain 1.3 kcal/mol in stability for each -CH2- group buried in folding, indicating the contribution of the hydrophobic effect to the stability of globular protein.

To gain a better understanding of the contribution of hydrophobic interactions to protein stability, the change in conformational stability was measured for 22 proteins with 36 to 534 residues. Hydrophobic interactions were found to contribute 60% and hydrogen bonds contribute 40% to protein stability. These findings indicated that the globular conformation of proteins is stabilized predominantly by hydrophobic interactions.

The initial aggregation process of lysozyme in $D_2O$ or $H_2O$ was studied to examine the relationship between the surface hydrophobicity and the aggregation rate of lysozyme. The initial aggregation rate of lysozyme in $H_2O$ is slower than in $D_2O$, implying that the interaction between lysozyme molecules is stronger in $D_2O$ than in $H_2O$. There is also a good correlation between the initial aggregation rate and

surface hydrophobicity. Hydrophobic interaction is hence an important active force in the initial aggregation process of lysozyme.

Tubulin is unstable as stored in solution and loses its ability to form microtubules rapidly. $D_2O$ was found to stabilize the protein against inactivation. In $H_2O$ buffer, tubulin is completely inactivated after 40 hours at 4 °C. But, in $D_2O$ buffer, no activity is lost after 54 hours. Therefore, $D_2O$ was proposed to exhibit its stabilizing effect on tubulin conformation that involves the disruption of hydrophobic forces.

A thermodynamic analysis of two globular proteins, egg lysozyme and bovine serum albumin, also revealed that both proteins tend to be more stable in $D_2O$ compared to $H_2O$. This finding revealed that $D_2O$ is a poorer solvent for nonpolar amino acids than $H_2O$, implying that the hydrophobic effect is larger in $D_2O$.

### *4.3.2 Nucleic Acids*

Investigations of thermal denaturation of DNA in $H_2O$ and $D_2O$ by electron microscopy as a function of temperature support the hypothesis that $D_2O$ plays a stabilizing role in strand separation during thermal denaturation of DNA. Moreover, deuterium oxide was found to increase fluorescence lifetime and intensity of the intercalating dyes (propidium iodide and ethidium bromide), when bound to nucleic acid structures. The accessibility of these dye molecules to the solvent environment once bound to DNA also leads to the differential enhancement effects of $D_2O$ on fluorescence intensity and lifetime of these probes.

Furthermore, the phase behavior of phage single-stranded DNA in the presence of monovalent and multivalent salts was investigated in both $H_2O$ and $D_2O$ solvents. DNA solubility depends on not only the nature of the salts and their concentrations but also the nature of the solvent. The appearance of attractive interactions between the monomers of the DNA chains in the solution is correlated with an adsorption of the chains at the air-water interface. The overall solubility of single-stranded DNA is decreased in $D_2O$ compared to $H_2O$, revealing a role of DNA hydration in addition to electrostatic factors.

### *4.3.3 Lipids*

Sphingosine-1-phosphate is currently considered to be an important signaling molecule in cell metabolism. Structurally related hydrophobic sphingolipids occur in either an aqueous dispersion or a bilayer environment. Sphingosine-1-phosphate behaves in aqueous media as a soluble amphiphile. Its aggregates display a thermotropic transition at approximately 60 °C, presumably due to the formation of smaller structures at higher temperatures.

The effects of substitution of $H_2O$ by $D_2O$ on the phase transitions of dipalmitoyl- and distearoyl-phosphatidylcholine bilayer membranes were investigated.

**Table 4.2** Typical effects of deuterium oxide and deuteration related to the strength of hydrophobic interaction

| Deuterium oxide medium | Biomolecular processes affected by relative strength of hydrophobic interaction |
|---|---|
| Protein | Larger hydrophobic effect in $D_2O$ medium. |
| | Hydrophobic effect contributing to globular protein stability. |
| | Increasing the rigidity of the protein native structure by hydrophobic interaction |
| | Hydrophobic interaction as an important active force in the aggregation of lysozyme. |
| | Stabilization of tubulin conformation involving hydrophobic forces. |
| | Involvement in stabilization of egg lysozyme and bovine serum |
| Nucleic acid | A stabilizing role during thermal denaturation of DNA. |
| | Decreasing the overall solubility of single- stranded DNA by hydrophobic forces |
| Lipid | Increasing the structural stability of deuterated lipid vesicles due to enhancing hydrophobic interaction |
| Deuteration | Biological processes affected by deuteration |
| Protein | Stabilization phycocyanin conformation as deuteration on exchangeable hydrogen, and decreasing nonpolar side-chain interaction as deuteration on non-exchangeable hydrogen |
| Nucleic acid | Involvement of hydrophobic interaction in gramicidin tryptophan side chain |
| Lipid | Enhancement of the structural stability of lipid vesicles |

There is no significant difference with respect to the main transition from the ripple gel phase to the liquid crystalline phase in $H_2O$ and $D_2O$ solvents. However, the pretransition temperature involving the lamellar gel phase is significantly raised by the substitution of $H_2O$ by $D_2O$ in both lipid bilayers. Such difference was interpreted in terms of the difference in hydrophobic interaction in $H_2O$ and $D_2O$ media.

Deuterium oxide effects related to hydrophobic interaction in biomolecules are briefly summarized in Table 4.2.

## 4.4 Deuteration Effects on Hydrophobic Interaction

Recent improvements in isotope labeling of RNA have strongly contributed to the high impact of nucleic magnetic resonance (NMR) and neutron scattering in determining RNA structure. Advances in isotope labeling of RNA focus on strategies for selective deuteration of nucleotides, which in combination with segmental isotope labeling paves the path for studying nucleic acid structure.

In protein studies, one of four tryptophan residues in gramicidin A analogues was selectively substituted by carbon-deuterated tryptophan and was incorporated into phospholipid liposomes. Raman spectra revealed that tryptophan side chains

are generally involved in strong hydrophobic interactions with the lipid acyl chains of the membrane and/or with another tryptophan residue.

### *4.4.1 Proteins*

Deuterated proteins can be isolated from organisms grown in $D_2O$. Algae and yeast can be grown in high concentration of deuterium oxide to make it possible to use them as a practical source of fully deuterated proteins. During adaptation to a deuterated environment, lag periods occur prior to growth of bacteria in deuterium medium.

For example, studies of protein phycocyanin isolated from algae revealed that the substitution of hydrogen by deuterium in exchangeable position stabilizes the protein conformation. In contrast, the introduction of deuterium in non-exchangeable hydrogen position appears to decrease nonpolar side-chain interaction.

### *4.4.2 Nucleic Acids*

Deuterated nucleic acids isolated from algae or yeast are essentially utilized for NMR studies. Advances in isotope labeling of RNA focus on strategies for selective deuteration of nucleotides. Selective deuteration of nucleotides combined with isotope labeling techniques paves the way for investigating nucleic acids. Nevertheless, using deuterated nucleic acid to investigate the impact of hydrophobic interaction in their structures is still lacking.

### *4.4.3 Lipids*

Deuterated lipids are synthesized by substituting hydrogen with deuterium in lipid hydrocarbon chains. Thermodynamic studies of deuterated phospholipids were conducted using deuterated lipid bilayers prepared from aqueous dispersions of 1,2-dimyristoyl-, 1,2-dipalmitoyl-, and 1,2-distearoyl-phosphatidylcholines. Investigations of the nature of interactions in deuterated lipids revealed that deuterium oxide increases the structural stability of deuterated lipid vesicles, due to an enhancement of hydrophobic interaction in $D_2O$.

Further thermodynamic studies of deuterated phospholipids were conducted using deuterated lipid bilayer vesicles prepared from aqueous dispersions of 1,2-dimyristoyl-d54-, 1,2-dipalmitoyl-d62-, and 1,2-distearoyl-d70-phosphatidylcholines. The temperature, the enthalpy, the free energy, the entropy, and the cooperative melting unit of the main lipid phase transition from the gel to the liquid-crystalline phase were determined. Steady-state polarization

measurements were also performed to elucidate the nature of interactions in these deuterated compounds. The results revealed that an enhancement of hydrophobic interaction in deuterated lipid vesicles leads to an increase in the structural stability of deuterated lipid vesicles.

The above-discussed deuteration effects on hydrophobic interaction in proteins, nucleic acids, and lipids are also summarized in Table 4.2.

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# Part III
# Implications of Deuterium Oxide and Deuteration in Biological Systems

# Chapter 5
# Deuterium Oxide and Deuteration Effects on Biomolecules

Water is vital to the stability, structure, dynamics, and function of biomolecules including proteins, nucleic acids, and lipids. Water also mediates biomolecular bindings through hydrogen bond and hydrophobic interaction in complex formation. Hence, changes in the aqueous environment, such as replacement of $H_2O$ with $D_2O$ as the solvent, are expected to affect the structure and function of biomolecules.

Alternatively, the role of water in biomolecular folding can be obtained from solvent perturbation experiments. The simplest perturbant for $H_2O$ is its isotopic form $D_2O$. Studies of biomolecules in deuterium oxide medium or deuteration of biomolecules can provide insights into not only the role of solvation but also hydrogen bonding and hydrophobic interaction.

Thermodynamically, deuterium oxide has been shown to stabilize biomolecular structures and functions. Kinetically, isotopic exchange of the solvent (referred to as solvent kinetic isotope effect) affects the reaction rate. Studies of the difference between reaction rates in $D_2O$ versus $H_2O$ can also provide clues as to the reaction's mechanism.

The behavior of water molecules at hydrophilic sites is different from that at hydrophobic sites. This dissimilar behavior promotes the anisotropy of the hydration shell of proteins, which is essential for the enzyme function. The interaction of solvent with a polypeptide chain is one of the primary factors that control protein folding and stability. Consequently, the replacement of $H_2O$ with $D_2O$ as the solvent is expected to affect the structure and function of proteins.

$D_2O$ has been known to stabilize attenuated viral RNA against thermal degradation. Studies of hydration dynamics of RNA revealed an increase in the stability of RNA structures in $D_2O$. $D_2O$ was also found to strengthen the hydrogen bonding network in the solvent and weaken the dynamical coupling of the hairpin to its solvation environment.

Investigations of the effect of $D_2O$ on phosphatidylcholine bilayers revealed no appreciable difference between the main phase transition temperature in $D_2O$ and $H_2O$. The pretransition phase transition temperature is significantly affected by the

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solvent substitution. $D_2O$ was also found to cause shrinkage of the molecular area of the phospholipid bilayer interface, due to the difference in bond strength between deuterium and hydrogen bonds.

Moreover, studies of deuteration effects on proteins revealed that, in general, the temperature of thermal denaturation of protein is lower in deuterated proteins than in normal proteins. Deuterated proteins were also found to be less resistant to the denaturant urea. Deuterated lipid bilayers were found to exhibit a broad transition in association with a lower temperature with a smaller enthalpy of the lipid phase transition. Deuteration was also found to affect lipid interactions with protein.

A procedure for preparing ribonucleotides and deoxyribonucleotides with deuterium incorporation from *E. coli* cells has been described. However, few studies were reported on the deuteration effect on the structure and interaction of nucleic acids.

## 5.1 $D_2O$ Solvent Effects on Biomolecules

The effects of deuterium oxide as the solvent on biomolecules, including proteins, lipids, and nucleic acids, are briefly discussed below.

### *5.1.1 Deuterium Oxide Effects on Proteins*

Differential interactions of polar and nonpolar groups with water are critical to the stability and interaction of protein and protein-ligand complex. The delineation of proteins and protein-ligand interactions require a direct account of the protein-solvent interactions. Water also participates in the catalytic functions of enzymes. Accordingly, in addressing the issue of $D_2O$ solvent effects, both structural and functional aspects should be considered.

#### 5.1.1.1 Protein Structure Effect

Biomolecules consist of water-exposed surfaces in particular at the protein surface. From the experimental point of view, substitution of $D_2O$ for $H_2O$ has been shown to affect the structural and dynamic properties of proteins. Elucidation of the mechanism of stabilization revealed that the aging of protein is significantly lower in $D_2O$ than in $H_2O$. Moreover, in $D_2O$, the hydrophobic effect is known to be stronger than in $H_2O$, which results in favoring the burial of nonpolar surfaces.

### Protein Stability

The native state of globular proteins was found to be more stable in $D_2O$ than $H_2O$, as shown in the studies of $D_2O$ effects on internal dynamics of proteins, such as ribonuclease T1, superoxide dismutase, $\beta$-lactoglobulin, liver alcohol dehydrogenase, alkaline phosphatase, and apo- and Cd-azurin. In most cases, $D_2O$ significantly increase the rigidity the native structure of protein. Such structure tightening by deuterium oxide is generally amplified at higher temperatures. These findings support the hydrophobic nature of the underlying interaction of proteins in deuterium oxide medium. A comparative study of the stability of several proteins also revealed that the substitution of $D_2O$ for $H_2O$ also affects the enthalpy of protein unfolding.

Moreover, the initial rate and final extents of polymerization of bovine brain tubulin were enhanced in $D_2O$. $D_2O$ also reduces the critical concentration for polymerization of brain tubulin. $D_2O$ is also known to promote the assembly of tubulin into microtubules in vitro, increase the volume of mitotic spindle microtubules, and inhibit mitosis. The direct involvement of $D_2O$ in the polymerization of tubulin may cause an increase in the length and number of microtubules of the mitotic spindles in the dividing cells.

Furthermore, the mechanism responsible for the ability of $D_2O$ to stabilize microtubule dynamics may involve enhancement of hydrophobic interaction in the microtubule lattice and/or the substitution of deuterium bonds for hydrogen bonds. Studies of thermal protective effect of $D_2O$ on rp26 protein (retinitis pigmentosa) in equine infectious anemia virus revealed that 80% $D_2O$ is able to provide thermal protection to rp26 protein up to 2 months of incubation at 45 °C.

### Protein Aggregation

Protein aggregation is a significant issue affecting the integrity of proteins. $D_2O$ has been shown to promote protein aggregation when used to substitute for $H_2O$. Using bovine serum albumin as a model, $D_2O$ was found to enhance the conformational stability of monomeric bovine serum albumin. Maintaining the stability of the monomeric form may be beneficial for the long-term storage of bovine serum albumin.

The issue of protein storage in deuterium oxide medium was also investigated for other proteins. Tubulin is an unstable protein when stored in solution and loses its ability to form microtubules rapidly. Tubulin protein polymerizes into microtubules to form a major component of the eukaryotic cytoskeleton. $D_2O$ was found to stabilize the protein against inactivation at both 4 and 37°C. Replacing 50% or more of $H_2O$ with $D_2O$ promotes microtubule polymerization and stabilizes microtubules against disassembly.

Such enhancement of tubulin polymerization by $D_2O$ could be the result of strengthening the hydrophobic interaction of tubulin molecules. The initial rate and final extent of polymerization of bovine brain tubulin were also enhanced in the

presence of $D_2O$. Thermodynamic analysis also suggested that such enhancement could be the result of strengthening intra- and/or intermolecular hydrophobic interactions of the tubulin molecules.

Peptide Stability

The effect of deuterium oxide on the stability of collagen model peptides was also analyzed. The transition temperatures of the protonated polypeptide (Pro-Pro-Gly) are 25.4 and 28.7°C in $H_2O$ and $D_2O$, respectively. This increase in the transition temperature of peptide in $D_2O$ is comparable to that of globular proteins.

#### 5.1.1.2 Protein Functional Effect

The interactions of solvent with polypeptide chains are a primary factor affecting the structure and function of proteins. The dissimilar behavior of water molecules at hydrophilic and hydrophobic sites that promotes the anisotropic hydration shell of proteins is essential for enzymatic functions. Protein structure changes due to the replacement of hydrogen with deuterium induce the inhibitory effects of $D_2O$ by reduction of the frequency of -OH bonds.

The mechanisms underlying the functional inhibitory effects of deuterium oxide are likely to provide insight into the significance of hydrogen bonds in biological functions. The effects of $D_2O$ on thermal inactivation of enzymes and protein-ligand interactions are further addressed below.

Enzyme Inactivation

Investigations of thermal inactivation with several membrane-bound enzymes, such as chitin synthase, cytochrome c oxidase, and ATPases, indicated that most of these enzymes could not be protected by $D_2O$ against thermal inactivation, except that *E. coli* ATPase is located at the surface of the membrane and cytochrome c oxidase.

Effects of $D_2O$ on the rate of hydrolysis of ATP by Na,K-ATPase were also investigated. High concentration of deuterium oxide inhibits the rate of ATPase reaction. However, activation of the enzyme was observed in the solution with less than 5% $D_2O$. Such effect of deuterium oxide also depends on the ratio between the concentrations of sodium and potassium ions in the medium.

Protein-Ligand Binding

Delineation of protein-ligand interaction requires a direct account of the changes in the interactions of protein with the solvent that accompany the folding or binding reactions. In the case of tacrolimus and rapamycin, these two nonpolar and

structurally related ligands each bind with high affinity to a common site on a binding protein. A large enthalpic destabilization of binding was observed in $D_2O$ relative to $H_2O$. Thermodynamic analysis suggests that a major contributor to the observed enthalpy of destabilization is the differential hydration between protein and ligand in $D_2O$ and $H_2O$ media.

### *5.1.2 $D_2O$ Solvent Effects on Lipids*

Membrane lipids delineate the barrier between the living cell and its surroundings. Membranes are also essential for regulating the machinery of life throughout many interfaces within the interior of cell. Physicochemical properties of biological membranes are strongly influenced by the specific character of the ions in their surrounding aqueous solutions. Comparison of the structure of membrane, such as that extracted from yeast cell grown in $H_2O$ and $D_2O$ culture media, revealed that the difference in molecular composition of deuterated and protonated lipid extracts induces distinguishable structural organization of the membrane lipids.

#### 5.1.2.1 Lipid Interface

The properties of biological membranes depend on the presence of aqueous solutions at their interface. A better understanding of the structural properties of water at the interface of lipid bilayers and the influence of lipid headgroup on the dynamics of water will improve the understanding of the membrane-specific effect. For example, at room temperature, $D_2O$ was found to reduce the surface occupied area of monoolein at the interface in comparison with $H_2O$. Such deuterium oxide effect was believed due to the difference in the strength of deuterium bond versus hydrogen bond.

Moreover, studies of $D_2O$ solvent effects on headgroup hydration, mobility of lipid diffusion, and lipid backbone packing in dimyristoyl- and dipalmitoyl-phosphocholine bilayers revealed significantly slower lipid dynamics and lower hydration of lipid headgroup region in $D_2O$ medium. These findings implied a slightly more rigid organization of phospholipid bilayers in $D_2O$ than H2O. However, dielectric dispersion measurements made on egg phosphatidylcholine and phosphatidylcholine or cholesterol bilayer membranes showed that the properties of the dielectrically distinct substructural layers of lipid bilayers are insensitive to replacement of $H_2O$ by $D_2O$.

#### 5.1.2.2 Lipid Phase Transition

Studies of the phase transitions of dipalmitoyl- and distearoyl-phosphatidylcholine bilayers revealed no significant difference in the main phase transition temperature when $D_2O$ is substituted for $H_2O$ as the solvent. However, the pretransition temperature is significantly increased by the substitution of $H_2O$ by $D_2O$. Such effect of pretransition temperature, not the main transition, was also observed in $D_2O$ medium in the studies of the phase transition of lipid bilayers prepared from dihexadecyl-phosphatidylcholine and dipalmitoyl- and distearoyl- phosphatidylcholines.

In spite of no effect on the lipid main transition temperature, $D_2O$ was shown to affect the enthalpies of the main transition in the above phosphatidylcholine bilayers. In addition, the substitution of $H_2O$ by $D_2O$ was found to cause shrinkage of the molecular area of phospholipid at bilayer interface, due to the difference in bond strength between deuterium and hydrogen bonds.

Examinations of thermotropic transitions of dihexadecyl-phosphatidylcholine bilayer dispersions in $H_2O$ and $D_2O$ showed that the transition temperature between interdigitated gel phase and ripple phase is lower in $D_2O$ than in $H_2O$, suggesting that the interdigitated gel phase is more stable in $H_2O$ than in $D_2O$.

Furthermore, monoolein (1-(cis-9-Octadecenoyl)-rac-glycerol) is fully hydrated by $H_2O$ to form a cubic phase. Substitution of $D_2O$ for $H_2O$ was found to lower the cubic phase to hexagonal phase transition temperature of monoolein. At about 90 °C, the cubic phase is transformed into an inverted hexagonal phase. The structural analysis indicated that $D_2O$ reduces the surface occupied area of monoolein at the interface by 12% in comparison with $H_2O$. This change is probably due to the difference in the strength of the deuterium bond and hydrogen bond.

### 5.1.3 *Deuterium Oxide Solvent Effects on Nucleic Acids*

Using four human digestive organ cancer cell lines, the cytotoxic and cytostatic activities of $D_2O$ were assessed. Oral administration of $D_2O$ resulted in a significant inhibition of the growth of Panc-1 tumor (a pancreatic cancer cell line), suggesting that $D_2O$ affects cytotoxic and cytostatic activities and may serve as a potential anti-cancer agent. Moreover, the nucleic acid synthesis inhibition assay suggested that the inhibition of DNA synthesis may be one of the mechanisms responsible for the antitumor effects of $D_2O$.

Furthermore, although the interference with DNA replication need not be a primary mechanism in the blockade of cell division by deuterium oxide, current hypotheses on the molecular basis of the blockade suggest that such interference might take place. Experiments revealed that sea urchin eggs incorporate thymidine into deoxyribonucleic acid from almost the beginning of development, but cells immersed in deuterium-enriched media do not. In addition, blockade of mitosis and inhibition of thymidine incorporation are relieved when the eggs are returned to normal water medium.

#### 5.1.3.1 Structural Stability

The thermal stability of DNA structures is associated with the properties of the water molecules around the DNAs. Due to the stronger deuterium bond than hydrogen bond, the melting temperatures of nucleic acids are expected to be higher in $D_2O$ than in $H_2O$. This has been confirmed in the case of salmon-sperm DNA over the pH range of 4.5–7.5. Investigation of DNA solutions from *E. coli* has also demonstrated different stability of DNA in $H_2O$ and $D_2O$, where the substitution of $D_2O$ for $H_2O$ in aqueous media was believed to enhance the helical stability in DNA.

The dissociation constant of the -$NH_2CO$- guanosine group in $H_2O$ and $D_2O$ was found to be 9.2 and 9.7, respectively, leading to conclude that the observed difference in the stability of DNA is mainly attributed to the different dissociation constants of -$NH_2CO$- and -$NH_2$ groups in $D_2O$ and $H_2O$ media. The difference in the stability of double-helix formation in $D_2O$ and $H_2O$ media was also supported by thermodynamic studies which revealed that the average enthalpy of double-helix formation by (dG-dC)3 is −62.7 kcal/mol of helix in $D_2O$, as compared to −56.9 kcal/mol of helix in $H_2O$.

#### 5.1.3.2 Functional Effects

The effects of deuterium oxide on cell growth were shown for rat basophilic leukemia cells cultured with 15 moles/liter $D_2O$. The result revealed a decrease in cell growth attributing to cells not doubling their DNA content. Further studies of the extent and pattern of the rearrangements of microtubule arrays in interphase and mitotic PtK2 cells showed that $D_2O$ influences the reassembly of the cytoplasmic microtubule complex of interphase cells. In the presence of 75% $D_2O$, the conversion of the cytoplasmic microtubule complex into the mitotic spindle was found to be retarded.

### 5.1.4 *Other $D_2O$ Solvent Effects*

Deuterium oxide was also found to influence the dynamics of mitosis and the morphology of the mitotic apparatus in HeLa cells. Two hours of incubation of HeLa cells with 1–25% of $D_2O$ was found to increase the frequency of multipolar divisions up to 20 times the control level. Substitution of 10% and 25% $D_2O$ for $H_2O$ induces changes in the proportions of mitotic phases, and 50% $D_2O$ strongly inhibits mitotic activity. Such antiproliferative effect of deuterium oxide on cells was also observed in PtK2 cell lines, where no mitotic figures were found after 2–3 days of incubation in the presence of 75% of $D_2O$ medium.

The thermostabilizing effect of deuterium oxide has also been demonstrated on viruses, such as oral poliovirus and influenza virus.

Deuterium oxide solvent effects on proteins and peptide, nucleic acid, and lipid are briefly presented in Table 5.1.

**Table 5.1** Deuterium oxide solvent effects on biomolecules

| Structure and function of biomolecules | Deuterium oxide solvent effects |
|---|---|
| Proteins and peptides | |
| Structural effect | Slow in the aging of protein |
| | Favorite of the burial of nonpolar surfaces |
| | Stabilization of the native state of proteins |
| | Enhancement of the rigidity of protein |
| | Promotion of protein aggregation |
| | Increasing transition temperature of peptide |
| Functional effect | No protection against thermal inactivation of most enzymes |
| | Inhibition of the rate of ATPase reaction |
| | Destabilization of ligand binding |
| Nucleic acids | |
| Structural stability | Enhancement of the melting temperatures of nucleic acids and the helical stability |
| Functional effects | Reduction in cell growth attributing to cells not doubling their DNA content |
| | Inhibition of mitotic activity |
| Lipids | |
| Lipid interface | Slowing dynamics and lowering hydration of lipid headgroup region |
| Lipid phase transition | Shrinking molecular area of lipid bilayer Interface |

## 5.2 Deuteration Effects on Biomolecules

The effects of deuteration on biomolecules, including protein, lipid, and nucleic acid, are briefly described below. Deuterated protein with deuteration up to 99%, such as phycocyanin, can be obtained from blue-green algae grown in $D_2O$, while the extraction of lipids from microorganisms grown in deuterated media can produce deuterated lipids, which has been applied to elucidate the structure, dynamics, and function of cell membrane. However, comparative studies of deuterated nucleic acids with normal nucleic acids are lacking in the literature.

### *5.2.1 Deuteration Effects on Proteins*

Comparative investigations of normal and deuterated phycocyanins, isolated from two blue-green algae (*Plectonema calothricoides* and *Phormidium luridum*), revealed that the temperature of thermal denaturation is about 5 °C lower in deuterated phycocyanin than in normal phycocyanin. The magnitude of the corresponding enthalpy is 18–36% lower in deuterated protein than normal protein.

In *P. luridum*, deuterated phycocyanin is about 8% higher in α-helix content, but in *P. calothricoides,* its α-helix content is not significantly higher. Moreover, the heat capacity change in protein unfolding is essentially the same (2 kcal/mol/K) for deuterated phycocyanin and normal phycocyanin. In addition, deuterated phycocyanin is less resistant to the denaturant urea than normal protein. The denaturant concentration at the midpoint of the denaturation curve is 0.6–1.2 mol/L lower in the deuterated protein than normal protein. The free energy of unfolding of deuterated protein is 1–1.5 kcal/mol less than for normal protein.

## 5.2.2 Deuteration Effects on Lipids

The cooperative melting unit of the main lipid phase transition from the gel to the liquid-crystalline phase was examined to elucidate the nature of interactions. Investigations of deuterium alkyl-chain substitution effects including lipid phase transition and lipid interaction are described below.

### 5.2.2.1 Phase Transition

Thermodynamic studies of deuterated phospholipids were conducted using deuterated lipid bilayer vesicles prepared from aqueous dispersions of 1,2-dimyristoyl, 1,2-dipalmitoyl-, and 1,2-distearoyl-phosphatidylcholines, where hydrogen in two lipid long alkyl chains was replaced with deuterium. The obtained phase transition properties of bilayer vesicles prepared from deuterated phospholipids were then compared with those of nondeuterated phospholipids.

Unlike a sharp phase transition observed in a nondeuterated lipid, a deuterated lipid exhibits a broad transition in association with a lower temperature and a small enthalpy and entropy of the transition. However, in the case of deuterated lipid extracts from the yeast, the structural features of fully deuterated lipid stacks were found to be comparable with those of their hydrogenous analogues. In addition, the deuterated and hydrogenous lipid extracts were similar in multi-lamellar arrangement, which implies the presence of a large number of polar phospholipid molecules.

Moreover, investigations of the effects of chain deuteration and headgroup deuteration on lipid structure and phase behavior of 1,2-distearoyl-, 1,2-dipalmitoyl-, and 1,2-dimyristoyl phosphatidylcholines revealed that the phase transition temperature is 4.3 °C lower for lipids with deuterated chains as compared to hydrogenated chains. In addition, deuterated chains cause a reduction in the lamellar repeat spacing and bilayer thickness.

Other studies include ethyl pyrene that forms excimer and dimethylaniline exciplexes in lipid vesicles prepared with 1,2-dimyristoyl phosphatidylcholine and its deuterated analogue. The results showed that deuterium substitution in lipid enhances the excimer yield. In the liquid-crystalline phase, the two hydrocarbon

tails in lipid dispersions are structurally comparable, while, in the gel phase, the tails in the deuterated lipid have a significantly less ordered configuration than those in non-deuterated lipid, implying that deuterated lipid is less rigid in its structure.

#### 5.2.2.2 Lipid Interaction

Studies of interactions of deuterated lipids were illustrated using ergosterol, the main sterol molecule found in the cell membranes of yeasts and other fungi, where the effects of ergosterol on lipid multilayers prepared with deuterated phospholipids were examined. The results showed that the effect of ergosterol on phospholipid extract rich in unsaturated acyl chains is different from that in membranes rich in saturated phospholipids.

Moreover, investigation of the effect of protein on the melting behavior of deuterated lipid, glycophorin isolated from human erythrocyte membranes, was reconstituted into lipid vesicles composed of binary mixtures of phosphatidylserine and acyl-chain perdeuterated dipalmitoyl phosphatidylcholine. The results indicated that acyl chain deuteration does not significantly alter lipid characteristics.

In addition, cytochrome b5 was also examined by reconstituting with a highly deuterated phospholipid to form ordered multilayers. Studies of the interaction of the membrane-binding domain of cytochrome b5 with the lipid bilayer revealed that the protein penetrates well into the bilayer.

### *5.2.3 Deuteration Effects on Nucleic Acids*

An efficient procedure for the preparation of ribonucleotides and deoxyribonucleotides with deuterium incorporation has been described. The obtained ribose intermediates were converted to ribonucleotides and deoxyribonucleotides via enzymatic reactions with an average deuterium content of 96%.

Moreover, deuterated DNA extracted from *E. coli* cells cultured in deuterated nutrient medium was also reported, where hydrogens covalently bonded to carbon atoms in the DNA sugars and bases were replaced by deuterium atoms. In that study, deuterated DNA confirms a network of water running along the inside edge of the major groove linking phosphate oxygen atoms, in which ordered water lies in the center of the major groove.

The above-described deuteration effects on protein, nucleic acid, and lipid are briefly summarized in Table 5.2.

**Table 5.2** Deuteration effects on biomolecules

| Deuterated biomolecules | Deuteration effects |
|---|---|
| Protein | |
| Denaturation | Less resistant to the denaturant urea for deuterated hycocyanin than normal protein |
| | Lower temperature and enthalpy of thermal denaturation for deuterated phycocyanin |
| Lipid | |
| Phase transition | Lowering temperature, enthalpy, and entropy of lipid phase transition |
| | Reduction in bilayer thickness for deuterated lipid chains |
| | Decreasing lipid ordered configuration |
| Lipid chain rigidity | Less rigid in the structure of deuterated lipids |
| Lipid interaction | Improvement of lipid bilayer-protein interaction |
| Nucleic acid | |
| Interaction with water | Confirmation of ordered water lying in the center of the major groove of deuterated DNA |

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# Chapter 6
# Effects of $D_2O$ and Deuteration on Biomembranes

Biological membranes not only delineate the barrier between the living cell and its surroundings to keep toxic substances out of the cell but also regulate the machinery of life throughout many interfaces within the interior of the cell. Biological membranes also contain receptors and channels that mediate cellular and extracellular activities and allow specific molecules such as ions, nutrients, wastes, and metabolic products to pass between organelles.

Physicochemical properties of biological membranes are strongly influenced by specific characters in their surrounding solutions. The effects of $D_2O$ on biological membranes include changes in membrane receptor binding and conformation, membrane-bound enzyme activities, and membrane potential and ion transport. Moreover, the mechanisms underlying the effects of deuterium oxide are likely to provide insight into the fundamental significance of hydrogen bonding and hydrophobic interaction in biological functions.

Hence, elucidation of the molecular mechanisms underlying $D_2O$ solvent and deuteration effects on cell membranes has been an active research area. $D_2O$ is able to activate or inhibit biological systems and thus influences the metabolism of tissues and organs. Deuterium oxide also affects protein structure, due to the replacement of hydrogen with deuterium. For example, comparative studies of thermal inactivation of membrane-bound enzymes in $H_2O$ and $D_2O$ revealed that deuterium oxide stabilizes *E. coli* ATPase as well as cytochrome c oxidase.

On the other hand, deuterium oxide seems to inhibit the activation process of the receptor potential. The effects of solvent substitution with deuterium oxide on gap junctional conductance were examined in the earthworm axon, which results showed that $D_2O$ causes a reduction in gap junctional conductance and a decrease in the amplitude of the receptor potential.

Moreover, $D_2O$ also affects membrane excitation on giant internodal cells of alga (*Chara gymnophylla*). The primary effect consists of membrane depolarization. A kinetic isotope effect on the rate of binding was also observed, indicating that deuterium substitution for protium affects $H^+$ transfer reaction across cell membranes.

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On the basis of these findings, an osmotic stress effect of $D_2O$ on membrane ion channels was hypothesized.

The presently discussed deuterium oxide and deuteration effects on biological membranes include the effects on membrane structures and functions, such as membrane potential, channel, polarization, transport, and receptor. In addition, the present chapter also covers deuteration isotope effects on membrane proteins, lipid bilayer phases, and essential fatty acids.

## 6.1 $D_2O$ Solvent Effects on Biomembranes

$D_2O$ has been used as a putative inhibitor of the plasma membrane $H^+$-ATPase and the plasma membrane redox system. Concentration of 50% $D_2O$ was found to inhibit $H^+$ secretion and the plasma membrane redox system. Studies of the effects of $D_2O$ on alga (*Chara gymnophylla*) cells showed that $D_2O$ causes membrane excitation followed by potassium leakage. To address the effect of $D_2O$ on membrane depolarization, a hypothesis was proposed to deal with an osmotic stress effect of $D_2O$ on membrane ion channels.

In dialyzed *Myxicola* axons, the substitution of $H_2O$ with $D_2O$ slows both sodium and potassium kinetics and decreases the maximum conductance. These results suggested that the rate-limiting process which produces a conducting channel involves detectable local changes in solvent structure. Such changes lead to slowing the $Na^+$ and $K^+$ currents in *Myxicola* axons by 40–50% at 5 °C. These findings imply that the rates of $K^+$ and $Na^+$ inactivation during a maintained depolarization are slowed in $D_2O$ medium.

Furthermore, in lipid studies, phospholipid bilayers hydrated with $D_2O$ express different biophysical properties as compared with hydration by $H_2O$. Studies of $D_2O$ isotope effects on headgroup hydration and mobility, lateral lipid diffusion, and lipid backbone packing revealed significantly slower dynamics and lower hydration of the lipid headgroup region. These findings imply that the deuterium oxide affects the order parameter of lipid bilayers, resulting in a more rigid organization for phospholipid bilayers.

### *6.1.1 Structures and Functions*

Studies of $D_2O$ effects on membrane functions, such as calcium release as measured with membrane potential in single muscle fibers of *Balanus nubilus*, demonstrated that deuterium oxide inhibits calcium release without affecting membrane potential. This finding is consistent with the proposal that $D_2O$ inhibits the coupling between excitation and contraction. In crayfish stretch receptor organ, substitution of $D_2O$ for $H_2O$ was found to produce a decrease in the amplitude of the receptor potential

to a level of 34% of the control, which implies that $D_2O$ has an inhibitory effect on the transduction process.

The elongation of subapical segments of maize (*Zea mays*) roots was also inhibited in $D_2O$ medium. Further studies showed that $D_2O$ is a putative inhibitor of the plasma membrane $H^+$-ATPase and the plasma membrane redox system. A 50% $D_2O$ was found to inhibit $H^+$ secretion and the plasma membrane redox system. The addition of more than 5% $D_2O$ causes the plasma membrane of roots to be transiently depolarized. However, despite the inhibiting effect of $D_2O$, the plant is still able to regulate the membrane potential.

Moreover, experiments on plasma membrane vesicles also revealed that $H^+$-pumping and ATPase activities are largely inhibited by 35–50% $D_2O$. But a 30 min exposure to $D_2O$ slightly reduces $O_2$ uptake and significantly increases ATP levels. Furthermore, the actions of saxitoxin were also investigated in frog myelinated nerve. Substitution of $D_2O$ for $H_2O$ increases the potency of saxitoxin by approximately 50%. The inhibition of sodium currents by 1 nM saxitoxin is doubled in $D_2O$. These findings are consistent with a hypothesis that attributes receptor-toxin stabilization to isotopic changes of hydrogen bonding to deuterium bonding.

In addition, association and dissociation rates constants of several steroids binding to glucocorticoid receptor protein were also determined in $H_2O$ and $D_2O$ media. Substitution of deuterium for hydrogen does not influence association rate constants. However, dissociation rate constants are decreased about twofold in deuterium oxide in the case of steroids that contain an 11-beta-hydroxyl group, which is known to be present in every optimal glucocorticoid agonist molecule. These results suggest that 11-beta-hydroxyl group participating in hydrogen bonding may play a role in glucocorticoid action.

Being able to activate or inhibit biological systems, $D_2O$ influences metabolism and functions of tissues and organs. Investigations were carried out to examine the effects of $D_2O$ on the functions of human platelets, including changes in membrane receptor conformation and binding strength of substances to their specific receptors. The results revealed that $D_2O$ causes a decrease in ADP- and collagen-induced aggregation, and retraction is also inhibited in $D_2O$ in a dose-dependent manner.

### 6.1.2 Membrane Potentials

Membrane potential is composed of transmembrane proton gradient and electrical potential of the protonmotive force. Proton gradient and electric potential are together referred to as electrochemical potential according to the Mitchell hypothesis. Proton electrochemical potential provides the driving force for translocation reactions of solutes across the cell membrane. For example, studies of active transport of lactose across *E. coli* membrane showed that the translocation reaction is retarded by a factor of greater than 3 in $D_2O$ medium, as compared to $H_2O$ medium. This finding implied that proton-transfer reaction is the rate-limiting step in the translocation of lactose across the *E. coli* membrane.

Moreover, in the crayfish stretch receptor organ, $D_2O$ was found to decrease the amplitude of the receptor potential to a level of 34% of the control, which suggested that $D_2O$ exhibits an inhibitory effect on the transduction process that is responsible for the receptor potential.

### *6.1.3 Ion Channels*

The effects of solvent substitution with deuterium oxide on gap junction channel were examined in the earthworm axon. Substitution with $D_2O$ reduces gap junction channel by 20%. This reduction reflects the changes that occur in solvent viscosity, resulting in influencing trans junctional ion mobility. Furthermore, the effects of deuterium oxide on the properties of endplate channels were studied in voltage-clamped muscle fibers from a frog. The single channel conductance was found to decrease in $D_2O$. Such decrease is greater than that could be accounted for by the increased viscosity of $D_2O$ solutions.

Experiments have also been performed to investigate the effects of deuterium oxide on acetylcholine-activated channels at the frog neuromuscular junction. The results indicated that the solvent can affect the properties of channel conductance and lifetime. In deuterium oxide solvent, the channel closing rate is speeded up, and the mean channel lifetime is smaller. A possible explanation of these findings is that the channel gating change in the presence of $D_2O$ makes the open state less stable.

The effects of substituting $D_2O$ for $H_2O$ on the voltage-activated $H^+$ selective conductance of rat alveolar epithelial cells were also examined. $D^+$ is able to permeate proton channels but with a conductance only about 50% that of $H^+$. This decrease in conductance in $D_2O$ suggested that $D^+$ interacts specifically with the channel during permeation.

In addition, investigations of internodal cells of freshwater alga and the isolated $Ca^{2+}$ channels revealed that both $Ca^{2+}$ and $Cl^-$ channels are activated by the effects of $D_2O$. A proposed mechanism of $D_2O$ action was based on an osmotic-like stress effect and higher D-bond energy compared to the H-bond.

Moreover, studies of the effects of $D_2O$ substitution of $H_2O$ on the permeation and gating of $Ca^{2+}$-activated potassium channels in alga (Chara gymnophylla) membrane showed that the single channel conductance is decreased by 15% in $D_2O$ as compared to $H_2O$. The blockade of channel conductance by cytosolic $Ca^{2+}$ is also weakened in $D_2O$. These findings suggested that voltage-dependent channel gating is affected by $D_2O$, primarily due to the change in $Ca^{2+}$ binding to the channel during the activation step.

The effects of solvent substitution on the steady-state kinetic properties of drugs (gallamine triethiodide) and related ions were also studied in $D_2O$ medium. Nonyltriethylammonium and $Ba^{2+}$ are known to block off $Na^+$ and $K^+$ channels. Significant isotope effects on the kinetics of blockage occur at 5 °C, which is consistent with processes involving solvent interaction.

Also, abundant evidences demonstrated that $D_2O$ modulates various secretory activities. The replacement of $H_2O$ with $D_2O$ in solutions of patch electrodes reduces $Ca^{2+}$ currents evoked by depolarizing voltage steps. This finding suggests that $D_2O$ gradient across the plasma membrane is critical for $Ca^{2+}$ channel kinetics. The differences in the zero-point vibrational energy between protonated and deuterated amino acids were believed to cause the dysfunction of $Ca^{2+}$ channels.

### *6.1.4 Membrane Transports*

In the studies of muscle fibers of *Balanus nubilus*, $D_2O$ was shown to inhibit calcium release but did not affect membrane potential. This finding is consistent with the postulate that deuterium oxide inhibits the coupling between muscle excitation and contraction. In another investigation, potassium absorption by the root of rice seedlings was found to decrease proportionally with $D_2O$ concentration. The translocation of the absorbed cations by roots to shoots is also decreased markedly in >40% $D_2O$ medium.

Examinations of the effects of $D_2O$ on the rate of hydrolysis of ATP by Na,K-ATPase revealed that a high concentration of $D_2O$ inhibits the rate of ATPase reaction. Such isotope effects depend on the ratio between $Na^+$ and $K^+$ concentrations in the medium, and the transport of ions by the enzyme is accompanied by dehydration of these ions.

Other studies of the effects of $D_2O$ on membrane transport were carried out in *E. coli* lactose permease reconstituted proteoliposomes, in which uphill $H^+$ translocation is coupled with downhill lactose transport. The results demonstrated that initial rates of carrier-mediated efflux down a chemical gradient are retarded over threefold in deuterium relative to protium over a pH (pD) range from 5.5 to 7.5. Counterflow was also examined in both $H_2O$ and $D_2O$ media to test the hypothesis that efflux is an order reaction limited either by the rate of deprotonation or by a pH-dependent equilibrium.

$D_2O$ solvent kinetic isotope effects affect membranous transport processes such as the rate of binding. Deuterium substitution for protium affects an $H^+$ transfer reaction within lactose permease of *E. coli*, which is associated with sugar binding. At neutral pH or pD, both the rate of sugar dissociation and the forward rate are slower in $D_2O$ than in $H_2O$ by a factor of 2. These results indicated that internal transfer of $H^+$ is involved in the kinetic isotope effect.

Moreover, the voltage-activated $H^+$ selective conductance of rat epithelial cells was investigated to study the effects of deuterium oxide on the conductance and the pH dependence of gating. $D^+$ is able to permeate proton channels but with a conductance about half that of $H^+$. Such reduction of conductance in $D_2O$ is more than that can be accounted for by the lower mobility of $D^+$ than $H^+$. These findings not only suggested $D^+$ interacting specifically with the channel during permeation but also supported the hypothesis that $H^+$ (or $D^+$) and not $OH^-$ is the ionic species that carry the current.

### *6.1.5 Lipid Phases*

Molecular dynamics simulation studies on dipalmitoyl-phosphatidylcholine bilayers revealed that the membrane core and the membrane-water interface are affected by replacing $H_2O$ by $D_2O$. The lifetimes of these interactions are much longer in $D_2O$ medium than those in $H_2O$ medium. A slightly better ordering of $D_2O$ molecules and average self-diffusion was also found due to the longer live deuterium-bonding to lipid headgroups in lipid bilayers.

Investigations of thermotropic transitions of dihexadecyl-phosphatidylcholine dispersions in $H_2O$ and $D_2O$ showed that transition temperature between interdigitated gel phase and ripple phase is lower in $D_2O$ than in $H_2O$, indicating that the interdigitated gel phase is more stable in $H_2O$ than in $D_2O$. However, the transition between the ripple phase to the fluid phase in $D_2O$ occurs at a temperature slightly higher than in $H_2O$.

Moreover, studies of dipalmitoyl-phosphatidylcholine dispersions revealed that the transition temperature between lamellar gel phase and ripple phase is higher in $D_2O$ than in $H_2O$. A difference in interfacial energies between these aqueous environments was also discussed to account for the shift of the transition temperature by the $D_2O$.

In addition, the bilayer phase transitions of three phospholipids (dipalmitoyl-, distearoyl-, and dihexadecyl-phosphatidylcholines) were also compared in $D_2O$ and $H_2O$ media. Both the pretransition and the main transition are significantly affected by $D_2O$. The substitution of $H_2O$ by $D_2O$ causes shrinkage of the molecular area of phospholipid at the bilayer interface, due to the difference in bond strength between deuterium and hydrogen bonds.

The above-discussed deuterium oxide effects on biological membranes are briefly summarized in Table 6.1.

## 6.2 Deuteration Effects on Biological Membranes

Deuteration of phospholipids is a common practice to elucidate membrane structure, dynamics, and function. The effects of deuteration on the organization and phase behavior of phospholipids were investigated to delineate the effects of chain deuteration and headgroup deuteration on lipid structure and phase behavior.

Moreover, polyunsaturated fatty acids of cellular, mitochondrial, retinal, and membranes are essential to neuronal function. They are susceptible to lipid peroxidation reaction. Selective deuteration of polyunsaturated fatty acids is a novel, non-antioxidant method to investigate lipid peroxidation-related oxidative stress. Such oxidative stress is a common feature of genetic and idiopathic neurological diseases.

The impacts of lipid deuteration on the general fitness of the bacteria and its membrane fluidity in relation to fatty acid chain composition were also examined. Such lipid deuteration protocol is likely applicable to other microorganisms for

**Table 6.1** Typical studies of deuterium oxide on biological membranes

| Membrane structures and functions | Deuterium oxide effects |
|---|---|
| Membrane functions | Inhibitions of calcium release, transduction process, $H^+$-pumping, or ATPase activities |
| | Enhancement of saxitoxin potency |
| | Reduction of dissociation rate constants of steroids binding to glucocorticoid receptor |
| Membrane potentials | Reduction of protonmotive force or receptor potential |
| | Inhibitory effect on transduction process |
| Ion channels | Reduction of gap junction channel, channel conductance, or $Ca^{2+}$ current |
| | Reduction of the channel open state stability |
| | Enhancement of the channel kinetics |
| Membrane transports | Inhibition of $Ca^{++}$ release or ATPase reaction rate |
| | Reduction of lactose transport across proteoliposomes |
| Lipid vesicles | Enhancement of the lifetime of membrane- water interaction |
| | Affecting lipid phase transition temperatures |
| | Shrinkage of the molecular area of phospholipid at bilayer interface |

in vivo NMR studies. The advantageous use of neutron scattering techniques for the determination of membrane structures was also described. Constituents of biological membranes showed much larger differences in neutron scattering than X-rays. Methods of contrast enhancement using $H_2O$ and $D_2O$ exchange and deuteration were outlined from studies of nerve myelin artificial membranes and retinal rod outer segments.

### *6.2.1 Membrane Components*

The deuteration of biomolecules is valuable in structural studies where specific regions of a complex system can be examined, especially membrane components. In mammalian membranes, the presence of cholesterol is crucial in modulating the properties of lipids and the interaction of lipid with proteins.

Partially deuterated cholesterol was prepared and incorporated with phosphatidylcholine lipid bilayers, including saturated and unsaturated lipids. Cholesterol was found to be distributed asymmetrically within the bilayer and positioned closer to the headgroups of the lipids than to the middle of the tail core.

Understanding fatty acid metabolism and lipid synthesis requires information about fatty acid and lipid formation within the cells. Deuterated substrates of α-linolenic acid and linoleic acid were used to determine the relative amounts of polyunsaturated fatty acids and specific phospholipids incorporated into cell plasma

membranes. The results revealed that the fatty acid treatments induced an increase in the amounts of these acids and their long-chain conversion products.

An enhanced level of phospholipid turnover of these fatty acids in lipids, such as phosphatidyl-cholines, ethanolamines, and inositols, was also observed in the cell plasma membrane. In addition, the study of organization and dynamics of phospholipids and their application to intact bacteria was carried out with *Vibrio splendidus* labeled with deuterated palmitic acid. *V. splendidus* is a marine bacterium which is highly adapted to dynamic salty ecosystems.

### *6.2.2 Deuterated Membranes*

*Halobacterium halobium*, purple membranes in which valines or phenylalanines are present in deuterated forms, were also investigated to assess the distribution of valine and phenylalanine in the purple membrane structure. The results revealed that valine is distributed toward the periphery of a single bacteriorhodopsin molecule, whereas phenylalanine is distributed toward its center. Moreover, the charged and polar groups of the bacteriorhodopsin molecule tend to lie at the molecular interior, away from contact with lipid, while the nonpolar surfaces are directed outward, making contact with the lipid regions.

Quinones are best known as carriers of electrons and protons during oxidative phosphorylation and photosynthesis. The localization of ubiquinone within lipid bilayers was investigated by neutron diffraction, where quinone was incorporated into stacked bilayers of perdeuterated dimyristoyl-phosphatidylcholine. Quinone at the center of the hydrophobic core was found to be parallel to the membrane plane. This localization is of importance for its function as a redox shuttle between the respiratory complexes.

### *6.2.3 Membrane Proteins*

Deuterated membranes from deuterated cells of *Micrococcus lysodeikticus* containing 85–90% of deuterium were isolated. The secondary structure of deuterated membrane proteins and the relative contents of deuterated lipids and proteins were found to be similar to those in the protonated ones.

In contrast, in deuterated phycocyanin isolated from blue-green algae, deuterated protein exhibits a thermal denaturation temperature lower than that of hydrogenated proteins. Deuterated phycocyanin was also found to be less resistant to the denaturant urea.

In peptides, selectively deuterated analogues of histidine, tyrosine, phenylalanine, and tryptophan have been synthesized by chemical exchange. These analogues have been used for growth of bacteria. In *E. coli*, active lactose repressor protein has been isolated from cells grown in deuterated amino acids to investigate proton electrochemical potential across *E. coli* membranes.

### 6.2.4 Lipid Bilayers

Lipid bilayers undergo a phase transition from gel phase to liquid crystalline phase. The temperature of gel-fluid phase transition was found to be 4 °C lower for lipids with deuterated chains compared to protonated chains, which is consistent with the result that deuterated fatty acids have a melting point 3–5 °C lower than nondeuterated ones. These findings reveal that deuterated lipid has a significantly less ordered configuration in gel phase than nondeuterated lipid, suggesting that deuterated lipid chains cause a reduction in the lamellar repeat spacing and bilayer thickness.

### 6.2.5 Essential Fatty Acids

Essential fatty acids promise to benefit age-related diseases, including Alzheimer's and other neurological conditions. Autoxidation of polyunsaturated fatty acids damages lipid membranes and generates toxic byproducts, which are implicated in neurodegeneration, aging, and other pathological conditions.

Deuterated essential fatty acid can be obtained by substituting hydrogen with deuterium at the bis-allylic methylene of essential fatty acids. Abstraction of bis-allylic hydrogen atoms is the rate-limiting step of polyunsaturated fatty acid autoxidation. This process is inhibited by replacing bis-allylic hydrogen with deuterium. The substitution leads to a significantly slower oxidation due to the kinetic isotope effect, resulting in inhibiting membrane damage.

The above approach has the advantage of preventing the harmful accumulation of reactive oxygen species by inhibiting the propagation of lipid peroxidation. Although in cells the presence of a relatively small fraction of deuterated polyunsaturated fatty acids was found to inhibit lipid peroxidation, the protection rendered by deuterated polyunsaturated fatty acid may depend on the structure of fatty acid.

The above-discussed deuteration effects on biological membranes are briefly summarized in Table 6.2.

**Table 6.2** Typical studies of deuteration on biological membranes

| Membrane structures and functions | Deuteration effects |
|---|---|
| Membrane components | Cholesterol distributes asymmetrically within the bilayer and positions closer to lipid headgroups |
| | Fatty acid induces an increase in the amounts of α-linolenic acid and linoleic acid |
| | Lipid deuteration impacts the membrane fluidity of a marine bacterium |
| Membrane proteins | Insignificant changes in secondary structure and relative content of lipids and proteins |
| Lipid phase transition | Lowering phase transition temperature |
| | Less ordered configuration in gel phase |
| | Lowering melting point of fatty acids |
| Essential fatty acids | Slower oxidation of unsaturated fatty acids |
| | Inhibition of lipid peroxidation |

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# Part IV
# Applications of $D_2O$ and Deuteration to Biochemical Reactions

# Chapter 7
# Biochemical Effects of Deuterium Oxide and Deuteration

The stability of a biomolecule is vital for its biological function. Proper conformation is partially driven by intermolecular interactions between biomolecule and water. Studies of deuterium oxide and deuteration effects can provide insight into the role of solvation, hydrogen bonding, and hydrophobicity in biomolecule-water interactions.

$D_2O$ is very useful in biochemical applications to investigate the structures and functions of biomolecules. Thermodynamic studies of biochemical effects in $D_2O$ solvent have been carried out to elucidate the role of water in the structure and conformation of biomolecules, such as the structural stability and the association-dissociation equilibrium of protein subunits. The stabilities of native proteins and protein-ligand complexes are maintained by differential interactions among polar and nonpolar atoms within proteins and ligands with water.

Moreover, the deuterium kinetic effect is the study of change in the reaction rate of chemical reaction in which hydrogen in the reactant is replaced by deuterium. It is the most common and well-studied isotope effect. The difference between the reaction rate in $D_2O$ solvent versus that in $H_2O$ often can help the elucidation of the reaction mechanism. Experimentally, the kinetic isotope effect is expressed as the ratio of rate constants for the reactions ($k_H/k_D$), where $k_H$ and $k_D$ refer to the kinetic rate constant in $H_2O$ and $D_2O$, respectively.

In addition, investigations of the biochemical effects of deuterium oxide and deuteration could also potentially delineate the pharmacokinetics of drugs that are metabolized by pathways involving carbon-hydrogen (C-H) bond. Such research could lead to new drug development or improve unfavorable pharmacokinetics by protecting metabolically vulnerable C-H bonds.

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## 7.1 Deuterium Oxide Thermodynamic Effects

The hydrophobic effect is known to be stronger than in $H_2O$, resulting in favor of the burial of nonpolar surfaces and van der Waals' packing in the biomolecule cores. In contrast, biomolecules present more water-exposed surfaces in $H_2O$ medium. Deuterium oxide thermodynamic effects on proteins, nucleic acids, and lipids are discussed below.

### *7.1.1 Proteins*

Proteins discussed in the following deuterium oxide thermodynamic effects include enzyme stability (lactate dehydrogenase, glutamate dehydrogenase, and butyrylcholinesterase) and protein inactivation (tubulin, tacrolimus and rapamycin, bacteriorhodopsin, and biotin repressor).

The substitution of $D_2O$ for $H_2O$ was found to increase the transition temperature and decrease the enthalpy of protein unfolding. However, as a result of entropic compensation for the decrease in enthalpy, the overall stability of proteins was believed to be largely unchanged based on the proposal of enthalpy-entropy compensation. Such compensation is attributed to changes in hydration of proteins in $D_2O$ compared to $H_2O$.

Further analyses of thermodynamic data for the transfer of model compounds from $H_2O$ to $D_2O$ show that the changes in the enthalpy of unfolding due to water isotopic substitution can be rationalized by changes in hydration of the buried nonpolar groups.

#### 7.1.1.1 Enzyme Stability

Thermodynamic studies of enzymes revealed that $D_2O$ stabilizes lactate dehydrogenase and inhibits subunit interchange in solution. In bovine liver, $D_2O$ appears to facilitate the association of monomers to form polymers. The stabilizing action of $D_2O$ reflects the increased strength of deuterium bonds and deuterium water bridges. Moreover, in thermal inactivation of the tetrameric form of butyrylcholinesterase in $H_2O$ and $D_2O$, a slight solvent isotope effect was observed, including a stabilizing effect and a shift in the transition temperature.

#### 7.1.1.2 Protein Folding

Tubulin, an unstable protein when stored in solution, loses its ability to form microtubules rapidly. $D_2O$ was found to stabilize the protein against inactivation. The deuterated solvent retards an aggregation process that occurs during incubation at 4

and 37 °C. $D_2O$ was believed to exhibit stabilization effect on tubulin conformation step by involving the disruption of hydrophobic forces.

Elucidation of the mechanism of stabilization of tubulin by $D_2O$ demonstrated that the rate of decrease of tryptophan fluorescence during aging of protein was significantly lower in $D_2O$ than in $H_2O$. Circular dichroism spectra of tubulin suggested the stabilization of the secondary structure in $D_2O$. The number of available cysteine residue was decreased to a lesser extent in $D_2O$ than in $H_2O$. Meanwhile, the temperatures of tubulin unfolding are higher in $D_2O$ than in $H_2O$: 58.6 and 62.2 °C in $D_2O$, as compared to 55.4 and 59.3 °C in $H_2O$.

#### 7.1.1.3 Thermal Unfolding

Investigations of thermal unfolding of *Drosophila* signal transduction protein in $H_2O$ and $D_2O$ solutions showed the stabilizing effect of $D_2O$ compared with $H_2O$. Moreover, tacrolimus and rapamycin are structurally related ligands; each bind with high affinity to a common site on a small binding protein called FK506. Studies of the enthalpies of tacrolimus and rapamycin binding to FK506 in $H_2O$ or $D_2O$ revealed a large enthalpic destabilization of binding in $D_2O$ compared to $H_2O$. A main contributor to the observed enthalpic destabilization is due to the differential hydration of protein-ligand binding by $D_2O$ and $H_2O$.

In addition, the thermal retinal isomerization of bacteriorhodopsin in purple membrane in $H_2O$ and $D_2O$ was also investigated. The enthalpy change in the thermal trans- to -cis isomerization reaction was found to be 24.7 and 20.1 kcal/mol in $H_2O$ and $D_2O$, respectively, while the Gibbs free energy in $D_2O$ is 0.4–0.7 kcal/mol lower than that in $H_2O$. Hydrogen bonding was believed to involve in the solvent isotope effect on the isomerization reaction of bacteriorhodopsin.

#### 7.1.1.4 Protein Interaction

Besides, thermodynamic studies were also performed for biotin repressor that forms a homodimer as a prerequisite to DNA binding to repress transcription initiation. Solvent reorganization was found to contribute significantly to the energetics of protein-protein interactions. The effect of replacing $H_2O$ with $D_2O$ on protein dimerization revealed a solvent isotope effect of −1.5 kcal/mol on the Gibbs free energy of dimerization. Such solvent isotope perturbations are consistent with a significant contribution of solvent release to the dimerization reaction.

#### 7.1.1.5 Peptide Stability

Furthermore, in (Pro-Pro-Gly)10, this collagen-like polypeptide forms a triple-helical structure in $H_2O$ solution with a melting temperature of 24.5 °C, which is increased to 40 °C in $D_2O$. Thermodynamic analysis showed that these findings are

due to an increase in the enthalpy of unfolding in $D_2O$ verse $H_2O$. A significant lowering of the potential energy was also found in hydrated polypeptide in $D_2O$ solvent.

## *7.1.2 Nucleic Acids*

Nucleic acids discussed in deuterium oxide thermodynamic effects include RNAase, double-helix (dG-dC)3, RNA, and DNA.

### 7.1.2.1 RNAase

Thermal denaturation of RNAase is accompanied by changes in the states of solvated molecules. The hydration of RNAase is greater in $D_2O$ than $H_2O$ medium.

### 7.1.2.2 RNA Stability

$D_2O$ has been known to stabilize attenuated viral RNA against thermal degradation. In $D_2O$, there is a considerable increase in the stability of the folded basin, which translates into a higher melting temperature in $D_2O$ when compared with $H_2O$. $D_2O$ strengthens the hydrogen bonding network in the solvent and lengthens inter-residue water-bridge lifetime.

### 7.1.2.3 Helix-Coil Transition

The helix-to-coil denaturation transition in DNA has also been studied in mixed solvents. The melting transition temperature was found to be 94 °C in 4% mass fraction DNA/deuterated solvent. DNA structural information obtained included the characteristics of inter-distance between hydrogen-containing (deoxyribose sugar-amine base) groups.

### 7.1.2.4 Double Helix Formation

Thermodynamic studies of double helix formation by (dG-dC)3 in $H_2O$ and $D_2O$ reported the enthalpies of −56.9 kcal/mol of helix in $H_2O$ and − 62.7 kcal/mol of helix in $D_2O$.

### *7.1.3 Lipids*

Deuterium oxide thermodynamic effects on lipid bilayer structure and phase transition as described below include dipalmitoyl-, distearoyl-, and dihexadecyl-phosphatidylcholines.

#### 7.1.3.1 Bilayer Structure

Studies of bilayer phase transitions in phospholipids (dipalmitoyl-, distearoyl-, and dihexadecyl-phosphatidylcholines) showed that the substitution of $H_2O$ by $D_2O$ affects the pretransition temperatures of lipid bilayers. Thermodynamic analysis also revealed that the substitution of $H_2O$ by $D_2O$ causes shrinkage of the molecular area of phospholipid at bilayer interface, due to the difference in bond strength between deuterium and hydrogen bonds.

#### 7.1.3.2 Phase Transition

Investigations of thermotropic transitions of dihexadecyl-phosphatidylcholine dispersions in $H_2O$ and $D_2O$ revealed that the lipid transition temperature between interdigitated gel phase and ripple phase is lower in $D_2O$ than in $H_2O$, but the transition between the ripple phase and fluid phase in $D_2O$ occurs at a temperature slightly higher than in $H_2O$.

In contrast, the transition temperature between the gel phase and ripple phase in dipalmitoyl-phosphatidylcholine dispersions was found to be higher in $D_2O$ than in $H_2O$, suggesting that the interdigitated gel phase is more stable in $H_2O$ than in $D_2O$.

### *7.1.4 Bacteria*

Comparative studies of solvent isotope effects on macromolecular dynamics were also performed in *E. coli* bacteria. The result revealed that the flexibility of *E. coli* bacteria is smaller in $D_2O$ than in $H_2O$.

In summary, the above-discussed deuterium oxide thermodynamics effects on proteins, nucleic acids, lipids, and bacteria are briefly included in Table 7.1.

## 7.2 Deuterium Oxide Kinetic Effects

The kinetic isotope effect is a phenomenon associated with isotopically substituted molecules that exhibit different reaction rates when one of the atoms in the reactants is replaced by one of its isotopes. Accordingly, the kinetic isotope effect is

**Table 7.1** Deuterium oxide thermodynamic effects on biomolecules

| Biomolecules | Deuterium oxide thermodynamic effects |
|---|---|
| *E. coli* bacteria | Flexibility is smaller in $D_2O$ than in $H_2O$ |
| Proteins | |
| Lactate dehydrogenase | $D_2O$ stabilizes the enzyme and inhibits subunit exchange |
| Drosophila signal transduction | $D_2O$ exhibits stabilizing effect |
| Tubulin | Stabilization of its secondary structure |
| Bacteriorhodopsin isomerization | Lower Gibbs free energy in $D_2O$ than in $H_2O$ |
| Biotin repressor | Lower Gibbs free energy of dimerization in $D_2O$ than in $H_2O$ |
| Nucleic acids/peptides | |
| (Pro-Pro-Gly)10 | Higher melting temperature in $D_2O$ |
| RNAase | Hydration is greater in $D_2O$ than in $H_2O$ |
| Viral RNA | $D_2O$ stabilizes thermal degradation |
| Lipids | |
| Dipalmitoyl-, distearoyl-, and dihexadecyl-phosphatidyl-cholines | $D_2O$ causes shrinkage of molecular area of lipid bilayers |
| Dipalmitoyl-phosphatidyl-choline dispersions | Lower lipid transition temperature between interdigitated gel and ripple phases in $D_2O$ than in $H_2O$ |

represented by the ratio of rate constants for the reactions involving the light ($k_{light}$) and the heavy ($k_{heavy}$) isotopically substituted reactants as follows:

$$\text{Kinetic isotope effect} = k_{light} / k_{heavy} \text{ or } k_H / k_D \tag{7.1}$$

where $k_H$ and $k_D$ denote rate constants for protium and deuterium reactants.

The issues related to deuterium oxide kinetic effects, including vibrational energies, kinetics effect classification, primary kinetic isotope effects, secondary kinetic isotope effects, and solvent kinetic isotope effects, are addressed below.

### *7.2.1 Vibrational Energies*

Isotope-induced change in reaction rate is a quantum mechanical effect that results from heavier isotope having lower vibrational frequencies as compared to its lighter counterpart.

A greater energetic input is needed for heavier isotope to reach the transition state and consequently to have a slower reaction rate.

Since deuterium has a greater atomic mass than hydrogen, the lower zero-point energy results in higher activation energy and a slower rate for C-D bond cleavage. Accordingly, cleavage of the carbon-deuterium (C-D) covalent bond requires

greater energy than the carbon-hydrogen (C-H) bond. The ratio of the rate of C-H verse C-D bond-cleavage has been reported to have a theoretical limit of 9 at 37 °C.

### 7.2.2 Kinetics Effect Classification

Studies of kinetics isotope effects are important not only in helping the understanding of reaction kinetics but also providing insight into the molecular basis of the reaction mechanism. The effect of reaction rate influenced by an isotopic exchange of the solvent, such as $D_2O$, is referred to as solvent kinetic isotope effect. The deuterium kinetic isotope effect is by far the most well-understood type of the kinetic isotope effect.

Generally, kinetic isotope effects can be divided into three categories: (a) primary kinetic isotope effect, (b) secondary kinetic isotope effect, and (c) solvent kinetic isotope effect, which are discussed below.

### 7.2.3 Primary Kinetic Isotope Effects

A primary kinetic isotope effect can be found when a bond to the isotopically labeled atom is being formed or broken. Therefore, a primary kinetic effect is indicative of breaking or forming a bond to the isotope at the rate-limiting step.

Primary kinetic isotope effects are typically measured for reactions where protium is exchanged for deuterium, where the lighter hydrogen reacts faster than the heavier isotope deuterium, and the energy required to cleavage the carbon-deuterium (C-D) covalent bond is greater than that for the carbon-hydrogen (C-H) bond. Such effects could potentially affect the pharmacokinetics of many drugs that are metabolized by pathways involving C-H bond scission.

### 7.2.4 Secondary Kinetic Isotope Effects

Secondary kinetic isotope effects involve an isotopic atom in which the bond changes during the reaction; however, no bond is broken or formed. The change in the reaction rate is also characterized by the difference in zero-point energies, as in the case of a hydrogen being substituted by a deuterium.

Nevertheless, the change caused by secondary kinetic isotope effect can be either normal or inverse. In normal isotope effects, the rate is slower with the heavy isotope, while inverse ones show faster rates with the heavier atom. Studies of secondary isotope effects have been carried out to determine reaction mechanisms and to elucidate the structure of their transition states.

### 7.2.5 Solvent Kinetic Isotope Effect

The steady-state kinetic parameters $k_{cat}$ and $k_{cat}/k_m$ were found to decrease when the reaction is performed in $D_2O$. For example, primary deuterium isotope effects as found for the reaction of secondary alcohols with nicotinamide adenine dinucleotide (DPN) to give reduced deuterionicotinamide adenine dinucleotide (DPNH) are approximately 1.18. While the secondary isotope effect for the conversion of DPN to DPNH is 0.89.

Moreover, the deuterium solvent kinetic isotope effect ($k_H/k_D$) of human skin fibroblast collagenase was also studied in native and denatured states. Reaction velocity on collagen in solution was slowed by 15–35% ($k_{light}/k_{heavy}$ = 1.2–1.5), as deuterium was substituted for hydrogen in the solvent buffer.

In an extension of the above investigations of reaction kinetics and reaction mechanism, deuterium oxide effects on enzyme-catalyzed reaction were also discussed in cytochrome P450s (CYP450s) catalyze metabolic reactions of many foreign compounds, where C-H bond cleavage is a major feature in these reactions. The presence of a significant primary deuterium kinetic isotope effect appears in many CYP450 reactions, which makes it possible to modulate the in vivo metabolism or toxicity of chemicals by deuterium substitution.

Research on deuterium oxide effects on enzymatic reactions can have many implications for biomedical applications, such as the alteration of drug metabolism. Accordingly, $D_2O$ effects on enzyme-catalyzed reactions are further discussed below.

## 7.3 $D_2O$ Effects on Enzyme-Catalyzed Reactions

Catalytic reactions may refer to a chemical step that is accelerated by lowering an energy barrier. As a catalyst, an enzyme accelerates a chemical reaction without changing the overall thermodynamics. Reaction rates and binding equilibria are sensitive to isotopic substitution, such as hydrogen is substituted by deuterium.

Increased atomic mass alters the bond vibrational environment of reactants. Isotope effects for enzymatic reactions cause a change in rate or equilibrium as a result of an atomic substitution. Primary, secondary, and solvent kinetics isotope effects can be used to elucidate the extent to which a change in binding reaction occurs.

Important parameters in the studies of enzyme-catalyzed reactions are $k_m$ and $k_{cat}$. $K_m$ refers to the substrate concentration at which the reaction velocity is 50% of its maximal value ($V_{max}$). While $K_{cat}$ is the turnover number, that is, the number of substrate molecules each enzyme site converts to product per unit time. One way to measure the catalytic efficiency of a given enzyme is to determine the ratio $k_{cat}/k_m$.

Since $k_{cat}$ describes how many substrate molecules are transformed into products per unit time by an enzyme and $k_m$ describes the affinity of the substrate to the active

site of the enzyme, the ratio of $k_{cat}/k_m$ allows to determine how effective the enzyme is on a particular substrate. The greater the ratio, the higher the rate of catalysis is. Conversely, the lower the ratio, the slower the catalysis is.

### 7.3.1 Primary Kinetic Isotope Effects

CYP450s catalyze oxidation reactions of more substrates than any other group of enzymes. Cleavage C-H bond is a major feature of CYP450 catalyzed reactions. The presence of a significant primary deuterium kinetic isotope effect is evident in these reactions, where hydrogen abstraction is at least partially rate-limiting.

Another enzyme involving primary kinetic isotope effect is tryptophan 2,3-dioxygenase, which is a hemoprotein that catalyzes the first step in the oxidative degradation of tryptophan. A primary $D_2O$ isotope effect of 4.4 on $V_{max}/K_m$ was observed at the optimum pH 7.0. This finding implies that the abstraction of the indole proton is potentially a rate-determining step.

Moreover, the pyruvate dehydrogenase from *Escherichia coli* was also studied in deuterium oxide, which revealed a primary kinetic isotope effect on its overall reaction. The Michaelis constant for pyruvate was nearly unchanged, but the maximum velocity in $H_2O$ and $D_2O$ differed. The proton of the enzyme's carboxylic group exchanges very fast in $D_2O$ solvent.

A number of efforts are in progress to utilize deuterium substitution to alter the metabolism of drugs catalyzed by xenobiotic metabolic enzymes.

### 7.3.2 Secondary Kinetic Isotope Effects

Secondary deuterium isotope effects have been investigated for butyrylcholinesterase – catalyzed hydrolysis of acetyl-L(3)-thiocholine, where L is hydrogen or deuterium. Kinetic isotope effect on $k_{cat}$ indicates that the Michaelis complex is a tetrahedral intermediate. The rate-limiting step is its decomposition step, which supports a mechanism for substrate-activated turnover of acetylthiocholine by butyrylcholinesterase.

Moreover, depending on the substrate concentration, the value of $k_{cat}/k_m$ is in the range of 0.95. These findings imply that the transition state for rate-determining decomposition of the tetrahedral intermediate is stabilized by protonic interactions.

### *7.3.3 Effects on Hydrolysis Reaction*

The steady-state kinetics of ATP hydrolysis was examined in the presence or absence of $D_2O$ as a function of temperature. The steady-state kinetic parameters kcat and kcat/km revealed that kcat/km was independent of temperature in the presence or absence of $D_2O$. Also, a normal isotope effect was observed in plots of kcat versus temperature in the presence of $H_2O$ and $D_2O$ for ATP hydrolysis, which were increased and then leveled off as temperature increased.

In summary, deuterium oxide kinetics effects on biomolecular reactions are presented in Table 7.2, where D and H denote deuterium and hydrogen, respectively.

## 7.4 Deuteration Effects on Biochemical Reactions

Deuteration effects on biochemical reactions include thermodynamic and kinetic effects are discussed below. As an example of the effects on the solvation dynamics, deuterated methanol ($CH_3OD$, $CD_3OH$, or $CD_3OD$) was found to exhibit slower solvation dynamics than normal methanol, where deuteration effect on the OH group occurs due to the effects on hydrogen bonding.

### *7.4.1 Thermodynamic Effects*

#### 7.4.1.1 Protein Stability

Using thermostable model systems, such as the villin headpiece subdomain and the binding domain of protein G, the effects of deuteration of non-exchangeable protons on protein global thermal stability, hydrophobicity, and local flexibility were

**Table 7.2** Deuterium oxide kinetics isotope effects

| Kinetics characters | Deuterium oxide isotope effects |
|---|---|
| Vibrational energy | Lower vibrational frequency in $D_2O$ than $H_2O$ |
| Zero-point potential | Lower zero-point energy for D than H |
| Energy to reach transition state | Higher activation energy for D than H |
| Cleavage of bond | Slower rate for C-D bond than C-H bond |
| Primary kinetic isotope effect | Slower rate constant for C-D bond cleavage than C-H bond |
| Secondary kinetic isotope effect | Slower kinetic rate for D than H |
| Solvent kinetic isotope effect | Decreased $k_{cat}$ and $k_{cat}/k_m$ in $D_2O$ |

$K_{cat}$ is the turnover number (the number of substrate molecule that each enzyme site converts to product per unit time)

$k_{cat}/k_m$ determines the effectiveness of the enzyme (the greater the ratio, the higher the rate of catalysis)

investigated as a function of temperature. The results indicated that deuteration increases motional amplitudes of sub-nanosecond motion in the binding domain of protein G but decreases those in the villin headpiece subdomain.

Moreover, *E. coli* enzyme I(Ntr) is the first protein in the nitrogen phosphotransferase pathway. The effects of substitution of all nonexchangeable protons by deuterium on the properties of I(Ntr) were examined, which revealed that the catalytic function remained unperturbed, but its stability was modulated by deuteration. The deuterated form exhibits a reduction of about 4 °C in thermal stability, where the aromatic residues are particularly sensitive to the effects of deuteration.

In addition, bovine glutamate dehydrogenase requires substrate-induced subunit interactions for maximum catalytic activity. Deuterated glutamate shows a steady-state isotope effect but has little effect on overall stability, whereas norvaline markedly stabilizes the protein.

#### 7.4.1.2 Nucleotide Binding

Investigation of the influence of specific nucleotide binding on the thermal unfolding of ATP synthase from the thermophilic *Bacillus* PS3 revealed an irreversible endothermic transition for ATP synthase in the absence of nucleotides. The thermal denaturation occurs at a transition temperature of 81.7 °C. An exothermic transition due to aggregation processes was also affected by nucleotide binding. Hydrogen/deuterium exchange was clearly affected by specific nucleotide occupancy. Binding induces conformational changes, which shields amide protons to more buried hydrogen-bonded structures.

#### 7.4.1.3 Lipid Transition

Studies with dielaidoylphosphatidyl-ethanolamine (DEPE) or deuterated dipalmitoyl-phosphatidylcholine (DPPC) showed that sphingosine-1-phosphate modifies the gel-fluid transition of the glycerophospholipids, shifting it to lower temperatures and decreasing the transition enthalpy. Low (<10 mol %) concentrations of sphingosine-1-phosphate also have a clear effect on the lamellar-to-inverted hexagonal transition of DEPE by increasing the transition temperature and stabilize the lamellar versus the inverted hexagonal phase.

### *7.4.2 Kinetic Effects*

#### 7.4.2.1 Protein Dynamics

The effects of $D_2O$ on the dynamics of proteins were assessed by buried Trp residues. The results obtained with proteins, including ribonuclease T1, superoxide dismutase, beta-lactoglobulin, liver alcohol dehydrogenase, alkaline phosphatase, and

apo- and Cd-azurin, demonstrated that in most cases $D_2O$ significantly increases the rigidity the native structure. With the exception of alkaline phosphatase, the kinetics of the structure tightening effect of deuteration is rapid compared with the rate of H/D exchange of internal protons. Structure tightening by $D_2O$ is generally amplified at higher temperatures, supporting a hydrophobic nature of the underlying interaction.

#### 7.4.2.2 Enzyme-Lipid Interaction

Cytochrome c oxidase and deuterated lipid palmitoleoyl-phosphatidylcholine were studied by deuterium NMR. These reconstituted particles are of uniform lipid and protein content. However, the spectra clearly show two environments: the less ordered environment and the more restricted environment. The amount of the restricted lipid is temperature dependent, and the rate of exchange of lipid between the free and restricted environments is slower.

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# Chapter 8
# Physical Methods for Investigating $D_2O$ and Deuteration Effects

Substitution of $D_2O$ for $H_2O$ has been shown to affect the structural and dynamic properties of biomolecules. Physical methods that are applied to study deuterium oxide and deuteration effects on the structure and function of biomolecules include nuclear magnetic resonance, infrared, ultraviolet and visible, and fluorescence spectroscopies, as well as other physical methods including neutron scattering, differential scanning calorimetry, circular dichroism, and molecular dynamic stimulation.

Nuclear magnetic resonance (NMR), a powerful tool for studying the physical characteristics of biomolecules, has been applied to determine how the atoms in a molecule are interconnected. When the radio frequency energy that detects the nuclei is removed, the nuclei relax back to their original state and emit an electromagnetic pulse, which is transformed into NMR spectrum. Deuterium NMR has a simple resolution than hydrogen NMR. Therefore, it is feasible to investigate deuterium solvent isotope and deuteration effects on biomolecules.

Infrared spectroscopy can also provide information concerning biomolecular structures, such as infrared bands corresponding to vibration in polypeptide backbone that are sensitive to the conformation. Moreover, a common use of the spectroscopic technique employs the ultraviolet region of the spectrum, where proteins and nucleic acids absorb strongly, such as the absorption of aromatic side chains of phenylalanine, tyrosine, and tryptophan in the region of 270–290 nm.

In fluorescence spectroscopy, after being raised to an excited state by absorption of radiant energy, the molecule returns to the ground state, where the molecule loses part of its energy of excitation, but reradiates a large part of energy which gives rise to the fluorescence. While circular dichroism, another important spectroscopic technique, involves the use of polarized light. When asymmetric biomolecules absorb light, they exhibit a preference for the absorption of left or right circularly polarized light. A right circularly polarized beam interacts differently than a left circular polarized beam, leading to a difference in circular dichroism absorption.

Besides, neutron scattering is also a powerful technique for determining the structure and dynamics of biomolecules in a variety of environmental conditions.

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**Table 8.1** Physical methods for studying deuterium oxide and deuteration effects on biomolecules

| ***Methods for studying $D_2O$ effects*** |
|---|
| Spectroscopies |
| Nuclear magnetic resonance spectroscopy |
| Infrared spectroscopy |
| Fluorescence spectroscopy |
| Ultraviolet spectroscopy |
| Other physical methods |
| Differential scanning calorimetry |
| Circular dichroism |
| Molecular dynamic simulation |
| ***Methods for investigating deuteration effects*** |
| Spectroscopies |
| Deuterium nuclear magnetic resonance |
| Infrared spectroscopy |
| Other physical methods |
| Neutron scattering |
| Differential scanning calorimetry |

Deuteration of biomolecules has a major impact on both quality and scope of neutron scattering experiments. Moreover, differential scanning calorimetric experiments have been performed to investigate thermal properties of biological compounds and their structural stabilities.

In theoretical study, molecular simulation provides useful information from microscopic view for understanding the properties of biomolecules. This technique concerns physical interactions and are performed on computers. Accuracy of force field parameters and simulation time scale are essential to biomolecular dynamic simulation.

The above physical methods for studying deuterium oxide and deuteration effects on biomolecules are summarized in Table 8.1.

## 8.1 Techniques for Studying Deuterium Oxide Effects

The applications of spectroscopic methods in studying deuterium oxide and deuteration effects on biomolecules, including nuclear magnetic resonance; infrared, ultraviolet and visible, and fluorescence spectroscopies; neutron scattering; and differential scanning calorimetry, are described below.

## 8.1.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

Proton NMR spectra of biological molecules are generally dominated by strong proton-proton and proton-carbon dipolar interactions. As a result, the spectra contain a large number of overlapping resonances that are difficult to analyze. To ensure that the solvent does not interfere with the NMR spectrum of the sample, $D_2O$ solvent has been widely used for NMR spectroscopy to null the signal interference from the solvent. Besides, deuterium NMR and proton NMR require different operating frequencies at a given magnetic field strength. Consequently, deuterium NMR absorptions are not detected under the conditions used for proton NMR.

Deuterium NMR investigations of membrane surface charge of the glycerol headgroup of dimyristoyl-phosphatidylglycerol showed the characteristics of random dispersions of liquid-crystalline lipids in a bilayer configuration. In membrane proteins, technical challenges are present for structural studies due to their requirement for lipid environment. NMR offers the potential of determining the structures of membrane proteins in their native environment of phospholipid bilayers under physiological conditions, where NMR enables to characterize the structure and dynamics of backbone and side chain of proteins alone or in complexes with other biopolymers.

Examples of the applications of NMR spectroscopy to investigate protein, nucleic acid, lipid, and membrane are presented as follows.

### 8.1.1.1 Lipid-Protein Complex

Deuterium magnetic resonance spectra of lipid-protein complex, containing cytochrome c oxidase isolated from beef heart mitochondria and the deuterated lipid palmitoleoyl-phosphatidylcholine, revealed two environments characterized by distinctly different order parameters. The less ordered environment shows a splitting similar to that of the pure lipid alone at a given temperature. While the more restricted environment appears to be induced by the presence of the protein.

### 8.1.1.2 Nucleic Acid

To provide information regarding the conformational flexibility of nucleic acids, deuterium NMR was carried out to study single-stranded and double-stranded polynucleotides with deuteration at the 8-position of the base in solution. Hydration to the extent of eight molecules of water per nucleotide results in the disappearance of the deuterium signal, which suggests that polynucleotides are hydrated to a degree of approximately eight molecules of water per base pair.

#### 8.1.1.3 Lipid

Using selectively deuterated samples, the order parameters for the methylene and methyl segments of phospholipid dispersions have also been investigated with deuterium NMR. Acyl chain segmental order parameters were determined for perdeuteriostearic acid intercalated as a molecular probe in the lamellar liquid crystalline phase of phosphatidylcholine-cholesterol-water mixtures. The deuterium NMR data also showed that cholesterol induces a high degree of order in the acyl chains of the phospholipid, while maintaining the general profile of high order near the head group as well as relatively low order in the middle of the bilayer.

Moreover, the effects of anesthetics (1-octanol and 1-decanol) on deuterated palmitoyl-phosphatidylcholine aqueous dispersions have been studied by deuterium NMR. 1-Octanol and 1-decanol were found to depress and broaden the main lipid transition. However, 1-alkanol had no significant effect on the profile of the carbon-deuterium bond order parameter.

The effects of alpha-tocopherol (vitamin E) on aqueous multilamellar dispersions of sn-2-substituted palmitoyl-phosphatidylcholine have also been investigated by deuterium NMR. The main gel to liquid-crystalline phase transition was found to be progressively broadened. Its onset temperature is lowered by increasing concentrations of alpha-tocopherol.

Deuterium NMR was also applied to map the phase boundaries of mixtures of cholesterol and perdeuterated 1,2-dipalmitoyl-sn-glycero-3-phosphocholine. Three distinct phases were identified, the liquid-crystalline phase, the gel phase, and the beta phase (a high cholesterol phase), where the liquid-crystalline phase is characterized by highly flexible phospholipid chains, the gel phase has much more rigid lipid chains, and the beta phase is characterized by highly ordered chains and symmetric reorientation.

#### 8.1.1.4 Membrane

Deuterium magnetic resonance of membranous systems differs from conventional proton NMR. Some hydrogen atoms in a membrane molecule are replaced by deuterium, which consists of a few resonances. Due to the smaller magnetic moment of the deuteron, the dipolar couplings are much reduced compared to the corresponding proton spectra.

The effect of ergosterol, an important component of fungal plasma membranes, on the physical properties of dipalmitoyl-phosphatidylcholine bilayers was also studied by deuterium NMR, where the sn-1 chain of lipid was deuterated. The phase diagram exhibits ordered plus disordered lipid regions, which demonstrates that liquid-ordered domains present in liquid crystalline membranes contain ergosterol.

### 8.1.2 Infrared (IR) Spectroscopy

Examples of the applications of IR spectroscopy to investigate body water, peptide, polysaccharide, lipid, and membrane are described below.

#### 8.1.2.1 Body Water

An IR spectroscopy using tracer doses of $D_2O$ for determining the total body water was reported. The practical advantages of this method are a simple analysis that permits repeated measurements over brief periods. Such administration of smaller oral $D_2O$ doses (5–6 g) can provide reliable total body water values without an undue buildup of background deuterium levels in the body.

#### 8.1.2.2 Peptide

The conformational characteristics of the Gly-l-Ala-methyl amide dipeptide in $D_2O$ solution were also investigated by infrared spectroscopy to determine the mutual orientation of the two C═O bonds and the dynamics due to solute-solvent interactions. The interconversion between the different conformations occurs on time scales longer than the vibrational lifetime. Such observation in spectra is attributed to the solvent dynamics.

#### 8.1.2.3 Polysaccharide

Infrared spectra in the carbonyl region have been obtained for aqueous solutions of glycosaminoglycans. In $D_2O$ solution, the uronic carboxylate and acetamido groups absorb at characteristic frequencies. In acidic solution, the amide bands remain substantially unmodified, whereas those of the carboxylate anion disappear and are replaced by a single band due to the undissociated carboxyl. Infrared data for the uronic acid and 2-acetamido-2-deoxyhexose content of glycosaminoglycans are in reasonable agreement with those expected from the established structures.

#### 8.1.2.4 Lipid

Aqueous dispersions of cholesterol-containing phosphatidyl-ethanolamine bilayers were also examined by infrared spectroscopy, which revealed that the thermotropic events observed with mixtures of low cholesterol content are analogous to the gel to liquid-crystalline phase transitions exhibited by the pure phosphatidyl-ethanolamine. Low levels of cholesterol in lipid bilayers cause progressive reductions in the temperature, enthalpy, and overall cooperativity of the lipid hydrocarbon chain-melting phase transition.

#### 8.1.2.5 Membrane

Infrared spectroscopy, deuterium NMR and calorimetry were employed to investigate the effect of ethanol on a model membrane composed of lipids mixed with N-palmitoyl-sphingosine, palmitic acid, and cholesterol. Ethanol was found to influence the membrane in a dose-dependent manner by disrupting packing and increasing lipid motion.

### *8.1.3 Fluorescence Spectroscopy*

Examples of the applications of fluorescence spectroscopy to study protein, peptide, lipid, and vaccine are presented as follows.

#### 8.1.3.1 Protein

Fluorescence spectroscopic studies of tubulin were aimed to elucidate the mechanism of stabilization of the protein by deuterium oxide. The rate of decrease of tryptophan fluorescence during aging of tubulin at 4 and 37 °C was significantly lower in $D_2O$ than in $H_2O$. As temperature increases, the rate of decrease of fluorescence at 335 nm and the change of circular dichroism at 222 nm were lesser in $D_2O$.

#### 8.1.3.2 Peptide

The lower critical solution temperature was investigated as a function of the chain length of elastin-like polypeptides in both $D_2O$ and $H_2O$. Differences in the lower critical solution temperature values with $D_2O$ and $H_2O$ were correlated with secondary structure formation of the polypeptide chains.

#### 8.1.3.3 Lipid

Fluorescence spectroscopy has also been applied to investigate phospholipid bilayers hydrated with $D_2O$. Different biophysical properties were compared with hydration by $H_2O$ on 1,2-dimyristoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine membranes. $D_2O$ solvent isotope effects on headgroup hydration and mobility, lateral lipid diffusion, and lipid backbone packing were also examined. Time-dependent fluorescence shift experiments showed significantly slower dynamics and lower hydration of the headgroup region for a bilayer hydrated with $D_2O$.

#### 8.1.3.4 Vaccine

The mechanisms underlying the thermal stabilization of poliovirus by $D_2O$ were investigated by fluorescence spectroscopy. Three serotypes of Sabin oral poliovirus vaccine strains were examined by fluorescence steady-state polarization, which revealed that the conformation of poliovirus capsid is sensitive to $D_2O$ medium. The rigidity of poliovirus conformation is increased in $D_2O$, indicating that the exposure of poliovirus tryptophan residues to water is modified by $D_2O$. The involvement of hydrogen bonding in the $D_2O$ solvent effect was also demonstrated by the greatly increased value of relative fluorescence intensity.

### *8.1.4 Ultraviolet (UV) Spectroscopy*

Examples of the applications of UV spectroscopy to investigate protein and peptide are described below.

#### 8.1.4.1 Protein

The absorption spectra of proteins are characterized by two absorptions in the ultraviolet region, one at 2800 A and the other near 2000 A. The former has been ascribed to the phenol, indole, and phenyl groups of tyrosine, tryptophan, and phenylalanine. The latter may be attributed to peptide bonds. In addition to the well-known absorption bands due to aromatic amino acid residues, several proteins exhibited a new absorption band at about 2650 A when dissolved in $D_2O$. This additional band was also found in $D_2O$ solution of clupein, a protein containing no aromatic amino acid residue. This 2650 A band was also found in $D_2O$ solutions of six of ten amino acid examined.

#### 8.1.4.2 Peptide

A time-dependent change in the UV absorbance at 285 nm of the phenol ring of tyrosine has been observed when tyrosine was transferred from $H_2O$ to $D_2O$ medium to determine the rate constant of the hydrogen-deuterium exchange reaction of the tyrosine OH group. The visible-ultraviolet spectra of 13 anilines have also been measured in $H_2O$ and $D_2O$. Spectra shifts of 180 $cm^{-1}$ for primary aniline and 40 $cm^{-1}$ for tertiary aniline are attributed to differences in solvation by specific hydrogen bonding at the amino group.

Other methods applied to investigate deuterium oxide and deuteration effects on biomolecules, including differential scanning calorimetry, molecular dynamic simulation, and circular dichroism, are also discussed.

### *8.1.5 Differential Scanning Calorimetry*

Examples of the applications of differential scanning calorimetry to study protein, peptide, nucleic acid, lipid, and virus are presented below.

#### 8.1.5.1 Protein

Differential scanning calorimetric experiments have been applied to study the structures and conformations of proteins and peptides. Studies of tubulin showed that the temperature of tubulin unfolding is 58.6 and 62.17 °C in $D_2O$ and 55.4 and 59.35 °C in $H_2O$, respectively. The results implied that $D_2O$ stabilizes thermal unfolding of tubulin.

The influence of $D_2O$ solvent substitution of $H_2O$ on the structural stability of globular proteins was also determined by differential scanning calorimetry. Two types of globular proteins: lysozyme with relatively strong internal cohesion (hard globular protein) and bovine serum albumin with conformational adaptability (soft globular protein) were analyzed thermodynamically. Both proteins tend to be more stable in $D_2O$ compared to $H_2O$. Such increase in protein stability was attributed to $D_2O$ being a poorer solvent for nonpolar amino acids than $H_2O$, which implies that the hydrophobic effect is larger in $D_2O$.

The conformational stability of beta-lactoglobulin is enhanced in $D_2O$ over that in $H_2O$, as shown by an increase in peak temperature by calorimetric thermogram. However, the effects of pH and salt concentration on the heat-induced aggregation are similar in $H_2O$ and $D_2O$, suggesting that the mechanism of heat-induced aggregation of beta-lactoglobulin is not significantly affected by the replacement of $H_2O$ with $D_2O$.

#### 8.1.5.2 Peptide

The thermal melting of a dicyclic 29-residue peptide that has helix-stabilizing side-chain to side-chain covalent links at each terminal was also investigated by differential scanning calorimetry to allow an analysis of the thermodynamics of helix formation. The helix unfolding was found to proceed with a small positive heat capacity increment, consistent with the solvation of nonpolar groups upon helix unfolding. These results suggested that hydrogen bonds are not the only factors responsible for the formation of the alpha helix. Hydrophobic interactions are also believed to play a role in its stabilization.

Thermodynamic data obtained by differential scanning calorimetry also revealed that there is a larger change in the lower critical solution temperature between $H_2O$ and $D_2O$ for elastin-like polypeptides, which is attributed to forming a larger amount of beta-turn or aggregate structure. Hydrogen bonding rather than hydrophobicity

was believed to be the key factor in the stabilization of the collapsed state of elastin-like peptides in $D_2O$ compared with $H_2O$.

Moreover, the thermal retinal isomerization of bacteriorhodopsin in the purple membrane was also investigated in $H_2O$ and $D_2O$. The changes in enthalpy from the initial state to the transition state were determined to be 24.7 and 20.1 kcal/mol in $H_2O$ and $D_2O$, respectively. The Gibbs free energy of trans to cis thermal isomerization reaction in $D_2O$ is 0.4–0.7 kcal/mol lower than that in $H_2O$. These deuterium solvent isotope effects imply that the hydrogen bonding in the transition state of bacteriorhodopsin is stronger than that in the initial state.

#### 8.1.5.3 Nucleic Acid

In the studies of nucleic acids, the thermodynamics of double-helix formation by (dG-dC)3 were measured in $H_2O$ and $D_2O$. The averages of the enthalpies measured by spectroscopy are −56.9 and −62.7 kcal/mol of helix in $H_2O$ and $D_2O$, respectively, as compared to calorimetric values of −59.6 and −65.8 kcal/mol of helix, which shows that spectrophotometric values are in good agreement with calorimetric results.

#### 8.1.5.4 Lipid

Differential scanning calorimetry has also been applied to study lipid bilayers. In the liquid-crystalline state of lipid bilayers, cyclosporin A causes an increase in the order parameters along the acyl chains. This finding suggested that cyclosporin A is located along the acyl chains of the phospholipid. Small decreases in the main transition temperature and associated enthalpy were observed in the presence of cyclosporin A.

Thermodynamic studies of deuterated phospholipids in $H_2O$ were also conducted using deuterated lipid bilayer vesicles prepared from aqueous dispersions of 1,2-dimyristoyl-d54-, 1,2-dipalmitoyl-d62-, and 1,2-distearoyl-d70-phosphatidylcholines. The effects of deuterium solvent and deuterium alkyl-chain substitution on deuterated phospholipids include the temperature, the thermodynamic parameters, and the cooperative melting unit of the main lipid phase transition from the gel to the liquid-crystalline phase. Examinations of interactions in deuterated compounds revealed that the deuterium solvent effect increases the structural stability of deuterated lipid vesicles, due to an enhancement of hydrophobic interaction in $D_2O$.

Moreover, thermotropic transitions of dihexadecyl-phosphatidylcholine dispersions in $H_2O$ and $D_2O$ were also investigated by differential scanning calorimetry. The transition temperature between interdigitated gel phase and ripple phase is lower in $D_2O$ than in $H_2O$. While the transition between the ripple phase and fluid phase in $D_2O$ occurs at a temperature slightly higher than in $H_2O$. On the other hand, in dipalmitoyl-phosphatidylcholine dispersions, transition temperature between

lamellar gel phase and ripple phase is higher in $D_2O$ than in $H_2O$. These results suggested that the interdigitated gel phase is more stable in $H_2O$ than in $D_2O$.

The incorporation of low levels of cholesterol into phosphatidyl ethanolamine bilayers causes progressive reductions in the temperature, enthalpy, and overall cooperativity of the lipid phase transition. These differences in the effects of cholesterol on phospholipid thermotropic phase behavior are attributed to stronger electrostatic and hydrogen-bonding interactions at the surfaces of phosphatidyl ethanolamine bilayers.

#### 8.1.5.5 Virus

Among a few calorimetric studies of virus, $D_2O$ was found to reduce the interaction of the virus with water, leading to a decline in the extent of water penetration into the poliovirus capsid. Maintaining the assembly and conformation of the virus and reducing the swelling of the virus capsid were found to be key factors in increasing the thermostability of poliovirus.

### *8.1.6 Circular Dichroism*

Examples of the applications of circular dichroism to study protein, peptide, and nucleic are discussed below.

#### 8.1.6.1 Protein and Peptide

Circular dichroism spectra were applied to elucidate the mechanism of stabilization of tubulin by deuterium oxide, which suggested a stabilization of protein secondary structure in $D_2O$. The number of cysteines was decreased to a lesser extent in $D_2O$ than in $H_2O$.

Studies of the thermal melting of a dicyclic 29-residue peptide, with helix-stabilizing side-chain to side-chain covalent links at each terminal, revealed that alpha-helical undergoes a reversible transition from the folded to the disordered state with increasing temperature. The temperature dependency of the ellipticity at 222 nm is well fit by a two-state model, indicating a cooperative melting transition from −10 to 100 °C.

Comparisons of the structural and dynamic features of polyglutamic acid and polylysine molecules were also carried out in $H_2O$ and $D_2O$. Distinctive differences in structure and transition energies between the enantiomeric polypeptides were detected in $H_2O$. But these differences were abolished in $D_2O$, suggesting that such deviation is induced in part by different hydration in $H_2O$ and $D_2O$.

#### 8.1.6.2 Nucleic Acid

Circular dichroism is also sensitive to the conformational states of nucleic acids. It has been applied to study various features of nucleic acid structures, such as structural arrangements and stabilization as well as transitions between structural states. Circular dichroism spectra of tri-alanine as measured in $D_2O$ and $H_2O$ showed a somewhat disordered flat beta-strand conformation. A thermodynamic analysis revealed that enthalpic contributions of about 11 and 17 kJ/mol stabilize polyproline in $D_2O$ and $H_2O$, respectively. Such isotope effect of a higher occupation of polyproline in $H_2O$ with respect to $D_2O$ strongly suggests that the hydrogen-bonding network involving the peptide and water molecules in the hydration shell plays a major role in stabilizing this conformation.

### *8.1.7 Molecular Dynamic Stimulation*

Although the molecular dynamic simulation method has been actively applied to investigate chemical systems, its applications to study biomolecules in deuterium oxide are still lacking. However, in the case of lipid, molecular dynamic simulation was carried out to examine the effects of substituting $D_2O$ for $H_2O$ on the properties of dipalmitoyl-phosphatidylcholine bilayer.

The results revealed that the membrane core and the membrane-water interface are affected by replacing $H_2O$ with $D_2O$. Nevertheless, membrane compactness, acyl chain order, and numbers of lipid-water bonds are much less affected. Moreover, the lifetimes of such interactions are much longer in $D_2O$ than in $H_2O$, implying a slightly better ordering in $D_2O$ compared with the $H_2O$. Such deuterium isotope effects have been assigned to the actions of the longer life of deuterium bonding to headgroups of the dipalmitoyl-phosphatidylcholine bilayer.

## 8.2 Physical Methods for Studying Deuteration Effects

Probing biomolecules using deuteration in which hydrogen atoms are replaced by deuterium is useful in a wide range of biophysical techniques. Selectively deuterated versions of biomolecules have many applications, especially in studying the structure and dynamics of biomolecules. Deuterium NMR and neutron scattering are particularly useful when parts of the biomolecular structure are deuterated. Besides, infrared spectroscopy was also applied to determine biomolecular structures at different stages of hydrogen/deuterium exchange. Investigations of biomolecules by these physical methods are briefly discussed below.

### *8.2.1 Neutron Scattering*

A characteristic property of the neutron is its sensitivity to detecting hydrogen and distinguishing it from its isotope deuterium. Neutron scattering is a powerful technique for determining the structure and dynamics of biomolecules in a variety of environmental conditions. Deuteration of biomolecules has a major impact on both quality and scope of neutron scattering experiments.

Neutron scattering was applied to investigate the structural characteristics of protein, lipid, and membrane, which are extracted from cells grown in standard and deuterated culture media. The distinctive molecular composition of deuterated and protiated extracts can induce different structural organization of their isolated biomolecules.

#### 8.2.1.1 Peptide and Protein

Neutron diffraction measurements of fully deuterated C-phycocyanin have been carried out to determine the average coherent structure factors and the corresponding radial distribution functions of this protein. The changes in distribution functions observed in hydrated samples depend strongly on the level of hydration and most of these changes are due to water-protein interactions.

Studies of halobacterium halobium, in which all valines or phenylalanines are present in deuterated form, showed that valine is distributed toward the periphery of a single bacteriorhodopsin molecule, while phenylalanine is distributed toward its center. These findings lead to the conclusion that the charged and polar groups of the bacteriorhodopsin molecule tend to lie at the molecular interior, away from contact with lipid. On the other hand, the nonpolar surfaces are directed outward, making contact with the lipid regions.

To study cytochrome b5 reconstituted into small lipid vesicles made of a highly deuterated phospholipid, small-angle neutron diffraction patterns were collected in a $H_2O$-$D_2O$ mixture from vesicles consisting of lipid and cytochrome b5. The results revealed that the protein penetrates the lipid bilayer, where the hydrophobic domain is distributed across it.

#### 8.2.1.2 Lipid

Cholesterol is a major component of mammalian cells, where it plays a critical role in membrane permeability, rigidity, and dynamics. Neutron scattering studies of perdeuterated cholesterol provide a unique way of probing the structural and dynamical properties of the lipoprotein complexes.

In characterization of the effect of ergosterol on lipid multilayers, deuterated phospholipids were extracted from the yeast *Pichia pastoris*. The effect of ergosterol on membranes showed that, in contrast to cholesterol in synthetic phospholipid membranes, the presence of ergosterol up to 30 mol % in yeast phospholipid membranes only slightly alters the multilayer structure.

Moreover, the structural arrangement of cholesteryl esters in human plasma low-density lipoproteins has also been studied by selective deuteration. Low-density lipoproteins were labeled by in vitro exchange with two different kinds of deuterated cholesteryl esters, one labeled in the fatty acyl chain and the other in the branched side chain of cholesterol. Neutron scattering data from deuterated and protonated low-density lipoproteins were compared to identify the locations of the fatty acyl and cholesterol side-chain moieties. The results revealed that the cholesteryl nuclei are situated more distantly from the center than the fatty acyl chains.

## *8.2.2 Deuterium Nuclear Magnetic Resonance*

*Hydrogen, deuterium, and tritium are three isotopes of hydrogen that are used in NMR spectroscopy, where each isotope resonates at different frequency ranges. Since the spectrometer transmits and receives over a limited frequency range, only one isotope is observed at a time. Deuterium magnetic resonance spectroscopy* is a noninvasive technique that allows the detection of *deuterated* biomolecules, including protein, lipid, and carbohydrate as described below.

### 8.2.2.1 Protein

Deuterium solvent isotope effects have been observed on the kinetics of lysozyme. The rates of distinct phases of enzyme folding were changed in $D_2O$ compared with $H_2O$, while the rates were essentially independent of whether backbone amide nitrogens were protiated or deuterated. The isotope effect on the rate-determining step was found to be qualitatively reversed at low pH, where changes in environments disappear as carboxylate groups are protonated.

Site-directed deuterium NMR spectroscopy was used to investigate the structure of the retinal within the binding pocket of bovine rhodopsin. The conformation of retinal bound to the G protein-coupled receptor rhodopsin is linked to its photochemistry, which initiates the visual process.

Deuterium NMR has been applied to investigate the effects on deuterated dimyristoyl phosphatidylcholine bilayers in the presence of proteins, including myelin proteolipid apoprotein, cytochrome b, and cytochrome c oxidase. The results revealed that the transition temperature of lipid bilayers between the gel and liquid crystal phases is reduced in the presence of these proteins, while lipid hydrocarbon chains are prevented from crystallizing by the protein incorporated in the membrane.

In addition, using membranes reconstituted with specifically deuterated dimyristoyl-phosphatidylcholines, the influence of myelin proteolipid apoprotein on the order and dynamics of lipid chain was also studied by deuterium NMR. In the lipid fluid phase, the protein was found to exhibit little influence on either the chain order or the population of gauche rotational isomers but strongly retard the chain dynamics.

#### 8.2.2.2 Lipid

Selectively deuterated N-palmitoyl sphingomyelins were investigated by deuterium NMR to elucidate the backbone conformation and the interaction of the sphingolipids with glycerophospholipids. Selective deuteration at the acyl chain carbons was performed. Profiles of C-D bond order parameters were derived for sphingomyelin and sphingomyelin-phosphatidylcholine mixtures. In the liquid-crystalline state, the N-acyl chain of sphingomyelin alone showed significantly more configurational order than the chains of homologous di-saturated or monounsaturated phosphatidylcholines.

Moreover, the dynamic molecular lipid chain conformations in fully hydrated dimyristoyl phosphatidylcholine-dimyristoyl glycerol mixtures were also investigated by deuterium NMR spectroscopy using mixtures of lipid compositions. The gel phase was found to exhibit a lamellar structure with the lipid chains rotating about the molecular long axis. In the fluid lamellar phase of myristoyl phosphatidylcholine-dimyristoyl glycerol mixture, the profile of segmental chain flexibility is similar to that in single-component phospholipid bilayers.

#### 8.2.2.3 Cellulose

In vivo generated deuterated bacterial cellulose cultivated from deuterated glycerol in $D_2O$ medium was analyzed by NMR spectroscopy for deuterium incorporation. Near equal deuteration at sites of the glucopyranosyl ring was found. Despite a high level of deuterium incorporation, no significant differences in the molecular and morphological properties were observed for the deuterated and protiated bacterial celluloses.

### *8.2.3 Infrared Spectroscopy*

Examples of the applications of infrared spectroscopy to investigate protein, peptide, and nucleotide are discussed below.

#### 8.2.3.1 Protein

FTIR spectroscopy was employed to determine secondary structures of proteins at different stages of hydrogen/deuterium exchange process. The results consistently indicate an improvement of secondary structure predictions for the α-helix, while the β-sheet fraction is better predicted in non-deuterated conditions.

#### 8.2.3.2 Lipid

FTIR spectroscopy is also a powerful and convenient technique for studying the structure and organization of membrane lipids in their various polymorphic phases. This spectroscopic technique yields information about the conformation and dynamics of all regions of the lipid molecule, simultaneously without the necessity of introducing extrinsic probes. FTIR was also used to study the thermotropic phase behavior of binary lipid mixtures composed of deuterated phospholipids and lipopolysaccharides.

Studies of the thermotropic behavior of hydrated multibilayers of deuterated 1,2-dipalmitoyl-sn-glycero-3-phosphocholine revealed no significant conformational disorder introduced into the acyl chains in the lipid gel phase. However, the pretransition effect is large in the central segments of the acyl chains, which demonstrates that the interchain interactions are more pronounced in this region than in the center of the bilayer.

Furthermore, investigation of mixtures of cholesterol with dimyristoyl phosphatidylserine or its deuterated lipid demonstrated that the orientation of deuterated methylene symmetric and asymmetric bands of the deuterated lipid is little influenced by cholesterol. In the polar region of the lipid, no effect of cholesterol on the dichroic ratios of carbonyl and phosphate vibrations was detected.

#### 8.2.3.3 Nucleotide

The influence of specific nucleotide binding on the thermal unfolding of ATP synthase from the thermophilic *Bacillus* was characterized by FTIR spectroscopy, which showed that hydrogen/deuterium exchange was affected by specific nucleotide occupancy.

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# Part V
# Exploration of Deuterium Oxide and Deuteration in Health-Related Research

# Chapter 9
# Living Cells Grown in Deuterium Oxide for Deuteration

The effects of heavy water on various organisms have been investigated. Some other organisms are unable to grow in heavy water, while others have no difficulties. Depending on the concentration, living cells tend to exhibit species specificity to their tolerance to deuterium oxide. Algae and bacteria can grow in almost 100% $D_2O$. Mice were reported to have a normal life span and normal body weight after drinking 30% $D_2O$.

However, tissue culture cells incubated at 43 °C show a dramatic decrease in cell survival compared with cells incubated at lower temperatures. Mitosis of a fertile egg of sea urchin is completely inhibited by 75% $D_2O$. Drinking 30% $D_2O$ was found to inhibit the tumor growth by a factor of 0.4–0.5, as compared to the growth of the same tumor in the control animal that drinks normal water.

While a small amount of $D_2O$ exhibits low toxicity toward mammals, a higher concentration of deuterium oxide (usually >20% of body weight) can be toxic to animals and animal cells. Mammals do not survive more than 35% of $D_2O$ substitution for normal water in their body fluids for a long period of time.

Replacement of water in the culture medium with deuterium oxide produces a dose-dependent reduction in hyperthermic cell death, suggesting that the mechanism of hyperthermic cell death involves a hydration-dependent phase transition in membrane lipids. This effect is called hyperthermic cell death.

Moreover, studies of the effects of deuterium oxide on cell growth and vesicle transport in rat basophilic leukemia cells revealed that cells cultured with 15 moles/l deuterium oxide showed decreased cell growth, which are attributed to cells not doubling their DNA content. At the cellular level, $D_2O$ may also affect membrane functions. However, the mechanism of the toxicity of $D_2O$ and the effects of $D_2O$ on cellular events have not been fully understood.

$D_2O$ is more toxic to malignant than normal animal cells. It was also found that deuterium oxide reduces salt- and ethanol-induced hypertension in rats. In addition, $D_2O$ was applied for measuring water spaces in humans and other animals. The

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adaptation to $D_2O$ medium was also found to regulate amino acids and proteins with a reduction in growth rate.

By optimizing expression media and cell culture conditions, high levels of deuterated carbonic anhydrases were produced with about 70% deuterium incorporation. The obtained deuterated proteins exhibit reduced solubility and thermal stability. The H/D kinetic isotope effect appears as the major limiting factor of cellular functions under deuteration.

The substitution of D for H in oxidation-sensitive positions of cellular components may protect against oxidative attack, such as deuterated nucleosides and proteins that are protected from oxidative damage. Oxygen-reactive species are toxicants that lead to protein damage and genomic instability. Most of the oxidative regions involve a cleavage of C-H bonds and H abstraction.

The subjects concerning the growth of living cells in $D_2O$, the isolation of deuterated biomolecules from cells grown in $D_2O$, the toxicity of $D_2O$ to living cells, the antineoplastic effect of $D_2O$, and other related subjects are discussed below.

## 9.1 Growth of Organisms in $D_2O$

Studies of the growth of different organisms in various concentrations of $D_2O$ revealed that the growth is increasingly inhibited in higher concentrations as compared to lower concentrations. The growth of tissue cultures in $D_2O$ is also temperature dependent. Tissue culture cells incubated at 43 °C showed a dramatic decrease in cell survival compared with cells incubated at lower temperatures.

There seems to exhibit species specificity to cell tolerance to heavy water. Some organisms are unable to grow in $D_2O$, while others have no difficulties. A broad variety of morphological and physiological changes have been observed in deuterium-treated cells and organisms, including changes in fundamental processes such as cell division or energy metabolism.

For example, in vivo replacement of hydrogen by deuterium induces a stress response, reduces cell growth, and impairs cell division in organisms such as *Chlamydomonas reinhardtii*, a green unicellular alga. The effects of deuterium on cell cycle progression in *C. reinhardtii* cultures were cultivated in about 80% $D_2O$ medium. Impaired cell cycle progression in deuterated cultures was observed, such as starch and lipid accumulations. This alga is a good model to study the effect of deuterium on growth and division.

Moreover, in the root tissue of winter rye seedlings equilibrated with $D_2O$, $D_2O$ was found to suppress the absorption of water and $K^+$. The higher the $D_2O$ concentration, the greater the suppression. In addition, *Lemna minor* fronds in 50% $D_2O$ rapidly undergo a loss of soluble protein, which is due to the inhibition of protein synthesis as well as an increase in protein degradation. In plants grown for 3–6 days in 50% $D_2O$, protein synthesis is inhibited by 20%, and the rate constant of degradation is 2–3 times that measured in $H_2O$.

The growth of algae, moss, yeast, bacteria, and animal cells in deuterium oxide medium is briefly described below.

### 9.1.1 Algae

Algae, such as *Chlorella vulgaris*, *Chlorella pyrenoidosa*, and *Scenedesmus obliquus*, can be grown in 99.6% deuterium oxide media. Their rate of growth is fast enough to make it possible to use them as a practical source of biosynthesized fully deuterated compounds.

In blue-green algae, *Plectonema calothricoides* and *Phormidium luridum*, normal and deuterated phycocyanins can be isolated to investigate the differences in structure and stability between normal and deuterated proteins. Moreover, alga *Chara gymnophylla* was grown in $D_2O$ for use in studying membrane excitation followed by potassium leakage.

### 9.1.2 Moss

Mosses are green plants somewhat similar to algae, except that they have a complex structure that resembles stems and leaves. They contain chlorophyll and can manufacture their own food. Moss *Funaria hygrometrica* is able to grow in up to 90% $D_2O$. Its ability to tolerate such high concentrations of $D_2O$ makes it an ideal system for producing deuterated metabolites.

### 9.1.3 Yeast

A species of yeast, *Schizosaccharomyces pombe*, was grown in $D_2O$ for studying glucose metabolism and growth retardation. High concentrations of $D_2O$ were found to alter glucose metabolism and growth retardation. After prolonged incubation in $D_2O$, cells display gross morphological changes and thicken cell walls. Yeast cells grown in 99% $D_2O$ were also found to exhibit cellular aggregates and cause colonies to become slightly uneven. $D_2O$-tolerant strains of yeast can also be applied to produce deuterated biomolecules.

### 9.1.4 Bacteria

The growth of bacteria, including *Azotobacter agilis* and *A. vinelandii*, in deuterium oxide medium revealed that lag periods occur prior to growth during adaptation to a deuterated environment. The growth of several other strains of bacteria is also inhibited by $D_2O$ medium. The degree of inhibition is strain specific. The incorporation of a small percentage of NaCl into the $D_2O$ medium was found to decrease such inhibition of growth. Deuterium oxide also induces the occurrence of mutants in some strains. The amount of deuterium incorporated into the bacteria is related to the induced mutagenic effect.

### 9.1.5 Animal Cells

The effects of deuterium oxide on cell growth were also investigated in rat basophilic leukemia cells cultured with 15 moles/liter deuterium oxide. The observed decrease in cell growth was attributed to cells not doubling their DNA content. However, such interference with DNA replication is not necessarily a primary mechanism in the blockade of cell division by $D_2O$.

Moreover, deuterium oxide (up to 25%) was found to accelerate and increase the growth of immature ganglia neurons in chicks and rodents, where living deuterated cells appear opaquer and heteromeric than control neurons. Besides, there is an abundance of granular and fibrillar elements in the cells.

In addition, fish has been reported to survive in 30% $D_2O$. Mice and dogs tolerate long-term replacement of at least 10%–15% of body fluid hydrogen with deuterium. However, toxicity is observed with acute or chronic exposure to $D_2O$ above 25%.

The growth of various organisms in $D_2O$ is briefly summarized in Table 9.1.

**Table 9.1** Growth of organisms in deuterium oxide medium

| Organism | Species |
|---|---|
| Algae | *Chlorella vulgaris*, *Chlorella pyrenoidosa, Scenedesmus obliquus*, *Chara gymnophylla, Plectonema calothricoides*, *Phormidium luridum* |
| Moss | *Funaria hygrometrica* |
| Yeast | *Schizosaccharomyces pombe* |
| Bacteria | *Azotobacter agilis*, *A. vinelandii* |

$D_2O$-tolerant organisms have potential to be applied to produce deuterated biomolecules

## 9.2 The Effects of Deuterium Oxide on Cells

The effects of $D_2O$, including cytotoxicity, cytostatic activity, antineoplastic effect, antimitotic activity, and nucleic acid synthesis, are briefly discussed below. Chronic applications of $D_2O$ were reported to cause toxic effects. For example, the 35% substitution of $H_2O$ with $D_2O$ was found to induce fatal effects in the mouse. Mitosis of a fertile egg of sea urchin was completely inhibited by 75% $D_2O$. Nevertheless, the mechanisms of the toxicity of $D_2O$ and the effects of $D_2O$ on cellular events have not been fully understood.

### *9.2.1 Cytotoxicity*

Cytotoxicity refers to toxicity to living cells that lead to cell damage. Substitution of protium (H) for deuterium (D) strongly affects biological systems. Higher eukaryotes such as plants and mammals hardly survive a deuterium content of greater than 30%. At the cellular level, $D_2O$ may also affect mitosis and membrane functions. While higher concentrations (> 20% of body weight) can be toxic to animals and animal cells, a lower concentration of $D_2O$ has been used for measuring water spaces in humans and other animals.

Replacement of hydrogen with deuterium by administrating 25% $D_2O$ in the drinking water was found to result in certain alterations in the reproductive potential of female mice. These alterations include a decrease in the number of pregnancies carried close to term, as well as a decrease in the survival of newborn mice.

Deuterium oxide also affects a variety of biological activities different from those of normal water. $D_2O$-mediated cytotoxicity was investigated by using a murine malignant astrocytoma cell line, which demonstrated that $D_2O$ was cytotoxic to malignant astrocytoma cells. The mechanism of the observed cytotoxicity may involve $D_2O$-induced apoptosis and cell-cycle modulation. $D_2O$-mediated cytotoxicity was also examined by using a malignant cell line and similar results were found. Moreover, at $D_2O$ concentrations of 10%–50%, the cytotoxic effect was found to be dose- and time-dependent.

Deuterium oxide was also shown to be active in various cancer cell lines in vitro and in vivo. The cytotoxic effects of $D_2O$ were investigated in three pancreatic cancer cell lines. $D_2O$ of 15%–27% led to the induction of apoptosis when compared to untreated controls. The mechanism of $D_2O$-mediated cytotoxicity was shown to involve the induction of apoptosis and cell accumulation.

Further reports on the effects of deuterium oxide on the survival of mice inoculated with tumor revealed that after tumor inoculation and drinking water containing 30% $D_2O$, the weight of the tumor was reduced to the values ranging from 22% to 65% of the control. An improved survival time of about 6 days was also found. Such effects were interpreted in terms of inhibition of tumor cell division and systemic toxicity.

### 9.2.2 Cytostatic Activity

Cytostatic activity refers to a cellular effect caused by a substance that inhibits cell growth and division. Drinking 30% $D_2O$ was found to inhibit the tumor growth by a factor of 0.4–0.5 compared to the growth of the same tumor in control animals that drink regular water. This finding implies that deuterium oxide at high concentration interferes with cell division and depresses the uptake of DNA precursors in mammalian cells.

The effects of cytostatic activity of cancer cell lines were also assessed in $D_2O$. Significant effects were found at concentrations higher than 30% $D_2O$. The results of $D_2O$ exhibiting cytostatic activities against human digestive organ cancer cell lines suggested that $D_2O$ may be a potential anticancer agent. In addition, heavy water was also found to effectively retard the growth of human carcinomas in mice given drinking water containing 30% $D_2O$.

Moreover, moderate deuteration in a combination of cytostatic drug was found to significantly increase the survival time of mice. The tumors were weighed 36%–90% less than those of control animals. Heavy water combined with cytostatic drug also showed synergistic effects in tumor variants, implying an underlying deuterium-induced prolongation of tumor-cell cycle times and a reduction of the growth fraction.

Furthermore, the effects of deuterium oxide on human pancreatic tumor cells were also studied. The cytotoxic effects of $D_2O$ were examined in three pancreatic cancer cell lines. $D_2O$ of 15%–27% was found to lead to the induction of apoptosis, when compared to the controls in the absence of $D_2O$. This finding suggested that $D_2O$ might offer an additional option for the treatment of pancreatic carcinomas.

### 9.2.3 Antineoplastic Effect

*Antineoplastic effect refers to agents that interfere with cell division, leading to cell death.* Antineoplastic chemotherapy drugs inhibit or prevent the growth and spread of neoplasms or malignant cells. Studies of the influence of deuterium oxide on the growth of human carcinomas revealed that deuterium-enriched water has an antiproliferative effect on transplantable mouse tumors. Drinking 30% $D_2O$ was found to effectively retard the growth of human carcinomas.

The antineoplastic effect of heavy water on the growth of human carcinoma was also compared to that of cytostatic drugs (5-fluoro-uracil or bleomycin). $D_2O$ was found to delay the growth of carcinoma variants. Deuterium oxide combined with either cytostatic drug also showed synergistic effects. These findings revealed an underlying deuterium-induced prolongation of tumor-cell cycle times and a reduction of the growth fraction.

### 9.2.4 Antiproliferative Effect

Antiproliferative substances are related to those used to retard the spread of malignant cells in surrounding tissues. Studies showed that deuterium-enriched water has an antiproliferative effect on transplantable mouse tumors. Deuterium-enriched water was found to exhibit an antiproliferative effect on transplantable mouse tumors without toxic side effects.

### 9.2.5 Antimitotic Activity

Antimitotic activity refers to compounds that exhibit arrest cell multiplication in mitosis. Depending on the conditions of deuteration, cell division in eggs of sea urchin (*Arbacia punctulata*) could be delayed or arrested reversibly. Such antimitotic action of deuterium oxide is attributed to its interference with cell division.

Moreover, dynamics and correctness of mitotic division of PtK1 cells grown in vitro in deuterium oxide were also assessed. The results revealed that incubation of PtK1 monolayers for 2 h in 25%–75% $D_2O$ media retards the interphase or prophase transition in a dose-dependent manner.

The influence of deuterium oxide on the dynamics of mitosis in HeLa cells grown in vitro showed that a 2 h incubation of HeLa monolayers with $D_2O$ (1%–25%) in the medium increases the frequency of multipolar divisions. Substitution of 10%–25% $D_2O$ for $H_2O$ induced changes in the proportions of mitotic phases; 50% $D_2O$ was strongly inhibited, and 75% $D_2O$ blocked the cell cycle. These results supported the antimitotic activity of deuterium oxide and confirmed that $D_2O$ influences the cell cycle before mitosis.

### 9.2.6 Nuclei Acid Synthesis

Nucleic acid synthesis involves chemical processes in which components of nucleic acids are synthesized, including phosphate, pentose sugar, and nitrogenous base. Using various cell lines, cytotoxic and cytostatic activities of $D_2O$ were assessed against four cancer cell lines. $D_2O$ was found to exhibit significant cytotoxic and cytostatic effects at a concentration > 30%. The inhibition of DNA synthesis may be one of the mechanisms responsible for the antitumor effects of $D_2O$ against human digestive organ cancer cell lines.

Moreover, rat basophilic leukemia cells showed decreased cell growth in deuterium oxide medium, which was attributed to cells not doubling their DNA content. Investigations of the effects of $D_2O$ on cell growth and vesicle transport in rat leukemia cells cultured with 15 moles/liter deuterium oxide also revealed a decrease in cell growth. The inhibition of DNA synthesis is one of the mechanisms responsible

**Table 9.2** Typical effects of deuterium oxide and deuteration on cells

| ***Deuterium oxide effect*** | ***Effects on living cells*** |
|---|---|
| Cytotoxicity | Inhibition of tumor cell division and systemic toxicity |
| | Cytotoxicity to malignant astrocytoma cells |
| | Induction of apoptosis and cell accumulation |
| Cytostatic activity | Inhibition of tumor growth by drinking 30% $D_2O$ |
| | Deuterium-induced prolongation of tumor-cell cycle times |
| Antineoplastic effect | Retard the growth of human carcinomas by 30% $D_2O$ |
| | $D_2O$ is more toxic to malignant than normal animal cells |
| Antiproliferative effect | $D_2O$ retards the spread of mouse malignant cells in surrounding tissues |
| Antimitotic activity | Interference with cell division |
| | $D_2O$ influences the cell cycle |
| Nuclei acid synthesis | Inhibition of DNA synthesis |
| ***Deuteration effect*** | ***Effects on lipids and pigments*** |
| Lipid peroxidation | Polyunsaturated fatty acids deuterated at bis-allylic sites are more resistant to autoxidation reactions |
| | Protection of deuterated yeast cells against the toxic effects of lipid autoxidation products |
| Antioxidant | Anti-oxidant and anticancer properties of deuterated anthocyanins |

for the antitumor effects of $D_2O$, suggesting that $D_2O$ may be a potential anticancer agent.

In summary, the effects of deuterium oxide on living cells, including cytotoxicity, cytostatic activity, antineoplastic effect, antiproliferative effect, antimitotic action, and nucleic acid synthesis, are presented in Table 9.2.

## 9.3 Isolation of Deuterated Biomolecules

*Living organisms such as algae (Chlorella vulgaris, Chlorella pyrenoidosa, or Scenedesmus obliquus*) can be grown in deuterium oxide media. When the rate of growth is great enough, it is possible to be used as a practical source of biosynthesized fully deuterated compounds, in which the hydrogen normally present is essentially completely replaced by the deuterium. Such isolated deuterated biomolecules make it possible to carry out a variety of novel experimental approaches to investigate the structures and functions of biomolecules essential to life.

For example, cholesterol is a major component of mammalian cells, which plays a critical role in membrane permeability, rigidity, and dynamics. Production of perdeuterated cholesterol in lipid-engineered *Pichia pastoris* was carried out in deuterated minimal medium. The isolated perdeuterated cholesterol provides a unique way of probing the structural and dynamical properties of the lipoprotein complexes.

### 9.3.1 Deuterated Proteins

Since the preparation and purification of algal proteins require large quantities of algae, many techniques have been developed for the mass culture of algae. Organisms cultured in $D_2O$ can be used to isolate a variety source of biological compounds, for example, fully deuterated protein phycocyanin and bacterial enzyme alkaline phosphatase.

Studies of thermal denaturation of normal and deuterated phycocyanins isolated from blue-green algae revealed that the values of thermal denaturation of deuterated proteins are about 5 °C lower than those in normal proteins and the enthalpy of denaturation in deuterated proteins are 18%–36% lower than those in normal proteins. Deuterated proteins are also less resistant to the denaturant urea than are normal proteins.

Moreover, a bacterial alkaline phosphatase and a plant ribonuclease have also been isolated to produce deuterated enzymes in a high degree of purity. Kinetic measurements and thermal studies indicate only slight differences in the tertiary structure of the fully deuterated enzymes as compared to the ordinary enzymes. Besides, the deuterated alkaline phosphatase also shows a decreased rate of catalysis.

Furthermore, the adaptation of a large part of *E. coli* proteome from growth on a protonated medium to a deuterated medium was investigated for proteins. The results exhibited strong regulation with a reduction in growth rate with no significant morphological effects. The proteins upregulated under deuteration are enzymes with hydrogen transfer functions, suggesting that the deuterium kinetic isotope effect is the major factor of cellular functions under deuteration.

### 9.3.2 Deuterated Nucleic Acids

To determine whether the replacement of hydrogen by deuterium in living organisms is accompanied by changes in cellular components, *Chlorella vulgaris* and *Scenedesmus obliquus* algae were grown separately in $H_2O$ and $D_2O$ medium. Cytoplasmic RNA was found to be more widely distributed and in higher quantities in deuterated algae than in nondeuterated algae. Also, the nuclei of deuterated cells are more irregular in shape, and mitosis appears with greater frequency in the deuterated organisms.

Moreover, fully deuterated DNA was also isolated from *E. coli* grown in a deuterated nutrient medium (99.6% $D_2O$ with fully deuterated substrates). The melting temperature of deuterated DNA was found to be essentially identical to that of nondeuterated DNA in $H_2O$ solution. This finding suggested that deuterated DNA and hydrogenated DNA exhibit similar thermostability.

### *9.3.3 Deuterated Lipids*

The extraction of lipids from microorganisms grown in deuterated media was demonstrated to produce deuterated lipids. Comparisons of the structures of membranes extracted from yeast cells grown in $H_2O$ and $D_2O$ media revealed that the molecular composition isolated from deuterated and protiated lipid extracts shows a difference in structural organizations of lipid membranes.

In addition, to prevent the harmful accumulation of reactive oxygen species and to inhibit the propagation of lipid peroxidation, a model system was developed by using deuterated polyunsaturated fatty acids to prevent the accumulation of lipid peroxides and to reduce the cumulation of reactive oxygen. The results demonstrated that deuterated polyunsaturated fatty acids can be used as a food supplement to deaccelerate the aging process, which can lead to extending lifespan.

Moreover, palmitic acid specifically deuterated at different carbon atoms has been incorporated into the membrane lipids of *Clostridium butyricum*. The ordering of the deuterated hydrocarbon chains in whole cells was compared to those of isolated cell membranes and membranes formed from the total phospholipid extract. The shape of the order profiles was found to be similar for all membranes. However, the values of the order profiles in whole cells and isolated membranes are lower than those of the liposomal lipids. In addition, the order profiles have the same characteristic shape as those found for the lamellar liquid-crystalline phases of synthetic diacyl-phospholipids.

## 9.4 Deuteration Effects on Lipid Peroxidation and Antioxidant

Lipid peroxidation is the reaction of oxidative degradation of lipids involving free radicals. Antioxidants are substances that are able to slow or prevent damage to cells caused by free radicals. Deuteration effects on lipid peroxidation and antioxidant are briefly presented below and are also in Table 9.2.

### *9.4.1 Lipid Peroxidation*

Autoxidation of polyunsaturated fatty acids damages lipid membranes and generates toxic by-products implicated in neurodegeneration. Abstraction of bis-allylic hydrogen atoms is the rate-limiting step of polyunsaturated fatty acid autoxidation, which is inhibited by replacing bis-allylic hydrogens with deuterium atoms.

Polyunsaturated fatty acids deuterated at bis-allylic sites are much more resistant to autoxidation reactions due to the isotope effect. Deuterated polyunsaturated fatty acids also confer protection to wild-type yeast subjected to heat stress. The

substitution leads to a significantly slower oxidation and inhibits membrane damage. To investigate the prevention of reactive oxygen species by inhibiting the propagation of lipid peroxidation, a model system was developed to mimic the human dietary requirement of omega-3 to study the role of deuterated polyunsaturated fatty acids. Moreover, deuterated trilinolenin was found to prevent the accumulation of lipid peroxides.

The above findings imply that deuterated polyunsaturated fatty acids can be used as a food supplement to decelerate the aging process. In cells, the presence of a relatively small fraction of deuterated polyunsaturated fatty acid among natural polyunsaturated fatty acids was found to effectively inhibit lipid peroxidation. Moreover, since deuterated polyunsaturated fatty acids are more stable compared to standard polyunsaturated fatty acids, they protect yeast cells against the toxic effects of lipid autoxidation products.

### *9.4.2 Antioxidant*

Anthocyanins are water-soluble phytochemical found in the plant. They are a type of flavonoid, which are known for beneficial health effects due to their antioxidant and anticancer properties. Deuterated anthocyanin was prepared using recombinant *E. coli* as a production host and deuterated glycerol and $D_2O$ in the culture media. The preparation resulted in the formation of deuterated cyanidin 3-*O*-glucoside, which is more stable at pH 7.0 as compared to nondeuterated one.

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# Chapter 10
# Deuterium Oxide and Deuteration Effects on Health Issues

Deuterium oxide exhibits a variety of biologic activities different from normal water, which makes it possible to gain a deeper insight into the role of water in biology by using deuterium oxide. Deuterium solvent isotope effects are derived from special properties of deuterium oxide as a solvent. As discussed above, differences in structures, functions, and activities for biomolecules are observed in $D_2O$ and $H_2O$ solvents. Deuterium oxide is also a promising component in the areas of chemistry, medicine, and biotechnology.

$D_2O$ has been used in studies related to human health, for example, biomedical applications as therapeutic agent against hypertension, cancer, cardiac, aging, and other diseases. $D_2O$ exhibits cytotoxic and cytostatic activities against cancer cell lines in the human digestive organ, leading to investigating deuterium oxide as a potential therapeutic agent against cancers. However, $D_2O$ in high concentrations is toxic to multicellular organisms. A concentration of >20% of body weight can be toxic to animal cells.

Investigations of dose-dependent effects of deuterium oxide in drinking water on systolic blood pressure and aortic calcium uptake revealed that the usual increase in systolic blood pressure and the associated increase in aortic calcium uptake in rats were lowered by deuterium oxide in a dose-dependent manner. In addition, $D_2O$ was also found to be more toxic to malignant cells than normal cells.

Deuterium oxide and deuteration effects have also gained attention due to their potential to affect the pharmacokinetic and metabolic profiles of drugs. In pharmaceutical industries, deuteration goes beyond simple progress of pharmacokinetic parameters of a drug. Beyond the progress of the pharmacokinetic parameters of a drug, deuterium oxide and deuteration can also provide an opportunity to study issues in terms of metabolism-mediated toxicity.

Deuterated drug might provide an opportunity when facing problems in terms of metabolism and drug interactions. The use of deuterium also offers the opportunity to reduce the dose of a drug and the discovery of new drugs. In the case of

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deutetrabenazine, this deuterated drug can provide a more favorable adverse effect profile than tetrabenazine in the therapeutic treatment of Huntington's disease.

The effects of deuterium on living organisms are generally negative. However, some of the $D_2O$ applications are of biotechnological potential. Deuterium oxide has a lower ionization constant than that of hydrogen oxide. The ratio of $D^+$ to $H^+$ is approximately 1–15,000 in biological fluids. Comparing $D_2O$ with $H_2O$, O-D bond length is shorter than O-H, and D-O-D angle is lesser than H-O-H. Deuterium forms stronger hydrogen bonds than protium. Hydrophobic interaction is also stronger in $D_2O$ than $H_2O$. Deuteration effects are derived from the compounds containing C-D bonds rather than C-H bonds, where C-D bonds are more stable than C-H bond.

Deuterium oxide and deuteration effects on human health, including diseases, health-related issues, deuterium-depleted water, and deuterated drugs, are discussed below.

## 10.1 Deuterium Oxide and Deuteration Effects on Diseases

As mentioned above, deuterium oxide and deuteration are a promising component in the areas of human health, pharmacology, and biotechnology. $D_2O$ has numerous therapeutic applications to various disease conditions such as hypertension, cancers, and cardiac and liver diseases. Deuterium-depleted water was found to be an anticancer agent with potential clinical application. Deuterium oxide has also been applied to determine body water.

### *10.1.1 Hypertension*

Hypertension, a common public health problem, could lead to degenerative diseases such as congestive heart failure, renal disease, and peripheral vascular disease. Factors related to hypertension and the effects of deuterium oxide are presented below.

#### 10.1.1.1 High Salt

There are strong evidences that excess dietary salt is a major factor contributing to the development of hypertension. Studies of systolic blood pressure and vascular calcium uptake suggested that increased calcium uptake mechanisms are associated with hypertension in salt-sensitive rats. Systolic blood pressure and uptakes of calcium are significantly higher in rats on the high salt diet for 6 weeks, as compared with those on the low salt diet.

Deuterium oxide was found to improve systolic blood pressure and aortic calcium uptake in hypertensive rats on high salt diet. Intake of 25% $D_2O$ normalizes

systolic blood pressure and aortic calcium uptake in hypertensive rats with high salt diet. This finding suggested that an increased cytosolic free calcium is involved in the pathophysiology of hypertension, and deuterium oxide helps to prevent hypertension by normalizing cytosolic free calcium.

#### 10.1.1.2 High Calcium

Increased calcium uptake in vascular tissues leading to elevated cytosolic free calcium has been implicated in the pathophysiology of hypertension. $D_2O$ appears to prevent hypertension by normalizing calcium uptake in vascular smooth muscle. A minimum dose of 10% deuterium oxide is needed to prevent elevated aortic calcium uptake, platelet cytosolic free calcium, and renal vascular changes in hypertensive rats.

Moreover, high calcium uptake induced by phenylephrine in rats via receptor-operated and KCl voltage-operated channels was found to be effectively reduced by $D_2O$ in a concentration-dependent manner. These findings revealed that the increased calcium uptake mechanism is associated with hypertension, and $D_2O$ acts like a calcium channel blocker that effectively normalizes vascular calcium uptake mechanisms.

#### 10.1.1.3 Alcohol Consumption

Systolic blood pressure, platelet cytosolic calcium, and aortic calcium uptake are significantly higher in rats given ethanol for 14 weeks. Ethanol-treated rats also showed smooth muscle hyperplasia with thickening of the wall and narrowing of the lumen in small arteries and arterioles of the kidney. $D_2O$ given to ethanol-treated rats was found to normalize their blood pressure, cytosolic free calcium, and aortic calcium uptake.

#### 10.1.1.4 $D_2O$-Containing Water

Investigations of the effects of 5, 10, or 20% deuterium oxide in drinking water on systolic blood pressure and aortic calcium uptake were carried out in hypertensive rats. The observed increases in systolic blood pressure and the associated aortic calcium uptake in hypertensive rats are lowered by $D_2O$ in a dose-dependent manner. A minimum dose of 10% deuterium oxide is needed to prevent the development of hypertension in rats.

The effects of 5% deuterium oxide in drinking water on systolic blood pressure were also examined in rats with fructose-induced hypertension. Deuterium oxide given together with fructose prevented the development of high blood pressure and the associated increases in platelet cytosolic calcium, aortic calcium uptake, and plasma triglycerides. Such parallel increases in systolic blood pressure, cytosolic

free calcium, and vascular calcium uptake suggested that an increased cytosolic free calcium is involved in the pathophysiology of hypertension, while $D_2O$ prevents this hypertension by normalizing cytosolic free calcium.

### *10.1.2 Cancers*

The antineoplastic effect of $D_2O$ on the growth of human carcinoma was compared to that of cytostatic drugs and 5-fluorouracil. Drinking water containing 30% $D_2O$ effectively retards the growth of the human carcinomas in mice, and $D_2O$ in combination with cytostatic drug showed synergistic effects on tumor. In addition, deuterium-enriched water was found to exhibit an antiproliferative effect on transplantable mouse tumors.

#### 10.1.2.1 Cancer Cell Lines

Heavy water was also shown to be active in various cancer cell lines in vitro and in vivo. $D_2O$ at concentrations higher than 10% was found to significantly inhibit the invasion of tumor cells. $D_2O$ also showed significant cytotoxic and cytostatic activities against human digestive organ cancer cell lines, suggesting that $D_2O$ may be a potential anticancer agent.

#### 10.1.2.2 Pancreatic Cancer

Pancreatic cancer constitutes an entity that is difficult to treat and mostly fatal. The effect of deuterium oxide in human pancreatic tumor cells and the cytotoxic effects of $D_2O$ were examined in three pancreatic cancer cell lines. $D_2O$ was found to induce apoptosis when compared to untreated controls. The possibility of $D_2O$ as a therapeutic agent against human pancreatic cancer was also investigated. In vitro, the growth of pancreatic cancer cells was found to be significantly inhibited by 10%–30% $D_2O$, suggesting that $D_2O$ may be applied for the therapeutic use against pancreatic cancer.

#### 10.1.2.3 Others

The effect of deuterium oxide on the survival of mice with ascites tumor has also been studied. Mice maintained on 25%–30% $D_2O$ drinking water showed an improved survival time of about 6 days. Such effect was interpreted in terms of inhibition of tumor cell division and systemic toxicity by $D_2O$. Deuterium-enriched water was also reported to exhibit an antiproliferative effect on transplantable mouse tumors.

After tumor inoculation, the mice were given drinking water containing 30% $D_2O$. Heavy water was found to effectively retard the growth of human carcinomas. Deuterium-enriched water also has an antiproliferative effect on transplantable mouse tumors. The influence of $D_2O$ on the growth of xenotransplanted human carcinomas was investigated. After tumor inoculation, the mice were given drinking water containing 30% $D_2O$, which effectively retarded the growth of the human carcinomas. The nucleic acid and protein synthesis inhibition assay suggested that the inhibition of DNA synthesis may be one of the mechanisms responsible for the antitumor effects of $D_2O$.

### *10.1.3 Cardiac Disease*

Studies of cardiac injury induced by ischemia and reperfusion in rats revealed that the dialysate myoglobin levels were increased in the presence of $D_2O$, suggesting that $D_2O$ might have a beneficial effect against cardiac injury in rats. Measurements of dialysate myoglobin levels during coronary occlusion and reperfusion showed that $D_2O$ attenuates myocardial myoglobin release.

To determine whether deuterium affects heart rate, flies *Drosophila melanogaster* were raised on protium and deuterated media, and their heartbeat was monitored. The rate of heartbeat decreased with increasing concentrations of deuterium. The higher the concentration of deuterium oxide, the less temperature-sensitive was the heart rate.

The effects of $D_2O$ on the myocardial injury-induced ischemia and reperfusion in rat heart were also examined. $D_2O$ was found to attenuate myocardial myoglobin release during coronary occlusion, reperfusion, and local perfusion. To confirm the effect of $D_2O$ on NaCN-induced myocardial injury, the dialysate myoglobin levels were measured in the absence and presence of $D_2O$. $D_2O$ was found to attenuate myocardial myoglobin release during 15–30 min of coronary occlusion and 0–30 min of reperfusion and 15–60 min of local perfusion of NaCN. These findings suggested that $D_2O$ might exhibit a beneficial effect of the myocardium against ischemia, reperfusion, and chemical hypoxia.

### *10.1.4 Liver Disease*

Drinking $D_2O$-$H_2O$ mixed water (30 to 70%) for 6 weeks was performed to investigate the effects of $D_2O$ on rat liver activities. The liver showed marginal increase in weight, while gradual decrease in the body weight was found. Moreover, acid phosphatase, glucose-6-phosphatase, and adenosine triphosphatase were decreased, while alkaline phosphatase was increased.

The effect of moderate deuteration of body fluids on bleomycin-induced lung damage in mice was also studied. Three weeks after bleomycin injection, deuterated

mice lacked signs of irreversible bleomycin-induced pulmonary damage. Lung parameters in deuterated mice were lower than those in non-deuterated mice.

These findings revealed that moderate concentrations of deuterium may prevent the development of fibrosing alveolitis in bleomycin-treated mice, possibly by reducing proliferation of alveolar fibroblasts. However, the toxicity of bleomycin is enhanced by ingestion of deuterium, which could result in morphological liver alterations.

### *10.1.5 Degenerative Eye Disease*

The reactivity of vitamin A leading to its dimerization is believed to play a key role in forming granular deposits in the retinal pigment epithelium, which is a cause of major degenerative eye diseases. A method to slow vitamin A dimerization by enriching it with the stable isotope deuterium at carbon 20 position was investigated to elucidate the pathogenesis of macular degenerations and to develop a therapeutic intervention.

The results showed that dimerization of deuterium-enriched vitamin A was considerably slower than that of vitamin A at natural abundance as measured in vitro. This study suggested that administration of vitamin A enriched with the stable isotope deuterium at carbon 20 position may be a useful approach to retard vitamin A dimerization and to slow the progression of degenerative eye diseases.

The above-discussed deuterium oxide and deuteration effects on various diseases in animal models are briefly summarized in Table 10.1.

**Table 10.1** Deuterium oxide and deuteration effects on diseases

| Diseases | $D_2O$ and deuteration effects |
|---|---|
| Hypertension | $D_2O$ improves hypertensive rats with high salt diet. |
| | 10% $D_2O$ prevents elevated aortic calcium uptake. |
| | $D_2O$ normalizes blood pressure of ethanol-treated rats |
| Cancers | $D_2O$ shows against digestive organ cancer cell lines. |
| | $D_2O$ inhibits growth of pancreatic cancer cells. |
| | 30% $D_2O$ retards the growth of carcinomas |
| Cardiac disease | $D_2O$ shows benefits against cardiac injury in rats. |
| | Heartbeat rate in flies is decreased with $D_2O$. |
| | $D_2O$ benefits the myocardium against ischemia, reperfusion, and chemical hypoxia |
| Liver disease | $D_2O$ affects liver enzyme activities. |
| | Deuterated mice lack signs of bleomycin-induced pulmonary damage |
| Degenerative eye diseases | Slower dimerization in deuterium-enriched vitamin A |

## 10.2 Deuterium Oxide and Deuteration on Health-Related Issues

Deuterium oxide increases the temperature tolerance of *Drosophila pseudoobscura* flies, when it is administered as a sucrose solution. This finding implies an increased resistance of biomolecules to thermal denaturation in deuterium oxide. Such conformational stability is attributed to the enhancement of hydrophobic interaction. Nevertheless, the increased strength of deuterated hydrogen bonds cannot be excluded.

Moreover, at the cellular level, $D_2O$ may affect mitosis and membrane function. A low toxicity of lower concentration of $D_2O$ toward mammals is reflected in its widespread use for measuring water spaces in humans and other animals. However, higher concentrations (usually >20% of body weight) can be toxic to animals and animal cells.

$D_2O$ is more toxic to malignant than normal animal cells but at concentrations too high for regular therapeutic use. The effects of deuterium oxide on other health-related issues, including aging, body water, deuterium-deleted water, lipid autooxidation, and stabilization of enzymes, are discussed below.

### *10.2.1 Aging*

Since O-D bond length is shorter than O-H, D-O-D angle is lesser than H-O-H, and deuteron bonds are stronger than proton bonds, an increase of deuterated macromolecules with aging would be expected. Once a deuterium is exchanged with a proton in a biomolecule, a conformational change in biomolecule may occur. A comparison of the mean life spans for populations of *Drosophila melanogaster* (a species of fly), which were maintained on media prepared with $H_2O$ and with 50% $D_2O$, revealed that deuteration decreases longevity.

Deuteration may also slow down electron transfer in the electron transport chain by interfering with proton-coupled electron transport reactions. In the course of ATP synthase and the electron transport chain, deuteration may interfere with the conformation and function of biomolecules, leading to deuterium oxide effects on aging.

### *10.2.2 Body Water*

The human body needs water to function the right way. *Body water is the total amount of fluid in a human body*. It helps to regulate the internal body temperature and strengthen muscles and moisturize skin. Being the primary building block for cells, *body water* is an important measure when it comes to a healthy body.

The human body contains an average of about 60% of water. However, the amount of water in the body changes slightly with age, sex, and hydration levels. The low toxicity of $D_2O$ toward mammals is reflected in its widespread use for measuring water spaces in humans and other animals. However, higher concentrations (usually >20% of body weight) can be toxic.

A tracer dose of deuterium oxide can be applied to determine the total body water using the deuterium NMR technique. The infrared spectrophotometric method using a tracer dose of $D_2O$ is also used to determine the total body water.

### *10.2.3 Deuterium-Depleted Water*

In contrast to the above-discussed inhibition of tumor cell division by a low concentration of $D_2O$, deuterium-depleted water was found to be a promising anticancer agent with potential for future clinical application. Deuterium-depleted water has a lower concentration of deuterium (less than 145 ppm) than that occurs naturally.

#### 10.2.3.1 Cancers

A clinical study was performed on patients with lung cancers who consumed deuterium-depleted drinking water as a nontoxic agent in addition to conventional chemotherapy and radiotherapy. The results revealed that such therapy prolongs the survival time of lung cancer patients, suggesting that deuterium-depleted drinking water may be used as a nontoxic anticancer dietary supplement. Thus, daily intake of deuterium depleted water (10–20 ppm lower deuterium content) may be a promising treatment for cancer patients.

Despite empirical evidence that deuterium-depleted water (25–125 ppm deuterium) has anticancer effect, the molecular mechanism remains unclear. A concept that is based on deuterium affecting cell signaling and tumor growth has been introduced.

#### 10.2.3.2 Cardiovascular System

Moreover, the effects of deuterium-depleted water on the cardiovascular system were also investigated. Blood pressure and selected biochemical parameters were measured in hypertensive rats. Deuterium-depleted water treatment was found to decrease the total cholesterol and triglyceride levels in rats.

#### 10.2.3.3 Depression

Environmental factors can significantly affect disease prevalence, including neuropsychiatric disorders such as depression. The link between deuterium content of water and depression was investigated to examine a correlation analysis between deuterium content of tap water and rates of depression. The effect of deuterium depletion on sleep electrophysiology was also evaluated in mice. Depressive-like features were reduced in mice fed with deuterium-depleted water. The result suggested that the deuterium content of water may influence the incidence of affective disorder-related pathophysiology and major depression.

#### 10.2.3.4 Obesity

The hypothesis that deuterium-depleted water can alleviate diet-induced obesity and its associated metabolic impairments was tested by rats fed with a high-fat diet. In obese animals treated with deuterium-depleted water, the tested results indicated a reduction in inflammation, decrease in the level of oxidative stress, and an enhancement of antioxidant enzyme activities.

### *10.2.4 Lipid Oxidation*

Due to the kinetic isotope effect, the substitution of hydrogen at the bis-allylic methylene of essential fatty acids with the isotope deuterium was found to significantly slow the lipid oxidation reaction. Such approach has the advantage of preventing the accumulation of reactive oxygen species by inhibiting the propagation of lipid peroxidation.

#### 10.2.4.1 Neurological Conditions

Oxidative damage resulting from increased lipid peroxidation is considered an important factor in the development of late-onset/age-related Alzheimer's disease. Alzheimer's disease involves progressive deposition of amyloid β-peptide, synapse loss, and neuronal death, which occur in brain regions critical for learning and memory. Considerable evidence suggests that lipid peroxidation contributes to synaptic dysfunction and neuronal degeneration.

Essential fatty acids are useful in fighting age-related diseases such as Alzheimer and other neurological conditions. Polyunsaturated fatty acids undergo autoxidation, which generates reactive carbonyl compounds that are toxic to cells in association with age-related neurodegenerative diseases. Their autoxidation process is initiated by the abstraction of bis-allylic hydrogen atoms.

Replacement of the bis-allylic hydrogen atoms with deuterium atoms arrests polyunsaturated fatty acid autoxidation due to the isotope effect. The replacement of just one bis-allylic hydrogen atom with deuterium was found to be sufficient to significantly inhibit lipid autoxidation.

#### 10.2.4.2 Polyunsaturated Fatty Acids

Recent findings suggest that lipid peroxidation can be inhibited by the replacement of polyunsaturated fatty acids (H-PUFA) with deuterated PUFA (D-PUFA). Investigations of the effect of dietary D-PUFA on amyloid β-peptide pathology concluded that a D-PUFA diet reduces the brain tissue concentration of amyloid β-peptide.

Moreover, D-PUFAs are more resistant to the reactive oxygen species-initiated chain reaction of lipid peroxidation than H-PUFAs. Investigation of the effect of D-PUFA treatment on lipid peroxidation and cognitive performance in mice established a model of oxidative stress-related cognitive impairment that exhibits Alzheimer's disease-like pathologies. D-PUFAs were found to represent a promising new strategy to broadly reduce rates of lipid peroxidation.

### *10.2.5 Stabilization of Enzymes*

$D_2O$ was found to protect enzyme cholinesterase against acid denaturation. Studies of irreversible thermal inactivation of the tetrameric form of butyrylcholinesterase revealed a stabilizing effect and a shift in the transition temperature in $D_2O$ medium. Moreover, *E. coli* ATPase located at the surface of the membrane and enzyme cytochrome c oxidase are also stabilized by deuterium oxide.

The above-discussed deuterium oxide and deuteration effects on health-related issues are summarized in Table 10.2.

## 10.3 Deuteration Effects on Drugs

Carbon-hydrogen bonding is particularly relevant for understanding important properties of drug molecules. Deuterated drug refers to the selective replacement of protium hydrogen in small molecule drugs with deuterium isotope. Deuteration of a drug is most likely to affect pharmacokinetic properties. The metabolism of drugs may be favorably influenced when deuterium is substituted for protium, resulting in improved safety, tolerability, or efficacy.

Development of deuterated drugs such as deutetrabenazine is briefly described below. A more detailed description of deuterium oxide and deuteration effects on pharmacology is discussed in the following chapter.

**Table 10.2** Deuterium oxide and deuteration on health-related issues

| Health-related issues | $D_2O$ or deuteration effect |
|---|---|
| Aging | Deuteration decreases longevity. |
| | $D_2O$ slows electron transport chain |
| Body water | < 20% $D_2O$ used for measuring body water. |
| | Tracer dose of $D_2O$ used to determine body water |
| Deuterium-depleted water | Potential anticancer agent for clinical applications. |
| | A promising treatment for cancer patients. |
| | Decrease in cholesterol and triglyceride levels in rats |
| Lipid autoxidation | Deuterated polyunsaturated fatty acids are more resistant to the reactive oxygen-initiated lipid peroxidation than hydrogenated ones |
| Neurotransmitter related enzyme | $D_2O$ protects cholinesterase against acid denaturation |

### *10.3.1 Development of Deuterated Drugs*

Deuterated compounds may offer advantages over nondeuterated forms through alterations in clearance. Deuteration may also redirect metabolic pathways in directions that reduce toxicities. To improve pharmacokinetics, metabolism, and excretion of medicine, deuterated drugs are being developed to treat various diseases.

Deuterated versions of existing drugs can exhibit improved pharmacokinetic or toxicological properties, due to the stronger deuterium-carbon bond that modifies their metabolism. Deuteration may change the pathway of drug metabolism, leading to increased duration of action and lower toxicity. Some deuterated drugs may also show different transport processes.

For example, deutetrabenazine is a deuterated form of tetrabenazine. It is the first deuterated medication that has been approved by the US Food and Drug Administration for therapeutic use to treat chorea in Huntington's disease. The substitution of deuterium for hydrogen at key positions in the tetrabenazine molecule allows a longer drug half-life and less frequent daily dosing. Deutetrabenazine also improves chorea in Huntington's disease with less adverse effects.

### *10.3.2 Deutetrabenazine*

Deuterated drugs that have been evaluated in clinical trials include tetrabenazine, paroxetine, and dextromethorphan. Deutetrabenazine is a derivative of tetrabenazine in which two trideuteromethoxy groups substitute two methoxy groups. It is the first deuterated drug to receive approval for the treatment of chorea associated with Huntington's disease. Chorea is the most common motor manifestation of Huntington's disease, an inherited neurodegenerative disorder that can interfere with daily activities, reduce the quality of life, and cause injury.

Deutetrabenazine has been shown to be as effective as tetrabenazine but has a lower risk of adverse effects. Moreover, the active metabolites of deutetrabenazine have a longer half-life than those of tetrabenazine, together with a greater overall absorption. Deutetrabenazine's favorable pharmacokinetic profile permits a lower dosage than tetrabenazine, thereby potentially improving the safety profile of deutetrabenazine versus tetrabenazine, while maintaining its efficacy.

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# Part VI
# Applications of Deuterium Oxide and Deuteration to Pharmacology

# Chapter 11
# Deuterium Oxide Effects on Thermostability of Vaccines

Live-attenuated vaccines such as polio, influenza, yellow fever, measle, and mump vaccines are made up of proteins, nucleic acids, lipids, and carbohydrates. The stability of these vaccines is a key factor to preserve their potency and efficiency. In addition to being affected by pH and ionic strength, vaccines also undergo changes on exposure to heat.

The thermal degradation rate of a vaccine is determined by the storage temperature. For instance, reconstituted measle vaccine rapidly loses potency at room temperature. At 22–25 °C, there is usually a 50% loss in potency in 1 h. At higher temperatures, the reconstituted vaccine loses potency even more rapidly.

Moreover, oral polio virus vaccine, an effective immunizing agent, is a living, attenuated virus. Like most viruses, it is unstable, except when held at very low temperatures in the frozen state. Polio vaccine strains lose infectivity, when they are stored above refrigerator temperatures. These losses increase rapidly at a higher temperature.

Developing vaccine formulations with good thermostability and immunogenicity remains a great challenge. Consequently, research to reduce vaccine storage problems in heat climates is an active area of research. To improve the stability of vaccine, a number of additives have been studied, including sugars (lactose and sorbitol), amino acids (histidine and alanine), and divalent cations ($Ca^{2+}$ and $Mg^{2+}$) in phosphate buffer. For instance, $MgCl_2$ has been incorporated to stabilize oral poliovirus vaccine.

Biomolecular stabilization has been applied to stabilize biologicals such as pirodavir, a class of antipicornavirus compounds. Pirodavir inhibits the thermal denaturation of viral capsid. A combination of pirodavir and deuterium oxide leads to an additive effect. Similarly, research to reduce poliovirus vaccine storage problems in heat climates in deuterium oxide medium has also been carried out.

Oral polio vaccine containing deuterium oxide, instead of ordinary water, was found to allow potency to be maintained for 7 days at 37 °C without significant loss. Moreover, the addition of deuterium oxide to $MgCl_2$ was even more promising,

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making the poliovirus vaccine more thermostable than in ordinary water containing $MgCl_2$ alone.

The addition of deuterium oxide to tissue culture cell harvests stabilizes oral poliovirus vaccine, prompting the examination of the effect of deuterium oxide on the stabilization of other viral vaccines. In live influenza vaccines, thermal stabilization with deuterium oxide was found to be greater than seen with standard stabilization with sucrose-phosphate-glutamate.

Such stabilization effect afforded by deuterium oxide also includes influenza A and B vaccines, yellow fever vaccine, and Newcastle disease virus vaccine. Nevertheless, in spite that deuterium is a stable isotope without radioactivity, concerns have been raised about clinical applications of deuterium oxide-containing vaccines.

## 11.1 Stabilizers of Vaccines

Viruses are grown in primary cells or on continuous cell lines, while bacteria are grown in bioreactors. Antigen that triggers the immune response is generated and isolated from the cells. Vaccines contain tiny fragments of their active component. The immune system recognizes the invading germ, virus, or bacteria. A vaccine works with the body to produce antibodies by the immune system to fight disease.

Vaccine is manufactured by adding adjuvants to increase the immune response of the antigen. Stabilizers are then added to enhance the storage life of the vaccine. Preservation of biological products is an important task in maintaining the quality of medical products. Potency and thermal stability are crucial for the control of the effectiveness of a vaccine.

Vaccines, such as polio, influenza, yellow fever, measle, and mump vaccines, consist of proteins, nucleic acids, lipids, and carbohydrates, which undergo changes when expose to heat. Hence, live-attenuated viral vaccines usually require cold chain maintenance during storage and distribution. In addition, the thermostability of vaccine in solution is also influenced by pH and ionic strength. The stability of vaccine must be determined empirically through various testing. Hence, a number of stabilizers are currently used to help retain the quality of vaccines.

Deuterium oxide has previously been reported to have a protective effect on biomolecules, cells, and organisms against thermal degradation. $D_2O$ has been applied to demonstrate its potential in the stabilization of vaccines such as polio, yellow fever, and others.

The potential of applying $D_2O$ in the stabilization of veterinary vaccines is also under investigations.

### 11.1.1 Preservation of Vaccines

Preservation of vaccines and other biologicals is one of the important tasks in maintaining the quality of the products from manufacture until they reach the users. Live-attenuated viral vaccines, serum immunoglobulins, plasma fractions, and clinical samples including tissues and body fluids are all materials that usually require cold chain maintenance during storage and distribution.

Among these medical products, the stability of vaccine is a key factor to preserve vaccine potency and efficiency, as its potency decays over time. Like many biological preparations, vaccines are unstable during storage. The instability can reduce the safety and efficacy of vaccines. The choice of stabilizers for viral vaccine formulation depends mainly on the structure and property of viral antigen in the vaccine or its active pharmaceutical ingredient.

### 11.1.2 Temperature Factor

Significant changes in the stability of vaccines may occur following their exposure to temperature stresses. For example, reconstituted measle vaccine rapidly loses potency at room temperature. At 22-25 °C, there is usually a 50% loss in potency in 1 h. At higher temperatures, measle vaccine loses potency even more rapidly and cannot be used. Moreover, vaccine, such as oral polio vaccine, loses infectivity when it is stored above refrigerator temperatures. Yellow fever vaccine is also subject to potentially adverse conditions under thermal stress. The sensitivity of vaccines to temperature has led to the development of storage requirements for them.

Enhancement of thermostability of vaccines can lead to the improvement of vaccine effectiveness. Knowledge of the stability of vaccine, particularly the rate of decline in potency at a given temperature, can be helpful in determining the vaccine storage requirements. Physical and chemical analyses enable the characterization of the degradative pathways of thermally labile vaccines, leading to the development of approach to the stabilization of them.

### 11.1.3 Additives to Stabilize Vaccines

As indicated above, potency and thermal stability are crucial for the control of the final vaccine product. Specifically, at room temperature, reconstituted measles vaccine rapidly loses potency with a 50% loss in potency in 1 h. At higher temperatures, the measle vaccine loses potency even more rapidly and cannot be used. To improve the thermal stability of vaccine, a number of additives have been investigated.

One conventional method is the addition of a stabilizer, such as 1 molar $MgCl_2$ or 35–50% sucrose to vaccines. Other successful formulation includes sugars,

amino acids, or divalent cations, such as lactose (4%), sorbitol (2%), histidine (0.01 M), and alanine (0.01 M) in phosphate buffered saline with $Ca^{2+}$ and $Mg^{2+}$. The vaccine produced with such stabilizers commonly loses about 20% of its effectiveness after being maintained at 37 °C for 14 days.

For example, when molar $MgSO_4$ is added, measles vaccine is more stable, losing about 0.3 log10 in 30 min at 50 °C. Yellow fever, an acute mosquito-borne viral fever, is preventable by use of the live-attenuated vaccine. To improve its stability, a number of additives have been systematically investigated, including sugars, amino acids, and formulated solution [lactose (4%), sorbitol (2%), histidine (0.01 M), alanine (0.01 M)] in phosphate buffered saline with $Ca^{2+}$ and $Mg^{2+}$].

Moreover, deuterium oxide has been known for a long time to exhibit a protective effect on biomolecules, cells, and organisms against thermal shock. For instance, effects of deuterium oxide on the thermostability of recombinant p26 protein were shown in carbonate-bicarbonate coating buffer prepared in 60% and 80% $D_2O$ for the diagnosis of equine anemia virus infection. A gradual increase in the stabilization effect of $D_2O$ at elevated temperature was observed, which provides the thermal protection to p26 protein up to 2 months of incubation at 45 °C.

The applications of deuterium oxide as the stabilizer for biomolecules and medical products against thermal destabilization are further elaborated below.

### *11.1.4 Deuterium Oxide as Stabilizer*

Understanding the basis of $D_2O$ action is an important step that may improve the way that thermolabile medical products are stored. $D_2O$ has been reported to stabilize attenuated viral RNA against thermal degradation. A considerable increase in the stability of its folded basin leads to a higher melting temperature in $D_2O$ as compared with $H_2O$.

The hydration dynamics of the RNA has also been explored in both $H_2O$ and $D_2O$ media, which revealed a considerable increase in the stability of the folded basin in $D_2O$ medium. Such increase in the stability of the folded basin in the $D_2O$ medium has been studied through intramolecular hydrogen bonding and flexibility of RNA structures. RNA hairpin contains a base pair motif, which is conserved in poliovirus-like enteroviruses.

Two different inactivation mechanisms are believed to be responsible for the thermolability of the vaccine. They are the denaturation of the viral capsid and the degradation of the viral RNA within the capsid. Research to reduce vaccine storage problems in heat climates in deuterium oxide medium has also been actively carried out.

The observation that the addition of deuterium oxide to tissue culture cell harvests stabilizes oral poliovirus vaccine prompts the examination of the effect of

deuterium oxide on stabilization of other viral vaccines, such as influenza A and B vaccines, yellow fever vaccine, and Newcastle disease virus vaccine. The implications of deuterium oxide for stabilization of these vaccines have important practical applications.

Despite $D_2O$ having been known to stabilize attenuated viral RNA against thermal degradation, its molecular mechanism of action is still under investigation. $D_2O$ is assumed to strengthen the hydrogen bonding network in the solvent, lengthen inter-residue water-bridge lifetime, and weaken dynamical coupling of the hairpin to its solvation environment.

In the following, thermal stabilization effects on various vaccines, including oral poliovirus vaccine, influenza virus vaccine, Newcastle disease virus vaccine, and measle vaccine, are discussed.

## 11.2 Thermostability of Polio Vaccine

Eradication of poliomyelitis is based on the mass administration of oral poliovirus vaccine, which is widely accepted as an effective immunizing agent. The vaccine has essentially prevented the appearance of new poliomyelitis cases in the world. Poliovirus vaccine contains a living, attenuated virus, which is considered the most thermolabile of all the common childhood vaccines.

Like most viruses, polio vaccine is unstable except when held at very low temperatures in the frozen state. The major difficulty in assuring good vaccine coverage, especially in hot climates, is the thermostability of the vaccine. Delivery of effective vaccines in the developing world, especially in tropical areas, is compromised when refrigeration cannot be assured.

### *11.2.1 Thermal Stabilization of Polio Vaccine by $MgCl_2$*

To maintain its potency, oral polio vaccine is stored and shipped frozen. After thawing, it is held in the refrigerator at no more than 10 °C for a period not to exceed a week. Attempts to minimize the losses of polio vaccine effectiveness generally rely on the addition of salts such as $MgCl_2$. A concentration of one molar $MgCl_2$ is currently used as stabilizer for oral poliovirus vaccine.

The $MgCl_2$-stabilized vaccine elicits an antibody response in humans equal to that of standard vaccine maintained in the frozen state, which is thawed just before administration. Although oral poliovirus vaccine can be held for long at 4 °C with $MgCl_2$ as the stabilizer, the vaccine cannot hold in 3 days at 37 °C and in 3 h at 45 °C.

### 11.2.2 Thermal Stabilization of Polio Vaccine by $D_2O$

As mentioned above, efforts to reduce polio vaccine storage problems in high heat climates were carried out. A most reported effective condition is the maintenance of the pH at 6.5 as well as the incorporation of deuterium oxide. Oral polio vaccine containing deuterium oxide instead of ordinary water allows potency to be maintained for 7 days at 37 °C without significant loss, which is a significant improvement in comparison with other additives, such as sucrose or $MgCl_2$, detergents, albumin, or antiviral compounds.

In spite that $D_2O$ has been shown to increase the thermostability of polio vaccine, especially at temperatures that are commonly encountered during transportation, safety considerations regarding the human use of deuterium oxide-containing bioproducts have been raised due to deuterium being an isotope of hydrogen, in spite that deuterium exhibits no radioactivity.

### 11.2.3 Thermal Stabilization of Polio Vaccine by $D_2O$ and $MgCl_2$

As discussed above, the effect of different stabilizers on the loss of infectivity of live polio vaccine at various temperatures has been examined, which revealed that maintaining at pH 6.5 and the use of deuterium oxide at a concentration of 90% are the most effective conditions. All other additives such as detergents, albumin, antiviral compounds, sucrose, and magnesium chloride (alone or in combination) exhibit no comparative effectiveness. Poliovirus vaccine is the most extensively studied vaccine that is stabilized by deuterium oxide.

The oral poliovirus vaccines prepared with three live-attenuated polioviruses (Sabin strains, serotypes 1, 2, and 3) are considered as most thermolabile of vaccines. Improved thermostabilizing effect was found in all three Sabin strains with a combined 87% $D_2O$ and one molar $MgCl_2$. Attempts were made to elucidate the mechanisms underlying the thermal stabilization of these three serotypes of poliovirus vaccine strains by $D_2O$ and $MgCl_2$.

Temperature-dependent fluorescence emission studies showed that the effects of $D_2O$ and $MgCl_2$ on the stability and conformation of poliovirus are correlated with those of the infectivity of poliovirus. The rigidity of poliovirus conformation is increased in the presence of $D_2O$-$MgCl_2$. The involvement of hydrogen bonding in the $D_2O$ effect was also demonstrated by the greatly increased value of relative fluorescence intensity.

With stronger hydrogen bonds, $D_2O$ protects virus proteins against denaturation and enhances the thermostability of poliovirus. $D_2O$ and $MgCl_2$ were also found to reduce the interaction of the virus with water, which leads to a decline in the extent of water penetration into the poliovirus capsid. These observations were found to be more profound in a combination of $D_2O$ and $MgCl_2$ than in $D_2O$ or $MgCl_2$ alone.

By inducing a conformation favorable to maintaining the poliovirus assembly and by reducing virus-water interaction to decrease water penetration into the poliovirus capsid, a combination of $D_2O$ and $MgCl_2$ enables to exert their thermal stabilization effect. Maintaining the assembly and conformation of the virus and reducing the swelling of the virus capsid are two mutually complementary factors. The latter can provide a favorable environment for the former, and the former, in turn, lead to the latter.

Although clinical data have shown the safety of deuterium oxide at the dosage that would be given with polio vaccine, reservations are raised about the public acceptability of polio vaccine with the addition of deuterium oxide. As a result, this area of research has not been pursued further. Nevertheless, this issue deserves further investigation.

## 11.3 Thermostability of Other Vaccines by $D_2O$

As indicated above, preservation of vaccines, viruses, and other biologicals is one of the critical tasks in maintaining the quality of medical products from manufacture until they reach the users. Live-attenuated viral vaccines usually require cold-chain maintenance. Deuterium oxide has been reported to have a protective effect on biomolecules and organisms against thermal shock.

In addition to the above-discussed poliovirus vaccine, $D_2O$ has also been demonstrated in the stabilization of other vaccines, including influenza vaccine, yellow fever vaccine, and Newcastle disease virus vaccine. Thermal stabilization effects on these vaccines by deuterium oxide are described below.

### *11.3.1 Influenza Vaccine*

Influenza vaccination represents the basis of influenza prevention. In manufacture, influenza vaccines are formulated as liquid, which is unstable at ambient temperatures and required to be stored and distributed under refrigeration. In order to maintain stabilization, influenza vaccines can be brought into the dry state using suitable stabilizers and drying processes. The resulting stabilized influenza vaccine powder is independent of cold-chain facilities.

However, the status of influenza vaccine development remains a challenge. The ultimate market is the introduction of a stable and effective dry-powder influenza vaccine. The above finding that the addition of deuterium oxide to tissue culture cell harvests stabilizes oral poliovirus vaccine leads to the examination of $D_2O$ effect on stabilization of influenza vaccine.

Investigations of the thermal stabilization effect of deuterium oxide on live-attenuated influenza A and B vaccines grown in embryonated eggs revealed that thermal stabilization with $D_2O$ is much greater than seen with standard stabilization

of these live influenza vaccines with sucrose-phosphate-glutamate. Such findings that $D_2O$ is able to stabilize influenza vaccine have important practical application.

### 11.3.2 Yellow Fever Vaccine

Yellow fever, an acute mosquito-borne viral fever, is preventable by the use of the live, attenuated vaccine produced using the 17D strain of yellow fever attenuated virus. The vaccine is used principally in tropical climates and is subject to potentially adverse conditions. Lyophilized vaccine without stabilizers deteriorates rapidly when exposed to temperatures above -20 °C.

A successful formulation used by various manufacturers employs sugars, amino acids, lactose (4%), sorbitol (2%), histidine (0.01 M), and alanine (0.01 M) in phosphate-buffered saline with divalent cations $Ca^{2+}$ and $Mg^{2+}$. Despite its stability when freeze-dried, yellow fever 17D vaccine is quite unstable after reconstitution.

Several stabilizers are currently used to help retain the quality of vaccines during transit. Deuterium oxide has previously been reported to have a protective effect on biomolecules, cells, and organisms. The stabilizing effect of $D_2O$ was also demonstrated in the yellow fever 17D vaccine virus strain. Analyzing its stability using different reconstitution solutions revealed that 90% $D_2O$ shows the best stabilizing effect for yellow fever vaccine under thermal treatment of 37 °C up to 24 h.

### 11.3.3 Newcastle Disease Virus Vaccine

Newcastle disease virus is a paramyxovirus that bears two envelope glycoproteins at the virion surface. The development of Newcastle disease virus vaccine is based on immunization with avian cells expressing glycoprotein. Then, recombinant cells constitutively expressing at the surface of this glycoprotein are generated. They are used to immunize chickens and birds.

The loss in vaccine potency due to inactivation of the virus was studied in vitro at various time intervals. The thermostability of Newcastle disease virus vaccine was compared by reconstituting commercially available vaccine with diluents prepared in $H_2O$ and 80% $D_2O$ at ambient temperature. Reconstitution of freeze-dried Newcastle disease virus vaccine in 80% $D_2O$ diluent was found to maintain the vaccine stability for 50 h or more at 35 °C temperatures without significant deterioration in potency.

The above-discussed $D_2O$ protection effects on various vaccines, including polio vaccine, influenza vaccine, yellow fever vaccine, and Newcastle disease virus vaccine, imply that the potential of using deuterium oxide to stabilize other biologicals is anticipated. For instance, it has also been known that deuterium oxide exhibits protective effects on attenuated viral RNA as discussed above.

**Table 11.1** Deuterium oxide effects on thermostability of vaccines

| Vaccine | $D_2O$ or deuteration effect |
|---|---|
| Polio vaccine | $D_2O$ exhibits significantly thermal stabilization effect |
| | More stabilized by a combination of $D_2O$ and $MgCl_2$ than $D_2O$ alone |
| Influenza vaccine | $D_2O$ is a greater stabilizer than standard stabilization with sucrose-phosphate-glutamate |
| Yellow fever vaccine | 90% $D_2O$ shows the best stabilizing effect |
| Newcastle disease | $D_2O$ maintains the vaccine stability for more |
| Virus vaccine | Than 50 h at 35 °C |
| Heroin vaccine | Deuterated vaccine produces greater efficacy than protium vaccine |
| Veterinary vaccines | Exploration of stabilization by $D_2O$ is under investigation |

In summary, deuterium oxide effects on thermal stabilization of various vaccines are briefly described in Table 11.1. These findings imply the potential development of thermal stabilization of veterinary vaccines by deuterium oxide. Future exploration in this area of research is needed.

## 11.4 Mechanism of $D_2O$ as Vaccine Stabilizer

An important issue in the applications of deuterium oxide to thermal stabilization of various vaccines is the elucidation of the mechanism of stabilization. As mentioned above, experimental evidence indicates that $D_2O$ reduces the interaction of the virus with water, leading to the enhancement of the rigidity conformation of the virus by increasing hydrophobic interaction of the virus and the decrease of the penetration of water into the virus capsid.

In vaccines, $D_2O$ was found to reduce the interaction of the virus with water, resulting in a decline in the extent of water penetration into the virus capsid. Consequently, the rigidity of virus conformation is increased in the presence of $D_2O$. The involvement of hydrogen bonding in the $D_2O$ effect was demonstrated by a significant increase in relative fluorescence intensity and changes in circular dichroism spectra. For example, the induction of conformation favorable to maintaining the poliovirus assembly and the reduction of virus-water interaction leads to decreasing water penetration into the poliovirus capsid. Consequently, $D_2O$ enables to exert its thermal stabilization effect on polio vaccine.

The proposed involvement of hydrogen bonding is consistent with the finding that $D_2O$ stabilizes attenuated viral RNA against thermal degradation by enhancing the stability of the folded basin. As monitored through intramolecular hydrogen bond, $D_2O$ strengthens the hydrogen bonding network of RNA and lengthens inter-residue water bridge lifetime.

Maintaining the virus assembly and conformation and reducing the swelling of the virus capsid are the two key factors that contribute to the increase in the

thermostability of poliovirus by deuterium oxide. Future investigations in vaccine systems other than poliovirus vaccine are needed to further elucidate the mechanism of $D_2O$ effect on thermostability of vaccines in general.

## 11.5 Stabilization by Deuteration: Heroin Vaccine

Development of a heroin vaccine is a major research focal point. An efficient vaccine against heroin is required to generate not only a potent immune response but also respond against heroin and its multiple psychoactive molecules. Selective deuteration of a heroin hapten and its impact on the immune response against heroin and its psychoactive metabolites was investigated and compared for deuterium and protium heroin haptens. The results revealed that the deuterated vaccine exhibited a greater efficacy as compared to the protium vaccine. These findings suggested that selective deuteration of heroin hapten could be useful for developing vaccine against heroin abuse.

## 11.6 Concerns About Deuterium as an Isotope

Although deuterium is a nonradioactive isotope of hydrogen and has been extensively applied to various medical fields, concerns have been raised in clinical research due to deuterium being an isotope. Further research is needed to address such a concern, particularly, the clinical applications of deuterium oxide to thermal stabilization of various vaccines.

Besides the concern of deuterium as an isotope, another issue that also requires attention is that deuterium oxide exhibits toxicity toward mammals at a concentration >20%. Accordingly, further research concerning the applications of $D_2O$ to health-related issues may also require to address its effects on cells, including cytotoxicity, cytostatic activity, antineoplastic effect, antimitotic activity, and nucleic acid synthesis.

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# Chapter 12
# Deuterium Oxide and Deuteration Effects on Pharmacology

A critical area of pharmacologic research involves the study of how a body processes a drug, including the time course of absorption, distribution, bioavailability, metabolism, and excretion. To improve the effectiveness of therapeutics of drugs, research has been carried out to prolong the residence time of drug in plasma to achieve greater efficacy and alter the rate of metabolism to afford greater tolerability.

Another critical area of pharmacologic research is the studies of the reactions between drugs and living systems, including the investigation of the body's biological responses to drugs as well as biochemical or molecular interactions between the drug and the human body.

The above two important areas of research are referred to as pharmacodynamics and pharmacokinetics, respectively. In pharmacodynamics, the drug molecule interacts with the target receptor to create the desired therapeutic effect, where the interaction is directly correlated to the drug concentration. While the pharmacokinetic process involves adsorption, distribution, metabolism, excretion, and toxicity of the drug. The present chapter is most focused on pharmacokinetics than pharmacodynamics.

Improvement of the half-life of a drug can lower the dose of the medication and limits the formation of toxic metabolites. If the drugs are metabolized and excreted too quickly during the pharmacokinetics, then they are unable to produce the desired therapeutic effect. Thus, change in metabolism may affect the duration of drug action and the toxicity of metabolites.

Deuteration is commonly used in the studies of metabolism of drugs or toxic substances in human health. To slow down the metabolic process and improve the half-life of the drugs, incorporation of deuterium in the drugs (referred to as deuterated drugs) has been carried out. In many cases, the metabolism of the drugs may be favorably influenced when deuterium is substituted for protium in drug molecules, resulting in improved safety, tolerability, or efficacy.

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If the deuterium is strategically located in a drug's chemical structure, the extra stability of the bond will be more resistant to metabolic breakdown, and the duration of drug action can be prolonged, leading to deuterated drugs often being more resistant to metabolic changes. Thus, due to the stronger deuterium-carbon bond that modifies their metabolism, deuterated versions of existing drugs can exhibit improved pharmacokinetic or toxicological properties.

Moreover, the deuteration effect also involves chemical reactions, such as changes in the rate of cleavage of covalent bonds to an atom located adjacent to deuterium in a reactant molecule. Such changes in the rate of bond breaking can lead to the primary kinetic isotopic effect, resulting in slowing down the metabolic pathway, reducing systemic clearance, and an improved half-life and bioavailability of a drug.

The effects of deuterium oxide on living systems can be classified into two categories: (a) solvent effect based on the properties of $D_2O$ molecule as a whole in particular its effects on the structure of water around biological macromolecules and (b) deuteration effect resulting from the ability of deuterium to replace with hydrogen in biological molecules.

The physical basis of deuterium oxide solvent effect is mainly due to the differences in polarities and noncovalent interactions, including ionic, Van der Waals interaction, hydrogen bonding, and hydrophobic interaction. While deuteration effects can affect noncovalent interactions between biomolecules and solvent. The sizes of deuterated versus nondeuterated molecules and the extent of noncovalent interactions depend on the site of deuteration within a biomolecule.

Moreover, since the C-D bond is stronger than the C-H bond, C-D bond is more resistant to enzymatic cleavage, such as oxidative processes catalyzed by cytochrome P450 or by other enzymes which are involved in metabolism where the C-H bond cleavage is the rate-limiting step. A strategy to take advantage of deuterium over hydrogen is in terms of atomic mass.

Deuterium forms bonds with carbon that vibrate at a lower frequency. Therefore, C-D bonds are thus stronger than C-H bonds. If a particular C-H bond in a drug molecule is known to be broken during metabolisms, then swapping that hydrogen for deuterium can slow the process down. Hence, deuterium versions of drugs may last longer in the body.

## 12.1 Pharmaceutical Processes

Potential effects of deuterium oxide and deuteration on pharmacokinetics and pharmacodynamics that drugs encounter in the body are summarized in Table 12.1. Pharmacologic research on deuterium oxide not only investigates its effect on the reactions between drugs and living systems but also biochemical or molecular interactions between the drug and the human body.

As mentioned before, pharmacokinetics studies how the body processes a drug, such as the time course of absorption, distribution, bioavailability, metabolism, and

**Table 12.1** Examples of pharmacological and biochemical processes that deuterium oxide and deuteration encounter

| Deuterium oxide/ deuteration effect | Pharmacological/biochemical process |
|---|---|
| Deuterium oxide solvent effects | Strengthening hydrogen bonding network |
| | Affecting degradation of biomolecules, enzyme catalytic reactions, and drug degradation reactions |
| | Toxicity to cells at $D_2O$ > 20% of body weight |
| Deuteration substitution effects | Stronger C-D bond than C-H bond |
| | Potential improvement of the safety, efficacy, and tolerability of a therapeutic agent |

excretion. Various factors affecting pharmacokinetics of drugs include the water solubility and the degree of drug dissociation. If the drug is metabolized and excreted too quickly, then it is no longer available to produce the desired therapeutic effect. In contrast, p*harmacodynamics* refers to the studies of the reaction between a drug and the living systems, including the body's response to drugs and the interactions between the drug and the human body.

## 12.2 Deuterium Oxide Solvent Effects

Deuterium oxide solvent effects occur when deuterium replaces hydrogen in many pharmacological and biological systems. Among them is the degradation rate of drug and its related mechanism. Distinctive physical and chemical properties of deuterium oxide in comparison with hydrogen oxide leads to variations in molecular properties of biomolecules as $H_2O$ is replaced with $D_2O$ as the solvent, such as hydrogen bonding and hydrophobic interaction.

For example, the strengthening of hydrogen bonding network by $D_2O$ was demonstrated in studying RNA stability, which revealed that deuterium oxide stabilizes attenuated viral RNA hairpin against its thermal degradation and lengthens its lifetime of inter-residue water bridge. A significant increase in the stability of RNA hairpin due to a higher melting temperature in $D_2O$ as compared with $H_2O$ was also found.

Other investigations of deuterium solvent effects on pharmacology and biomolecules include enzyme-catalyzed reactions and the degradation of drug-related metabolism. For instance, $D_2O$ affects catalytic reactions of cytochrome P450s (CYP450s), the most important enzyme in the activation metabolism of drugs. Moreover, $D_2O$ was found to affect hydroxide ion-catalyzed degradation reaction of penicillin. At below pH (pD) 6, penicillin exhibits a ratio of 1.53 for the rate constants for the H - and D-substituted reactants.

In contrast to the above beneficial effects, potential toxic effect of deuterium oxide on living cells was also examined. At a higher concentration (usually >20% of body weight), deuterium oxide can be toxic to human cells. Nevertheless, at a low concentration, deuterium oxide can be helpful to health-related issues, such as its effects on human diseases and its application to measure body water.

Examples of pharmacological and biochemical processes that deuterium oxide encounters are briefly summarized in Table 12.1.

## 12.3 Deuteration Effects

Since many drugs are carbon-based compounds, carbon-hydrogen bonding is particularly important for understanding the properties of drug molecules. A deuterated drug is a drug in which the hydrogen atom in one or more of the carbon-hydrogen bonds in its chemical structure is replaced by deuterium-carbon bonds.

Selective replacement of hydrogen atoms with deuterium (deuteration) in drug has a benefit of retaining the pharmacologic profile of active drug compound. In addition, it also has an impact on drug metabolism, leading to potential benefit to improve the safety, efficacy, and tolerability of a therapeutic agent.

In spite that the complexity of biological systems can make the applications of deuteration to drugs highly unpredictable, deuterated drugs with well-defined pharmacological effects can potentially provide an efficient approach to creating new medicines that meet important medical requirements (Table 12.2).

**Table 12.2** Potential $D_2O$ and deuteration effects on drug molecules

| $D_2O$/deuteration | Effect on drug molecules |
|---|---|
| Deuterium oxide as solvent | Deuterium bonding versus hydrogen bond |
| | Affecting the degradation rate of drug |
| | Slower rate of metabolic breakdown |
| | Affecting catalytic reactions of drug metabolism enzymes |
| Deuteration | Stronger C-D bond than C-H bond |
| | Slowing down metabolic process |
| | Changing the pathway of drug metabolism |
| | Increasing half-life of drug |
| | Lowering the dose of medication |
| | Reducing toxic metabolites |

### 12.3.1 Effects of Deuteration on C-H Bond

A larger atomic mass of deuterium (D) implies a C-D bond having a lower vibrational frequency and lower zero-point energy than a corresponding C-H bond. A lower zero-point energy translates to a higher activation energy, which leads to a slower reaction rate constant for C–D bond. Because a C-D bond is more stable than a C-H bond, a chemical bond is harder to break with a heavier deuterium isotope.

When the cleavage of a C-H bond is the rate-determining step, a slower rate of bond cleavage can result in deuterium kinetic isotope effect. Such an effect has the potential to affect biological processes of many drugs that are metabolized by involving C-H bond. As a result, the substitution of C-H bond by C-D bond can lead to enhancing pharmacokinetic profile of a drug.

Selective deuteration has a unique effect of retaining the pharmacologic profile of drugs. Due to the higher mass of deuterium (D) compared with hydrogen (H), the C-D bond is much more resistant toward oxidative processes, such as shown in cytochrome P450-catalyzed reactions.

Since it is harder for a metabolizing enzyme to break the C-D bond, the replacement of H with D can slow the related reaction as bond breaking is a rate-determining step. Slowing down the metabolic pathway can lead to reduced systemic clearance, which results in an improved half-life and bioavailability of drugs.

### 12.3.2 Deuteration Kinetic Isotope Effect

Deuteration kinetic isotope effect is the relative change in the rate of a chemical reaction when one of the atoms in the reactants is substituted with one of its isotopes. A lower zero-point energy translates to a higher activation energy for C-D bond cleavage and a slower reaction rate (*k*). The primary deuteration isotope effect is expressed as $k_H/k_D$, the ratio of the reaction rate constants of C–H versus C–D bond cleavage.

The deuteration effect on drugs is associated with an increase in the kinetic isotope effect of pharmaceutical substances. The substitution of C-H bond by C-D bond leads to enhancing the pharmacokinetic profile of drugs, which allows developing pharmaceuticals that exhibit improvement in pharmacokinetics, decreasing their toxicity, and changing the mechanism of their action. Deuterated drugs may also offer advantages over nondeuterated drugs through alterations in clearance and metabolic pathways.

Accordingly, the goals of deuterated drugs are to improve half-life, lower the dose of the medication, limit the formation of toxic metabolites, and enhance the metabolic profile. Since deuteration is an effective tool for the enhancement of a drug's metabolic profile, medicinal chemists have also used deuterated drug candidate to slow enzyme metabolisms, especially those changes mediated by the enzyme cytochrome P450s.

Moreover, the deuterated forms of drugs often have different actions than the protonated forms by showing different transport processes. Deuteration may also change the pathway of drug metabolism, referred to as metabolic switching. Changes in metabolism may lead to increased duration of action and lower toxicity. For example, deuteration can lower the genotoxicity of anticancer drugs, such as tamoxifen.

Selective replacement of hydrogen with deuterium can also lead to increased bond strength. In turn, deuteration can enhance the biological half-life and metabolic stability of the drug. Meanwhile, selective replacement of hydrogen with deuterium has the unique benefit of retaining the drug pharmacologic profile.

### 12.3.3 Deuteration Effects on Pharmacokinetics

Two broad areas of clinical pharmacology are pharmacokinetics and pharmacodynamics. Pharmacokinetics is concerned with what the body does to the drug, including absorption, distribution, metabolism, and excretion of drugs, while pharmacodynamics is involved with the body's biological response to drugs.

The pharmacokinetic properties of drugs may be altered by deuteration effects. To gain information on the application of deuteration to alter drug pharmacokinetics, it is important to understand the drug clearance mechanism, the metabolic enzymes involved**,** and the differences in systemic clearance mechanisms.

Drug metabolism involves the breaking of chemical bonds. Selective replacement of hydrogen with deuterium can lead to increased bond strength, which in turn increases the half-life and metabolic stability of the drug. Factors that delay or inhibit the breaking of chemical bonds can lead to increasing the half-life of a drug.

Researchers in the pharmaceutical industry use deuterium as a tool to improve drug properties. Deuterated drugs that have been evaluated in clinical studies are such as paroxetine, tetrabenazine, and dextromethorphan. Since deuteration of a drug is most likely to affect pharmacokinetic rather than pharmacodynamic properties, deuteration has gained attention on its potential effects on the pharmacokinetic metabolic profiles.

The kinetic isotope effect was explored by studying reaction mechanisms as the cleavage of an isotope bond is the rate-determining reaction. An understanding of enzymatic reaction mechanism, metabolic clearance of drug, and systemic clearance mechanisms is critically important to the pharmacokinetic application of deuteration kinetic isotope effects.

One important factor for most drugs is the rate of metabolism. Slowing the rate of metabolism of a drug will increase its half-life. The dosage of drugs is designed such that the blood concentration of the drug falls between below the required threshold and the high exhibiting toxicity. Knowledge of the dose, time, and frequency keeps the blood level of the drug within the therapeutic window as much as possible throughout the course of therapy.

The applications of deuteration as a strategy to alter drug pharmacokinetics are elaborated below, using cytochrome oxidases, anthocyanins, and peptide drugs as examples.

#### 12.3.3.1 Enzymes

Investigations of deuterium isotope effects on pharmacokinetics of enzyme CYP450s (CYP3A4 and CYP2C19) with specifically deuterated form revealed the complexity of deuteration as an approach for improving their pharmacokinetics when enzymes are involved in metabolic clearance. Pharmacologists have also used the deuterium kinetic isotope effect to slow the CYP450 metabolism of the deuterated versions of drug candidates.

Interestingly, the magnitude of the intrinsic clearance isotope effect is dependent on the position and extent of deuteration. With one chemotype, no intrinsic clearance deuterium isotope effect was found, whereas with the other chemotype, the rate-limiting step was isotopically sensitive.

#### 12.3.3.2 Anthocyanins

Anthocyanins are plant metabolites that have valuable applications as natural food colorants. Due to their antioxidant and anticancer properties, anthocyanins are also known for their beneficial health effects. Stable versions of these molecules are applied to investigate pharmacokinetic properties of anthocyanins and to obtain the effectiveness of their therapeutic results.

Deuterated anthocyanin from *Escherichia coli* was produced in the culture medium containing deuterated glycerol and $D_2O$, which resulted in the formation of deuterated cyanidin 3-glucoside, an anthocyanin. Studies of the deuterium exchange of O-D and C-D bonds revealed that deuterated cyanidin 3-glucoside is more stable as compared to nondeuterated one.

#### 12.3.3.3 Peptide Drugs

The main problem for peptide-based drugs is their low degree of cellular uptake. Due to the conformational flexibility and the interaction of proteases with backbone amide bonds, peptide drugs tend to exhibit poor pharmacokinetic properties. To improve this problem, *stapled peptides* were developed, which bring an external brace to force the peptide structure into an α-helical conformation by using hydrocarbon cross-linking strategy.

Studies of the conformational dynamics of peptide drugs revealed that in stapled peptides, amide exchange is reduced depending on staple placement and deuteration kinetics is correlated directly with the rate of proteolysis for improving the properties of peptide drugs.

## 12.4 Deuterated Drug Molecules

As discussed above, deuterium is gaining increasingly applied as a component of active pharmaceutical ingredients, in which selective hydrogen atoms in a drug molecule are replaced with deuterium atoms. The kinetic isotope effect of deuterated drugs allows a new direction in the development of pharmaceuticals. While deuterium drugs retain the potency of their hydrogen analogs, they often exhibit different pharmacokinetic properties in comparison with protonated ones, such as improved safety, tolerability, or efficacy.

Deuterated-containing drugs may also offer advantages in metabolic pathways, including lowering metabolic rate and more resistant to metabolic changes. Beyond the improvement of such pharmacokinetic parameters, deuteration in medicinal chemistry may also provide an opportunity to improve problems in terms of metabolism-mediated toxicity, drug interactions, and bioactivation in addition to lowering the degree of epimerization and reducing the dose of administered drug.

Examples of pharmacological effects on deuterated drugs, including paroxetine, delta-tocotrienol, 7-ethoxy coumarin, enzalutamide, vismodegib, deutetrabenazine, and 2′,3′,5′,5″-tetradeuterated uridines, are briefly discussed below.

### *12.4.1 Paroxetine*

In deuterated paroxetine, the two hydrogen atoms at the methylenedioxy carbon are substituted with deuterium atoms. Although deuterated paroxetine shows similar selectivity for serotonin receptor and neurotransmitter uptake inhibition as unmodified paroxetine, human liver microsomes clear deuterated paroxetine faster than nondeuterated one.

Moreover, deuterated paroxetine is metabolized more rapidly and exhibits a lower pharmacokinetic accumulation index than nondeuterated one. These results revealed that deuteration can improve the metabolism profiles of existing pharmacotherapies of paroxetine without affecting its intrinsic pharmacology.

### *12.4.2 Delta-tocotrienol*

Delta-tocotrienol is a member of vitamin E family. The deuterium substituted delta-tocotrienol derivative, $d_6$-delta-tocotrienol, was synthesized to examine the metabolic stability of delta-tocotrienol. The result showed that deuterated delta-tocotrienol improved its metabolic stability as compared to the nondeuterated one.

### 12.4.3 7-Ethoxy Coumarin

7-Ethoxy coumarin was employed as a model substrate in the reactions catalyzed by enzyme CYP450s. Kinetic isotope effects were applied to probe the aspects of CYP450 kinetics. Enzymes CYP450 1A2 and 2E1 are known to be major catalysts of 7-ethoxy coumarin O-deethylation in human liver microsomes.

In addition, human CYP450 1A2 was known to catalyze 3-hydroxylation of 7-methoxy coumarin at appreciable rates, where the C-H bond-breaking step is a major contribution to the reaction rate. Deuterium isotope effects were applied to estimate the intrinsic kinetics of 7-methoxy or 7-ethoxy coumarin dealkylation reaction.

### 12.4.4 Enzalutamide

Enzalutamide is a competitive inhibitor of the androgen receptor and is used for the treatment of metastatic castration-resistant prostate cancer. To attenuate the N-demethylation pathway, hydrogens of the N-CH3 moiety are replaced by deuterium atoms. The results revealed that deuterated d3-enzalutamide shows similar pharmacological activities but exhibits more favorable pharmacokinetic properties. The maximum velocity and Michaelis constant of d3- enzalutamide in human liver microsomes were found to be 72.9% lower than those of the nondeuterated compound, and the exposure of the N-demethyl metabolite is many folds lower.

### 12.4.5 Vismodegib

Vismodegib is a drug used for the treatment of basal cell carcinoma. Analogs of vismodegib with deuterium for hydrogen replacement at certain metabolically active sites were found to have better pharmacokinetic properties than nondeuterated vismodegib. By prolonging blood circulation half-life time, the deuterated vismodegib also alters better blood circulation behavior as compared to the nondeuterated one.

### 12.4.6 Deutetrabenazine

The pharmacokinetics and safety of deutetrabenazine in comparison with tetrabenazine were investigated to evaluate the impact of deuteration on pharmacokinetics of their active metabolites (alpha-dihydrotetrabenazine and beta-dihydrotetrabenazine) with respect to their metabolite profile, safety, and tolerability. Deutetrabenazine

**Table 12.3** Pharmacological effects of deuterated drug molecules

| Deuterated drug | Pharmacological effects |
|---|---|
| Deutetrabenazine | Better pharmacokinetic profiles |
| | Longer half-lives for active metabolites |
| Delta-tocotrienol | Improvement of metabolic stability |
| Enzalutamide | Favorable pharmacokinetic properties |
| 7-Ethoxy coumarin | Kinetic isotope effects for probing |
| | CYP450 catalyzed reaction |
| Paroxetine | Improvement of metabolism profiles lowering pharmacokinetic accumulation |
| 2′,3′,5′,5″-tetradeuterated uridines | Synthesizing deuterium-substituted nucleic acid probes |
| Vismodegib | Prolonging blood circulation half-life time |
| | Better blood circulation |
| Peptide drugs | Affecting rates of proteolysis to improve drug properties |
| | Better pharmacokinetic properties |

was found to result in a better pharmacokinetic profile and an increase in the ratio of active to inactive metabolites. These findings confirmed that deutetrabenazine exhibits longer half-lives for active metabolites as compared with tetrabenazine.

### *12.4.7 2′,3′,5′,5″-Tetradeuterated Uridines*

2′,3′,5′,5″-Tetradeuterated uridine derivatives are synthesized from 2,3,5,5′-selectively tetradeuterated ribose. The deuterium content of the tetradeuterated uridines is over 92%. These derivatives are expected as building blocks for the synthesis of deuterium-substituted nucleic acid probes for tracking the pharmacokinetics of nucleic acid drugs.

In summary, the above-discussed pharmacological effects of deuterated drugs are briefly presented in Table 12.3.

## 12.5 Metabolic Actions of Deuterated Drugs

Since a carbon-deuterium (C-D) bond is more stable than a carbon-hydrogen (C-H) bond, the extra stability of C-D bond will be more resistant to metabolic breakdown, and the duration of drug action will be prolonged if the deuteration is strategically located in a drug's chemical structure.

One of the most important contributing to the half-life of drugs is the rate of drug metabolism. Factors that slow the rate of drug metabolism will enhance its half-life. Since drug metabolism involves the breaking of chemical bonds, inhibition or

delaying the breaking of chemical bonds will increase the drug half-life. To slow down the metabolic process and improve the half-life of drugs, researchers have incorporated deuterium in the drug structures.

Moreover, due to having a longer half-life than nondeuterated counterparts, deuterated drugs would produce different metabolites. The improved half-life may lower the dose of the medication, so as to limit the formation of toxic metabolites. Consequently, the toxicity of many known drugs can be modified by selective deuteration.

## 12.6 Deuterabenazine: First Approved Deuterated Drug

As discussed above, researchers are interested in replacing C-H bond with C-D bond in drug molecules to extend the lifetime of active drugs, so as to improve their pharmacokinetic and toxicological properties for better efficacy with reducing clinical dosage. One of the deuteration ideas is to prolong the residence time of the active drug species in plasma to achieve greater efficacy and to avoid its adverse side effects. Using deuterium to replace hydrogen in drug molecules was also sought to inhibit metabolic conversion to less active or inactive molecules.

Deutetrabenazine, a version of tetrabenazine, is the first deuterated drug molecule approved by US Food and Drug Administration to use for treating chorea associated with Huntington's disease. Chorea is the most common motor manifestation of Huntington's disease. The disease may interfere with daily activities. Tetrabenazine has been used for the treatment of such movement disorders.

In deuteration, the two methoxy groups are replaced by two trideuteromethoxy groups in deutetrabenazine. Differences in pharmacokinetics are shown in comparison of deutetrabenazine with tetrabenazine, including alteration of the rate of metabolism, leading to greater tolerability and improving dosing regimen.

Moreover, deutetrabenazine hinders oxidative metabolism of the methoxy groups, thus providing the primary kinetic isotope effect. In addition to being as effectiveness as tetrabenazine in the treatment of Huntington's disease, deutetrabenazine also has a lower risk of adverse effects than tetrabenazine.

Deuteration of tetrabenazine to deutetrabenazine is presented in Fig. 12.1, where the two methoxy groups on its aryl ring and the methoxys are demethylated to the phenol or catechol. Such replacement of $OCH_3$ with $OCD_3$ alters pharmacokinetic and toxicological properties of this drug.

Comparative assessment of deutetrabenazine and tetrabenazine on their pharmacokinetics and safety has been performed. Deutetrabenazine is as effective as tetrabenazine in the treatment of chorea but has a lower risk of adverse effects. The effects of food on absorption of the deuterated active metabolites, α- and β-dihydrotetrabenazines, were also examined. Both studies confirmed longer half-life for active metabolites and lower metabolites in comparison of deutetrabenazine with tetrabenazine.

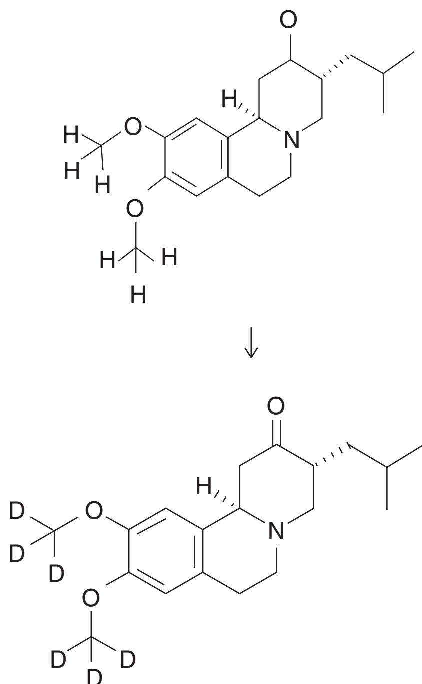


**Fig. 12.1** Deuteration of tetrabenazine to deutetrabenazine

Moreover, enzymes CYP450s are most responsible for the activation metabolism of most drugs. Other enzymes in drug metabolism include monoamine oxidase, alcohol dehydrogenase, and aldehyde oxidase. Deutetrabenazine was found to exhibit a lower rate of metabolism of its active metabolites, α- and β-dihydrotetrabenazines, by CYP2D6 enzyme.

In addition, the pharmacokinetics and safety of deutetrabenazine and its metabolites were also assessed by examining the effects of paroxetine, a potent CYP2D6 inhibitor. Paroxetine administration was found to increase the exposure of the deuterated active metabolites, α- and β-dihydrotetrabenazines. Such increases were shown to be less than those previously reported for tetrabenazine. These findings implied that deutetrabenazine reduces the burden of drug interaction.

## 12.7 Other Deuterated Drugs

Among other deuterated drugs are dextromethorphan/quinidine and paroxetine. *Dextromethorphan*/quinidine belongs to a class of medications called central nervous system agents, which is used to treat agitation in patients with Alzheimer-type dementia. Paroxetine is a class of medications called selective serotonin-reuptake inhibitors, which is used to treat depression.

Deuterated (d6)-dextromethorphan/quinidine is a compound under recent clinical development. Deuteration appears to prolong dextromethorphan's plasma half-life and facilitate brain penetration. Its pharmacokinetic and pharmacodynamic profiles and safety issues were under discussion. A clinical development program of deuterated (d6)-dextromethorphan/quinidine for Alzheimer-type dementia agitation has been initiated.

Moreover, the two hydrogen atoms at the methylenedioxy carbon of paroxetine were substituted with deuterium. This new chemical entity is called CTP-347, which demonstrates similar selectivity for the serotonin receptor and the neurotransmitter uptake inhibition as unmodified paroxetine. However, human liver microsomes clear CTP-347 faster than unmodified paroxetine. In addition, CTP-347 is metabolized more rapidly in humans and exhibits a lower pharmacokinetic accumulation index than paroxetine.

## 12.8 Future of Deuterated Drugs

Drugs are mainly metabolized in the liver, which is often carried out through breaking C-H bonds in drug molecules. The placement of hydrogen with deuterium can also have a significant effect on slowing down the liver's clearance mechanism. The ideas of deuteration also include prolonging the residence time of the active drug species in plasma to achieve greater efficacy and to avoid adverse side effects. Ongoing clinical trials suggest that a number of other deuterated drug molecules are being developed and evaluated for the treatments of various diseases.

As discussed above, deuteration is an effective method for the enhancement of metabolic profile of drugs. Selective replacement of hydrogen with deuterium leads to increased bond strength, which in turn increases the biological half-life and the metabolic stability of drugs. Accordingly, many known drugs can be modified by selective deuteration, leading to improving their pharmacokinetic properties.

Moreover, the incorporation of deuterium into pharmacologically active agents offers potential benefits resulting in a decrease in the production of toxic metabolites and improvements in efficacy, tolerability, or safety. Deuterated drugs can also resist metabolic degradation and remain active for longer in the body than the non-deuterated ones.

In conclusion, deuterium oxide and deuteration have potential utilization in pharmaceutical industries, leading to developing new medications. An increase in recent interests in deuterated medicines is reflected by a greater number of patent filings. Deuterium-modified drugs that are being developed include dextromethorphan, ruxolitinib, ivacaftor, pioglitazone, and linoleic acid. Early clinical results have been encouraging. The advancement of these deuterated drug candidates should play an important role in the applications of deuterium oxide and the developments of deuterated drugs for clinical applications.

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