Friedrich Siebert and Peter Hildebrandt

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Friedrich Siebert and
Peter Hildebrandt
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in Life Science

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The Authors

Friedrich Siebert
Institut für Molekulare Medizin und Zellforschung
Albert-Ludwigs-Universität Freiburg
e-mail: frisi@biophysik.uni-freiburg.de

Peter Hildebrandt
Institut für Chemie
Technische Universität Berlin
e-mail: hildebrandt@chem.tu-berlin.de

Cover Picture
Vibrational spectroscopy, i.e. Raman (bottom) and infrared (top) spectroscopy, has considerably contributed to the understanding of the function of proteins, here of the light-driven proton pump bacteriorhodopsin.

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Contents

Preface *IX*

1 Introduction *1*
1.1 Aims of Vibrational Spectroscopy in Life Sciences *2*
1.2 Vibrational Spectroscopy – An Atomic-scale Analytical Tool *3*
1.3 Biological Systems *4*
1.4 Scope of the Book *7*
1.5 Further Reading *9*
References *10*

2 Theory of Infrared Absorption and Raman Spectroscopy *11*
2.1 Molecular Vibrations *12*
2.1.1 Normal Modes *15*
2.1.2 Internal Coordinates *18*
2.1.3 The *FG*-Matrix *19*
2.1.4 Quantum Chemical Calculations of the *FG*-Matrix *23*
2.2 Intensities of Vibrational Bands *25*
2.2.1 Infrared Absorption *25*
2.2.2 Raman Scattering *28*
2.2.3 Resonance Raman Effect *32*
2.3 Surface Enhanced Vibrational Spectroscopy *38*
2.3.1 Surface Enhanced Raman Effect *39*
2.3.2 Surface Enhanced Infrared Absorption *43*
References *60*

3 Instrumentation *63*
3.1 Infrared Spectroscopy *63*
3.1.1 Fourier Transform Spectroscopy *64*
3.1.1.1 Interferometer *67*
3.1.1.2 Infrared Detectors *69*
3.1.2 Advantages of Fourier Transform Infrared Spectroscopy *70*
3.1.3 Optical Devices: Mirrors or Lenses? *71*
3.1.4 Instrumentation for Time-resolved Infrared Studies *72*

3.1.4.1 Time-resolved Rapid-scan Fourier Transform Infrared Spectroscopy *72*
3.1.4.2 Time-resolved Studies Using Tunable Monochromatic Infrared Sources *74*
3.1.4.3 Time-resolved Fourier Transform Infrared Spectroscopy Using the Step-scan Method *74*
3.1.5 Time-resolved Pump-probe Studies with Sub-nanosecond Time-resolution *76*
3.2 Raman Spectroscopy *79*
3.2.1 Laser *80*
3.2.1.1 Laser Beam Properties *81*
3.2.1.2 Optical Set-up *83*
3.2.2 Spectrometer and Detection Systems *84*
3.2.2.1 Monochromators *84*
3.2.2.2 Spectrographs *86*
3.2.2.3 Confocal Spectrometers *87*
3.2.2.4 Fourier Transform Raman Interferometers *89*
References *97*

4 Experimental Techniques *99*
4.1 Inherent Problems of Infrared and Raman Spectroscopy in Life Sciences *99*
4.1.1 The ''Water'' Problem in Infrared Spectroscopy *99*
4.1.2 Unwanted Photophysical and Photochemical Processes in Raman Spectroscopy *101*
4.1.2.1 Fluorescence and Raman Scattering *102*
4.1.2.2 Photoinduced Processes *104*
4.2 Sample Arrangements *105*
4.2.1 Infrared Spectroscopy *106*
4.2.1.1 Sandwich Cuvettes for Solution Studies *106*
4.2.1.2 The Attenuated Total Reflection (ATR) Method *108*
4.2.1.3 Electrochemical Cell for Infrared Spectroscopy *113*
4.2.2 Raman and Resonance Raman Spectroscopy *116*
4.2.2.1 Measurements in Solutions *116*
4.2.2.2 Solid State and Low-temperature Measurements *117*
4.3 Surface Enhanced Vibrational Spectroscopy *118*
4.3.1 Colloidal Suspensions *119*
4.3.2 Massive Electrodes in Electrochemical Cells *120*
4.3.3 Metal Films Deposited on ATR Elements *122*
4.3.4 Metal/Electrolyte Interfaces *123*
4.3.5 Adsorption-induced Structural Changes of Biopolymers *127*
4.3.6 Biocompatible Surface Coatings *128*
4.3.7 Tip-enhanced Raman Scattering *130*
4.4 Time-resolved Vibrational Spectroscopic Techniques *131*
4.4.1 Pump–Probe Resonance Raman Experiments *132*
4.4.1.1 Continuous-wave Excitation *133*

4.4.1.2 Pulsed-laser Excitation *138*
4.4.1.3 Photoinduced Processes with Caged Compounds *141*
4.4.2 Rapid Mixing Techniques *141*
4.4.2.1 Rapid Flow *144*
4.4.2.2 Rapid Freeze–Quench *145*
4.4.3 Relaxation Methods *146*
4.4.4 Spatially Resolved Vibrational Spectroscopy *148*
4.5 Analysis of Spectra *149*
References *151*

5 Structural Studies *155*
5.1 Basic Considerations *155*
5.2 Practical Approaches *158*
5.3 Studies on the Origin of the Sensitivity of Amide I Bands to Secondary Structure *161*
5.4 Direct Measurement of the Interaction of the Amide I Oscillators *167*
5.5 UV-resonance Raman Studies Using the Amide III Mode *169*
5.6 Protein Folding and Unfolding Studies Using Vibrational Spectroscopy *171*
References *178*

6 Retinal Proteins and Photoinduced Processes *181*
6.1 Rhodopsin *183*
6.1.1 Resonance Raman Studies of Rhodopsin *185*
6.1.2 Resonance Raman Spectra of Bathorhodopsin *188*
6.1.3 Fourier Transform Infrared Studies of the Activation Mechanism of Rhodopsin *195*
6.1.3.1 Low-temperature Photoproducts *197*
6.1.3.2 The Active State Metarhodopsin II (MII) *201*
6.2 Infrared Studies of the Light-driven Proton Pump Bacteriorhodopsin *206*
6.3 Study of the Anion Uptake by the Retinal Protein Halorhodopsin Using ATR Infrared Spectroscopy *214*
6.4 Infrared Studies Using Caged Compounds as the Trigger Source *217*
References *222*

7 Heme Proteins *227*
7.1 Vibrational Spectroscopy of Metalloporphyrins *228*
7.1.1 Metalloporphyrins Under D_{4h} Symmetry *228*
7.1.2 Symmetry Lowering *231*
7.1.3 Axial Ligation *232*
7.1.4 Normal Mode Analyses *233*
7.1.5 Empirical Structure–Spectra Relationships *234*
7.2 Hemoglobin and Myoglobin *236*
7.2.1 Vibrational Analysis of the Heme Cofactor *237*

7.2.2 Iron–Ligand and Internal Ligand Modes *239*
7.2.3 Probing Quaternary Structure Changes *240*
7.3 Cytochrome *c* – a Soluble Electron-transferring Protein *244*
7.3.1 Vibrational Assignments *245*
7.3.2 Redox Equilibria in Solution *246*
7.3.3 Conformational Equilibria and Dynamics *248*
7.3.4 Redox and Conformational Equilibria in the Immobilised State *253*
7.3.5 Electron Transfer Dynamics and Mechanism *260*
7.3.6 The Relevance of Surface-enhanced Vibrational Spectroscopic Studies for Elucidating Biological Functions *267*
7.4 Cytochrome *c* Oxidase *268*
7.4.1 Resonance Raman Spectroscopy *268*
7.4.2 Redox Transitions *271*
7.4.3 Catalytic Cycle *274*
7.4.4 Oxidases from Extremophiles and Archaea *277*
References *278*

8 Non-heme Metalloproteins *283*
8.1 Copper Proteins *284*
8.2 Iron–Sulfur Proteins *290*
8.3 Di-iron Proteins *296*
8.4 Hydrogenases *300*
References *302*

Index *305*

Preface

Vibrational spectroscopy and life sciences, how do they fit together? For more than 30 years vibrational spectroscopy was the classical tool used for the study of small molecules and an analytical tool to characterise unknown chemical compounds, and therefore, it is not obvious that these two subjects would indeed fit together. Nevertheless, the fact that K. P. Hofmann asked us to write a book on the application of vibrational spectroscopy in life sciences, within the newly created series *Tutorials in Biophysics*, clearly demonstrates that this subject has reached a mature stage.

The success of vibrational spectroscopy in life sciences is certainly due, largely, to technical developments leading, for instance, to the commercial availability of lasers for Raman spectroscopy and rapid-scan interferometric detection systems for Fourier transform infrared (IR) spectroscopy. In this way, the sensitivity of vibrational spectroscopy increased considerably, allowing experiments that were hitherto unimaginable to be carried out. However, it is still not clear how these developments made it possible for the basic questions on protein function to be addressed, considering that proteins are very complex systems consisting of thousands of atoms.

Thus, the main goal of this tutorial is to provide arguments as to why vibrational spectroscopy is successful in biophysics research. Both of us have had the privilege of taking active roles in these exciting scientific developments right from the beginning. Thus, it should be understood that the material in this book has been influenced by our personal experiences. When we started to devise the content of the book, we soon realised that, when considering the application of vibrational spectroscopy in life sciences, we had to focus on *molecular* biophysics. This meant leaving out the exciting fields in which vibrational spectroscopy is used as a diagnostic tool for the identification of bacteria, cancerous cells and metabolites in living cells. In addition, within the field of molecular biophysics, we had to make compromises, mainly dictated by space limitations. We, therefore, decided to restrict the applications of vibrational spectroscopy to selected classes of proteins and enzymes for the benefit of an instructive illustration of the *principles* of the most important methodologies. The selection of examples was – inevitably – subjective and governed by didactic considerations. Thus, not all colleagues who have made important contributions to this field could be adequately referenced.

As an additional consequence, the vibrational spectroscopy of other classes of proteins, lipids and nucleic acids and of lipid–protein and nucleic-acid–protein interactions, had to be omitted. However, we are convinced that scientists interested in these systems will be able to extract the principle ideas of the various vibrational spectroscopic methods described in the applications to proteins and enzymes.

The present tutorial introduces the *fundamentals* of Raman and infrared spectroscopy, including the concept of molecular vibrations and a basic theoretical treatment of IR absorption and Raman scattering. It further describes, in more detail, instrumental and sampling techniques. The book is intended for students and scientists with backgrounds in life sciences and in physics and chemistry. Hence, in this respect we also had to make compromises to accommodate the interests and backgrounds of a readership coming from very different disciplines.

The book was completed with ''a little help from our friends''. We would like to thank P. Hamm (Zürich), J. Bredenbeck (Frankfurt) and T. A. Keiderling (Chicago) for their advice on the chapter on structural studies and for providing figures for this chapter. Further thanks are due to R. Vogel (Freiburg), for his help in preparing several figures. We thank G. Büldt, (Jülich) for providing figures of the ground and M-state structures of bacteriorhodopsin. Support and assistance in various aspects by M. Böttcher, J. Grochol, A. Kranich, M. A. Mroginski, H. Naumann, D. v. Stetten, N. Wisitruangsakul and I. Zebger (Berlin) are gratefully acknowledged. Special thanks are due to D. H. Murgida (Berlin/Buenos Aires) for continuous critical discussions, providing important stimuli for the book. In particular, we wish to thank I. Geisenheimer (Berlin) for the great work on producing the artwork for the figures. Thanks must also be given to C. Wanka from Wiley for her patience and support. Last but certainly not least, we wish to thank our wives, D. Siebert-Karasek and K. Graf-Hildebrandt, for their steady support and encouragement and specifically for their indulgence when this book occupied our evenings and weekends.

February 2007 *Friedrich Siebert, Peter Hildebrandt*

1
Introduction

Vibrational spectroscopy is a classical technique and one of the oldest spectroscopic methods. Its origins can be traced back two centuries to William Herschel, who discovered infrared (IR) radiation in the electromagnetic spectrum of the sun. At the beginning of last century, IR radiation was being used increasingly to measure interactions with matter, thereby producing the first vibrational spectra. In the 1920s, the discovery of the Raman effect, named according to the Indian scientist Chandrasekhara V. Raman, led to a second area of vibrational spectroscopy. Around that time, the potential of IR and Raman spectroscopy to elucidate molecular structures was soon acknowledged, although technical constraints limited the applications to fairly small molecules. Many of these studies have been considered in the famous textbook by Herzberg (Herzberg 1945), which is still a standard reference for spectroscopists and a rich source of information.

It took a fairly long time until vibrational spectroscopy was introduced into biological studies. This was not only due to the limited sensitivity and poor performance of spectrometers, detectors, and light sources in those early days, but also the state-of-the-art of preparing and purifying biological samples up to a grade that was appropriate for spectroscopic experiments was nowhere near as advanced as it is nowadays. In both spectroscopy and biology, the progress in methodology and technology started to grow exponentially in the 1960s. Important milestones in the exciting development of vibrational spectroscopy were certainly the invention of lasers and their use as light sources in Raman spectroscopy and the development of interferometers for measuring IR spectra. Thus, experiments with large and rather complex molecular systems became possible, and the application of Raman and IR spectroscopy to biomolecules afforded astonishing results, which had not previously been anticipated. The enormous success of the union between vibrational spectroscopy and the life sciences prompted many researchers from very different disciplines to adopt various IR and Raman spectroscopic techniques for the study of biological systems, thereby constituting a highly interdisciplinary research area at the interface between physics, chemistry, and biology.

1.1 Aims of Vibrational Spectroscopy in Life Sciences

The physiological functions of biological macromolecules are determined by the structural organisation at different hierarchical levels, which are the sequence of the individual building blocks in a biopolymeric chain (primary structure), the fold of the chain (secondary structure), and the spatial arrangement of various secondary structural elements within a chain (tertiary structure). Finally, two or more biopolymeric chains may constitute the quaternary structure. In this way, highly complex three-dimensional structures are formed, which have been optimised through evolution to carry out specific biological functions. For example, proteins that possess very similar primary, secondary, and tertiary structures, such as the bacterial retinal proteins bacteriorhodopsin, sensory rhodopsin, or halorhodopsin, can exert very different functions (i.e., signal transduction, proton or anion transport) due to subtle structural differences in critical parts of the proteins. Conversely, the same elementary chemical reaction can be catalysed by structurally different enzymes. A typical example is the reduction of molecular oxygen by the heme-copper enzyme cytochrome *c* oxidase or by the copper enzyme laccase.

The most challenging task in contemporary molecular biophysics, therefore, is the elucidation of the structure–function relationship of biological macromolecules. However, in view of the powerful techniques used in structural biology, i.e., X-ray crystallography, NMR spectroscopy, and cryogenic electron microscopy, which can provide detailed structures of macromolecules, one might ask what the current and future contributions of vibrational spectroscopy to this field could be.

Of course, knowledge of the three-dimensional structure of a biopolymer is important in the understanding of the functional mechanism as it guides the development of realistic hypotheses. However, a comprehensive elucidation of reaction mechanisms on a molecular level requires structural information usually beyond the resolution of the classical methods used in structural biology. For instance, the positions of hydrogen atoms and protons in the three-dimensional structure and van-der-Waals, hydrogen bonding, or electrostatic intermolecular interactions, which are essential for biochemical and biophysical processes, can only be *assumed* but not determined by X-ray crystallography. NMR spectroscopic techniques could be an alternative, but size limitations impose severe constraints because three-dimensional structures are currently restricted to biopolymers smaller than about 50 kDa.

Biological processes involve a series of structurally different states, such that a full understanding of the reaction mechanism requires knowledge of the initial and final states and of the intermediate species. Identification of intermediate states and the description of their molecular properties are only possible on the basis of techniques that can provide structural data as a function of time. Extending X-ray crystallography to the time-resolved domain is associated with substantial experimental difficulties and, moreover, is restricted to those instances where the crystals are not destroyed during the reaction sequence.

In all these respects, vibrational spectroscopy offers a variety of advantages. Firstly, vibrational spectroscopy can contribute to the elucidation of details in the molecular structures and intermolecular interactions that go far beyond the resolution of even highly resolved crystal structures. Secondly, unlike NMR spectroscopy, vibrational spectroscopy is in principle not restricted by the size of the sample and thus can afford valuable information for small biomolecules in addition to complex biological systems. Thirdly, vibrational spectroscopic methods are applicable regardless of the state of the biomolecule, i.e., they can be used to study biomolecules in solutions, in the solid and crystalline state, or in monolayers. Thus, it is possible to adapt the techniques according to the specific requirements of the sample and the biophysical questions to be addressed. In this sense, vibrational spectroscopy offers the potential to probe molecular events under conditions that are closely related to the physiological reaction environment. Fourthly, this versatility also allows combining vibrational spectroscopy with various time-resolved approaches. Thus, detailed information regarding the dynamics of biological systems can also be obtained, down to the femtosecond time scale.

Thus, it is one of the central objectives of this book to demonstrate that vibrational spectroscopic methods represent powerful tools, which are complementary to the techniques used in structural biology.

1.2 Vibrational Spectroscopy – An Atomic-scale Analytical Tool

Vibrational spectroscopy probes the periodic oscillations of atoms within a molecule. These oscillations do not occur randomly but in a precisely defined manner. This can easily be understood by taking into account that an N-atomic molecule has $3N$ degrees of freedom, of which three refer to translations and three (two) correspond to rotations in the case of a nonlinear (linear) molecule structure. The remaining degrees of freedom represent $3N - 6$ ($3N - 5$) vibrations of a nonlinear (linear) molecule, the so-called normal modes. In each normal mode every atom oscillates in-phase and with the same frequency, albeit with different amplitudes. The frequency, however, the first principle observable in vibrational spectroscopy, has a sensitive dependence on the forces acting on the individual atoms and on the respective masses. These forces do not only result from the chemical bonds connecting the individual atoms but also include contributions from nonbonding interactions within the molecule and with the molecular environment. In this way, the frequencies of the normal modes constitute a characteristic signature of the chemical constitution, the structure, and electron density distribution of the molecule in a given chemical environment, i.e., all of the parameters required for a comprehensive atomic-scale description of a molecule. These parameters also control the second important observable parameter in the vibrational spectrum, the intensities of the bands, which, unlike the frequencies, are not independent of the method by which the vibrational spectrum is probed.

The two main techniques used to obtain vibrational spectra, IR and Raman spectroscopy, are based on different physical mechanisms. In IR spectroscopy, molecules are exposed to a continuum of IR radiation and those photons that have energies corresponding to the frequencies of the normal modes can be absorbed to excite the respective vibrations. The wavelength range of the IR radiation corresponding to the frequencies of molecular vibrations extends typically between 2.5 and 50 μm. In Raman spectroscopy, these so-called vibrational transitions are induced upon inelastic scattering of monochromatic light by the molecule, such that the frequency of the scattered light is shifted by the frequency of the molecular vibration. For a given molecule, absorption- and scattering-induced vibrational transitions are associated with different probabilities, hence IR and Raman spectra may display different vibrational band patterns, which are an additional source of information about the structural and electronic properties of the molecule.

1.3 Biological Systems

The size of the biological systems that are the targets of vibrational spectroscopy in the life sciences can vary substantially. They range from building blocks of biopolymers (e.g., amino acids or lipids) or cofactors of proteins up to protein assemblies, membranes, or DNA–protein complexes. Concomitant with the increasing size of the system, the number of signals in the spectrum, i.e., the vibrational modes, increases with the number of atoms involved. Thus, only for small molecules with less than 50 atoms, corresponding to ca. 150 normal modes, is it usually possible to resolve all the individual vibrational bands in the IR and Raman spectra. For biopolymers such as proteins or nucleic acids, the number of vibrational modes is prohibitively large, resulting in complex spectra with many overlapping bands of slightly different frequencies. This is also true for bands originating from the same modes of the individual building blocks as these entities may be in a slightly different environment. Accordingly, it is not obvious how detailed information, for example, on the interaction of a substrate in the catalytic centre of an enzyme, or on the minute structural changes occurring in the protein during the enzymatic process, can be derived from vibrational spectra of large biological systems. The question should be rephrased: how can vibrational spectroscopy be made to be selective for those molecular groups of the macromolecule that one is interested in?

For proteins, there are two basic principles by which the desired selectivity is accomplished. In Raman spectroscopy, the wavelength of the monochromatic light, which is used for inelastic scattering, is selected to be in resonance with an electronic transition of a chromophoric group of the protein, which may either be a cofactor or a chromophore of the apoprotein. Under these *resonance* conditions, the probability of the scattering-induced transitions, and thus the intensity of the Raman scattered light originating from vibrational modes of the chromo-

phore, is selectively enhanced by several orders of magnitude. Then the resonance Raman spectrum displays the vibrational bands of the chromophore exclusively, whereas the Raman bands of the optically transparent matrix remain largely invisible. This selectivity is associated with an enhanced sensitivity and thus drastically reduces the protein concentration required for high quality spectra.

A more general method uses the ''function'' of the system, that is, its natural reaction, as a selectivity tool. The underlying idea is simple: the molecular groups involved in the function represent only a small fraction of the total system. As an example, we refer to the membrane protein bacteriorhodopsin, which acts as a light driven proton pump. This function is associated with only relatively small structural changes as shown in Fig. 1.1a and b (Sass et al. 2000). On stabilising two well-defined functional states of protein, in this instance the parent state BR_{570} and an intermediate M_{410}, the difference between the respective spectra only displays contributions from those groups undergoing molecular changes during the $BR_{570} \rightarrow M_{410}$ transition, because all bands that remain unchanged cancel each other out (Fig. 6.19). Correspondingly, the spectra are greatly simplified and, moreover, only reflect the *functionally relevant* structural changes. This method is called reaction-induced difference spectroscopy. The term ''reaction'' is implied in a very general sense. It can refer to ligand binding, substrate binding and transformation, light-induced reactions, and electron transfer in redox-reactions.

Both methods, i.e., resonance Raman and IR difference spectroscopy, can be extended to time-resolved studies, such that it is possible to probe the dynamics of molecular changes in real time during the reactions and processes of the system.

However, the scope of Raman and IR spectroscopy in the life sciences is broader as it is not restricted to the analyses of minute structural changes. For many proteins and for other biological systems including nucleic acids and membranes, these techniques may provide valuable information about more global structural properties. The individual building blocks of proteins, i.e., the amino acids, are linked via the same chemical entities as are the peptide bonds. Likewise, nucleic acids also form a backbone of repetitive units of sugar–phosphate linkages. As some of the vibrational modes of these units depend on the folding of the biopolymeric chain, vibrational spectra can give insights into the secondary structures of proteins and nucleic acids. Also, bilayer membranes exhibit global structural properties, which result from the periodic arrangement of lipid molecules possessing the same conformation. Characteristic vibrational marker bands for these conformations may be monitored to determine extended structural changes associated, for instance, with phase transitions.

The considerable progress that has been achieved in experimental Raman and IR spectroscopy in recent years is not adequately paralleled by the development of universal strategies for extracting the structural information from the spectra. Still, empirical approaches prevail that are based on the comparison with experimental data for related systems and model compounds. In many instances, isoto-

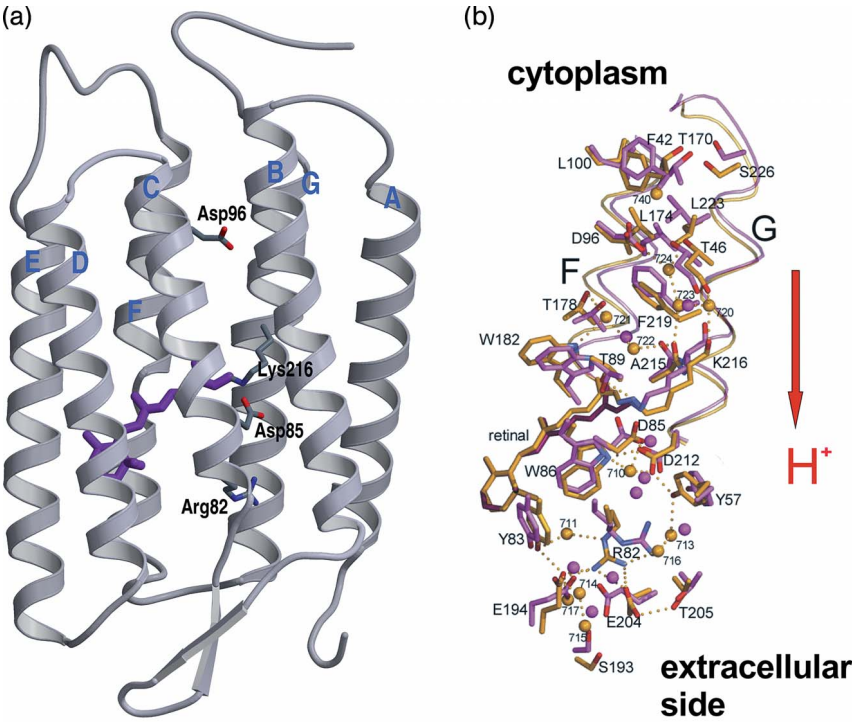

Fig. 1.1 (a) Three-dimensional structure of bacteriorhodopsin in the parent state BR_{570}. The seven transmembrane helices are indicated by the letters A to G. The chromophore is shown in purple. The retinal binding lysine Lys216, the proton acceptor for Schiff base deprotonation, Asp85, and the proton donor for Schiff base reprotonation are indicated. In addition, Arg82 pointing towards the retinal binding site is shown. The C-terminus is up (intracellular side), the N-terminus down (extracellular side), proton pumping is from the intracellular to the extracellular side. Oxygen atoms are coloured in red, nitrogen atoms in blue. Coordinates from crystal structure 1CWK of the protein data bank were used (courtesy of G. Büldt). For details of the mechanism of bacteriorhodopsin see Chapter 6.2. (b) Differences in the crystal structures of the ground and M states of bacteriorhodopsin in the neighbourhood of helices F and G. Ground state is shown in purple, M state in yellow. Oxygen atoms are coloured in red, nitrogen atoms in blue. Resolved water molecules are depicted as purple and yellow balls for the ground and M states, respectively. The direction of proton pumping is indicated. In the M state, the light-induced isomerisation of the chromophore retinal from all-*trans* to 13-*cis* is clearly seen. A distinct molecular change concerns Arg82 (R82), which now points downwards. This is thought to cause proton release from a site close to the extracellular surface and to increase the pK_a of the retinal Schiff base for its reprotonation in the next N state. Several water molecules have been displaced in the M state. However, protonation of Asp85 (D85) and deprotonation of the Schiff base, as deduced from infrared and Raman spectroscopy, cannot be deduced from the M structure as protons cannot be seen directly (adapted from Sass et al. 2000).

pic labelling is an indispensable tool in vibrational spectroscopy for assigning bands to specific modes. This experimental approach is straightforward for molecules including protons that can be exchanged by deuterons in ^2H_2O solutions. For all other isotopic substitutions (e.g., ^{15}N, ^{18}O, ^{13}C, covalently bound ^2H) synthetic work is required either by organic chemists or by microorganisms producing the compounds of interest in isotopically enriched media. These time-demanding and costly procedures are not applicable in each instance, but have been shown to contribute substantially to the vibrational analyses of protein cofactors and building blocks of nucleic acids, proteins, and membranes. Furthermore, it should be emphasised that NMR studies on proteins also require, in most instances, isotopic labelling with ^{13}C, ^{15}N, and ^2H.

The vibrational analyses of proteins are also supported by genetic engineering such that specific bands can be assigned to individual amino acid residues. This approach strongly benefits from the tight interactions of spectroscopists and biologists, inasmuch as the functional consequences of individual mutations have to be assessed as a prerequisite for unambiguous interpretations of the spectra in terms of structure–function relationships.

These empirical approaches typically only focus on small segments of the vibrational spectra and thus the major part of the structural information contained in the spectra remains obscured. More comprehensive methods are based on the classical treatment of the vibrational eigenstate problem. In the past, these normal mode analyses have been the domain of a few specialists, and, in fact, only a small number of biomolecules, i.e., cofactors of proteins such as tetrapyrroles or retinals, have been treated by these tedious methods. The popularisation of quantum chemical programs, the development of efficient program codes, and the increasing availability of powerful personal computers, have all contributed to reducing the exclusivity of theoretical methods and to open up novel possibilities for comprehensive and reliable vibrational analyses. Although a sound application of these methods requires knowledge of theoretical chemistry, they will no doubt develop to become a standard tool to be employed routinely by experimentalists also.

1.4 Scope of the Book

During recent years vibrational spectroscopy has become an important tool in biophysical research, both for structural and functional studies. Whereas in the beginning this research area was the domain of physicists and physico-chemists, who not only had to master the methodological challenges but also to become acquainted with the concepts and emerging problem in the life sciences, more and more biologists have now recognised the high potential of these techniques to elucidate the molecular functioning of biomolecules. Therefore, the main goal of this book is to introduce the basic concepts of vibrational spectroscopy to ''newcomers'' to this area, and to students specialising in this particular discipline of

molecular biophysics, in addition to advanced scientists with a non-spectroscopic background and to spectroscopists who intend to work with biological systems. Specific emphasis is given to the practical aspects of Raman and IR spectroscopy, which, when applied in the life sciences, usually has to be adapted to the specific needs and demands of the systems to be studied. This is reflected by an extensive description of the instrumental and sampling techniques (Chapters 3 and 4) in the first part of the book. Conversely, we restricted the treatise of the theoretical background to the elementary relationships, avoiding lengthy mathematical derivations (Chapter 2). Generally, we will separate more elaborate explanations and derivations from the body of the text. Thus, the main content of the various chapters is easier to follow, and the more specialised or difficult parts can be read later, or even be omitted. For a better understanding of these chapters, a basic knowledge of physics, especially optics and molecular physics, and of general physical chemistry would be helpful.

A basic knowledge of biochemistry, in particular with respect to the structure and processes of proteins, is desired for the second part of the book. Textbooks on biophysics, biophysical chemistry, and biochemistry usually provide an excellent basis. This part includes four chapters (Chapters 5–8) devoted to applications of vibrational spectroscopic methods to the study of biomolecules. Instead of covering the broad range of biological molecules comprehensively, this part is restricted to structural studies of proteins (Chapter 5) and to specific classes of proteins (Chapters 6–8). These chapters are considered to *illustrate* the application of dedicated methods and to point out what type of information they may provide. In this respect, proteins (and among them specific representatives such as rhodopsin or cytochrome *c*) represent the most versatile targets because they have been studied by a large variety of different vibrational spectroscopic techniques and, in some instances, even served as models for methodological developments. We will, therefore, describe not only well-established approaches but also new and emerging techniques that promise to become important analytical tools in the future. The restriction to principle aspects of the applications also implies that only exemplary results are reported. For comprehensive accounts, the reader is referred to original and review articles. According to the concept outlined above, and due to general space restrictions imposed on this book, other important biological systems, such as nucleic acids, lipids, and carbohydrates, will not be covered. However, the methodological approaches usually applied to these systems are equally well covered, on the basis of the specific example proteins.

The applications of vibrational spectroscopy discussed in this book are restricted to problems in *molecular* biophysics. They do not include approaches for characterising bacteria, tissues, and cell cultures, even though Raman and IR spectroscopic analyses of such highly complex systems are of significant importance in microbiological and medical applications. As these studies do not focus on the molecular properties of biomolecules, they are beyond the scope of this book.

For the readers having a background in physics and physical chemistry, we want to demonstrate that, despite the complexity of biological macromolecules,

vibrational spectroscopy is a potent tool for the study of their structural properties and their functions at a molecular level. Biologists and biochemists, on the other hand, should be encouraged to utilise the fairly sophisticated IR and Raman spectroscopic techniques and to exploit their specific advantages for studying biological systems. Eventually, we hope that the reader can assess the potential and limitations of vibrational spectroscopy in molecular life sciences and be able to judge whether the system he or she is interested in could be successfully studied using vibrational spectroscopy.

1.5 Further Reading

Vibrational spectroscopy is a method which has developed over many years. Thus a number of excellent books have been published that cover certain aspects, and a few of these monographs should be mentioned here. The book by Colthup provides an excellent introduction to general vibrational spectroscopy (Colthup et al. 1975). It also contains a treatment of the basic theoretical concepts. Lin-Vien et al. have presented a collection of data for organic molecules (Lin-Vien et al. 1991), directed to provide a basis for the identification chemical compounds. However, as the spectral properties of chemical groups are discussed fairly thoroughly, this book also serves as a reference for many more applications. The book by Nakamoto offers similar information on inorganic and coordination compounds (Nakamoto 1986). A compilation of spectra of amino acids serves as a very useful reference (Barth 2000). The effect of isotopic labelling on molecular vibrations is discussed by Pincas and Laulicht (Pinchas and Laulicht 1971). A basic introduction into practical, theoretical, and applied aspects of Raman spectroscopy is given by Smith and Dent (Smith and Dent 2005). A comprehensive treatise of Raman spectroscopy including various applications has been edited by Schrader (Schrader 1995). The technical aspects of Fourier transform spectroscopy, particularly important for IR spectroscopists, are covered in great detail in the book by Griffith and de Haseth (Griffith and de Haseth 1986). An up-to-date account of the theory and practice of surface enhanced Raman spectroscopy is presented in a recent book that includes contributions from various research groups (Kneipp et al. 2006). For the theory of vibrational spectroscopy, we wish to recommend the excellent books by Herzberg (Herzberg 1945) and Wilson et al. (Wilson et al. 1955). Albeit published half a century ago, they are indispensable textbooks and reference books for all vibrational spectroscopists. The book by Long also includes major treatise on the theory of Raman spectroscopy with particular emphasis on polarisation effects (Long 1977). Biological applications of vibrational spectroscopy are described in the textbooks by Carey (Raman) (Carey 1982), Twardowski and Anzenbacher (Raman and IR) (Twardowski and Anzenbacher 1994).

Collections of review articles on specialised topics of vibrational spectroscopy, also on biomolecular applications, can be found in the book series *Advances in Spectroscopy* (edited by Clark and Hester) and in the three-volume edition *Biologi-*

cal Applications of Raman Spectroscopy (Spiro 1987, 1988). A selection of articles on infrared spectroscopy of biomolecules have been published in a book of the same title (Mantsch and Chapman 1996), and more specialised articles on biomolecular infrared and Raman spectroscopy have appeared recently in a book from the series *Practical Spectroscopy* (Gremlich and Yan 2001).

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2
Theory of Infrared Absorption and Raman Spectroscopy

Molecular vibrations can be excited via two physical mechanisms: the absorption of light quanta and the inelastic scattering of photons (Fig. 2.1) (Herzberg 1945). Direct absorption of photons is achieved by irradiation of molecules with polychromatic light that includes photons of energy matching the energy difference $h\nu_k$ between two vibrational energy levels, the initial (i, e.g., ground state) and the final (f, e.g., first excited state) vibrational state.

$$h\nu_k = h\nu_f - h\nu_i \tag{2.1}$$

As these energy differences are in the order of 0.5 and 0.005 eV, light with wavelengths longer than 2.5 μm, that is infrared (IR) light, is sufficient to induce the vibrational transitions. Thus, vibrational spectroscopy that is based on the direct absorption of light quanta is denoted as IR absorption or IR spectroscopy.

The physical basis of IR light absorption is very similar to light absorption in the ultraviolet (UV)–visible (vis) range, which causes electronic transitions or combined electronic–vibrational (vibronic) transitions. Thus, UV–vis absorption spectroscopy can, in principle, also provide information about molecular vibrations. However, for molecules in the condensed phase at ambient temperature, the vibrational fine structure of the absorption spectra is only poorly resolved , if at all, such that vibrational spectroscopy of biomolecules by light absorption is restricted to the IR range.

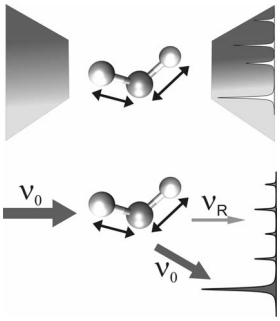

Fig. 2.1 Illustration of the excitation of molecular vibrations in IR (top) and Raman (bottom) spectroscopy. In IR spectroscopy, the vibrational transitions are induced by absorption of light quanta from a continuous light source in the IR spectral region. Vibrational Raman transitions correspond to inelastic scattering (ν_R; thin arrow) of the incident monochromatic light (ν_0) whereas the elastic scattering (ν_0) is represented by the thick arrow.

In contrast to IR spectroscopy, the scattering mechanism for exciting molecular vibrations requires monochromatic irradiation. A portion of the incident photons is scattered inelastically such that the energy of the scattered photons ($h\nu_R$) differs from that of the incident photons ($h\nu_0$). According to the law of conversation of energy, the energy difference corresponds to the energy change of the molecule, which refers to the transition between two vibrational states. Thus, the energy differences

$$h\nu_0 - h\nu_R = h\nu_f - h\nu_i \tag{2.2}$$

lie in the same range as the transitions probed by the direct absorption of mid-IR quanta, although photons of UV, visible, or near-infrared light are used to induce scattering. This inelastic scattering of photons was first discovered by the Indian scientist C. V. Raman in 1928 and is thus denoted as the Raman effect.

Vibrational transitions may be associated with rotational transitions that can only be resolved in high resolution spectra of molecules in the gas phase and is, therefore, not relevant for the vibrational spectroscopy of biomolecules. Thus, vibration–rotation spectra will not be treated in this book.

Depending on the molecule, the same or different vibrational transitions are probed in IR and Raman spectroscopy and both techniques provide complementary information in many instances. Hence, IR and Raman spectra are usually plotted in an analogous way to facilitate comparison. The ordinate refers to the extent of the absorbed (IR) or scattered (Raman) light. In IR absorption spectroscopy, the amount of absorbed light is expressed in units of absorbance or, albeit physically less correct but frequently used, in terms of the optical density. In contrast, Raman intensities are measured in terms of counts per second, i.e., of photons detected per second. As this value depends on many apparatus-specific parameters, in most instances only relative intensities represent physically meaningful quantities. Thus, the Raman intensity scale is typically expressed in terms of arbitrary units or the scale is even omitted. The energy of the vibrational transition, expressed in terms of wavenumbers (cm^{-1}), is given on the abscissa, corresponding to the frequency of the absorbed light ν_{abs} in IR spectroscopy and to the frequency difference between the exciting and scattered light, $\nu_0 - \nu_R$, in Raman spectroscopy.

The principle sources of information in vibrational spectroscopy are the energies of the vibrational transitions and the strength of their interaction with the IR or UV–vis radiation, i.e., the band intensities. Classical mechanics constitutes the basis for describing the relationship between vibrational frequencies and the molecular structure and force fields whereas quantum mechanics is indispensable for understanding the transition probabilities and thus the intensities of vibrational bands in the IR or Raman spectra.

2.1 Molecular Vibrations

As the starting point for introducing the concept of harmonic vibrations, it is instructive to consider molecules as an array of point masses that are connected

with each other by mass-less springs representing the intramolecular interactions between the atoms (Wilson et al. 1955). The simplest case is given by two masses, m_A and m_B, corresponding to a diatomic molecule A–B. Upon displacement of the spheres along the x-axis from the equilibrium position by Δx, a restoring force F_x acts on the spheres, which according to Hooke's law, is given by

$$F_x = -f\Delta x \tag{2.3}$$

Here f is the spring or force constant, which is a measure of the rigidity of the spring, that is, the strength of the bond. The potential energy V then depends on the square of the displacement from the equilibrium position

$$V = \frac{1}{2} f \Delta x^2 \tag{2.4}$$

For the kinetic energy T of the oscillating motion one obtains

$$T = \frac{1}{2}\mu(\Delta\dot{x})^2 \tag{2.5}$$

where μ is the reduced mass defined by

$$\mu = \frac{m_A \cdot m_B}{m_A + m_B} \tag{2.6}$$

Because of the conservation of energy, the sum of V and T must be constant, such that the sum of the first derivatives of V and T is equal to zero, as expressed by Eq. (2.7):

$$0 = \frac{dT}{dt} + \frac{dV}{dt} = \frac{1}{2}\frac{d(\Delta\dot{x}^2)}{dt} + \frac{1}{2} f \frac{d(\Delta x^2)}{dt} \tag{2.7}$$

which eventually leads to the Newton equation of motion

$$\frac{d^2\Delta x}{dt^2} + \frac{f}{\mu}\Delta x = 0 \tag{2.8}$$

Equation (2.8) represents the differential equation for a harmonic motion with the solution given by a sine or cosine function, i.e.,

$$\Delta x = A \cdot \cos(\omega t + \varphi) \tag{2.9}$$

where A, ω, and φ are the amplitude, circular frequency, and phase, respectively. Combining Eq. (2.9) with its second derivative one obtains

$$\frac{d^2\Delta x}{dt^2} + \omega^2\Delta x = 0 \tag{2.10}$$

such that comparison with Eq. (2.8) yields

$$\omega = \sqrt{\frac{f}{\mu}} \tag{2.11}$$

Equation (2.11) describes what one intuitively expects: the circular frequency of the harmonic vibration increases when the rigidity of the spring (or the strength of the bond) increases but decreases with increasing masses of the spheres. In order to express the circular frequency in wavenumbers (in cm^{-1}), Eq. (2.11) has to be divided by $2\pi c$ (with c given in cm s^{-1}):

$$\tilde{\nu} = \frac{1}{2\pi c}\sqrt{\frac{f}{\mu}} \tag{2.12}$$

In contrast to the straightforward treatment of a two-body system, including a third sphere corresponding to a triatomic molecule clearly represents a conceptual challenge (Wilson et al. 1955). Let us consider a bent molecule such as H_2O as an example (Fig. 2.2). Following the same strategy as for the diatomic molecule, we analyse the displacements of the individual atoms in terms of the restoring forces. There are two questions to be answered. (a) What are the displacements that lead to vibrations? (b) Are all possible displacements allowed?

In the Cartesian coordinate system, each atom can be displaced in the *x*-, *y*-, and *z*-directions, corresponding to three degrees of freedom. Thus, a molecule of N atoms (α) has in total $3N$ degrees of freedom, but not all of them correspond to vibrational degrees of freedom. If all atoms are displaced in the *x*-, *y*-, and *z*-directions by the same increments, the entire molecule moves in a certain direction, representing one of the three translational degrees of freedom. Furthermore, one can imagine displacements of the atoms that correspond to the rotation of

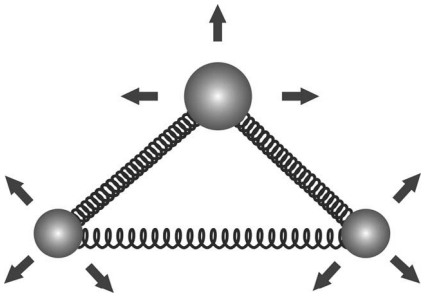

Fig. 2.2 Illustration of the vibrating H_2O molecule represented by spheres that are connected via springs of different strengths. The tighter springs linking the large sphere (oxygen) with each of the small spheres (hydrogen) symbolises the chemical bonds between two atoms, whereas the looser spring refers to weaker interactions between two atoms that are not connected via a chemical bond.

the molecule. It can easily be seen that a nonlinear molecule (i.e., where the atoms are not located along a straight line) has three rotational degrees of freedom, whereas there are only two for a linear molecule. Thus, the remaining $3N-6$ and $3N-5$ degrees of freedom correspond to the vibrations of a nonlinear and a linear molecule, respectively. For the treatment of molecular vibrations in terms of Cartesian coordinates, the rotational and translational degrees of freedom can be separated by choosing a rotating coordinate system with its origin in the centre of mass of the molecule.

As an important implication of these considerations, we note that the vibrational degrees of freedom and thus the number of molecular vibrations are uniquely determined by the number of atoms in the molecule. In our example of a nonlinear three-atomic molecule there are just 3 ($= 3 \cdot 3 - 6$) vibrational degrees of freedom. Thus, molecular vibrations do not represent random motions but well-defined displacements of the individual atoms. Consequently, one may intuitively expect that these vibrations, which are denoted as normal modes, are characteristic of a given molecule. The primary task of the normal mode analysis is to decode the relationships between normal modes, specifically their frequencies, and molecular properties.

2.1.1 Normal Modes

To determine the normal mode frequencies, we begin by expressing the kinetic and potential energy in terms of the displacements of the Cartesian coordinates for each atom α (Wilson et al. 1955). For the kinetic energy one obtains [see Eq. (2.5)]

$$T = \frac{1}{2}\sum_{\alpha=1}^{N} m_\alpha \left[\left(\frac{\mathrm{d}\Delta x_\alpha}{\mathrm{d}t}\right)^2 + \left(\frac{\mathrm{d}\Delta y_\alpha}{\mathrm{d}t}\right)^2 + \left(\frac{\mathrm{d}\Delta z_\alpha}{\mathrm{d}t}\right)^2 \right] \tag{2.13}$$

At this point it is convenient to introduce so-called mass-weighted Cartesian displacement coordinates, which are defined according to

$$\begin{aligned} q_1 &= \sqrt{m_1}\Delta x_1, \quad q_2 = \sqrt{m_1}\Delta y_1, \quad q_3 = \sqrt{m_1}\Delta z_1 \quad \text{for atom } \alpha = 1 \\ q_4 &= \sqrt{m_2}\Delta x_2, \quad q_5 = \sqrt{m_2}\Delta y_2, \quad q_6 = \sqrt{m_2}\Delta z_2 \quad \text{for atom } \alpha = 2 \end{aligned} \tag{2.14}$$

and correspondingly for all other atoms such that one obtains $3N$ mass-weighted Cartesian displacement coordinates. Substituting Eq. (2.14) in Eq. (2.13) simplifies the expression for the kinetic energy to

$$T = \frac{1}{2}\sum_{i=1}^{3N} \dot{q}_i^2 \tag{2.15}$$

To derive the appropriate expression for the potential energy, V, is more complicated as it has to take into account all possible interactions between the

individual atoms, which primarily include the bonding interactions but also non-bonding (electrostatic, van-der-Waals) interactions. For the three-atomic water molecule in Fig. 2.2 this implies that the displacement of one hydrogen atom depends on the attractive and repulsive forces of both the central oxygen and the second hydrogen atom. Within the framework of the sphere–spring model we therefore also have to connect both hydrogen ''spheres'' via a spring which, however, is less rigid than those connecting the hydrogen spheres with the oxygen.

It is convenient to expand the potential energy in a Taylor series in terms of the displacement coordinates Δx_i, Δy_i, Δz_i, which can be also expressed in terms of the coordinates q_i defined in Eq. (2.14).

$$V = V_0 + \sum_{i=1}^{3N} \left(\frac{\partial V}{\partial q_i}\right)_0 q_i + \frac{1}{2} \sum_{i,j=1}^{3N} \left(\frac{\partial^2 V}{\partial q_i \partial q_j}\right)_0 q_i q_j + \cdots \tag{2.16}$$

The first term refers to the potential energy at equilibrium, which we can set equal to zero as we are interested in changes to V brought about by displacements of the individual atoms. At equilibrium, infinitesimal changes in q_i do not cause a change in V, such that the second term is also zero. For small displacements q_i within the harmonic approximation, higher order terms can be neglected, such that Eq. (2.16) is simplified to

$$V \cong \frac{1}{2} \sum_{i,j=1}^{3N} \left(\frac{\partial^2 V}{\partial q_i \partial q_j}\right)_0 = \frac{1}{2} \sum_{i,j=1}^{3N} f_{ij} q_i q_j \tag{2.17}$$

where f_{ij} are the force constants.

In books on classical mechanics it is shown that, in the absence of external and non-conservative forces, Newton's equations of motion can be written in the following form:

$$\frac{\mathrm{d}}{\mathrm{d}t} \frac{\partial T}{\partial \dot{q}_j} + \frac{\partial V}{\partial q_j} = 0 \tag{2.18}$$

which yields

$$\ddot{q}_j + \sum_{i=1}^{3N} f_{ij} q_i = 0 \tag{2.19}$$

Equation (2.19) is equivalent to Eq. (2.10) for the diatomic harmonic oscillator, except that it represents not just one but a set of $3N$ linear second-order differential equations for which we can write the general solution, in analogy to Eq. (2.9),

$$q_i = A_i \cos(\sqrt{\lambda} t + \varphi) \tag{2.20}$$

Inserting Eq. (2.20) into Eq. (2.19) yields

$$-A_j\lambda + \sum_{i=1}^{3N} f_{ij}A_i = 0 \tag{2.21}$$

which corresponds to $3N$ linear equations for A_j. These equations only have a solution different from zero if the $3N \cdot 3N$ determinant vanishes (secular equation):

$$\begin{vmatrix} f_{11}-\lambda & f_{12} & f_{13} & \cdots\cdots & f_{1,3N} \\ f_{21} & f_{22}-\lambda & f_{23} & \cdots\cdots & f_{2,3N} \\ f_{31} & f_{32} & f_{33}-\lambda & \cdots\cdots & f_{3,3N} \\ \cdots\cdots & \cdots\cdots & \cdots\cdots & \cdots\cdots & \cdots\cdots \\ f_{3N,1} & f_{3N,2} & f_{3N,3} & \cdots\cdots & f_{3N,3N}-\lambda \end{vmatrix} = 0 \tag{2.22}$$

There are $3N$ solutions for λ corresponding to $3N$ frequencies $\lambda^{1/2}$. As the summation has been made over all $3N$ degrees of freedom, 6 (5) of these solutions refer to translational and rotational motions of the nonlinear (linear) molecules and, therefore, must be zero. Thus, Eq. (2.22) yields only $3N-6$ ($3N-5$) non-zero values for λ. The proof for this is lengthy and is not shown here (Wilson et al. 1955). The non-zero solutions correspond to the so-called normal modes.

Once the individual λ_k values have been determined, the amplitudes A_i for each normal mode have to be determined on the basis of in Eq. (2.21).

$$\begin{aligned} &(f_{11}-\lambda_k)A_{1k} + f_{12}A_{2k} + \cdots\cdots + f_{1,3N}A_{3N,k} = 0 \\ &f_{21}A_{1k} + (f_{22}-\lambda_k)A_{2k} + \cdots\cdots + f_{2,3N}A_{3N,k} = 0 \\ &\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \\ &f_{3N,1}A_{1k} + f_{3N,2} + \cdots\cdots + (f_{3N,3N}-\lambda_k)A_{3N,k} = 0 \end{aligned} \tag{2.23}$$

As Eq. (2.23) represents a set of homogeneous equations, only relative amplitudes can be obtained and a normalisation is required, as will be discussed below. The amplitudes A_{ik} describe the character of a normal mode as they quantify the displacements of each atom i in each normal mode k. Eqs. (2.20 and 2.23) imply that in a given normal mode k all atoms vibrate in-phase and with the same frequency $(\lambda_k)^{1/2}$, but with different amplitudes. Thus, it is always an approximation, albeit a useful one in many instances, to characterise normal modes of polyatomic molecules in terms of specific group vibrations, i.e., if only one coordinate dominates the normal mode.

Although the treatment of normal modes in the Cartesian coordinate system is straightforward, it has the disadvantage of distributing all information for a given normal mode among $3N$ equations. In particular, for describing probabilities of vibrational transitions [see Eq. (2.2)] a more compact presentation is desirable. For this purpose, the mass-weighted Cartesian coordinates q_i are converted into normal coordinates Q_k via an orthogonal transformation according to

$$Q_k = \sum_{i=1}^{3N} l_{ik} q_i \tag{2.24}$$

The transformation coefficients l_{ik} are chosen such that T and V, expressed as a function of Q_k, adopt the same form as Eqs. (2.15 and 2.16) and the potential energy does not depend on cross products $Q_k \cdot Q_{k'}$ (with $k \neq k'$). The solution of Newton's equation of motion thus leads to

$$Q_k = K_k \cos(\sqrt{\lambda_k} t + \varphi_k) \tag{2.25}$$

with arbitrary values of K_k and φ_k. The representation of molecular vibrations in normal coordinates is particularly important for the quantum mechanical treatment of the harmonic oscillator (Box 2A).

2.1.2 Internal Coordinates

The normal coordinate system is, mathematically, a very convenient system and, moreover, is required for the quantum chemical treatment of vibrational transitions. However, it is not a very illustrative system as molecular vibrations are usually imagined in terms of stretching or bending motions of molecules or parts of molecules. Such motions cannot be intuitively deduced from a normal coordinate or the array of mass-weighted Cartesian coordinates (Wilson et al. 1955). It is, therefore, desirable to introduce a coordinate system that is based on "structural elements" of molecules, such as bond lengths and angles, and torsional and out-of-plane angles. These so-called internal coordinates are derived from Cartesian displacement coordinates $(\Delta x_\alpha, \Delta y_\alpha, \Delta z_\alpha)$ on the basis of the geometry of the molecule.

The displacement of each atom α is defined by the vector $\vec{\rho}_\alpha(\Delta x_\alpha, \Delta y_\alpha, \Delta z_\alpha)$, which is related to the internal coordinate S_t according to

$$S_t = \sum_{\alpha=1}^{N} \vec{s}_{t\alpha} \cdot \vec{\rho}_\alpha \tag{2.26}$$

The vector $\vec{s}_{t\alpha}$ is chosen such that it points in the direction of the largest displacement of $\vec{\rho}_\alpha$ corresponding to the greatest increase in S_t. This statement is best illustrated on the basis of the most simple internal coordinate, the bond stretching coordinate (Fig. 2.3). A stretching coordinate is defined by two atoms ($\alpha = 1, 2$). Thus, for this coordinate the displacement of all other atoms is zero and the sum in Eq. (2.26) only refers to two terms. The largest displacement from the equilibrium positions occur along the axis of the bond assumed to be the x-axis but in opposite directions for atom 1 and 2. Expressing $\vec{s}_{t1}$ and $\vec{s}_{t2}$ in terms of unit vectors we thus obtain

$$\vec{s}_{t1} = \vec{e}_{21} = -\vec{e}_{12} \quad \text{and} \quad \vec{s}_{t2} = \vec{e}_{12} \tag{2.27}$$