CHAPTER 3
EXPERIMENTAL TECHNIQUES

A brief description of methods of sample preparation, experimental techniques and the instruments used to obtain the vibrational and electronic spectra are presented in this chapter.

3.1 Sample Preparation

Free base octaethylporphyrin (H$_2$OEP), cobalt(II) meso-tetraphenylporphyrin (Co$^{III}$TPP) and horse heart cytochrome-c (cyt-c) were commercially purchased from Porphyrin Products Inc. (USA), Aldrich Chem. Co., and Sigma Chemical Co., respectively. Cytochrome-c (Sigma type VI) was further purified by column chromatography, while other porphyrins were directly used as received. The nitrogenous ligand 2-methylimidazole (2-MeIm) and electron acceptor p-benzoquinone (p-BQ) were obtained from Aldrich Chemical Co. The nitrogenous base was recrystallized from benzene before use. Dichloromethane, dichloroethane and tetrachloroethane were of reagent grade and purified by washing with concentrated H$_2$SO$_4$, 15% solution of potassium carbonate, large volume of water, dried over anhydrous calcium chloride, distilled and then stored over molecular shives. Methanol and carbon disulphide were also reagent grade and purified by storing over anhydrous potassium carbonate and anhydrous calcium carbonate respectively for about 18 hrs, filtered and glass distilled. All other solvents used were of HPLC grade and used without further purification. Aqueous solutions of cyt-c were prepared with triple distilled, deionized water.
Chemical oxidation and reduction of porphyrin were performed by adding the appropriate oxidizing or reducing agents to the porphyrin solutions. The oxidizing agent used in this work is dilute solution of bromine and sodium dithionite is used as reducing agent. Diacid derivative of free base octaethylporphyrin diacid was prepared by addition of conc. HCl, dilute HClO₄ and dilute bromine to the solutions of the free base.

3.2 Degassing of Solutions

Free and dissolved air contained in the solution is removed using freeze-pump-thaw cycles. The solution for Raman studies is first transferred to a quartz cell fitted with accessories for evacuation and cooling. The sample is frozen by liquid nitrogen and the cell is evacuated with the help of a vacuum pump with fore pressure of 10⁻⁴ Torr. After sufficient evacuation, the cell is allowed to warm up to room temperature. The solution is purged with nitrogen gas for few minutes and the above cycle is repeated. The entire cycle is repeated at least three times to ensure proper degassing and removal of dissolved oxygen from the solution. After degassing, the solution is transferred to a Raman cell which is connected to the evacuating cell with vacuum tight joint. The unit is then mounted on a stand for laser excitation and Raman measurements under anaerobic conditions.

3.3 Measurement of Raman Spectra

Raman spectra of different porphyrins were recorded in a 90° scattering geometry with a Raman spectrometer (SPEX Ramalog 1403 and JOEL 400D)
equipped with a thermoelectrically cooled RCA 31034 photomultiplier and photon counting arrangement. The spectrometer control and data processing were achieved with the help of a microprocessor based SPEX “Datamate”. Excitation lines were obtained from HeCd (Liconix, model 4240 and Kinmon Electronics, model CDR80MGE), Krypton Ion (Spectra-Physics, model 164) and Argon Ion lasers (Spectra-Physics, model 165-09 and NEC, model GLG3200).

The excitation wavelength dependence of photoreduction of cytochrome-c was measured using high photon flux from Okazaki large spectrograph (Japan).

3.3.1 Helium-Cadmium Laser (Liconix Model 4240)

This laser consists of two units: a laser head and a power supply. Three cables, one for control functions and other two for the high voltage supply to the laser tube connect the head and supply.

The optical section of laser head is formed by two mirrors mounted on adjustable plates and held at a precise separation by three invar rods that run along the length of the laser tube which is made of pyrex glass. The mirrors are adjusted to reflect the laser light down the bore of the pyrex glass laser tube and to allow the emission of a precise amount of light as an output laser beam. Functionally, the helium-cadmium laser is a helium filled plasma discharge tube containing cadmium metal within a reservoir and is terminated in a vertically polarized Brewster windows of the resonating cavity. The coherent laser emission occurs by a mechanism similar to that of the helium neon laser. The laser action takes place between the energy levels of Cd atoms while He gas helps in creating population inversion in Cd atoms.
This laser head is a air cooled system and requires no external cooling. The maximum output power at 441.6 nm is about 40 mW.

The power supply unit utilizes AC power from the primary source and converts it into different DC levels for maintaining constant output power by controlling gas pressure (both helium pressure and cadmium vapor pressure) and associated current regulations in different electronic circuits.

3.3.2 Argon Ion Laser (Spectra-Physics Model 165-09)

The Argon ion laser is a CW laser consisting of laser head and Exciter. The laser head is made up of a beryllium oxide (BeO) plasma tube closed at both ends by fused-silica Brewster's angle windows, a solenoid for providing necessary magnetic field and an optical resonator. The plasma tube is mounted in an optical cavity resonator formed by a spherical reflector at the output and a prism assisted by a flat mirror (to select wavelengths) at the high reflector end. The whole assembly of the resonator is held solidly against quartz rods with springs to minimize microphonic frequency shifts. The plasma tube is supported on a kinematically adjustable mount and is adjusted in such a way that the plasma tube is exactly centered. The external thumb wheel controls are provided for wavelength selection and for changing the intra-cavity aperture. The laser gives polarized light and by using the polarizer the plane of polarization can be changed to the desired plane for recording the polarized Raman spectra.

The Exciter is fully equipped with the necessary electronic circuits to create, sustain and regulate the ion discharge in the plasma tube and to control the output power from the laser by simultaneously regulating the solenoid current. An
arrangement is provided to have a desired constant output optical power. The Exciter is fed with a stabilized three phase 380 V (phase to phase) power line. This unit requires cooling of the transistor pass bank in the exciter, the solenoid and the BeO plasma tube which is achieved by circulating distilled and deionized water at 15° C.

3.3.3 Krypton Ion (Spectra-Physics, model 164)

The construction and basic unit of Krypton Ion laser is in general similar to that of Argon Ion laser. The main advantage of Krypton Ion laser lies in the availability of more number of lasing lines at reasonable output powers. But Krypton Ion lasers are not as stable in power output or discharge characteristics as Argon Ion lasers. The effect of gas pressure change during warmup is significant, especially if output is on lines higher than 647.1 nm. There is a tendency for the laser to operate closer to plasma instability points when output is at 568.2, 530.9, 350.7, or 356.4 nm. To minimize pressure change effects and avoid plasma instabilities during operation, specific steps have to be followed in the turn-on procedure.

3.3.4 Okazaki Large Spectrograph

Okazaki Large Spectrograph is a computer-operated, large-scale spectrograph recently built at the National Institute for Basic Biology at Okazaki, Japan. The design and performance of the spectrograph are described below.¹

The spectrograph is composed principally of 5 parts: light sources, a monochromator, automatically-controlled boxes for sample irradiation, a carrier system for the boxes, and a microcomputer system for control. The spatial arrangement of these components is shown in Fig. 3.1.
Two Xe short arc lamps (30 and 6 kW) and a 450 W medium pressure Hg lamp form the light sources. A Monk-Gillieson mounting is adopted as the optical arrangement so that different biological specimens can be irradiated simultaneously with a continuous range of wavelengths from 250 to 1000 nm. The diffraction grating of the monochromator has 1200 grooves mm\(^{-1}\) and first order diffracted light is used. Seven kinds of bandpass or sharp-cut-off filters are used to remove higher-order diffracted light and other stray light. A stage is installed so that samples or boxes containing samples are exposed to light from the monochromator. Sample is placed in a computer-controlled ‘sample box’ having programmed parameters such as wavelength, photon fluence rate, photon fluence and timing. Through data communication between a host computer and the corresponding parts of the whole system of the spectrograph control over the following processes are done automatically: selection of the light source, opening and closing of the shutter, control of the entrance slit width, automatic transportation of the sample boxes to desired wavelength positions, irradiation conditions in terms of photon fluence rate, photon fluence and timing, etc. At present, up to 12 sample boxes can be placed at once. Photon fluence rates and the timing of irradiations in each boxes are controlled independently. Wavelength calibration is provided by a Hg lamp and an automatic sample box.

### 3.3.5 SPEX Model Ramalog 1403 Laser Raman Spectrometer

Fig. 3.2 shows the relevant optical diagram of the instrument. This spectrometer (SPEX 1403 double monochromator) is a f/7.8 instrument with spectral coverage from 31000 cm\(^{-1}\) to 11000 cm\(^{-1}\) with an accuracy of ± 1 cm\(^{-1}\) in the
10000 cm\(^{-1}\) range. The spectral repeatability of \(\pm 0.2\) cm\(^{-1}\) and resolution of 0.15 cm\(^{-1}\)

at 5791 Å (Hg line, FWHM) can be achieved by this instrument. The gratings used in this instrument are of holographic type having rulings with 1800 grooves/mm and blazed at 5000 Å. The gratings are mounted on a modified Czerny-Turner mount [Fig. 3.3] using the following fundamental grating equation:

$$m\lambda = d (\sin \alpha + \sin \beta) \quad 3.1$$

where

- \(m\) = spectral order
- \(\lambda\) = wavelength
- \(d\) = grating spacing
- \(\alpha\) = angle of incidence
- \(\beta\) = angle of diffraction

For simplicity, let us put

$$\alpha = \theta + \phi$$

and

$$\beta = \theta - \phi$$

where \(\theta\) is the angle of grating rotation measured from zero as shown in Fig. 3.3, and is a constant angle, depending on the instrument’s design. Therefore equation 3.1 can be rewritten as

$$m\lambda = 2d \sin \theta \cos \phi \quad 3.2$$

The Raman peaks are measured in terms of wavenumber shift in cm\(^{-1}\) on a linear X-axis by utilizing a cosecant drive for grating rotation with \(\phi = 10^\circ\) and thus \(\cos \phi = 0.984\) (manufacturer’s supplied values).
To record the Raman spectra, the laser beam is deflected upward (90°) by a mirror and focused on to the sample to a spot of diameter 10 μm by fused silica condensing lens. Scattered radiation from the sample then passes through a polarization analyzer (optional), a device based on birefringence and total reflection or on dichroism. Use of polarization analyzer provides direct information about the state of polarization of the observed Raman bands. For powdered sample or for samples in KBr matrix (pellet), the use of polarization analyzer becomes redundant because of the random orientation of the constituent molecules or microcrystals of the sample. The scattered radiation is then collected by an elliptical mirror (f/1.4) and focused onto the entrance slit (S3) [Fig. 3.2] of the spectrometer after deflecting from the mirror (M7) and passing through a polarization scrambler. The polarization scrambler converts the plane-polarized scattered radiation to a circularly polarized radiation before it reaches the spectrometer and thus cancels variations in spectrometer response that result from polarization-dependent efficiencies. The polychromatic scattered radiation focused onto the entrance slit gets dispersed by the 1800 lines/mm holographic gratings (G2 and G3). Thus, finally a nearly monochromatic light signal of a particular wavenumber (cm⁻¹) selected by spectrometer control reaches the exit slit (S7) of the double monochromator. This exit slit can be coupled to the third monochromator Model SPEX 1442V.

3.3.6 The Third Monochromator Model SPEX 1442V

This device is used for reducing stray light in Raman scattering where the weak spectral features are not clearly resolved in the vicinity of the intense Rayleigh line. This device functions as a variable band pass, variable frequency filter. The
third monochromator is a modified Czerny-Turner spectrograph attached to the exit slit of the double monochromator. The light entering the third monochromator is nearly monochromatic at the particular tuned frequency of the double monochromator with some stray light at the exciting frequency. This light is further dispersed and finally made to fall onto the exit slit of the third monochromator to which the photomultiplier tube is attached. This final dispersion and adjustment of the exit slit allows only the desired components of the incoming monochromatic light to pass between the slit blades of the exit slit of the third monochromator while the stray radiation at other frequencies are obstructed by the slit blades. The third monochromator can operate in the fixed mirror mode, in the scanning grating mode, or in the stationary grating mode. The fixed mirror mode essentially converts the system to a double monochromator and so maximizes the signal intensity. It is ideal getting high resolution for low intensity signal. The scanning grating mode is suggested for substances plagued by unwanted scattered light over the complete Raman spectrum. The stationary grating mode is especially valuable for identifying Raman lines close to the excitation frequency (Rayleigh line).

3.3.7 Spectrometer Control and Data Processing

The spectrometer control (frequency scanning) and data processing are achieved with a 8-bit dedicated micro-computer SPEX ‘DATAMATE’. With the help of the in-built software, it is possible to manipulate the spectral data by background subtraction, integration, addition, division, frequency range and intensity range expansion/reduction, differentiation, etc., whenever necessary. The incoming spectral data as well as the manipulated data array can be stored in the 4K data point
storage in any of the eight variable length files. The stored data can be plotted on a stripchart recorder or transfer to external peripherals, e.g., floppy disc or to a PC through the standard RS 232 port for further manipulation. Applying the radiometric corrections from the in-built 1K EAROM it is possible to erase the unavoidable wavelength dependent distortions to the spectral data from the spectrometer optics. Using the programming option, the entire spectrometer control as well as data collection and manipulation could be completely automated. The data storing facility could be bypassed and real time spectra could be recorded directly on the stripchart recorder.

The raw data is obtained from the output of the pre-amplifier (PC Dam). The anode of the photomultiplier tube is the input of the PC Dam. The pre-amplifier gain is 400. The high voltage (-1750 volts) required for operating the photomultiplier tube is also supplied by the Datamate with a stability of ± 0.002% after half an hour warm up.

The photomultiplier tube Model RCA 31034 used with the instrument is a 2" diameter, head-on, 11-stage QUANTACON photomultiplier having a gallium arsenide chip as its photocathode, an ultraviolet transmitting glass window and in-line copper beryllium dynode structure. This tube is cooled to -30° C by a thermoelectric cooling device and has almost linear absolute response in the 3000 Å to 8500 Å wavelength range. It operates with a current gain of 10^6 with a maximum dark pulse summation of 12 CPS (count per second).
3.3.8 Scanning of Raman Spectra

There are a number of difficulties associated with recording the Raman spectra of colored samples under resonance conditions. The most prominent include: (a) the optimization of the concentration of the sample to minimize re-absorption of the scattered light by the sample and at the same time allowing the scattering to be maximum; (b) the local heating of the sample due to absorption of the exciting light which may give rise to thermal lens effect and also lead to thermal decomposition of the sample; and (c) the strong background due to fluorescence from impurities in the compound or in the solvent or intrinsic fluorescence from the sample itself.

The first point can be taken care by using samples of different concentrations until a good quality spectrum is obtained. To avoid local heating effect, Kiefer and Bernstein\(^2,3\) had developed a technique which involves continuous rotation of the sample with respect to the laser beam. In this cases, as the sample rotates continuously, the small portion of the sample from which light scattering takes place remains in the laser beam only for a short period of time and thereby reducing the local heating and thermal decomposition. To reduce the fluorescence background, Raman spectra can be measured in the solid form in KBr pellet. In solution, purification of the compound and solvents by standard methods is an effective way of avoiding fluorescence coming from impurities.

To record the Raman spectra of liquid solution, 1-2 ml solution of respective porphyrin at an appropriate concentration is taken in a cylindrical quartz cell and positioned in a proper mount. The laser beam at selected wavelength is then made to strike the bottom of the cell very near to its perimeter. In this way, self-absorption of the scattered light is minimized. The spectra were routinely calibrated with known
CH$_2$Cl$_2$ lines in the lower (100-50 cm$^{-1}$) and with indene in the higher frequency region (1200-1700 cm$^{-1}$) and some times with known Raman lines of solvents that are being used. Other spectral parameters such as laser power, integrating time, wavenumber increment, slit width, etc., are adjusted time to time in order to optimize signal to noise ratio.

3.4 Electronic Absorption Spectra

The electronic absorption spectra were recorded on a Varian Carey 2300 UV-VIS-NIR and on a Hitachi 124S spectrophotometers.

3.4.1 Varian Carey 2300 UV-VIS-NIR Spectrophotometer

This spectrophotometer is a double-beam recording instrument for measuring light intensity at specified wavelengths in a continuous manner. The model employs two lamp sources: a tungsten-halogen lamp for VIS-NIR wavelengths (3152-340 nm) and a deuterium source for UV wavelengths (185-340 nm). Light from the appropriate source is directed through a band pass or long wave pass filter to the single grating monochromator. The monochromatic beam is split into two components of equal intensity, one beam passing through the reference and the other through the sample. The intensity of light emerging from the reference/sample cells is detected by a photomultiplier tube for UV/VIS wavelengths and a lead sulphide detector for NIR wavelengths. The current pulses generated by light from the sample/reference cells are amplified and recorded in transmission or absorbance mode. In the absorbance mode the amplified signal is analyzed by a log converter.
which transforms the light transmission current values to optical density and absorbance is equal to log 1/T (T = transmission). The resolution of the spectrophotometer is 0.07 nm with a wavelength repeatability of ± 0.05 nm and a wavelength accuracy of ± 0.2 nm in the UV-VIS region.
References


Fig. 3.1 Spatial arrangement of the Okazaki large spectrograph. A, monochromator room; B, irradiation room; C, sample box preparation room; D, optical fiber room; E, microcomputer room; F, power supply room. A1, 30 kW Xe short arc lamp; A2, 6 kW Xe short arc lamp; A3, rotatable condensing mirror; A4, medium pressure Hg lamp; A5, shutter; A6, heat-absorbing filter; A7, entrance slit; A8, plane mirror; A9, condensing mirror; A10, double-blazed plane grating; A11, window. B1, focal curve stage; B2, sample box; B3, x-axis frame; B4, y-axis frame; B5, arm, hanging under and moving along the y-axis frame; B6, origin of the automatic carrier system; B7, interface for entrance slit control and mirror cover drive; B8, interface for connector drive. C1, trolley; C2, control panel; C3, CRT terminal; C4, printer. D1, optical fiber bundle; D2, optical fiber outlet unit; D3, panel for monitoring the fluence rate data from the photodiode to the host computer. E1, host computer; E2, data typewriter; E3, CRT terminal; E4, printer; E5, NC; E6, NC interface. F1, air cooling unit for lamps; F2, power supply for lamps; F3, control panel for lamps; F4, water cooling unit for lamps.
Fig. 3.2 Optical diagram of a Spec Model Ramalog spectrometer, including the UVISIR illuminator and the third monochromator. M1, M2, M6, M12, M13, M14, M15, plane mirrors; M3, M4, M7, M8, M9, M10, M11, concave mirrors; M5, elliptical mirror; S1 – S6, slits; L1, fused silica condenser lens; L2, field lens; S, sample; G1, G2, G3, gratings; PMT, photomultiplier tube.
Fig. 3.3 Mechanical cosecant drive mechanism for the Czerny-Turner mount of gratings for the Spex Model 1403 Ramalog spectrometer. The nut N moves along lead screw LS while the slide B moves along a bar held at right angles to the gratings G1. The grating rotates as the arm A moves along the bar. M7 and M8 are mirrors.