CHAPTER 5

NATIVE FLUORESCENCE SPECTROSCOPIC CHARACTERIZATION OF BLOOD PLASMA AND TISSUES DURING RAT LIVER REGENERATION

5.1 INTRODUCTION

As mentioned earlier, optical spectroscopy provides a means to characterize various physical and chemical changes occurring in cells and tissues and thereby offers exciting possibilities for developing novel noninvasive photonic diagnostic instruments and approaches (Alfano and Yao 1981). For the past several years, this analytical procedure has been based mainly on the use of exogenous fluorophores, such as porphyrin derivatives, exploiting either the selective labeling of biological targets or their preferential accumulation in cell and tissue components in order to discriminate normal from pathological conditions. Recently, the intrinsic photophysical properties of biomolecules and biostructures have been considered as possible parameters, which may be related to the morphofunctional state of the biological substrate (Monici et al 1995).

Based on these facts, Chapter 3 described the native fluorescence spectroscopic characterization of blood plasma under normal and different pathological conditions such as oral cancer, gastrointestinal cancer and non malignant liver diseases. These studies revealed that the native fluorescence spectra of blood plasma of cancerous and liver diseased subjects exhibit characteristic red fluorescence in the spectral region 610 – 670 nm, which may be attributed to endogenous porphyrins. Several researchers have also reported on this characteristic emission in the red region in the case of cancerous tissues. This characteristic red emission of cancerous tissues was reported to be due to endogenous porphyrins, which are known to accumulate preferentially in tumor tissues (Yuanlong et al 1987 and Wagnieres et al 1998).
The affinity of porphyrins for neoplastic tissue is so striking that it has been studied for the past several years by many researchers. It was realized at the outset that this affinity of neoplastic tissues for porphyrins has been proved to be generally true for all tumours and hence this class of substances could be utilized to improve the existing methods of cancer detection and therapy. Since regenerating and embryonic tissues are similar in some respects to neoplastic tissues, it is desirable to know whether these tissues would also concentrate porphyrins (Figge et al 1948). It has been shown that certain porphyrins show a preferential retention or uptake not only in malignant tumours but also in embryonic, lymphatic and regenerating tissues.

The great capacity of regeneration exhibited by the liver of animals in response to injury is well known (Michalopoulos and DeFrances 1997). The best experimental model for the study of liver regeneration is, that introduced by Higgins and Anderson (1931), a simple operation called partial hepatectomy (PH) in which two thirds of the liver of a rat are removed intact, without damage to the lobes left behind. The residual lobes make up for the mass of the removed lobes, though the resected lobes never grow back. The whole process lasts 5 to 7 days. Partial hepatectomy is the most often used stimulus to study liver regeneration because, compared with other methods that use hepatic toxins (such as CCl₄), it is not associated with tissue injury and inflammation and the initiation of the regenerative stimulus is precisely defined (removal of liver lobes) (Michalopoulos and DeFrances 1997). Among the different theories proposed to explain this huge regeneration capacity of the liver, the appearance of serum factor(s) is responsible for the extraordinary cell multiplication rate observed. Supporting this hypothesis, several authors have performed experiments, both in vivo and in vitro, showing the appearance of a liver-growth factor activity in blood of animals subjected to partial hepatectomy (Diaz-Gil et al 1986). Fisher et al., demonstrated that the human factor responsible for liver regeneration is found in portal blood and its concentration increases as a great amount of liver is removed (Fisher et al 1971).

In this context, an attempt is made to study the native fluorescence characteristics of blood plasma and tissues during the regeneration phase of the rat liver. The main purpose of this study is to check whether the blood plasma of animals undergoing liver regeneration exhibit similar spectral characteristics, in particular, the characteristic red emission from endogenous porphyrins, as observed in the case of cancerous subjects.
The assessment of liver growth in human patients after the removal of malignancy and during the progression of cirrhosis is extremely important as a prognostic tool. The current methods of measuring hepatocyte growth factor (HGF) either by radio immunoassay or by Enzyme Linked Immuno Sorbent Assay (ELISA) are quite expensive and time consuming. In this regard, the present study is also aimed at estimating the potentiality of native fluorescence spectroscopic characteristics of blood plasma and tissues as a possible method for assessment of liver regeneration.

5.2 STEADY STATE NATIVE FLUORESCENCE SPECTROSCOPIC CHARACTERISTICS OF BLOOD PLASMA AND TISSUES DURING RAT LIVER REGENERATION

The steady state native fluorescence emission and excitation spectra of acetone extract of blood plasma and liver tissues of Wistar strain Albino rats were measured at different excitation / emission wavelengths in the visible region, before and at several time intervals after partial hepatectomy (PH). For each post PH time, four different trials were made and blood and tissue samples were collected up to 240 hrs of post PH time. Fluorescence spectra were also measured for the blood plasma and liver tissues collected from control animals, not subjected to partial hepatectomy. The details of the experimental animals studied and the methods of sample preparation are given in Chapter 2.

The fluorescence spectra of blood plasma and liver tissues were measured at different post PH time intervals, viz. 2, 4, 6, 8, 12, 16, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 hrs. However, the presentation here is limited to only the results of 2, 6, 12, 16, 24, 96, 168 and 240 hrs of post PH time, as the spectra measured at these time intervals were found to represent the prominent characteristic spectral changes occurring during liver regeneration. The results of the native fluorescence emission spectral characteristics of blood plasma and liver tissues at 380, 405, 420 and 450 nm excitation and the corresponding fluorescence excitation characteristics at 630 nm emission at different post PH time periods are presented in the following sections. The fluorescence excitation spectra at 460 and 520 nm emission were also measured for the blood plasma and liver tissues at different post PH time periods. However, as
these spectra exhibited similar spectral characteristics before and after partial hepatectomy, only
the representative excitation spectra of blood plasma and liver tissues are presented here at
these emission wavelengths.

5.3 STEADY STATE NATIVE FLUORESCENCE CHARACTERISTICS OF
BLOOD PLASMA-ACETONE EXTRACT DURING RAT LIVER
REGENERATION

The steady state native fluorescence emission spectra of blood plasma of
experimental animals measured at different post PH time periods, at 380, 405 and 420 nm
excitation and the excitation spectral characteristics at 630 nm emission are presented in the
following sections. To compare the spectral characteristics before and after PH, the
corresponding fluorescence emission and excitation spectra of control animals and the
difference spectra between control and experimental animals are also shown in each figure.

5.3.1 Emission Characteristics at 380 nm Excitation

The normalized average fluorescence emission spectra of blood plasma-acetone
extract at 380 nm excitation are shown in Figures 5.1a-d and 5.2a-d at 2, 6, 12, 16, 24, 96, 168
and 240 hrs respectively. From the figures, it can be seen that the average fluorescence
spectrum of control animals exhibits an emission peak around 410 nm whereas the spectra of
animals subjected to PH have a primary emission peak around 410-415 nm which shows a red
shift of about 4-5 nm with respect to controls at 12 and 16 hrs of post PH time. This red shift is
not observed in the fluorescence spectra of blood plasma at other post PH time periods. The
observed emission peaks in the fluorescence spectra of control and experimental animals in the
region 410-415 nm and the red shifted emission peaks at 12 and 16 hrs of post PH time may be
tentatively assigned to lipofuscins, plasma lipids and / or Pyridoxal phosphate Schiff base

In addition to the primary emission peak, the fluorescence spectra of experimental
animals exhibited a secondary emission peak around 620 - 625 nm, which is not observed in the
case of control animals. This secondary emission peak in the red region may be attributed to
Figure 5.1 Normalized average fluorescence emission spectrum of blood plasma of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 2 hrs, (b) 6 hrs, (c) 12 hrs and (d) 16 hrs, at 380 nm excitation.
Figure 5.2 Normalized average fluorescence emission spectrum of blood plasma of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 24 hrs, (b) 96 hrs, (c) 168 hrs and (d) 240 hrs, at 380 nm excitation.
endogenous porphyrins. It is also be noted that the intensity of this secondary emission peak shows a wide variation at different PH time periods. It is found to attain a maximum at 16 hrs of post PH time and is less intense at 6 hrs and also after 24 hrs of post PH time, as shown by Figures 5.1 and 5.2. The difference spectra between control and experimental animals show negative peaks around 430-450 nm and 620-625 nm, at different post PH times.

5.3.2 Emission Characteristics at 405 nm Excitation

Figures 5.3a-d and 5.4a-d show the normalized average fluorescence spectra of blood plasma of control animals and experimental animals at different post PH times, measured at 405 nm excitation. It is observed from the figures that the average spectrum of control animals at 405 nm excitation shows a broad emission peak centered around 455 nm. However, the average spectra of experimental animals exhibit primary emission peaks in the range 440-470 nm, at different post PH time. It is found that the fluorescence spectra of blood plasma of experimental animals at 12, 16, 24 and 96 hrs show significant red shifts of about 10-15 nm with respect to that of control animals. These primary emission peaks around 440-470 nm observed in the fluorescence spectra of control and experimental animals may be attributed to NAD(P)H. The possible reason for the observed red shift in the primary emission peak of experimental animals with respect to controls may be due to the conformational and / or microenvironmental changes of NAD(P)H occurring during the regenerating phase of the rat liver.

The average fluorescence spectra of experimental animals also exhibit distinct secondary peaks in the red region around 620-625 nm, possibly due to endogenous porphyrins. This secondary red emission is not observed in the fluorescence spectra of control animals, at 405 nm excitation. It can be seen from Figures 5.3 and 5.4 that the intensity of this secondary emission peak in the red region attains a maximum value at 16 hrs of post PH time and it is less intense at 240 hrs of post PH time. The difference spectra between control and experimental animals show negative peaks around 620-625 nm at different post PH time periods. At 6 hrs and 168 hrs of post PH time, the difference spectra between control and experimental animals show a positive peak around 480 nm, which indicates the blue shift in the primary emission peak of the experimental animals with respect to controls.
Figure 5.3  Normalized average fluorescence emission spectrum of blood plasma of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 2 hrs, (b) 6 hrs, (c) 12 hrs and (d) 16 hrs, at 405 nm excitation.
Figure 5.4  Normalized average fluorescence emission spectrum of blood plasma of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 24 hrs, (b) 96 hrs, (c) 168 hrs and (d) 240 hrs, at 405 nm excitation.
5.3.3 Emission Characteristics at 420 nm Excitation

Figures 5.5a-d and 5.6a-d show the normalized average native fluorescence spectra of blood plasma of control animals and experimental animals at different post PH time periods, at 420 nm excitation. It can be seen that the average fluorescence spectrum of control animals shows a primary emission peak around 470 nm while that of experimental animals show primary emission peaks in the region 470-485 nm. As observed in the case of 405 nm excitation, at 420 nm excitation, the average fluorescence spectra of experimental animals show a marked red shift of about 10-15 nm in the primary emission peak at 12, 16, 24 and 96 hrs of post PH time, with respect to control animals. These primary emission peaks observed around 470-485 nm in control and experimental animals, may be attributed to NAD(P)H and / or vitamins (Ramanujam 2000). The possible reason for the red shift in the primary emission peak after partial hepatectomy may be due to the conformational and / or microenvironmental changes occurring during the regenerating phase of the rat liver.

The characteristic red emission around 620-625 nm possibly due to endogenous porphyrins is also observed in the fluorescence spectra of experimental animals at 420 nm excitation, as in the case of 380 and 405 nm excitations. As mentioned earlier, the intensity of this secondary peak is found to be maximum at 16 hrs of post PH time and it is almost diminished at 240 hrs of post PH time. The intensity of this secondary peak shows a wide variation in the fluorescence spectra at other post PH time periods. At 420 nm excitation, the difference spectra between control and experimental animals show broad negative peak in the region 485-525 nm and an additional negative peak around 620-625 nm. The isosbestic points are observed around 475 nm in the difference spectra, at different post PH time periods.

5.3.4 Emission Characteristics at 450 nm Excitation

Figures 5.7a-d and 5.8a-d represent the normalized average fluorescence emission spectra of control animals and experimental animals at different post PH time periods, at 450 nm excitation. It can be seen from the figures that the average spectrum of control animals exhibit a primary emission peak around 500 nm. However, in the case of experimental animals subjected to partial hepatectomy, the position of the primary emission peak varies at different
Figure 5.5  Normalized average fluorescence emission spectrum of blood plasma of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 2 hrs, (b) 6 hrs, (c) 12 hrs and (d) 16 hrs, at 420 nm excitation.
Figure 5.6  Normalized average fluorescence emission spectrum of blood plasma of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 24 hrs, (b) 96 hrs, (c) 168 hrs and (d) 240 hrs, at 420 nm excitation.
post PH time periods and it is located around 485-515 nm. It is found that the primary emission peak in the fluorescence spectra of blood plasma at 2 hrs and 96 hrs of post PH time exhibit a marked blue shift of about 15 nm with respect to that of control animals, as shown by their corresponding difference spectra. These primary emission peaks in the region 485-515 nm may be attributed to emission from flavins. The observed blue shift in the emission spectra of regenerating animals suggests the possible changes in the conformation and/or microenvironment of flavins during liver regeneration. Further, it is observed that the fluorescence spectra at 16 hrs of post PH time exhibit the characteristic red fluorescence around 630-640 nm, which may be attributed to endogenous porphyrins. It should be noted that this characteristic red emission is not observed in the fluorescence spectra of experimental animals at other post PH time periods, before and after 16 hrs, at 450 nm excitation.

5.3.5 Excitation Characteristics at 460 and 520 nm Emission

Figures 5.9a and 5.9b show the representative normalized fluorescence excitation spectra of blood plasma of experimental animals subjected to partial hepatectomy at 460 and 520 nm emissions respectively. As mentioned earlier, the control animals and experimental animals show similar spectral characteristics at these emission wavelengths and hence only the representative spectra are shown here. Figures 5.9 (a) and (b) show that at 460 nm emission, the fluorescence excitation spectra of blood plasma shows a well defined excitation peak around 345 nm and at 520 nm emission, the excitation peak is located around 445 nm. These excitation peaks may be assigned to NAD(P)H and flavins respectively. These peaks observed in the fluorescence excitation spectra at 460 and 520 nm emissions suggest that the origins of the emission peaks observed around 440-460 nm and 500-515 nm are possibly due to NAD(P)H and flavins respectively.

5.3.6 Excitation characteristics at 630 nm emission

In order to confirm the origin of the secondary red emission peak observed in the fluorescence spectra of blood plasma of experimental animals at 380, 405 and 420 nm excitations, the fluorescence excitation spectra of blood plasma were measured at 630 nm emission, for both control and experimental animals. Figures 5.10a-d and 5.11a-d show the normalized average fluorescence excitation spectra of control animals and experimental animals
Figure 5.7 Normalized average fluorescence emission spectrum of blood plasma of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 2 hrs, (b) 6 hrs, (c) 12 hrs and (d) 16 hrs, at 450 nm excitation.
Figure 5.8 Normalized average fluorescence emission spectrum of blood plasma of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 24 hrs, (b) 96 hrs, (c) 168 hrs and (d) 240 hrs, at 450 nm excitation.
Figure 5.9 Representative normalized fluorescence excitation spectrum of blood plasma of animals subjected to PH at (a) 460 and (b) 520 nm emission.
Figure 5.10  Normalized average fluorescence excitation spectrum of blood plasma of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 2 hrs, (b) 6 hrs, (c) 12 hrs and (d) 16 hrs, at 630 nm emission.
Figure 5.11 Normalized average fluorescence excitation spectrum of blood plasma of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 24 hrs, (b) 96 hrs, (c) 168 hrs and (d) 240 hrs, at 630 nm emission.
at different post PH time periods. It is observed that the average fluorescence excitation spectrum of control animals exhibit less distinct excitation peaks around 455 and 530 nm. It is also observed that the excitation spectra of experimental animals exhibit significant spectral differences with respect to control animals. In the case of experimental animals subjected to partial hepatectomy, the fluorescence excitation spectra of blood plasma show a distinct primary emission peak around 390 nm with additional peaks around 460 and 530 nm. The primary emission peak observed around 390 nm in the excitation spectra of experimental animals may be assigned to the Soret absorption band of porphyrins. The secondary excitation peaks observed around 455-460 nm and 530 nm in the spectra of control and experimental animals might be attributed to the Q-band absorption of porphyrins (Yuanlong et al 1987). The difference spectra between control and experimental animals may show a distinct negative peak around 390 nm and small peaks around 460-490 and 530 nm, at different post PH time periods. It can be seen from the Figures 5.6 and 5.7 that the Soret band absorption is more intense in the excitation spectra for post PH time periods before 24 hrs when compared to the spectra at post PH time periods at and beyond 24 hrs. This is evident from the difference spectra between control animals and experimental animals, which show that the negative peak around 390 nm is more intense at post PH time periods before 24 hrs.

5.4 STEADY STATE NATIVE FLUORESCENCE CHARACTERISTICS OF ACETONE EXTRACT OF LIVER TISSUES DURING REGENERATION

As in the case of blood plasma, fluorescence emission and excitation spectra were also measured for the acetone extract of liver tissues of both control and experimental animals, at 380, 405, 420 and 450 nm excitations and 460, 520 and 630 nm emissions, at different time intervals after partial hepatectomy. The results of these emission and excitation spectra are presented in this section.

5.4.1 Emission Characteristics at 380 nm Excitation

Figures 5.12a–d and 5.13a–d show the normalized average fluorescence spectra of liver tissues at 380 nm excitation, for control animals and experimental animals at different post
Figure 5.12  Normalized average fluorescence emission spectrum of liver tissue extract of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 2 hrs, (b) 6 hrs, (c) 12 hrs and (d) 16 hrs, at 380 nm excitation.
Figure 5.13 Normalized average fluorescence emission spectrum of liver tissue extract of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 24 hrs, (b) 96 hrs, (c) 168 hrs and (d) 240 hrs, at 380 nm excitation.
PH time periods. From the figures, it can be seen that at 380 nm excitation, the average fluorescence spectrum of control animals exhibit two distinct emission peaks around 420 and 512 nm and the intensity of 512 nm peak is found to be less intense when compared to that at 420 nm. In the case of control animals, the average fluorescence spectra at 380 nm excitation show prominent emission peaks around 412-425 nm and 512-517 nm. Further, it is observed that the intensity of these two emission peaks vary with different post PH periods. For instance, in the fluorescence spectra of experimental animals at 2, 6 and 12 hrs of post PH time, the emission peak around 512-517 nm is more prominent than that around 412-425 nm. However, at PH periods greater than 16 hrs, the peak around 412-425 nm becomes more prominent than that around 512-517 nm in the fluorescence spectra of experimental animals. It is also noted that at and beyond 16 hrs of post PH time, the intensity of the emission peak around 512-517 nm is significantly less and only a hump is observed in this region until 96 hrs of post PH time. However, it is seen that at 168 hrs and in particular at 240 hrs of post PH time, the fluorescence spectra of experimental animals exhibit similar spectral signatures as that of control animals, indicating that the regeneration of liver is maximum at a post PH period of 240 hrs. This is evident from comparing the difference spectra between control and experimental animals at different post PH time periods.

The emission peaks observed around 412-425 nm and 512-517 nm observed in the fluorescence spectra of liver tissues of control and experimental animals at 380 nm excitation may be attributed to emission from lipofuscins and flavins respectively. It can be seen from the Figures 5.12a-d and 5.13a-d, that lipofuscin fluorescence is more dominant at 16 hrs of post PH time when compared to control animals and experimental animals at other post PH time periods. Similarly, it is also noted that the fluorescence spectra of experimental animals at 2, 6 and 12 hrs of post PH time exhibit small humps around 575 and 620-625 nm which may be assigned to emission from lipofuscins and endogenous porphyrins. At 380 nm excitation, the difference spectra between control and experimental animals show negative peaks around 515 and 575 nm at 2, 6 and 12 hrs of post PH time, whereas at other post PH time periods, the difference spectra show a positive peak around 515-520 nm.
5.4.2 Emission Characteristics at 405 nm Excitation

Figures 5.14a–d and 5.15a–d represent the normalized average fluorescence spectra of control animals and experimental animals at different post PH time periods, at 405 nm excitation. It is observed that the average fluorescence spectrum of control animals shows a distinct emission peak around 510 nm, which may be attributed to emission from flavins. In the case of experimental animals, the average fluorescence spectra of liver tissues show two emission peaks around 515 and 575 nm with a hump around 620-625 nm, at 2, 6 and 12 hrs of post PH time. In these cases, it is also observed that the intensity of the emission peak around 575 nm is more when compared to the peak at 515 nm. Beyond 12 hrs of post PH time, it is found that the fluorescence spectra of liver tissues show only a single distinct emission peak around 510 nm as in the case of control animals. The difference spectra between control and experimental animals, at 405 nm excitation, show well defined negative peaks around 575 and 620-625 nm, at 2, 6 and 12 hrs of post PH time. At 16 hrs of post PH time, the difference spectrum shows a small negative peak around 580 nm and beyond 16 hrs, no significant spectral differences were observed between the average fluorescence spectra of control animals and experimental animals subjected to partial hepatectomy.

The emission peaks observed around 515 nm observed in the fluorescence spectra of experimental animals may be attributed to emission from flavins. Similarly, the emission peak observed around 575 nm in the case of experimental animals may be attributed to lipofuscins. It is found that the possible lipofuscin emission peak around 575 nm is not observed in the fluorescence spectra of control animals and it is observed only in the spectra of experimental animals subjected to partial hepatectomy at 2, 6 and 12 hrs of post PH time. Further, it is observed that the intensity of this emission peak around 575 nm is more intense at 12 hrs than at 2 and 6 hrs of post PH time. At 16 hrs, no distinct emission peak is observed around 575 nm and only a hump is seen in this region.

5.4.3 Emission Characteristics at 420 nm Excitation

The normalized average fluorescence spectra of control animals and experimental animals at different post PH time periods are shown in Figures 5.16a–d and 5.17a–d, at
Figure 5.14  Normalized average fluorescence emission spectrum of liver tissue extract of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 2 hrs, (b) 6 hrs, (c) 12 hrs and (d) 16 hrs, at 405 nm excitation.
Figure 5.15  Normalized average fluorescence emission spectrum of liver tissue extract of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 24 hrs, (b) 96 hrs, (c) 168 hrs and (d) 240 hrs, at 405 nm excitation.
Figure 5.16 Normalized average fluorescence emission spectrum of liver tissue extract of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 2 hrs, (b) 6 hrs, (c) 12 hrs and (d) 16 hrs, at 420 nm excitation.
Figure 5.17  Normalized average fluorescence emission spectrum of liver tissue extract of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 24 hrs, (b) 96 hrs, (c) 168 hrs and (d) 240 hrs, at 420 nm excitation.
227 nm excitation. It is found that the average fluorescence spectrum of control animals, exhibits a distinct emission peak at 515 nm, which may be assigned to emission from flavins. In the case of experimental animals, the fluorescence spectra at 420 nm excitation show two emission peaks, around 515-525 nm and around 575 nm at 2, 6 and 12 hrs of post PH time. A hump around 625-630 nm possibly due to endogenous porphyrins is also observed in the fluorescence spectra of experimental animals, at 2, 6, 12 and 16 hrs of post PH time. At the post PH time of 16 hrs, the average fluorescence spectrum of control animals exhibit an emission peak around 515 nm and a hump around 575 nm. After 16 hrs of post PH time, the fluorescence spectra of experimental animals exhibit only a single emission peak around 515 nm as observed in the case of control animals. These emission peaks around 515-525 nm observed in the case of experimental animals may be attributed to flavins and those around 575 nm may possibly be assigned to lipofuscins. The difference spectra between control and experimental animals at 420 nm excitation show distinct negative peaks around 580 nm and small negative peaks around 625-630 nm, at 2, 6 and 12 hrs of post PH time. At 16 hrs, the difference spectrum shows a positive peak around 530 nm and a negative peak around 580 nm. Beyond 16 hrs, no significant spectral differences were observed between the fluorescence spectra of control animals and experimental animals.

5.4.4 Emission Characteristics at 450 nm Excitation

As mentioned earlier, the fluorescence spectra of both control and experimental animals at different post PH time periods exhibit similar spectral characteristics at 450 nm excitation. Hence, only the representative normalized fluorescence spectrum of liver tissues is shown in Figure 5.18 at 450 nm excitation. In this case, a well defined primary emission peak is observed around 515 nm, which may be assigned to emission from flavins.

5.4.5 Excitation Characteristics at 460 and 520 nm Excitation

As in the case of 450 nm excitation, the fluorescence excitation spectra of liver tissues of control and experimental animals did not show any spectral differences at 460 and 520 nm emissions. Hence, the representative normalized fluorescence excitation spectra of liver
tissues are shown in Figures 5.19 (a) and (b), for 460 and 520 nm emission respectively. Figure 5.19 (a) shows that at 460 nm emission, the fluorescence excitation spectrum of liver tissues shows a distinct excitation peak around 340 nm which may be attributed to NAD(P)H. Similarly, it is found that the fluorescence excitation spectrum at 520 nm emission exhibits an excitation peak around 440 nm, which may be attributed to flavins.

![Normalized fluorescence emission spectrum of liver tissue extract of animals subjected to PH, at 450 nm excitation](image)

**Figure 5.18** Representative normalized fluorescence emission spectrum of liver tissue extract of animals subjected to PH, at 450 nm excitation

### 5.4.6 Excitation characteristics at 630 nm excitation

Figures 5.20a–d and 5.21a–d show the normalized average fluorescence excitation spectra of control and experimental animals at different post PH time periods, at 630 nm emission. It can be seen from the figures that the average fluorescence excitation spectrum of control animals shows a well defined excitation peak around 440 nm and a hump around 515 nm. However, in the case of experimental animals, the fluorescence excitation spectra at 630 nm emission shows excitation peaks around 405 and 540 nm and a hump around 450 nm, at 2, 6 and 12 hrs of post PH time. At 16 hrs, the excitation peak is located around
Figure 5.19 Representative normalized fluorescence excitation spectrum of liver tissue extract of animals subjected to PH at (a) 460 and (b) 520 nm emission.
Figure 5.20  Normalized average fluorescence excitation spectrum of liver tissue extract of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 2 hrs, (b) 6 hrs, (c) 12 hrs and (d) 16 hrs, at 630 nm emission.
Figure 5.21 Normalized average fluorescence excitation spectrum of liver tissue extract of control animals (green) and animals subjected to PH (red) with their corresponding difference spectrum (blue) at a post PH time of (a) 24 hrs, (b) 96 hrs, (c) 168 hrs and (d) 240 hrs, at 630 nm emission.
415 nm and in this case, the excitation peak around 540 nm and the hump around 450 nm are not observed. Beyond 16 hrs of post PH time, it is found that the fluorescence excitation spectra of experimental animals exhibit similar spectral characteristics as that of control animals, except that the hump around 515 nm is not observed in the case of experimental animals. The difference spectra between control and experimental animals shows negative peaks around 405 and 540 nm at 2, 6, 12 and 16 hrs of post PH time. At 16 hrs of post PH time, the difference spectrum shows a negative peak around 405 nm and a positive peak around 475 nm. At other post PH periods, the difference spectra show a broad positive peak centered around 500-505 nm and in some cases a negative peak around 400 nm is also observed.

The excitation peak observed around 450 nm in the case of control animals may be attributed to flavins. Similarly, in the case of experimental animals, the excitation peak observed around 405-415 nm may be attributed to the Soret band absorption and the peak around 540 nm may correspond to the Q band absorption of endogenous porphyrins respectively. It should be noted that the presence of the excitation peak at 450 nm and the absence of any excitation peak around 405 nm in the excitation spectra of control animals suggest that flavin emission may be predominant and emission due to endogenous porphyrins may be absent in the case of control animals. Also, in the case of experimental animals at post PH time periods before 24 hrs, the excitation peaks observed in the Soret and Q band regions indicate the presence of endogenous porphyrins in liver tissues of animals subjected to partial hepatectomy. The less pronounced hump around 450 nm observed in the fluorescence excitation spectra of experimental animals at 2, 6 and 12 hrs of post PH time suggests that flavin emission is less in the case of experimental animals when compared to control animals.

5.5 DISCUSSION

The liver has a remarkable capacity to regenerate after injury and to adjust its size to match its host. Within a week after partial hepatectomy, the hepatic mass is back essentially to what it was prior to surgery. Some additional interesting observations regarding regeneration of liver includes the following: In a few cases where baboon livers have been transplanted into people, they quickly grow to the size of a human liver. When the liver from a large dog is transplanted into a small dog, it loses mass until it reaches the size appropriate for a small dog.
Hepatocytes or fragments of liver transplanted in extrahepatic locations remain quiescent but begin to proliferate after partial hepatectomy of the host. These types of observations have prompted considerable research in the mechanisms responsible for hepatic regeneration, because understanding the processes involved will likely assist the treatment of a variety of serious liver diseases and may have important implications for certain types of gene therapy. A majority of this research has been conducted using rats and utilized the model of partial hepatectomy, but a substantial body of confirmatory evidence has accumulated from human subjects (Michalopoulos and DeFrances 1997). Traditionally, the clinical assessment of liver function during regeneration has relied on indirect measurement based on blood proteins and plasma biochemical indices (Mann et al 2001). It is well known that different growth factors such as Hepatocyte growth factor (HGF), Tumor necrosis factor (TNF-α), Epidermal growth factor (EGF) responsible for the extraordinary cell multiplication rate observed during liver regeneration appear in the serum / plasma (Michalopoulos and DeFrances 1997).

Based on these facts, the fluorescence emission and excitation characteristics of blood plasma and liver tissues of animals subjected to partial hepatectomy were studied and compared with that of control animals, at different excitation / emission wavelengths. The results of these studies reveal that the fluorescence emission and excitation characteristics of blood plasma as well as liver tissues are significantly different before and after partial hepatectomy. Further, the fluorescence spectral characteristics differ at different time periods after partial hepatectomy. It is observed that the emission characteristics observed in the fluorescence spectra of blood plasma of control and experimental animals may be attributed to intrinsic fluorophores such as plasma lipids, lipofuscins, vitamins, NAD(P)H, FAD and endogenous porphyrins. The NAD(P)H emission peak is found to exhibit significant red shifts in experimental animals after partial hepatectomy, when compared to control animals. These wavelength shifts may be attributed to the conformational and / or microenvironmental changes of NAD(P)H such as oxidation state, binding site, which might occur during liver regeneration.

Further, it has been found that when hematoporphyrin was injected into mice, which had been incised or otherwise traumatized, the porphyrin became concentrated at the site of injury and near the regenerating margins of incisions. These experiments revealed that growing
tissues in general have an affinity for hematoporphyrin (Figge et al 1948). This observation is in close agreement with the results of the present study in which it is observed that the blood plasma of experimental animals exhibit well defined emission peaks in the red region around 620-625 nm, which may be attributed to the presence of endogenous porphyrins. The emission peak due to endogenous porphyrins is found to appear as early as 2 hrs after PH and attains a maximum value at about 16 hrs of post PH time. Beyond 16 hrs, this porphyrin emission is found to be less pronounced in the spectra of control animals. At 240 hrs after PH, the intensity at 620-625 nm becomes comparable to that of control animals, which might indicate maximal recovery of liver in response to partial hepatectomy.

From the present study, it is observed that liver tissues of control animals and animals subjected to partial hepatectomy exhibit significantly different spectral features when compared to blood plasma. For instance, the possible NAD(P)H emission around 440-460 nm, which is found to be dominant in the fluorescence emission spectra of blood plasma at 405 and 420 nm excitations are not observed in the case of liver tissues. In contrast, the fluorescence spectra of liver tissues are dominated by emissions in the wavelength range of 500-520 nm assigned to emission from flavins and 575-580 nm possibly the emission from lipofuscins. Further, it is found that the emission spectra of control animals are dominated by flavin emission whereas in the case of experimental animals at post PH time periods before 16 hrs, the predominant emission is due to lipofuscins. After 16 hrs of post PH time, the emission spectra of experimental animals appear similar to that of control animals. This may indicate the maximal recovery of liver in response to injury, i.e. partial hepatectomy.

At this juncture, it is worth to mention the following: The fluorescence properties of lipofuscin appear to be subject to differences of opinion but most studies agree that maximal excitation of lipofuscin fluorescence occurs at near-visible ultraviolet wavelengths (330 – 390 nm). Autofluorescent emission can vary from blue (420 nm) to an orange-yellow in colour or encompass a wide spectral range (450-650 nm). In a study of the relationship between lipofuscinogenesis and glutathione concentration, cultured rat cardiomyocytes fluoresced at 550 nm for 450-490 nm excitations (Billinton and Knight 2001). These reported results suggest that the emission peaks observed around 575-580 nm may have originated from lipofuscins in
the rat hepatocytes. Moreover, the reason for the dominant lipofuscin emission in experimental animals subjected to partial hepatectomy and the absence of the same in the case of control animals may be explained by the following reported results. Sigal et al., have reported that partial hepatectomy caused multiple changes in the rat liver, including accumulation of polyploid hepatocytes along with prolonged depletion of diploid hepatocytes. Remnant hepatocytes in the partially hepatectomized liver showed increased autofluorescence and cytoplasmic complexity on flow cytometry, which are associated with lipofuscin accumulation during cell aging, and underwent apoptosis more frequently (Sigal et al 1999).

Although, both the blood plasma and liver tissues of animals subjected to PH exhibit the characteristic red emission, the amplitude of this emission around 625 nm is more pronounced in the blood plasma than in the liver tissues. A comparison between the fluorescence characteristics of blood plasma of cancerous patients and liver diseased subjects mentioned in Chapter 3 and the results of the present study on blood plasma and liver tissues during the regenerating phase of the rat liver, suggest the following: Though the fluorescence spectra of blood plasma of diseased subjects (cancer, liver disease) and partially hepatectomized rats exhibit characteristic emission in the red region around 610-630 nm which is possibly due to endogenous porphyrins and its derivatives, the observed peak emission wavelengths are different for cancerous subjects, liver diseased cases and liver regenerating animals. In the case of oral and gastrointestinal cancerous subjects, the emission due to porphyrins is observed around 630 nm whereas in the case of liver diseased subjects this red emission is blue shifted and appears around 615 nm. In partially hepatectomized rats, the emission peak in the red region due to endogenous porphyrins is located around 620-625 nm. These observations reveal the possibilities of suggesting that either different endogenous porphyrins might contribute to the fluorescence emissions observed in the case of cancerous subjects, liver diseased cases and regenerating tissues or the microenvironment of endogenous porphyrins may be different in these cases. However, detailed studies are mandatory in this direction to elucidate the possible reasons for the observed spectral differences in the present study.