METALLOPORPHYRIN - CATALYSED CHEMILUMINESCENCE IMMUNOASSAY

by

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DEDICATION

I dedicate this thesis to my parents and my wife Linda.

ABSTRACT

The rapid growth of the diagnostics market is creating an ever-increasing demand for more sensitive, faster and cheaper detection systems. Horseradish peroxidase (HRP) is a relatively common enzyme with wide applications in chemiluminescence immunoassay.

It has been the intention of this work to make a contribution to this field by developing metalloporphyrin mimics of horse radish peroxidase enzyme. This was thought to have a two-fold importance. First it would offer a cheaper and more robust alternative to HRP and second, the simpler structure would facilitate investigations of the mechanism of the enzyme action which in turn could be used for improving enzyme performance.

To develop an artificial replacement for HRP a wide range of substituted metallo-tetraphenylporphyrins were synthesised and their structure-activity relationships in catalysing the oxidation of luminol by hydrogen peroxide were probed. The chemiluminescence resulting from the oxidation of luminol offered a reliable and quantifiable means of comparing the activities.

At the end of the screening programme tetra(aminophenyl)porphyrin manganese^{III} chloride ($Mn(T_{NH2}PP)Cl$ was identified as the best all-round catalyst and reaction mechanisms were proposed which go some way towards explaining this choice.

The practicality of using $Mn(T_{NH2}PP)Cl$ as a replacement for HRP in chemiluminescence immunoassay was demonstrated when the dose response curve produced a lower detection limit of $1x10^{-12}$ moles/ml and the functionalised metalloporphyrin was successfully conjugated to human IgG.

As in the case of HRP and μ-peroxidase the catalytic performance of $Mn(T_{NH2}PP)Cl$ was found to be improved with the use of enhancers. Thus up to 20-fold increases in signal levels were observed when 1-methylimidazole was used as enhancer.

Additionally it was also found that the free-base porphyrin and some metalloporphyrins are capable of chemiluminescence upon oxidation by hydrogen peroxide under alkaline conditions. The analysis of the chemiluminescence spectrum revealed the existence of S_1 and S_2 emission bands. Although under intense excitation energies the unusual phenomenon of S_2 fluorescence emission has been reported for few limited cases, this is the first reporting of S_2 chemiluminescence emission and was produced under relatively mild oxidation conditions.

An attempt was also made to open up the HRP molecule by unfolding the polypeptide chains surrounding the central heme by means of ultrasonic irradiation. Although the activity of the enzyme was reasonably enhanced when it was exposed to moderate irradiations, the technique needs to be refined before it can have any investigative value.

It is thought that the sensitivity of the artificial enzyme developed in the course of this research can be greatly improved by further optimisation of the reaction conditions thereby making its use as an HRP replacement viable.

CHAPTER I

INTRODUCTION

1.1 INTRODUCTION

The immune system, when working properly, protects the human body very effectively from the ravages of infection. When it is not working properly, it presents some of the most serious and challenging diagnostic and therapeutic problems which physicians and patients face.

Diagnostics tests help characterise and monitor various immune system parameters. The emergence of new diseases and the development of different disease management regimes are creating the need for more sophisticated requirements within the immuno-diagnostics market. These factors coupled with the emergence of more refined alternative non-isotopic technologies, are expected to generate an enormous growth in demands for faster, cheaper, safer and of course superior detection systems.

1.2 IMMUNOASSAY

1.2.1 Historical

During the last two decades of the nineteenth century famous experiments set up the basis for the actual antigen-antibody reaction, used as a tool for infectious disease diagnosis[1].

The presence of specific neutralising activity (antibody) in the sera of immunised animals was first recognised by Emil Von Behring and Shibasoburo Kitasato[2], during their experiments on diphtheria toxin; while Ehrlich and Morgenroth[3] demonstrated the presence of specific serum factors neutralising the infectious agents.

The precipitation of antigen in vitro by an appropriate antiserum was reported by Rudolf Kraus[4] in 1897. The discovery of the precipitin reaction arose from the demonstration of the precipitate formed when cell-free filtrates of typhoid cultures were mixed with the corresponding antiserum.

The quantitative nature of the antigen-antibody reaction was first exploited by Heideberger and Kendall[5] (1935) who found that it is possible to isolate and estimate the immunoprecipitate formed by adding increasing amounts of an antigen to a fixed amount of antibody. This finding led to introduction of the first quantitative assay to medicine thus permitting detection of less than 0.1μg antigen in solution.

1.2.2 New Branch of Science

Since their discovery, researchers have utilised the specificity of serum reactivity to detect and to identify substances of various biological origin, both microbial and non-microbial, founding a new branch of science, serology, that is the study of reactions between serum antibody and antigens.

At the present time , antigen-antibody reactions are utilised in many other fields of medical science , such as haematology (blood typing for transfusions), endocrinology (hormone titration in blood and urine), surgery (tissue typing for quantitative transplantation), oncology (detection and identification of neoplastic cells), forensic medicine (identification of species and individuals), and anthropology (comparative studies among races).

The principal application still remains the diagnosis of infectious diseases for which a variety of in vitro serodiagnostic tests are used. Following the type of reaction involved, the immunodiagnostic tests can be grouped as:

1) Precipitation assays (performed in test tubes, in agar or other semi-solid or solid substrates).

2) Agglutination assays and related techniques (for example, Coomb's test, passive agglutination, Rose-Waaler test, haemoagglutination initiation and coagglutination).

3) Haemolysis and bacteriolysis (for example complement fixation test).

4) Serum neutralisation (toward cytopathogenic viruses, bacteria and toxins).

5) Reactions with labelled antibodies (immunofluorescence, immunochemiluminescence, radio-immunoassays, enzyme-immunoassays).

6) Immunological reactions for special applications (immuno- electron microscopy, Western blotting, immunodot blot).

1.2.2.1 Labelled Immunoassay

Labelled immunoassay consists of two main components - a binding agent which can specifically and avidly bind to the analyte which is to be measured , and a signal-generating entity whose signal can be detected easily and with great sensitivity. The binding agent is usually an antibody. There is a wide range of signal- generating components varying from those which give a radioactive signal to those which create fluorescence / chemi-luminescence or those which give rise to an enzymatically produced colour change.

1.2.2.1.1 Radio Immunoassay

Since 1969, radioimmunoassay (RIA) has been widely used in clinical laboratories for the measurement of immunochemicals in biological fluids. Although RIA procedures have the advantage of sensitivity and specificity, they suffer from the chemical instability of the labelled antigen which leads to a short shelf life for reagent kits, problems associated with handling radioisotopes in the laboratory, and the disposal of radioactive waste.

1.2.2.1.2 Enzyme Immunoassay

Here labelled antigens or antibodies are produced by covalent coupling to an enzyme which is capable of converting a colourless substrate to a coloured product[6]. The intensity of colour formation is thus a function of the amount of bound label and hence is a measure of analyte concentration. Sensitivity is limited in assay of this type but it can be improved using fluorogenic or chemiluminogenic substrates. Alternatively, enzyme amplification can be used.

1.2.2.1.3 Fluorescence Immunoassay

Fluorescent molecules, such as fluorescein, have been used successfully as labels for a number of years. Among the disadvantages of this technique are the high background due to scattered incident light and endogenous fluorescence from the sample, resulting in loss of sensitivity. This can be improved by using pulsed-light, time-resolved fluorometry in which lanthanide chelates are used as labels.

1.3 CHEMILUMINESCENCE

A chemical substance that absorbs energy can undergo a variety of changes. An increase in temperature, for example can excite the electrons of a substance to higher energy levels. In this "excited " state, the electrons may become unstable and they must lose the excess energy to regain their original ground level stability. This loss of excess energy may be achieved through a variety of photophysical and photochemical processes.

Most chemical reactions release energy as heat via vibrational excitation of ground state products, and even when electronically excited states are produced, most of them decay via non-radiative processes.

A relatively unusual way for excited molecules to release excess energy is by emission of light. Processes in which excited state molecules release light are termed luminescent and can be displayed by the Jablonski diagram[7] (fig 1.1).

Luminescence is concerned primarily with the emission of visible or near-visible radiation (200-1500 nm) when electrons in excited orbitals decay to ground state, the light arising from the potential energy of electronic transitions within atoms or molecules.

Many types of luminescence have been identified, which can be distinguished by the source responsible for generating or releasing the light. These sources can be associated with heating, irradiation, electrical phenomena, structural rearrangements and chemical reactions. Luminescence associated with chemical reactions is by far the most common and can be

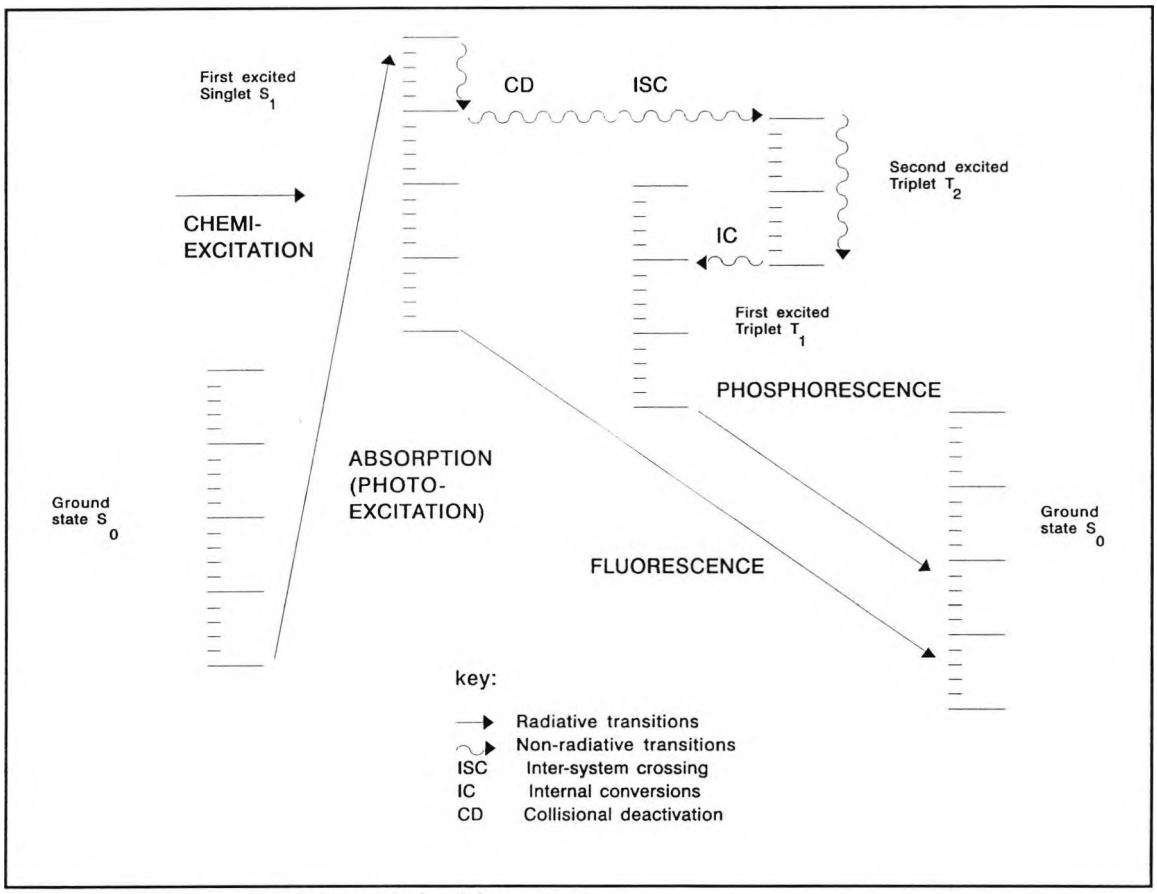


Figure 1.1 - Jablonski diagram

produced from a chemical or biological source. Hence, the terms chemiluminescence and bioluminescence.

Chemiluminescence can therefore be defined as the emission of light from electronically excited states , formed as a result of a chemical reaction .

The range of wavelengths of light emitted from a chemiluminescent source is large, from the near ultraviolet to the infra- red, but the 400-700 nm range is the most common. The colour of the emitted light is dependent on the molecule oxidised, and the amount of energy released . In general, the more energetic reactions give light of short wavelength , e.g. blue , whereas a less energetic reaction will produce green or red light.

1.3.1 Chemiluminescent Reactions

Although chemiluminescent reactions are relatively uncommon, hundreds of reactions have been identified in the liquid, gas, and solid phases. The range covers inorganic and organic reactions, as well as the interface between organic and inorganic chemistry, the organometallic reactions. Some examples of such reactions are shown in Tables[7].1.1 and 1.2.

Several mechanisms have been described to explain the manner by which chemical energy is provided for chemiluminescent reactions; these include reactions involving peroxide decomposition, singlet oxygen, ion radicals, and chemically initiated electron exchange.

Classification of chemiluminescent reactions may be based either on the class of compound, or on the mechanism of the luminescent reaction, or on the phase in which luminescence occurs, i.e. gas, liquid, or solid[8].

Table 1.1 - Examples of Organic Chemiluminescence	
Reaction	**Colour**
Solid	
Rubrene peroxide dissociation by heat	red
Liquid	
Organomagnesium halides (Grignard reagents), in ether + O_2	ultraviolet
Oxidation of luminol in aq. alkali	blue
Oxidation of lucigenin by alkaline H_2O_2	blue-green
Oxidation of lophine in alcoholic NaOH	yellow
Pyrogallol in alkaline H_2O_2	reddish
Gas	
Carbon monoxide flame	blue
Ether flame	blue

Table 1.2 - Examples of Inorganic Chemiluminescence	
Reaction	**Colour**
Solid	
Oxidation of phosphorus in air (vapour just above surface)	blue
Oxidation of alkali metals in air	whitish
Liquid	
H_2O_2 or strong acids on slaked lime	white
Alkali metal hydroxide + acids	whitish
$PbAc_2 + H_2SO_4$	whitish
Siloxene ($Si_6H_6O_3$) + H_2O_2, oxidised by HNO_3, H_2O_2 or permanganate	red
H_2O_2 + NaOCl	red
Gas	
$NO + O \rightarrow NO_2^*$ (air afterglow)	yellow-green
$N + N (+M) \rightarrow N_2^*$ (nitrogen afterglow)	yellow
$^1O_2 \rightarrow {}^3O_2$	red-infrared
$2\ O_2 \rightarrow 2\ {}^3O_2$	red
$O_3 \rightarrow O_2$	ultraviolet
O_3 and atomic oxygen with other gases (e.g. nitrogen oxides, olefins, SO_2, H_2S)	various
Sodium + halogens	yellow
Phosphine ($PH_3 + P_2H_4$) oxidation	blue
$H + H + S_2 \rightarrow H_2 + (S_2)^*$	ultraviolet

Some of the best known chemiluminescent compounds are depicted in fig 1.2[7].

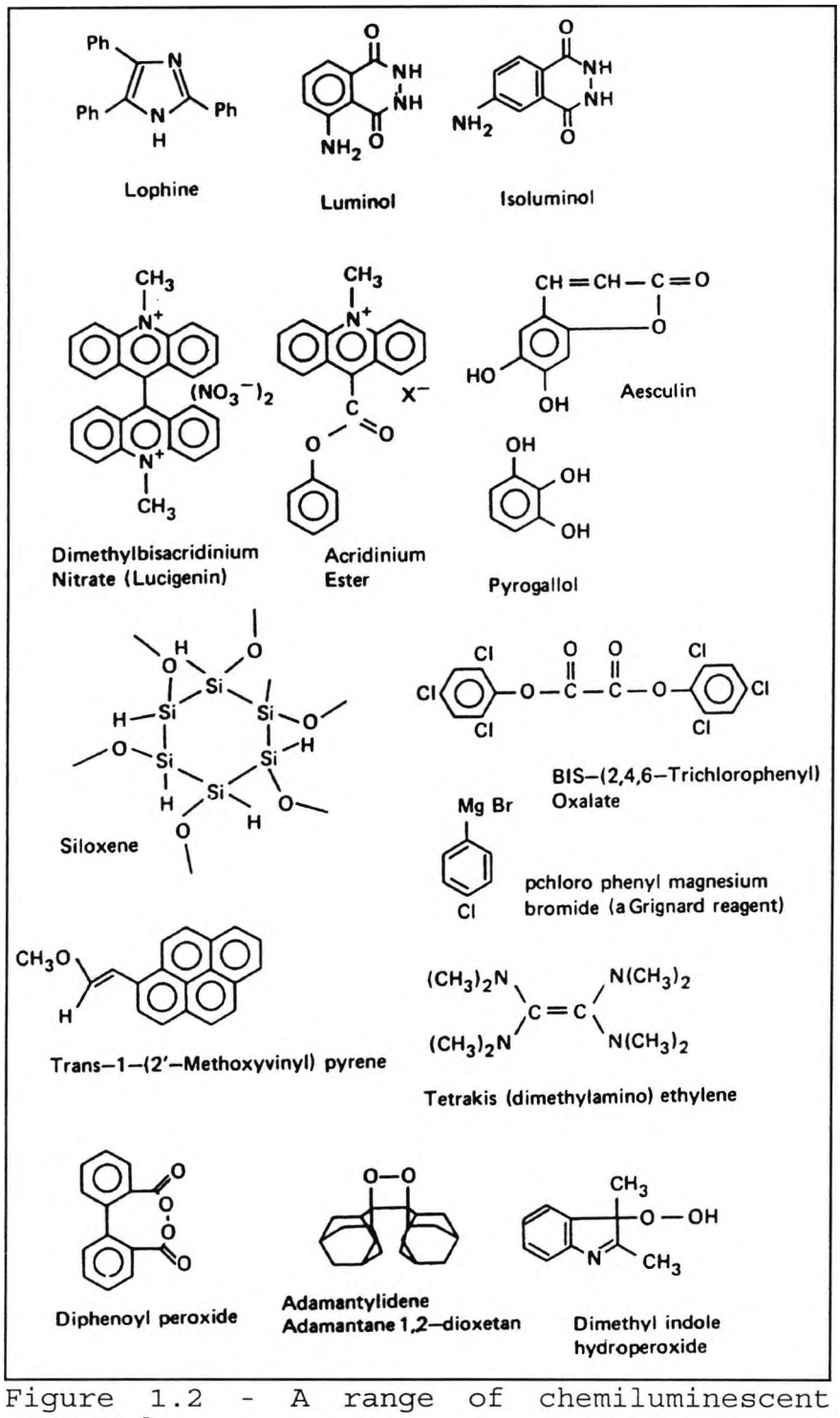
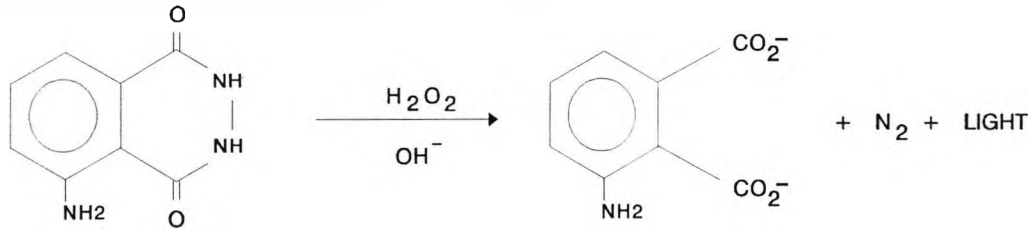


Figure 1.2 - A range of chemiluminescent compounds.

1.3.1.1 Oxidation of Luminol

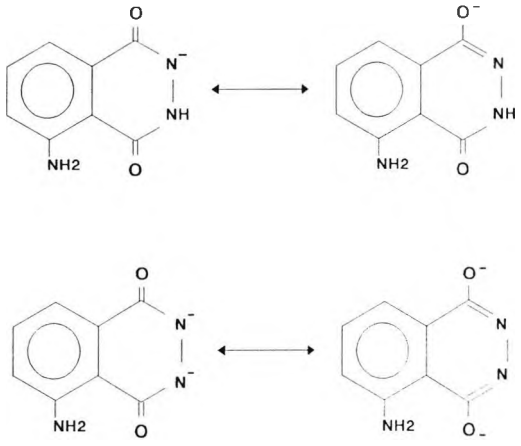
Although luminol (5-amino-phthalhydrazide) is the most studied chemiluminescent compound, the precise mechanism of its reaction under aqueous conditions is still controversial. The overall reaction is as follows:



The starting point for luminol oxidation is an anion , since base is an essential catalyst. Luminol monoanion is present in aqueous alkaline solution, almost as the sole ionic luminol species. In such systems an oxidising agent such as hydrogen peroxide and a catalyst such as a transition metal ion (often as a complex) or an enzyme such as peroxidases are required for the chemiluminescent reaction.

Luminol dianion on the other hand, has been shown to exist in aprotic solutions (eg. DMSO or DMF) as the predominant anionic species. In such systems, in the presence of a strong base, molecular oxygen can attack the dianion to form a peroxide and additional catalyst is not required for chemiluminescence.

In mixed aqueous/DMSO solutions, both the mono- and dianion are observed. In aqueous solution , the first critical intermediate on the chemiluminescent pathway of luminol is a hydroperoxide. At high pH, the anion expels nitrogen and yields the monoprotonated



dicarboxylate.

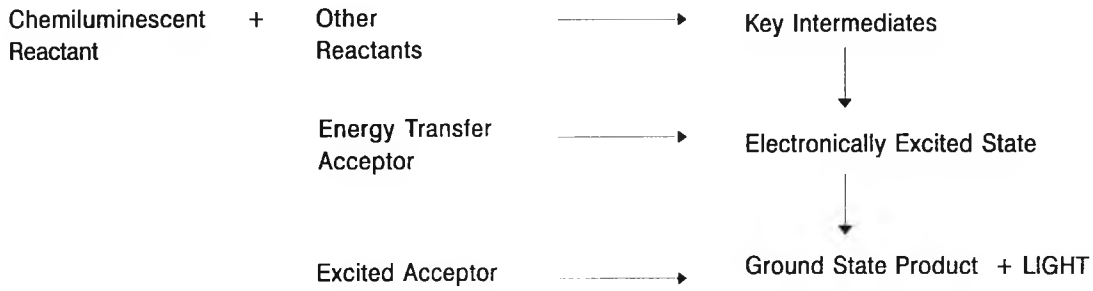
1.3.2 Measurement

The quantum yield of chemiluminescence emission is dependent on several parameters related to the physicochemical properties of the substance in question. The intensity of emission is given by[9]:

$$\mathbf{I} = \phi_{CL}\ \mathbf{K}\ \mathbf{C_0}\ \mathbf{e^{(-Kt)}}$$

where ϕ_{CL} is the chemiluminescent quantum yield, **K** is the reaction rate constant, $\mathbf{C_0}$ is the amount of substance initially present and **t** is time. Since the substance is consumed in the reaction, a constant rate of consumption as a small percentage of the amount of substance present leads to a quantifiable light intensity over a period of time. Therefore the intensity is proportional to the absolute amount of the substance present.

Chemiluminescence is a multistep process, the final quantum yield is the product of the chemical and physical efficiency of the processes involved .



Depending on the chemistry, the emission may be seen either as a glow lasting for many hours or as a flash lasting for less than a second. Thus both the nature of chemiluminescence emission and the nature of the chemistry influence the manner in which the emission is to be detected or quantified. Whilst strong chemiluminescence is often observable to the naked eye, many applications require the quantitative detection of much lower intensities.

The most common methods of quantifying light intensity at low levels employs photomultiplier tubes. Theoretically one molecule of chemiluminescent substance gives rise to one photon but in practice quantum yields are often far less than unity.

For monitoring a chemiluminescent reaction the photomultiplier tube must be incorporated into a suitable instrument, where the reaction vessel will be in optical contact with the detector in a light-tight housing. In the case of long-lived chemiluminescent reactions it is possible to initiate the reaction externally and then place the reaction vessel in the light-tight chamber. For fast reactions, initiations must take place in situ and the chamber must possess a facility for the introduction of initiator substances with rapid mixing.

Depending on the reaction kinetics, there are a number of options available for the processing of the signal from a luminometer[9] as illustrated by figure 1.3.

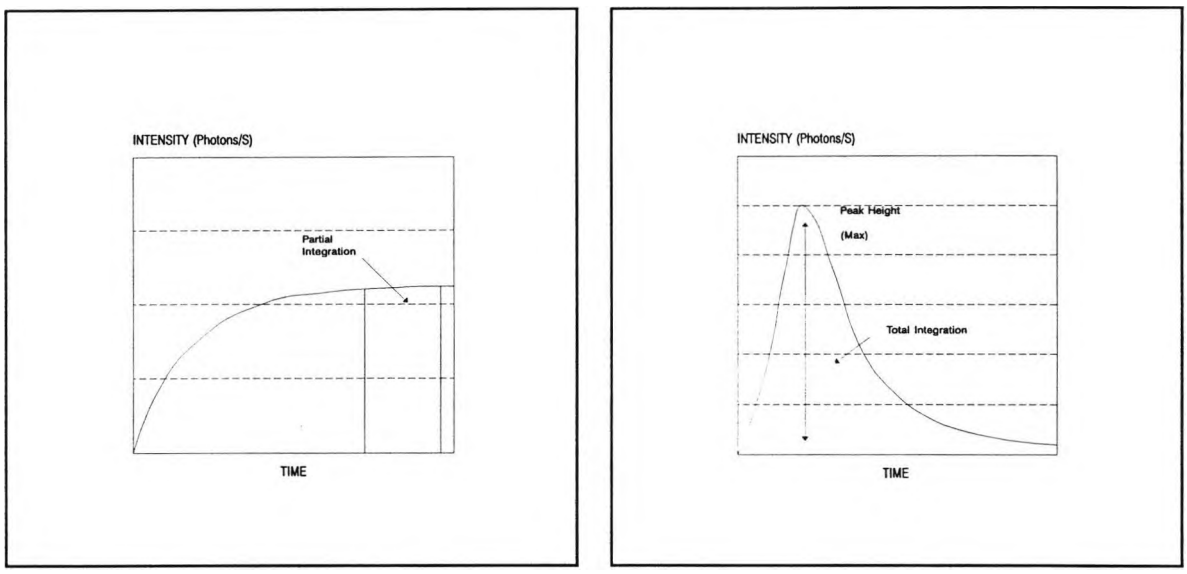


Fig 1.3 - Chemiluminescent reaction profiles

Integrated photon counts over a period of time are the most common parameter used for the measurement as this parameter is more robust, particularly with fast reactions.

1.4 CHEMILUMINESCENCE IMMUNOASSAY

Although in isolation the phenomena of chemiluminescence and the technique of immunoassay are well established, the interface of the two disciplines is less well appreciated, and has only been the subject of serious interest in the last decade.

There are two methods of interfacing a chemiluminescent reaction to an immunochemical reaction[9].

1) Direct coupling

2) Indirect coupling

In the former method a high quantum yield chemiluminescent molecule is coupled to an immunoreactant which serves as the label. In order to serve as a chemiluminescent label, a

species must satisfy the following criteria:

I) possess a high quantum yield which is maintained upon coupling to the appropriate component of the immunochemical reaction;

II) should not alter the immunochemical properties of the immunoreactant;

III) possess the appropriate functional groups for coupling or be easily derivatised;

IV) should yield stable conjugates;

V) should suffer minimal interference from its environment;

VI) should have its light-emitting reaction triggered under relatively mild conditions;

VII) should be rapidly and accurately quantifiable by measurement of its emission intensity.

It is not surprising that only a few groups of compounds are capable of satisfying the above criteria. The most successful include acridinium esters and the hydrazides.

Early attempts concentrated on the use of luminol as the label. The covalent coupling of luminol to antibodies resulted in substantial loss of quantum yield of light emission from the molecule, thus limiting the usefulness of this label[6]. Modification of the basic luminol structure to give aminobutylethyl- isoluminol[10] allowed the resulting aliphatic amine group to be used for coupling purposes with less loss of quantum yield.

More recently, improvements in phthalhydrazide systems have been gained by using molecules of higher quantum yield (naphthalhydrazides), together with carefully optimised oxidation systems[11]. In this way, it has been possible to develop chemiluminescent immunoassays based on phthalhydrazide labels for routine laboratory immunoassay.

The chemiluminescent labels which have yielded the most sensitive, robust assays are those based on acridinium salts[12]. Acridinium salts undergo a chemiluminescent reaction in the presence of dilute hydrogen peroxide without the need for a catalyst. The mechanism is well established and during the last few years, many immunoassays based on these labels

have been described.

Indirect coupling is based on the use of another component of the chemiluminescent reaction, as the label as an alternative to coupling the chemiluminescent molecule itself.

The most commonly used chemiluminescent reactions for the indirect coupling approach have been those of phthalhydrazides. Under aqueous conditions phthalhydrazide chemiluminescence requires the presence of a catalyst in addition to the oxidant. The most commonly used catalysts are heme chelates or hemeoproteins which include μ-peroxidase and horse radish peroxidase (HRP).

HRP on its own is not a particularly good catalyst for luminol chemiluminescence. Thus in direct interface assays it has been replaced by μ-peroxidase. In indirect systems it is only useful if it is used in conjunction with an enhancer.

The enhancement by p-iodophenol, etc. increases chemiluminescence and at the same time suppresses background, allowing the system to compete very favourably with the best immunoassay systems available.

HRP is by no means the only example of chemiluminescence immunoassay using the "indirect" approach and a wide range of other systems have been reported[13], including the use of glucose-6-phosphate dehydrogenase, galactose dehydrogenase, alcohol dehydrogenase, maleate dehydrogenase, glutamic oxaloacetic transaminase and the use of alkaline phosphatase and β-D- galactosidase involving stable dioxetane chemiluminescent substrates[14].

The fact that HRP labels have been used in colorimetric enzyme immunoassays for many years has caused this system to receive greater interest than other potential systems.

1.4.1 HRP-Catalysed Oxidation of Luminol

HRP-luminol- H_2O_2 is by far the most common chemiluminescence immunoassay system. Here luminol radicals ($L^{\cdot -}$ and $LH^{\cdot}$) are formed and subsequently converted to azaquinone (L), 3-(or 6-) amino-2-formyl benzoate (AFB) and peroxide adduct (LO_2H^-). The peroxide adduct is the precursor of the emitter, i.e. 3- aminophthalate (3-AP). The major reactions involved are shown in fig 1.4[15].

The production of the precursor of light emitter, is through two pathways , the superoxide pathway (reaction **I**) and the azaquinone pathway (reaction **F**). The sum of the rates of reaction **I** and reaction **F** is proportional to the light emission. It has been shown that the superoxide pathway is predominant at low H_2O_2 and high OH^-, while the azaquinone pathway is predominant under opposite conditions.

A	$HRP + H_2O_2$	→	Comp I
B	Comp I + LH^-	→	Comp II + $L^{\cdot -}$
C	Comp II + LH^-	→	HRP + $L^{\cdot -}$
D	Comp I + $L^{\cdot -}$	→	Comp II + L
E	$2L^{\cdot -}$	→	L + LH^-
F	L + HO_2^-	→	LO_2H^-
G	$L^{\cdot -}$ + O_2	→	L + $O_2^{\cdot -}$
H	L + $O_2^{\cdot -}$	→	$L^{\cdot -}$ + O_2
I	$L^{\cdot -}$ + $O_2^{\cdot -}$	→	LO_2H^-
J	LO_2H^-	→	3-AP + N_2 + LIGHT
K	L + OH^-	→	AFB
L	$LH^{\cdot}$ + $O_2^{\cdot -}$	→	LH^- + O_2

Fig 1.4 - Reactions in the HRP-luminol-H_2O_2 system.

In applications where HRP or HRP-conjugates are being assayed, steady state conditions are obtained by using high concentrations of luminol and H_2O_2 well above the HRP concentration. Under such conditions the contribution from the azaquinone pathway is predominant and is linearly related to HRP concentration. The relative contribution from the superoxide is low (especially at high HRP concentration) but is not negligible at low HRP concentration.

A change of the pathway, for example at intermediate HRP concentrations, can result in non-linearity between the sum of LO_2H^- production (and therefore light emission) and HRP concentration.

In Enzyme Immunoassay (EIA) applications the predominance of the azaquinone pathway and consequently a linear relationship between HRP-conjugate and light emission is achieved through the use of high substrate levels.

1.5 PORPHYRINS AND RELATED COMPOUNDS

The unequalled ability of enzymes to exhibit molecular recognition and to catalyse selective reactions has led to diverse efforts to understand the reaction mechanisms and to design synthetic systems with similar capabilities.

It has long been known[16] that the active site of some of the most important enzymes and pigments which participate in redox reactions contain porphyrin prosthetic groups.

Iron protoporphyrin IX (heme) is the central component of haemoglobin, myoglobin, catalase, peroxidases, and many of the cytochromes. The participation of heme proteins in the oxygen transport, peroxide reduction and disproportionation, the mitochondrial electron transport chain, and drug metabolism (cytochrome P-450) stresses the biological importance and diverse roles of the iron porphyrins.

It is therefore not surprising that a lot of interest has been generated in porphyrin chemistry . This has largely been directed towards understanding the mechanism of catalysis and in recent years has led to the utilisation of metalloporphyrins as catalysts in a variety of oxidation reactions, including olefin epoxidation and alkane hydroxylation, in attempts to mimic enzymic activity of cytochrome P-450.

Porphyrins are formally derived from porphin[17] (fig 1.5) by substitution of some or all of the peripheral positions with various side-chains. The porphin nucleus consists of four "pyrrole-type" rings joined by four methine bridges to give a macrocycle.

The porphin macrocycle is highly conjugated and a number of resonance forms can be written. There are nominally 22 π-electrons, but only 18 of these can be included in any one delocalisation pathway; this conforms with Huckel's 4n + 2 rule for aromaticity. Porphyrins are highly coloured, with distinct and characteristic absorption spectra. Their main absorption bands have very high extinction coefficients, and the intense "Soret" band, found around 400 nm is characteristic of the macrocycle.

In the classic system of nomenclature, the peripheral positions are numbered from 1 to 8 and the "interpyrrolic" methine positions, usually termed "meso" are designated α , β, γ and δ.

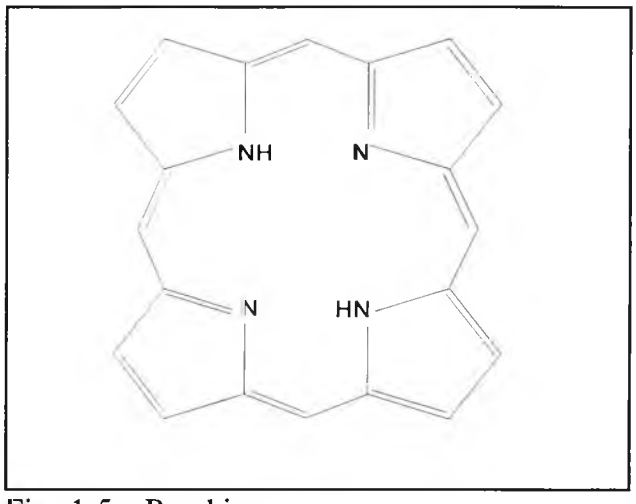


Fig. 1.5 - Porphin

The porphyrins are named and classified on the basis of their side-chain substituents, e.g. etioporphyrins, mesoporphyrins uroporphyrins, coproporphyrins and protoporphyrins. Of these the protoporphyrins are by far the most abundant in nature[18]. Protoporphyrins contains four methyl groups, two vinyl groups and two propanoic acid groups.

Fifteen different isomeric protoporphyrins differing in the sequence of substitution of the above groups in eight available side-chain positions can be written. Of these many possible forms, one, protoporphyrin IX (fig 1.6) is the only form found in nature.

Protoporphyrin forms quadridentate chelate complexes with iron, magnesium, zinc, nickel, cobalt, and copper ions, in which the metal is held by four coordination bonds. Such a chelate complex of protoporphyrin with Fe(II) is called protoheme or more simply heme; a similar complex with Fe(III) is called hemin or hematin.

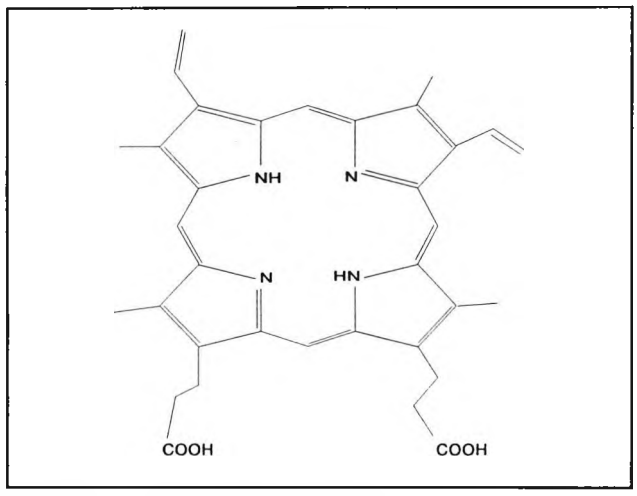


Fig. 1.6 - Protoporphyrin IX

In heme, the four ligand groups of the porphyrin form a square- planar complex with the iron; the remaining fifth and sixth coordination positions of the iron are perpendicular to the plane of the porphyrin ring. In the heme proteins myoglobin and haemoglobin the fifth position is occupied by an imidazole group of a histidine residue and the sixth position is either unoccupied or occupied by oxygen (oxyhemoglobin and oxymyoglobin) or other ligands, such as carbon monoxide. In nearly all the cytochromes, on the other hand, both the fifth and sixth positions of the iron are occupied by the R groups of specific amino-acid residues of the proteins. These cytochromes therefore cannot bind with ligands like oxygen and carbon monoxide.

In the normal function of haemoglobin and myoglobin the iron atom does not undergo change in valency as oxygen is bound and lost; it remains in the Fe(II) state. In cytochromes, however, the iron undergoes reversible changes between Fe(II) and Fe(III) forms; cytochromes serve as electron carriers, whereas haemoglobin and myoglobin act as ligand

(oxygen) carriers.

The most studied synthetic porphyrins are the tetraphenylporphyrins (TPP) (fig 1.7) and their metallated salts (MTPP).

Synthetic TPPs are easily prepared, bind a variety of metals (M = Fe, Co, Cr, Mn, Ru, etc.), and have been used extensively to model the natural heme prosthetic group protoporphyrin IX. The bulky aryl substituents reduce the tendency of aggregation and help to protect the meso carbon from oxidation[19,20]. In addition , the phenyl groups can be substituted with a variety of substituents, enabling controlled changes in physical and chemical properties of the macrocycle. Further more relevant substituents can be functionalised to facilitate the conjugation of TPP to other molecules, particularly proteins.

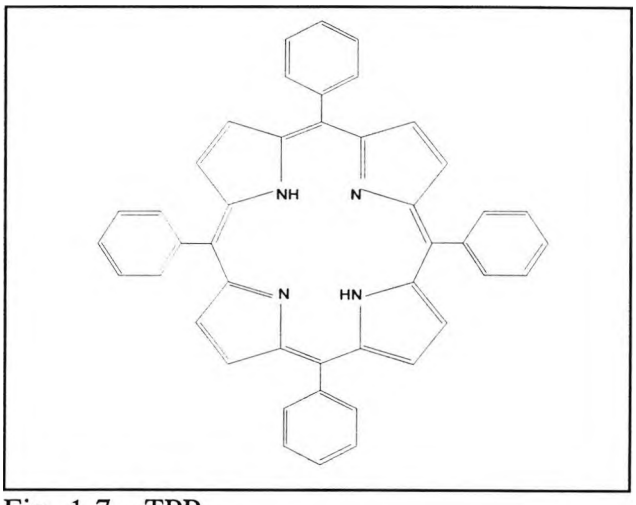


Fig. 1.7 - TPP

1.6 INSTRUMENTATION

A wide spectrum of instrumentation is available to measure the production of light from either chemical or biological sources. Commercial instruments vary from simple photometers to sophisticated multichamber, multichannel photon counters.

The luminescence detecting system used in this study was comprised of a basic luminometer, a dispenser, and output signal-recording devices.

1.6.1 Luminometer

The luminescence resulting from the oxidation of luminol was measured on an LKB-Wallac 1250 Luminometer. This basic luminometer comprised of a measuring head and an electronics unit. In the measuring head (Dark Chamber) a photomultiplier tube (R105-LKB) converted the light produced by the sample or by the built-in ^{14}C standard photon source into an electrical signal.

The sensitivity range of operation for the above photomultiplier was in the 400-600 nm range, which was well suited for the measurement of luminescence from luminol (λ_{Max} = 425 nm).

In the luminometer described above there is no provision for spectral selection other than that arising from the spectral responsiveness of the chosen photomultiplier tube. In this work optical filters and a monochromator were used to achieve narrower spectral bands.

Through the use of an LKB-Wallac 1291-002 Dispenser, the above system was capable of initiating the reaction inside the light-tight chamber of the luminometer. This enabled the measurements of short-lived chemiluminescent flashes as well as long-lasting chemiluminescent glows.

The dispenser, through its pump action, was capable of the addition of 100-400 μl reagent with a coefficient of variation (C.V.%) of less than 1%. Mixing was achieved by forced-jet injection of the reagent into the reaction mixture.

This signal was shaped and amplified by the electronics unit of the luminometer, so as to produce an output suitable for the recording of the signal. The signal so generated, was collected by a computer (analyser) and a chart recorder.

A special computer programme was developed in-house with the help of Dr. Martin Hilchenbach of City University, on a BBC computer. The programme (shown in the Appendix II) was developed with the following features:

* Capturing of 10 data point per second in a digital format.
* Recording of the data on floppy disk.
* Collection of data for fixed and continuous periods.
* Integration between any time points.
* Reporting the max, min and cumulative signal values and their corresponding time points.
* Presentation of the results in graphic format.

The task of direct and continuous output signal monitoring was performed by a J.J. potentiometric chart recorder, which was also connected to the luminometer.

1.6.2 Calibration

1.6.2.1 Luminometer

In order to standardise the photomultiplier, the luminometer was calibrated on a daily basis by adjusting the Gain and Background Sub-controls, against the inbuilt ^{14}C standard which was designed to produce a constant 10 mV signal.

The sensitivity of the luminometer was varied by changing the gain control setting (0-10). The relationship between gain control and signal output was found to be linear between settings 4-10. Provisions were made in the software for the automatic correction of the gain settings between the linear range. This feature enabled relatively accurate comparison of signals at different gain settings.

1.6.2.2 Dispenser

The ultra-sensitive nature of the chemiluminescence technique required a precise reagent delivery mechanism. The uncalibrated dispensing precision of the dispenser was $\pm$ 20%. On calibration, the instrument was capable of dispensing with a precision of 0.5-1%. Clearly, regular calibration was an important requirement of the experimental procedure.

The normal dispensing volumes used in this study were 100 and 200 μl. Thus for each volume, the instrument was calibrated fortnightly by means of calculating the volume of the dispensed reagent (water) from its weight. After adjustment, the dispensing precision of the instrument was checked by dispensing 10 aliquots into a 4ml polystyrene sample cuvette, and

calculating their volumes from their weights.

The precision of the instrument for dispensing each volume was expressed in terms of the Coefficient of Variation (C.V.%) calculated from the formula:

$$C.V.\% = \frac{SD}{Mean} x100$$

1.6.2.3 Pipettes

A combination of four Socorex Micropipettes with disposable tips was used for the preparation and dispensing of the reagents. Each pipette was calibrated on a monthly basis in accordance with the procedures outlined above for the dispenser.

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CHAPTER II

SYNTHESIS AND CONJUGATION OF METALLOPORPHYRINS

2.1 INTRODUCTION

2.1.1 Synthesis of Free Base Tetraphenyl Porphyrins

By far the most common and convenient method of preparation of tetraphenyl porphyrin and its derivatives, involves the condensation of pyrrole with benzaldehyde/ substituted benzaldehyde under acidic conditions.

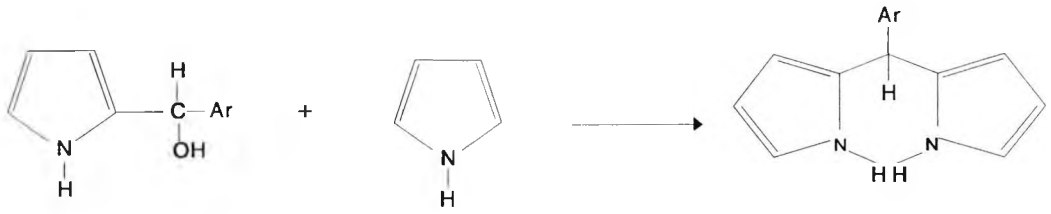
In this work two different versions of the above condensation were employed. The first is the classic method and is known as Alder's method. The second is a recent development of Alder's method and is described by Lindsey.

2.1.1.1 Alder's Method[1]

In this method pyrrole and the corresponding benzaldehyde are condensed in refluxing propanoic acid . It is proposed that the reaction proceeds via four chemical steps.[2,3]

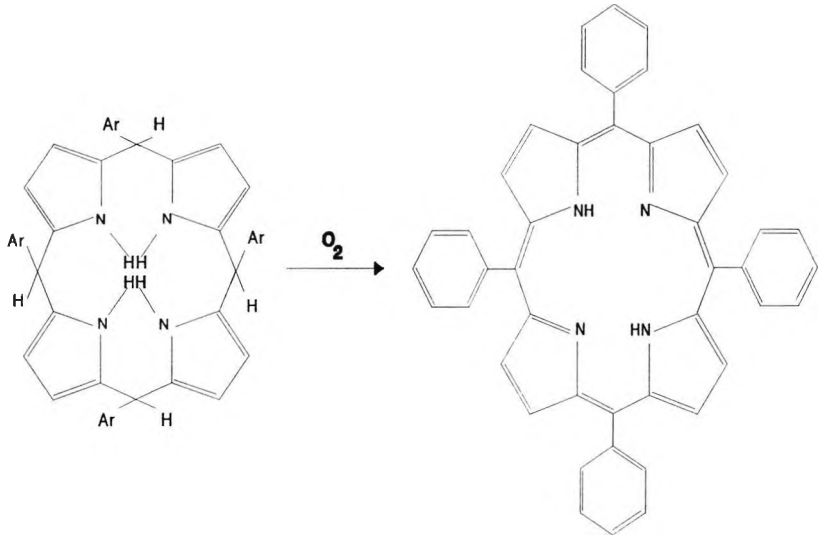
(i) An addition step to produce a carbinol.

(ii) A condensation step involving the carbinol and another molecule of pyrrole.



Followed by further condensation steps.

(iii) The closure of the open-chain tetrapyrrole to form a cyclic tetrapyrrole or porphyrinogen.



(iv) Oxidation by atmospheric oxygen to give the porphyrin.

Although the Alder reaction affords yields of 20% with simple benzaldehydes and can be performed on a large scale, it suffers from several limitations:

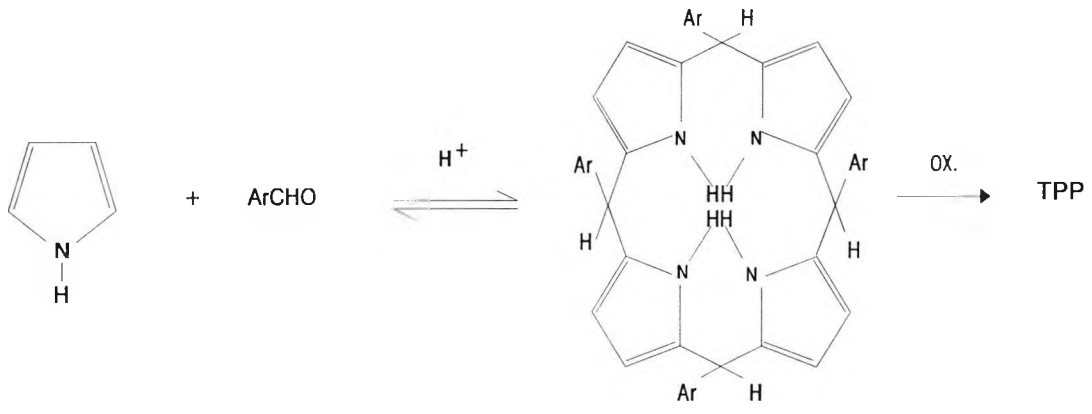
- First, the reaction fails completely with benzaldehydes bearing sensitive functional groups.
- Second, intractable purification problems arise for porphyrins which do not readily crystallize or precipitate from the tar-laden propionic acid.
- Third, the yields obtained are often not reproducible and are very low for benzaldehydes

bearing electron-withdrawing and sterically hindered functional groups.

In this project, Alder's method was used to synthesise TPP, $T_{NO2}PP$, $T_{COOH}PP$ and their derivatives.

2.1.1.2 Lindsey's Method[4,5]

This more recent method of porphyrin synthesis uses a milder reaction condition. Here pyrrole and substituted benzaldehyde are allowed to react with trace acid catalysis at room temperature to afford the tetraphenyl porphyrinogen at thermodynamic equilibrium. The addition of an oxidant irreversibly converts the porphyrinogen to the corresponding porphyrin.



In this method porphyrins bearing sterically hindered, electron-withdrawing and sensitive functional groups can be synthesised in 40% yields, under mild, clean and convenient conditions. However the method is not suitable for bulk synthesis (> 1 g) as equilibrium is best reached at 10^{-2} M concentrations.

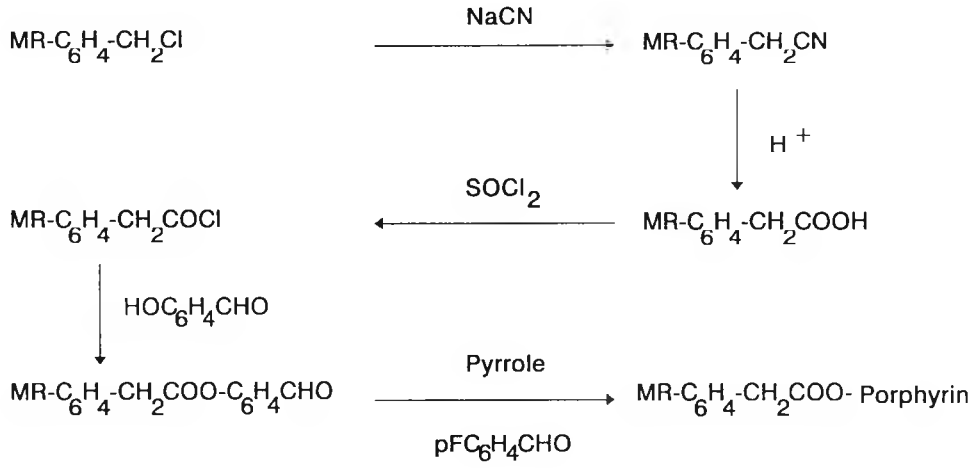
In this work Lindsey's method was used to synthesise TMP and $T_{DCl}PP$.

2.1.2 Synthesis of Unsymmetrical TPP on Solid Phases

Symmetrical TTPs are prepared and separated relatively easily in high yields (40%), using the classical methods of preparation and separation. In the case of unsymmetrical TPPs however, the classical methods give poor yields (< 2%) and the desired product can only be isolated from the complex reaction mixture by extensive chromatographic separations. The synthesis of mono-functionalised $M_{NO2}TPP$ and $M_{COOH}TPP$ carried out in this project being typical examples.

To overcome the above difficulties the technique used by Lenzoff and Svirskaya[6] was adapted by synthesising the unsymmetrically functionalised TPP on an insoluble solid polymer support, which provides a suitable means of "fishing out" the minor component from a complex reaction mixture.

The insoluble solid polymer support used in the synthesis of $Mono_{OH}Tri_{PentaF}PP$ in this project was a 1% cross-linked Merrifield Resin (MR = chloromethylated styrene / divinyl-benzene copolymer), which was activated to bear the benzaldehyde carrying the mono-functionalised group ($HO\text{-}C_6H_4CHO$), prior to the mixed aldehyde condensation, as follows:



Once separated from the reaction mixture, the porphyrin was cleaved off from the Merrifield resin to leave a relatively pure mono-functionalised porphyrin. Additionally the polymer-bounded porphyrin was metallated and used directly as a catalyst.

2.1.3 Synthesis of Metallo-TPP

Metallation of TPPs are best achieved by metallating the corresponding TPP with the required metal salt according to the overall reaction:

$$M^{II} + TPP(H_2) \longrightarrow MTPP + 2H^+$$

The synthetic procedure consist simply of allowing the porphyrin and the divalent metal salt to react in a medium such as N, N- dimethylformamide (DMF), where both the porphyrin and the metal salt are soluble (while the solubility is low at room temperature, at the reflux point most TPPs and metal salts such as halides, acetates, hydroxides and

carbonates are soluble in DMF).

The above reactions can achieve 100% conversion to the metal product in as little as 5 min with a stoichiometric amount of the metal salt. One of the advantages of DMF as a reaction medium stems from its relatively large liquid range and high boiling-point (- 61 to 153°C). The high reflux temperature not only facilitates rapid reaction, but readily displaces the formed acid product thereby driving the reaction to stoichiometric completion. On the other hand, the low melting point often allows the product to be directly crystallized out of the reaction medium and the product can be used without the need for elaborate separation and purification techniques.

In this project, the above method was used to incorporate Fe and Mn into TPP and its derivatives.

2.2 EXPERIMENTAL

2.2.1 Tetraphenylporphyrin (TPP)

Freshly distilled pyrrole (56 ml, 0.8 mole) and 80 ml (0.8 mole) of reagent grade benzaldehyde were added to 3 l of refluxing reagent grade propanoic acid. After refluxing for 30 min, the solution was cooled to room temperature and filtered, and the filter cake was washed thoroughly with methanol. After a hot water wash, the resulting purple crystals were vacuum dried in a desiccator to give the product.

Yield = 17.3 g

Found: uv/vis (DMF) (nm) 416, 514, 548, 590, 646

Lit[7]: uv/vis (DMF) (nm) 417, 514, 548, 590, 647

2.2.2 Tetraphenylporphyrin Iron^{III} Chloride (Fe[TPP]Cl)

Metallation was achieved[8,9] by allowing the divalent metal salt to react in refluxing DMF with TPP. Thus 0.61g ($1x10^{-4}$mole) of TPP and 0.4g of $FeCl_2.4H_2O$ were refluxed in 100ml of DMF for 2hr.

The reaction was monitored by uv/vis spectroscopy and was stopped when the band for free base porphyrin (415 nm) was completely replaced by the iron porphyrin (418 nm). The solution was cooled in an ice-water bath for 15 min and cold water was added (100ml). The resulting crystalline precipitate was collected by filtration , washed with water and finally dried in vacuo over phosphorus pentoxide. Recrystallisation was carried out from 1,2-dichloroethane/hexane (1/1 v/v).

Yield: 0.63g

Found: uv/vis ($CHCl_3$) (nm) 380, 418, 510, 690

Lit[9]: uv/vis ($CHCl_3$) (nm) 380, 417, 511, 690

2.2.3 Tetra(4-sulphonatophenyl)porphyrin ($T_{SO3H}PP$)

The sulphonated porphyrin was prepared according to Dolphin's method[10] in which TPP is sulphonated in conc. sulphuric acid, followed by neutralisation and dialysis.

Powdered TPP (2 g) was suspended in concentrated sulphuric acid (50 ml) in a 250ml round-bottomed flask, equipped with a drying tube. The mixture was heated in a steam bath for 6 hr. and then left to stand overnight. At the beginning of the reaction the viscous mixture was occasionally swirled to dissolve the porphyrin. Water (150 ml) was then added and the mixture was allowed to cool to room temperature. The resulting green protonated porphyrin was collected by filtration and washed with a little acetone. The porphyrin and Celite were suspended in water (150 ml) and neutralised with a saturated solution of sodium bicarbonate until the green solution turned purple. The mixture was filtered to remove Celite and unreacted TPP, and the filtrate was dialysed four times to remove inorganic contaminants.

The dialysis was carried out using cellulose dialysis tubing (Gallenkamp PJC-400-050L) which had been previously boiled in distilled water for 15 min and then rinsed. Each dialysis was carried out for 3 hr against a 40-fold excess of stirred distilled water. The solution was concentrated to about 50 ml, filtered and then evaporated to dryness. The resulting tetrasodium meso- tetra(4-sulfonatophenyl) porphyrin was recrystallised from methanol- ethanol and dried for 1 hr at 110°C to give the dodecahydrated salt.

Yield = 2.3g

Found: uv/vis (H_2O) (nm) 412, 516, 553, 580, 635

Lit[10]: uv/vis (H_2O) (nm) 414, 516, 553, 579, 633

2.2.4 Tetra(4-sulphonatophenyl)porphyrin Manganese^{III} Chloride

$Mn[T_{SO3H}PP]Cl$

To 0.25 g of $T_{SO3H}PP$ in 20ml of distilled water was added excess (0.2 g) of manganese II chloride . The resulting solution was heated at 80°C for 24hr. At the end of the time (the end was decided by uv/vis spectroscopy) the solution was reduced in volume under reduced pressure and separated on a Dowex-50-W x 8 cation exchange column to remove the excess salt. The filtrate was evaporated to dryness to give the pure metalloporphyrin.

Yield = 0.15g

uv/vis (H_2O) (nm) 373, 399, 470

2.2.5 Tetra(sulphonatochlorophenyl)porphyrin ($T_{SO2Cl}PP$)

To 0.1g of T_{SO3H} TPP 10ml (excess) chlorosulphonic acid was added drop-wise. After the addition, the mixture was heated under nitrogen to 120°C in a sand bath for 3hr. At the end of the reaction time the solution was poured drop-wise into ice, keeping the temperature at about 0°C by addition of more ice. After completion, the mixture was allowed to reach room temperature and the resulting sulphonyl chloride derivative was extracted with chloroform. The extracts were combined, concentrated and dried overnight with anhydrous $CaCO_3$, followed by evaporation to dryness to give the final product.

Yield: 0.45g

2.2.6 5-Mono(p-nitrophenyl)10.15.20-triphenylporphyrin[9]

$M_{NO2}TPP$

A 1l three-necked flask containing 800ml of propanoic acid was warmed to refluxing. To it was added 25g (0.24 mole) of benzaldehyde and 12.1g (0.08 mole) of p-nitrobenzaldehyde followed by dropwise addition of 21.4g (0.32 mole) of freshly distilled pyrrole.

The mixture was refluxed for 20 min. After cooling, tarry precipitates were washed with acetone/ diethyl ether mixture (1/10 v/v) and the insoluble portion was collected on a glass filter. The solution was then washed with methanol. The precipitate was extracted with an acetone/ diethyl ether mixture (1/1 v/v). The soluble part was evaporated to dryness and washed with methanol and hot water. Finally the product was vacuum dried in a desiccator.The yield was 8.3g.

The above residue was purified by column chromatography on silica gel using toluene/hexane mixtures. After the first and second elutes using a toluene/ hexane (1/1 v/v) mixture, the elute using a toluene/ hexane (2/1 v/v) mixture was collected and evaporated to dryness. The product was further purified on silica gel by repeating the above procedure.

Yield = 0.85 g

Found: uv/vis (DMF) (nm) 418, 514, 551, 590, 642

Lit[9]: uv/vis (DMF) (nm) 418, 514, 550, 591, 641

2.2.7 5-Mono(p-aminophenyl)10,15,20-triphenylporphyrin[3,9]

$M_{NH2}TPP$

To 0.60g of $M_{NO2}TPP$ was added 25ml of concentrated HCl. After adding 3.0g (excess) of $SnCl_2$. $2H_2O$, the solution was kept at 65°C for 25 min, neutralised with concentrated aqueous ammonia and extracted with chloroform. The chloroform layer was filtered, concentrated and further washed with dilute ammonium hydroxide solution, washed with 200ml of water twice, and evaporated to dryness. The residue was separated on silica gel using a chloroform/ toluene (1/9 v/v) mixture as a solvent and the main component (which emerged last, a long way behind the first) was collected and evaporated to dryness.

Yield = 0.2g

Found: uv/vis (DMF) (nm) 420, 521, 562, 595, 655

Lit[9]: uv/vis (DMF) (nm) 421, 520, 560, 596, 656

2.2.8 5-Mono(p-isothiocyanophenyl)10,15,20-triphenylporphyrin

$M_{NCS}TPP$

40 mg of $M_{NH2}TPP$ was dissolved in 10ml of dry acetone and to the stirred solution was added an excess of thiophosgene (4 drops). The resulting mixture was filtered after a few minutes and the filtrate evaporated to dryness, resulting in a dark purple oily component. Attempts at purifying the resulting product by preparative TLC using cyclohexane/chloroform (1/2 (v/v)) was only partially successful as the product seemed too unstable to be purified by this technique.

Yield = 8 mg

I.R. ($CHCl_3$) (cm^{-1}) Isothiocyanate peak at 1995-2100

uv/vis ($CHCl_3$) (nm) 420, 515, 550, 592

2.2.9 5-Mono(p-carboxyphenyl)10.15.20-triphenylporphyrin

$M_{COOH}TPP$

4 - carboxybenzaldehyde (14 g, 0.093 mole) and benzaldehyde (39.5 g, 0.372 mole) were heated to reflux in 1.1 l of propanoic acid. Freshly distilled pyrrole (31.2 g, 0.464 mole) was then added and the reflux continued for 30 min. The reaction mixture was allowed to cool and kept at 4°C for 24 hr. At the end of the time it was filtered and the resulting black/ purple crystals were washed with methanol, followed by hot water to give 40g of crude porphyrin (mixture of TPP, Mono-, Di-, Tri- and Tetra-carboxyphenylporphyrin).

10 g of the crude porphyrin mixture was purified by dry column chromatography using chloroform as the eluting solvent. The first and second bands (found to be TPP and impurities respectively) were discarded. The very slow-moving third band containing the mono-substituted porphyrin was further purified on a second column to give the pure product.

Yield = 0.28g

uv/vis (DMF) (nm) 418, 514, 548, 589, 645

2.2.10 5-Mono(chlorocarboxyphenyl)10.15.20-triphenylporphyrin

$M_{COCl}TPP$

The acid chloride of M_{COOH} TPP was prepared by dissolving it (0.23 g, 4x 10^{-4} mole) in a mixture of 50 ml of benzene and 5ml of oxalyl chloride. The resulting solution was stirred at room temperature for 3hr. At the end of the reaction time the solvent was removed under reduced pressure. The residue was redissolved in 25ml of benzene and once again taken to dryness under reduced pressure to remove traces of oxalyl chloride. The acid chloride so obtained was then used immediately without further purification.

Yield = 0.24g

IR (DMF) (cm^{-1}) -COCl peak at 1719

2.2.11 5-Mono(isothiocyanatophenyl)10.15.20-triphenylporphyrin

$M_{CONCS}TPP$

0.10g of M_{COCl} TPP was dissolved in 10ml of acetone. To the stirred solution was added 2ml of a saturated solution of potassium thiocyanate. After leaving to stand at room temperature overnight, the mixture was filtered and the product was extracted with chloroform. The extracts were combined, concentrated , dried over anhydrous Na_2SO_4, filtered and the solvent removed under reduced pressure to give the final product, which was used without further purification.

Yield = 70mg

IR (DMF) (cm^{-1}) Isothiocyanate peak at 1995-2100

uv/vis (DMF) (nm) 418, 510, 545, 585, 642

2.2.12 5-Mono(carboxysuccinimidophenyl)10,15,20-triphenylporphyrin

$M_{CS}TPP$

A solution of M_{COOH} TPP (0.1 g, 1.52 $x10^{-4}$ moles), dicyclohexyl carbodiimide (0.031 g,1.52 x 10^{-4} mole) and N- hydroxysuccinimide (0.0175 g, 1.52 x 10^{-4} mole) in 10 ml of dichloromethane/ dry THF (1/1 v/v) was left to stand overnight at room temperature. At the end of the time , the urea precipitate was filtered off and the filtrate evaporated to dryness under reduced pressure. The resulting active ester was used for labelling without further purification.

Yield = 65 mg

2.2.13 Tetra(nitrophenyl)porphyrin[11] ($T_{NO_2}PP$)

P-Nitrobenzaldehyde (101 g, 0.67 mole) was dissolved in refluxing propanoic acid (2 l). Freshly distilled pyrrole (46.5 ml, 0.67 mole) was then slowly added to the boiling solution. The resulting dark solution was heated under reflux for 20min, chloroform (250ml) was added, and the solution was allowed to cool, preventing the separation of the tarry by- products. The resulting mixture was cooled in an ice-bath to 35°C and the purple crystalline product was isolated by filtration and washed well with chloroform until the washings were essentially colourless.

The crude product so obtained was dried at 100°C for several hours and was used without further purification as it was insoluble in most organic solvents and hence not easily purifiable.

Yield = 15.3g

Found: uv/vis (DMF) (nm) 410, 517, 550, 595, 650

Lit[11]: uv/vis (DMF) (nm) 409, 518, 551, 594, 652

2.2.14 Tetra(aminophenyl)porphyrin[11] ($T_{NH2}PP$)

Tetra (nitrophenyl)porphyrin 12g was dissolved in concentrated hydrochloric acid (600 ml) at room temperature, followed by the addition of excess $SnCl_2.2H_2O$ (50 g). The resulting green mixture was quickly heated to 65-70°C for 25min, then cautiously neutralised with concentrated aqueous ammonia. Chloroform (1 l) was added to the hot suspension and the mixture was stirred for 1 hr.

The chloroform layer was separated, the aqueous layer was extracted several times with chloroform and the extracts were combined and filtered. The chloroform solution was then reduced in volume, washed with aqueous ammonia and water and dried over anhydrous sodium sulphate. Ethanol (150 ml) and heptane (100 ml) were added, and the mixture was slowly reduced in volume on a rotary evaporator to produce a very dark crystalline product, which was isolated and washed well with methanol. A small analytical sample was recrystallised from $CHCl_3$/MeOH and dried in air.

Yield = 6.9 g

Found: uv/vis ($CHCl_3$) (nm) 421, 516, 550, 590, 652

Lit[11]: uv/vis ($CHCl_3$) (nm) 422, 516, 550, 591, 653

2.2.15 Tetra(nitrophenyl)porphyrin Manganese^{III} Chloride[11]

Mn[T_{NO2}PP]Cl

To 1 g of T_{NO2}PP in refluxing DMF (250 ml) was added 0.5 g of manganese II chloride and the reaction was allowed to proceed. After 30 minutes the end of the reaction was confirmed by withdrawing a small aliquot and running a uv/vis spectrum. The reaction vessel was removed from the heat source and cooled in an ice-water bath for 15 min. 250 ml of chilled water was then added to the solution and the resulting precipitate was collected by filtration. The product so obtained was washed with water and then air dried . A small sample was purified by column chromatography using benzene as the eluent.

Yield = 0.96 g

uv/vis (DMF) (nm) 390, 472

2.2.16 Tetra(aminophenyl)porphyrin Manganese^{III} Chloride

Mn[T_{NH2}PP]Cl

To 1 g of T_{NH2}PP in refluxing DMF (100 ml) was added 0.5 g of manganese II chloride and the reaction was allowed to proceed. After 30 minutes the end of the reaction was confirmed by withdrawing a small aliquot and running a uv/vis spectrum. The reaction vessel was removed from the heat source and cooled in an ice-water bath for 15 min. 100 ml of chilled water was then added to the solution and the resulting precipitate was collected by filtration, washed with water and air dried. Purification was achieved by column chromatography using chloroform as the eluent.

Yield = 0.90 g

uv/vis (DMF (nm) 374, 395, 470

2.2.17 Tetra(p-isothiocyanatophenyl)porphyrin Manganese^{III} Chloride

Mn[T_{NCS}PP]Cl

100 mg (1.3 x 10^{-5} mol) of Mn[T_{NH2}TPP]Cl was dissolved in 10ml of dry acetone and to the stirred solution was added 30 mg (2.6 x 10^{-4} mol) of thiophosgene (2 x -NH_2 equivalent *). After 15 minutes the mixture was filtered and the filtrate was evaporated to dryness under reduced pressure (at 15°C) to give a dark purple oily product. The product was recrystallized from dry cyclohexane / chloroform (1/2 v/v) and was used immediately used for conjugation.

* The use of only 2 x -NH_2 equivalent of thiophosgene for the activation of -NH_2 groups, assured that for the majority of Mn[T_{NH2}TPP]Cl molecules, activation was restricted to 1 or 2 -NH_2 groups.

Yield = 67 mg

I.R. ($CHCl_3$) (cm^{-1}) Isothiocyanate peak at 1995-2100

2.2.18 Tetra(p-carboxyphenyl)porphyrin ($T_{COOH}PP$)

4 - Carboxybenzaldehyde (75 g, 0.50 mole) was heated to reflux in 1.2 l of propanoic acid. Freshly distilled pyrrole (33.5 g, 0.50 mole) was then slowly added and the reflux continued for 30min. The reaction mixture was allowed to cool and kept at 4°C overnight. The following morning it was filtered and the resulting black/purple crystals were washed with methanol followed by hot water to give 57g of crude porphyrin.

10 g of the above porphyrin was purified by dry column-chromatography using chloroform as the eluting solvent. The very slow moving band containing the porphyrin was further purified on a second column to give the pure product.

Yield = 3.24g

Found: uv/vis (Pyr) (nm) 422, 516, 552, 591, 647

Lit[12]: uv/vis (Pyr) (nm) 422, 517, 552, 591, 649

2.2.19 Tetra(p-carboxyphenyl)porphyrin Manganese^{III} Chloride

$Mn[T_{COOH}PP]Cl$

To 1 g of $T_{COOH}PP$ in refluxing DMF (100 ml) was added 0.5 g of manganese II chloride and the reaction was allowed to proceed. After 30 minutes the end of the reaction was confirmed by withdrawing a small aliquot and running a uv/vis spectrum. The reaction vessel was removed from the heat source and cooled in an ice-water bath for 15 min. Addition of 100 ml of chilled water resulted in the precipitation of the product which was collected by filtration, washed with water and air dried. Purification was achieved by column chromatography using chloroform as the eluent.

Yield = 0.85 g

uv/vis (DMF) (nm) 387, 405, 468

2.2.20 Tetra(carboxysuccinimidephenyl)porphyrin Manganese^{III} Chloride

Mn[T_{CS}TPP]Cl

A solution of Mn[T_{COOH}PP]Cl (100 mg, 1.13 x 10^{-5} mol), dicyclohexyl carbodiimide (DCC) (46 mg, 2.26 x 10^{-5} mol ,(2 x -COOH equivalent*)) and N- hydroxysuccinimide (26 mg, 2.26 x 10^{-5} mol, (2 x -COOH equivalent*) in 10 ml of dichloromethane/ dry THF (1/1 v/v) was left to stand overnight at room temperature.

At the end of the time , the precipitate (urea) was filtered off and the filtrate was evaporated to dryness under reduced pressure. The resulting active ester was used for labelling without further purification.

* The use of only 2 x -COOH equivalent of DCC and N- hydroxysuccinimide for the activation of -COOH groups, insured that for the majority of Mn[T_{COOH}TPP]Cl molecules, activation was restricted to 1 or 2 -COOH groups.

Yield = 61 mg

2.2.21 Tetramesitylporphyrin[4] (TMP)

A 2-l three neck round-bottomed flask fitted with a septum, reflux condenser and nitrogen inlet port was filled with 1l of chloroform (distilled from K_2CO_3), mesitaldehyde 1.48 ml (10 mmol) and pyrrole 0.69 ml (10 mmol). After flushing the solution with nitrogen for 5 min, BF_3-etherate 1.32 ml (3.3 mmol) was added via a syringe. (The room temperature reaction was monitored by removing small aliquots, and oxidising with excess 2,3 dichloro-5,6 - dicyanobenzoquinone (DDQ), followed by uv absorption spectroscopy). At the end of 1hr, p-chloranil 1.84g (7.5 mmol) was added in powder form and the reaction was gently refluxed for 1hr.

The solvent was then evaporated to dryness and the crude dry product was washed with methanol to remove the highly soluble polypyrrylmethenes. The resulting dark green crystalline product was shown by TLC to be pure and was used without further purification.

Yield = 0.487 g

Found: uv/vis (CH_2Cl_2/ EtOH (3/1 v/v)) (nm) 417, 513, 546, 590, 645

Lit[4]: uv/vis (CH_2Cl_2/ EtOH (3/1 v/v)) (nm) 418, 514, 547, 590, 647

2.2.22 Tetramesitylporphyrin Manganese^{III} Chloride Mn[TMP]Cl

To 0.2 g of TMP in refluxing DMF (25 ml) was added 0.2 g of manganese II chloride and the reaction was allowed to proceed. After 45 minutes the end of the reaction was confirmed by withdrawing a small aliquot and running a uv/vis spectrum.

Precipitation of the metallated porphyrin product from the above reaction mixture was achieved by cooling, followed by the addition of 25 ml of chilled water. The product was collected by filtration, washed with water and after air drying it was purified by column chromatography using chloroform as the eluent.

Yield = 0.13 g

uv/vis (CH_2Cl_2) (nm) 370, 478, 586, 620

2.2.23 Tetra(2.6 dichlorophenyl)porphyrin[2] ($T_{DCl}PP$)

A solution of 10^{-2}M pyrrole (2.68 g) and 10^{-2}M 2,6,- dichlorobenzaldehyde (7.00 g) in dichloromethane (4l) was stirred under nitrogen for 1hr with 10^{-2}M trifluoroacetic acid (4.56 g) present as a catalyst. After 1hr the temperature of the solution was increased to 39°C, p-chloranil (3 molar equivalents, 29.51 g) was added and the solution was stirred for a further 1hr. The solvent was then removed under vacuum to leave a black solid residue, which was washed free of its polypyrrylmethenes with methanol. Final purification of the product was achieved by column chromatography using benzene as the eluent.

Yield = 0.112 g

uv/vis (CH_2Cl) (nm) 418, 473, 511, 587

2.2.24 Tetra(2.6 dichlorophenyl)porphyrin Manganese[III] Chloride

Mn[$T_{DCl}PP$]Cl

To 0.08 g of $T_{DCl}PP$ in refluxing DMF (10 ml) was added 0.1 g of manganese II chloride and the reaction was allowed to proceed for 2hr. The end of the reaction was confirmed spectrophotometrically by withdrawing a small aliquot and running a uv/vis spectrum. Precipitation of the metallated porphyrin product from the reaction mixture was achieved by the cooling, followed by the addition of 25 ml of chilled water.

The product was collected by filtration, washed with water and after air drying was purified by column chromatography using chloroform as the eluent.

Yield = 0.065 g

uv/vis (DMF) (nm) 369, 477, 580

2.2.25 Tetra(pentafluorophenyl)porphyrin Manganese^{III} Chloride

Mn[T_{PF}PP]Cl

To 0.1 g of T_{PF}PP in refluxing DMF (20 ml) was added 0.1 g of manganese II chloride and the reaction was allowed to proceed. After 1 hr the end of the reaction was confirmed spectrophotometrically by withdrawing a small aliquot and running a uv/vis spectrum.

At the end of the time, the reaction vessel was removed from the heat source and cooled in an ice-water bath for 15 min. 20 ml of chilled water was then added to the solution and the resulting precipitate was collected by filtration. The product obtained was washed with water and then air dried . A small sample was purified by column chromatography using chloroform as the eluent.

Yield = 0.82 g

uv/vis (CH_2Cl_2) (nm) 367, 469, 573

2.2.26 Tetra(4-methoxyphenyl)porphyrin Manganese^{III} Chloride

Mn[T_{OMe}PP]Cl

To 0.5 g of T_{OMe}PP in refluxing DMF (50 ml) was added 0.3 g of manganese II chloride and the reaction was allowed to proceed. After 30 minutes the end of the reaction was confirmed by withdrawing a small aliquot and running a uv/vis spectrum. The reaction vessel was removed from the heat source and cooled in an ice-water bath for 15 min. Addition of 50 ml of chilled water resulted in the precipitation of the product which was collected by filtration, washed with water and air dried. Purification was achieved by column chromatography using chloroform as the eluent.

Yield = 0.90 g

uv/vis (DMF) (nm) 372, 475, 575

2.2.27 Synthesis of Unsymmetrical TPP on Solid Phases

2.2.27.1 Synthesis of Solid Support Phase[13]

2.2.27.1.1 Synthesis of Cross-Linked Resin Bearing -CH_2CN Groups

MR-$C_6H_4CH_2CN$

The 1% cross-linked Merrifield resin (MR)(chloromethylated styrene/divinylbenzene copolymer) was chosen as the solid phase. 20 g of the resin was suspended in a mixture of freshly distilled DMF (40 ml) and water (8 ml). To this was added a solution of sodium cyanide (8 g) in DMF (80 ml) and water (16 ml) and the mixture was heated at 115-120°C for 20hr. The resulting brown coloured mixture was filtered hot, and the resin was washed successively with water (40 ml x 6), dioxane-water (1:3 (v/v) x 5), dioxane-water (1:1 (v/v) x 5), dioxane-water (3:1 (v/v)x 5), dioxane (x 5), dioxane-ethanol (2:1 (v/v) x 5), dioxane-ethanol (1:2 (v/v) x 3), ethanol (x3) and ether (x 3). The resin was finally dried in vacuo over phosphorus pentoxide.

Yield = 19.1 g

IR (KBr) cm^{-1} 2250 (-CN)

The absence of the -CH_2Cl groups at the end of this conversion was shown as follows. 10 mg of the prepared resin was suspended in a mixture of DMF (0.2 ml) and 20% aqueous sodium hydroxide (0.2 ml), and the mixture was heated for 10min at 120°C. After cooling the supernatant liquid was made acidic by the addition of 1M nitric acid. Upon addition of an aqueous silver nitrate solution to it, no precipitation took place. In contrast, the starting material, the Merrifield resin, gave a massive precipitation of white silver chloride on similar treatment.

2.2.27.1.2 Synthesis of Cross-Linked Resin Bearing -CH_2COOH Groups

MR-$C_6H_4CH_2COOH$

The Merrifield resin bearing -CH_2CN groups (19 g) in a mixture of concentrated sulphuric acid (60 ml) , acetic acid (60 ml) and water (60 ml) was heated under stirring at 115-120°C for 10hr. At the end of the reaction time the slightly yellow resin was collected by filtration, washed successively with water (40 ml x 6), dioxane-water (1:3 (v/v) x 5), dioxane-water (1:1 (v/v) x 5), dioxane-water (3:1 (v/v) x 5), dioxane (x 5), dioxane-ethanol (3:1 (v/v) x 5), dioxane-ethanol (1:3 (v/v) x 3), ethanol (x 3) and ether (x3). The resin was finally dried in vacuo over phosphorus pentoxide.

Yield = 18g

IR (KBr) (cm^{-1}) 1705 (CO), lack of -CN peak at 2250

2.2.27.1.3 Synthesis of Cross-Linked Resin Bearing -CH_2COCl Groups

MR-$C_6H_4CH_2COCl$

17 g of MR-CH_2COOH was mixed with toluene (distilled and dried over Na) (80ml) and thionyl chloride (80 ml) and the mixture was heated under exclusion of moisture ($CaCl_2$ tube) with mechanical stirring.

After 24hr of refluxing when the strong evolution of gaseous HCl subsided, the mixture was filtered (care was taken not to introduce moisture during this filtration and subsequent washing steps by working in a dry air cabinet under a stream of dry nitrogen).

The resin washing was carried out successively with anhydrous toluene (x5) and ether (x3). The resin was finally dried over phosphorus pentoxide in vacuo, and was kept in a tightly stoppered sample bottle in a desiccator until required for use.

Yield = 18.5 g

Found: IR (KBr) (cm^{-1}) 1738 (CO)

Lit[13]: IR (KBr) (cm^{-1}) 1735 (CO)

2.2.27.1.4 Synthesis of Cross-Linked Resin Bearing -CH_2COO-C_6H_4CHO Groups (MR- $C_6H_4CH_2COO$- C_6H_4CHO)

A mixture of 15 g of Merrifield resin containing 1mmol of benzoyl chloride per gram of polymer and 4-hydroxy-benzaldehyde 5.5 g (45 mmol) in anhydrous pyridine (50 ml) was stirred in a tightly stoppered flask. After allowing the reaction to proceed at room temperature for 24hr, methanol (3 ml) was added and the stirring was continued for a further 20hr. The resin was then collected by filtration, and washed successively with pyridine (x 4), pyridine-water (3:1 (v/v) x 3), pyridine-water (1:1 (v/v) x 3), pyridine-water (1:3 (v/v) x 3), water (x 3), water-ethanol (1:1 (v/v) x 2), ethanol (x3), and ether (x 3). The resin was then dried over phosphorus pentoxide in vacuo.

Yield = 14.3 g

IR (KBr) (cm^{-1}) 1701 (-CHO), 1742 (-CO-O-)

2.2.27.2 5-Mono(hydroxyphenyl)10.15.20-tri(pentafluorophenyl) porphyrin on Solid Support[6,14]

MR- $M_{OH}Tri_{pF}$ PP

2.2.27.2.1 Formation of MR-Porphyrin

To 12 g of MR-$C_6H_4CH_2COO$-C_6H_4CHO and pentafluorobenzaldehyde, 4.7 g (24mmole) in refluxing propanoic acid (200 ml) was added 1.6 g (24 mmol) of pyrrole. After 30 min refluxing, filtration resulted in a black polymer which was readily washed free of pentafluorophenyl porphyrin and polypyrroles by repeated extraction in a Soxhlet extractor with chloroform to yield the polymer-bound 5-Mono(hydroxyphenyl) 10,15,20 tri-(pentafluorophenyl) porphyrin. The polymer-porphyrin was finally dried over phosphorus pentoxide in vacuo.

Yield = 14.7

IR (KBr) (cm^{-1}) 990 present in porphyrin & MR-porphyrin but absent from MR

2.2.27.2.2 Cleavage of MR-Porphyrin

8 g of MR- $M_{OH}Tri_{pF}$ PP was stirred at room temperature in 50 ml of a saturated solution of potassium carbonate in methanol for 24hr. At the end of the time the mixture was filtered, washed with methanol followed by chloroform until the washings were colourless. The filtrate was then evaporated to dryness to give the crude product.

Purification of the porphyrin was achieved by a quick filtration through a silica column using chloroform as eluent, followed by preparative thin-layer chromatography using benzene / ether/chloroform (7:2:1) as eluent to give the pure product.

Yield = 255 mg

uv/vis (DMF) (nm) 409, 512, 545, 586

2.2.28 Merrifield Resin-5-Mono(hydroxyphenyl)10.15.20 tri(pentafluorophenyl)porphyrin Manganese^{III} Chloride

Mn[MR-$M_{OH}Tri_{PF}$PP]Cl

To 4 g of MR- $M_{OH}Tri_{pF}$ PP in refluxing DMF was added 1 g of manganese (II) chloride tetrahydrate and the mixture was refluxed for 2hr. At the end of the time, the mixture was filtered , washed with DMF, and then with ether. The polymer-bound metalloporphyrin so obtained was finally dried in vacuo.

Yield = 3.9 g

AA [Mn] = 8.6x 10^{-4} mol/g

2.2.295-Mono(hydroxyphenyl)10.15.20-tri(pentafluorophenyl)porphyrin-Manganese^{III} Chloride

Mn[$M_{OH}Tri_{pF}$ PP] Cl

To 0.1 g of $M_{OH}Tri_{pF}$ PP in refluxing DMF (20 ml) was added 0.1g of manganese II chloride and the reaction was allowed to proceed. After 3hr the end of the reaction was confirmed spectrophotometrically by withdrawing a small aliquot.

The reaction vessel was removed from the heat source and cooled in an ice-water bath for 15 min. 20 ml of chilled water was then added to the solution and the resulting precipitate was collected by filtration. The product so obtained was washed with water and then air dried. The resulting metalloporphyrin was purified by column chromatography using chloroform as the eluent.

Yield = 80 mg

uv/vis (DMF) (nm) 369, 469, 567

2.2.30 Tetra(4-hydroxy,3,5 methoxyphenyl)porphyrin-Manganese^{III} Chloride

Mn[$T_{4\text{-}OH,3,5\text{-}OMe}$PP]Cl

To 0.1 g of $T_{4\text{-}OH,\ 3,5\text{-}OMe}$PP in refluxing DMF (10 ml) was added 0.1 g of manganese II chloride and the reaction was allowed to proceed. After 2hr the end of the reaction was confirmed spectrophotometrically by withdrawing a small aliquot.

The reaction vessel was removed from the heat source and cooled in an ice-water bath for 15 min. 10 ml of chilled water was then added to the solution and the resulting precipitate was collected by filtration. The product so obtained was washed with water and then air dried . The resulting metalloporphyrin was purified by column chromatography using chloroform as the eluent.

Yield = 86 mg

uv/vis (DMF) (nm) 368, 402, 474, 574, 614

2.2.31 Immobilization of Mn[T_{NH2}PP]Cl on Nylon [15,16]

To 2 g of Nylon 6/6 beads (5 mm diameter) [poly (hexamethyleneadipamide)] [-$NH(CH_2)_6NHCO(CH_2)_4CO$-]$_n$ in a stoppered test tube was added 10 ml of a 1M solution of triethyloxonium tetrafluoroborate in dichloromethane. The mixture was agitated for 4hr using a mechanical agitator.

At the end of the time, the excess alkylating agent was removed by filtration and the nylon beads were washed well with cold methanol, followed by water.

A saturated solution of Mn[T_{NH2}PP]Cl in 10 ml of methanol was added to the activated nylon above and after thorough mixing the mixture was allowed to incubate at room temperature overnight. At the end of the incubation period the nylon beads were filtered free of the excess metalloporphyrin and washed with methanol. The beads were then soaked in a stirred solution of methanol to wash the nylon beads free of the loosely bound metalloporphyrins. The above procedure was repeated several times until all the washings were completely colourless. The nylon-bound metalloporphyrin was stored in methanol and kept in a refrigerator until required for use.

Yield = 1.8 g

2.2.32 Conjugation of Functionalised Porphyrins With γ-Globulin

The following activated porphyrins were used for the labelling of human IgG: $M_{CONCS}TPP$, $M_{CS}TPP$, $M_{NCS}TPP$, $T_{SO2Cl}TPP$, $Mn[T_{CS}TPP]Cl$, $Mn[T_{NCS}TPP]Cl$

In each case 0.500 mg of the porphyrin was taken up in 0.1ml of DMF (the low solubility of the above porphyrins in the labelling buffer necessitated the addition of a small amount of DMF) and to the resulting solution was added 0.4ml of labelling buffer (sodium carbonate- sodium bicarbonate buffer solution pH 9.4: 19.48g sodium carbonate and 26.56 sodium bicarbonate made up to one litre in isotonic saline (8.78g NaCl per litre)). 1.00 mg of human IgG in 0.5ml of labelling buffer was then added to the above porphyrin solution to give an overall volume of 1 ml.

The above solutions were swirled and left in a refrigerator (4°C) to stand overnight. At the end of the time the unreacted porphyrins were separated from the protein mixture by means of ion-exchange chromatography. Thus the mixtures were applied to a Sephadex (G-25) column and were eluted with eluting buffer (phosphate buffer solution pH 7.5: 3.90g sodium dihydrogen orthophosphate.$2H_2O$ and 6.70g disodium hydrogen ortho-phosphate. $7H_2O$ in 2.5l of isotonic saline (containing 21.94g NaCl)). The bands containing labelled and unlabelled protein were identified and collected. The labelled IgGs were at their best if assayed immediately, but they were stable for a few days if refrigerated under a sealed environment.

uv/vis (nm) (eluting buffer) 275 (IgG) , 400 - 650 (various MTPP peaks).

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CHAPTER III

CHEMILUMINESCENCE

OF

METALLOPORPHYRINS

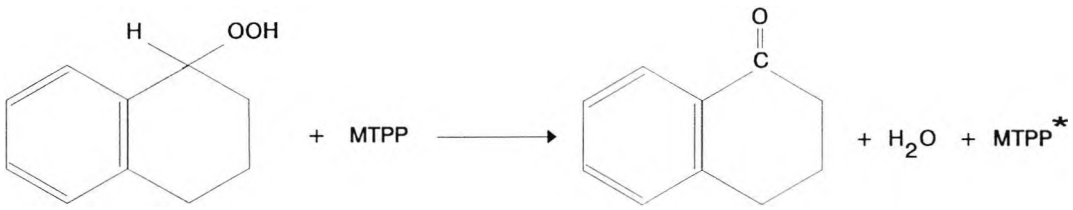
3.1 INTRODUCTION

Since their discovery nearly 100 years ago , an enormous range of reactions have been reported to be chemiluminescent. The range covers inorganic and organic reactions, as well as the interface between organic and inorganic chemistry, the organometallic reactions.

There are many hundreds of inorganic and organic chemical reactions which are known to produce light. The inorganic elements which can participate in chemiluminescent reactions range from the alkali metals and heavy metals, such as mercury and lead , to non-metals such as O, S, N, P, As and the halogens. In the organic series, apart from aliphatic compounds in flames, most are aromatic substances. Important exceptions are the aliphatic oxalate esters and stable aliphatic dioxtanes.

Examples of organometallic reactions which chemiluminesce include the oxidation of phenylmagnesium iodide (a Grignard reagent), metallophthalocyanins, metalloporphyrins and their derivatives.

The chemiluminescence of metalloporphyrins in the presence of peroxides was discovered by Helberg[1,2], who observed a red emission when magnesium phthalocyanine was added to hot tetralin. Further work showed that tetralin hydroperoxide (THP) , present as an impurity in the solvent, is required for chemiluminescence, and the emission is accompanied by the breakdown of both the peroxide and the metalloporphyrin.
The catalysed breakdown of THP results mainly in tetralone and water:



(where * signifies an electronically excited molecule.)

Among the metalloporphyrins and related compounds which show chemiluminescence is chlorophyll itself [3]. The role of chemiluminescence in photosynthesis is yet to be defined, but if this reaction is made to run backwards, water will split and a hydroperoxide will be obtained. This in hypothetical terms can be looked at for photosynthetic studies.

3.1.1 Objectives

Metalloporphyrins have been used as " Artificial Enzymes " to mimic the natural enzymes in a variety of reactions[4-15]. In one such experiment carried out in our laboratories, it was brought to our attention that MTPPs were capable of chemiluminescence when oxidised by hydrogen peroxide under alkaline conditions.

The study of this phenomenon was thought to be important as it would shed further light into the mechanism of chemiluminescence, as well as the mechanism of enzyme activity of metalloporphyrins.

3.1.2 Method

Experiments were designed and undertaken to identify:

1) The criteria for the chemiluminescence of metalloporphyrins.

2) The chemiluminescent species and the wavelengths of the emitted light.

As in the other sections, the porphyrin studied was TPP and its derivatives. Due to the large number of possibilities which could arise from the combination of simultaneously varying the two major variables, namely the central metal ligand and the substituents on the phenyl rings, it was decided to change only one variable at a time and keep the other constant.

The chemiluminescent reactions were initiated as a result of oxidation of the MTPPs under alkaline conditions. In all cases the oxidising reagent was hydrogen peroxide and for reasons of comparison, the volume, the concentration and the pH of the reaction mixtures were kept constant for all the MTPPs under investigation.

3.2 EXPERIMENTAL

The reaction of hydrogen peroxide with MTPPs under alkaline conditions was studied under standard operating procedures.

3.2.1 Operating Procedures

3.2.1.1 Preparation of Reagents

The optimum MTPP and hydrogen peroxide concentrations were obtained by independently varying the hydrogen peroxide concentration over a 0.03 - 3% w/v range and the concentration of a model metallotetraphenyl porphyrin (Mn[TPP]Cl) over a $1x\ 10^{-3}$ to $1x\ 10^{-7}$ molar range.

MTPPs were dissolved in DMSO to a 1.00×10^{-4} molar concentration, hydrogen peroxide (30% w/v) was diluted in Millipore water in a ratio of 1:100 to give a concentration of 0.3% w/v and the reaction mixtures were made alkaline by the addition of 0.1 molar aqueous sodium hydroxide solution.

3.2.1.2 Initiation of Chemiluminescence

To 100 μl of the MTPP in a luminometer sample bottle was added 100 μl of aqueous sodium hydroxide and the mixture, after shaking, was quickly placed in the dark chamber of the luminometer. The injection of 100 μl of hydrogen peroxide initiated the chemiluminescence, which was recorded by means of the chart recorder and computerised

data collection system discussed earlier.

Reaction Mixture

Reagent	Concentration	Volume
MTPP/DMSO	1x 10^{-4} molar	100 μl
NaOH aq.	0.1 molar	100 μl
H_2O_2/ H_2O	0.3% w/v	100 μl
	Total Reaction mixture volume =	300 μl

3.2.2 The Criteria for Chemiluminescence of Metalloporphyrins

3.2.2.1 The Role of the Central Metal Ligand

Unsubstituted TPP and MTPPs were tested for chemiluminescence. Thus, TPP and M-TPPs : Fe[TPP]Cl, Mn[TPP]Cl, Zn[TPP], Co[TPP], Ru[TPP]CO, V[TPP]O, Ni[TPP], Pd[TPP] and Cu[TPP] were either synthesised (as described in the experimental section of Chapter II), or obtained from the sources outlined in Appendix I.

Reagents and solvents used were of high purity.

3.2.2.2 The Role of Substituents

The role of phenyl ring substituents on chemiluminescence was studied on Mn[T_SPP]Cl where S = substituent:

penta F, 2,6 di Cl, 4 -COOH, 4 -SO_3H,

4 -NO_2, 4 -NH_2, 4 -OMe, and Mesityl.

The above substituted MnTPPs were synthesised as described in Chapter II.

3.2.3 The Chemiluminescent Species and Spectra

In order to identify the light-emitting species, absorption, fluorescence and chemiluminescence spectral measurements of the MTPPs were undertaken. Absorption spectra were obtained on a Phillips recording spectrophotometer. Fluorescence measurements were made on a Perkin Elmer spectrophotofluorometer.

For both absorption and fluorescence spectroscopy, the respective cuvettes were ¾ filled (3 ml) with the MTPP solution and the base line was recorded after the addition of 1 ml of water. The process was then repeated with the addition of 1 ml of hydrogen peroxide solution (instead of water) to a fresh solution of MTPP and any changes in the spectra in general and in Soret bands in particular against time were recorded.

As no access to a spectrophoto - chemiluminometer was available, chemi-luminescence wavelengths were measured singly, through the use of photographic filters (from Lee Filters Ltd., Andover, England).

Although these filters had relatively broad bands, they were thin and flexible and fitted onto the luminometer cell window, thereby only allowing the light from the chosen region in the spectrum to reach the photomultiplier, without much loss in the intensity. Thus by choosing the appropriate red and blue filters the light emitted from the metalloporphyrins was effectively divided into a blue (400-550 nm) and a red (550-800 nm) region, respectively.

Later, more precise measurements were obtained on a purpose built spectrometer in which the light passed through a monochromator before being detected by the R105-LKB photomultiplier in conjunction with a multi-channel analyser. The signal-to-noise ratio was greatly improved with the continuous cooling of the photomultiplier tube by a cold water circulation system.

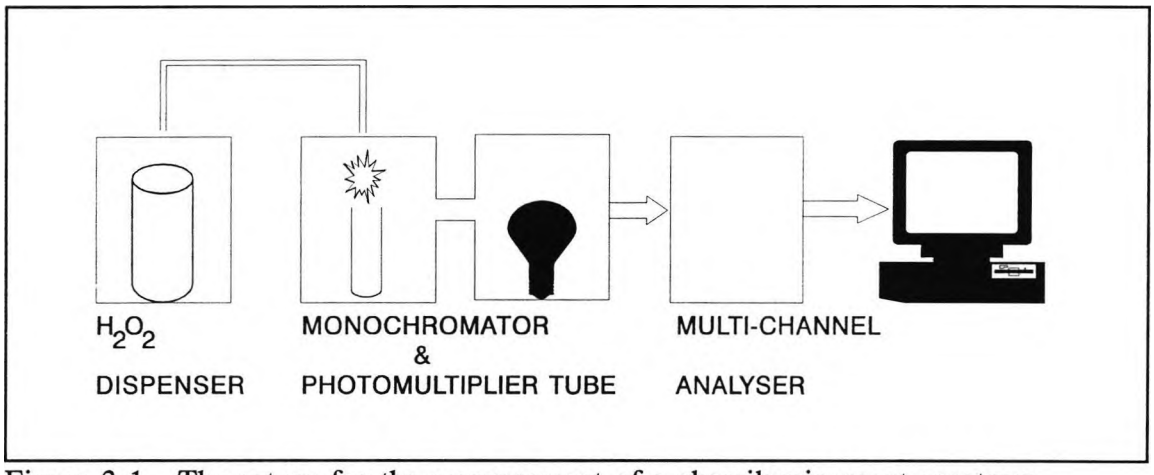


Figure 3.1 - The set up for the measurement of a chemiluminescent spectrum.

3.3 RESULTS

The general features of the reaction of hydrogen peroxide with MTPPs under alkaline conditions were the decomposition of both peroxide and MTPP, and the chemiluminescence of porphyrin.

3.3.1 Peroxide Decomposition

The MTPP catalysed decomposition of the hydrogen peroxide was easily observable from the evolution of oxygen gas bubbles. Although no systematic attempts were made to quantify the peroxide decomposition rates as a function of different catalysts, it was apparent that the rate of hydroperoxide decomposition varied from catalyst to catalyst. Neither unmetallated TPP, nor a mixture of metal and unmetallated TPP, showed an accelerating effect on the rate of hydroperoxide breakdown.

Because of the widely different solubilities and stabilities of the MTPPs, it was difficult to compare the effectiveness of the various TPP metal complexes which were tested. However, the order of catalytic activity was semi-quantitatively determined by means of monitoring the unchanged hydrogen peroxide concentration using Quantofix ether peroxide monitoring sticks, as obtained from Aldrich.

Using the above monitors, MTPP catalytic activity for peroxide decomposition was found to be in the following order:

For unsubstituted MTPP

$$\mathbf{Mn > Co, Fe > Ni > Cu , Zn >> H}$$

MnTPP being the best catalyst.

For the substituted MTPP

$$\mathbf{NH_2 > H > NO_2 > Cl}$$

-NH_2 substituted MTPP being the best catalyst.

3.3.2 Metalloporphyrin Decomposition

The oxidative breakdown of metalloporphyrins by hydrogen peroxide was demonstrated by the gradual disappearance of the characteristic metalloporphyrin absorption bands in general and the Soret band in particular (fig 3.2).

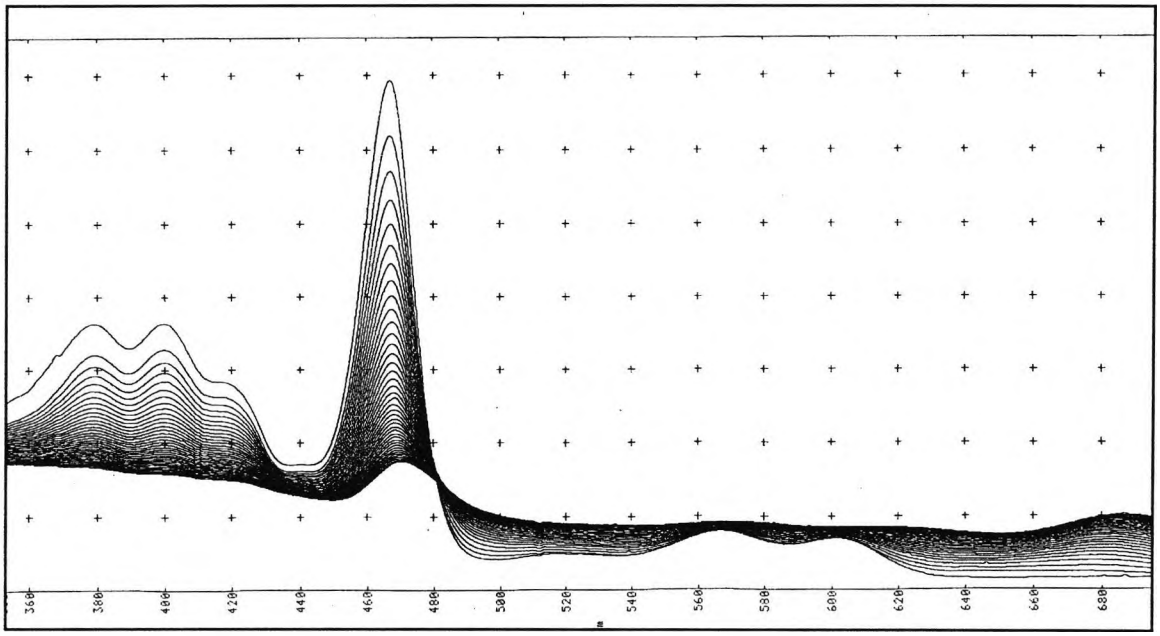


Figure 3.2 - Changes in absorption spectra of Mn(TPP)CL (solvent DMSO/H_2O_2) against time (5 min scan intervals).
X Axis = Wavelength (nm), Y Axis = Absorbance

By following the rate of the disappearance of the Soret band, a good indication of the rate of the decomposition of the metalloporphyrin in question was obtained. Similarly, by applying the same principle the relative stabilities of metalloporphyrins to peroxides were compared against each other.

The results indicated that the rates of MTPP oxidation were governed among other factors, by the type of central metal ligand, the substituents on the phenyl rings, and steric factors as well as on the concentration of the oxidant.

For unsubstituted MTPPs under fixed conditions, the stability varied with the central metal ligand and these were in the following order:

$$\mathbf{Mn < Fe < Co < Zn, Cu , Ni << H}$$

MnTPP being the least oxidation resistant metalloporphyrin and unmetallated TPP being totally resistant to oxidation, under the above experimental conditions.

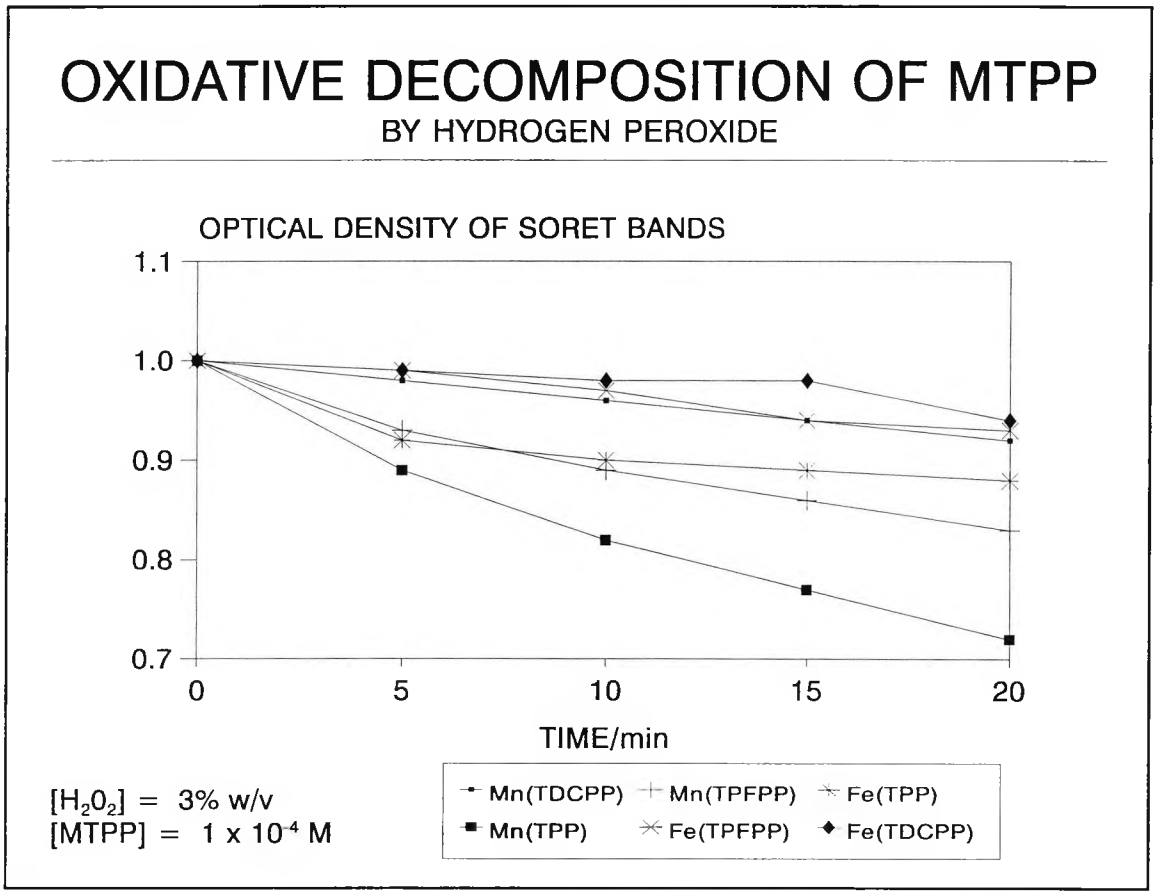


Figure 3.3

For a given metal and under fixed conditions, the rate of decomposition and therefore the stability against hydrogen peroxide of substituted MTPPs was dependent on charge-stabilising ability of the substituents as well as on their steric effects (fig 3.3).

Charge-stabilising ability of the functional groups on the phenyl rings decreased in the order of:

$$NH_2 > H > SO_3H > COOH > Cl$$

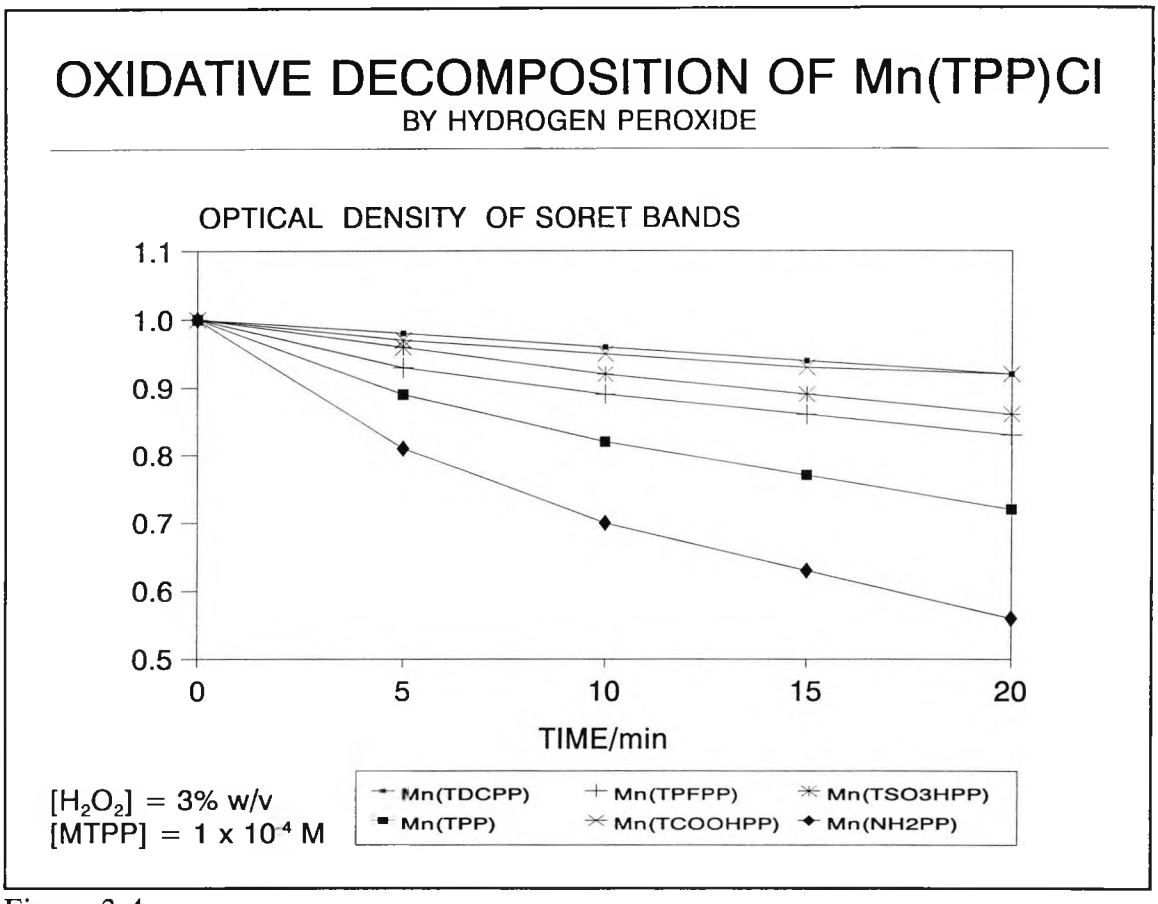


Figure 3.4

The above order was in complete agreement with the stability order of functionalised metalloporphyrins to oxidation. The -Cl substituted MTPP being the most stable and -NH_2 being the least stable metalloporphyrin to oxidation (fig. 3.4).

Among the MTPPs tested, those with sterically hindered functional groups such as 2,6- dichlorophenyl and mesityl were found to be the most oxidation resistant .

The rates of oxidative breakdown of MTPPs were also affected by the concentration of the peroxide and the pH of the solution. At low $[H_2O_2]$ the MTPPs were found to be decaying only slowly whereas at high $[H_2O_2]$ the rate of decomposition was much higher (fig. 3.5).

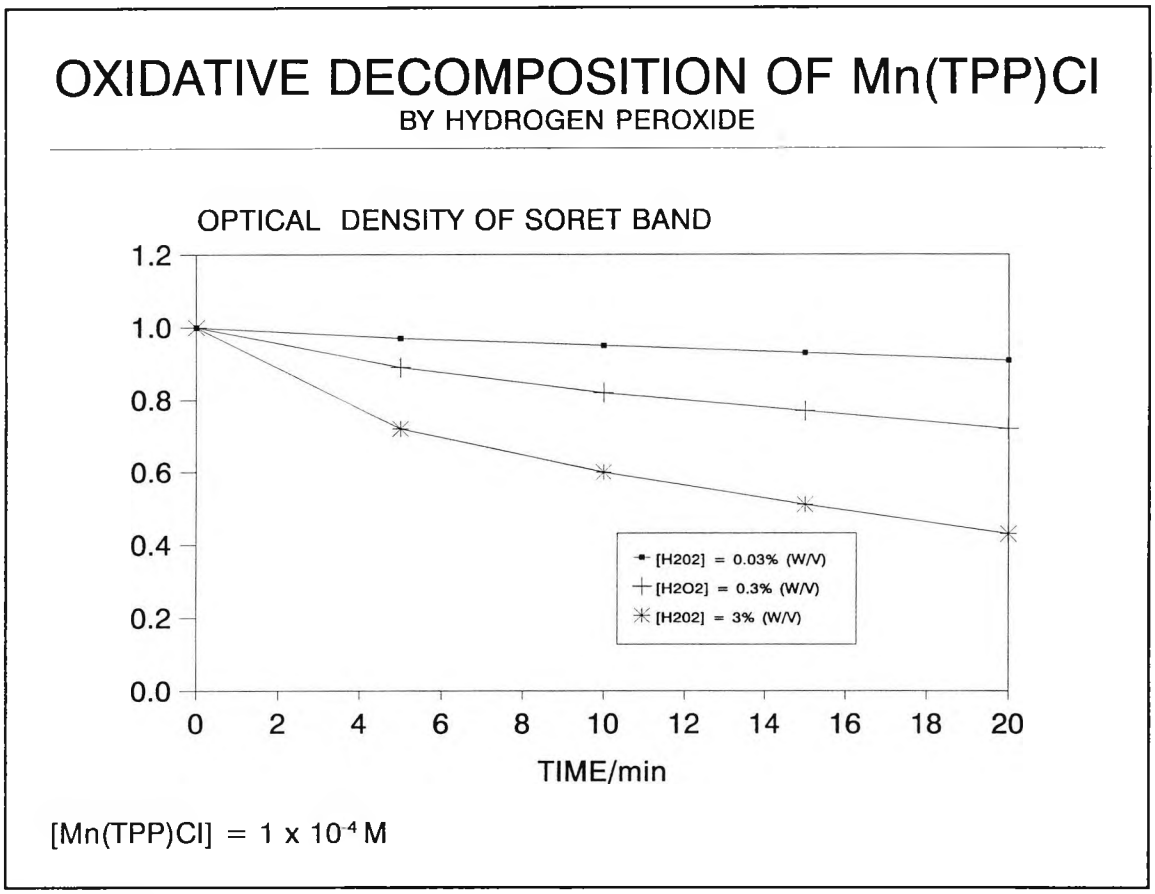


Figure 3.5

Experiments also indicated that the rate of decomposition increased with an increase in pH (ie. increase in $[^- OH]$) of the solution (fig. 3.6).

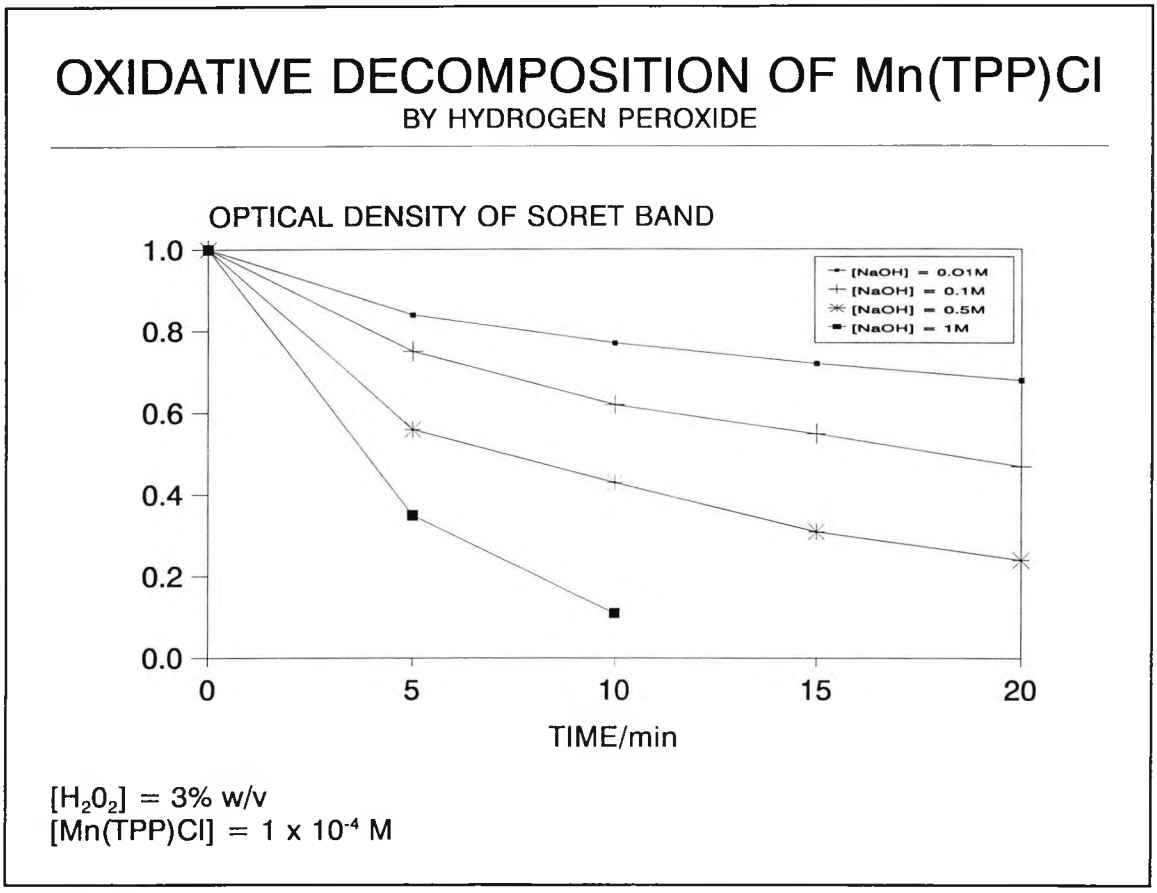

OXIDATIVE DECOMPOSITION OF Mn(TPP)Cl
BY HYDROGEN PEROXIDE
OPTICAL DENSITY OF SORET BAND
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
TIME/min
[NaOH] = 0.01M
[NaOH] = 0.1M
[NaOH] = 0.5M
[NaOH] = 1M
[H2O2] = 3% w/v
[Mn(TPP)Cl] = 1 x 10-4 M

Figure 3.6

3.3.3 Luminescence of MTPP

3.3.3.1 Chemiluminescence of MTPP

Fig. 3.7 shows a typical chemiluminescence initiation and decay run of MTPPs. Under the above mentioned experimental conditions, the initiation resulted in a rapid burst of light, followed later by a somewhat slower decay.

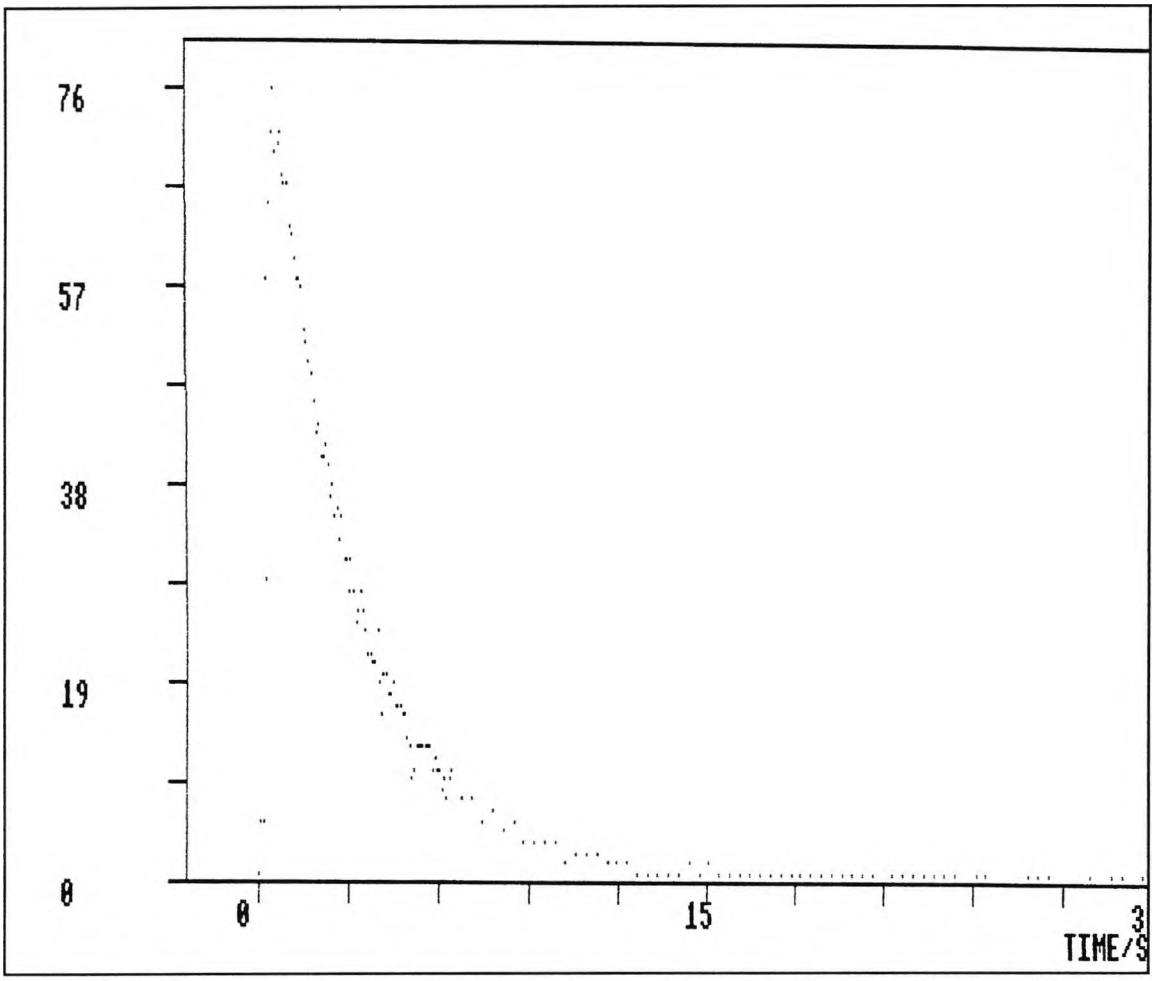


Figure 3.7- Time profile of chemiluminescence from Mn(T_{NH2}PP)Cl as oxidised by hydrogen peroxide/NaOH(aq.).
X Axis = Time/sec, Y axis = Light Intensity (photoncounts)/ Arbitrary units

Comparing the luminescence intensities of MTPPs proved to be difficult because of the vast differences in their solubilities, and optimum reaction conditions . Of the

unsubstituted TPPs studied:

Mn(TPP)Cl, Zn(TPP), Fe(TPP)Cl, Co(TPP), V(TPP)O, Ru(TPP)CO, Cu(TPP), Ni(TPP), Pd(TPP)Cl and TPP only three gave sufficient luminescence to be measured, namely Mn(TPP)Cl, Fe(TPP)Cl and Zn(TPP). Among the iron-porphyrins showing luminescent reactions were μ- peroxidase and HRP.

ZnTPP was shown to exhibit weak chemiluminescence (close to the detection limit) under the above conditions. When a mixture of μ- peroxidase / tris buffer and ZnTPP / DMSO was oxidised in alkaline conditions by hydrogen peroxide and its output compared with that of a mixture of μ- peroxidase / tris buffer and DMSO there was a three-fold difference in their chemiluminescence intensity indicating the involvement of ZnTPP in the chemiluminescence.

μ-P/Tris + DMSO + NaOH (aq) + H_2O_2 ⟶ 33 mv

μ-P/Tris + ZnTPP/DMSO + NaOH (aq) + H_2O_2 ⟶ 95 mv

Tris + ZnTPP/DMSO + NaOH (aq) + H_2O_2 ⟶ 8 mv

Tris + DMSO + NaOH (aq) + H_2O_2 ⟶ 0 mv

Most substituted Mn(III) and Fe(III) tetraphenyl porphyrins tested showed some degree of luminescence. The former MTPP was studied in more detail and comparisons with the Fe(III) counterpart was only made in one case (Table 3.1).

S = SUBSTITUENT	Relative Light Intensity	
	$Mn(T_SPP)Cl$	$Fe(T_SPP)Cl$
NH_2	2.0	-
H	1.0	0.8
Me OH	0.6	-
4-SO_3H	0.7	-
4-COOH	0.3	-
3,5 oMe, 4,6 Me	0.1	-
2,3,4,5,6 F	0.1	-
2,6 Cl	0.0	-

Table 3.1

The limiting components in all these chemiluminescent reactions were found to be MTPPs. These when added to the spent reaction mixtures at the end of chemiluminescent reactions initiated new chemiluminescence, whereas similar additions of the oxidant or hydroxide (with separate runs) did not initiate any such new reactions.

The intensity and rates of initiation and decay of light from the above MTPPs, among other factors were determined by the concentration of the MTPPs and the peroxide solutions.

Fig. 3.8 shows the variations in light intensity with changes in hydrogen peroxide concentrations. The optimum range of concentrations for maximum chemiluminescence under the above mentioned experimental conditions was found to be between 0.3 - 1.5 (w/v %).

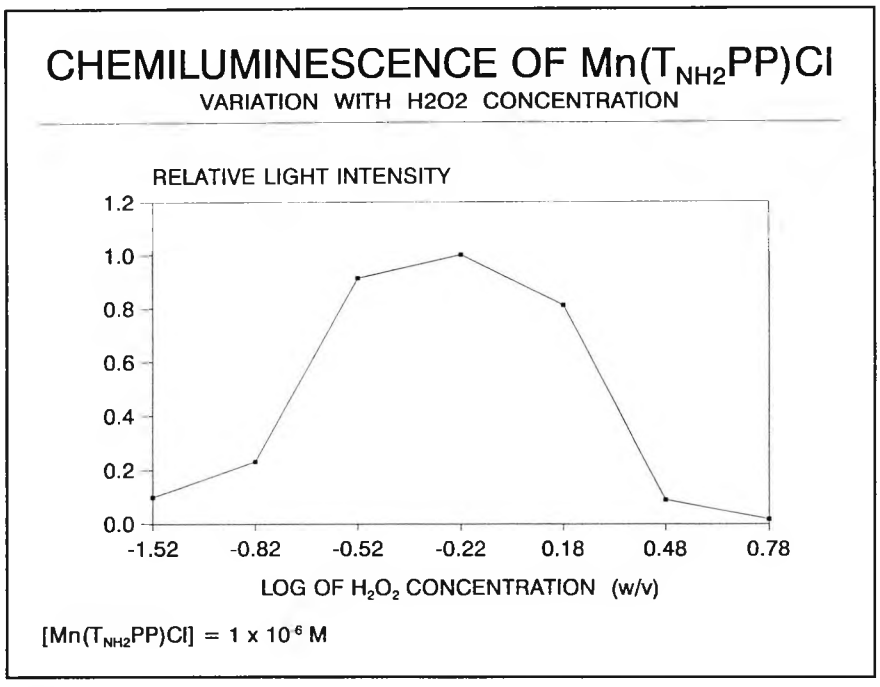


Figure 3.8

It was also observed that an increase in the concentration of peroxide solution resulted in a more rapid light initiation and decay. Figures 3.9 and 3.10 show the variations in light intensity and the rates of emission at different MnTPP concentrations.

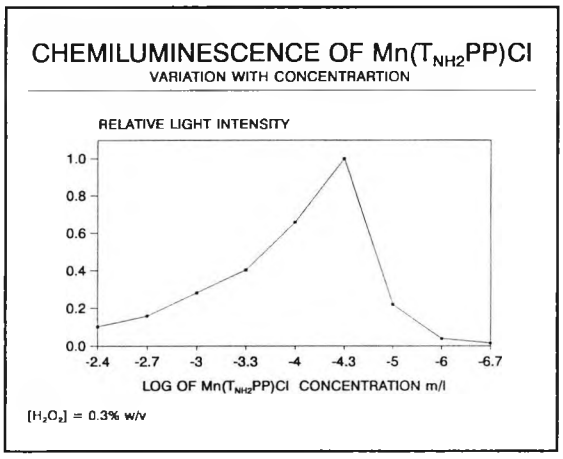


Figure 3.9

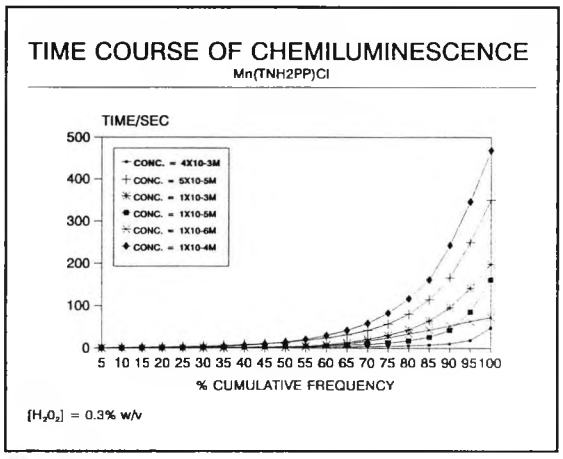


Figure 3.10

The highest light output was observed at concentrations between 1 x 10^{-4} to 1 x 10^{-5}M of MnTPP.

3.3.3.1.1 Chemiluminescence Spectra

Studies with red and blue filters indicated that the chemiluminescence from the MTPPs and peroxidase, was composed of red and blue regions, the relative intensities of which varied according to the conditions and the metalloporphyrin used.

Fig 3.11 and 3.12 show the transmission spectra of Primary Red (106) and Light Blue (118) filters as used in these experiments. The former allowed transmissions below 600 nm and the latter only transmitted light of wavelength between 400 - 550 nm.

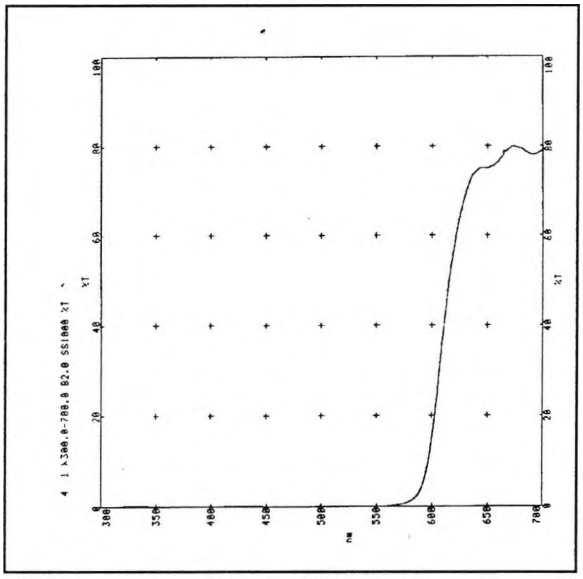


Figure 3.11- Transmission spectrum of Primary Red filter.

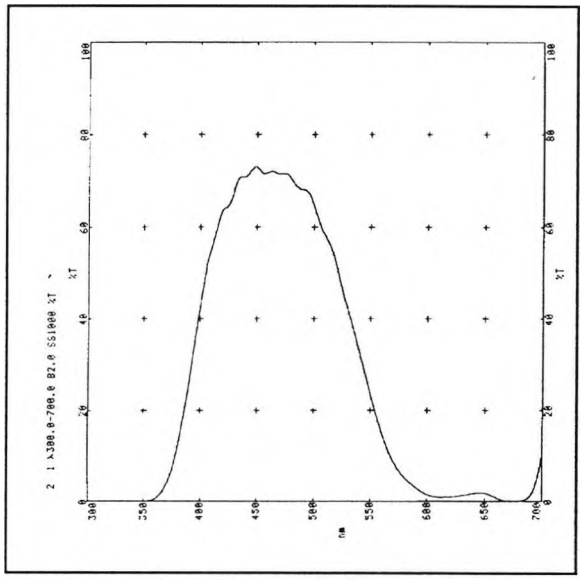


Figure 3.12 - Transmission spectrum of Light Blue filter.

Fig 3.13 shows the total blue and red light intensities of $Mn(T_{NH2}PP)Cl$ at various MTPP concentrations.

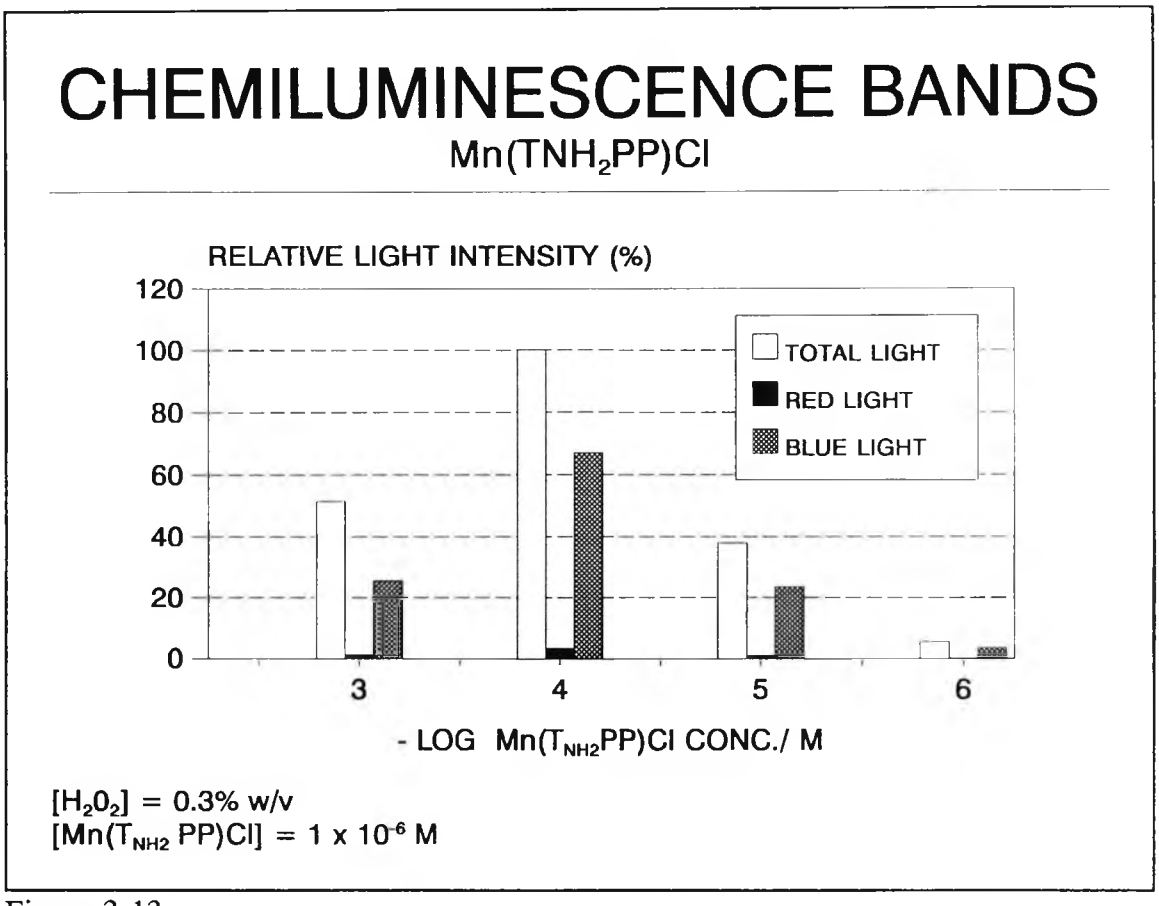


Figure 3.13

In order to obtain a better picture of the spectra, and to identify the emitting species the phenomenon was further investigated by means of a multichannel analyser linked to a monochromator. Due to the fast decaying and weak nature of the luminescent reaction it was necessary to use a fresh sample for each wavelength under investigation.

This spectroscopic method, despite giving valuable information, was very time-consuming, wasteful from the material point of view and of low sensitivity, thus limiting its use to those metalloporphyrins with strong emissions.

Fig 3.14 shows a typical chemiluminescence spectrum.

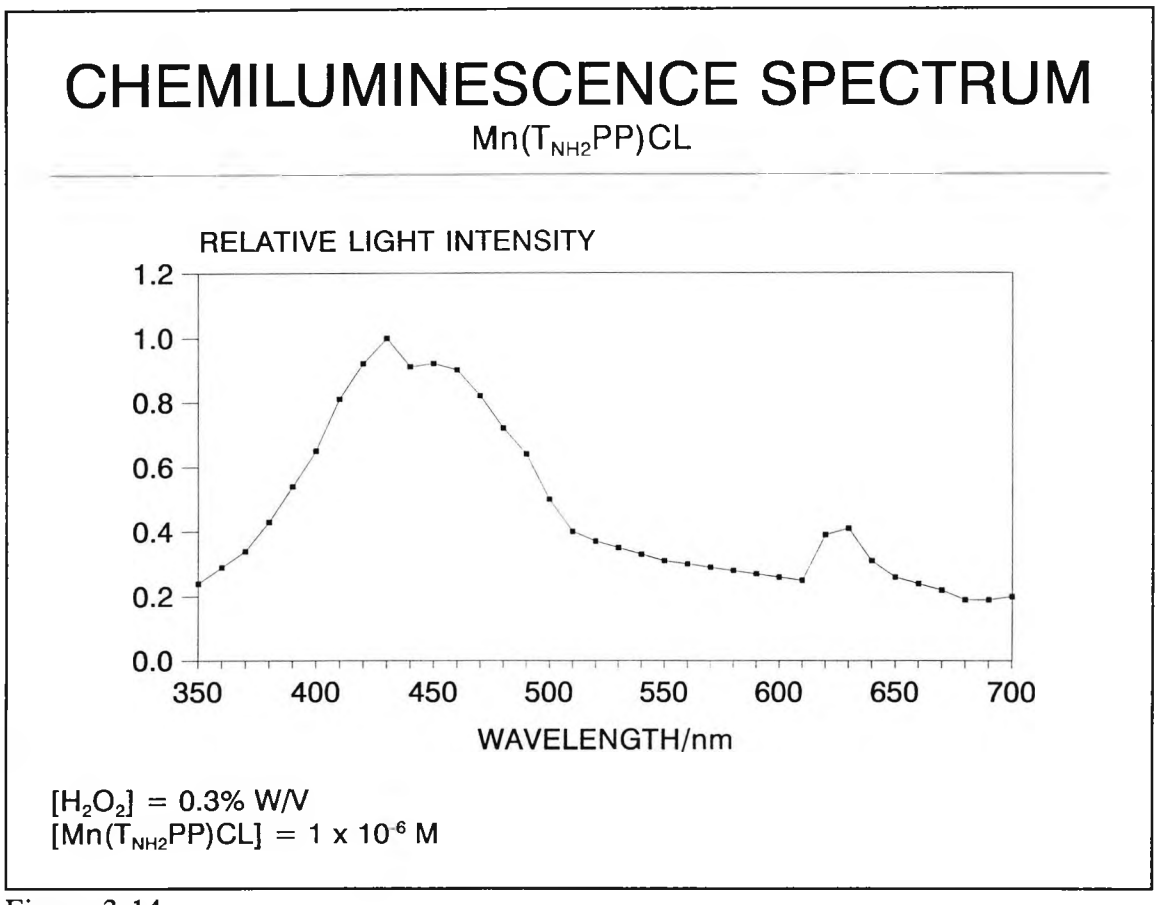


Figure 3.14

For reasons of comparison dimol emission from $^1O_2$ was generated by mixing alkaline aqueous hydrogen peroxide with sodium hypochlorite and its spectrum was recorded by means of the spectro-chemiluminometer described in fig 3.1.

Fig 3.15 shows characteristic red $^1O_2$ dimol emission bands from the above reaction.

The effects of the addition of DABCO, β- carotene and sodium azide on the luminescence of Mn(III) and Fe(III) TPPs were investigated. The results showed that the total and the red light output were only slightly increased by DABCO and to some extent decreased by β- carotene and sodium azide. This compared with the much more pronounced changes of the hydrogen peroxide/sodium hypochlorite system.

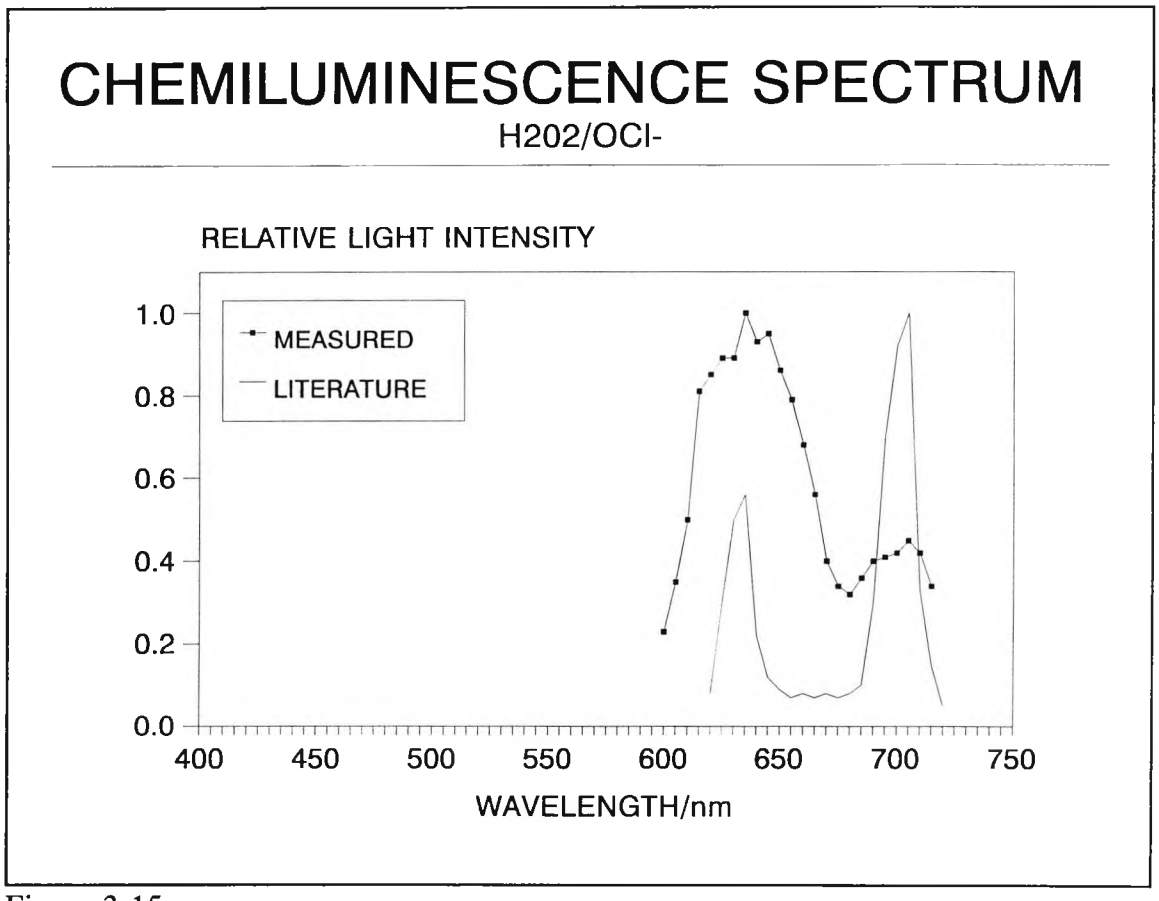

CHEMILUMINESCENCE SPECTRUM
H202/OCl-
RELATIVE LIGHT INTENSITY
MEASURED
LITERATURE
1.0
0.8
0.6
0.4
0.2
0.0
400
450
500
550
600
650
700
750
WAVELENGTH/nm

Figure 3.15

3.3.3.2 Fluorescence Spectra

Mn and Fe porphyrins being paramagnetic are non-fluorescent. To identify the source of chemiluminescence in these porphyrins, absorption and fluorescence emission spectra of the constituent porphyrin, tetra phenyl porphyrin was obtained (fig 3.16) .

The fluorescence spectrum of TPP was in good agreement with absorption and chemiluminescence spectra. This implied that the fluorescence and chemiluminescence emission had not originated from any non-porphyrin impurities.

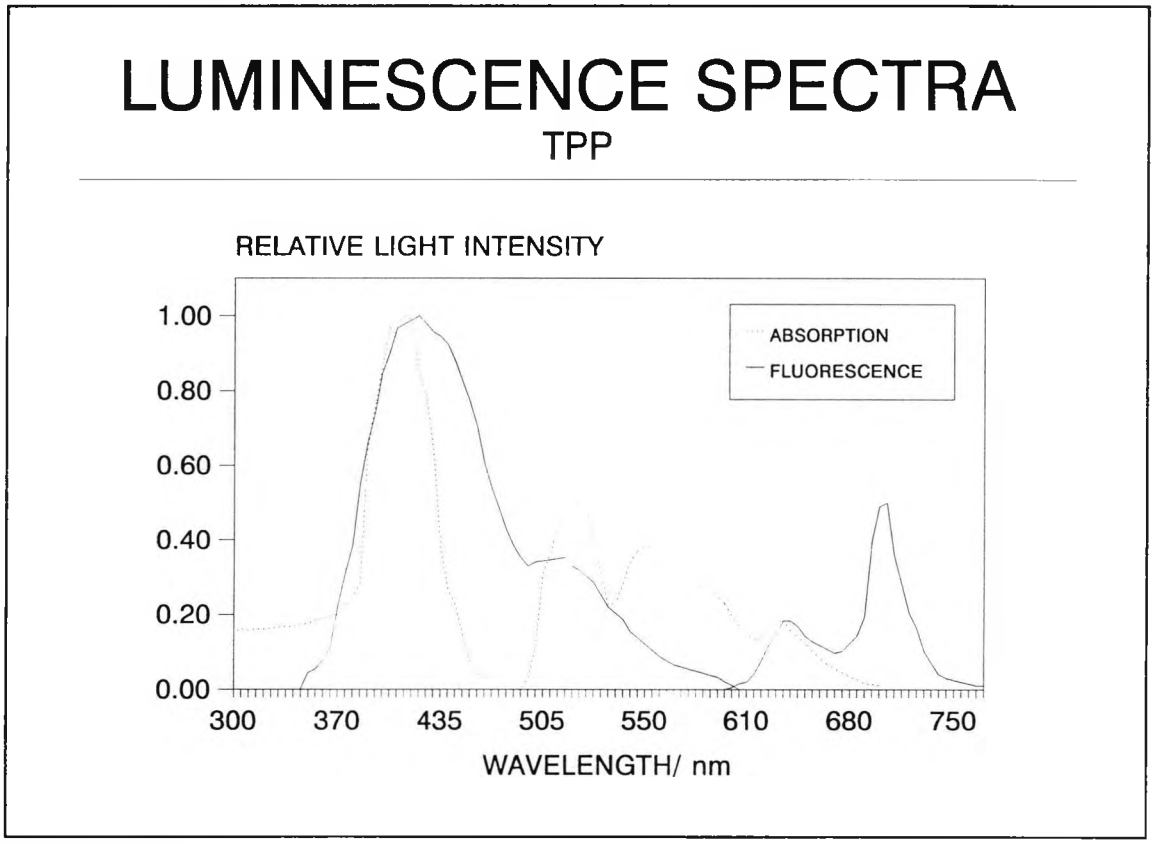


Figure 3.16

3.4 DISCUSSION

The general features of the reaction between MTPP and hydrogen peroxide under alkaline conditions were the decomposition of both the peroxide and the MTPP, and chemiluminescence of the MTPP.

Among the first questions to be asked is whether the decomposition of the peroxide is truly catalysed by MTPP or is it the result of a stoichiometric reaction between the two. There is certainly no doubt that a certain fraction of the total peroxide decomposed is used to break down the MTPP.

$$H_2O_2 \xrightarrow{\text{MTPP}} \text{Decomposition}$$

$$\text{MTPP} \xrightarrow{H_2O_2} \text{Decomposition}$$

The presence of a metallo - TPP complex was found to be a strict requirement for the decomposition.

$$H_2O_2 \xrightarrow{\text{M + TPP}} \text{NO Decomposition}$$

$$\text{TPP} \xrightarrow{H_2O_2} \text{NO Decomposition}$$

Linschitz[16] estimated that for the ZnTPP / THP (tetralin hydroperoxide) system, over approximately the first (peroxide) half-life, about thirty molecules of peroxide were decomposed for every molecule of ZnTPP which disappeared. This high ratio, which was independent of variations in the initial concentration ratio of peroxide and catalyst, could not be explained in terms of a stoichiometric reaction between peroxide and MTPP.

The degree of catalysis was found to vary from one metalloporphyrin to another. Although no systematic attempt was made to quantify it, the order of catalytic activity for peroxide decomposition was semi-quantitatively found to be in the following order:

For unsubstituted MTPP:

Mn > Co, Fe > Ni > Cu , Zn > > H

MnTPP being the best catalyst.

For the substituted MnTPP:

NH_2 > H > NO_2 > Cl

-NH_2 substituted MTPP being the best catalyst.

The overall best catalyst being Mn(T_{NH2} PP)Cl.

The metal requirement in the above reaction suggests that the first step for peroxide decomposition, MTTP oxidation and chemiluminescence is the complexing of the peroxide at the central metal ion.

Fe, Mn and Co complexes are capable of participating in a redox cycle, whereas Zn and Cu complexes are not (these complexes undergo ligand redox cycles). Assuming the structure of the complexes gives enough room for coordination of the central atom with the hydroperoxide, only in the former case (Fe, Mn and Co complexes) is the peroxide capable of complex formation with the MTPP. NiTPP complexes are capable of ligand oxidation/ reduction as well as valency change and therefore this resulted only in partial complex formation.

Evidence for ground-state complex formation being involved in the catalytic cycle has

been obtained by a spectroscopic study in a model system. Such complexes are typically characterised by a shift of the maximum of the porphyrin Soret absorption band relative to that of the non-complexed porphyrin [17].

The above mechanism would explain the experimental findings that the H_2O_2 and MTPP decompositions proceeded faster in higher pH regions, as a high pH would encourage the formation of peroxo complexes of HOO^- with the catalyst.

The fact that Cu and Zn complexes were also capable of catalysing the H_2O_2 decomposition, be it at much reduced rates, would indicate that the ligands may also participate in the formation of HOO^- complexes.

In non-polar solvents the formation of a charge-transfer complex is followed by the oxidation of the metalloporphyrin by electron transfer.

$$H_2O_2 + MTPP \longrightarrow MTPP^+\text{-}OH^- + OH^\cdot$$

Various reaction pathways may then follow, resulting in the overall breakdown of the MTPP and hydrogen peroxide and the chemiluminescence of MTPP. However, in all the pathways it is thought that electronic excitation of the metalloporphyrin results from the removal of $^\cdot OH$ from the charge-transfer complex to form H_2O, and the return of the electron into the π- system of the porphyrin.

$$2MTPP^+\text{-}OH^- \longrightarrow 2MTPP^* + H_2O + \tfrac{1}{2}O_2$$

$$MTPP^* \longrightarrow MTPP + h\nu$$

For a given MTPP under fixed conditions, the rate and degree of complex formation determines the rate and degree of the above mentioned reactions. Complex formation itself

is dependent on the charge-stabilising ability of the substituents as well as on their steric effects.

Electron donating and therefore charge-stabilising ability of the functional groups on the phenyl rings decreased in the order:

$$\mathbf{NH2 > H > SO_3H > COOH > Cl}$$

This was the reverse order of their stability to oxidation.

The -Cl substituted MTPP, being the most electron-withdrawing, had a de-stabilising effect on the intermediate complex, hindering its formation. The net result being the resistance of -Cl substituted MTPPs to oxidation.

The $-NH_2$ substituted MTPP on the other hand, being the most electron-donating, had a stabilising effect on the complex and encouraged its formation, which in later reactions resulted in the decomposition of the MTPP itself. $Mn(T_{NH2}PP)Cl$ was therefore the least stable MTPP.

Using the same argument, the order of catalytic activity can also be explained in terms of the ability of the MTTP to form stable complexes with peroxide. The best catalyst being the one which encouraged complex formation, which is the prerequisite for the catalytic decomposition of peroxide.

$Mn(T_{NH2}PP)Cl$ was found to be the best catalyst for the decomposition of H_2O_2, but was itself the target for attack by H_2O_2, which eventually resulted in its decomposition. Thus

$Mn(T_{NH2}PP)Cl$ cannot be classed as a "true catalyst" for the decomposition of H_2O_2.

The chemiluminescence of ZnTPP and Peroxidase has already been reported by several workers and it has been suggested that the peroxide decomposition rate parallels the luminescence intensity, both rates showing second order kinetics over most of the course of reaction. Both peroxide decomposition and luminescence intensity show a rapid initial phase followed by a slow and steady rate.

In our work, although ZnTPP was shown to chemiluminesce, the experimental conditions were not suitable for obtaining a strong emission (the original work by Linschitz used a sealed tube at temperatures between 135-150°C to initiate ZnTPP chemiluminescence). But in the presence of HRP a much stronger emission was obtained which could not be explained in terms of the combination of the chemiluminescence from HRP and ZnTPP. This suggests that perhaps the HRP catalyses the chemiluminescent reaction of the porphyrin.

Porphyrins have already been shown to be substrates for peroxidase. Morehouse[18] reported the one-electron oxidation of porphyrins to porphyrin π-cation radicals by peroxidase. Voltammetric analysis was then used to show that the π-cation radicals decay via a disproportionation process, yielding the corresponding porphyrin di-cation:

$$2H_2P \xrightarrow{HRP} 2H_2P^{\cdot+} \rightleftharpoons H_2P + H_2P^{2+}$$

The porphyrin di-cations being strong electrophiles, react with water to form

isoporphyrins and meso dihydroxyporphyrins.

$$H_2P^{2+} \xrightarrow{2[OH]^-} H_2P(OH)_2 \longrightarrow \text{RING-OPENED PRODUCTS}$$

These products are unstable and undergo further reactions by ring-opening, resulting in destruction of the porphyrin chromophore and bleaching of the absorption spectra. In the same manner it can be argued that in our work ZnTPP was also the substrate for HRP, yielding Zinc porphyrin π- and di- cation radicals prior to its disintegration.

$$2ZnTPP \xrightarrow{HRP} 2ZnTPP^{\cdot+} \rightleftharpoons ZnTPP + ZnTPP^{2+}$$

$$ZnTPP^{2+} \xrightarrow{2[OH]^-} ZnTPP(OH)_2 \longrightarrow \text{RING-OPENED PRODUCTS}$$

Although the mechanism of ZnTPP and peroxidase chemiluminescence is not entirely understood it is proposed that electronic excitation of these porphyrins (including peroxidase) results from the return of the electron into the π-system of the porphyrin.

The return of an electron to $ZnTPP^{\cdot+}$ (and possibly $ZnTPP^{2+}$) can produce enough energy to raise the porphyrin to a higher energy level. ZnTPP being a porphyrin which is capable of fluorescence, loses its excess energy and reaches ground level stability by emission. The emission spectrum of ZnTPP from the Linschitz system showed an S_1 emission band with a 650 nm maximum matching the fluorescence spectrum of ZnTPP under similar conditions.

$$ZnTPP^* \longrightarrow ZnTPP + h\nu$$

Mn and Fe TPPs are among the non-fluorescing porphyrins. Our experimental results

indicating their luminescence was therefore treated with suspicion.

Luminescence spectra from the above metalloporphyrins showed maxima at around 420, 650 and 700 nm. These maxima which were very similar for both the metallo-porphyrins, did not match with the absorption spectra of either of the metalloporphyrins, but were closely associated with those of the constituent porphyrin, ie. TPP (and $T_{NH2}PP$), indicating that chemiluminescence may have originated from the unmetallated TPP present in small amounts as impurities.

The unusual fact about the chemiluminescence spectra of these porphyrins was the presence of the blue emission band at around 420 nm. Although blue fluorescence emission, which is attributed to the Soret band fluorescence from the second excited singlet state; $S_2 \longrightarrow S_0$, has been reported[19] for several metalloporphyrins such as Y(III), Th(IV) and Lu(III) complexes of TPP and free-base TPP[20], and more recently for metal-free phthalocyanine and metallo- phthalocyanins[21], it was not expected to be observed under our experimental conditions. The condition under which S_2 emission was reported required intense laser excitation in EPA at room temperature.

The fact that S_2 emission of free-base porphyrins is too weak to be detected unless intense laser excitations are used indicates that somehow in our work the necessary excitation energy was generated to result in S_2 and S_1 emissions without the need for high energy excitations. ie. the reaction resulting in emission was somewhat catalysed.

In the work described in Chapter IV it has been reported that Mn and Fe TPPs can

mimic the catalytic activity of peroxidase. It is therefore reasonable to suggest that (similar to the case of HRP-catalysed chemiluminescence of ZnTPP) in MnTPP and FeTPP samples the observed chemiluminescence had originated from the oxidation of TPP, as catalysed by the above metalloporphyrins.

Red and blue components of chemiluminescence emissions of a variety of haemoproteins including HRP have been reported by Liu[22], who on the basis of experimental results suggested that the red band was due to dimol emission of singlet oxygen, and the blue band emission was proposed to be the luminescence from the excited carbonyl groups produced as a result of haem fragmentation.

Although in our investigations, the chemiluminescence spectra of peroxidase were not studied , our results based on the studies of metalloporphyrins contradict Liu's findings. Instead it is proposed that the blue band is the S_2 emission and the red band is the S_1 emission of the excited haem.

Mis-identification of the red band as a dimol was an easy mistake to make as this band in TPPs with maxima at around 650 and 700 nm is very similar in position to that of dimol of singlet oxygen with maxima of 634 and 703 nm.

The fact that in our work, under certain experimental conditions, the red band emission from TPP was also slightly enhanced by DABCO (enhancer of dimol emission) and slightly quenched by sodium azide and β- carotene (quenchers of dimol emission), as compared to almost total enhancement and quenching of true dimol emission from the

H_2O_2/OCl^- reaction, suggests that under certain conditions dimol emission may also be present in addition to the S_1 emission of porphyrin. But in all cases the latter emission was by far the most predominant band.

The blue band was identical to the characteristic Soret absorption band and the S_2 fluorescence emission band of TPP, indicating that the luminescent molecule is the excited unchanged porphyrin itself, which is excited in the course of its catalytic function rather than its breakdown.

3.5 CONCLUSION

The reactions of a series of substituted MTPPs with hydrogen peroxide in the absence of other substrates were studied.

The results showed that those MTPPs which are capable of redox cycles catalyse the decomposition of hydrogen peroxide into H_2O and O_2. The catalytic activities of MTPPs have been discussed in terms of their ability to form complexes with hydrogen peroxide.

In addition it was also shown that MTPPs themselves are substrates for oxidative decomposition by hydrogen peroxide. This decomposition was also catalysed by the MTPPs which are capable of redox cycles. The fact that the best catalysts were found to be the ones that were least resistant to oxidation gave an insight into the mechanism of the reaction which is further discussed in Chapter VIII.

An unusual side-reaction to the MTPP decomposition was chemiluminescence for those TPPs and MTPPs which were fluorescent. Analysis of chemiluminescence spectra on a purpose built spectrometer revealed the existence of S_1 and S_2 emission bands. The observation of S_2 emission is a fairy unusual phenomenon as it requires very intense excitation energies and has only been reported for a few cases.

The high energy species responsible for the S_1 and S_2 emissions are thought to be porphyrin π- cation radicals and di-cations. These species are intermediates to the disintegrating porphyrin and their formation under mild reaction conditions is attributed to the catalytic action of MTPPs.

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CHAPTER IV

METALLOPORPHYRINS AS MODELS FOR PEROXIDASE

4.1 INTRODUCTION

Peroxidases are classified as the group of enzymes which catalyse the oxidation of numerous organic and inorganic compounds by hydrogen peroxide or other peroxides of the ROOH type. Many peroxidases with diverse physiological functions are found in nature [1].

In the plant kingdom peroxidases exist in everything from fruits to cereals. Most worthy of note, however, is the root of horseradish plant in which the enzyme is highly concentrated. Peroxidases are also widely distributed in animal tissues. Among the most studied are lactoperoxidase, myeloperoxidase of the leucocytes which takes part in the phagocytic process, and thyroid peroxidase which is involved in the biosynthesis of the hormones thyroxine and tri-iodothyronine.

Several moulds, yeasts and fungi also contain peroxidase. For example, chloroperoxidase, which is obtained from the mould Caldariomyces fumago, is efficient in catalysing the oxidation of chloride ions and the peroxidase of the fungus, Arthromyces ramosus is reported to have enzymic characteristics superior to HRP[2].

4.1.1 The Native Enzyme

Horseradish peroxidase (HRP) is a glycohemoprotein isolated from the horseradish plant. Although its physiological role has not entirely been understood due to the large number of reactions it catalyses, it is one of the better studied peroxidases since it is found in high concentration in horseradish roots and can therefore be readily isolated. Commercial

preparations of high purity enzyme can be purchased.

The enzyme contains 1 mole of ferriprotoporphyrin IX as its heme prosthetic group and has a molecular weight of about 40,000. Approximately 18% of this weight is due to the covalently bound carbohydrate moiety. Isoenzyme C, which is the dominant isoenzyme of the horseradish root, consists of 308 amino-acid residues, 8 carbohydrate side-chains attached through asparagine residues, and a heme group[1].

4.1.2 Mechanism

Horseradish peroxidase (HRP) reacts with hydrogen peroxide to form the intermediates called Compound I and Compound II. Kinetic studies, together with chemical and physical measurements on the oxidation state of the native enzyme and Compounds I and II , indicated that the initial reaction of hydrogen peroxide with HRP produces Compound I, and both of the oxidising equivalents of hydrogen peroxide are transferred to the enzyme. Compound I can return to its native resting state by two successive one electron reduction steps. Compound II is produced by the first electron transfer of substrate (AH_2) to Compound I.

$$\mathbf{HRP + H_2O_2 \longrightarrow Compound\ I + H_2O}$$

$$\mathbf{Compound\ I + AH_2 \longrightarrow Compound\ II + AH^{\cdot}}$$

$$\mathbf{Compound\ II + AH_2 \longrightarrow HRP + AH^{\cdot}}$$

$$\mathbf{Compound\ II + AH^{\cdot} \longrightarrow HRP + A + H_2O}$$

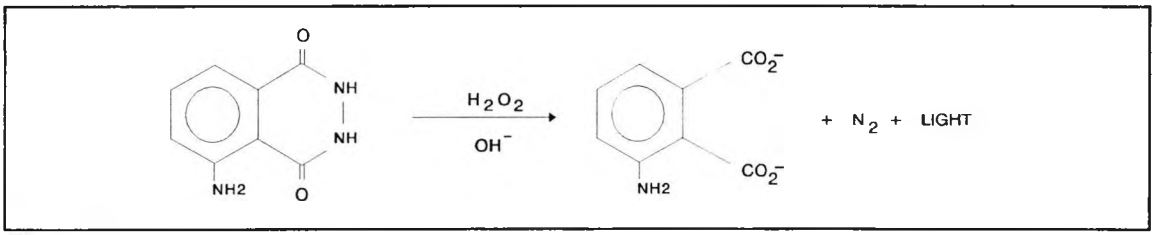
Compound I has been characterised as an oxo iron(IV) porphyrin radical species on the basis of Mossbauer[3], EPR[4], and NMR[5] studies. Compound II is best characterised as a neutral oxo iron (IV) complex[6] resulting from a one electron reduction of the porphyrin π-radical of Compound I. The axial coordination site in HRP is occupied by a histidine ligand[7].

4.1.3 HRP as a Catalyst of Luminol

One of the most popular uses of HRP is in Enzyme Immunoassay. In recent years, enzyme immunoassay with chemiluminescent detection has gained popularity. In particular, the reaction of enhanced chemiluminescence oxidation of luminol by hydrogen peroxide catalysed by HRP is widely applied[8]. With this in view the investigation of the reaction mechanism and ways of improving the sensitivity of the assays are of great interest.

4.1.4 Oxidation of Luminol

Although luminol (5-amino-phthalhydrazide) is the most studied chemiluminescent compound, the precise mechanism of its reaction under aqueous conditions is still controversial. The overall reaction is given by equation 4.1.



Equation 4.1

The starting point for luminol oxidation is an anion, since base is an essential catalyst. Luminol monoanion is present in aqueous alkaline solution, almost as the sole ionic luminol species (fig. 4.2 (a)). In such systems an oxidising agent such as hydrogen peroxide and a catalyst such as a transition metal ion (often as a complex) or an enzyme such as peroxidase are required for the chemiluminescent reaction[9].

Luminol dianion on the other hand, has been shown to exist in aprotic solutions (eg. DMSO or DMF) as the predominant anionic species (fig. 4.2 (b)). In such systems, in the presence of a strong base, molecular oxygen can attack the dianion to form a peroxide and additional catalyst is not required for chemiluminescence.

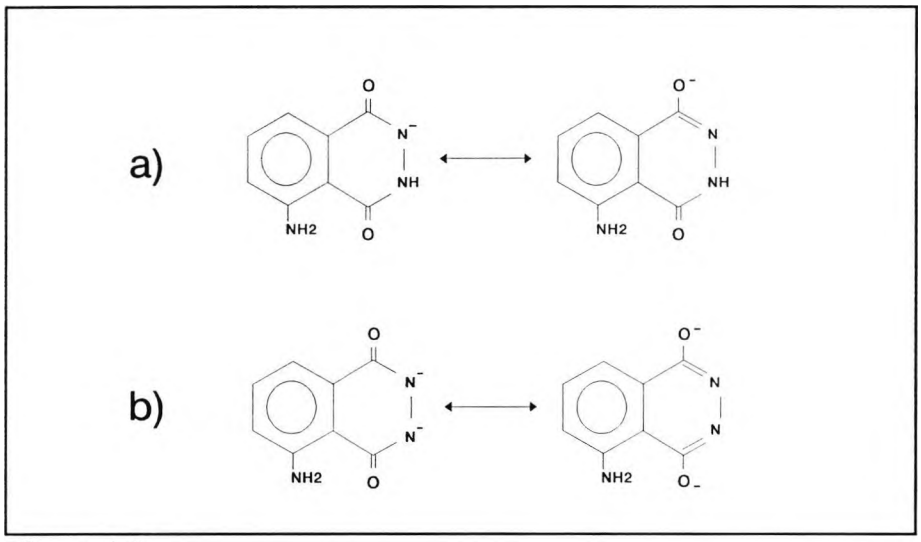


Figure 4.2

In mixed aqueous/DMSO solutions, both the mono- and dianion are observed. In aqueous solution, the first critical intermediate on the chemiluminescent pathway of luminol is a hydroperoxide. At high pH, the anion expels nitrogen and yields the monoprotonated dicarboxylate.

4.1.5 Peroxidase Model

The molecular mechanisms of biological oxygen activation are the focus of sustained attention. Due to the complexity of these studies, investigators have often resorted to studying synthetic models.

Synthetic enzyme models offer the advantages of increased precision of measurement compared with what is possible with enzymes. Also, they are comparatively easy to control and experimental conditions may be varied.

The facts that the active site of HRP contains a single iron- porphyrin prosthetic group, and synthetic metalloporphyrins are capable of catalysing a wide variety of oxidation reactions, make metalloporphyrins ideal models for peroxides.

4.2 OBJECTIVE

During a project involving porphyrins, it was brought to our attention that metalloporphyrins are capable of catalysing a variety of oxidation reactions. In this work metalloporphyrins are used as models for HRP with the view of further elucidating the mechanism of the peroxidase catalytic reactions. It is also intended that the study will find synthetic alternatives to HRP.

4.3 METHOD

To investigate the catalytic activity, it was decided to systematically probe appropriate structure - activity relationships. In this work **M**etallo **T**etra **P**henyl**P**orphyrins (MTPP) were chosen as the model synthetic catalyst for the oxidation of luminol by hydrogen peroxide.

MTPPs were chosen as their structures and properties are well studied, they are relatively easy to prepare and functionalise, and they possess distinct and measurable chemical and physical properties which can be varied by structural changes.

The bulky aryl substituents reduce the tendency of the natural heme to agglomerate in solution and help protect the meso carbons from oxidation. In addition, the phenyl groups can be substituted with a variety of substituents .

Thus it was decided to compare the catalytic activity of different MTPPs in the oxidation reaction of luminol by hydrogen peroxide. The reaction being chemiluminescent offered an easy and quantifiable means of comparing catalytic activities.

Condensation of pyrrole with benzaldehydes offered a relatively easy way of TPP preparation. By using substituted benzaldehydes, the above method was extended to the preparation of substituted TPPs (see Chapter II).

Metallo-TPPs were best prepared by metallating the corresponding TTPs through their reaction with the required metal salts (Chapter II). For a given porphyrin macromolecule

such as TPP, catalytic activities are influenced by factors such as the central metal ligand, substitutions, reaction buffer, buffer pH, ligands, concentrations of the reagents, etc..

This project concentrated on the study of catalytic activity as related to the porphyrin structure. Therefore it investigated primarily the effect of the central metal ligand and substitutions on the phenyl rings. As the combination of the simultaneous alterations of all the variables would have given rise to a large number of MTPPs, it was decided to only vary one component at a time.

From the first trials, the critical importance of the central metal ligand to the catalytic activity was evident. To establish the role of the metal ligand in the catalytic activity, unsubstituted TPP was metallated with a number of common transition group metals, and its catalytic activity was studied as a function of its central metal. The effect of substitution on the catalytic activity was then studied on the metallo-TPP showing the best catalytic activity.

The following substituted groups were chosen on the basis of their availability, ease of preparation, ease of functionalisation and conjugation to proteins.

$-NH_2$, -COOH, -OH, SO_3H

4.3.1 Criteria

To be a successful replacement for HRP, the MTPPs had to satisfy the following requirements:

1) They must have a high catalytic activity.

2) They must be soluble in aprotic media.

3) They must be stable at low concentrations.

4) They should possess easily functionalisable groups.

In order to compare the activities of different MTPPs the use of uniform reaction media and conditions were essential. This proved to be difficult as the solubilities of the TPPs varied widely and optimum reaction conditions were necessary to provide the true potential of each catalyst.

The kinetics of the light generation and the magnitude of the signal itself are both significantly influenced by the choice of buffer composition, pH and molarity.

A wide range of reaction buffers are employed in immuno- diagnostics of which borax, tris, and phosphate are the most common. In this project 0.1 molar tris buffer, pH 8.4 was chosen as the universal reaction medium[10]. This buffer gives rise to greater dose response linearity and low-end sensitivity under the pH studied[11]. The pH of the buffer was fixed at 8.4 as this was the optimum pH for luminescence of luminol and the solubility of porphyrins under investigation.

4.3.2 Optimisation of the Concentration of Reaction Components

Preliminary observations had indicated that the light output, among other factors, is critically dependent on the concentration of the reaction components. In order to compare the true catalytic potentials of these MTPPs it was decided to optimise the concentration of the reaction components, prior to the start of the systematic investigation.

Fe(TPP)Cl was chosen as the model MTPP for the optimisation exercise. The concentration of each of the other two components, namely luminol and H_2O_2, were varied separately to establish the optimum concentrations of the components.

The concentrations of the MTPPs were fixed at 1 x 10^{-6} moles / litre. At this concentration all the MTPPs under investigation were found to be soluble in tris buffer. In line with enzyme immunoassay techniques which use HRP, MTPPs were used as the limiting component as compared to luminol and H_2O_2, which were provided in excess.

To establish the optimum luminol concentration, a stock solution of luminol (10 mg/ml) in NaOH was diluted in tris buffer to obtain concentrations covering 0.01 to 5.64 x 10^{-6} molar range. 3%, 0.3% and 0.03% dilutions of H_2O_2 were also prepared in tris buffer.

The optimum concentration of luminol was first established by fixing the Fe(TPP)Cl and H_2O_2 concentrations at 1x10^{-6} moles/litre and 0.3% w/v, respectively, and varying the luminol concentration over the 0.01 to 5.64 x 10^{-6} molar range.

The results, which are presented graphically in fig. 4.1 indicated that under the above conditions the highest-signal-to-noise ratio was obtained at a luminol concentration of 5.64 x 10^{-4} (0.1 mg/ml). This was chosen as the optimum luminol concentration.

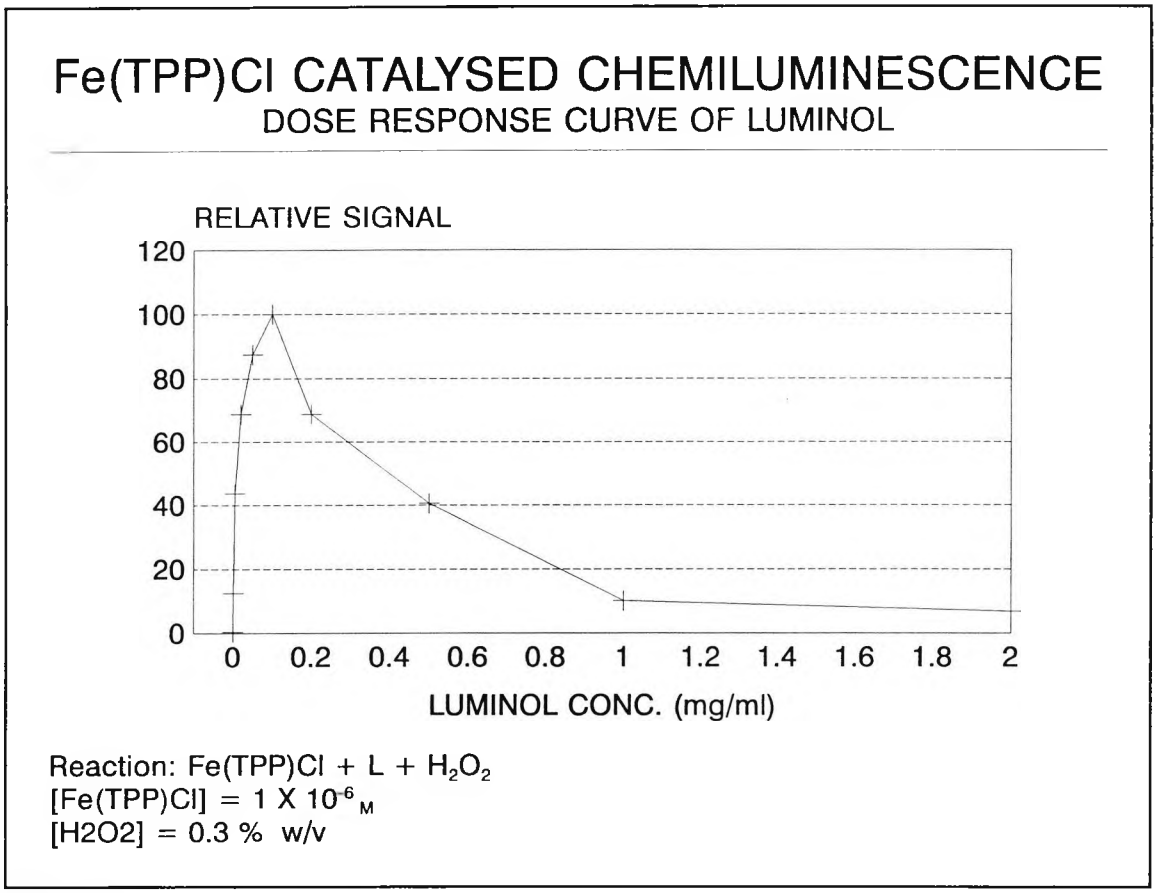


Figure 4.1

H_2O_2 concentration was varied over the 0.03 - 3% w/v concentration to optimise the reaction against H_2O_2 concentration. All three concentrations of the peroxide resulted in similar overall light yields. However, 0.3% H_2O_2 was chosen on the grounds of its slower reaction kinetics which made the measurements more reproducible.

The reaction concentrations were therefore chosen to be as follows:

MTPP $1x10^{-6}$ moles/litre

Luminol 5.64 x 10^{-4} moles / litre

H_2O_2 0.3% w/v

Under the above reaction conditions, the background luminescence from the system (ie luminol plus H_2O_2, prior to the addition of the MTPP) was found to be negligible.

4.4 ASSAY PROTOCOL

4.4.1 Sample Preparation Protocol

A stock solution of luminol was prepared by dissolving 1mg of luminol in 1 ml of 0.1 molar NaOH to give a concentration of 5.64 x 10^{-3} moles/litre. This solution was diluted ten fold to 5 .64 x 10^{-4} in 0.1 molar tris buffer pH 8.4 before use.

A hydrogen peroxide solution was prepared freshly on the day of the experiment by diluting a 30% w/v of hydrogen peroxide solution 100-fold in tris buffer, to give a working strength solution of 0.3% w/v.

0.01 moles of the respective MTPP was dissolved in 20 ml of DMSO to give a stock solution of 5 x 10^{-4} moles/litre. This was further diluted in tris buffer to give a working strength MTPP concentration of 1 x 10^{-6} moles/litre.

The amount of DMSO in the reaction mixture was lower than 0.2 % and it did not influence the measurement.

4.4.2 Protocol for The Initiation of Chemiluminescence

To 100 μl of the MTPP in a luminometer sample bottle was added 100 μl of working strength luminol solution and the mixture, after gentle shaking, was quickly placed in the dark chamber of the luminometer. The injection of 100 μl of hydrogen peroxide initiated the chemiluminescence, which was recorded by means of the chart recorder and computerised data collection system discussed earlier (Chapter I).

Reaction Mixture

Reagent	**Concentration**	**Volume**
TPP/ Tris	1x 10^{-6} molar	100 μl
Luminol/ Tris	5.64x 10^{-4} molar	100 μl
H_2O_2/ Tris	0.3% w/v	100 μl
	Total reaction mixture volume =	300 μl

4.5 PROCEDURE

In order to determine the criteria for the catalytic activity of tetraphenylporphyrins it was necessary to study the effect of the major variables.

The two major variables determining the activity of TPPs are:

a) The central metal ligand.

b) The substituent groups on the phenyl rings.

As the permutations arising from the simultaneous change of all the variables would have resulted in an unacceptably large number of samples, it was decided to change only one variable at a time.

4.5.1 The Role of the Central Metal Ligand

In order to determine the role of the central metal in the catalytic activity of MTPPs, transition metal complexes of TPP were screened for their ability to catalyse the oxidation of luminol .

Thus, TPP and MTPPs : Fe[TPP]Cl, Mn[TPP]Cl, Zn[TPP], Co[TPP], Ru[TPP]CO, V[TPP]O, Ni[TPP], Pd[TPP] and Cu[TPP] were either synthesised (as described in the Experimental section - Chapter II), or obtained from the sources outlined in the Appendix I .

Reagents and solvents used were of high purity.

4.5.2 Results

Fig. 4.2 shows a typical chemiluminescence initiation and decay run of luminol as oxidised by H_2O_2 and catalysed by an MTPP. Under the above experimental conditions, the initiation resulted in a rapid burst of light followed by a slower decay.

For each metal complex the light output, following the initiation of the reaction, was measured for 180 seconds. The results were expressed as the average integral intensity (in arbitrary units) per second.

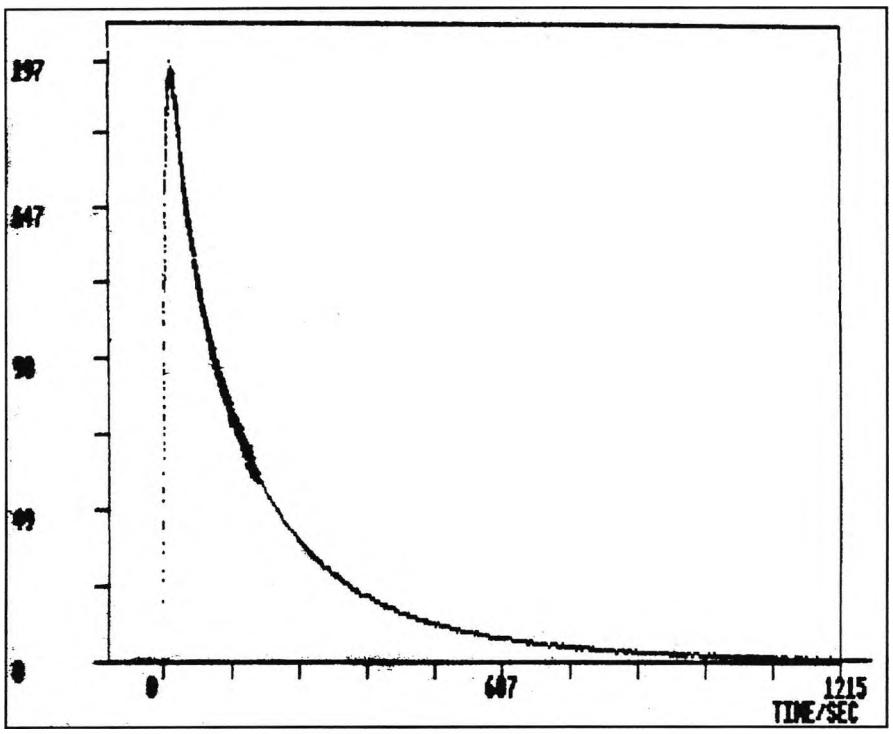


Figure 4.2 - Time profile of chemiluminescence from luminol, as oxidised by H_2O_2 and catalysed by $Mn(T_{NH2}PP)Cl$.
X axis units = Time/sec
Y axis units = Light intensity (photon count)/ Arbitrary units

A summary of the results is given in Table 4.1.

MTPP	AVERAGE INTENSITY (Normalised)
TPP	1
V(TPP)O	1
Mn(TPP)Cl	2913
Fe(TPP)Cl	88
Co(TPP)	12
Ni(TPP)	3
Cu(TPP)	1
Zn(TPP)	1
Ru(TPP)CO	40
Pd(TPP)	2
SOLVENT	0

Table 4.1 - Average chemiluminescent light intensity from luminol + H_2O_2, as catalysed by a variety of MTPPs.

TPP and its complexes with metals: Zn, V, Cu, Ni and Pd showed almost no catalytic activity. The Co complex showed a small degree of catalytic activity. Only Mn, Fe and Ru complexes showed significant amounts of catalytic activity.

4.5.3 The Role of Phenyl Ring Substituents on Catalytic Activity

The manganese complex of TPP was found to be by far the best catalyst for the oxidation of luminol by hydrogen peroxide. The effect of phenyl ring substituents on chemiluminescence was therefore studied on Mn[T_SPP]Cl, where S = substituents :

H, penta F, 2,6 di-Cl, 4 -COOH, 4 -SO_3H,

4 -NO_2, 4 -NH_2, 3,5-OMe-4-OH, and mesityl.

The above substituted MnTPPs were synthesised as described in Chapter II .

4.5.4 Results

In Tables 4.2 and 4.3 the catalytic results of Mn(TPP)Cl are given for the oxidation of luminol by hydrogen peroxide. In order to be able to compare the effect of substituents on the catalytic activity, the results are normalised against that of the unsubstituted catalyst.

UNSYMMETRICAL (MnTPP)Cl SUBSTITUENTS

RING SUBSTITUENTS	AVERAGE INTENSITY (Normalised)
mono p-OH,Tri penta F	0.2
m- NO_2	0.5
m- COOH	0.8
m- NH_2	1.4

Table 4.2 - Average chemiluminescent light intensity from luminol + H_2O_2, as catalysed by unsymmetrically substituted Mn(TPP)Cl (Results are normalised against Mn(TPP)Cl).

SYMMETRICAL (MnTPP)Cl SUBSTITUENTS

RING SUBSTITUENTS	AVERAGE INTENSITY (Normalised)
p- NO_2	0
2,4 di Cl	0
penta- F	0.3
p- COOH	0.7
H	1
Mesityl	1.5
p-SO_3H	1.9
3,5-OMe-4-OH	3
p-NH_2	9.1

Table 4.3 - Average chemiluminescent light intensity of from luminol + H_2O_2, as catalysed by symmetrically substituted Mn(TPP)Cl (Results are normalised against Mn(TPP)Cl).

4.5.5 Dose Response Curve of $Mn(T_{NH2}PP)Cl$

The relationship between catalytic activity and concentration of $Mn(T_{NH2}PP)Cl$ was determined by diluting the working strength metalloporphyrin repeatedly and measuring its catalytic activity. This represented the dose response curve of $Mn(T_{NH2}PP)Cl$ and is given in fig 4.3.

The low end detection limit of $Mn(T_{NH2}PP)Cl$ was found to be $1X\ 10^{-12}$ moles/ml.

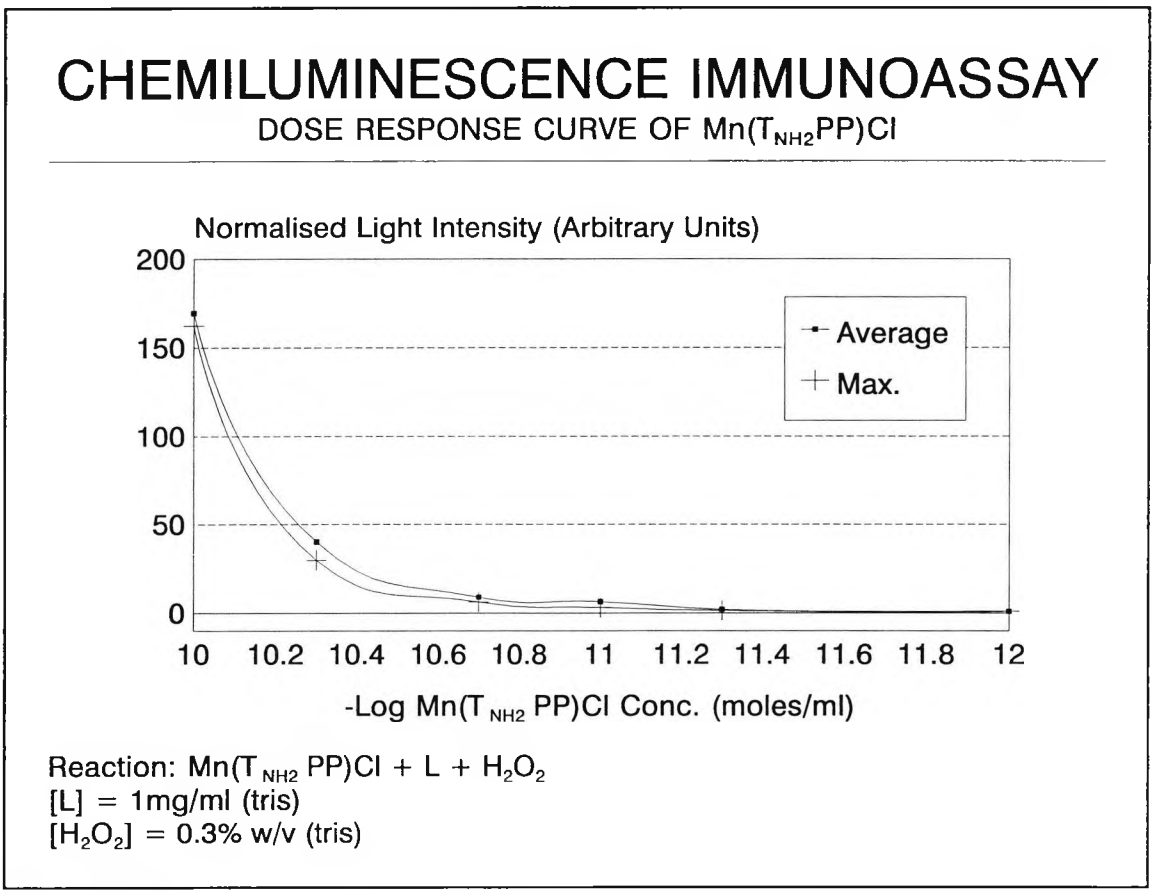


Figure 4.3

4.6 DISCUSSION

A variety of MTPPs were shown to catalyse the oxidation of luminol by hydrogen peroxide. The critical role of the central metal ligand in catalysis was depicted by the fact that TPP and its complexes with the metals Zn, V, Cu, Ni and Pd showed no catalytic activity whereas Co, Ru complexes and Mn , Fe complexes showed small or significant amounts of catalytic activity (see Table 4.1).

The above observations combined with the fact that metalloporphyrins themselves are substrates for oxidation by hydrogen peroxide whereas metal-free TPP is resistant to such oxidation (see Chapter III) indicate that MTPP oxidation is the primary route to the catalytic process.

The porphyrin ring oxidation (and reduction) is governed by the activation or deactivation of the π-electron conjugated system through the electrostatic action of the central ligands. Depending on the central ligand, metalloporphyrins are capable of two kinds of oxidation processes, either valency change of the central metal atom or ligand oxidation or both. Table 4.4 outlines the oxidation states[12] and electronic configurations of each metal complex used in this study.

Metal	Atomic Number	Z_{ox}	Z_s	Electronic Config.	Metal Oxidation
V	23	4	4	d^1	N/A
Mn	25	2,3,4	3	d^4	III → IV
Fe	26	1,2,3,4	3	d^5	III → IV
Co	27	1,2,3	2	d^7	II → III
Ni	28	2,3	2	d^8	II → III
Cu	29	2	2	d^9	N/A
Zn	30	2	2	d^{10}	N/A
Ru	44	2,3	2	d^6	II → III
Pd	46	2,4	2	d^8	II → IV

Table 4.4 - Oxidation states and electronic configurations of some common MTPPs.
Z_{ox} = Possible metal oxidation numbers of metalloporphyrins. Underlined figures indicate "stable oxidation states" (stable within metalloporphyrins under aerobic conditions).
Z_s = The oxidation states of the MTPPs in this study.

It is clear that TPP and its complexes with V , Cu, Zn are unable to take part in any redox reaction as they do not possess central ligands with a variable valency. For these MTPPs oxidations are ligand-centred and the oxidation potential is proportional to the electronegativity of the central ion[13-15]. A plots of oxidation potentials vs. electronegativity for this group of MTPPs yields a linear relationship[16] (fig. 4.4).

In the case of MTPPs with variable oxidation numbers, i.e. Mn, Fe, Co, Ni, Ru and Pd, the first oxidation is metal-centred and can subsequently be followed by first and second ligand oxidations at higher potentials.

The oxidation potentials of these complexes, when plotted against the appropriate ionisation potentials (third in the case of M(II)TPP and fourth in the case of M(III)TPP), reveal a direct relationship[17] (fig 4.5). The ligand oxidation potentials for these groups of MTPPs were shown to be approximately independent of the metal ions.

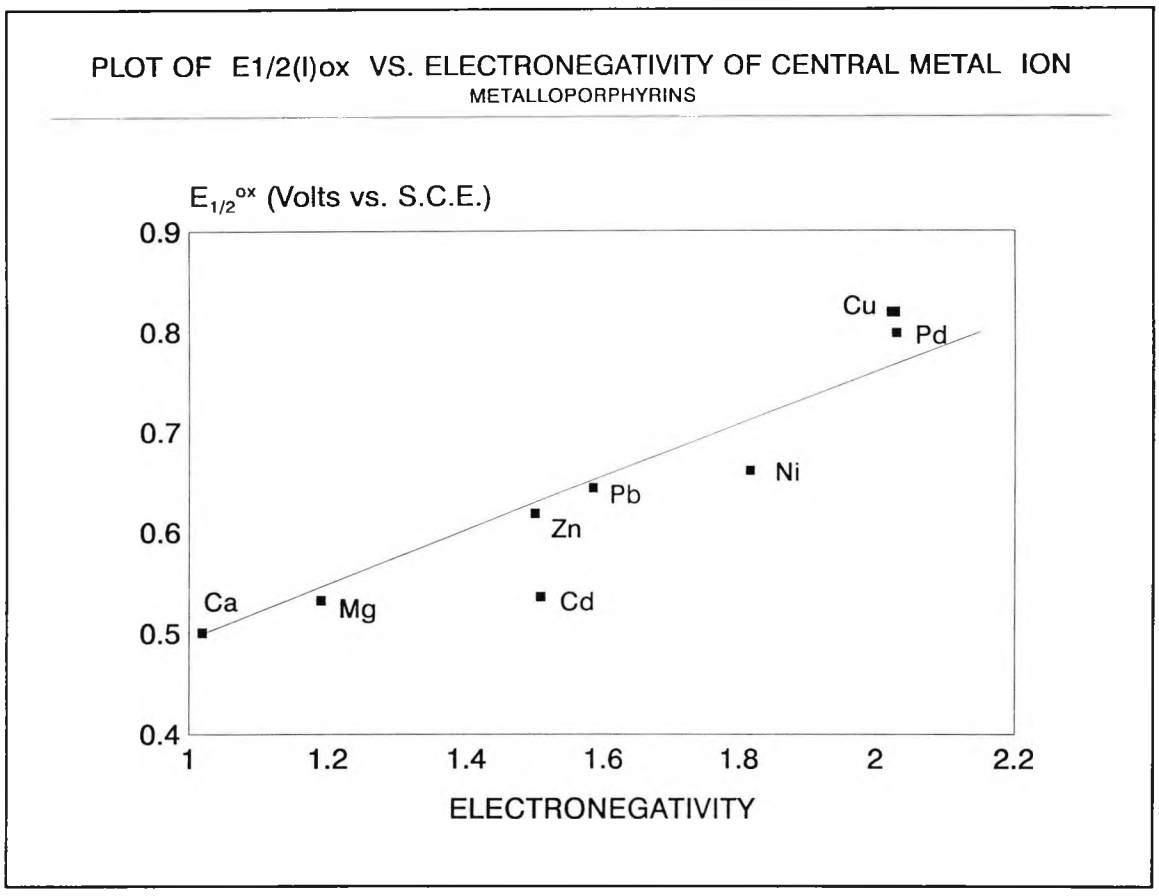


Figure 4.4

Comparison of the catalytic activities of various MTPPs indicates that catalysis can only occur with metalloporphyrins that undergo metal-centred oxidation. Evidently, even among the MTPPs which show catalytic activity the identity of the metal ion matters greatly for the catalysis. Compare for example , Mn(TPP)Cl, the light output of which is nearly 250 times larger than Co(TPP).

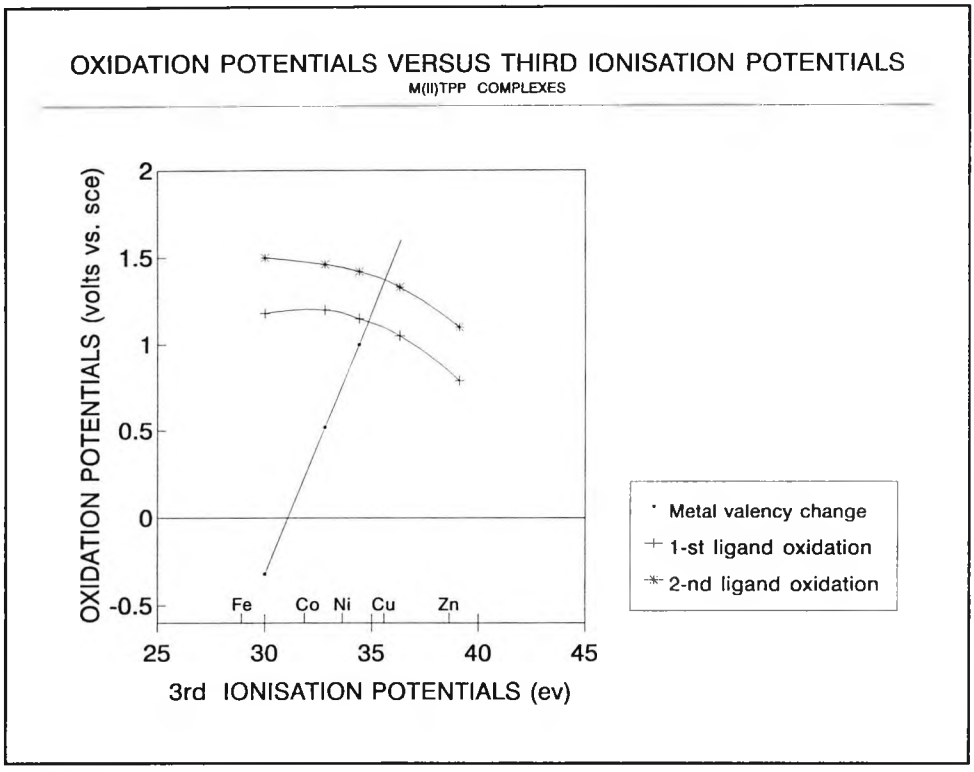


Figure 4.5

Catalytic activity was therefore found to be dependent on the central metal ion in the complex and its magnitude was correlated with the oxidation potential of the catalyst. In general the higher the oxidation potential of the complex the lower its catalytic activity.

← Increase in catalytic activity

Mn > Fe > Co > Ni > Cu , Zn

Increase in oxidation potential →

The catalytic activity of MTPPs was also found to be affected by substitution on the phenyl ring. Compare for example, Mn(T_{NH2} PP)Cl, the light output of which is nearly 9

times larger than Mn(TPP)Cl. This is because the oxidation potentials of these complexes can also be modified by substituents on the periphery of the molecule. Here electron-donating or electron-withdrawing substituents are able to affect the basicity of the porphyrin nitrogen atoms[18-20], which in turn, affect redox potentials and catalytic activities.

This is possible as the π-orbitals of the phenyl rings are able to overlap significantly with the porphyrin π-orbitals, even though the phenyl rings are prevented from being coplanar with the porphyrin ring by unfavourable steric interactions between ortho-H of the phenyl groups and pyrrole-H of the porphyrin ring.

The magnitude of interaction of the central metal with the porphyrin ring can be measured using the Hammett free energy relationship[21] :

$$\Delta E_{1/2} = 4\sigma\rho$$

The equation describes shifts in half-wave potentials as a result of changing substituents (X). In each case, σ, a substitution constant, measures the electron-donating or electron-withdrawing characteristics of X, and ρ, the reaction constant, measures the sensitivity of the reaction to electron-donating or electron withdrawing characteristics of substituents.

The symmetrically substituted TPPs have substituents on each of the four phenyl rings. 4σ is used as a total substituent constant. This represents the sum of inductive and resonance effects of all substituents, and is dependent on the kind and position of the four substituents.

In the above equation ρ is given in volts and its value depends on the kind of electroactive group, the composition of the supporting electrolyte, and temperature[22]. Fig 4.6 shows one such relationship for the reaction:

$$[Fe^{III}\ TPP] \rightarrow [Fe^{IV}\ TPP]^{2+} + e$$

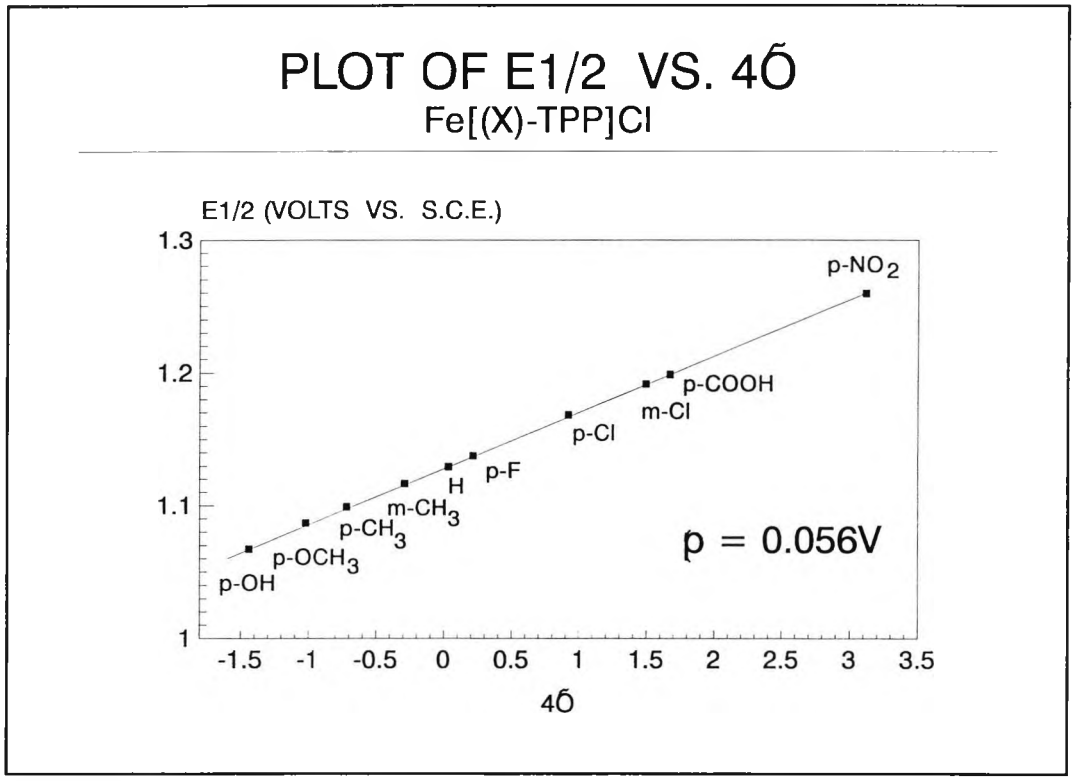


Figure 4.6

It is evident that porphyrin rings that bear a negative ring charge should easily undergo chemical oxidation and vice versa.

Electron-donating groups on the porphyrin ring increase the electron transfer rate constant for all reactions. This indicates that the high electron density on the porphyrin ring or central metal is important for a fast reaction.

The order of catalytic activity of substituted metalloporphyrins was found to be as follows:

← Increase in catalytic activity

$$NH_2 > H > COOH > NO_2$$

← Increase in electron-donating power

For the mono-substituted porphyrins 1 electron is used in the calculation of the Hammett relationship. In our work it was found that the behaviour of mono-substituted MnTPPs was similar to that of the tetra-substituted, only less pronounced . To this effect the extra effort and expense in synthesising and investigating the unsymmetrical metalloporphyrins could not have been justified and our efforts were concentrated on the more accessible tetra- substituted porphyrins.

Many studies have indicated that high valent oxo-metalloporphyrin species are key oxidising intermediates in the catalytic cycles of several heme-containing oxygenase enzymes[23]. The existence of such species has been confirmed by spectroscopic evidence, where a shift in absorbance is observed.

In order to use any catalyst for oxidation, it is important that the catalyst be oxidatively robust. Unfortunately this was not the case with simple metalloporphyrins. Despite the fact that the manganese and other MTPPs catalysed the oxidation of luminol by hydrogen peroxide, they cannot be classified as true catalysts as they are themselves

substrates for hydrogen peroxide[24] .

This was especially true for the best catalysts which were found to be those with the lowest oxidation potentials and therefore the easiest to be oxidised. Here in parallel to the catalytic reaction, extensive and rapid metalloporphyrin destruction took place which lessened catalytic activity.

The destruction of MTPPs in the absence of oxidisable substrates was particularly rapid and is discussed separately in Chapter III.

Porphyrin metal complexes undergo oxidation at the meso position, producing highly reactive species which are susceptible to reaction with the surrounding medium and to further oxidation, leading finally to irreversible ring opening and break up of the metalloporphyrin.

It has been shown[25] that the stability of metalloporphyrins to oxidation can be greatly enhanced by protecting the enzyme from oxidative attack by substituents which cause steric hindrance and/or increase the oxidation potential through their electron-withdrawing properties.

The latter approach was already shown not to be a viable option for achieving enzyme robustness, as it was achieved at the expense of catalytic activity. The former approach was tested by substituting the Mn(TPP)Cl with mesityl and pentafluoro groups. These complexes have been shown to be oxidatively robust [26-28] .

Unfortunately here also catalytic robustness was achieved at the expense of catalytic activity which dropped dramatically. This indicated that the formation of activated metalloporphyrin, which is a prerequisite to the luminol oxidation, was subject to steric inhibition.

4.7 CONCLUSION

The catalytic activities of over 20 metalloporphyrins for the oxidation of luminol by hydrogen peroxide were studied. Catalytic activity was found to be dependent on the central metal ion in the complex and correlated with the oxidation potentials of the catalyst.

The catalytic activity was further modified by substitution on the periphery of the metalloporphyrin. In general it was found that electron-donating substituents on the phenyl ring increased the rate of the reaction.

The catalysts themselves, were found to be substrates for oxidation by hydrogen peroxide, and suffered from irreversible oxidation of the porphyrin ring, which destroyed the catalyst. As in the case of HRP, part of the catalytic activity was used for the decomposition of hydrogen peroxide , which was chemically catalysed by the catalase-like activity of the complex.

Among the metalloporphyrins investigated, $Mn(T_{NH2}PP)Cl$ was found to possess the best catalytic activity and offered potential as a replacement for HRP. The low end detection limit of $Mn(T_{NH2}PP)Cl$ was found to be 1×10^{-12} moles/ml. It is thought that this could be improved greatly by the following :

1) Optimising the concentration of the luminol and H_2O_2 for a lower enzyme concentration.

2) The use of enhancers as described in Chapter VII.

3) The use of a more modern and sensitive luminometer, as the one used in this work was

manufactured in 1978 in the early days of chemiluminescence and did not have the sensitivity of modern luminometers.

4) The use of pure compounds in general and metalloporphyrins in particular. The purity of $Mn(T_{NH2}PP)Cl$ used in this work was thought to be about 95%. This would compare unfavourably with the purities of > 99% sought for immunodiagnostics applications.

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CHAPTER V

ENHANCED CHEMILUMINESCENCE

5.1 INTRODUCTION

HRP is widely used as a marker in chemiluminescence immunoassay. Such an application is limited by a low light intensity and a high level of background reactions. These limitations were largely overcome by finding out the effects of increasing light intensity on simultaneous peroxidase-catalysed oxidation of luminol and some easily oxidised substrates[1,2].

The use of so called enhanced chemiluminescent reaction allows one to achieve the requirements close to those of an ideal detection system, ie. a high light intensity with a long steady-state region and a low level of background, providing a low detection limit and a high specificity of enzyme determination.

In this work we examine the receptivity of metalloporphyrin-catalysed chemiluminescent reactions to enhancers, in the hope of identifying enhancers capable of improving the sensitivity of the system as well as shedding further light on the mechanism of the reaction.

5.2 CHOICE OF ENHANCERS

A number of compounds with potential for enhancement of MTPP-catalysed chemiluminescent oxidation of luminol were screened. The choice of compounds to be screened was based on the findings of colleagues working in this field as well as published and patented information.

Studies by Thorpe et al[3] had identified a number of phenols including p-iodophenol and p-phenylphenol as powerful enhancers capable of increasing the light emission from the HRP-catalysed oxidation of luminol by up to 1000-fold.

Other enhancers for HRP-catalysed luminol oxidation include[4]: p-hydroxycinnamic acid, 1,6-dibromonaphth-2-ol, 1-bromonaphth-2- ol, and 6-hydroxybenzothiazole. From work carried out in our department by Dewar[5], imidazoles and DABCO were identified as useful enhancers for μ-peroxidase-catalysed reactions.

5.3 METHOD OF SCREENING

Mn(T_{NH2}PP)Cl was chosen as the model metalloporphyrin. Sample preparation and assay protocol were in accordance with the procedure given in Chapter IV.

The compounds were obtained from a variety of commercial sources, and were screened across a range of concentrations in tris buffer (0.1 M, pH 8.4). The range of enhancer concentration was determined by its solubility.

Luminescent reactions are very sensitive to pH changes. To avoid the masking of the results by pH variations, the pH values of all enhancer solutions were corrected to 8.4 by the addition of appropriate amounts of 5M NaOH or HCl, before the addition of the enhancer to the reaction mixture.

Assay protocol was as follows:

Reaction Mixture

Reagent	Concentration	Volume
$Mn(T_{NH2}PP)Cl$/ Tris	1.00x 10^{-6} molar	100 μl
Luminol/ Tris	5.64x 10^{-4} molar	100 μl
Enhancer/Tris	0 - 5 molar	100 μl
H_2O_2/ Tris	0.3% w/v	100 μl
	Total Reaction mixture volume =	400 μl

The time interval between the addition of enhancer and initiation of the reaction by the addition of peroxide was kept constant to avoid any variations which may have arisen from side reactions.

5.4 RESULTS AND DISCUSSION

For ease of comparison the results have been presented graphically in pages 146-151. For each enhancer the % increase in light signal and background noise as well as the signal-to-noise ratio were plotted against enhancer concentration.

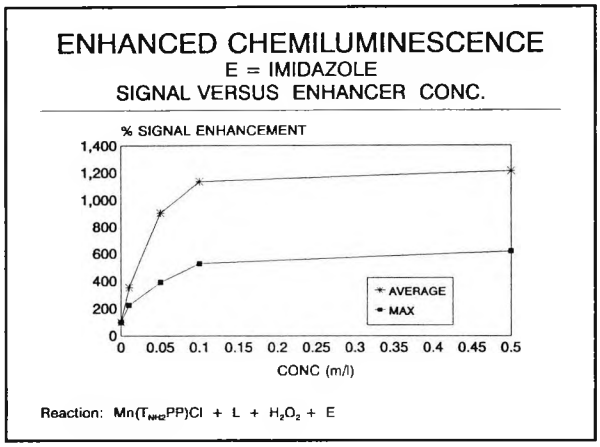
To distinguish between the changes in overall light output as compared against changes in reaction kinetics, signals are expressed both in units of Average light Intensity (Average) and Max Light Intensity (Max).

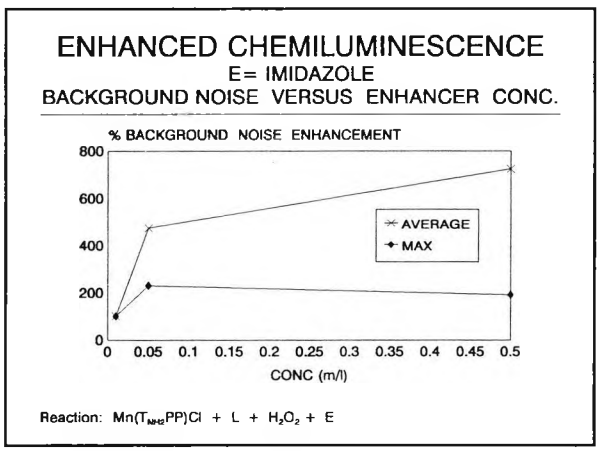
The results are summarised in Table 5.1.

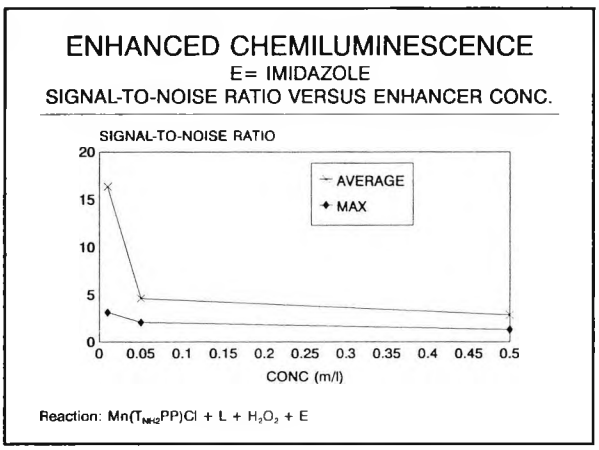
COMPOUND	PROPERTIES	COMMENTS	CONCLUSION
IMIDAZOLE (Im)	known enhancer	Addition of Im resulted in up to 14 folds increase in average signal intensity, while the max. intensity increased by 10 folds. At conc. 0.05 M the average intensity reached a plateau and further addition of Im only resulted in increases in max intensities. The background noise also increased with the addition of Im. Signal to noise ratio decreased for the average intensity and increased for the max.	A reasonable degree of signal enhancement was observed but its use as an enhancer will be limited as signal to noise ratio was actually reduced by the addition of Im. Highest signal enhancement : 14 fold
1 METHYL IMIDAZOLE (1 Me Im)	known enhancer	Addition of 1 Me Im increased the average signal intensity by 20 folds and the max. signal intensity by 15 folds. At concentrations > than 1 M the enhancement started to decrease. Addition of 1 Me Im resulted in an increase in average background noise while the max. background noise was little affected. The addition of 1 Me Im increased the signal to noise ratio.	At 1 molar concentration the signal to noise ratio was enhanced by 3 fold for the Average signal and by up to 5 fold for Max signal. 1 MeIM offers potentials as a reasonable enhancer. Highest signal enhancement:20 fold Highest signal/noise enhancement: 5 fold
2 METHYL IMIDAZOLE (2 Me Im)	known enhancer	Addition of 2 Me Im resulted in a rapid increase in average intensity. At its optimum concentration of 1 M the average signal intensity increased by 30 folds. Further increases in concentration resulted in increase in the rate of the reaction as seen by the 200 folds increase in the max signal intensity at concentration of 5 M. Addition of 2 Me Im resulted in an increase in average background noise while the max background noise was little affected. Addition of 2 Me Im resulted in a decrease in signal to noise ratio for the average signal while the signal to noise for the max signal increased.	Despite the fact that up to 30 fold increase in signal was achieved up on the addition of 2MeIm , its use as an enhancer will be limited as the signal to noise ratio was actually decreased. Highest signal enhancement: 30 fold
DABCO	anti-oxidant	At low concentrations (< 0.2 M) addition of DABCO resulted in modest increases in average and max. signal intensities. At higher concentrations the enhancements were reversed.	Weak enhancement activity was observed. Highest signal enhancement: 16 fold Highest signal/noise enhancement: 2 fold
p-IODO PHENOL	known enhancer	Addition of p-Iodo phenol, even at very low concentrations resulted in signal quenching. At 0.01 M concentration an 80% reduction in signal was observed.	Not suitable as an enhancer.
UFOXANE	sulphonated lignin	Addition of ufoxane even at very low concentrations resulted in a quenching of signals. At concentration of 0.01 mg/ml a 95% quenching effect was observed.	Not suitable as an enhancer.
ß CYCLO DEXTRIN		At very low concentrations addition of ß Cyclo-dextrin resulted in small increases in average and max signal intensities. At concentration of 1 x 10^{-5} mg/ml, a 3 fold increase in signal was observed. At higher concentrations no change in signal levels were observed.	Very weak enhancement activity was observed. Highest signal enhancement: 3 fold

Table 5.1 - The effect of some enhancers on the $Mn(T_{NH2}PP)Cl$-catalysed chemiluminescent reaction of luminol and H_2O_2.

IMIDAZOLE

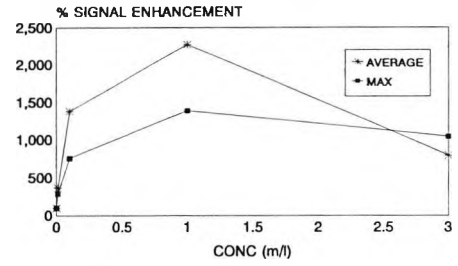

ENHANCED CHEMILUMINESCENCE
E = IMIDAZOLE
SIGNAL VERSUS ENHANCER CONC.
% SIGNAL ENHANCEMENT
1,400
1,200
1,000
800
600
400
200
0
0 0.05 0.1 0.15 0.2 0.25 0.3 0.35 0.4 0.45 0.5
CONC (m/l)
AVERAGE
MAX
Reaction: Mn(T$_{NH2}$PP)Cl + L + H_2O_2 + E


ENHANCED CHEMILUMINESCENCE
E= IMIDAZOLE
BACKGROUND NOISE VERSUS ENHANCER CONC.
% BACKGROUND NOISE ENHANCEMENT
800
600
400
200
0
0 0.05 0.1 0.15 0.2 0.25 0.3 0.35 0.4 0.45 0.5
CONC (m/l)
AVERAGE
MAX
Reaction: Mn(T$_{NH2}$PP)Cl + L + H_2O_2 + E


ENHANCED CHEMILUMINESCENCE
E= IMIDAZOLE
SIGNAL-TO-NOISE RATIO VERSUS ENHANCER CONC.
SIGNAL-TO-NOISE RATIO
20
15
10
5
0
0 0.05 0.1 0.15 0.2 0.25 0.3 0.35 0.4 0.45 0.5
CONC (m/l)
AVERAGE
MAX
Reaction: Mn(T$_{NH2}$PP)Cl + L + H_2O_2 + E

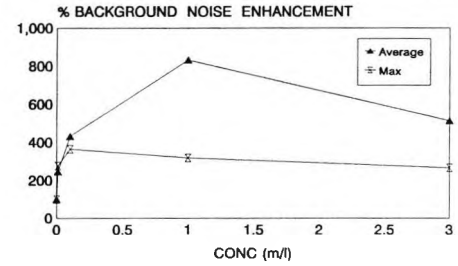
1-METHYLIMIDAZOLE

ENHANCED CHEMILUMINESCENCE
E = 1 Me IMIDAZOLE
SIGNAL VERSUS ENHANCER CONC.



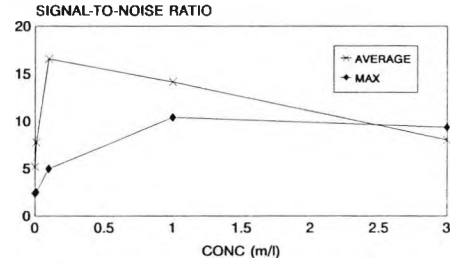
Reaction: $Mn(T_{NH2}PP)Cl + L + H_2O_2 + E$

ENHANCED CHEMILUMINESCENCE
E= 1 Me IMIDAZOLE
BACKGROUND NOISE VERSUS ENHANCER CONC.



Reaction: $Mn(T_{NH2}PP)Cl + L + H_2O_2 + E$

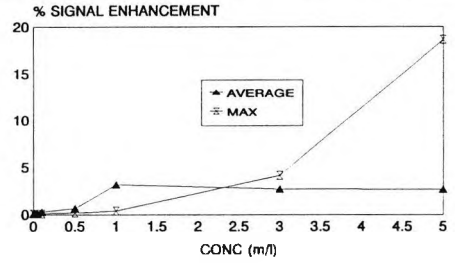
ENHANCED CHEMILUMINESCENCE
E= 1 Me IMIDAZOLE
SIGNAL-TO-NOISE RATIO VERSUS ENHANCER CONC.



Reaction: $Mn(T_{NH2}PP)Cl + L + H_2O_2 + E$

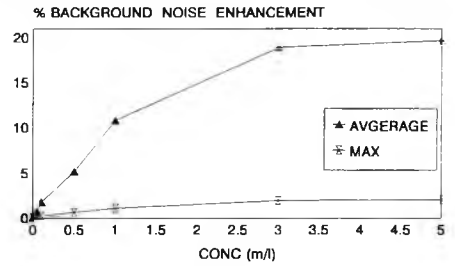
2-METHYLIMIDAZOLE

ENHANCED CHEMILUMINESCENCE
E= 2-Me IMIDAZOLE
SIGNAL VERSUS ENHANCER CONC.



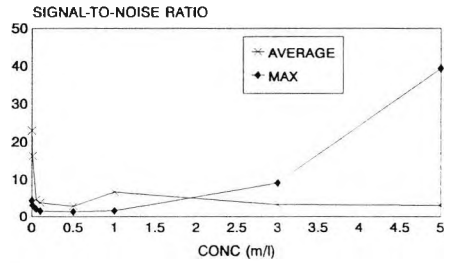
Reaction: $Mn(T_{NH2}PP)Cl + L + H_2O_2 + E$

ENHANCED CHEMILUMINESCENCE
E=2-Me IMIDAZOLE
BACKGROUND NOISE VERSUS ENHANCER CONC.



Reaction: $Mn(T_{NH2}PP)Cl + L + H_2O_2 + E$

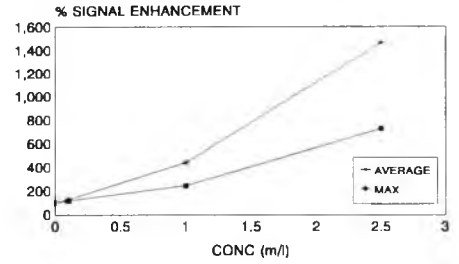
ENHANCED CHEMILUMINESCENCE
E= 2-Me IMIDAZOLE
SIGNAL-TO-NOISE RATIO VERSUS ENHANCER CONC.



Reaction: $Mn(T_{NH2}PP)Cl + L + H_2O_2 + E$

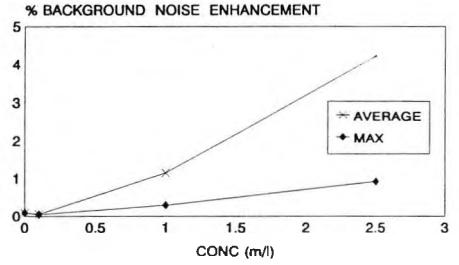
DABCO

ENHANCED CHEMILUMINESCENCE
E = DABCO
SIGNAL VERSUS ENHANCER CONC.



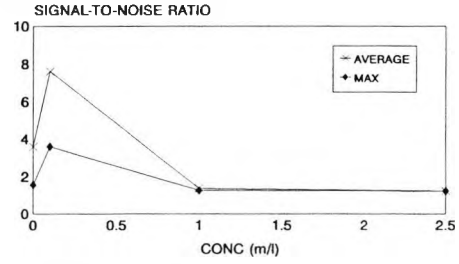
Reaction: $Mn(T_{NH2}PP)Cl + L + H_2O_2 + E$

ENHANCED CHEMILUMINESCENCE
E= DABCO
BACKGROUND NOISE VERSUS ENHANCER CONC.



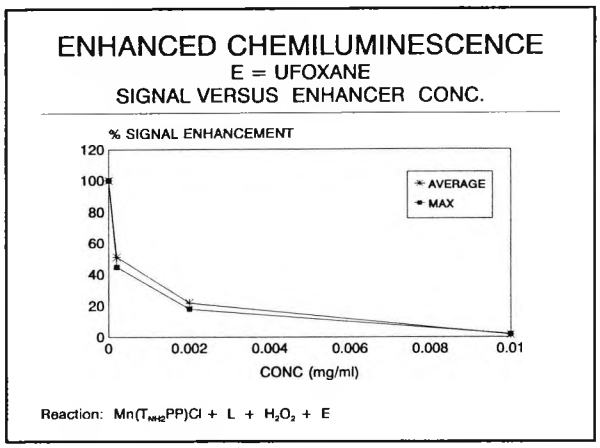
Reaction: $Mn(T_{NH2}PP)Cl + L + H_2O_2 + E$

ENHANCED CHEMILUMINESCENCE
E= DABCO
SIGNAL-TO-NOISE RATIO VERSUS ENHANCER CONC.

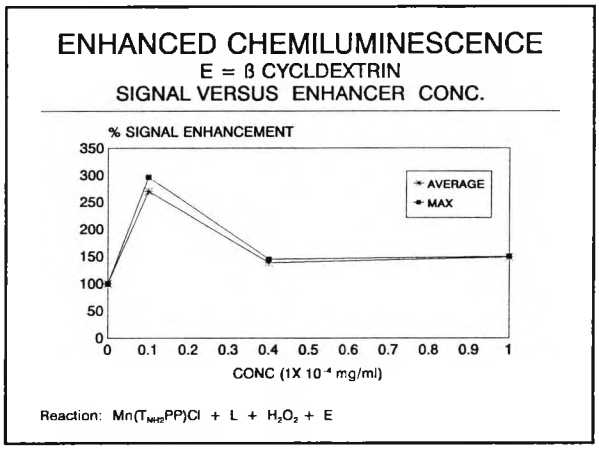


Reaction: $Mn(T_{NH2}PP)Cl + L + H_2O_2 + E$

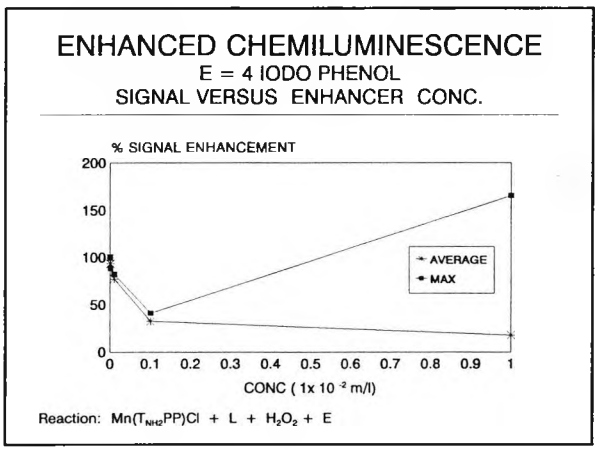
UFOXANE



ß - CYCLO-DEXTRIN



4 - IODOPHENOL

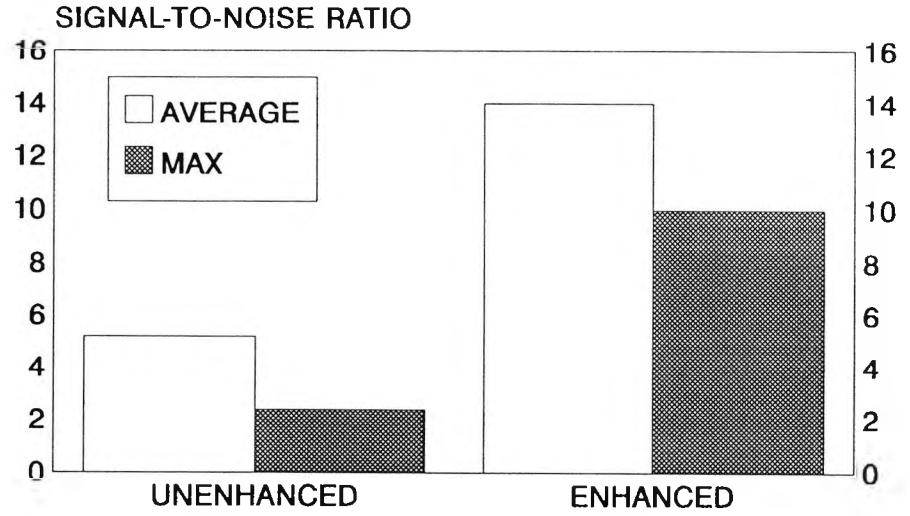


BEST OVERALL ENHANCER

ENHANCED CHEMILUMINESCENCE

E= 1-Me IMIDAZOLE

SIGNAL-TO-NOISE RATIO



Reaction: $Mn(T_{NH2}\ PP)Cl + L + H_2O_2 + E$

5.5 CONCLUSION

At the end of a screening programme, a number of compounds were identified which enhanced the catalytic activity of $Mn(T_{NH2}PP)Cl$ in the oxidation of luminol. The most significant enhancements were produced by 1- methyl imidazole and DABCO where up to 5- and 2-fold increases in signal to noise ratios were achieved, respectively.

Some compounds previously reported to enhance chemiluminescent or bioluminescent systems did not enhance the chemiluminescence of our system under the experimental conditions studied, suggesting they operated differently.

The work carried out in this project was not detailed enough to identify the mode of enhancer action, but the results indicated that enhancers were "enzyme" specific. It was also shown that the catalytic activity of metalloporphyrins was enhanced by the same category of compounds which were found to be effective with μ-peroxidase as compared to HRP.

The issue of specificity would suggest that no single mechanism can be put forward to explain the enhancement mechanism. The multistep nature of enzyme-catalysed chemi-luminescent reactions, with rate-limiting steps and competing side reactions creates several areas in which improvements in efficiency can produce increases in light emission.

In general terms, enhancement was achieved by helping the enzyme to re-cycle and/or by protecting it from the oxidative destruction at the expense of enhancer. The mechanism of the most successful HRP enhancer, p-iodo phenol (PIP), is discussed in Chapter VIII.

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CHAPTER VI

IMMUNOASSAY

6.1 INTRODUCTION

Having developed artificial enzymes which satisfy the basic criteria for enzyme immunoassay, namely the possession of high and easily quantifiable activity, their suitability for immunoassay needed to be verified.

Apart from the high catalytic activity, enzymes which are candidates for immunoassay applications need to satisfy the following criteria:

I) They should possess the appropriate functional groups for coupling or be easily derivatised;

II) They should maintain a high activity upon coupling to the appropriate component of the immunochemical reaction;

III) They should not significantly alter the immunochemical properties of the immuno-reactant;

IV) They should yield stable conjugates;

V) They should suffer minimal interference from their environment, e.g. buffer ions, components of the analytical sample matrix.

The full evaluation of criteria II- V is outside the scope of this study, as it requires immunodiagnostics and clinical expertise. In this chapter we set out to explore the suitability of our most successful "enzymes", from only a chemical perspective for future chemiluminescence immunoassay applications.

6.2 CONJUGATION

Most of the porphyrins investigated in our work were designed with conjugation in mind. It was however, very convenient to find that the most successful "enzymes", were also easily functionalisable.

The following metalloporphyrins were thought to possess high enough activities to merit their conjugation to antibody for further investigation:

$Mn(T_{NH2}PP)Cl$, $Mn(T_{COOH}PP)Cl$, $Mn(T_{SO3H}PP)Cl$

6.2.1 Activation of Reactive Sites

There are many types of linkage of a probe to a protein. The reactive sites on the surface of a protein vary from type to type, but the principal approach is to produce a covalent bond between the probe and the protein. Such a bond is strong enough to prevent the separation of the probe from the protein under normal handling conditions and insures that there is no electrostatic interaction to affect the electrophoretic point and the overall conformation of the protein, which is essential for its specificity.

The principal reactive sites on a peptide chain are the lysyl terminal amines of lysine residues (fig 6.1 a), the thiols of cysteine residues (fig 6.1 b) and the phenolic hydroxyls of tyrosine residues (fig 6.1 c). The lone pairs on the amine nitrogen, thiol sulphur and

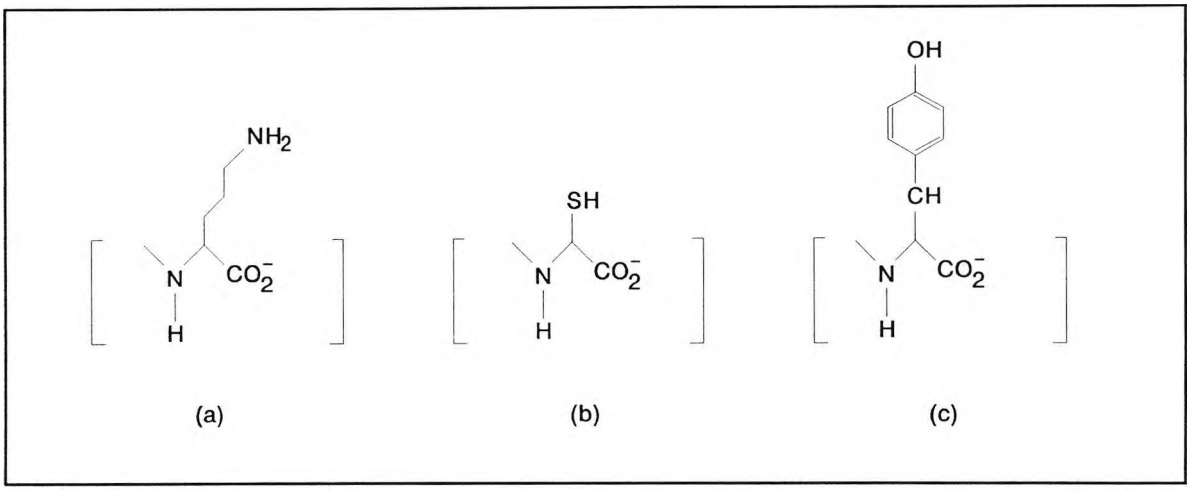


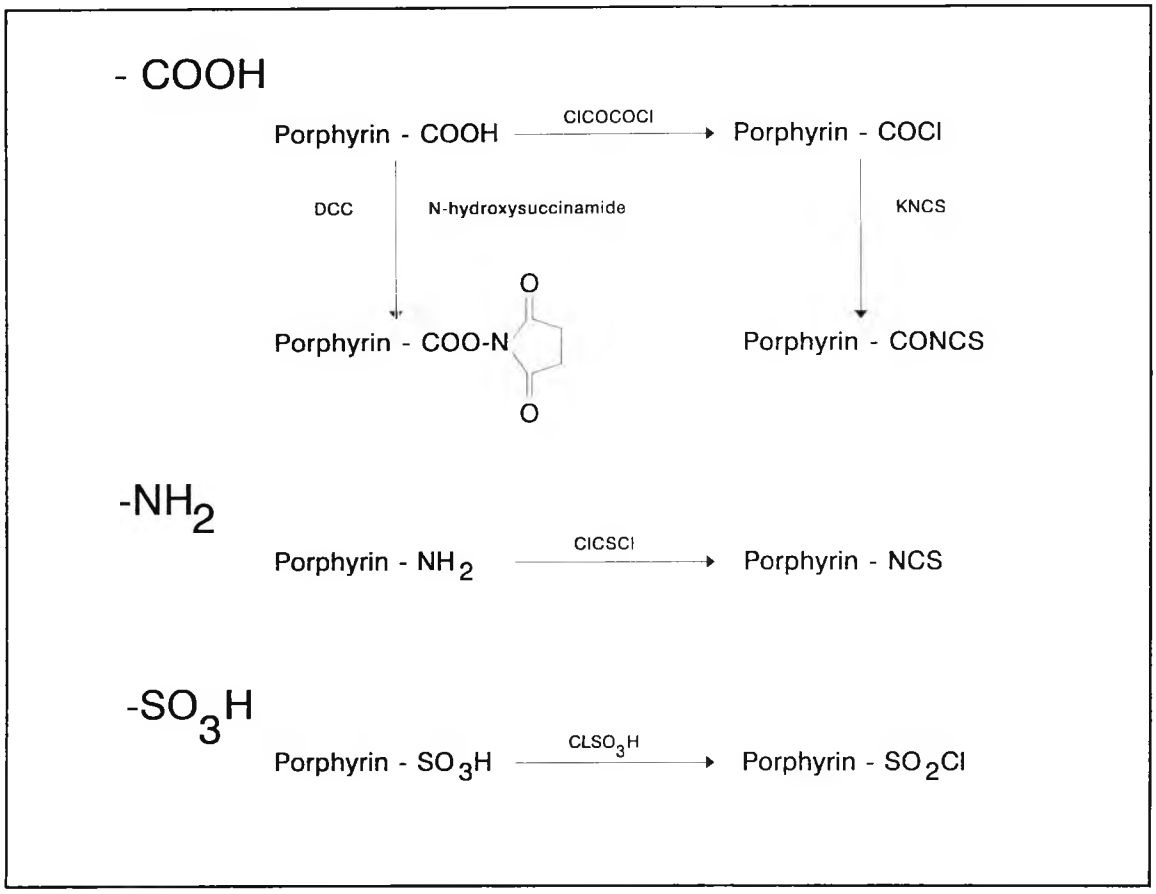
Figure 6.1

hydroxyl oxygen, being nucleophilic will attach themselves to any electrophilic group.

The principal reactive sites used for the conjugation of our enzymes were -COOH, $-NH_2$, and $-SO_3H$ groups. These groups can easily be activated to provide the sites for nucleophilic attack by the reactive groups of proteins, resulting in the formation of covalent bonds.

In this work, labels were prepared for conjugation with the lysyl terminal amines of lysine. To this end, porphyrins and metalloporphyrins bearing the reactive groups - COOH, - NH_2 and - SO_3H were activated according to Scheme 6.1.

To avoid over labelling, the activation of the functional group was limited to one (out of four) by limiting the amount of activating agent. Although this was not a reliable technique as some molecules may have had more than one functional group activated at the expense of other molecules, it was thought to be an acceptable alternative for this work.



Scheme 6.1

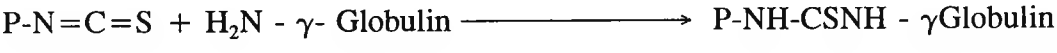
The experimental details of functional group activation are given in Chapter III.

6.2.2 Labelling

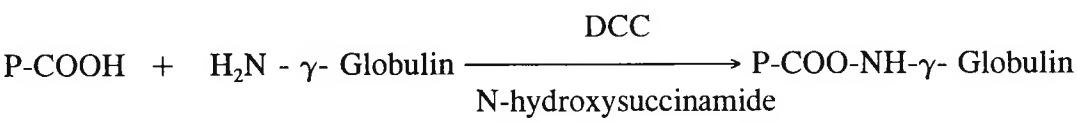
In this work human γ- Globulin or IgG was chosen as the standard immunoglobulin for labelling. Human IgG was chosen because of its accessibility and surface lysine amino-acid abundance.

The activated groups, on incubation with IgG, in labelling buffer (carbonate buffer pH 9.4) reacted with the lysyl amines of lysine to form the following linkages:

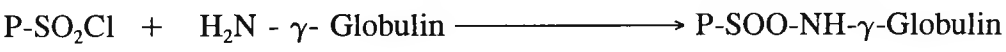
I) Isothiocyanates ——————→ Thiourea Linkage



II) Succinimido Ester ——————→ Ester Linkage



III) Sulphonyl Chloride ——————→ Sulphonamide Linkage



Where P = Porphyrin

6.2.3 Separation

Having conjugated the enzyme with the immuno-reactant, any unreacted enzyme must be separated from the reaction mixture, before the measurement. It is obvious that the method of separation should not affect enzyme activity. Commercial immunoassay kits make use of antibody coated surfaces such as tubes, beads or microwells.

In our work at the end of the incubation time the reaction mixtures were separated by gel-permeation chromatography using Sephadex as the gel and eluting buffer (phosphate buffered saline pH 7.5) as the eluent.

Conjugation was confirmed by the presence of IgG (characteristic peak at 275 nm) and of labelled porphyrin (characteristic Soret band peak at approximately 420 nm) in the uv/vis spectrum.

6.3 ASSAY OF IgG

Having confirmed the conjugation, the eluent containing the conjugate was diluted by 10 fold in tris buffer to obtain the working strength conjugate. The activity of conjugate was then checked by the luminometer in accordance with the procedure adopted throughout this study.

All three conjugates gave reasonable luminometer signals. As expected the signal from $Mn(T_{NH2}PP)Cl$ labelled γ-Globulin was an order of magnitude higher than $Mn(T_{COOH}PP)Cl$ and $Mn(T_{SO3H}PP)Cl$.

In order to assay the immuno-reactant quantitatively, it would have been necessary to calibrate the signals against known concentrations of the immuno-reactant-$Mn(T_{NH2}PP)Cl$ conjugate. This was not possible within the scope of this project. However, a rough idea of the concentration of the IgG- $Mn(T_{NH2}PP)Cl$ conjugate was obtained from the dose response curve of the unconjugated $Mn(T_{NH2}PP)Cl$ (as detailed in Chapter IV).

When the signals from the above conjugate were fed into the dose response curve , and the signal was translated into concentration, the result (1.6×10^{-11} moles) had a reasonable order of magnitude, which testified to the feasibility of the technique (fig. 6.2).

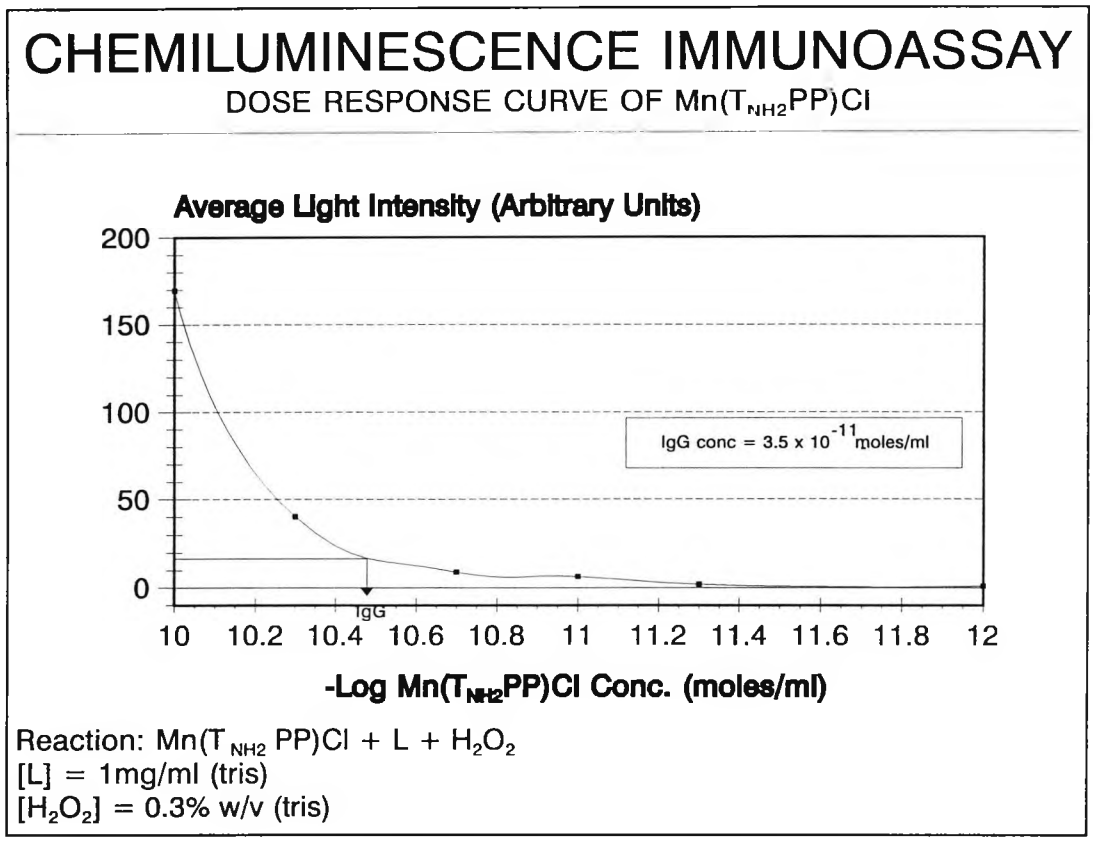


Figure 6.2

6.4 Immobilisation of Porphyrin on Solid Support

In previous chapters it was shown how Merrifield resin could be used for the synthesis of unsymmetrical porphyrins. Having made the resin bound porphyrin (as the precursor to the unsymmetrical porphyrin product) it was thought appropriate to extend the study to the effect of immobilisation on the catalytic activity and stability of the metalloporphyrin.

It was proposed that by immobilising metalloporphyrins, they may be shielded from the oxidative attack of peroxide resulting in an increased stability[1]. The advantages of such a system would be enormous in commercial oxidation of a large range of products and would

provide an easy way of fishing the "enzyme" out of the reaction mixture for reuse.

In the field of immunochemistry the immobilisation of "enzymes" on solid support would offer an easy way of separating the conjugate from the rest of the reaction mixture.

The nylons are a family of linear polymers consisting of repeating assemblies of methylene groups joined together by secondary amide linkages.

Nylons are readily available in a wide variety of physical forms, their structures are mechanically strong and non- biodegradable, rendering possible their prolonged exposure to biological media. Additionally nylons can be functionalised to support biochemicals in an environment which is conducive to the stability of the protein. For these reasons, among their many uses, nylons have found use as a support matrix for the immobilisation of enzymes. Immobilisation being achieved by covalently linking the enzyme's proteins to the nylon polymer. Native nylon of high molecular weight has a few free end groups, and so it must be pre-treated in order to generate potentially reactive centres that are capable of interacting covalently with enzyme molecules. Several types of nylon are available commercially, differing only in the number of methylene groups in the repeating alkane segments. In this work, nylon 6/6 [poly(hexamethylene adipamide)] [-$NH(CH_2)_6NHCO(CH_2)_4$ $CO-]_n$, was the type used for the immobilisation of " Artificial Enzyme ", $Mn[T_{NH2}PP]Cl$.

6.4.1 Merrifield Resin

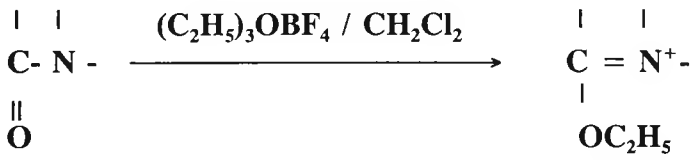
The Merrifield resin bearing the unsymmetrical porphyrin 5 Mono (hydroxy phenyl) 10,15,20 Tri (penta fluoro phenyl) porphyrin was metallated according to:

$$MR\text{-}M_{OH}T_FPP \xrightarrow{MnCl_2 \,/\, DMF} Mn[MR\text{-}M_{OH}T_FPP]Cl$$

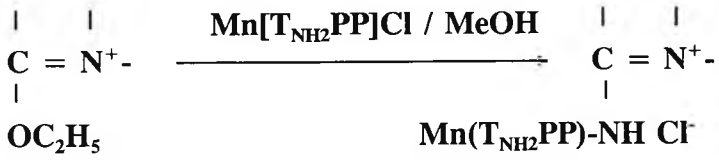
to give the manganese salt as detailed in Chapter II.

6.4.2 Nylon 6/6

Reactive centres were introduced onto the nylon by O-alkylation of the support's secondary amide bonds, yielding the reactive imidate salt of the nylon without depolymerisation of the support, as follows:



Thus the treatment of nylon with the powerful alkylating agent, triethyloxonium tetrafluoroborate in dichloromethane, yielded the corresponding imidate salts. On incubation, the imidate salts reacted directly with the nucleophilic amine of the metalloporphyrin to form the $Mn[T_{NH2}PP]Cl$ coupled nylon.



6.4.3 Catalytic Activity

The catalytic activity of immobilised porphyrins was tested by placing two nylon beads or 10mg of Merrifield resin bearing the appropriate metalloporphyrins in the luminometer reaction tube and measuring the luminescence of the luminol/H_2O_2 system according to procedures adopted throughout this study. For comparison the procedure was repeated with nylon and Merrifield resin without the bound metalloporphyrins.

To study the reusability of the metalloporphyrins when bound to the solid supports, the spent reaction mixtures were aspirated and after a few wash and aspiration cycles (tris buffer), fresh luminol and H_2O_2 were added to initiate a new chemiluminescent reaction.

6.4.4 Result and Discussion

The catalytic activity of dry MR-[Mn($M_{OH}T_FPP$)Cl] was close to the detection limit of the instrument. The activity was slightly increased if the resin was allowed to soak in DMF before the measurement of the activity.

The catalytic activity of Nylon-[Mn(T_{NH2}PP)Cl] was a few orders of magnitude

higher than that of Merrifield resin, and could easily be measured (fig 6.3). The background luminescence from the unbound resin was however very high.

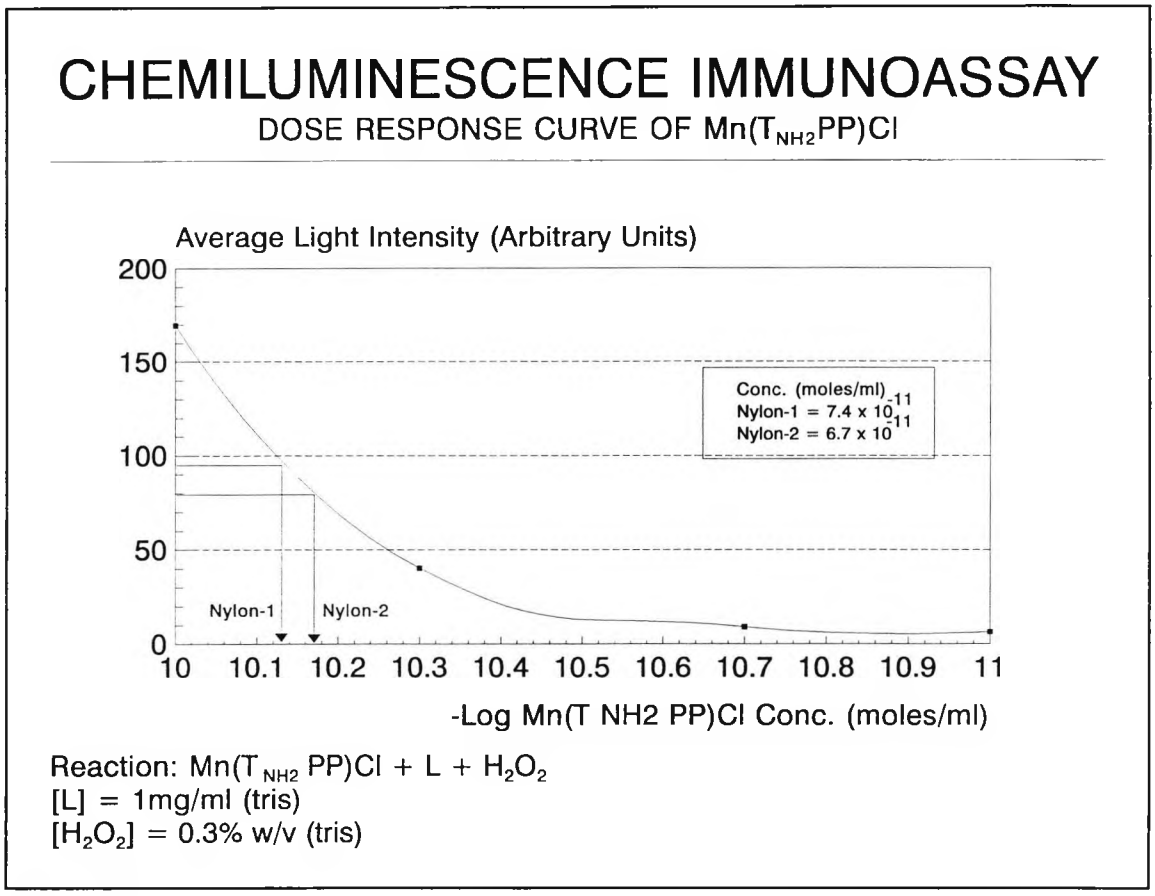


Figure 6.3

Although the above results are in agreement with earlier findings that Mn($M_{OH}T_FPP$)Cl is a very poor catalyst whereas Mn(T_{NH2}PP)Cl possesses very high activity, no clear comparisons could be made between the activities of free and immobilised metalloporphyrins as the activities could not be quantitatively related to enzyme concentrations.

The activity of recycled MR-[Mn($M_{OH}T_FPP$)Cl] was found to be too weak to be measured and compared with the fresh sample.

The activity of recycled Nylon-[Mn(T_{NH2}PP)Cl] was found to have decreased by 20% from the fresh sample. Repeated washing and recycling of Nylon-[Mn(T_{NH2}PP)Cl] , resulted in between 15-25 % loss per cycle (fig 6.4).

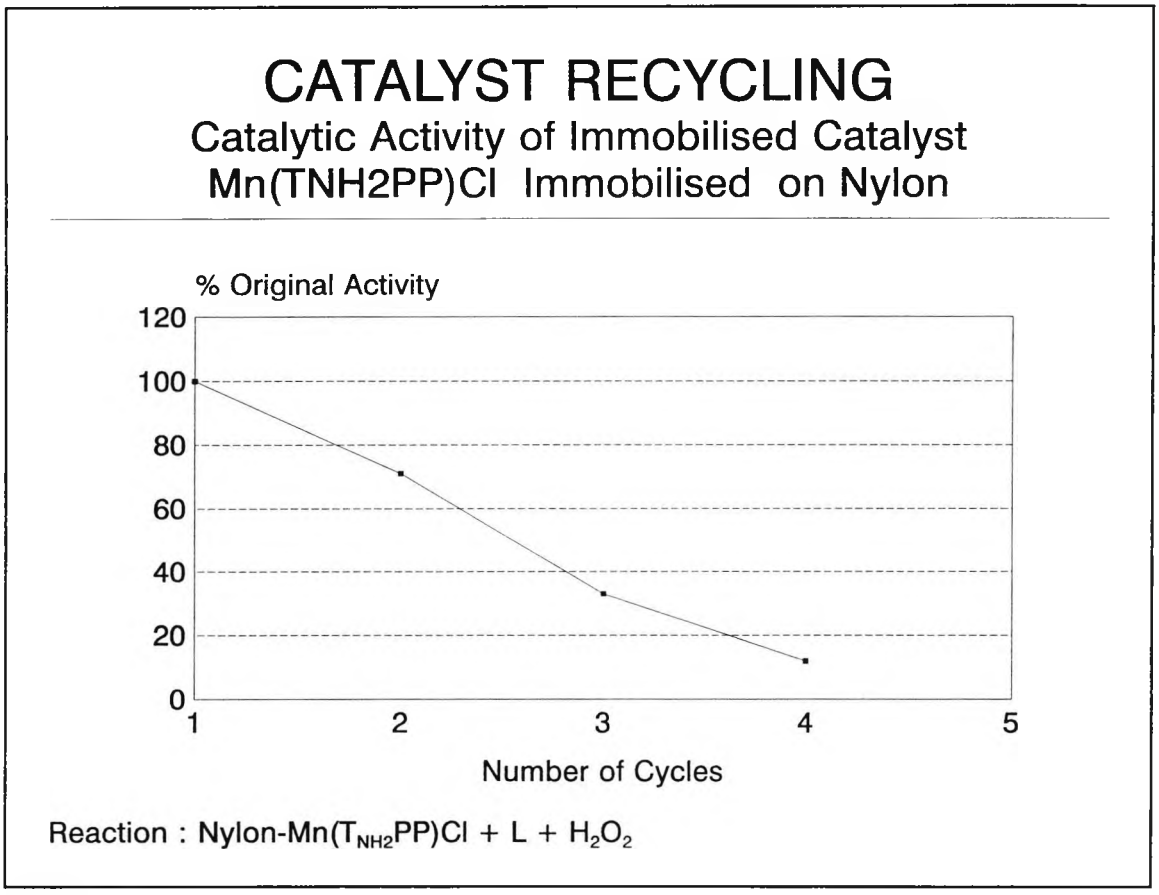


Figure 6.4

6.5 CONCLUSION

The metalloporphyrins: $Mn(T_{NH2}PP)Cl$, $Mn(T_{COOH}PP)Cl$, and $Mn(T_{SO3H}PP)Cl$ after activation were successfully conjugated with IgG. The resulting conjugates were shown to possess catalytic activities. The conjugate with the highest catalytic activity was found to be $Mn(T_{NH2}PP)Cl$. This was in agreement with the results for free metalloporphyrins.

In the absence of a conjugate dose response curve the conjugates could not be assayed quantitatively. However, when signals were fed into the dose response curve of the free $Mn(T_{NH2}PP)Cl$, the results were of reasonable order of magnitude.

$Mn(T_{NH2}PP)Cl$ and $Mn(M_{OH}T_FPP)Cl$ were successfully immobilised onto nylon beads and Merrifield resin, respectively. The catalytic activities of the two immobilised metalloporphyrins were in line with their free form.

Recycled Nylon-[$Mn(T_{NH2}PP)Cl$] was used repeatedly for the initiation of luminescence in fresh luminol/H_2O_2 reaction mixtures. Repeated recycling resulted in between 15-25 % loss in catalytic activity per cycle.

The work carried out in this chapter demonstrated the feasibility of metalloporphyrins in general and $Mn(T_{NH2}PP)Cl$ in particular as an alternative to peroxidase for chemiluminescence immunoassay. Further work is needed for the optimisation of conjugation, purification and a detection system before $Mn(T_{NH2}PP)Cl$ can be confirmed as a viable alternative.

6.6 REFERENCE

1) Y. Saito, M. Mifune, T. Kawaguchi, J. Odo, Y. Tanaka, M.Chikuma, and H. Tanaka, Chem. Pharm. Bull., 34, 2885, (1986).

CHAPTER VII

ULTRASONIC IRRADIATION OF HRP

7.1 INTRODUCTION

Despite extensive studies, the three-dimensional structure of HRP remains unresolved. The most striking structural feature of HRP seems to be the way the protein folds around the heme so as to bury the iron centre and all but the active site of the enzyme. In order to obtain information about the role of the protein-folding on catalytic activity, numerous chemical modification experiments have been performed on HRP and its isoenzymes[1].

It was the intention of this work to obtain basic information about the role of the HRP proteins in catalytic activity without entering the complex field of three-dimensional enzyme conformation for which we were not equipped.

A general method of determining the structural features that guide protein-folding is to unfold the protein by denaturation. Heat, pH and chemical treatments are among the most common procedures used for the denaturation experiments.

Ultrasonic radiations are among the vigorous and high energy techniques used for the rupturing of plant and bacteria cell walls. These radiations are capable of denaturation as well as breaking covalent bonds in the backbones of the polypeptide chain.

In this work it was proposed that by applying various degrees of high energy radiations, one could obtain HRPs at various unfolded and deproteinated stages which could be used for comparing catalytic activities.

7.2 METHOD

20ml of a $5x10^{-3}$ mg/ml solution of HRP in tris buffer (0.1 M, pH 8.4) was sonicated for 20 minutes at various power settings. At suitable intervals 200μl aliquots of the sonicated enzyme were withdrawn, diluted by 10-fold in tris buffer and used for the comparison of catalytic activities by measuring the chemiluminescence of the luminol/H_2O_2 system as discussed in earlier chapters.

Reaction Mixture

Reagent	**Concentration**	**Volume**
HRP/ Tris	$1x\ 10^{-4}$ mg/ml	100μl
Luminol/ Tris	6.78×10^{-5} molar	100 μl
H_2O_2/ Tris	0.3% w/v	100 μl
	Total Reaction mixture volume =	300 μl

7.3 INSTRUMENT

Name: Vibra Cell
Model: 50 Watt

7.4 RESULTS

The results of a typical HRP sonication experiment are shown graphically in figures 7.1-7.3. In general the findings indicate that sonication of HRP for short periods (< than 2 minutes) can result in an enhancement of catalytic activity. The consequence of prolonged sonications were detrimental to catalytic activity.

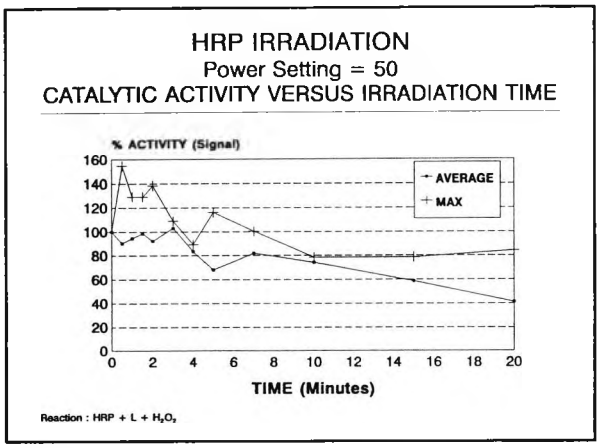


Figure 7.1

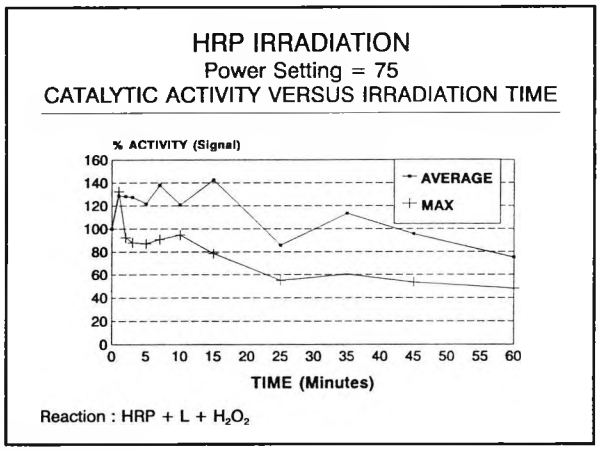


Figure 7.2

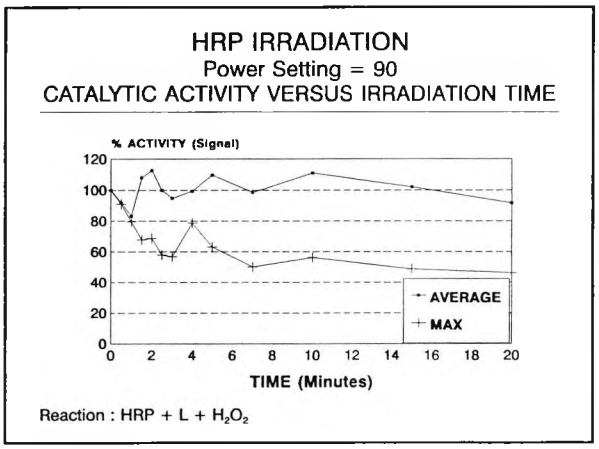


Figure 7.3

7.5 DISCUSSION

In earlier chapters it was shown that metalloporphyrins are capable of catalysing oxidation reactions through an HRP-like mechanism. This finding together with the fact that the heme of HRP is also capable of catalysing some oxidation reactions, albeit less efficiently than HRP, questions the role of the polypeptide chains in the catalytic process. Furthermore μ-peroxidase, which contains the same heme as HRP but smaller amounts of polypeptide chains shows different catalytic behaviour to HRP.

In a tentative approach to investigating the role of the polypeptide chain it was decided to probe the effects of ultrasonic unfolding and opening-up of the polypeptide chain on catalytic activity.

This method, in addition to the unfolding of the polypeptide chain, offers the potential of chain fragmentation at higher power settings. Thus in theory it may be possible to "chop" sections of the chain and to investigate the "dismantling" enzyme.

The results indicate that the activity of the enzyme was enhanced by up to 25% when the enzyme was sonicated for very short periods. As expected prolonged sonication resulted in deactivation of the enzyme.

The fact that any polypeptide chain unfolding/fragmentation which may have occurred was too uncontrollable to be studied effectively, questioned the usefulness of the above technique as an experimental tool. However, repeated experiments at various power settings

over long sonication periods suggested that:

1) HRP was a remarkably robust enzyme, capable of resisting the destructive power of ultrasonic radiation for prolonged periods.
2) Sonication resulted in loss of enzyme precision. This may be due to the random unfolding/fragmentation of individual HRP molecules.
3) Short term sonication resulted in the enhancement of HRP activity. This may have been due to the fact that by applying moderate levels of high energy radiation the polypeptide chains unfold, making the active centre of the enzyme more accessible.

7.6 CONCLUSION

The catalytic activity of HRP was shown to be enhanced upon moderate exposure to ultrasonic radiation. This was explained in terms of unfolding of the polypeptide chains of the enzyme making the active site more accessible to the substrate.

The technique of polypeptide chain unfolding and fragmentation by means of high energy ultrasonic radiation needs to be improved and made controllable before it can be used as a useful tool for structure probing.

7.7 REFERENCE

1) S. Ferguson-Miller, D.L. Brautigan, and E. Margoliash, The Porphyrins (Ed. D. Dolphin), Academic Press, London and New York, 7, 150, (1979).

CHAPTER VIII

CONCLUSION

8.1 INTRODUCTION

In an attempt to develop good artificial mimics of HRP, the peroxidase-like activity of a large number of metalloporphyrins was systematically investigated in terms of their ability to catalyse the chemiluminescent oxidation of luminol by hydrogen peroxide.

At the end of this study artificial enzymes of high activity were identified, which offer potential as models in mechanistic studies as well as replacement of peroxidase as the enzyme of choice in a number of applications.

In the course of the investigations it was shown that decomposition of the peroxide and the MTPP was a general feature of the MTPP-catalysed oxidation of luminol by hydrogen peroxide.

The fact that these unwelcome "side reactions" were most prominent in the case of the most successful artificial enzymes indicated the close link between the catalytic activity and hydrogen peroxide/MTPP decomposition.

Closer experimental observations and literature searches revealed the complexity of the reaction mechanism. There has been an explosion of new information about biological redox processes which by and large contain porphyrins as the their active nucleus. The activities, structures, reactions and uses of metalloporphyrins, peroxidases and other closely related catalysts and pigments have been the subject of hundreds of publications in the last decade.

In this chapter we attempt to discuss the mechanism of the reaction by basing our suggestions on our experimental findings and the work of other researchers.

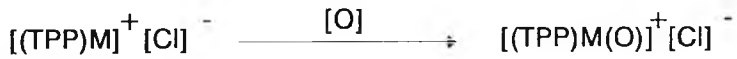
8.2 MECHANISM

It has long been known that peroxidase enzyme action, which involves the catalysed oxidation of a wide range of substrates including luminol by peroxo species, occurs through the formation of reaction intermediates. These intermediates arise via oxidation of the enzyme by the peroxo compound and, in turn, function as oxidants of selected substrates regenerating enzyme. Any discussions about the mechanism of the reaction will therefore need to consider:

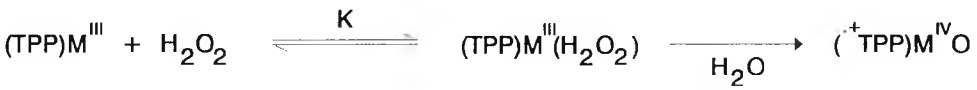
1) The mechanism of the oxidation of metalloporphyrins by oxygen transfer from hydrogen peroxide.
2) The mechanism of the oxidation of substrates by the resultant hypervalent metal-oxo porphyrin product.

8.2.1 The Mechanism of Oxidation of Metalloporphyrins

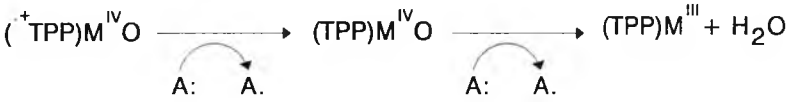
The first step in the oxidation is ligation of H_2O_2 to the metal (III) and transfer of an "oxene equivalent" to the metalloporphyrin such that the oxygen becomes an axial ligand of the metal and the metalloporphyrin undergoes two-electron oxidation[2].



It is conventional to refer to the product of the two-electron oxidation of a metal(III)-oxo porphyrin as "compound I". Two reaction routes are now available to compound I . A "peroxidase" route and a "catalase" route.



Peroxidase:



Catalase:

In the peroxidase route, the activated catalyst will transfer the "oxene equivalent" to the substrate, thereby facilitating oxidation of the substrate.

In the catalase route, the activated catalyst will facilitate the reduction and consequent decomposition of H_2O_2.

In nature the options are quite separate and are carried out by dedicated enzymes. For example, catalase and HRP, despite having a common prosthetic group and similar

"Compound I", are quite specialised in their tasks. The former is responsible for the decomposition of H_2O_2 to H_2O and O_2 and with a turnover number of 1×10^6 is among the most efficient enzymes, whereas the turnover number of peroxidase for the same task is less than 2000[1]. On the other hand HRP is much more efficient in catalysing the oxidation of a variety of substrates as compared to catalase.

Common to both of these enzymes is their prosthetic group, ferric protoporphyrin(IX). The difference in their activities is explained by the differences in the axial ligation of the central iron (III) moiety. For HRP, the fifth ligand to iron is nitrogen (histidine imidazole) whereas that for catalase is oxygen (tyrosine hydroxyl).

In our artificial enzymes the distinction between the two routes seemed to be less clear as the best catalyst for the decomposition of H_2O_2 was also found to be the best catalyst for the oxidation of luminol, namely $Mn(T_{NH2}PP)Cl$.

In the absence of substrate it is clear that the catalase was the only route available, leading to the decomposition of hydrogen peroxide. In this work the rate of decomposition was not determined experimentally but it is thought to be a few orders of magnitude smaller than that of catalase. Sigel[1] reported the catalase activity of a similar Fe complex (tetrasulphthalocyanine) to be in the region of 480.

No experimental results are available on the competition between the catalase and peroxidase routes in the presence of substrate, but the success of $Mn(T_{NH2}PP)Cl$ in catalysing the oxidation of luminol would suggest that under the stated experimental conditions , the

peroxidase is by far the dominant route.

The structure of Compound I is dependent on the nature of the metal species and the presence or absence of a strongly basic axial oxo ligand. When an oxo ligand is present, Compound I may have the structure of a metal(V)-oxo porphyrin, a metal(IV)-oxo porphyrin π cation radical or a metal(III)-oxo porphyrin di-cation (fig 8.1).

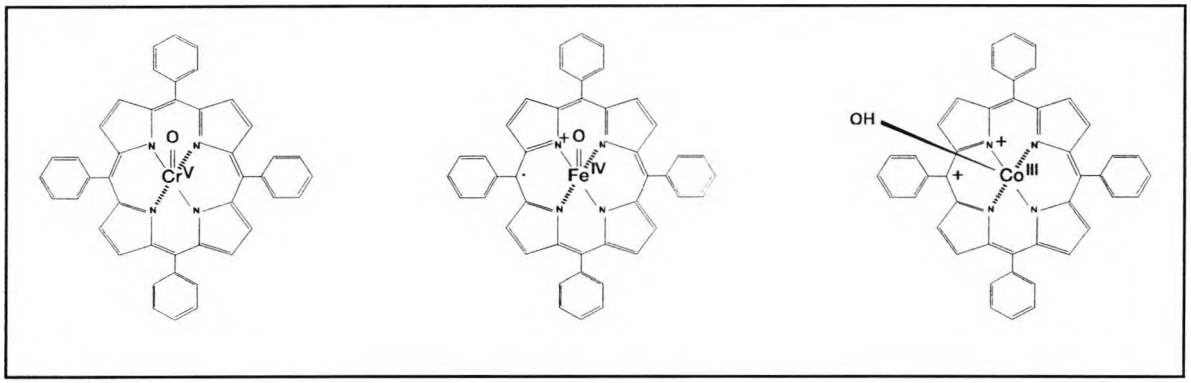


Figure 8.1

In the case of HRP (axial ligand imidazole) and catalase (axial ligand tyrosine-O-), much evidence exists to support the structure of Compound I as being an iron(IV)-oxo porphyrin π-cation radical[3-6].

In the reaction of manganese(III) tetraphenylporphyrin with H_2O_2 the oxene equivalent transfer and eventual peroxide decomposition has been reported to proceed according to:

$$\mathbf{(TPP)Mn^{III}(X) + H_2O_2 \longrightarrow (TPP)Mn^{V}(O)(X) + H_2O}$$

$$\mathbf{(TPP)Mn^{V}(O)(X) + H_2O_2 \longrightarrow (TPP)Mn^{III}(X) + O_2 + H_2O}$$

Where X = H_2O, OH^- , or imidazole

For X = imidazole and in the absence of substrate, the manganese (IV)-oxo species may be

predominant due to the reaction depicted by the following equation:

$$(TPP)Mn^{III}[ImR] + (TPP)Mn^{V}(O)[ImR] \longrightarrow 2(TPP)Mn^{IV}(O)[ImR]$$

At high substrate concentrations there will scarcely be any accumulation of manganese(IV) -oxo species due to the trapping of manganese(V)-oxo porphyrin by the substrate.

This is in contrast to the iron(III) tetraphenylporphyrin where the iron(IV)-oxo species is the sole intermediate product.

$$(TPP)Fe^{III}(X) + H_2O_2 \longrightarrow (^{+}TPP)Fe^{IV}(O)(X) + H_2O$$

$$(^{+}TPP)Fe^{IV}(O)(X) + H_2O_2 \longrightarrow (TPP)Fe^{III}(X) + O_2 + H_2O$$

Where X = H_2O, OH^- , or imidazole

Bruice and co-workers have reported[7] that for the reaction of manganese(III) porphyrin with H_2O_2 , the rate-determining oxene equivalent transfer is not subject to general catalysis at any pH. For iron(III) porphyrin on the other hand catalysis by some nitrogen-centred bases was observed.

To make matters more complicated, it has been reported[8] that the addition of imidazole enhances the rate of the reaction by five to six-fold.

One would have thought that the role of the imidazole is to function as a general base at the central metal, but from the contradictory results it is obvious that the role of general

catalysis in oxygen transfer to metal(III) porphyrins is not well understood and much remains to be done to gain an understanding of the role of general catalysis in these systems.

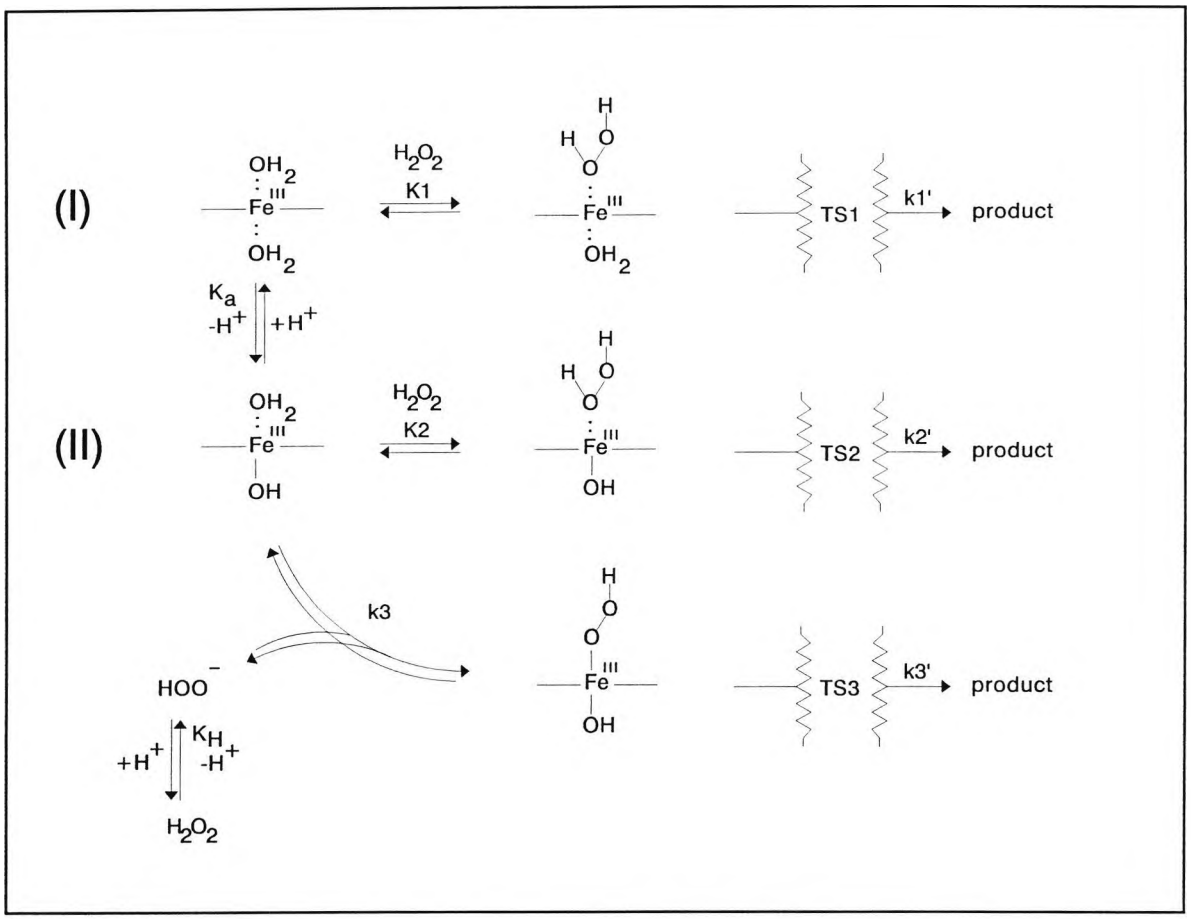
The difference in the oxo species of Mn(III) and Fe(III) may well have a part to play in the differences between the catalytic activities of Mn(TPP)Cl and Fe(TPP)Cl.

Studies on Fe (III) porphyrin have shown that three different mechanisms of decomposition of hydrogen peroxide by metalloporphyrin are operative between pH 1 and 12[7]. The three reaction paths are related to one another by their acid-base dissociation constants. A simple reaction sequence is depicted in scheme 8.1.

At low pH the reacting species are the metal(III) porphyrin bis- aquo complex I and H_2O_2; at intermediate pH the hydroxyl-ligated metal(III) porphyrin complex II and H_2O_2; and at high pH, complex III and the hydroperoxy anion[9].

The fact that the decomposition generally proceeds smoothly in higher pH regions can be explained by the formation of the more stable peroxo complex of HOO^- with the catalyst. Once HOOH ligates to the metal(III), the acid-base dissociation constant of HOOH is decreased by ca. 10^6. Also the axial ligand (X) may be exchanged for HOH in the course of reaction of the metal(III) porphyrin with HOOH and ligated HOH would have an acid-base dissociation constant[1].

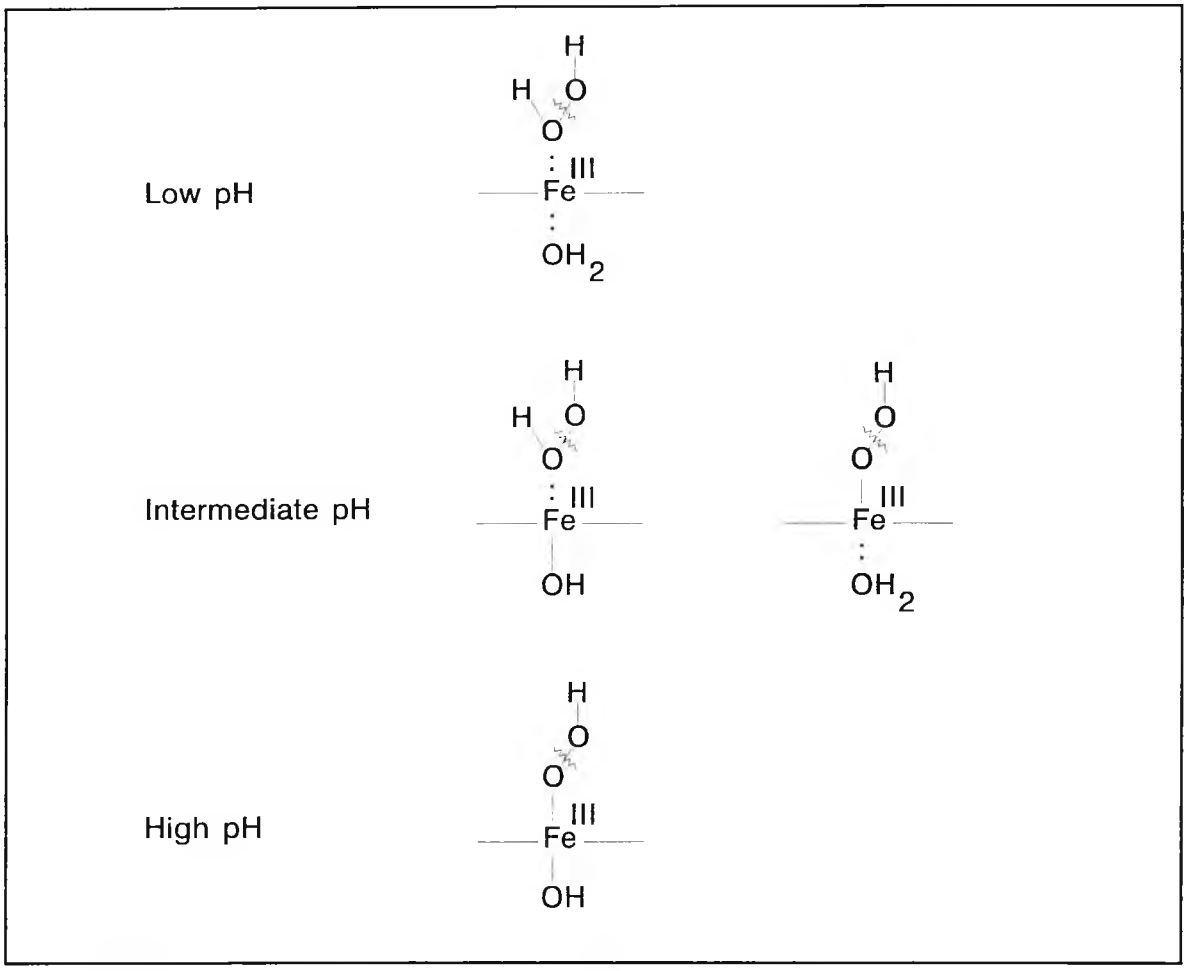
The possible modes of O-O bond cleavage at the three different pH regions are shown in fig 8.2.



Scheme 8.1

The formation of compound I is the prime route to catalytic activity. The ease of its formation and stability will determine the catalytic value of the metalloporphyrin. It is therefore not surprising to find that there is a correlation between the magnitude of catalytic activity and the one-electron oxidation potential of the catalyst.

Factors which alter the oxidation potential of the catalyst such as the nature of the central metal, the basicity of the porphyrin nitrogens (including the nature, position and size of substituents on the phenyl rings), the nature of the solvent (including pH, coordinating ability, dielectric constant) and the nature of the ligands, also alter the magnitude of catalytic activity.



Scheme 8.2

Of these factors the nature of the central metal and the substituents on the phenyl rings were found to be the most important in determining catalytic activity. In general the lower the oxidation potential of the complex the higher its catalytic activity.

← Increase in catalytic activity

Mn > Fe > Co > Ni > Cu , Zn

Increase in oxidation potential →

NH_2 > H > COOH > NO_2

← Increase in electron-donating power

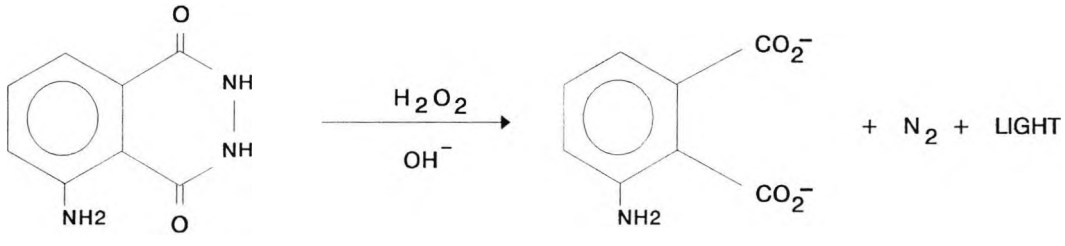
It is now easy to see why Mn(T_{NH2}PP)Cl was found to possess the highest catalytic activity.

8.2.2 The Mechanism of Oxidation of Substrates

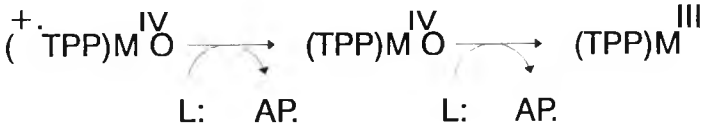
8.2.2.1 Unenhanced

Having discussed the mechanism of metalloporphyrin activation, ie. two-electron oxidation of a metal(III)-oxo porphyrin into "Compound I", a mechanism needs to be suggested to account for the oxidation of the substrate as well as the catalyst recycling to the unoxidised metal (III) porphyrin.

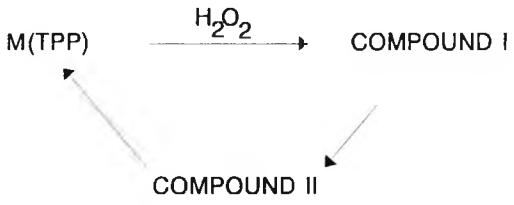
In our work for obvious reasons , luminol was the substrate of our choice. Although the step-by-step mechanism of luminol oxidation is not entirely understood there is no doubt that luminol undergoes a two-electron oxidation to produce aminophthalic acid and light.



Compound I with two oxidising equivalents , is capable of oxidising two luminol molecules. This is done in two stages. In the first stage, Compound I loses one oxidising equivalent to one molecule of luminol, which is oxidised.



The resulting intermediate, a product of one-electron reduction of the Compound I is known as "Compound II". The loss of the remaining oxidising equivalent from Compound II to a second molecule of luminol restores the metalloporphyrin to the resting, metal(III) - porphyrin format.



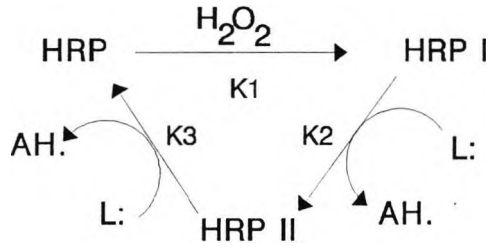
8.2.2.2 Enhanced

The multistep nature of enzyme-catalysed chemiluminescent reactions, with rate-limiting steps and competing side reactions creates several areas in which improvements in efficiency can produce increases in light emission. Many compounds previously reported to enhance chemiluminescent or bioluminescent systems did not enhance the chemiluminescence of our system under the experimental conditions studied.

A number of compounds, most distinctly from the imidazole group were shown to enhance the catalytic activity of MTPPs.

The fact that enhancers were found to be enzyme specific would suggest that they operate via different mechanisms. It was not within the scope of this work to identify a reaction mechanism for each enhancer, and any mechanistic discussion can only have a general approach.

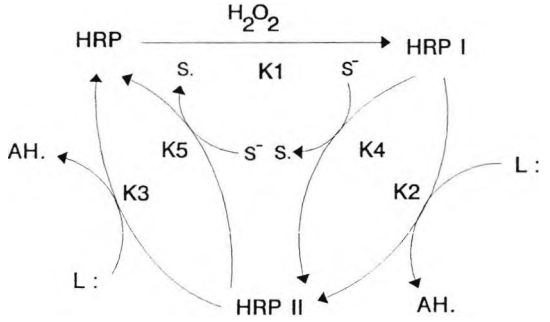
In the absence of data for the reaction constants in our system ie. MTPP + luminol + Enhancer, mechanistic discussions will be based on HRP + luminol + p-iodophenol (PIP), as it is of a similar nature and is the only system to date for which a mechanism has been proposed.



$K1 = 1.8 \times 10^7\ M^{-1}C^{-1}$
$K2 = 8.0 \times 10^5\ M^{-1}C^{-1}$
$K3 = 1.2 \times 10^4\ M^{-1}C^{-1}$

In the above reaction light emission is limited by the relatively slow reaction of Compound II with luminol which produces a luminol radical and regenerates HRP. This results in a buildup of Compound II and the disruption of the delicate recycling process.

It is thought that PIP may increase light emission by accelerating the conversion of Compound II into active HRP, by rapid reaction with Compound II, resulting in products



$K1 = 1.8 \times 10^7\ M^{-1}C^{-1}$
$K2 = 8.0 \times 10^5\ M^{-1}C^{-1}$
$K3 = 1.2 \times 10^4\ M^{-1}C^{-1}$

For S= PIP
$K4 = 2.8 \times 10^7\ M^{-1}C^{-1}$
$K5 = 1.2 \times 10^6\ M^{-1}C^{-1}$

such as radicals, which may then subsequently react with luminol to produce further luminol radicals and thereby further light emission.

A simplified scheme depicting the unenhanced and enhanced HRP-catalysed chemiluminescent reactions is given below[10].

$$HRP + H_2O_2 \rightarrow \text{Compound I} + H_2O$$

$\downarrow$

Unenhanced

Compound I + Luminol	→ Compound II + Luminol radical
Compound II + Luminol	→ HRP + Luminol radical

Enhanced

Compound I + Enhancer	→ Compound II + Enhancer radical
Compound II + Enhancer	→ HRP + Enhancer radical
Enhancer radical + Luminol	→ Enhancer + Luminol radical

$\downarrow$

$$\text{Luminol radical} + O_2 \rightarrow \text{Oxidised Luminol} + \text{Superoxide radical}$$

$$\text{Luminol radical} + \text{Superoxide radical} \rightarrow \text{Luminol endoperoxide}$$

$$\text{Luminol endoperoxide} \rightarrow N_2 + \text{Excited 3-aminophthalate dianion}$$

$$\text{Excited 3-aminophthalate dianion} \rightarrow \text{3-Aminophthalate dianion} + \text{LIGHT}$$

8.2.3 The Mechanism of Porphyrin Chemiluminescence

In Chapter III, it was shown that $Mn(T_{NH2}PP)Cl$ was also capable of catalysing the oxidation of unmetallated and some metallated porphyrins. The relatively unusual phenomenon accompanying such reactions was the release of low intensity, high energy

chemiluminescence.

The prime condition for this type of chemiluminescence was found to be the ability of the porphyrin to fluoresce and among the metalloporphyrins tested, ZnTPP as expected was found to be the strongest emitter.

The most probable route for the oxidation of TPP (and ZnTPP) is as follows:

$$2TPP \xrightarrow[H_2O_2\ ,\ OH^-]{Mn(T_{NH2}PP)Cl} 2TPP^{\cdot+} \rightleftharpoons TPP + TPP^{2+}$$

It is thought that the return of an electron to $TPP^{\cdot+}$ (and possibly TPP^{2+}) can produce enough energy to raise the porphyrin to higher energy levels. The subsequent loss of this excess energy for the fluorescing porphyrin is accompanied by chemiluminescence.

$$TPP^{\cdot+} \xrightarrow{e^-} TPP^* \longrightarrow TPP + h\nu$$

The question which needs to be answered is how a relatively weak oxidising agent such as H_2O_2 provides the necessary oxidising power for the formation of the mono- and possibly di- π cations of TPP, given the high energy requirements [11].

$$TPP \xrightarrow{1.00\ V} TPP^{\cdot+} \xrightarrow{1.20\ V} TPP^{2+}$$

The answer probably lies in the ability of $Mn(T_{NH2}PP)Cl$ to catalyse the reaction and this most probably occurs as follows:

$$2TPP + (^{\cdot+}TPP)Mn^{IV}(O) \longrightarrow (TPP)Mn^{III} + 2TPP^{\cdot+} + (O)$$

$$TPP + (TPP)Mn^{IV}(O) \longrightarrow (TPP)Mn^{III} + TPP^{\cdot+} + (O)$$

8.3 CONCLUDING REMARKS

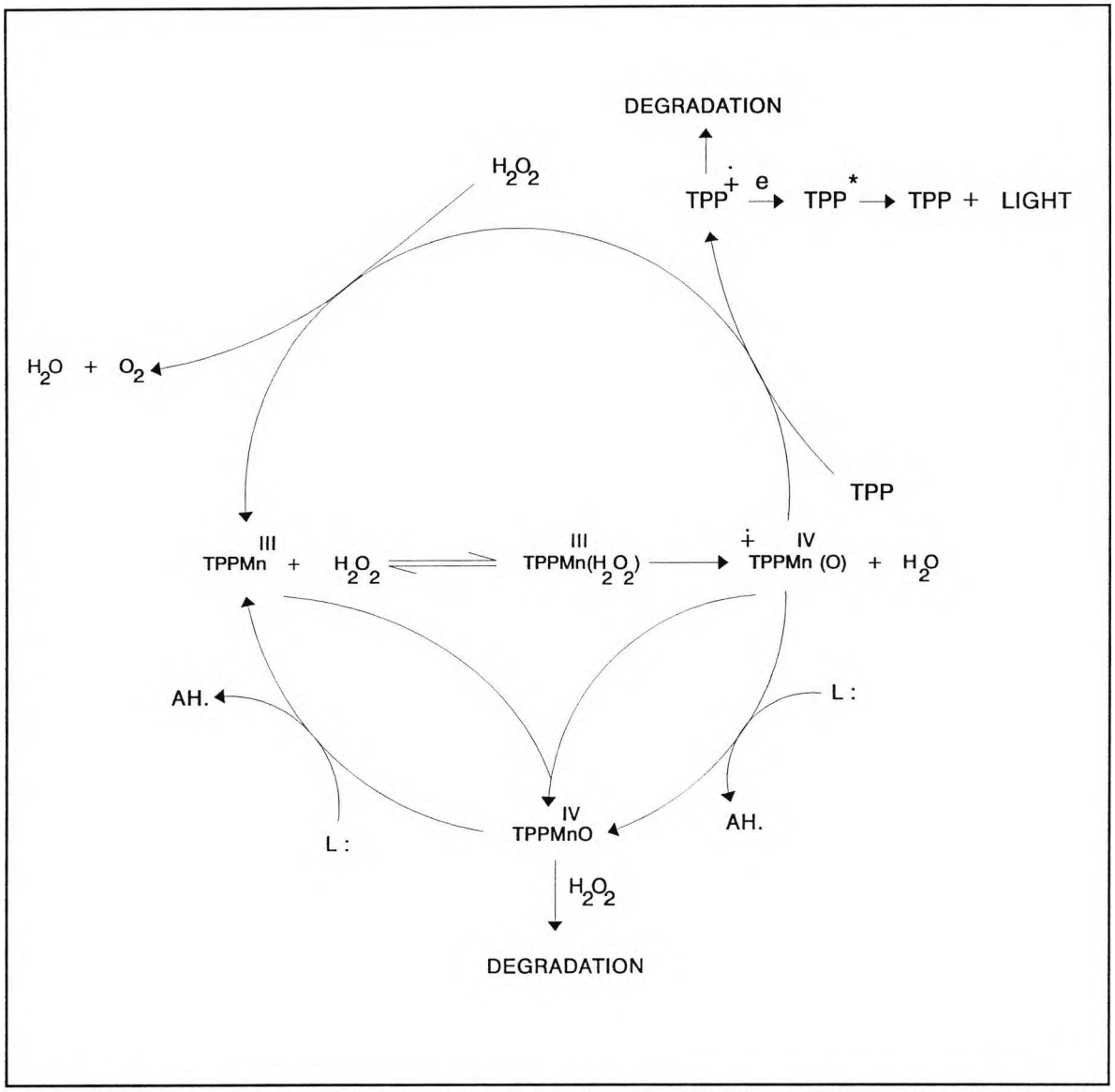
At the end of this project it was shown that a number of metalloporphyrins were good mimics for the peroxidase in catalysing the oxidation of luminol by hydrogen peroxide.

More detailed investigations on the most successful catalyst, $Mn(T_{NH2}PP)Cl$, revealed that these catalysts are also capable of catalysing the oxidative breakdown of hydrogen peroxide and other porphyrins. These side reactions adversely influence the efficiency of the catalyst.

It was also found that the catalytic activity of $Mn(T_{NH2}PP)Cl$ can be significantly enhanced by the addition of "enhancers", most notably from imidazole and its derivatives.

Among the more important findings was the superior activity of Mn porphyrins as compared to other metals, especially iron. This, together with the understanding which was gained about the role of other factors influencing the catalytic activity, including the nature of the substituents and the effect of enhancers, has gone some way towards furthering our understanding of the mechanism of the reaction. Scheme 8.1 summarises the inter-relation between all of the reactions discussed in this chapter.

Although in this work $Mn(T_{NH2}PP)Cl$ was successfully used as a replacement for HRP for chemiluminescence immunoassay on a model system with impressive sensitivity, further work is needed to elucidate the mechanism of the reactions and thereby improve assay performance.



Scheme 8.2 - A summary of the reactions involved in $Mn(T_{NH2}PP)Cl$-catalysed oxidation of luminol by hydrogen peroxide.

8.4 REFERENCES

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APPENDIX I

Sources of Chemicals

Aldrich Chemical Co. Ltd.

Tetraphenylporphyrin iron(III) chloride, tetraphenylporphyrin manganese(III) chloride, tetraphenylporphyrin nickel(II), tetraphenylporphyrin ruthenium(II) carbonyl, tetraphenyl porphyrin vanadium(IV) oxide, tetraphenylporphyrin zinc, tetrakis(4-methoxyphenyl) porphyrin, Sephadex, Merrifield's peptide resin, nylon 6/6, benzaldehyde, 4-nitrobenzaldehyde, 4-carboxybenzaldehyde, pyrrole, imidazole, 1-methylimidazole, 2-methyl imidazole, DABCO, p-iodo phenol, ufoxane, ß-cyclodextrin, oxalyl chloride, chlorosulphonic acid, potassium thiocyanate, dicyclohexyl carbodiimide, N-hydroxysuccinimide, thiophosgene,BF_3 etherate, DDQ, p-chloranil, 2,6 dichlorobenzaldehyde, trifluoroacetic acid, triethyloxonium tetrafluoroborate, mesitaldehyde, sodium cyanide, dioxane, phosphorus pentoxide, pentafluorobenzaldehyde.

Sigma Chemical Co. Ltd.

Horseradish peroxidase, human IgG.

Fisons Chemical Co. Ltd.

Dichloromethane, chloroform, benzene, methanol, carbon tetrachloride, glacial acetic acid, propanoic acid, 1,2- dichloromethane, isopropanol, ethanol, methanol, toluene, diethyl ether, THF, DMF, DMSO, heptane,acetone, dioxane, hexane, cyclohexane.

B.D.H. Chemical Co. Ltd.

30% aq. hydrogen peroxide, kieselgel, iron (II) chloride, manganese (II) chloride.

Mid Century Chemical Co. Ltd. P.O. Box 217, Posen, Illinois 60469, U.S.A.

Tertrakis 4-carboxy phenyl porphyrin.

Dr. Lionel Milgrom, Kingston Polytechnic

Tetrakis (3,5 methoxy,4 hydroxy phenyl) porphyrin.

Dr.Martin Walker. City University

$T_{pF}PP$, $Fe(T_{pF}PP)Cl$, $Fe(T_{DC}PP)Cl$.

APPENDIX II

Computer Programme for Data Capture and Processing

```
*LIST
  10 MODE7
  20*
  30 REM VAR
  40 J=0:I=0:K=0:L=0:G=0
  50 SI=16:GA=8.4
  60 BA=20
  70 EE=4000:EF=2000
  80 DIMM%(EE)
  90 DD=10
 100 DIMT(DD)
 110 DIMJ(DD)
 120 DIMR(DD)
 130 DIMS(DD)
 140 DIMD(DD)
 150 CU=23
 160 DIMFR(CU)
 170 DIMW(CU)
 180 EI=1:ZW=2
 190 FF=10
 200 DIMC(FF)
 210 FORJ=0TOFF:C(J)=INT(EXP(J)):NEXT
 220 REM INPUT
 230 PRINT"DISC (D...)"
 240 INPUT"FILENAME    ";N$
 250 IFLEFT$(N$,1)="D"THENN$=MID$(N$,2,7):GOSUB780:GOTO600
 260 IFLEN(N$)>7THENGOTO220
 270  INPUT"GAIN (SC)   ";GA
 280  IFGA>100RGA<1GOTO270
 290 INPUT "START Y/(N)";A$
 300 IFA$="N"GOTO220
 310 REM MEASURE
 320 J=1:P=1
 330 M%(J)=ADVAL(4)/SI
 340 IF(M%(J)-BA)<0GOTO330
 350 TIME=0:PRINT
 360 *FX 15,1
 370 J=J+1:M%(J)=ADVAL(4)/SI:PRINT" ";J,M%(J)
 380 FORQ=1TO80:NEXT
 390 B=INKEY(1):IFB=-1AND(J-EF)<0GOTO370
 400 T(P)=TIME:J(P)=J
 410 IFB=-1THENB1=C(1):GOTO430
 420 B=VAL(CHR$(B)):B1=C(B)
 430 P=P+1
 440 J=J+1:Y=0:FORI=1TOB1:Y=Y+ADVAL(4)/SI:FORQ=1TO80:NEXT:NEXT:M%(J)=INT(Y/
5):PRINT" ";J,M%(J)
 450 B=INKEY(0):IFB=-1AND(J-EE)<0AND(P-DD)<0GOTO440
 460 IFB>48GOTO400
 470 T(P)=TIME:J(P)=J
 480 REM END OF MEAS
 490 PRINT
 500 REM VAR N$,GA,P,T(P),J(P),M%(J)
 510 PRINT"TIME ";INT(T(P)/100);" (SEC)"
 520 PRINT"MEM  ";J(P)
 530 INPUT"SAVE ";A$
 540 IFA$="N"GOTO220
 550 GOSUB880
 560 *.
 570 INPUT"SAVE AGAIN (ANOTHER DISC)";A$
 580 IFA$="Y"GOTO550
```

```
590 GOTO600
600 REM CALC
610 PRINT
620 A$="Y":REM INPUT"CALC ";A$
630 IFA$="N"GOTO220
640 A$="Y":REM INPUT"PRINT";A$
650 VDU3:IFA$="Y"THENVDU2
660 PRINT
670 PRINT"NAME ";N$
680 PRINT"GAIN ";GA;:GF=(GA/8.4)^(1/.22):GF=1/GF:PRINT" G-FACTOR ";INT(GF*1
00
690 PRINT"TIME (SEC) AND MEMORY"
700 FORL=1TOP:PRINTT(L)/100,J(L):NEXT
710 GOSUB980
720 VDU3
730 INPUT"INTEGRATE ";A$
740 IF A$="Y" THENGOSUB1490
750 PRINT"END BACK TO MEASURE";A$
760 IFA$="N"GOTO750
770 GOTO220
780 A=OPENIN(N$)
790 INPUT£A,N$,GA,P
800 FORI=0TOP
810 INPUT£A,T(I),J(I)
820 NEXT
830 FORI=0TOJ(P)
840 INPUT£A,M%(I)
850 NEXT
860 CLOSE£A
870 RETURN
880 A=OPENOUT(N$)
890 PRINT£A,N$,GA,P
900 FORI=0TOP
910 PRINT£A,T(I),J(I)
920 NEXT
930 FORI=0TOJ(P)
940 PRINT£A,M%(I)
950 NEXT
960 CLOSE£A
970 RETURN
980 REM CALC
990 FORG=1TOP:R(G)=J(G)-J(G-1):S(G)=T(G)-T(G-1):D(G)=S(G)/R(G):NEXT
1000 REM FIND MAX
1010 Y1=-100000:J1=0:Y2=10^12:J2=0
1020 FORG=1TOP:FORJ=J(G-1)+1TOJ(G)
1030 Y=M%(J)
1040 IF(Y-Y1)>0THENY1=Y:J1=T(G-1)+(J-J(G-1))*D(G)
1050 IF(Y-Y2)<0THENY2=Y:J2=T(G-1)+(J-J(G-1))*D(G)
1060 NEXT
1070 NEXT
1080 PRINT
1090 PRINT"MAX ";INT(Y1*GF*10)/10;"  TIME (SEC) ";INT(J1)/100
1100 PRINT"MIN ";INT(Y2*GF*10)/10;"  TIME (SEC) ";INT(J2)/100
1110 PRINT
1120 REM INT
1130 Y=0:J(0)=1:FORG=1TOP
1140 FORJ=J(G-1)+1TOJ(G)
1150 Y=Y+(M%(J)+M%(J-E1))*D(G)/ZW
1160 NEXT
1170 NEXT
1180 J(0)=0
1190 MA=Y
1200 PRINT"INT (INT*SEC) ";INT(Y*GF)/100;" TIME (SEC) ";INT(T(P))/100
1210 PRINT"AVERAGE (INT) ";INT(Y*GF*10/T(P))/10
1220 PRINT
1230 REM CULM FREQUENCY
1240 W=.05:L=0:W1=.05:W2=.99
1250 J(0)=1
1260 V=0:FORG=1TOP
1270 FORJ=J(G-1)+1TOJ(G)
```

```
      V=V+(M%(J)+M%(J-EI))*D(G)/ZW
      IF(V/Y-W)>=0THENL=L+1:FR(L)=T(G-1)+(J-J(G-1))*D(B):GOSUB1470:W(L)=W:W=W+W1

1300 IFW>=1.03THENJ=J(G)+1:G=P+1:GOTO1320
1310 IFW>=1THENW=W2
1320 NEXT
1330 NEXT
1340 J(0)=0
1350 PRINT"CULM FREQ"
1360 W=.05
1370 PRINT"  %","(SEC)"
1380 FORK=1TOL
1390 PRINT" ";INT(W(K)*100),"  ";INT(FR(K))/100
1400 W=W+W1
1410 IFW>=1THENW=W2
1420 NEXT
1430 PRINT
1440 PRINT"AVERAGE 99% (INT) ";INT(MA*GF*10/FR(L))/10;" (SEC) ";INT(FR(L))/100
1450 PRINT
1455 GOSUB1700
1460 RETURN
1470 IF(V/Y-(W+W1))>=0THENW=W+W1:GOTO1470
1480 RETURN
1490 PRINT"START ";0
1500 INPUT"END   ";EN
1510 S=S*100:EN=EN*100
1520 IFEN>T(P)THENEN=T(P)
1530 V=0:FORG=1TOP
1540 FORJ=J(G-1)+1TOJ(G)
1550 IF(T(G-1)+(J-J(G-1))*D(G)-EN)>0THENJ=J(G)+1:G=P+1:GOTO1570
1560 V=V+M%(J)*D(G)
1570 NEXT
1580 NEXT
1590 VDU2
1600 PRINT
1610 PRINT"INT FROM 0 TO (SEC) ";EN/100
1620 PRINT"INT (INT*SEC) ";INT(V*GF)/100
1630 PRINT"AVERAGE       ";INT(V*GF*10/EN)/10
1640 PRINT
1650 VDU3
1660 RETURN
1700 REM
1710 N1$="F"+N$
1720 A=OPENOUT(N1$)
1730 PRINT£A,FR(L),100
1740 PRINT£A,1000/FR(L),800/100
1750 FOR K=1 TO L
1760 PRINT£A,FR(K)*1000/FR(L),INT(W(K)*100)*800/100
1770 NEXT
1780 CLOSE£A
```