ENHANCED CHEMILUMINESCENT ASSAYS FOR HORSERADISH PEROXIDASE AND THEIR APPLICATION IN IMMUNOASSAYS

by

GARY HAROLD GREGORY HENRY THORPE

A thesis submitted to the
Faculty of Science
of the
University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

Department of Clinical Chemistry
University of Birmingham
Edgbaston
Birmingham
B15 2TH
England

August 1986

UNIVERSITY OF
BIRMINGHAM

University of Birmingham Research Archive

e-theses repository

This unpublished thesis/dissertation is copyright of the author and/or third parties. The intellectual property rights of the author or third parties in respect of this work are as defined by The Copyright Designs and Patents Act 1988 or as modified by any successor legislation.

Any use made of information contained in this thesis/dissertation must be in accordance with that legislation and must be properly acknowledged. Further distribution or reproduction in any format is prohibited without the permission of the copyright holder.

SYNOPSIS

Certain 6-hydroxybenzothiazole and phenol derivatives enhance light emission from the horseradish peroxidase catalysed oxidation of cyclic diacyl hydrazides such as luminol. These reactions produce novel, enhanced chemiluminescent assays for horseradish peroxidase labels used in immunoassays. Enhanced assays are rapid and sensitive, light emission is relatively prolonged, decays slowly, and the light intensity can be a 1000-fold that of unenhanced reactions. The reactions have been partially characterised and their emission spectra shown to be remarkably similar to unenhanced reactions. Two hypotheses are proposed to explain the mechanism of enhancement.

The utility of enhanced chemiluminescent assays for horseradish peroxidase conjugates was demonstrated in a range of immunoassays, both immunoextraction and competitive, in conjunction with a variety of solid supports such as plastic beads, tubes and microtitre plates. The rapid and simply performed enhanced chemiluminescent assays have been used both to replace conventional colorimetric end-points, and in the development of rapid immunoassays. No interferences were encountered with clinical samples in enhanced chemiluminescently monitored immunoassays and results correlated well with those determined colorimetrically. Characteristics of enhanced reactions enable simplification of the design of instrumentation required for routine immunoassays, and allow quantitation of light emission using either photomultiplier tubes or instant photographic film. Such systems should facilitate the introduction of chemiluminescently monitored immunoassays into clinical laboratories.

ACKNOWLEDGMENTS

I would like to thank Professor T P Whitehead for giving me the opportunity of studying for a higher degree and for his help and encouragement throughout this research. I also wish to particularly express my gratitude to Dr Larry Kricka for his expert advice, helpful discussions and encouragement.

My thanks are also due to Richard Stott for technical advice and performing extensive modification to the computer software controlling the Berthold LB 950 luminometer, and to Roger Bunce and John Gibbons for designing and constructing various instruments used in this study. In addition, I should like to express my thanks to the staff of the Wolfson Research Laboratories, particularly Lesley Williams, Susan Moseley and Eileen Gillespie, and to staff at the Queen Elizabeth Hospital and Birmingham University who have been of great assistance.

I am indebted to many individuals including Dr N Baggett, Professor M A DeLuca, Dr F A Ala, Dr D R Stanworth, Helen Wood and Margot Wheaton, for supplying reagents or clinical samples, and to Dr S James and R K Percy for assistance in patenting various aspects of this research.

Finally, I should like to thank Amanda Griffiths for typing an initial draft of part of this study, and Adele Rawlins for preparing the remaining drafts and for her care and patience in typing the final version.

Abbreviations used in the text

Ab	- Antibody
ABTS	- 2,2'-Azino-di-(3-ethylbenzthiazoline-6-sulphonate)
ADP	- Adenosine 5'-diphosphate
AFP	- Alpha-fetoprotein
Ag	- Antigen
AMP	- Adenosine 5'-monophosphate
ASA	- American Standards Association
ATP	- Adenosine 5'-triphosphate
BSA	- Bovine serum albumin
CEA	- Carcinoembryonic antigen
CMV	- Cytomegalovirus
CV	- Coefficient of variation
DMSO	- Dimethyl sulphoxide
EIA	- Enzyme immunoassay
EMIT	- Enzyme multiplied immunoassay technique
FMN	- Flavin mononucleotide
HB_sAg	- Hepatitis B surface antigen
HCG	- Human choriogonadotrophin
HRP	- Horseradish peroxidase
IgE	- Immunoglobulin E
IgG	- Immunoglobulin G
NAD	- β-Nicotinamide adenine dinucleotide
NADH	- Reduced β-nicotinamide adenine dinucleotide
PAG	- Polyacrylamide gel
PBS	- Phosphate-buffered saline
PBST	- Phosphate-buffered saline containing Tween
PMT	- Photomultiplier tube
PTFE	- Poly(tetrafluoroethylene)
RZ	- Reinheitszahl value (ratio of absorbances at 403 and 275 nm)
T_4	- Thyroxine
Tris	- Tris(hydroxymethyl)aminomethane
Tween	- Tween 20, (monolaurate oxyethylene sorbitan)
WRL	- Wolfson Research Laboratories

Luminescence - The emission of light from atoms or molecules in electronically excited states on decay to ground state. In this thesis the term luminescence is used to denote bioluminescence and chemiluminescence it does not denote fluorescence although this is a type of luminescence.

CONTENTS

Chapter 1	Introduction	1
1.1	Rationale	1
1.2	Luminescence	2
1.3	Immunoassay	4
1.3.1	The contribution of labels to immunoassay sensitivity	5
1.3.2	Labels employed in immunoassays	9
1.4	The application of luminescence in immunoassay	11
1.4.1	The advantages and current limitations of chemiluminescence and bioluminescence in immunoassays	11
1.4.2	Immunoassays monitored using chemiluminescence or bioluminescence	18
1.4.2.1	Immunoassays employing chemiluminescent labels	20
1.4.2.2	Immunoassays employing luciferase labels	30
1.4.2.3	Immunoassays employing catalysts of chemiluminescent reactions as labels	31
1.4.2.4	Immunoassays employing cofactors of bioluminescent reactions as labels	33
1.4.2.5	Immunoassays employing chemi- or bioluminescent detection of products from conventional enzyme labels	35
1.4.2.6	Immunoassays using chemiluminescent monitoring of fluorophor-labelled conjugates	38
1.5	Summary	40
Chapter 2	The chemiluminescent and bioluminescent determination of horseradish peroxidase conjugates	
2.1	Introduction	41
2.1.1	Objectives	41
2.1.2	Techniques for peroxidase quantitation	41
2.1.2.1	Chemiluminescence and bioluminescence	43
2.1.2.2	Colorimetry	43
2.1.2.3	Fluorimetry	45
2.1.2.4	Potentiometry	47
2.1.3	Chemiluminescent and bioluminescent quantitation of peroxidase	48
2.1.3.1	Peroxidase mediated chemiluminescence with luminol and related hydrazides	48
2.1.3.2	Peroxidase mediated chemiluminescence with phenol derivatives	52
2.1.3.3	Peroxidase mediated luminescence with luciferins	53
2.1.4	Instrumentation	54
2.2	Experimental Materials and methods	57
2.2.1	Reagents	57
2.2.2	Instrumentation	58

2.2.3	Chemiluminescent and bioluminescent quantitation of peroxidase conjugates	61
2.2.3.1	The luminol, hydrogen peroxide reaction	61
2.2.3.2	The firefly luciferin enhanced luminol, hydrogen peroxide reaction	61
2.2.3.3	The luminol, perborate reaction	62
2.2.3.4	The isoluminol, hydrogen peroxide reaction	62
2.2.3.5	The Pholas dactylus luciferin, O_2 reaction	62
2.2.3.6	The purpurogallin, p-phenylenediamine, hydrogen peroxide reaction	63
2.2.3.7	The pyrogallol, hydrogen peroxide reaction	63
2.2.4	Colorimetric quantitation of peroxidase conjugates	63
2.2.4.1	o-Phenylenediamine, hydrogen peroxide	63
2.2.4.2	ABTS, hydrogen peroxide	64
2.3	Results	64
2.3.1	Reactions involving luminol	64
2.3.1.1	Unenhanced reactions	64
2.3.1.2	The firefly luciferin enhanced luminol, hydrogen peroxide reaction	67
2.3.2	The Pholas dactylus luciferin, O_2 reaction	71
2.3.3	The pyrogallol, hydrogen peroxide reaction	71
2.3.4	The purpurogallin, p-phenylenediamine, hydrogen peroxide reaction	71
2.4	Discussion	72
2.4.1	Advantages of the firefly luciferin enhanced luminol-hydrogen peroxide reaction for HRP-conjugate quantitation	72
2.4.2	Interpretation of luminescent assay performance	79
2.4.3	Summary	82
Chapter 3	Enhancement of light emission from horseradish peroxidase conjugate catalysed chemiluminescent reactions	
3.1	Introduction	84
3.1.1	Objectives	84
3.1.2	Enhanced reactions	84
3.1.2.1	Enhancement of luminescent reactions	84
3.1.2.2	Enhancement of reactions involving horseradish peroxidase	90
3.2	Experimental Materials and methods	102
3.2.1	Reagents	102
3.2.2	Investigation of enhanced chemiluminescent reactions	104
3.2.2.1	Identification of enhancers	104
3.2.2.2	The synergistic action of enhancers	105

3.2.2.3	Specificity of enhancement	106
3.2.2.4	Influence of pH	108
3.2.2.5	Influence of enhancer concentration	108
3.2.2.6	Influence of catalyst	109
3.2.2.7	Luminescent emission spectra of enhanced reactions	109
3.3	Results	111
3.3.1	Identification of enhancers	111
3.3.1.1	6-Hydroxybenzothiazole derivatives	112
3.3.1.2	Halophenols	115
3.3.1.3	Naphthols and halonaphthols	118
3.3.1.4	Reported enhancers of luminescent reactions	118
3.3.1.5	Enhancers of colorimetric HRP catalysed reactions	120
3.3.1.6	Miscellaneous compounds	120
3.3.1.7	The degree of enhancement produced by individual enhancers	123
3.3.2	The synergistic action of enhancers	123
3.3.3	Specificity of enhancement	125
3.3.3.1	Chemiluminescent compound	125
3.3.3.2	Oxidant	129
3.3.4	Influence of pH	129
3.3.5	Influence of enhancer concentration	133
3.3.6	Influence of catalyst	133
3.3.6.1	Horseradish peroxidase isoenzymes	133
3.3.6.2	Haemproteins	135
3.3.7	Luminescent emission spectra of enhanced reactions	135
3.4	Discussion	139
3.4.1	Characteristics and advantages of enhanced chemiluminescent reactions	139
3.4.2	Relationship between enhancer structure and degree of enhancement	144
3.4.3	Comparison of enhanced chemiluminescence with other enhanced systems	146
3.4.3.1	Comparison with other chemiluminescent and bioluminescent systems	146
3.4.3.2	Comparison with enhancement produced by structural modification of HRP, environmental factors or coupling reactions	147
3.4.3.3	Similarities to other enhanced colorimetric or chemiluminescent reactions	149
3.4.4	Possible mechanisms for enhanced chemiluminescent reactions	153
3.4.4.1	The 'enhancer radical' hypothesis	154
3.4.4.2	The 'inactive intermediate' hypothesis	157
3.4.5	Interpretation of experimental findings in relation to the proposed mechanisms	160
3.4.6	Evidence for possible involvement of enhancer radicals in enhanced chemiluminescent reactions	164
3.4.7	Summary	167

Chapter 4	Development of an immunometric assay for serum alpha-fetoprotein employing a horseradish peroxidase labelled specific antibody	
4.1	Introduction	169
4.1.1	Objective	169
4.1.2	Choice of assay	169
4.1.3	Assay design	171
4.2	Experimental Materials and methods	175
4.2.1	Reagents	175
4.2.2	Instrumentation	176
4.2.3	Standardised assay procedure	176
4.2.3.1	Coating polystyrene beads with anti AFP	176
4.2.3.2	Assay procedure	177
4.2.3.3	Colorimetric quantitation of bound anti AFP-HRP	178
4.2.4	Investigation of factors affecting the assay system	178
4.2.4.1	Source of antibody immobilised onto beads	178
4.2.4.2	Influence of temperature and time on coating polystyrene beads with antibody	179 179
4.2.4.3	Influence of incubation time of AFP samples with antibody coated beads	179
4.2.4.4	The influence of spring position on bead agitation	180
4.2.4.5	The influence of bead storage prior to antibody-HRP conjugate quantitation	180
4.2.5	Assay performance and validation	180
4.3	Results	181
4.3.1	Investigation of factors affecting the assay system	181
4.3.1.1	Influence of source of antibody for immobilisation onto beads	181
4.3.1.2	The influence of temperature and time of incubation on antibody immobilisation	183
4.3.1.3	The influence of incubation time on AFP binding to antibody coated beads	183
4.3.1.4	The influence of spring position on bead agitation	183
4.3.1.5	The influence of bead storage prior to quantitation of bound HRP-conjugate	185
4.3.2	Assay performance and validation	185
4.4	Discussion	188
Chapter 5	Luminescent quantitation of peroxidase conjugates in an immunometric assay for alpha-fetoprotein	
5.1	Introduction	191

5.1.1	Objectives	191
5.1.2	The advantages of luminescently monitored immunoassays	191
5.1.3	Previously reported luminescently monitored immunoassays employing peroxidase conjugates	192
5.1.4	The incorporation of luminescent procedures into immunoassays	195
5.1.4.1	Ideal requirements	195
5.1.4.2	Interferences encountered in luminescently monitored immunoassays	195
5.1.4.3	Initiation of luminescent reactions on solid supports	200
5.2	Experimental	202
5.2.1	Automatic injection of reagents onto antibody coated beads	202
5.2.2	The influence of bead position on light detection	203
5.2.3	Incorporation of luminescent assays for HRP-conjugate bound to beads in the AFP immunometric assay	204
5.2.4	Enhanced chemiluminescent assay of plasma-based AFP standards and patients' specimens	206
5.3	Results	207
5.3.1	Automatic injection of reagents onto antibody coated beads	207
5.3.2	The influence of bead position on light detection	207
5.3.3	Incorporation of luminescent assays for HRP-conjugate bound to beads in the AFP immunometric assay	210
5.3.4	Assay of plasma-based AFP standards and patients' specimens using an enhanced end-point	215
5.4	Discussion	215
5.4.1	Problems identified when initiating or monitoring luminescent reactions on polystyrene beads	215
5.4.1.1	Bead position	217
5.4.1.2	Irreproducible initiation of luminescent reactions	218
5.4.1.3	Storage of beads prior to luminescent quantitation	219
5.4.2	The performance of luminescent assays in the AFP immunometric assay using aqueous standards	220
5.4.3	The performance of enhanced chemiluminescent assays in the AFP immunometric assay using plasma-based standards	221
5.4.4	A comparison of the enhanced chemiluminescent and colorimetric AFP assay	222
5.4.5	Possible improvements in the enhanced chemiluminescent AFP assay	224

5.4.6	Summary	226
Chapter 6	Immunoassay systems employing enhanced chemiluminescent end-points to quantitate horseradish peroxidase conjugates	
6.1	Introduction	227
6.1.1	Objectives	227
6.1.2	Immunoassays employing peroxidase conjugates	227
6.1.3	Exploitation of enhanced chemiluminescent quantitation of HRP-conjugates	229
6.2	Experimental Materials and methods	234
6.2.1	Reagents	234
6.2.2	Incorporation of enhanced chemiluminescent end-points into commercially available immunoassay systems using beads or tubes	235
6.2.2.1	Light measurement	235
6.2.2.2	Assay of anti rubella virus IgG with a Rubazyme kit	237
6.2.2.3	Assay of carcinoembryonic antigen with a CEA-EIA kit	239
6.2.2.4	Assay of immunoglobulin E with an Enzygnost IgE kit	240
6.2.2.5	Assay of total thyroxine with an Enzymun-Test T_4 kit	242
6.2.2.6	Rapid assay of digoxin with an Enzymun-Test Digoxin kit	244
6.2.2.7	Rapid assay of hepatitis B surface antigen with an Auszyme II kit	244
6.2.3	Enhanced chemiluminescent end-points in immunoassay systems using black microtitre plates	245
6.2.3.1	Light measurement	245
6.2.3.2	Assay of cytomegalovirus specific IgG	246
6.2.4	Photographic monitoring of enhanced chemiluminescent immunoassays	247
6.2.4.1	Light measurement	247
6.2.4.2	Assay of serum ferritin	249
6.2.4.3	Assay of cytomegalovirus specific IgG	249
6.3	Results	250
6.3.1	Assay of anti rubella virus IgG	250
6.3.2	Assay of carcinoembryonic antigen	250
6.3.3	Assay of IgE	254
6.3.4	Assay of total thyroxine	260
6.3.5	Assay of digoxin	260
6.3.6	Assay of hepatitis B surface antigen	263
6.3.7	Assay of cytomegalovirus specific IgG	263
6.3.8	Photographically monitored assay of serum ferritin	267

6.3.9	Photographically monitored assay of cytomegalovirus specific IgG	269
6.4	Discussion	271
6.4.1	Incorporation of enhanced chemiluminescent end-points into commercially available immunoassays	271
6.4.2	Incorporation of enhanced chemiluminescent end-points into assays performed on microtitre plates	276
6.4.3	Enhanced chemiluminescent quantitation of conjugates in rapid immunoassays	278
6.4.4	Presentation of reagents employed in enhanced chemiluminescent end-points	280
6.4.5	Photographic monitoring of enhanced chemiluminescent immunoassays	280
6.4.6	Summary	284
References		287

ILLUSTRATIONS

Figures

1.1	The principles of a two-site immunometric assay	7
1.2	The principles of a competitive immunoassay	7
1.3	Techniques for employing chemiluminescent or bioluminescent reactions to detect labels in heterogeneous assays	21
1.4	Chemiluminescent compounds commonly used as labels in immunoassay	23
2.1 a,b	Compounds capable of participating in HRP catalysed reactions	46, 49
2.2	The Wolfson Research Laboratories' luminometer	59
2.3	Kinetics of light emission from luminol-H_2O_2 reactions	65
2.4	Kinetics of light emission from luminescent reactions	66
2.5	Levels of light emission in luminescent assays for antibody-HRP conjugate	68
2.6	Absorbance changes in colorimetric assays for antibody-HRP conjugate	69
3.1	6-Hydroxybenzothiazole derivatives screened for enhancer activity	113
3.2	Variation of pH with light emission from HRP-conjugate catalysed chemiluminescent reactions with luminol and hydrogen peroxide	132
3.3	Influence of 6-hydroxybenzothiazole or p-iodophenol concentration on light emission from the HRP-conjugate catalysed oxidation of luminol	134
3.4	The influence of p-iodophenol on light emission from the luminol-H_2O_2 reaction catalysed by basic HRP isoenzymes or an acidic HRP isoenzyme	137
3.5	The influence of p-iodophenol on light emission from the luminol-H_2O_2 reaction catalysed by lactoperoxidase or haemoglobin	138
3.6	Spectral energy distribution of the enhanced and unenhanced HRP-conjugate catalysed oxidation of luminol	140
3.7	Spectral energy distribution of the p-iodophenol enhanced and unenhanced HRP-conjugate catalysed oxidation of cyclic diacyl hydrazides	141
3.8	Schematic illustration of unenhanced and enhanced chemiluminescent reactions according to the 'enhancer radical' hypothesis	155
3.9	Reactions occurring in unenhanced and enhanced chemiluminescent systems according to the 'enhancer radical' hypothesis	156
3.10	Schematic illustration of unenhanced and enhanced chemiluminescent reactions according to the 'inactive intermediate' hypothesis	158
3.11	Reactions occurring in unenhanced and enhanced	159

	chemiluminescent systems according to the 'inactive intermediate' hypothesis	
4.1	Prototype Kone immunoassay timing module employed in the AFP immunometric assay	170
4.2	Temperature controlled incubation block, and coils supporting antibody coated beads	170
4.3	Binding of ^{125}I labelled AFP to antibody coated polystyrene beads	182
4.4	The influence of temperature and incubation time on binding of anti AFP to polystyrene beads	182
4.5	The influence of AFP concentration and its incubation time with antibody coated beads on antibody-HRP conjugate uptake	184
4.6	The influence of spring position on bead agitation	184
4.7	The influence of bead storage prior to quantitation of bound antibody-HRP conjugate	186
4.8	Dose-response curves in the immunometric assay using PBS or plasma-based AFP standards	187
5.1	High speed photography of automatic injection of reagent into a cuvette containing an antibody coated polystyrene bead	208
5.2	The influence of bead position on light detection	209
5.3	Dose-response curves obtained by employing luminol-oxidant chemiluminescent end-points in the AFP immunometric assay	211
5.4	Dose-response curves obtained by employing a purpurogallin-p-phenylenediamine-H_2O_2 chemiluminescent or a colorimetric end-point in the AFP immunometric assay	212
5.5	Dose-response curves obtained by employing either a pyrogallol-H_2O_2 or a Pholas dactylus luciferin-O_2 chemiluminescent end-point in the AFP immunometric assay	212
5.6	Dose-response curves obtained by employing 2,4-dichlorophenol enhanced chemiluminescent luminol-H_2O_2 end-points in the AFP immunometric assay	213
5.7	Kinetics of the light emitted using a purpurogallin-p-phenylenediamine-H_2O_2 chemiluminescent end-point in the AFP immunometric assay	214
5.8	Kinetics of the light emitted using a firefly luciferin enhanced luminol-H_2O_2 chemiluminescent end-point in the AFP immunometric assay	214
5.9	Dose-response curve obtained by employing a firefly luciferin enhanced luminol-H_2O_2 chemiluminescent end-point in the AFP immunometric assay	216
5.10	Comparison of serum AFP concentrations determined using an immunometric assay with either a firefly luciferin enhanced luminol-H_2O_2 chemiluminescent or a colorimetric end-point	216
6.1	Immunoassay solid supports used in conjunction with enhanced chemiluminescent end-points	236

6.2	Components of a camera luminometer	248
6.3	Kinetics of light emission using the firefly luciferin or the p-iodophenol enhanced chemiluminescent reaction in an immunometric assay for rubella specific IgG employing beads as solid support	251
6.4	Comparison of serum rubella antibody levels using an immunometric assay with either a p-iodophenol enhanced chemiluminescent or a colorimetric end-point	253
6.5	Standard curves for carcinoembryonic antigen using an immunometric assay employing either a colorimetric or a manually initiated p-iodophenol enhanced chemiluminescent end-point	255
6.6	Standard curves of human IgE in an immunometric assay using tubes as solid support and quantitation of peroxidase labels with either a colorimetric or firefly luciferin enhanced chemiluminescent end-point	257
6.7	Kinetics of light emission of various IgE standards using a p-iodophenol enhanced chemiluminescent end-point	258
6.8	Correlation between p-iodophenol enhanced chemiluminescent immunometric and colorimetric immunometric assay results for IgE on a series of 87 serum specimens	259
6.9	Dose-response curves for serum thyroxine using a colorimetric or a firefly luciferin enhanced chemiluminescent immunoassay	261
6.10	Standard curves of digoxin in an immunoassay using tubes as solid support and quantitation of peroxidase labels with either a colorimetric or a p-iodophenol enhanced chemiluminescent end-point	262
6.11	Standard curves for hepatitis B surface antigen in an immunometric assay employing beads as solid support and either a colorimetric or a p-iodophenol enhanced chemiluminescent end-point	264
6.12	Kinetics of light emission in a 6-hydroxybenzothiazole enhanced chemiluminescent immunometric assay for CMV specific IgG performed on black microtitre plates	265
6.13	A firefly luciferin enhanced chemiluminescent immunometric assay for a series of ferritin standards using instant photographic film to monitor light emission	268
6.14	Analysis of a series of patients' samples and ferritin standards using a photographically monitored firefly luciferin enhanced chemiluminescent immunometric assay	268
6.15	A 6-hydroxybenzothiazole enhanced chemiluminescent immunometric assay for CMV specific IgG monitored by use of instant photographic film	270

Tables

1.1	Desirable properties of labels and non-radioisotopic immunoassays	12
1.2	The advantages and current limitations of luminescence in immunoassays	13
1.3	Representative heterogeneous immunoassays monitored using chemiluminescent or bioluminescent reactions	19
1.4	Heterogeneous immunoassays using antigens labelled with luminol derivatives, and pre-extraction of samples	25
1.5	Heterogeneous immunoassay systems using luminol or luminol derivatives as labels, and no pre-extraction of samples	26
2.1	Detection limits of techniques for horseradish peroxidase estimation	42
2.2	Advantages and limitations of luminescent procedures for peroxidase quantitation	44
2.3	HRP-conjugate catalysed light emission from the oxidation of luminol alone, firefly luciferin alone and the combined reagents	70
2.4	The advantages and disadvantages of luminescent assays for HRP-conjugates	73
3.1	Enhancement of light emission from chemiluminescent systems	86
3.2	Enhancement of light emission from bioluminescent systems	87
3.3	Enhancement of light emission from luminescent reactions involving horseradish peroxidase	94
3.4	Enhancement of non-luminescent reactions involving horseradish peroxidase	96
3.5	Enhancement of horseradish peroxidase catalysed non-luminescent reactions proposed to occur by cyclic oxidation and reduction of phenol derivatives	97
3.6	Benzothiazoles screened for ability to increase light emission from the HRP-catalysed oxidation of luminol	113
3.7	Influence of benzothiazole derivatives on light emission from the luminol, hydrogen peroxide system in the presence and absence of horseradish peroxidase conjugate	114
3.8	Halophenols screened for ability to increase light emission from the HRP-conjugate catalysed oxidation of luminol	116
3.9	Enhancement of light emission from the HRP-conjugate catalysed oxidation of luminol by addition of halophenols	117
3.10	Naphthols and halonaphthols screened for ability to increase light emission from the HRP catalysed oxidation of luminol	119
3.11	Compounds exhibiting enhancement of light emission in luminescent reactions, screened for ability to	119

	increase light emission from the HRP catalysed oxidation of luminol	
3.12	Compounds exhibiting enhancement of non-luminescent HRP catalysed reactions screened for ability to increase light emission from the HRP catalysed oxidation of luminol	121
3.13	Additional peroxidase substrates screened for ability to increase light emission from the HRP catalysed oxidation of luminol	122
3.14	The degree of enhancement produced on addition of various enhancers to the HRP-luminol-H_2O_2 reaction	122
3.15	Light emission from components of enhanced chemiluminescent reactions	124
3.16	Increases in light emission from the horseradish peroxidase conjugate catalysed chemiluminescent oxidation of cyclic diacyl hydrazides by 6-hydroxybenzothiazole derivatives	126
3.17	Increases in light emission from the horseradish peroxidase conjugate catalysed chemiluminescent oxidation of cyclic diacyl hydrazides by addition of para-substituted phenols	127
3.18	The influence of various compounds on the light emission from the HRP-conjugate catalysed oxidation of purpurogallin at pH 6.5	128
3.19	The influence of oxidant on enhancement of light emission from the luminol-oxidant-HRP-conjugate reaction by 6-hydroxybenzothiazole derivatives	130
3.20	The influence of oxidant on light emission from reactions involving luminol in the presence and absence of para-substituted phenols	131
3.21	Light emission from the peroxidation of luminol catalysed by HRP isoenzymes in the absence and presence of enhancers	136
3.22	Enhancers common to HRP catalysed enhanced colorimetric and chemiluminescent reactions	150
5.1	Heterogeneous immunoassays employing chemiluminescent detection of horseradish peroxidase labels	193
6.1	Commercially available immunoassay kits employing HRP labels	230
6.2	Responses from controls in the Rubazyme assay using colorimetric or enhanced chemiluminescent end-points	252
6.3	Within-batch precision of light measurements in an assay for rubella virus specific IgG employing a p-iodophenol enhanced chemiluminescent end-point	252
6.4	Within-batch precision of light measurements in a CEA assay employing a p-iodophenol enhanced chemiluminescent end-point	256
6.5	Within-batch precision of serum IgE measurement using an immunometric assay with a p-iodophenol enhanced chemiluminescent or a colorimetric end-point	259

6.6 Light emission from bound HRP-conjugate in assays for CMV specific IgG performed on black microtitre plates and using a 6-hydroxybenzothiazole enhanced chemiluminescent end-point 266

CHAPTER 1

INTRODUCTION

1.1 Rationale

The sensitivity and specificity with which compounds of clinical interest can be quantitated using immunological methods have proved invaluable in medical research and routine diagnosis. Immunological techniques using radioactively labelled antigens (radioimmunoassay) (Yalow and Berson, 1959) and radioactively labelled antibodies (immunoradiometric assay) (Miles and Hales, 1968) have revolutionised the analysis of many biologically important substances such as proteins, hormones and drugs.

Despite the advantages of these techniques over earlier methods they still suffer from limitations derived from the radioactive nature of the label. In order to overcome the problems encountered with the stability and handling of radioactive reagents, and to penetrate the sensitivity limits currently achievable, alternatives to radiolabelling have been aggressively sought. At present the use of enzyme labels, or labels monitored using chemiluminescence or time resolved fluorescence appear to be emerging in preference to other techniques. Chemiluminescent and bioluminescent reactions have great potential in analytical chemistry as they enable rapid and very sensitive quantitation of diverse analytes. A range of compounds participating in such reactions have recently been employed in immunoassays to produce labelled compounds of high specific activity which can be rapidly and sensitively detected. Considerable effort has been devoted to the development of immunoassays based on chemiluminescent compounds such as luminol. However, horseradish peroxidase, one of the commonest enzyme labels, takes part in both chemiluminescent and bioluminescent reactions and has only had

limited application in immunoassay systems monitored in this manner. An observation on light emission from the horseradish peroxidase catalysed oxidation of luminol suggested conventional chemiluminescent assays for horseradish peroxidase could be significantly improved and light emission enhanced. This study was designed to characterise novel enhanced chemiluminescent assays and evaluate their applicability and performance in the quantitation of horseradish peroxidase labelled conjugates in immunoassay systems.

1.2 Luminescence

Luminescence is the emission of light by an atom or molecule as an electron is transferred to the ground state from a higher energy state. In chemiluminescent and bioluminescent reactions the free energy of a chemical reaction provides the energy required to produce a reaction intermediate or product in an electronically excited state, subsequent decay back to the ground state being accompanied by emission of light (Peng, 1976; Rauhut, 1979). Care should be taken not to confuse chemiluminescence with fluorescence in which incident radiation is needed to promote molecules to an excited state. Chemiluminescence is generally observed in oxidation reactions of certain classes of organic molecule. Bioluminescence is a form of chemiluminescence found in biological systems and is distinguished from chemiluminescent reactions by the necessary mediation of a protein, or enzyme (luciferase), (Cormier et al., 1973; McCapra, 1976). Generally the quantum yield, ie the number (or rate) of molecules emitting light divided by the number (or rate) of

molecules reacting, is approximately 1 to 5% for chemiluminescent reactions, whereas quantum yields approaching 100% are encountered in the more efficient bioluminescent reactions. Although the efficiencies of chemiluminescent reactions are relatively low, they are still capable of producing very sensitive assays with low detection limits using simple instrumentation. It is possible to measure extremely low levels of light using photomultiplier tubes as light emission is generated chemically, sensitivity is usually limited by background light emission rather than by light measuring capability. In order to use chemiluminescent or bioluminescent reactions analytically, the reactions must be performed under conditions in which the magnitude of light output is a function of the concentration of the reactant to be determined. The techniques differ from colorimetry or fluorimetry as measurement of light from chemi- or bioluminescent reactions is equivalent to the measurement of a reaction rate whereas in spectrophotometry or fluorimetry the readings are a direct measure of a substance present in the reaction medium (Schram, 1981).

Chemical and biochemical reactions producing light, as well as their mechanisms and applications, are the subject of numerous reviews (Campbell and Simpson, 1979; Seitz, 1981; Kricka and Thorpe, 1983). Many substances capable of participating in chemiluminescent reactions are known, perhaps the best known being cyclic diacyl hydrazides such as luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Rauhut, 1979; Roswell and White, 1978). Bioluminescence reactions are widespread in nature, but only a few bioluminescent reactions have so far been studied in

detail (Cormier, et al., 1975) The most extensively studied reactions are the firefly luciferase-luciferin reaction which can be used to assay ATP, and the marine bacterial luciferase-oxidoreductase reaction which can be used for sensitive quantitation of NADH (Wannlund and DeLuca, 1982).

The extreme sensitivity and speed of luminescent reactions, together with the ability to couple reactions of a large number of analytes to measurable species in luminescent reactions, have enabled the application of bio- and chemiluminescent reactions in numerous assay systems, including immunoassay applications, in analytical and clinical chemistry (Whitehead et al., 1979; Kricka and Thorpe, 1983).

1.3 Immunoassay

Immunoassay is a widely used analytical technique in the clinical laboratory. The technique takes advantage of the ability of antibodies to bind a specific antigen, and the antibody:antigen interaction can be monitored by adding either labelled antigen or antibody. The majority of currently used immunoassays are heterogeneous systems which require separation of immune complex from free antigen or antibody to monitor the reaction. Systems requiring no such separation to estimate the proportion of antibody bound antigen are known as homogeneous assays. Descriptions of the immunoassay configurations commonly employed and their relative advantages and disadvantages have been reviewed (Maggio, 1980).

Immunoassay systems can be divided into two classes distinguished

by whether immune complex is monitored by

a) Observation of antibody using labelled antibody - immunometric assays

b) Observation of analyte using labelled antigen - immunoassays.

Although the two forms of assay share many methodological features, they rely on different concepts, and the use of non-isotopic techniques such as chemiluminescence to quantitate high specific activity conjugates has differing influences on assay sensitivity and performance (Ekins, 1981a,b).

1.3.1 The contribution of labels to immunoassay sensitivity

Immunoassays employing labelled antibody: In these assays antigen is reacted with an excess of labelled antibody, unreacted antibody is removed and the label activity of the immune complex measured. The labelled complex is proportional to antigen concentration (Eqn. 1).

Eqn. 1

$$Ag + Ab\text{-}L \rightleftharpoons Ag{:}Ab\text{-}L + Ab\text{-}L$$

$$\downarrow$$

Separate and measure L

(L = label)

The rate of complex formation is given by k[Ag][Ab] where k, is the associative rate constant and [Ag] and [Ab] are the antigen and antibody concentrations. The theoretical sensitivity of such an assay is one molecule of antigen and maximal sensitivity is attained using an amount of antibody approaching infinity (Ekins, 1985). An example of this type of assay is the sandwich or two-site immunometric assay in which antigen is reacted with excess

solid phase bound antibody and the bound antigen determined by adding a labelled specific second antibody (Figure 1.1). In such assays the antigen must possess two spacially separated and molecularly distinct antibody binding sites, however, the use of two binding sites can impart a high degree of specificity to the systems. As the antibody is normally introduced in considerable excess, these systems are relatively free from environmental influences which alter the equilibrium constant of the reaction.

The practical sensitivity achievable in immunometric assays is governed by the minimum number of labelled antibody molecules bound to analyte which can be distinguished from any 'noise' or background that may exist. The two factors determining the detection limit of antigen bound labelled antibody are:

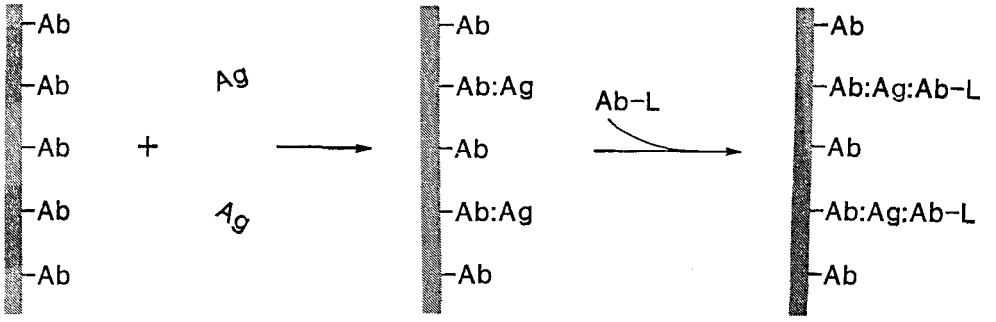
1. The specific activity of the labelled antibody, ie, the number of observable events/unit time for each antibody.
2. The background or noise level and its statistical variation.

Different labels possess different characteristics for each of these parameters and to exploit the potential sensitivity implicit in labelled antibody techniques it is necessary to develop high specific activity labels capable of emitting perceptible, precisely measurable signals against a negligible background signal using short observation periods.

Immunoassays employing labelled antigen: These immunoassays are based on competition between labelled and unlabelled antigen for a limited number of antibody binding sites. The label present in the bound fraction can be determined and related to the native

Figure 1.1

The principles of a two-site immunometric assay



EXCESS ANTIBODY IMMOBILISED ONTO SOLID SUPPORT

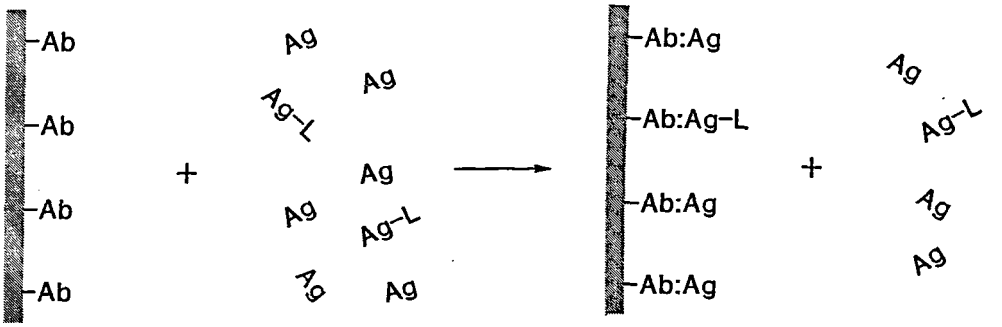
ADD ANTIGEN IN SAMPLE

ADD EXCESS LABELLED ANTIBODY CONJUGATE

MEASURE BOUND, LABELLED CONJUGATE

Figure 1.2

The principles of a competitive immunoassay



LIMITED QUANTITY OF ANTIBODY IMMOBILISED ONTO SOLID SUPPORT

ADD ANTIGEN IN SAMPLE,AND LABELLED ANTIGEN

MEASURE BOUND, LABELLED ANTIGEN

(OR MEASURE UNBOUND, LABELLED ANTIGEN)

antigen concentrations (Eqn. 2).

Eqn. 2

$$Ag + Ag\text{-}L + Ab \rightleftharpoons \underbrace{Ab{:}Ag\text{-}L}_{\text{Separate and measure L}} + Ab{:}Ag + Ag\text{-}L + Ag$$

As the technique depends on competition for binding sites by antigen, maximal sensitivity is achieved as antibody concentration approaches zero. The ultimate sensitivity of such a 'saturation' assay is governed by the equilibrium constant (K) between antigen and antibody, and is given by: E/K where E is the relative error in the experimental estimate of the amount of analyte in the complex (Ekins, 1981b). The principle of a typical competitive immunoassay is shown in Figure 1.2. The sensitivity attainable may be expressed as:

$$\frac{1}{K}\sqrt{E_s^2 + E_e^2}$$

where E_s and E_e are the signal detection and manipulation components of the error. The error in the measurement of the bound moiety consists of the error in the measurement of the signal from the label and error introduced by the various manipulations such as pipetting antibody and labelled antigen, and the separation of antibody:antigen complexes from free antigen.

The equilibrium constants of the most avid antigen:antibody reactions seldom exceed 10^{12} l/mol and as experimental errors never fall below 1%, the maximal sensitivity theoretically attainable is of the order 1×10^{-14} mol/l (Ekins, 1985). The assay design makes these immunoassays especially vulnerable to

influences which alter the equilibrium constant and kinetics of antibody:antigen reactions. Increases in the specific activity of labelled antigen and the sensitivities with which labels can be detected have only a limited influence on assay sensitivity as this is constrained by the manipulative errors.

1.3.2 Labels employed in immunoassays

Most immunoassays carried out in clinical chemistry employ a radioactive isotope (eg, ^{125}I) as a label. Despite the advantages of radioactive labels, such as their small size and the fact that nuclear decay is unaffected by factors present in serum, they still suffer from serious limitations. Radioactivity can be detected with great sensitivity, but, for example, for every 100 disintegrations of ^{125}I that occur in a minute, 1.25×10^7 atoms stand mute (Schall, Jr. and Tenoso, 1981). The signal cannot be stopped or started and in immunoradiometric assays using counting times of 1 to 2 minutes, a minimum of approximately 10^6 labelled molecules are required to yield a signal statistically distinguishable from background (Ekins, 1981b). Although, for these reasons radioactive labels produce conjugates of low specific activity, they provide a clear distinct signal for detection. Measurement however, involves relatively complex equipment. Other drawbacks include the natural chemical instability of the labels, the degradative effects that decay emanations have on nearby molecules (frequently resulting in impairment of biological activity) and the need to discard unused but outdated reagents. The danger of ionising radiation arising from nuclear decay is a minor hazard to people performing assays as the amount of radioactivity

involved is relatively small, but restrictive laws and regulations exist for the use, distribution and disposal of radioactive material.

The disadvantages of radioactive labels have stimulated a search for alternatives to the radioactive label. Alternatives that have been investigated include enzymes (Wisdom, 1976), organic free radicals (Leute et al., 1972), fluorescent molecules (Ullman et al., 1976; Watson et al., 1976), organometallic compounds (Cais et al., 1977), metal atoms (Leuvering et al., 1980), bacteriophages (Makela, 1966), cofactors (Carrico et al., 1976), synthetic particles (Ngo and Lenhoff, 1981) and erythrocytes (Alder and Lau, 1971). A comprehensive list of these labels, their detection and performance is given in several reviews (Schall, Jr. and Tenoso, 1981; Kricka, 1985). The use of non-isotopic labels such as enzymes, fluorophors or chemiluminescent molecules enable the production of conjugates with high specific activities. Care, however, is needed as the majority of non-isotopic labels are vulnerable to environmental effects which modulate the activity of the label and may be associated with high noise or background levels generated by components of the assay system other than the label. Certain chemiluminescent labels enable the production of high specific activity conjugates as they can all be triggered to emit light simultaneously. Several labels such as enzymes are not consumed during label quantitation and are capable of signal amplification. A single molecule of enzyme label for instance can catalyse the conversion of many molecules of substrate to form an observable product.

Similar effects are also seen using chemiluminescent or bioluminescent reactions in which a catalyst repeatedly produces light emission from substrates, or an enzyme label generates products that can subsequently be assayed using such techniques. Maximal benefit from this amplification is gained if the detection system employed to measure product is as sensitive as possible. Non-isotopic labels can be detected with great sensitivity, eg reported practical detection limits for chemiluminescent labels are 10^{-18} - 10^{-20} moles compared with 10^{-16} and 6 x 10^{-18} moles for ^{3}H and ^{125}I respectively (Weeks et al., 1983c). The application of luminescence in immunoanalysis is presently in a phase of rapid expansion and is discussed in detail in Section 1.4.2.

As many labels, including certain chemiluminescent labels, respond to their chemical or physical environments methods have been devised to modulate the properties of these labels by antigen:antibody interaction and achieve easily automatable, separation-free, homogeneous immunoassays. Such assays may however be subject to interference and are unlikely to be used in ultrasensitive applications. The desirable features of an 'ideal' label and non-isotopic immunoassay are listed in Table 1.1. At present no one label has shown itself to be the clear successor to the radioactive label.

1.4 The application of luminescence in immunoassay

1.4.1 The advantages and current limitations of chemiluminescence and bioluminescence in immunoassays

Chemi- or bioluminescently monitored labels fulfil many of the

Table 1.1

Desirable properties of labels and non-radioisotopic immunoassays

Labels should:

- be capable of rapid, precise and sensitive detection
- form stable, high specific activity conjugates
- be available in high purity at a reasonable cost
- retain activity after conjugation
- allow multiple labelling of conjugates
- produce minimal disturbance of the properties of the antigen or antibody
- exhibit minimum background interference from biological samples
- provide some degree of amplification

Non-radioisotopic immunoassays should:

- offer sensitivity, precision and reliability similar or superior to existing techniques
- present no health hazard
- possess potential for automation
- utilise low cost, compact, easily maintained equipment
- offer heterogeneous and homogeneous configurations

Table 1.2

The advantages and current limitations of bio- and chemiluminescence in immunoassays

Advantages:

- Sensitivity and speed of label detection
- Potential for multiple labelled, high specific activity conjugates
- Low cost instrumentation
- Amplification step often possible
- Wide range of linear response
- Stable, non-toxic reagents
- Homogeneous assays possible

Current limitations:

- Interferences
- Loss of label activity after conjugation
- Limited availability of pure, quality controlled reagents
- Lack of automated instrumentation

properties of the ideal label and have been successfully used to produce antibody or antigen conjugates of high specific activity. The advantages and current limitations of chemi- and bioluminescence in immunoassays are discussed in detail below and summarised in Table 1.2.

Advantages

The sensitivity of immunoassays is determined in part by the lower limit for detection of the label, and bio- and chemiluminescence offer extremely sensitive methods of detection for certain labels. Reported detection limits for luminol (Simpson et al., 1979), peroxidase (Puget et al., 1977), ATP (Webster et al., 1981), NADH (Stanley, 1974), glucose-6-phosphate dehydrogenase (Wannlund et al., 1982) and acridinium ester labelled antibodies (Weeks et al., 1983a) are 10^{-18}, 5×10^{-19}, 10^{-16}, 10^{-16}, 10^{-18} and 8×10^{-19} moles respectively. High specific activity conjugates can be produced and multiple labelling is often possible. Conjugates incorporating chemiluminescent labels which are consumed and only participate once in chemiluminescent reactions, can be stimulated to react almost completely within a few seconds. Other labels, including catalysts of luminescent reactions such as horseradish peroxidase, are not consumed, allowing an amplification step and the possibility of producing prolonged light emission. Chemi- and bioluminescent reactions can be monitored very rapidly. Measurement of peak light emission from chemiluminescent labels can often be made in approximately 5 seconds, and the intensity of light emission at a fixed period of time after initiation in more prolonged reactions can generally be made in under 30

seconds. In addition, the equipment required for measurement of light emission is relatively simple (essentially a light-tight detection chamber and a light detector) and there is no need for light sources, beam splitting, monochromators or sophisticated optical systems. Light emission from immunoassays performed on a variety of solid supports can be monitored and chemi- and bioluminescent reactions often exhibit a wide range of linear response. ATP estimation with the firefly luciferase-luciferin system for instance is linear over six orders of magnitude (Seitz and Neary, 1977). Chemi- and bioluminescent reagents are generally not toxic and the preparation of luminescent conjugates involves methods similar to those employed in the preparation of enzyme labelled conjugates (Kricka and Carter, 1982). Unlike radiolabelled compounds, luminescent conjugates are stable on storage and can be produced in bulk - which has important implications for the cost, quality of reagents, and reproducibility of luminescent assays (Puget et al., 1977).

Antibody binding to molecules labelled with luminescent compounds may markedly alter the reactivity of the luminescent label and homogeneous as well as heterogeneous assays are therefore possible (Boguslaski and Schroeder, 1981).

Current limitations

The major problems encountered using luminescence in immunoassays are that several reactions are prone to interference, and some loss of activity can occur when luminescent labels are covalently coupled to antigens and antibodies. In addition, few of the commercially available luminometers are appropriate for use in a

clinical laboratory dealing with large batches of specimens.

Luminescent reactions are prone to interference by a variety of compounds (Isacsson and Wettermark, 1974; Schroeder et al., 1978; Wong and Salin, 1981; Whitehead et al., 1979), and the nature of the interference varies from luminescent system to system. Care must be taken in the selection of pure reagents, including the purity of the water used to prepare buffers, as small amounts of impurity may give high background luminescence and reduce the sensitivity of label detection (Barnard et al., 1985). Biological fluids may contain variable amounts of endogenous substances that may catalyse, enhance or inhibit the luminescent reaction, or absorb the light energy emitted. Substances such as haemoglobin, haemproteins or metal ions can catalyse several chemiluminescent reactions and metabolites such as NADH or ATP which may be present can directly participate in bioluminescent reactions. Inhibition may occur by protein quenching (Boguslaski and Carrico, 1980), radical scavenging (Olsson and Thore, 1981) or by interference with enzymes directly or indirectly participating in luminescent reactions. Dilution, preliminary extraction of samples or heterogeneous assays employing efficient washing procedures help to eliminate such interferences.

Although chemiluminescent labels, such as isoluminol derivatives, can be coupled to low molecular weight compounds without loss of luminescent efficiency, covalent linkage of luminol or isoluminol derivatives to protein can drastically reduce the quantum yield of the reaction (Woodhead and Weeks, 1985). Over 90% of the chemiluminescent activity of luminol can be lost during coupling

(Campbell and Simpson, 1979). Similar effects were encountered in initial attempts to prepare luciferase conjugates, however conjugates retaining 60 to 70% of the luciferase's bioluminescent activity have recently been synthesised (Wannlund et al., 1980; Wannlund and DeLuca, 1981). The efficient preparation of enzymatically active horseradish peroxidase conjugates has been widely reported in both histochemistry and enzyme immunoassay (Tijssen and Kurstak, 1984).

Light from consumable chemiluminescent labels is emitted as a rapid, relatively low intensity flash and therefore rapid and highly reproducible initiation of individual reactions, directly in front of the photodetector is required to achieve good precision. Although this potential limitation has not prevented the successful development of a range of assays using such labels, measurement of light emission and the design of fully automated luminometers suitable for routine clinical laboratory use, is generally simplified if light is emitted from labels as a relatively constant high intensity glow. In such reactions the reagent injection is no longer a critical step and mixing of reagents directly in front of the photodetector can be completely eliminated. The commercial availability of suitable fully automated luminometers with appropriate data handling capacity, and pure quality controlled reagents, are obvious prerequisites for the introduction of luminescently monitored immunoassays into routine clinical laboratories.

1.4.2 Immunoassays monitored using chemiluminescence or bioluminescence

Chemiluminescent and bioluminescent reactions have been exploited in several ways to detect labels used in immunoassays. It is possible to distinguish 6 general categories of labelling systems, with common advantages and limitations. Possible labels include:

1) Chemiluminescent molecules which emit light and are consumed during detection.
2) Luciferases which catalyse light emission from luciferins in biological systems.
3) Catalysts of chemiluminescent reactions, for example horseradish peroxidase.
4) Cofactors of bioluminescent reactions, for example ATP.
5) Certain enzymes, commonly employed as labels in enzyme immunoassay, the products of which can be determined using chemi- or bioluminescent reactions.
6) Fluorescent molecules which can be determined by chemical excitation using chemiluminescent reactions.

Many such labels have been successfully applied to assays using clinical specimens and have been shown to be viable alternatives to radioactive labels and to produce results which correlate well with other techniques. Several other labels, such as metal complexes, have only been demonstrated in principle. Representative heterogeneous immunoassays employing labels which are detected using bio- or chemiluminescent reactions are shown in Table 1.3. The labels employed can vary in several respects.

Table 1.3

Representative heterogeneous immunoassays monitored using chemiluminescent or bioluminescent reactions

LABEL	ANALYTE	SENSITIVITY	REFERENCE
CHEMILUMINESCENT MOLECULES			
Luminol	Rabbit IgG	< 5 ng/ml	Simpson et al., 1979
4-Aminobutyl-N-ethyl isoluminol	Progesterone	1.7 pg/tube	De Boever et al., 1984
Acridinium ester	Thyroid stimulating hormone	3 μU/l	Woodhead and Weeks, 1985
LUCIFERASES			
Firefly luciferase	Methotrexate	0.5 pmoles	Wannlund et al., 1980
CATALYSTS OF CHEMILUMINESCENT REACTIONS			
Haemin	B_2-Microglobulin	10 ng/ml	Ikariyama et al., 1983
Horseradish peroxidase	Cortisol	5 pg/tube	Roda et al., 1984a
COFACTORS OF BIOLUMINESCENT REACTIONS			
ATP	Thyroxine	< 20 ng/ml	Grachev et al., 1983
CONVENTIONAL ENZYMES			
Glucose oxidase	17α-Hydroxyprogesterone	0.5 pg/tube	Arakawa et al., 1982
Pyruvate kinase	Transferrin	<0.1 μg/tube	Fricke et al., 1982
Glucose-6-phosphate dehydrogenase	Trinitrotoluene	10 attomoles	Wannlund et al., 1982

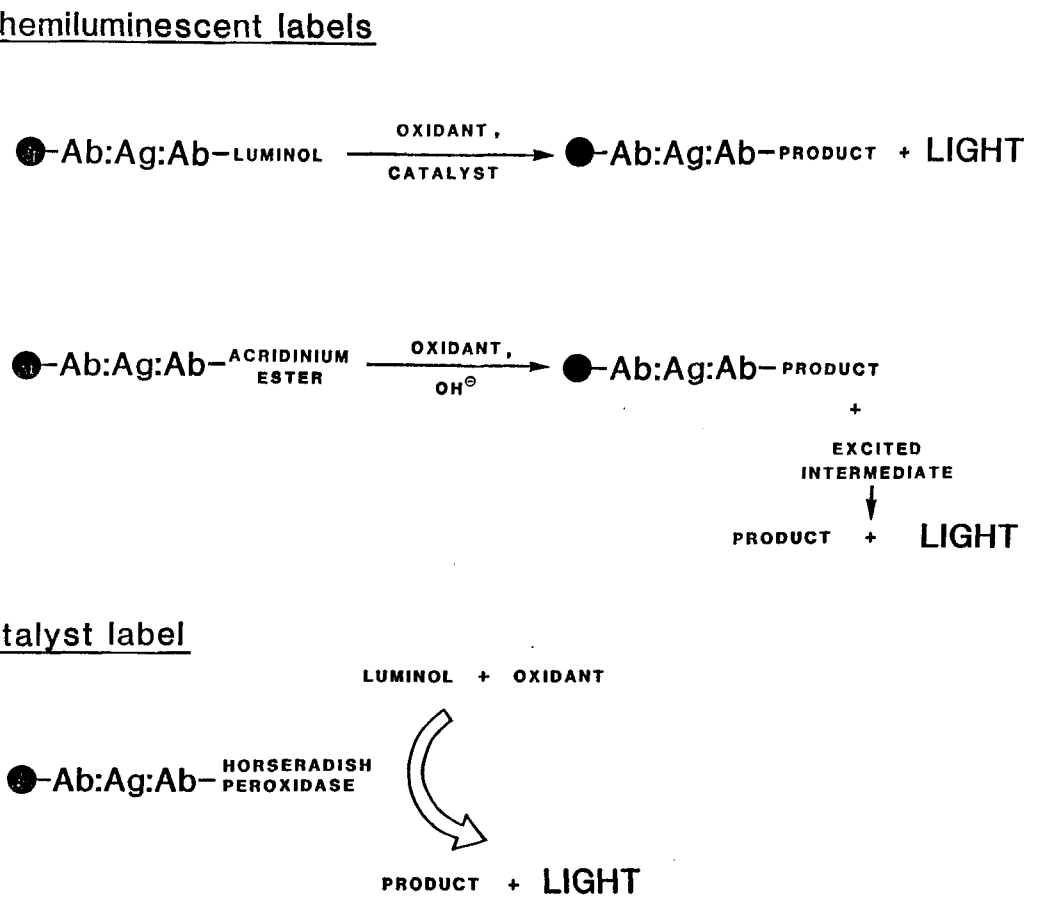
They may be either consumed in the light emitting reaction or remain intact and provide an amplification step. The principles underlying bio- or chemiluminescent detection of several labels used in immunoassay are schematically illustrated in Figure 1.3. Labels can vary in size from relatively small molecules such as luminol, 20 molecules of which can be incorporated onto a single antibody (Simpson et al., 1979), to larger molecules including enzymes such as horseradish peroxidase or luciferase which can incorporate several molecules of antigen onto their surface. Labels are generally quantitated by measuring a suitable parameter of light emission eg, peak intensity, partial integral, etc, after the addition of the appropriate reagents (Seitz, 1981). Novel chemiluminescent labels such as 1,2-dioxetanes have however been proposed which simply require thermal activation to induce light emission (Wynberg et al., 1981).

1.4.2.1 Immunoassays employing chemiluminescent labels

Studies on the application of chemiluminescence in monitoring immunoassays have centred on the use of chemiluminescent labels such as luminol or luminol derivatives, and more recently on the use of acridinium esters. The labels appear to be viable alternatives to radioactive labels and have been used in sensitive assays, for analytes in biological specimens, which were fully validated against other techniques. Conjugates produced are of high specific activity and stable. As chemiluminescent labels are relatively small molecules, multiple labelling of conjugates is often possible. Chemiluminescent end-points are initiated directly in front of photodetectors by injection of oxidant. Rapid and reproducible initiation is

Figure 1.3

Techniques for employing chemiluminescent or bioluminescent reactions to detect labels in heterogeneous assays



required as light emission exhibits rapid kinetics and occurs as a rapid flash. Measurement of peak light emission, or partial integration, can generally be made in under 30 seconds. Unlike counting of radioactive labels, initiation of end-points assumes great importance and there is no chance to repeat measurements. As chemiluminescent labels are consumed in light emitting reactions there is no amplification step in the end-point (Figure 1.3). Heterogeneous and homogeneous assays are however possible.

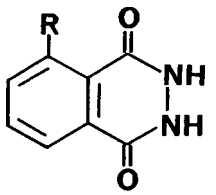
Heterogeneous immunoassays

Luminol and luminol derivative labels: In the presence of oxidant and a catalyst, luminol and its derivatives are oxidised to yield chemiluminescence. The relatively low chemiluminescent efficiency of luminol is significantly reduced after conjugation to other molecules via its amino group. In contrast, with isoluminol which is only 10% as efficient as luminol, substitution of the amino group by alkyl chains enhances the light output (Schroeder and Yeager, 1978; Schroeder et al., 1978). Luminol and a variety of isoluminol derivatives containing alkyl chains of varying lengths (Pazzagli et al., 1981a,c) and terminating with a primary amino group, have been employed in immunoassays (Figure 1.4). Considerable efforts have been devoted to study of the influence of label structure on conjugate performance, and into oxidation procedures which provide the lowest detection limits and minimise problems such as quenching of the signal.

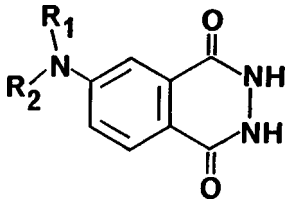
Since early reports demonstrating the feasibility of using luminol or isoluminol derivative labels in immunoassays (Maier,

Figure 1.4

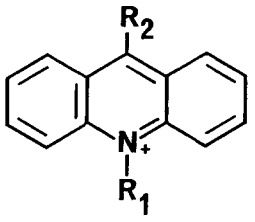
Chemiluminescent compounds commonly used as labels in immunoassay



Luminol derivatives
R = NH_2 - Luminol
R = N = N^+X^- - Diazoluminol



Isoluminol derivatives
R_1 and R_2 = H - Isoluminol
R_1 = C_2H_5 R_2 = $(CH_2)_2NH_2$ - 2-Aminoethyl-N-ethyl isoluminol
R_1 = C_2H_5 R_2 = $(CH_2)_4NH_2$ - 4-Aminobutyl-N-ethyl isoluminol
R_1 = C_2H_5 R_2 = $(CH_2)_5NH_2$ - 5-Aminopentyl-N-ethyl isoluminol
R_1 = C_2H_5 R_2 = $(CH_2)_6NH_2$ - 6-Aminohexyl-N-ethyl isoluminol



Acridine derivatives
R_1 = CH_3 R_2 = $COOR_3$ where R_3 is an alcohol
eg a sterol - Acridinium ester
R_1 = CH_3 R_2 = N-methyl acridine - Lucigenin
(9, 9'-bis-(N-methylacridinium nitrate)

1977; Pratt et al., 1978; Schroeder et al., 1978; Simpson et al., 1979; Woodhead et al., 1979) over 30 heterogeneous assay systems, for steroids, hormones and other analytes, using such labels have been reported. Representative assays employing luminol derivatives as chemiluminescent labels are collected in Tables 1.4 and 1.5. The vast majority of assays are competitive immunoassays using steroid conjugates labelled with a single molecule of 4-aminobutyl-N-ethyl isoluminol. Despite problems of quenching and poor chemiluminescent efficiency when labels are associated with proteins and peptides, there are reports of several successful assays employing such conjugates. Separation of antibody bound and free fractions in immunoassays using luminol derivative labels can be achieved using either liquid or solid phase reagents. Liquid phase separation, via a second antibody or dextran coated charcoal, has been used in assays for analytes such as progesterone (Pazzagli et al., 1981d), cortisol (Messeri et al., 1981) or 17 α-methyltestosterone (Jansen et al., 1985b). The separation step does not however successfully remove interferences from serum and urine samples and a preliminary extraction step is required. Several immunoassays achieve separation using reagents adsorbed or covalently bound to solid supports such as plastic tubes or beads and small polyacrylamide beads. Although extraction steps have been employed in assays for serum or urinary analytes (Table 1.4) a range of assays requiring no initial purification of serum, plasma, or urine samples are possible (Table 1.5). The chemiluminescent assays have excellent sensitivity and correlate well with standard methods such as radioimmunoassay. Although problems with

Table 1.4

Heterogeneous immunoassays using antigens labelled with luminol derivatives, and pre-extraction of samples

ANALYTE	SAMPLE	SEPARATION PROCEDURE	SENSITIVITY	REFERENCE
4-AMINOBUTYL-N-ETHYL ISOLUMINOL LABELS				
Progesterone	Plasma	Polystyrene tubes	10pg/tube	Kim et al., 1982b
"	"	Dextran coated charcoal	5pg/tube	Pazzagli et al., 1981d
"	"	Tubes	4pg/tube	Kohen et al., 1981
Unconjugated oestriol	Serum	Amerlex particles	1pg/tube	Klinger et al., 1982
Oestradiol -17β	Plasma	Polypropy-lene tubes	1.5pg/tube	Kim et al., 1982a
"	"	Beads	5pg	De Boever et al., 1983
Melatonin	Serum	Dextran coated charcoal	4pg/tube	Gupta et al., 1985
Cortisol	Urine, plasma	"	10pg/tube	Messeri et al., 1981
"	"	"	10 pg/tube	Pazzagli et al., 1981b
17α -methyl testosterone	Urine	Polystyrene tubes	10pg/tube	Jansen et al., 1985
6-AMINOHEXYL-N-ETHYL ISOLUMINOL LABELS				
Thromboxane B_2	Plasma	Polypropy-lene tubes	4.1pg/tube	Weerasekera et al., 1983b

Table 1.5

Heterogeneous immunoassay systems using luminol or luminol derivatives as labels, and no pre-extraction of samples

ANALYTE	SAMPLE	SEPARATION PROCEDURE	SENSITIVITY	REFERENCE
LUMINOL				
Mumps virus antibody	Serum	Polystyrene beads	-	Konishi et al., 1980
DIAZOLUMINOL				
Transferrin Caeruloplasmin Orosomucoid Ferritin Thyroxine binding globulin Thyroglobulin Gentamicin Alpha-fetoprotein Cortisol	Serum	Beads	-	Wood, 1984; Wood et al., 1984
2-AMINOETHYL-N-ETHYL ISOLUMINOL				
Oestrone-3-glucuronide*	Urine	Polystyrene beads	1.45 pg/tube	Weerasekera et al., 1982
4-AMINOBUTYL-N-ETHYL ISOLUMINOL				
Thyroxine*	Serum	Sephadex column	20 ng/ml	Schroeder et al., 1979
Choriogonadotrophin*	Urine	Polyacrylamide beads, Tubes	2 IU/l	Barnard et al., 1984
Luteinising Hormone*	Urine	Tubes	0.2 U/l	Brokelbank et al., 1984
Oestriol 16α glucuronide*	Urine	Polystyrene tubes	3.8 nmol/l	Barnard et al., 1981b
Testosterone 17β-D-Glucuronide*	Urine	Polystyrene tubes	8 pg/tube	Vannucchi et al., 1983
Cortisol*	Urine, Plasma	Dextran coated charcoal	0.18 nmol/l	Tommasi et al., 1984
Cortisol*	Urine, Plasma	Polystyrene beads	< 25 nmol/l	Strasburger et al., 1982
Cortisol*	Serum	Tubes	54 pg/tube	Lindstrom et al., 1982
Progesterone*	Serum	Polyacrylamide beads	1.7 pg/tube	De Boever et al., 1984
6-AMINOHEXYL-N-ETHYL ISOLUMINOL				
Hepatitis B surface antigen	Serum	Microtitre plate	2 ng/ml	Schroeder et al., 1981
Pregnanediol-3-α glucuronide*	Urine	Tubes	15 pg/tube	Barnard et al., 1981a
			30 pg/tube	Eshhar et al., 1981
4-AMINOBUTYL-N-ETHYL NAPHTHALENE DICARBOXYLIC ACID HYDRAZIDE				
Transferrin*	Seminal plasma	Antibody precipitation	2 ng/tube	Caldini et al., 1986

Assays employed: labelled antibody or *labelled antigen

imprecision can be encountered, the coefficient of variation for intra-assay replicates is typically in the range 5 - 10% (Seitz, 1984).

Chemiluminescent reactions for label detection have been performed under conditions ranging from pH 8.6 to pH 13.0. Light emission can be measured within 30 seconds of oxidant addition, and rapid chemiluminescent end-points are used in a range of assays (Wood et al., 1984). In several immunoassays however, the end-point measurement is preceded by an incubation at alkaline pH and high temperature, to improve sensitivity and the intensity of light emission (Barnard et al., 1985). Incubation with sodium hydroxide, before label oxidation, facilitates dissociation of antibody bound complex and may break up the labelled conjugate by alkaline hydrolysis resulting in the release of the chemiluminescent moiety. Incubations ranging from 15 to 60 minutes at temperatures up to 80^{o}C have been employed, although optimal conditions in terms of temperature, time and sodium hydroxide concentration appear to vary for individual assays. Such procedures are however inconvenient, introduce an additional source of imprecision, and dramatically increase the time required to perform the chemiluminescent end-point.

Acridinium ester labels: Woodhead and Weeks (1985) have recently exploited acridinium esters as chemiluminescent labels in several immunoassays, and although similar in several respects to luminol derivatives, they offer additional advantages. Acridinium esters have higher chemiluminescence efficiencies than luminol and as chemiluminescent reactions can be initiated simply by adding

dilute alkaline hydrogen peroxide without any requirement for a catalyst, problems of background emission and interference are minimised. Reactions of acridinium ester labels involve oxidative cleavage to yield a dioxetanone intermediate before the formation of the excited product molecule, N-methyl acridone. As a result of cleavage the N-methyl acridone is dissociated from hapten or protein prior to light emission (Figure 1.3) so that quenching of light emission in conjugates is avoided (Woodhead and Weeks, 1985) and labelled conjugates, particularly those with proteins, retain a relatively high quantum yield. As light emission is very rapid, virtually complete in 300 milliseconds, end-points can be performed in 10 to 20 seconds without any complicated chemical hydrolytic procedures.

Acridinium ester labels have been successfully used, in conjunction with cellulose and paramagnetic particles as solid support, in several sensitive immunometric assays performed on unextracted serum specimens, and in a competitive immunoassay for progesterone using plasma extracts. Assays for thyroid stimulating hormone (Woodhead and Weeks, 1985), alpha-fetoprotein (Weeks et al., 1983b), ferritin (Miller et al., 1985) and progesterone (Richardson et al., 1985) were validated with clinical specimens using alternative techniques such as radioimmunoassay. The potential performance of acridinium ester based assays is illustrated by the thyroid stimulating hormone immunometric assay which has an excellent sensitivity of 0.003 mU/l and a working range of 0.015 to greater than 60 mU/l.

Certain limitations of luminol labels are also shared by

acridinium esters. The labels are consumed, provide no amplification step, and as light is emitted as a relatively low intensity flash, individual reactions require rapid and reproducible initiation in front of sensitive photodetectors. In addition, conjugates must be used and stored under appropriate conditions to avoid pseudo-base formation and dark reactions which can result in loss of chemiluminescent activity.

Homogeneous immunoassays

Light emission from several isoluminol conjugates can be enhanced (or the kinetics changed) in the presence of specific antibody or binding protein (Schroeder et al., 1976b; Kohen et al., 1985). Enhancement is inhibited by homologous ligand in a dose-dependent manner and thus chemiluminescent assays requiring no separation of antibody bound and free ligands are possible. Assays based on this principle have been developed for progesterone (Kohen et al., 1979; Pazzagli et al., 1982), oestriol-16 α glucuronide (Kohen et al., 1980a), oestriol (Kohen et al., 1985) and total oestrogens (Messeri et al., 1984). Similar homogeneous assays for cortisol in which homologous antibody delayed light emission from a cortisol-aminopentyl-ethyl isoluminol conjugate (Kohen et al., 1980b) and for oestriol in which peak emission is unaltered but total light emission increased (Kohen et al., 1985) have also been reported. Homogeneous assays are simple to perform, amenable to automation and sensitivities comparable to radioimmunoassay can be achieved. However, labels are consumed, enhancement does not occur with all antibodies and conjugates, and most importantly the utility of the assays is limited as they are particularly prone to interference even using extracts of

plasma or diluted urine (Kohen et al., 1985).

1.4.2.2 Immunoassays employing luciferase labels

The feasibility of using firefly luciferase or bacterial luciferase as labels in immunoassays was first demonstrated by Wannlund and DeLuca (1981) and subsequently refined by Jablonski (1985). Luciferase labels, similar in size to conventional enzyme labels such as glucose-6-phosphate dehydrogenase, can be sensitively detected using bioluminescent reactions which are potentially more specific than chemiluminescent reactions although the necessary reagents are more expensive. Luciferase labels are not consumed in the bioluminescent end-point and therefore an amplification step is possible (Figure 1.3). Measurement of light emission can be made in approximately 10 seconds and high speed initiation of reactions is unnecessary as the kinetics of emission are slower than with chemiluminescent labels. Luciferase conjugates are stable and can be stored frozen, at 0 to 4°C, or lyophilised in a similar manner to conjugates used in enzyme immunoassays.

Although luciferases are susceptible to inactivation during conjugation procedures, bacterial luciferase-antibody conjugates (Jablonski, 1985) and firefly luciferase-trinitrotoluene conjugates retaining approximately 90% bioluminescent efficiency (Wannlund and DeLuca, 1982) can be prepared. Bacterial luciferase labels have been employed in a heterogeneous immunometric assay for serum rubella specific IgG (Jablonski, 1985), and firefly luciferase conjugates in competitive immunoassays capable of detecting 1 pmole trinitrotoluene

(Wannlund and DeLuca, 1982) and 0.5 pmole methotrexate (Wannlund et al., 1980). There are however, no reports of studies using serum samples.

1.4.2.3 Immunoassays employing catalysts of chemiluminescent reactions as labels

The chemiluminescent oxidation of molecules such as luminol is catalysed by a variety of compounds including horseradish peroxidase, complexed transition metals, haemin, haemproteins and metal ions (Ewetz and Thore, 1976; Campbell and Simpson, 1979). These compounds can be detected very sensitively using excess chemiluminescent substrate and oxidant, and several have been used as labels in heterogeneous immunoassays. Catalysts produce conjugates of high specific activity as they may not be consumed and a large amplification step is sometimes introduced with individual labels repeatedly stimulating light emission (Figure 1.3). Chemiluminescent catalyst labels are stable and range in size from relatively small molecules, such as complexed transition metals, to relatively large haemproteins such as horseradish peroxidase. Several catalysts possess functional groups available for labelling and retain catalytic activity after conjugation. Measurement of light emission can generally be made in under 60 seconds and the kinetics of emission are such that there is little reliance on initiation directly in front of photodetectors. Potential limitations of catalytic labels are that some light emission can occur from chemiluminescent substrates and oxidant in the absence of catalysts, and this background emission can limit sensitivity. In addition, several

catalysts of chemiluminescent reactions such as haemoglobin are encountered in biological fluids and non-specific binding of serum components to immunoassay solid supports must be avoided to eliminate interferences reducing sensitivity.

Numerous naturally occurring substances such as cytochrome c, ferritin, catalase, haemoglobin and microperoxidase are advocated for use in the patent literature (Forgione and Henderson, 1983; Hara et al., 1983a,b) and the feasibility of using small stable molecules including metal chelates such as haemin as labels, has been demonstrated in model competitive and immunometric assays for human serum albumin (Ikariyama et al., 1982; Ikariyama et al., 1984) and β_2-microglobulin (Ikariyama et al., 1983). No studies with biological specimens were however reported. Hara and colleagues have demonstrated the feasibility of using chemically synthesised metal complexes such as iron (III) 4,11,18,25-tetracarboxyphthalocyanine (Hara et al., 1983b), iron (III) 2,9,16,23-tetrakis(chlorocarbonyl)phthalocyanine (Hara et al., 1983a) or iron (III) 1,8,15,22-tetrakis(chloroformyl)phthalo-cyanine (Hara et al., 1985) as labels in assays for albumin. Precision was relatively poor and studies did not employ clinical specimens.

Horseradish peroxidase is widely used as a catalyst of chemiluminescent reactions involving luminol or other chemiluminescent substrates and has been used as a label in a range of chemiluminescently monitored heterogeneous immunoassays (Chapter 5, Table 5.1). There are several chemiluminescent assays for horseradish peroxidase (Chapter 2) ranging from assays

in which enzymatic activity is retained, to those at high pH where haem acts as the label and the remaining protein simply provides functional groups for covalent attachment to other molecules. The performance of chemiluminescent assays for horseradish peroxidase labels in heterogeneous immunoassays is outlined in Chapter 5. A variety of separation procedures are employed and although some problems have been encountered with interferences and limited sensitivity due to background light emission, sensitive assays which used unextracted serum samples and correlated well with other techniques have been reported. Although horseradish peroxidase is a larger molecule than chemiluminescent labels and catalysts such as haemin or metal chelates, numerous conjugates with both small molecules such as steroids, and large protein molecules including antibodies have been prepared. The stability and preparation of horseradish peroxidase conjugates has been studied in enzyme immunoassay and the development of assays monitored using chemiluminescent techniques is greatly assisted by the commercial availability of HRP-conjugates.

1.4.2.4 Immunoassays employing cofactors of bioluminescent reactions as labels

Several cofactors of bioluminescent reactions, including ATP and NAD, can be extremely sensitively detected using bioluminescent reactions and have been advocated as labels in immunoassays (Boguslaski et al., 1983) (Figure 1.3). Relatively prolonged light emission is possible and purified bioluminescent reagents to quantitate ATP or NAD are available commercially. The feasibility of using derivatives of NAD and ATP as labels has

been demonstrated in heterogeneous and homogeneous assays but the systems have not found widespread application. Conjugation of cofactors can lead to considerable decreases in activity and ATP derivatives appear only to be active after liberation of ATP from conjugates. The possibility of amplifying signals from cofactor labels, via cycling reactions (Carrico et al., 1976), has yet to be fully realised in bioluminescently monitored immunoassays.

Grachev and colleagues (1983) have successfully employed ATP labelled thyroxine and myoglobin conjugates, which are easily prepared and stable, in competitive heterogeneous immunoassays using a limited number of serum specimens. Although ATP can be rapidly assayed using the firefly luciferase-luciferin reaction, an incubation period of 90 minutes is required to release ATP into solution from conjugates prior to the bioluminescent end-point. Derivatives of NAD, which can be detected using the bacterial bioluminescent system, have been employed in competitive, heterogeneous immunoassays for oestriol-16 - glucuronide (Kohen et al., 1985) and progesterone (Hughes et al., 1984). Prior to rapid detection with the bacterial bioluminescent system, NAD conjugates require reduction to NADH (using for example glucose-6-phosphate and glucose-6-phosphate dehydrogenase) in incubations which take a minimum of 10 minutes. Studies involving clinical specimens have yet to be reported.

The activity of cofactor labels in conjugates can be modulated by binding of homologous antibody and it is possible to develop homogeneous assays. Carrico and co-workers (1978) have developed an assay for dinitrophenol, using an ATP labelled conjugate, the

activity of which is reduced on antibody binding. However, some problems were encountered from endogenous ATP in samples, degradation of conjugates by endogenous enzymes, and interference of bioluminescent reagents by constituents of samples (Carrico et al., 1978). Homogeneous assays for biotin, dinitrophenol (Schroeder et al., 1976a), progesterone (Hughes et al., 1984) and oestriol-16α glucuronide (Kohen et al., 1985) using NAD derivative labelled conjugates have also been described, but the assays suffer from similar limitations to homogeneous assays using ATP-conjugates and there are no reports of studies with biological samples.

1.4.2.5 Immunoassays employing chemi- or bioluminescent detection of products from conventional enzyme labels

The products of several conventional enzyme labels used in immunoassay can be very sensitively assayed using chemiluminescent or bioluminescent reactions. Enzyme conjugates can be prepared without significant reduction in catalytic activity, they are not consumed, and significant amplification is produced as many enzymes have turnover numbers that yield more than 100,000 product molecules per minute per enzyme (Mattiasson and Borrebaeck, 1980). Products from enzyme catalysed reactions are produced in solution and chemi- and bioluminescent reactions can be performed away from solid supports (Figure 1.3). As the technique is not limited by quenching (Wood et al., 1982) it is possible to use both protein and lower molecular weight conjugates. Several enzyme labels including oxidases, dehydrogenases and kinases have been employed in sensitive

heterogeneous immunoassays by measurement of their products, H_2O_2, NADH and ATP, respectively. The chemi- and bioluminescent reactions employed do not generally require initiation directly in front of photodetectors and several immunoassays have been validated against other techniques, using clinical samples. Although such end-points are rapid, a potential limitation of the technique is the incubation of enzyme labels with substrate, often required to build up appropriate concentrations of product. Fifty minute and overnight incubations are employed in several assays, significantly increasing the overall time required to quantitate labels.

Glucose oxidase labels catalyse the oxidation of glucose and the formation of hydrogen peroxide which can be assayed chemiluminescently using luminol and an appropriate catalyst, or an oxalate ester-fluorophor combination. Sensitive heterogeneous assays have been developed using conjugates of glucose oxidase with relatively small molecules such as 17 α-hydroxyprogesterone (Arakawa et al., 1982; Tsuji et al., 1984) and thyroxine (Arakawa et al., 1985), and with specific antibodies in assays for alpha-fetoprotein (Maeda and Tsuji, 1985) and human choriogonadotrophin (Botti et al., 1982). Acceptable precision was achieved, studies were performed using unextracted serum specimens and dried blood spotted onto filter paper, and results were shown to correlate with those obtained using alternative techniques. Although chemiluminescent end-points can be performed in under 30 seconds, they are proceeded in several assays by lengthy incubations with glucose to accumulate hydrogen peroxide. Similar sensitive chemiluminescently monitored immunoassays employing β-D-

galactosidase labels have been described for phenytoin, digitoxin, alpha-fetoprotein and thyroid stimulating hormone (Takayasu et al., 1985). Lactose and glucose oxidase were used as substrate and coupling enzyme respectively, and hydrogen peroxide, generated in a 2 hour incubation at 37°C, determined chemiluminescently using bis(2,4,6-trichlorophenyl)-oxalate in the presence of a fluorescent dye.

The production of NADH by labels such as glucose-6-phosphate dehydrogenase can be followed using the marine bacterial bioluminescent system which is 25,000 times more sensitive for NADH detection than spectrophotometry (Stanley, 1974). The feasibility of assays based on this system has been demonstrated in a competitive heterogeneous immunoassay for trinitrotoluene (Wannlund et al., 1982). NADH formed by the enzyme label during a 10 minute incubation with substrate was determined bioluminescently, and the assay had a detection limit for trinitrotoluene of 10^{-17} moles. Stanley (1979) has also demonstrated the potential of monitoring homogeneous EMIT assays, employing oxidoreductase labels, in a similar manner in an assay for phenytoin. Immunoassay systems employing immobilised reagents which do not require a separation step to remove excess free glucose-6-phosphate dehydrogenase labels have recently been reported (Terouanne et al., 1986). The assays employ an immunosorbent which consists of bacterial luciferase, FMN oxidoreductase and specific antibody coimmobilised onto Sepharose. Glucose-6-phosphate dehydrogenase labels coupled to antigen specifically bound to the antibody produces NADH which is

directly used by the nearby bioluminescent enzymes to produce light. NADH produced in solution however is directly oxidised without emitting light.

Pyruvate kinase conjugates capable of converting ADP to ATP can be quantitated using the firefly luciferase-luciferin reaction. Such conjugates have been utilised in heterogeneous immunoassays for immunoglobulin (Reichard and Miller, 1981) transferrin (Fricke et al., 1982) insulin, insulin antibodies and gentamicin (Wood et al., 1982). Clinical specimens were analysed and the assays shown to be comparable to radioimmunoassay both in sensitivity and reproducibility. Although an incubation with ADP and phosphoenol pyruvate can be used to accumulate ATP prior to the bioluminescent end-point, the rate of ATP production by labels can be determined by measurement of light emission between 30 and 60 seconds after initiation of reactions (Fricke et al., 1982).

1.4.2.6 Immunoassays using chemiluminescent monitoring of fluorophor-labelled conjugates

Chemiluminescent reactions have been used to excite chemically, conventional fluorophor-labelled conjugates in immunoassays (Boguslaski and Carrico, 1980; Carrico and Boguslaski, 1984). Chemically excited intermediates transfer energy to fluorophors which then emit light thus obviating the need for photogenic excitation of the label. Stable and easily prepared fluorescent labels can be employed, and as the labels are not consumed they can be repeatedly stimulated producing amplified signals. Reactions are generally free from protein quenching (Boguslaski

and Carrico, 1980) but background chemiluminescence and fluorescence from contaminants can be problematical (Seitz, 1984). Filters are also required to ensure that only light emission arising from the fluorophor is detected. Boguslaski and Carrico have demonstrated the feasibility of detecting lissamine rhodamine B labels using a reaction between hydrogen peroxide and an oxalate ester in heterogeneous and homogeneous assays for sisomicin (Boguslaski and Carrico, 1980; Carrico and Boguslaski, 1984). Background light emission was however high and studies with clinical specimens were not reported.

Conjugates employing chemiluminescent labels such as aminobutyl-ethyl isoluminol, can also be employed in homogeneous assays involving non-radiative energy transfer. The principle of the assays is that the wavelength of emitted light shifts when chemiluminescently labelled haptens or proteins bind to specific fluorescein-labelled antibody (Campbell and Patel, 1983). Although sensitive assays for serum IgG and cyclic AMP extracted from biological samples have been developed and shown to correlate well with radioimmunoassay (Patel and Campbell, 1983) the technique has several potential limitations. Antigen and antibody labelled conjugates are required, and light levels are low with little amplification. Light emission must be monitored simultaneously at two different wavelengths and the ratio of the two measurements determined. In addition, relatively complex instrumentation involving two photomultiplier tubes and appropriate interference filters is required.

1.5 Summary

Sensitive quantitation of labels in immunoassays can be performed rapidly using chemiluminescent or bioluminescent reactions. The detection of horseradish peroxidase labels is one of a number of ways in which such reactions have been exploited in end-points in heterogeneous immunoassays. Despite the potential advantages over other techniques, the use of conventional luminescent end-points to quantitate horseradish peroxidase labels has not found widespread applicability, mainly due to problems of interference and high background light emission. An observation which suggested horseradish peroxidase catalysed light emission from luminol could be increased prompted this study designed to investigate 'enhanced chemiluminescent' assays for horseradish peroxidase labels and to determine their applicability in immunoassay.

CHAPTER 2

THE CHEMILUMINESCENT AND BIOLUMINESCENT DETERMINATION OF HORSERADISH PEROXIDASE CONJUGATES

2.1 INTRODUCTION

2.1.1 Objectives

The advantages of employing catalysts of luminescent reactions as labels (eg HRP) and their subsequent luminescent determination in immunoassay systems have been discussed in Chapter 1. The objectives of this chapter were:

1) to compare the various luminescent methods for the quantitation of HRP-conjugates in solution, with particular emphasis on their sensitivity, kinetics of light emission, interferences, signal to background ratio, and compatibility with the available luminometer.

2) to compare the luminescent techniques with those methods such as colorimetry conventionally used to quantitate HRP-conjugates.

2.1.2 Techniques for peroxidase quantitation

The enzyme horseradish peroxidase (EC No 1.11.1.7) catalyses the oxidation of a wide range of substrates (Figures 2.1a and b) and it can be quantitated by monitoring such reactions under conditions where peroxidase is the limiting reagent. A variety of techniques including colorimetry, fluorimetry, the use of ion-specific electrodes, and luminescence (bio- and chemiluminescence) can be employed to monitor these reactions. Typical detection limits for HRP using colorimetric, fluorimetric or luminescent assays are collected in Table 2.1. This data should however be interpreted cautiously since HRP is unstable in dilute solutions potentially producing variable, and underestimated results (Puget et al., 1977). The high turnover

Table 2.1

Detection limits of horseradish peroxidase assays

Reagents	Principle	Detection limit (moles)	Reference
Pholas dactylus luciferin/O_2	Bioluminescence	4×10^{-19}	Puget *et al.*, 1977
Purpurogallin/p-phenylenediamine/H_2O_2	Chemiluminescence	5×10^{-16}	Halmann *et al.*, 1979
ABTS/H_2O_2 (20 hour incubation)	Colorimetry	5×10^{-16}	Groome, 1980
3-(p-Hydroxyphenyl)propionic acid/H_2O_2	Fluorimetry	6.5×10^{-16}	Zaitsu and Ohkura, 1980
Luminol/H_2O_2/pH 5.0	Chemiluminescence	1×10^{-15}	Campbell and Simpson, 1979
Luminol/H_2O_2/pH 8.0	Chemiluminescence	1×10^{-15}	Arrio *et al.*, 1980
Purpurogallin/H_2O_2	Chemiluminescence	2×10^{-15}	Velan *et al.*, 1979
MBTH/DMAB/H_2O_2	Colorimetry	6×10^{-15}	Ngo and Lenhoff, 1980
Luminol/perborate, NaOH preincubation	Chemiluminescence	7×10^{-15}	Ewetz and Thore, 1976
Pyrogallol/H_2O_2	Chemiluminescence	2×10^{-14}	Velan *et al.*, 1979 Halmann *et al.*, 1979
ABTS/H_2O_2 (30 minute incubation)	Colorimetry	2×10^{-14}	Velan *et al.*, 1979
Luminol/H_2O_2/pH 9.0	Chemiluminescence	3×10^{-13}	Puget *et al.*, 1977 Slawinska *et al.*, 1979

Abbreviations ABTS - 2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonate)
MBTH - 3-methyl-2-benzothiazoline hydrazone
DMAB - 3-(dimethylamino)benzoic acid

number, stability, and its ease of coupling to antigens or antibodies (without significant loss of reactivity) has lead to the widespread use of HRP in the production of conjugates for immunoassay systems (Schuurs and van Weeman, 1977; Groome, 1980). The techniques available for HRP quantitation together with their advantages and disadvantages are discussed below.

2.1.2.1 Chemiluminescence and bioluminescence

The luminescent quantitation of peroxidase can be achieved using cyclic hydrazides (eg luminol or related molecules; Section 2.1.3.1), phenol derivatives (eg pyrogallol or purpurogallin; Section 2.1.3.2), or complex biological molecules (luciferins) obtained from bioluminescent systems (eg luciferins isolated from Pholas dactylus or Cypridina; Section 2.1.3.3). Individual luminescent systems differ widely in their detection limits, specificity, reagent availability, and the magnitude and kinetics of light emission. General advantages and limitations of luminescent methods for HRP quantitation are summarised in Table 2.2. The main advantages are the speed and sensitivity with which HRP can be quantitated; the main disadvantages are that some methods are relatively non-specific and susceptibile to interference.

2.1.2.2 Colorimetry

Peroxidase can be quantitated colorimetrically using an oxidant (eg hydrogen peroxide) and one of a number of substrates which form coloured products (Ishikawa, 1980; Saunders, 1979). Enzyme turnover enables signal amplification and an additional advantage of colorimetric assays is that

Table 2.2

Advantages and limitations of luminescent procedures for peroxidase quantitation

Advantages

- Speed of analysis
- Sensitivity
- No lengthy, temperature controlled incubations
- Non-toxic reagents
- Wide range of linear response
- Potentially simple instrumentation

Limitations encountered with some luminescent procedures

- Limited specificity and potential interference
- Limited reagent availability
- Background light emission
- Rapid kinetics and requirement for rapid, reproducible mixing

conventional spectrophotometers can be used. The disadvantages include the need for lengthy, accurately timed, temperature controlled incubations.

Colorimetric systems differ in their sensitivity, specificity, product stability and optimal conditions. o-Phenylenediamine (Figure 2.1a (1)) offers a very sensitive assay for peroxidase (Gallati and Brodbeck, 1982) however although it is commonly employed in commercially available immunoassay kits, it is photosensitive, generally requires a 30 minute incubation and is a known mutagen (Riad-Fahmy et al., 1983). 2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonate) (ABTS) (2) involves less risk, is stable, and although its reaction product is unstable (Ishikawa, 1980) it enables sensitive peroxidase determination using incubations of 0.5-2 hours. Neither of these chromogens however are specific for HRP and substances such as haemoglobin can catalyse their oxidation (Marklund, 1978).

Attempts to increase the sensitivity of colorimetric HRP assays have involved the use of extended incubations (20 hours) (Groome, 1980), oxidative coupling of compounds such as 3-methyl-2-benzothiazolinone hydrazone (3) and 3-(dimethylamino)benzoic acid (4) (Ngo and Lenoff, 1980), tetramethylbenzidine (5), and the use of non-ionic detergents such as Tween to prevent enzyme inhibition (Porstmann et al., 1981a,b) and surface inactivation (Berkowitz and Webert, 1981).

2.1.2.3 Fluorimetry

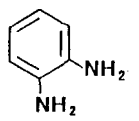
Several compounds serve as fluorogenic substrates for HRP

Figure 2.1a

Compounds capable of participating in HRP catalysed reactions

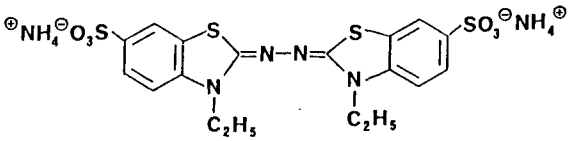
Colorimetric and fluorogenic substrates:

COLORIMETRIC SUBSTRATES



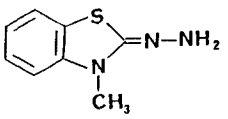
o-Phenylenediamine

(1)



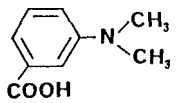
ABTS

(2)



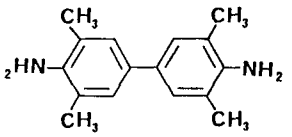
3-Methyl-2-benzothiazolinone hydrazone

(3)



3-(Dimethylamino)benzoic acid

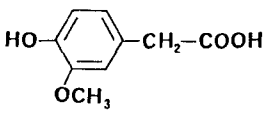
(4)



Tetramethylbenzidine

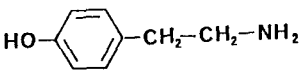
(5)

FLUOROGENIC SUBSTRATES



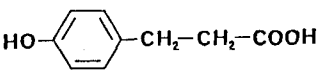
Homovanillic acid

(6)



Tyramine

(7)



3-(p-Hydroxyphenyl)propionic acid

(8)

(Ishikawa, 1980; Zaitsu and Ohkura, 1980) forming the basis of rapid and sensitive fluorometric HRP assays. Para-substituted phenolic compounds such as homovanillic acid (6), tyramine (7), tyrosine and p-methyl phenol have been employed and the fluorophores characterised as 2,2'-dihydroxydiphenyl derivatives. Greater sensitivity has been achieved using 3-(p-hydroxyphenyl)propionic acid (8) or p-tyrosol (Zaitsu and Ohkura, 1980). The former substrate has been used to quantitate HRP-conjugates within 30 minutes in immunoassays for insulin and human choriogonadotrophin using polystyrene beads as the solid support (Zaitsu et al., 1982a,b).

A homogeneous enzymatic fluorescence immunoassay for serum IgG, using HRP-antibody conjugates and laser induced fluoroescence of dichlorofluorescein produced by the HRP-catalysed oxidation of leuco-diacetyldichlorofluorescein with H_2O_2, has also been reported (Kelly and Christian, 1982).

Fluorometric assays for HRP are sensitive and rapid (as the rate of formation of fluorescent products can be monitored) however the technique is prone to interference and requires more complex instrumentation than either colorimetry or luminescence.

2.1.2.4 Potentiometry

A fluoride-selective electrode has been used to determine HRP or HRP-conjugates by detecting fluoride ion released from the HRP catalysed oxidation of p-fluoroaniline and a range of other substrates (Alexander and Maitra, 1982; Siddiqi, 1982). An iodide-selective electrode has also been used to monitor iodide oxidation by HRP-Hepatitis B surface antigen antibody conjugate

(Boitieux et al., 1979) or HRP-oestradiol conjugates (Boitieux, 1981). The ion-selective methods are sensitive, and much less prone to the interferences encountered in other types of HRP assays (Boitieux et al., 1979). Monitoring the rate of change of electrode potential enables quantitation in approximately 2 minutes, however, the recovery time of the electrode between samples is long and this extends the time per assay to approximately 6 minutes (Alexander and Maitra, 1982). Difficulties may also be encountered automating and coupling the technique to the numerous solid supports employed in immunoassay systems.

2.1.3 Chemiluminescent and bioluminescent quantitation of peroxidase

2.1.3.1 Peroxidase mediated chemiluminescence with luminol and related hydrazides

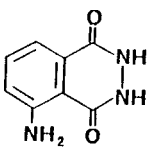
The chemiluminescent reactions of luminol (Figure 2.1b (9)) and related hydrazides (10, 11) have been extensively studied (Merenyi and Lind, 1980; McCapra and Leeson, 1979; Rauhut, 1979). All appear to be oxidations and a range of oxidising systems can be employed to produce light emission. Hydrogen peroxide acts in this manner, however, a third component which serves as a catalyst and/or co-oxidant is required (Seitz, 1981). HRP acts as a catalyst in the oxidation of cyclic hydrazides and several sensitive assays for its determination have been described (Table 2.1). Numerous compounds such as metal ions or haem containing compounds can also act as catalysts, indeed sensitive luminescent assays for these compounds have been developed (Campbell and

Figure 2.1b

Compounds capable of participating in HRP catalysed reactions

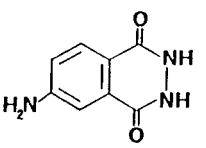
Chemiluminescent substrates:

DIACYL HYDRAZIDES



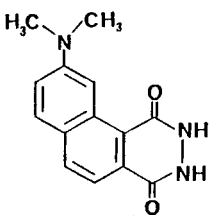
Luminol

(9)



Isoluminol

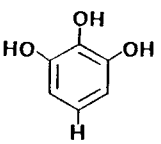
(10)



7-Dimethylaminonaphthalene-1,2 -dicarboxylic acid hydrazide

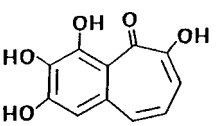
(11)

PHENOL DERIVATIVES



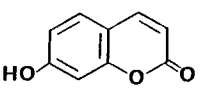
Pyrogallol

(12)



Purpurogallin

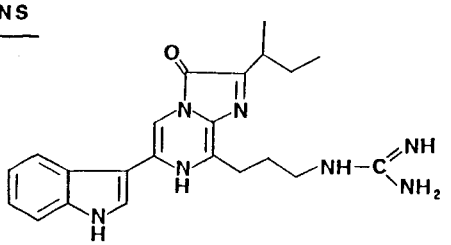
(13)



Umbelliferone

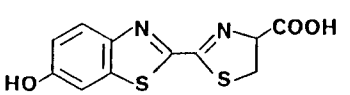
(14)

LUCIFERINS



Cypridina luciferin

(15)



Firefly luciferin

(16)

Simpson, 1979; Isacsson and Wettermark, 1974). Complex biological fluids, and even buffers (Arrio et al., 1980), may contain a variety of catalysts (Freeman and Seitz, 1978; Seitz and Hercules, 1973), these must be removed or conditions chosen to minimise their influence if sensitive and specific HRP quantitation is to be achieved.

Luminescent quantitation of HRP using luminol or luminol analogues has been carried out under a wide range of reaction conditions, (eg pH 5-13) (Campbell and Simpson, 1979; Ewetz and Thore, 1976), and using a variety of reagent concentrations and oxidants. Individual procedures differ in the kinetics and magnitude of light emission, specificity for HRP, and background light emission.

The chemiluminescent reactions of luminol have been subject to numerous mechanistic studies however the precise reaction mechanism for light production has not been determined (White and Roswell, 1985). A generally accepted reaction scheme involves the initial oxidation of luminol to a radical (Chapter 3, Section 3.4.4), then depending on the experimental conditions this reacts rapidly with oxygen or hydrogen peroxide to yield a peroxide intermediate. This decomposes to an excited state 3-aminophthalate which decays to the ground state emitting light. Mechanistic studies of the HRP catalysed oxidation of luminol by hydrogen peroxide under several conditions (pH 6.5, 8.0 and 10.0) have been carried out and it appears to be a complex process involving several intermediates (Cormier and Prichard, 1968; Wong and Salin, 1981; Misra and Squatrito, 1982, Chapter 3 Section

3.4.4). The involvement of superoxide radical, hydroxyl radical and singlet oxygen in the reactions leading to chemiluminescence depends upon the reaction conditions employed. Mechanisms may also be influenced by the effect of pH on enzyme activity and the dissociation of haem from the enzyme at high pH.

HRP assays employing luminol and luminol analogues such as isoluminol (10) can be broadly divided into (a) those carried out at high pH where haem dissociates from HRP and (b) those at pHs where the enzymatic activity of HRP is retained. Generally light emission is greater at high pH where haem dissociation is likely to occur, and pre-incubation with sodium hydroxide has been employed to enhance this dissociation as binding of haem to protein decreases its catalytic ability (Ewetz and Thore, 1976). The high pH reaction is, however, particularly prone to interference because of the presence of other haem containing compounds such as haemoglobin, and metal ions in biological fluids (Olsson et al., 1982). The technique is also sensitive to variations in mixing and exhibits a high background light emission. At near neutral pH light emission is generally lower but enzymatic activity is retained and although not completely eliminated, interferences from other compounds and background light emission are reduced.

Chemiluminescent quantitation of HRP-conjugates using luminol has been successfully employed in several immunoassay systems (Chapter 5). The synthesis of cyclic hydrazides such as 7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide (11) has produced luminol analogues which show significantly higher

quantum yields compared with luminol (Schroeder and Yeager, 1978) allowing more sensitive HRP quantitation. An isolated report of its application in immunoassay systems has claimed significant increases in sensitivity (Gundermann et al., 1982).

2.1.3.2 Peroxidase mediated chemiluminescence with phenol derivatives

The HRP catalysed oxidation of several phenol derivatives is accompanied by light emission. A variety of polyphenols such as pyrogallol (12), purpurogallin (13), resorcinol, gallic acid and umbelliferone (14) are suitable substrates for the HRP-H_2O_2 complex and have been employed in sensitive luminescent assays for HRP and HRP-conjugates (Halmann et al., 1979; Slawinska et al., 1979). General mechanisms, similar to those for luminol have been proposed although the exact nature of the emitters remain unidentified (Arrio et al., 1980; Slawinska et al., 1979). The magnitude and kinetics of light emission are influenced by numerous experimental factors, and compromises in the levels of light achieved are required to obtain suitable signal to background ratios (Slawinska et al., 1979). The signal to background ratio, which limits the sensitivity of HRP quantitation is higher for polyphenols than for luminol, however, under the conditions generally used (pH 6-7) light emission decays rapidly.

Sensitive HRP quantitation can be achieved using purpurogallin and this can be improved by the addition of p-phenylenediamine which enhances light emission eight-fold (Halmann et al., 1979). As yet no mechanism for this enhanced light emission has been

proposed. Chemiluminescence accompanying the oxidation of phenol derivatives is also accelerated by heavy metal ions, eg Fe^{3+}, microperoxidase and compounds such as formaldehyde (Seitz, 1981). In addition, enzymes with peroxidase activity such as Balanoglossid luciferase may be used instead of HRP (Dure and Cormier, 1963).

2.1.3.3 Peroxidase mediated luminescence with luciferins

In the presence of hydrogen peroxide, in the pH range 8-10, HRP can induce light emission from a variety of heterocyclic compounds which act as luciferins in bioluminescent systems. Balanoglossus biminiensis luciferin (possibly an N-substituted indole derivative, Dure and Cormier, 1963), the Cypridina luciferin (15) analogue 2-methyl-6-phenyl-3,7-dihydroimidazo-[1,2a]pyrazin-3-one (Kobayashi et al., 1981), and firefly luciferin (4-5-dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazole-carboxylic acid (16) act as luminescent substrates for HRP. The latter generates sharp bursts of light emission and has been employed for sensitive quantitation of HRP in solution (Slawinska et al., 1979). A novel luminescent procedure for HRP quantitation involving the simultaneous use of synthetic firefly luciferin and luminol analogues is described in Section 2.2.3.2.

A simple and extremely sensitive assay for HRP (less than 1×10^{-18} moles) and HRP-conjugates has been reported using luciferin from the luminescent mollusc Pholas dactylus (Michelson, 1978; Puget et al., 1977). A recent study has however failed to reach the sensitivity claimed (Longeon et al., 1986). The luciferin, a glycoprotein of molecular weight 35,000, is readily oxidised by

HRP with the emission of light in the absence of H_2O_2. Oxygen is necessary for the reaction and HRP acts as an oxygenase. The existence of a small molecular weight prosthetic group which undergoes oxidation and is the true luciferin has been proposed but attempts to separate the molecule have been unsuccessful (Michelson, 1978). Structural and mechanistic studies have been performed on the luciferin, but further studies and applications have been limited by the scarcity of colonies of the once prevalent mollusc. Light emission appears relatively independent of pH and at near neutral pH relatively constant levels of light emission can be obtained. In common with several other luminescent procedures employed for HRP quantitation, light emission can also be evoked by systems such as those involving certain metal ions or superoxide radicals (Michelson, 1978).

2.1.4 Instrumentation

Until recently most luminescent measurements were made using either 'home made' apparatus or modified commercial equipment. Instruments so modified include fluorimeters, scintillation counters, centrifugal analysers and continuous flow apparatus (Stanley, 1982; Whitehead *et al.*, 1979). Purpose designed, fully automated, sensitive equipment is now, however, becoming available commercially. Detection limits for the luminescent assay of HRP depend, in part, upon the sensitivity of the instrumentation employed. In this study a luminometer designed and built in this laboratory was used to quantitate light emission. As the design did not incorporate all the features available to achieve maximal sensitivity, it was not possible to determine the absolute detection limits for the different

luminescent assays.

Extension of luminescent HRP determination into immunoassay systems and improvement of their detection limits requires instrumentation both fully compatible with the solid supports employed and of high sensitivity, capable of monitoring small variations in light emission.

Instrumental sensitivity is dependent on various factors including the relative sensitivity of the detector, light collection efficiency and to a lesser extent the signal to noise ratio of the electronics (Campbell and Simpson, 1979; Rawlins and Peacock, 1982; Seitz, 1981). The signal to noise ratios achievable with solid state detectors is restricted by the small current produced and their requirement for high impedence amplifiers with high gain, and this limits instrumental sensitivity. Instrumentation of the highest sensitivity is produced using photomultiplier tubes for light detection. Choice of photomultiplier tube influences sensitivity, end-window tubes are more sensitive than side-window tubes, and the whole detector active surface should be employed to maximise the signal to noise ratio. Care should be taken to choose photomultiplier tubes matched to the wavelength of emitted light, and with a high signal to dark current ratio (Stanley, 1982). This ratio may be further improved by reduction of the dark current by cooling the tube during operation (Berthold and Kubisiak, 1982). Photocurrent determination or photon counting can be employed with photomultiplier tubes but the latter can only be used with low light levels and has a limited linear range of response.

Production of optimal signal levels from the detector for the following processing electronics should be achieved with minimal noise contribution to the overall system.

Careful design of the optics of the light collecting system improves instrumental sensitivity by ensuring that the highest possible percentage of emitted light strikes the detector. High detection efficiency is achieved by placing the light source very close to the detector, and can be further increased by placing suitably designed reflective elements around the source to direct light onto the detector, or by light conductors optically connected to the reaction cell and photocathode surface (Brolin et al., 1984). Such features help fulfil the major aim of luminometer design which is to achieve a maximal photon flux to detector area ratio. The use of multiple detectors can increase the effective count rate, however, the overall signal to noise ratio remains constant.

The precision and sensitivity of luminescent measurements are influenced by the method employed to mix reagents and initiate reactions (Arrio et al., 1980; Campbell and Simpson, 1979). Discrete luminometers (most compatible with immunoassay systems) have employed several techniques, reagents may be added gently and swirled or stirred to achieve complete mixing, or, alternatively, force of injection can serve to induce mixing (Seitz, 1981). Very fast luminescent reactions (ie a flash of light) require both rapid, efficient, highly reproducible mixing in front of the detector, and instrumentation with a short response time. The potential imprecision of initiating such

reactions may be increased when they are carried out in association with solid supports. The use of luminescent reactions that emit light in a relatively constant manner (ie glow) avoids the requirement for instrumentation employing *in situ* mixing. Such reactions enable simple initiation of luminescent reactions outside luminometers and the possible use of simpler instrumentation. The procedures are also less prone to variation and are compatible with numerous immunoassay solid supports.

2.2 EXPERIMENTAL

Materials and Methods

2.2.1 Reagents

Synthetic crystalline firefly D-luciferin, luminol, isoluminol, pyrogallol, purpurogallin, p-phenylenediamine, o-phenylenediamine, hydrogen peroxide (30% w/v), bovine serum albumin (Cohn fraction V 96-99%), Tris-HCl, glycine and monolaurate oxyethylene sorbitan (Tween 20) were purchased from Sigma Chemical Company, Dorset, BH17 7NH. Luminol was purified by conversion to its sodium salt, recrystallisation from sodium hydroxide and decolourization with charcoal.

2,2'-Azino-di-(3-ethyl-benzothiazoline sulphonate) was supplied by Boehringer Mannheim GmbH, West Germany. Rabbit anti-human AFP-HRP conjugate (2.64 mg protein/ml; 24 µmol/l HRP) was obtained from Dakopatts a/s, Denmark. Dilutions of conjugate were prepared in distilled water and used immediately after preparation. *Pholas dactylus* luciferin (4.8 mg protein/ml) was kindly supplied by Dr A M Michelson (Fondation Edmond de

Rothschild, Paris, France) via Dr A Voller (Nuffield Laboratories of Comparative Medicine, Institute of Zoology, London); the material was stored at -20°C prior to use. All solutions were prepared using distilled, deionised water. All other chemicals were of Analar quality and obtained from either Sigma Chemical Company or BDH Chemicals Ltd, Atherstone.

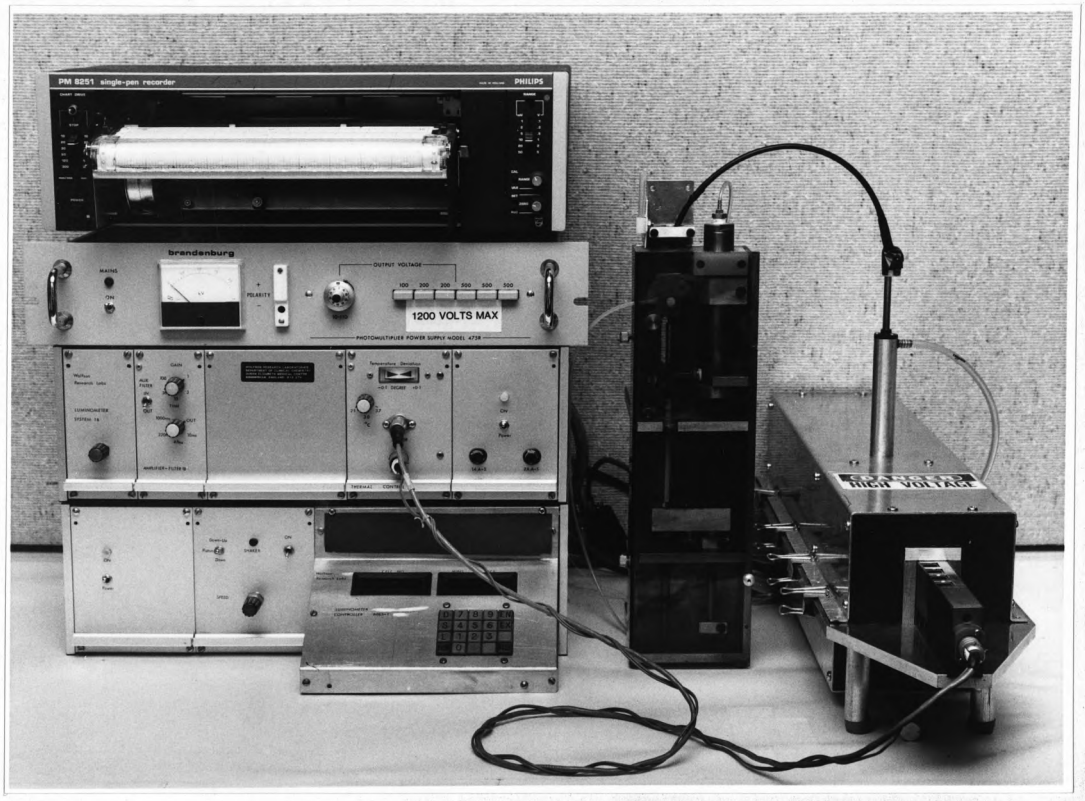
2.2.2 Instrumentation

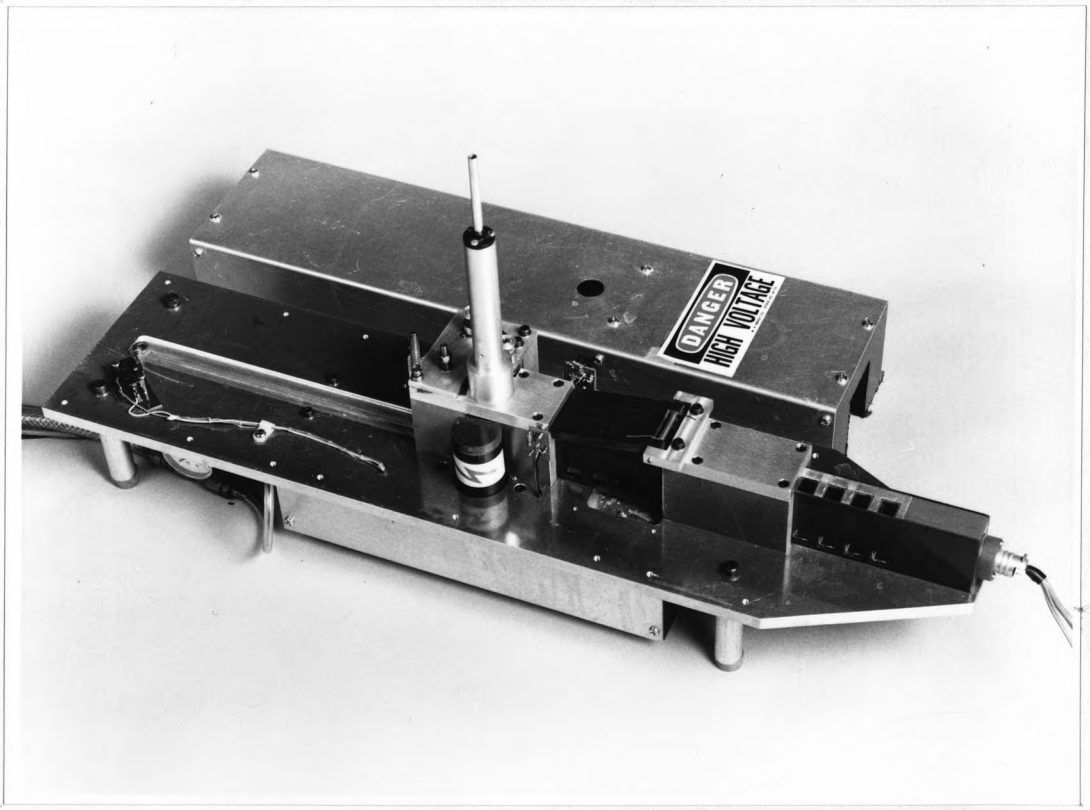
Light measurement

Light measurements were performed using a luminometer (Figure 2.2) constructed in the Wolfson Research Laboratories. The instrument (Bunce et al., 1980), is based on a side-window photomultiplier tube (EMI type 9781A, 94 μA/lumen) using photocurrent measurement. The analogue output from the PMT was displayed graphically on a Philips PM8251 chart recorder. The luminometer employed a moveable, thermostatically controlled carrier which enabled reaction vessels to be successively registered, in a stepwise manner, in front of the PMT under microprocessor control. Disposable square plastic cuvettes (1 x 1 x 4.5 cm) (W Sarstedt UK Ltd, Leicester) were used as reaction vessels. Light passed through the optically clear surface of the cuvette and a window in the side wall of the carrier, and was detected by the PMT closely positioned against, and overlapping, the window. Although sequential measurements of 16 cuvettes could be made, a single cuvette position was employed for all measurements to avoid any slight variation in their alignment in front of the photodetector and differences in the windows in the cuvette holder which could introduce imprecision in light

Figure 2.2

The Wolfson Research Laboratories' luminometer





collection.

Initiation of reactions in front of the photodetector could be achieved by forcible injection of a final luminescent co-reagent to a solution of all the other components, or by injection of buffer or reagent mixture, to mix small volumes of reagents placed in separate corners of the square reaction cuvette. Automatic injection of a single reagent, into a cuvette positioned in front of the PMT, was possible using a pneumatically operated dispenser. This comprised a 1 ml disposable plastic syringe (Gillette Surgical, Middlesex) in conjunction with a 3-way valve (AHS/International, Belgium) and a 0.25 mm internal diameter pipette delivery tip (Micromedic Systems Inc, Philadelphia). The system enabled pre-set volumes of reagent to be drawn from a reservoir and injected at adjustable speeds into the reaction vessel.

Care was taken to avoid the adjustable dispenser tip becoming misaligned, and to avoid any carryover produced by luminescent reagents attached to the tip. The dispenser tip was carefully positioned to ensure reproducible mixing and prevent the injection stream hitting the reaction vessel wall prior to reaching other reagents. In order to minimise variations in mixing, and imprecision in pipetting the small reagent volumes required to keep them separate in the reaction vessels, individual components of luminescent reactants were combined wherever possible in a similar manner to Ikariyama et al., 1983.

Colorimetry

Colorimetric measurements were performed on a Gilford 240

recording spectrophotometer.

2.2.3 Chemiluminescent and bioluminescent quantitation of peroxidase conjugates

2.2.3.1 The luminol, hydrogen peroxide reaction

pH 8.0 A solution of sodium luminol (1.25 mmol/l) and hydrogen peroxide (2.7 mmol/l) was prepared in Tris buffer pH 8.0 (10 mmol/l) several hours before use. Peroxidase conjugate dilution (10 µl) was placed in a cuvette, the luminescent reaction initiated in front of the photomultiplier tube by the automatic injection of luminol-hydrogen peroxide solution (900 µl) and the light emission recorded.

pH 12.9 Solutions of sodium luminol (1 mmol/l) and hydrogen peroxide (0.52 mol/l) were prepared in distilled deionised water. Luminol (10 µl), hydrogen peroxide (10 µl) and peroxidase conjugate dilution (10 µl) were placed in separate corners of a cuvette. The luminescent reaction was initiated in front of the photomultiplier tube by the automatic injection of 900 µl sodium hydroxide (0.125 mol/l) and the light emission recorded.

2.2.3.2 The firefly luciferin enhanced luminol, hydrogen peroxide reaction

A solution of sodium luminol (1.25 mmol/l) and hydrogen peroxide (2.7 mmol/l) as prepared in Tris buffer (10 mmol/l, pH 8.0) several hours before use. Synthetic firefly luciferin (1 mg/ml, 3.6 mmol/l) in 0.1 mol/l pH 8.0 phosphate buffer was prepared at the same time and stored protected from light prior to use in a small, capped plastic microcentrifuge tube. Firefly luciferin

(10 µl) and peroxidase conjugate dilution (10 µl) were placed in separate corners of a cuvette, the luminescent reaction initiated in front of the photomultiplier tube by the automatic injection of luminol-hydrogen peroxide solution (900 µl) and the light emission recorded. Light emission was also determined in the absence of luminol or firefly luciferin.

2.2.3.3 The luminol, perborate reaction

A solution of sodium luminol (0.11 mmol/l) and sodium perborate (5.6 mmol/l) was prepared in sodium hydroxide (0.125 mol/l) several hours before use. Peroxidase conjugate dilution (10 µl) was placed in a cuvette, the luminescent reaction initiated in front of the photomultiplier tube by the automatic injection of luminol-perborate solution, pH 12.9 (900 µl) and the light emission recorded.

2.2.3.4 The isoluminol, hydrogen peroxide reaction

Isoluminol, 30 µl, (31 µmol/l in 1 mol/l glycine/sodium hydroxide buffer pH 9.0), and peroxidase conjugate dilution, 10 µl, were placed in separate corners of a cuvette. The luminescent reaction was initiated in front of the photomultiplier tube by the automatic injection of 900 µl hydrogen peroxide (0.1 mmol/l in 0.1 mol/l glycine/sodium hydroxide buffer pH 10.5 containing 0.1 mmol/l EDTA) and light emission recorded.

2.2.3.5 The Pholas dactylus luciferin, O_2 reaction

Freshly thawed Pholas dactylus luciferin was diluted to 48 µg/ml with phosphate buffer (0.125 mol/l, pH 7.8) containing sodium chloride (25 mmol/l). Luciferin solution (500 µl) was added manually to peroxidase conjugate dilution (10 µl) in a cuvette,

then mixed and immediately placed in front of the photomultiplier tube. The light emission against time was recorded.

2.2.3.6 The purpurogallin, p-phenylenediamine, hydrogen peroxide reaction

Solutions of p-phenylenediamine (25 mmol/l) and hydrogen peroxide (0.12 mol/l) were prepared in potassium phosphate buffer (0.15 mol/l, pH 6.5). Purpurogallin (9.1 mmol/l) was prepared in methanol. The reagents were prepared up to four hours before use and stored in the dark. Purpurogallin (55 µl), p-phenylenediamine (20 µl) and peroxidase conjugate dilution (10 µl) were placed in separate corners of a cuvette, the luminescent reaction initiated in front of the photomultiplier tube by the automatic injection of hydrogen peroxide (900 µl) and peak light emission recorded.

2.2.3.7 The pyrogallol, hydrogen peroxide reaction

Solutions of pyrogallol (0.29 mol/l) and hydrogen peroxide (0.12 mol/l) were prepared in potassium phosphate buffer (0.15 mol/l, pH 6.5) and stored in the dark before use. Pyrogallol (55 µl) and peroxidase conjugate dilution (10 µl) were placed in separate corners of a cuvette, the luminescent reaction initiated in front of the PMT by the automatic injection of hydrogen peroxide (0.9 ml), and peak light emission recorded.

2.2.4 Colorimetric quantitation of peroxidase conjugates

2.2.4.1 o-Phenylenediamine

Aliquots of peroxidase conjugate dilutions (10 µl) were incubated for 30 minutes at room temperature (approximately 20^{o}C) in the

dark, with 0.5 ml of substrate solution (0.1 mol/l potassium citrate buffer, pH 5.5, containing 6 mmol/l hydrogen peroxide and 2.0 mg of o-phenylenediamine). Reactions were stopped by the addition of 2 ml hydrochloric acid (1 mol/l) and the absorbance at 492 nm measured against a water blank.

2.2.4.2 ABTS

Stock ABTS and hydrogen peroxide solutions were prepared by dissolving ABTS (0.55 g) in 500 ml acetate buffer (0.1 mol/l, pH 4.2) containing Tween 20 (0.01%), and by diluting 2.83 ml hydrogen peroxide (30% w/v) to 100 ml with distilled water. Immediately before use, hydrogen peroxide solution (1 ml) was mixed with ABTS (100 ml) and 0.9 ml of this mixture incubated with 10 µl aliquots of peroxidase conjugate dilutions at 37°C for 30 minutes. After the 30 minute incubation, the absorbance at 405 nm was measured against a water blank.

2.3 RESULTS

Rapid quantitation of HRP-conjugate was possible with all the luminescent assays investigated, but the kinetics and magnitude of light emission for individual assays differed. Typical light-time curves for reactions in the presence or absence of HRP-conjugate are shown in Figures 2.3 and 2.4. The sensitivity with which conjugate could be detected using the luminescent assays varied, as did the levels of light emitted in the presence of fixed quantities of HRP-conjugate (Figure 2.5).

2.3.1 Reactions involving luminol

2.3.1.1 Unenhanced reactions

Figure 2.3 illustrates the kinetics of light emission from the

Figure 2.3

Kinetics of light emission from luminol-H_2O_2 reactions

a) in the presence of 1:10,000 HRP-conjugate and

b) in the absence of HRP-conjugate

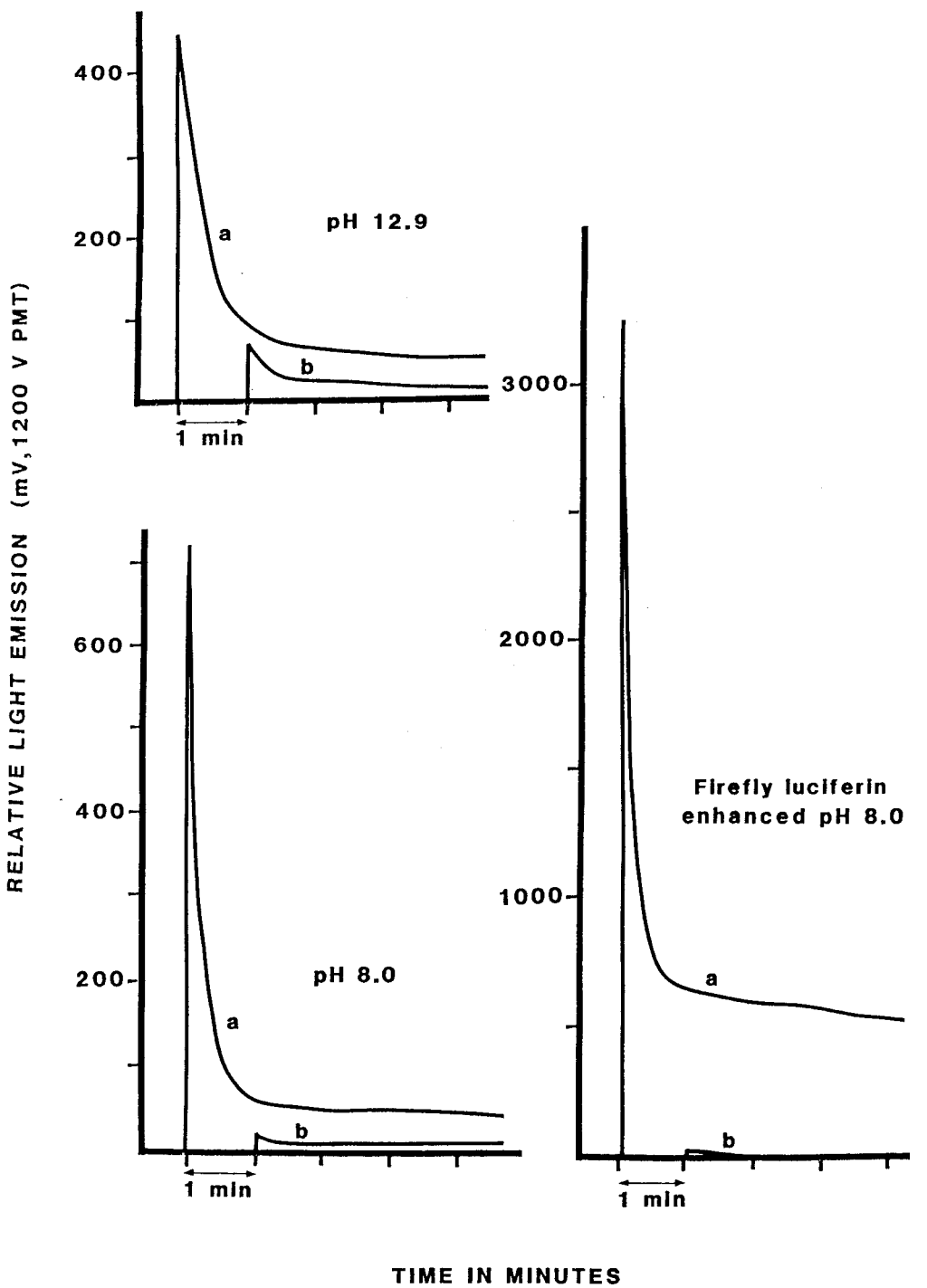
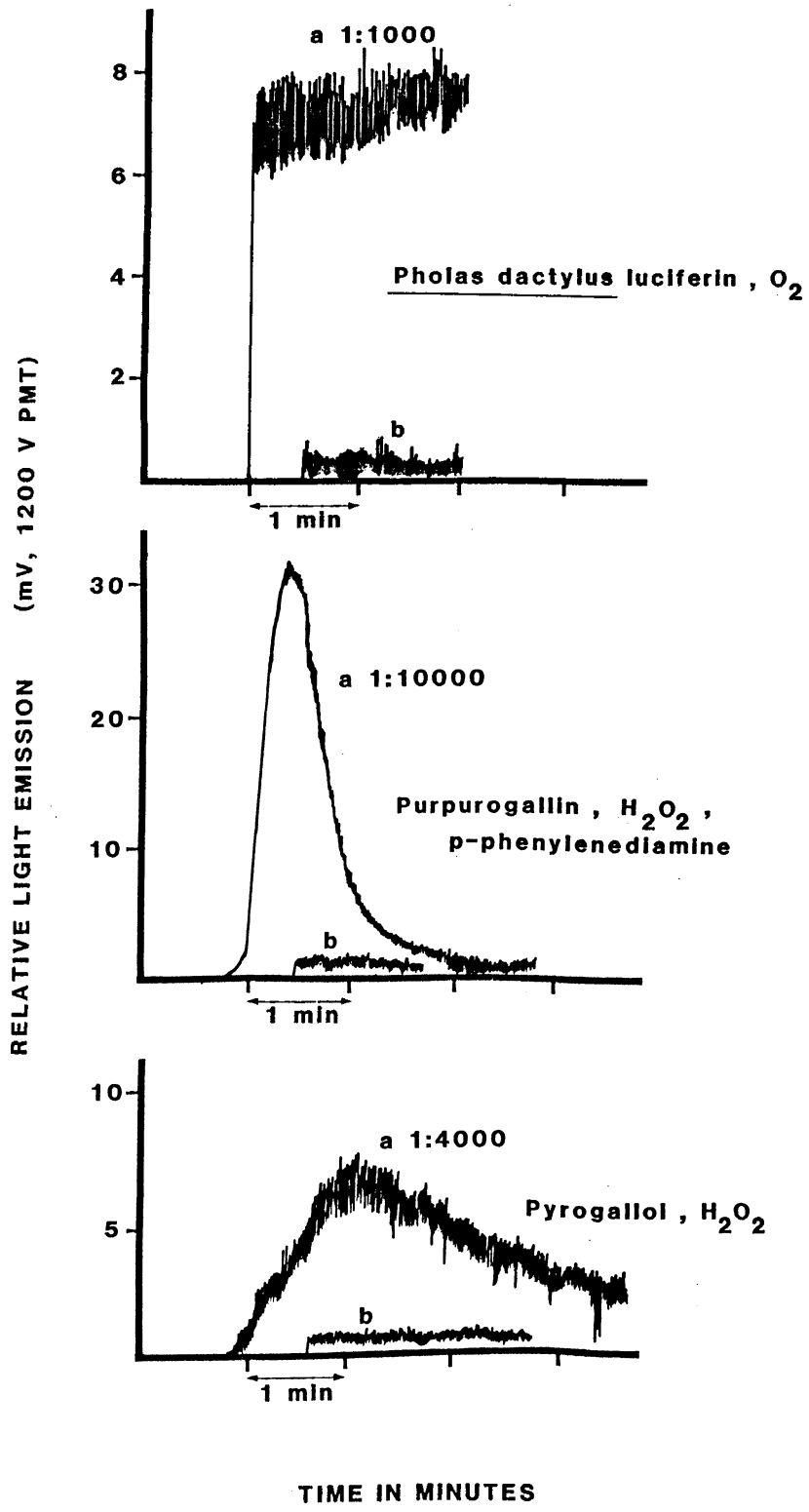


Figure 2.4

Kinetics of light emission from luminescent reactions a) in the presence, and b) in the absence of HRP-conjugate (dilutions of HRP-conjugate employed are indicated).



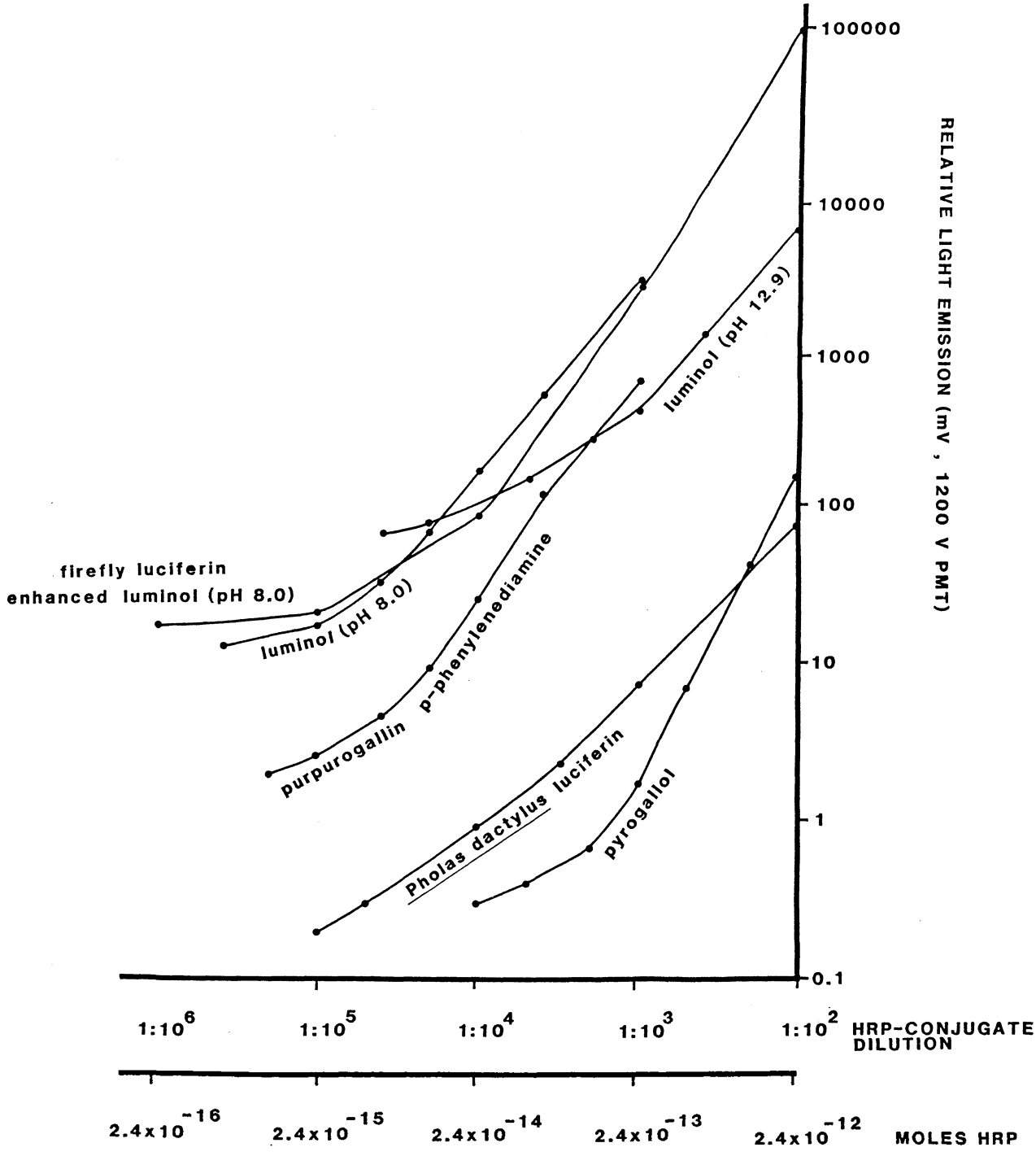
luminol-H_2O_2 reaction at pH 8 and 12.9. Initial rapid kinetics of light emission (an initial flash) which appeared dependent on the mixing procedure employed, were seen in reactions at both pHs. After approximately 1 minute, relatively constant and prolonged light emission was obtained for over 5 minutes. Levels of light emission were relatively high, however the sensitivity of the reaction at high pH was limited by very high background light emission and poor reproducibility. Similar kinetics were obtained with perborate as oxidant, however unlike other luminescent assays, those at high pH were particularly prone to interference by constituents in 10 µl of a 0.01 % BSA solution. The isoluminol-H_2O_2 reaction at pH 10.5 showed similar kinetics to the reactions described previously however levels of light emitted were over an order of magnitude lower and the sensitivity was poor.

2.3.1.2 The firefly luciferin enhanced luminol-H_2O_2 reaction

Figure 2.3 shows the dramatic increase in light emission seen by the addition of firefly luciferin to the luminol-H_2O_2-HRP-conjugate reaction at pH 8.0. Similar kinetics to that in unenhanced reactions were observed, an initial flash followed by a prolonged glow, and light emission even when recorded 30 seconds after initiation was high and easily detected (Figures 2.3 and 2.5). With such measurements sensitivity was good (at least 2×10^{-15} moles HRP) and approached that of the optimised 30 minute colorimetric o-phenylenediamine or ABTS reactions (Figure 2.6). HRP-conjugate catalysed light emission (with H_2O_2 as oxidant) from firefly luciferin alone, luminol alone, and the

Figure 2.5

Levels of light emission in luminescent assays for antibody-HRP conjugate



Peak light emission occurring in the first 2 minutes after initiation is plotted for all assays except the firefly luciferin enhanced luminol-H_2O_2 reaction in which light intensity 30 seconds after initiation is recorded.

Figure 2.6

Absorbance changes in colorimetric assays for antibody-HRP conjugate

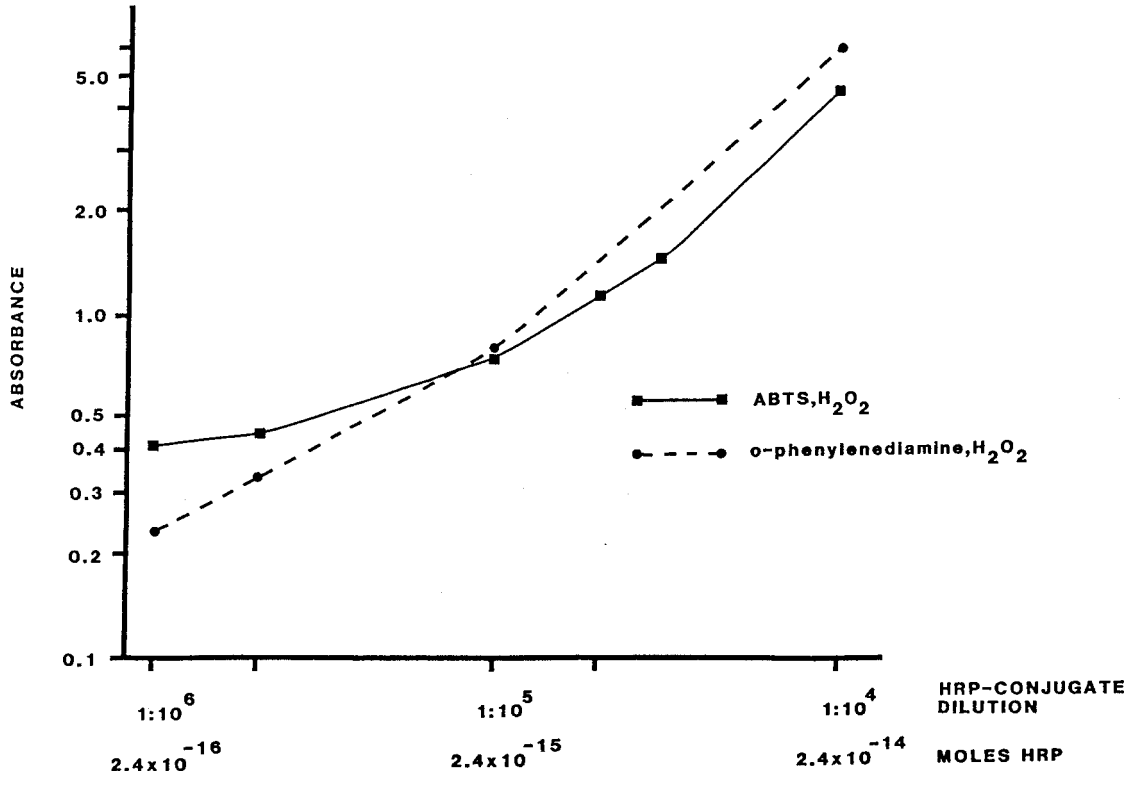


Table 2.3

HRP-conjugate catalysed light emission from the oxidation of luminol alone, firefly luciferin alone and the combined reagents

Reaction components	Light emission* (mV)	
	No HRP-conjugate	With HRP-conjugate
Luminol + H_2O_2	1.5	77
H_2O_2 + firefly luciferin	< 0.1	2
Luminol + H_2O_2 + firefly luciferin	0.25	570

Final concentrations; luminol (1.23 mmol/l), H_2O_2 (2.67 mmol/l), firefly luciferin (39 µmol/l); pH 8.0

* Light intensity 30 seconds after initiation was recorded, measurement of total light emission produced similar results.

combined reagents are shown in Table 2.3. Light emission from firefly luciferin and luminol together significantly exceeds that from either substance alone and the increased light emission produced is clearly not a purely additive effect.

2.3.2 The *Pholas dactylus* luciferin-O_2 reaction

Figure 2.4 illustrates the relatively constant and prolonged light emission from the *Pholas dactylus* luciferin-O_2 reaction. Levels of light emission were extremely low and difficult to distinguish from instrumental noise at low conjugate concentrations (Figure 2.5).

2.3.3 The pyrogallol-H_2O_2 reaction

Light emission catalysed by HRP-conjugate increased slowly to a maximum approximately 1 minute after initiation, a relatively slow decay of light emission then occurred (Figure 2.4). The intensity of light emitted was extremely low even in the presence of high concentrations of conjugate (Figure 2.5).

2.3.4 The purpurogallin-p-phenylenediamine-H_2O_2 reaction

Initial kinetics of light emission in the purpurogallin-p-phenylenediamine-H_2O_2 reaction were much slower than those involving luminol. After a slow initial rise, light emission increased rapidly to a distinct peak approximately 30 seconds after initiation and then decayed rapidly to near background levels (Figure 2.4). Sensitive HRP-conjugate quantitation was possible and the intensity of light emission higher than reactions using pyrogallol or *Pholas dactylus* luciferin although lower than those employing luminol (Figure 2.5).

2.4 DISCUSSION

2.4.1 Advantages of the firefly luciferin enhanced luminol-hydrogen peroxide reaction for HRP-conjugate quantitation

Enhancement of light emission from the luminol-H_2O_2 reaction by the addition of synthetic firefly luciferin at pH 8 enabled rapid, relatively simple and sensitive quantitation of HRP-antibody conjugate in solution. In common with conventional luminescent assays for HRP it is rapid (under two minutes), it eliminated the lengthy, accurately timed and temperature controlled incubations required using colorimetry and allowed quantitation of conjugate concentrations commonly encountered in immunoassays. Unlike the quantitation of luminescent labels such as isoluminol derivatives it allowed signal amplification and did not require any lengthy pre-incubation (such as 30 minutes at 60°C) prior to the luminescent reaction (Weerasekera et al., 1983a). The enhanced system offered advantages over conventional HRP assays which possessed limitations such as the limited availability of bioluminescent reagents (eg Pholas dactylus luciferin); and the susceptibility to interference, rapid decay of light emission and low light intensity in conditions in which interference was minimal. In addition, initiation and light emission from the enhanced assay were such that it appeared particularly convenient for use in conjunction with immunoassay solid supports. The properties of individual luminescent assays are summarised in Table 2.4. The advantages of the enhanced assay are outlined below, further features of the reaction and the possible mechanism of enhancement are discussed in Chapter 3.

Table 2.4

The advantages and disadvantages of different luminescent assays for HRP-conjugates

ASSAY SYSTEM	ADVANTAGES	DISADVANTAGES
Pholas dactylus luciferin/ O_2/pH 7.8	Rapid Relatively constant light emission Potential high sensitivity Near neutral pH Simple one reagent addition	Low levels of light emission Very limited reagent availability Uses a complex biological glycoprotein
Pyrogallol/ H_2O_2/pH 6.5	Rapid Steep dose-response curve (response is dependent on the square of the peroxidase concentration) High signal to background ratio Near neutral pH Relatively slow kinetics	Limited sensitivity Low levels of light emission
Purpurogallin/ p-phenylene-diamine/H_2O_2/ pH 6.5	Rapid High sensitivity High signal to background ratio Near neutral pH Relatively slow initial kinetics	Rapid decay of light emission Relatively low levels of light emission Reagents unstable and toxic
Luminol/H_2O_2/ pH 12.9	Rapid High levels of light emission Dependent on haem content not enzyme activity	Low specificity Prone to interference Rapid kinetics High background light emission Low signal to background ratio Dependent on rapid, reproducible mixing High pH
Luminol/ perborate/ pH 12.9	As above Slower kinetics	As above Timed, multiple reagent addition Timed preincubation with sodium hydroxide
Isoluminol/ H_2O_2/pH 10.5	-	Low levels of light emission
Luminol/H_2O_2/ pH 8.0	Rapid Relatively high sensitivity Prolonged light emission Near neutral pH	Low signal to background ratio Initial light emission dependent on mixing
Luminol/ firefly luciferin/ H_2O_2/pH 8.0	Rapid High sensitivity Good signal to background ratio Prolonged relatively constant light emission Relatively high, enhanced levels of light emission Near neutral pH Synthetic firefly luciferin readily available	Initial light emission dependent on mixing when using high speed initiation

Reagent availability: The components of the enhanced reaction, luminol, hydrogen peroxide and synthetic firefly luciferin are all commercially available. This is not the case for assays employing bioluminescent reagents such as Pholas dactylus or Cypridina luciferin where both reagents, and biological sources from which they can be extracted, are extremely rare. Acyl hydrazides with higher quantum yields than luminol such as 7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide were also unavailable for initial studies however their general properties were anticipated to be of a similar nature and relatable to those for luminol.

Reagent stability: Reagents employed in the enhanced reaction were relatively stable. Components of several luminescent assays are light sensitive and as a general precaution all solutions were stored in the dark. Under such conditions luminol solutions can be safely stored for 6 months at $4^{o}C$ in a stoppered bottle (Campbell and Simpson, 1979). When employed as a component of the firefly bioluminescent system, aqueous solutions of luciferin can be stored at $4^{o}C$ for periods of 1 - 2 months if protected from light, preferably under a nitrogen atmosphere (Bowie, 1978). Synthetic firefly luciferin stored for such periods, simply protected from light in a sealed plastic tube, retained its ability to enhance light emission from the HRP-luminol-H_2O_2 reaction. Components of chemiluminescent assays such as p-phenylenediamine and purpurogallin also exhibit light and air sensitivity requiring storage at 0 - $4^{o}C$ (Naveh et al., 1981). Although generally employed at room temperature, Pholas dactylus luciferin is a complex biological glycoprotein and is stored at

-20°C (Michelson 1978).

Reagent toxicity: Reagents employed in the enhanced reaction are not known to be toxic unlike components of other assays such as p-phenylenediamine and pyrogallol. The assay is also performed under relatively mild conditions in contrast to the highly alkaline conditions employed in some other procedures.

Kinetics of light emission: Under the conditions employed light emission from the enhanced reaction was prolonged and relatively constant for many minutes. This feature of the reaction, also seen in the assay employing Pholas dactylus luciferin, greatly simplifies requirements for initiation and light measurement. Although an initial relatively rapid flash occurred in the enhanced reaction this was at least in part produced by the initiation and injection procedure employed. Similar initial kinetics were obtained with the luminol-H_2O_2 reaction at near neutral and high pH, however, in these assays much poorer discrimination between conjugate concentrations was obtained when light emission became more constant. Light emission from the p-phenylenediamine-purpurogallin and pyrogallol systems showed less rapid initial kinetics but the rate of light production increased and then decayed relatively rapidly producing a distinct peak of light emission. With all procedures slight variations in the kinetics of light emission were observed at different conjugate concentrations, light emission generally decaying more rapidly at high HRP concentrations.

Although extremely rapid measurements could be made when light

was emitted as a flash, such assays are reliant on the rapidity, efficiency and reproducibility of mixing (Schram, 1981; Schroeder and Vogelhut, 1984). Such measurements are generally subject to poor precision with conventional injection methods (Seitz, 1981) and unlike reactions which glow, those with rapidly changing kinetics require accurate timing of when measurements are made to avoid considerable variations in the levels recorded. In addition, the reactions require initiation in front of the photodetector and, even if optimised for reactions in solution, problems would be exaggerated in the quantitation of conjugate bound to solid support surfaces. The ability to monitor the relatively constant portion of the enhanced luminescent time curve circumvented these problems.

Intensity of light emission: Light emitted in the enhanced reaction was relatively intense and thus readily quantitated. This was in contrast to the pyrogallol, purpurogallin-p-phenylenediamine and Pholas dactylus luciferin reactions, in which at high conjugate dilutions light emission was extremely low and difficult to differentiate from instrumental noise. Light emission from the luminol-H_2O_2 reaction at high pH was relatively high, however, it had a high level of background emission and gave poor discrimination between conjugate concentrations. The ability to generate high levels of light emission (whilst retaining a good signal to background ratio) with enhanced chemiluminescence provides the option of using simpler, less expensive instrumentation, possibly making the assay more robust. In addition, it avoids the elaborate precautions required to monitor reactions in which very low

levels of light are emitted. These include procedures to minimise background luminescence such as by: dark adaption of vials, the avoidance of fluorescent light and performing measurements in the dark, screening tubes and vials for non-specific activity, and the use of ultrapure reagents (Stevens et al., 1980). Reactions with emission of low levels of light are limited by dark current noise at the detection limit whereas at higher light levels reactions may be partially limited by 'sample flicker' noise. Sample flicker arises from variations in chemical and physical factors within the system such as rate of injection, which part of the solution emits light first, and where this light strikes the photodetector (Marino and Ingle, 1981).

Sensitivity: The enhanced reaction produced a sensitive assay for peroxidase conjugates. Using the luminometer available the assay appeared more sensitive than other luminescent assays investigated (at least 2×10^{-15} moles HRP) and allowed quantitation of the amounts of HRP-conjugate encountered in typical colorimetrically monitored enzyme immunoassays. An absolute detection limit was not determined because of the limited sensitivity of the luminometer, and the instability of HRP at low concentrations. The sensitivity achieved with a luminescent procedure is determined by several interdependent variables including the signal to background ratio. The sensitivity using diacyl hydrazides such as luminol is generally poorer than with other techniques, because this ratio is limited by a relatively high background light emission (Seitz, 1981).

The ability to enhance HRP catalysed light emission by the addition of firefly luciferin however, improves signal, and as shown in greater detail in Chapter 3, firefly luciferin also reduces background light emission.

Susceptibility to interference: The enhanced reaction was not prone to significant interference from biological components such as those present in an impure BSA sample. Quantitation of HRP-conjugates must be relatively specific as it is often performed when conjugate is associated with variable amounts of components from biological samples or trace impurities in the reagents employed. No luminescent assay is absolutely specific for HRP and susceptibility to interference varies with the system and conditions employed. Luminescent reactions performed at alkaline pH (where luminescence is dependent on haem content rather than enzymic activity) were shown to be extremely prone to interference. This is presumably due to interference from haem proteins such as haemoglobin or myoglobin, although under alkaline conditions interference from metal ions and autoxidation may also be significant (Burdo and Seitz, 1975). The ability to enhance light emission from HRP under conditions where gross interference from components of biological samples is not encountered is a particularly attractive feature of the luciferin enhanced reaction.

Initiation of reactions: Initiation of the enhanced reaction was by simple one-step addition of premixed reagents and was therefore compatible with a range of luminometers. The WRL luminometer could not perform multiple, timed injection of

reagents in front of the photodetector and was therefore unsuitable for assays such as the luminol-perborate reaction at high pH where this was required. The ability to employ premixed reagents simplifies initiation (Wood et al., 1985), reduces background luminescence (Ikariyama et al., 1983) and helps improve mixing precision. The successful quantitation of HRP-conjugates in immunoassays by initiating the luminescent reaction with a combined luminol-H_2O_2-enhancer solution is described in Chapter 6.

2.4.2 Interpretation of luminescent assay performance

Examination of the results obtained enabled a comparison of individual assays and identification of their general features. They do however require qualification as the full potential sensitivity of individual assays may not have been realised using the instrumentation available in this study. Access to other instrumentation or luminescent calibration standards, was not possible at this time. This complicated interpretation and exact comparison with results obtained in other studies where other instrumentation was employed. Features of both the instrumentation and interdependent variables which may have limited the sensitivity or reproducibility achieved are discussed below.

Instrumental sensitivity: All assays are influenced by the luminometer employed, particularly by photodetector sensitivity and the efficiency of light collection. The WRL luminometer employed photocurrent measurements from a side-window photomultiplier tube positioned close to the reaction vessel. No

attempt was made to reflect emitted light back onto the detector. The photomultiplier tube had adequate spectral response at the wavelengths of light emitted in the assays (quantum efficiency 20 - 12 % between 400 and 485 nm) and possessed a reasonable signal to noise ratio although it was not specifically chosen for low background.

The WRL luminometer was estimated to be two orders of magnitude less sensitive than other luminometers which employed photon counting from cooled, end-window photomultiplier tubes together with optimised geometry of sample housing and light collection. This explained the difficulty in detecting and discriminating between the low levels of light emitted in the isoluminol (Pronovost and Baumgarten, 1982) and Pholas dactylus luciferin reactions (Puget et al., 1977).

Initiation of reactions and mixing of reagents: The precision and sensitivity achievable with certain luminescent assays may have been limited by the reproducibility and efficiency with which they could be initiated. This is particularly so for assays with rapid kinetics requiring initiation and mixing in front of the photodetector to monitor the initial flash or peak light emission (Schroeder and Vogelhut, 1984). In contrast, assays with relatively constant light emission and employing manual initiation outside the luminometer were mainly limited by instrumental sensitivity. Certain design features of the WRL luminometer imposed constraints on the overall mixing process thus restricting its efficiency in comparison with that achievable with other instrumentation. The injector employed was

only capable of precise delivery of volumes between 200 and 1000 µl, therefore restricting the minimum volume injectable to values greater than those in other luminometers. For this reason it was difficult to achieve rapid, reproducible mixing of the relatively large reagent volumes without reagent splashing out of the reaction vessel, solution passing to areas of the cuvette not visible to the photodetector, and variable formation of bubbles in the solution - all factors which could contribute to poor precision and sensitivity. Reaction vessel shape can also influence mixing (Rich et al., 1981) and the square, flat bottomed cuvettes employed may have produced less efficient and reproducible mixing than the cylindrical round bottomed tubes conventionally used. The square cuvettes also imposed constraints on the minimum volume of reagents which could be mixed together whilst retaining a constant position relative to the photodetector.

Reagent concentration and reaction conditions: The reagent concentrations and reaction conditions employed in luminescent assays can influence the performance and the characteristics of light emission. The pH, concentrations of chemiluminescent substrate or oxidant, and the buffer used can affect sensitivity, the intensity and kinetics of light emission (ie a flash versus a glow), quantum yield, background light emission, sensitivity to variations in mixing and susceptibility to interferences (Seitz, 1981; Cormier and Prichard, 1968). Assays optimised for maximum sensitivity on a particular luminometer often require modification when used with another luminometer (Stevens et al., 1980). Although conditions used were based on previously

reported systems, improvements may have been possible as no extensive optimisation was performed and instrumental sensitivity and design prevented the use of certain reaction conditions and reagent concentrations. The problem of which conditions to employ is illustrated in assays using luminol or its analogues and an oxidant to quantitate HRP. Diacyl hydrazide concentrations of 12 nmol/l - 3.3 mmol/l and oxidant concentrations of 10 µmol/l to 3.3 mmol/l have been used. In addition the reactions have been performed at pH 5, 7, 8, 9, 10.5 and 12.7 with measurement of peak light intensity, the level of light emission after a fixed time, or integration of light emission (Ewetz and Thore, 1976; Puget et al., 1977; Tsuji et al., 1978; Arakawa et al., 1979; Campbell and Simpson, 1979; Olsson et al., 1979a,b; Slawinska et al., 1979; Arrio et al., 1980).

2.4.3 Summary

Light emission from the HRP-conjugate catalysed oxidation of luminol is enhanced by synthetic firefly D-luciferin. Preliminary investigation of this enhanced reaction and comparison with other chemiluminescent and bioluminescent assays for HRP-conjugates indicated the novel assay appeared well suited for future application in immunoassays. Light emission was prolonged and decayed slowly and therefore there was no need to rapidly initiate individual reactions directly in front of the photodetector. In addition, the relatively intense light emission was easily and rapidly measured and the reaction could be performed simply under mild conditions. Further characterisation of enhanced reactions and examination of the

ability of molecules other than firefly luciferin to act as chemiluminescent enhancers is described in Chapter 3.

CHAPTER 3

ENHANCEMENT OF LIGHT EMISSION FROM HORSERADISH PEROXIDASE CONJUGATE CATALYSED CHEMILUMINESCENT REACTIONS

3.1 INTRODUCTION

3.1.1 Objectives

The main objective was to develop enhanced chemiluminescent procedures for the quantitation of HRP-conjugates, which would be suitable for incorporation into immunoassay systems.

In order to achieve this it was necessary to -

1) characterise and establish the scope of peroxidase catalysed enhanced chemiluminescent reactions (eg firefly luciferin-luminol-hydrogen peroxide-HRP) with regard to:
   (a) type of chemiluminescent compound, oxidant and peroxidase
   (b) type of molecule which can act as enhancer
2) establish the mechanism of enhanced peroxidase catalysed luminescent reactions.

3.1.2 Enhanced reactions

The fortuitous observation that synthetic firefly luciferin significantly enhanced light emission from the HRP catalysed luminescent luminol-H_2O_2 reaction prompted a search of the literature for other examples of enhanced reactions. Both HRP catalysed reactions and general luminescent reactions can undergo enhancement and the proposed mechanisms were examined in an attempt to formulate a working hypothesis for the mechanism of firefly luciferin enhancement.

3.1.2.1 Enhancement of luminescent reactions

The efficiency of luminescent reactions

The intensity of chemiluminescence at any time depends on the chemiluminescence efficiency and the number of molecules reacting per unit time. The chemiluminescence efficiency is dependent on

the efficiency of excited state production, the proportion of molecules in an excited state, the chemical yield of the particular reaction product and the luminescence efficiency (the ratio of photons emitted to the number of electronically excited molecules formed) (Seitz, 1981; Schroeder and Vogelhut, 1984). The luminescence efficiency also includes the efficiency of any energy transfer process. In theory a single molecule of chemiluminescent reactant could produce one electronically excited molecule which could then decay to emit one photon of light. Chemiluminescent reactions are however not this efficient and although a quantum yield of 1 einstein/mole is theoretically possible, the ratio of the number of emitted photons to the number of reacting molecules is seldom greater than 1 % of this theoretical value (Gorus and Schram, 1979). The reported quantum efficiency for typical peroxidase catalysed chemiluminescent reactions are: luminol/H_2O_2 - 0.01 (Cormier and Prichard, 1968) and purpurogallin/H_2O_2 - 1.5×10^{-8} (Slawinska et al., 1979).

Enhancement of luminescent reactions not involving horseradish peroxidase

Enhancement of light emission is a phenomenon which has been observed in a wide range of chemiluminescent and bioluminescent reactions. Typical examples are collected in Tables 3.1 and 3.2.

Addition of chloride or bromide ions to the chromium (III) catalysed luminol-H_2O_2 reaction, increases light emission eight-fold (Bause and Patterson, 1979). The proposed mechanism of enhancement involves the formation of a chromium-bromide-peroxide complex in which the bromide facilitates electron transfer in the

Table 3.1

Enhancement of light emission from chemiluminescent systems

Luminescent system	Enhancers	Magnitude of enhancement (-fold)	Reference
Luminol-H_2O_2-Chromium (III)	Chloride or bromide ions	8	Bause and Patterson, 1979
Luminol-H_2O_2	Organic compounds such as: p-chlorobenzenediazonium fluoroborate, tribromonitromethane, 1-chloro-2,4-dinitrobenzene	5-250,000	Yurow and Sass, 1977
Antibody-bound isoluminol-steroid conjugates-H_2O_2-microperoxidase	Sodium hydroxide	-	Collins et al., 1983
Isoluminol-steroid conjugate-H_2O_2-microperoxidase	Specific antibody	6	Kohen et al., 1980a
Isoluminol-biotin-H_2O_2-lactoperoxidase	Avidin (biotin specific binding protein)	10	Schroeder et al., 1976b
Lucigenin-H_2O_2-NaOH	Micelle producing surfactants such as cetyltrimethylammonium bromide	4	Paleos et al., 1981
Short chain N-alkylamino luminol-H_2O_2-persulphate	"	0.7	Rauhut et al., 1966
Lucigenin-H_2O_2-NaOH	Cyclodextrin	20	Grayeski and Woolf, 1984

Table 3.2

Enhancement of light emission from bioluminescent systems

Luminescent system	Enhancers	Magnitude of enhancement (-fold)	Reference
Renilla luciferase-luciferin-O_2	Green-fluorescent protein	3	Ward and Cormier, 1978
Pholas dactylus luciferase-luciferin-O_2	Reducing agents such as, pyrogallol, ascorbic acid, or 1,2-dihydroxy-benzene	3-6	Michelson and Isambert, 1973
Firefly luciferase-luciferin -ATP-O_2	Non-ionic surfactants such as Triton X-100, polyvinylpyrrolidone or polyethylene glycol	3-6	Kricka and DeLuca, 1982

reaction with luminol. Enhancement of light emission from the luminol-H_2O_2 reaction at alkaline pH has been achieved by the addition of various organic compounds generally containing a diazonium or oxonium group, or various combinations of carbonyl, cyano, nitro, or halo groups (Yurow and Sass, 1977). It is proposed that the induction of light emission may involve nucleophilic attack of hydroperoxide anion on the electrophilic organic compounds followed by oxidation of luminol by intermediate hydroperoxides of varying oxidising power.

Two to five-fold enhancement of light emission, with virtually no increase in background emission, has been achieved by combining, pseudoperoxidase, sodium hydroxide and hydrogen peroxide reagents prior to initiation of chemiluminescence from 6-(N-(4-aminobutyl-N-ethyl) isoluminol) hemisuccinamide labels in solid phase immunoassays (Wood et al., 1985). Generation of OOH^- ions which optimise the light producing reaction via intermediate dioxetane production, may be responsible for increased light production.

Pre-incubation of antibody-bound steroid isoluminol conjugates with sodium hydroxide in heterogeneous immunoassays increases light emission (Collins et al., 1983). This may arise from either dissociation of the antibody complex or physico-chemical changes in the label. Increased light emission is also produced when certain isoluminol-labelled antigens bind to specific antibodies (Kohen et al., 1980a) or binding proteins (Schroeder et al., 1976b). This forms the basis of several non-separation immunoassays and it is suggested that enhancement occurs because a more hydrophobic environment is established for the emitting

species.

Organised media such as micellar solutions have been shown to increase the quantum yields of chemiluminescent reactions of lucigenin with hydrogen peroxide (Paleos et al., 1981; Grayeski and Woolf, 1984). Solutions containing lamellar aggregates of didodecyl dimethyl ammonium bromide increase the efficiency of the lucigenin reaction by a factor of twelve and increased emission is thought to arise from an increase in the efficiency of the excitation step of the reaction caused by solubilisation of an intermediate in the less polar environment of the organised media. Beta-cyclodextrin, a rigid conical carbohydrate molecule with a hollow hydrophobic interior, enhances light emission from the lucigenin reaction (Grayeski and Woolf, 1984). The enhancement is attributed to an increase in excitation efficiency and rate of the reaction through inclusion of a reaction intermediate in the cyclodextrin cavity.

Radiationless or resonance energy transfer has been proposed to account for the three-fold enhancement of light emission when Renilla green fluorescent protein is added to the Renilla luciferase-luciferin system (Ward and Cormier, 1978). In this process an excited donor molecule transfers its excitation energy directly to an acceptor molecule with which it is in resonance. As the efficiency of excited state production may be greater than the fluorescence quantum yield of the donor molecule, it is possible to increase the overall fluorescence quantum yield of a resonance energy transfer system by the selection of a suitable acceptor molecule. Energy from excited sulphur dioxide can also

be transferred to fluorescent molecules and very weak chemiluminescence from the permanganate-sulphite system can be enhanced by a factor of 330 on addition of fluorescent molecules such as riboflavin phosphate (Yamada et al., 1983).

Light emission from the Pholas dactylus luciferase catalysed oxidation of its luciferin by oxygen can be enhanced by reducing agents such as pyrogallol or 1,2-dihydroxybenzene (Michelson and Isambert, 1973). The mechanism proposed involves either an induction of free radical chain reactions or modification of enzyme bound product to facilitate its dissociation. Stimulation of light emission from the firefly luciferase reaction by solvents such as non-ionic surfactants (Kricka and DeLuca, 1982) may involve a similar mechanism. It is proposed that the polymers bind to the enzyme in such a manner that the product molecule, which is inhibitory and normally tightly bound, becomes more readily dissociated.

3.1.2.2 Enhancement of reactions involving horseradish peroxidase

The mechanism of HRP catalysed reactions

Horseradish peroxidase is a monomeric glycohaemoprotein (molecular weight 40,000) containing ferriprotoporphyrin IX as its non-covalently bound prosthetic group. The enzyme exists as acidic and basic isoenzymes with differing pH optima and substrate specificities (Marklund et al., 1974). HRP catalyses a wide variety of reactions, acting as an oxidoreductase or an oxidase, and is thought capable of one or two electron oxidations depending on the substrate employed (Josephy et al., 1982a,b).

The enzyme is relatively specific for the hydrogen acceptor (commonly H_2O_2 or perborate) however a variety of inorganic (Aviram, 1981) or organic compounds (Sasson et al., 1982) including numerous phenols and aromatic amines can act as hydrogen donors. The oxidation of similar substrates by H_2O_2 is catalysed by lactoperoxidase (another haem containing enzyme) and the reaction proceeds via intermediates analogous to those demonstrated for HRP.

Three distinct stages have been defined in peroxidase catalysis (Dunford, 1982).

$$HRP + ROOH \xrightarrow{k_1} \text{Compound I} + ROH + H_2O$$

$$\text{Compound I} + AH_2 \longrightarrow \text{Compound II} + AH.$$

$$\text{Compound II} + AH_2 \xrightarrow{k_4} HRP + AH.$$

1 Interaction of the enzyme with a hydroperoxide (ROOH) giving a spectrophotometrically identifiable derivative (compound I) which retains both oxidising equivalents of ROOH.

2 Reduction of compound I in a one electron step to give compound II and a radical derived from the reductant.

3 Reduction of compound II in a second one electron step with substrate to regenerate native enzyme.

The rate constant for the reaction of substrate with compound I is usually 40-100 times larger than that for the reaction of compound II with substrate (k_4), both however are considerably less than the rate of compound I formation (k_1). The radicals formed by reaction of substrates with compounds I or II are often

highly reactive and can combine, disproportionate and/or undergo other reactions, forming the basis of colorimetric, luminescent or fluorimetric assays (Dunford, 1982; Sawahata and Neal, 1982). In colorimetric reactions the free radical produced may itself be coloured or it can recombine to give a coloured product or products. Similar radicals produced from chemiluminescent substrates such as luminol, polyphenols or luciferins can undergo further reactions producing intermediates in excited states which then decay with the emission of light (Wong and Salin, 1981; Misra and Squatrito, 1982). Electronically excited species in HRP catalysed substrate oxidations can be generated in the active site (protected from external quenching) or outside where free radicals from several substrates have been shown to exist freely in solution at concentrations up to twenty times that of the enzyme (Augusto and Bechara, 1980; Duran et al., 1981).

HRP possesses a sterically restricted, hydrophobic active site into which substrates readily partition. The site is in the vicinity of the haem prosthetic group and it is assumed the substrate binds with aromatic amino acid residues, probably by a stacking mechanism. Attraction by other amino acid residues may facilitate proton removal, and electron transfer may take place through the interaction between pi-orbitals of haem and the substrates (Hosoya et al., 1983).

Enhancement of light emission from luminescent reactions involving horseradish peroxidase

The multistep nature of peroxidase catalysed luminescent reactions, with rate limiting steps and competing side reactions,

creates several areas in which improvements in efficiency can produce increases in light emission. Several examples of enhancement of light emission in peroxidase catalysed reactions are collected in Table 3.3.

Pretreatment of peroxidase with sodium hydroxide prior to quantitation using the luminol-perborate reaction at high pH enhances light emission (Ewetz and Thore, 1976). The increased ability to induce light emission presumably reflects the degree of dissociation of protoporphyrin from the protein, but specificity is poor because similar reactions occur with other haemproteins. Above certain peroxidase concentrations azide addition results in enhancement of light emission from the peroxidase-purpurogallin-H_2O_2 system (Halmann et al., 1981). Dimerization of peroxidase can occur and the active sites are probably less accessible to bulky chemiluminescent substrates such as purpurogallin. Purpurogallin has a higher affinity for the monomeric form of the enzyme, and by forming a complex with peroxidase, azide inhibits the dimerization process enabling the monomeric form to survive at higher peroxidase concentrations.

In a deuterated solvent the relative light emission of the peroxidase-luminol-H_2O_2 reaction is increased, possibly reflecting the greater radiative lifetime of the excited aminophthalate molecule in the solvent (Wong and Salin, 1981). The addition of hydroxyl radical scavengers such as mannitol to the peroxidase-luminol-H_2O_2 reaction also enhances light emission by removing hydroxyl radicals and preventing their interference in the light producing steps (Misra and Squatrito, 1982).

Table 3.3

Enhancement of light emission from luminescent reactions involving horseradish peroxidase

Luminescent system	Enhancers	Magnitude of enhancement (-fold)	Reference
Luminol-perborate-peroxidase	Sodium hydroxide	0.5	Ewetz and Thore, 1976
Luminol-H_2O_2-peroxidase	Hydroxyl radical scavengers such as mannitol, benzoate and histidine	3	Misra and Squatrito, 1982
Luminol-H_2O_2-peroxidase	Deuterated water	0.35	Wong and Salin, 1981
Purpurogallin-H_2O_2-peroxidase	Azide	7	Halmann et al., 1981
Pyrogallol-H_2O_2-peroxidase	o-Phenylenediamine	-	Ahnstrom and Nilsson, 1965
Purpurogallin-H_2O_2-peroxidase	p-Phenylenediamine	8	Halmann et al., 1979

The peroxidase catalysed chemiluminescent oxidation of pyrogallol by peroxide is activated by addition of o-phenylenediamine (Ahnstrom and Nilsson, 1965). The velocity of the luminescent process is accelerated and, although total light emission is unchanged, peak intensity is increased. Activation is proposed to arise from the coupled oxidation of pyrogallol (or a reaction product) by reaction products initially derived from the enhancer (Ahnstrom and Nilsson, 1965). Incorporation of p-phenylenediamine into the peroxidase-purpurogallin-H_2O_2 system also results in an eight-fold enhancement of light emission (Halmann et al., 1979).

Enhancement of non-luminescent reactions involving HRP

Enhancement of HRP catalysed reactions is not confined to luminescent reactions, and enhancement of a wide range of HRP catalysed colorimetric reactions have been reported. Typical examples are summarised in Tables 3.4 and 3.5. Numerous compounds produce enhancement and several mechanisms of enhancement have been proposed.

The oxidation of guaiacol by HRP-H_2O_2 is increased by salts such as K, Na and Li chlorides and sodium sulphate. There is a linear relationship between activation of HRP and the molarity of the salt and it is suggested the peroxidase protein is affected in some way by their presence (Whitaker and Tappel, 1962).

The oxidation of substrates such as dianisidine, p-phenylenediamine or diaminobenzidine by HRP-H_2O_2 is enhanced under certain conditions by nitrogenous compounds such as

Table 3.4

Enhancement of non-luminescent reactions involving horseradish peroxidase

System	Enhancers	Degree of enhancement (-fold)	Reference
o-Methoxyphenol/H_2O_2/HRP	KCl, NaCl, LiCl or sodium sulphate	1.27	Whitaker and Tappel, 1962
Dimethoxybenzidine/H_2O_2/HRP p-Phenylenediamine/H_2O_2/HRP Diaminobenzidine/H_2O_2/HRP	Ammonia, pyridine or imidazole	up to 160	Fridovich, 1963 Straus, 1982
o-Dianisidine/H_2O_2/HRP	2-Methylimidazole		Claiborne and Fridovich, 1979
o-Dianisidine/H_2O_2/HRP	Palmitic acid	0.4	Mattoo and Modi, 1975
"	Polyoxyethylene-octylphenol or	2.7	Porstmann et al., 1981a,b
"	Polyoxyethylene-sorbitolester	8.7	
Aldehydes/O_2/HRP	Enol phosphates	-	Gallardo et al., 1984
4-Aminoantipyrine/phenol/H_2O_2/HRP	Antibody binding, HRP aggregation	16	Hoshino et al., 1985
NADH/O_2/HRP	p-Hydroxycinnamic acid		
"	Vanillin alcohol		
"	4-Hydroxy-3-methoxy cinnamic acid		
"	4-Hydroxy-3-methoxy cinnamyl alcohol	up to 4.4	Gross et al., 1977
"	4-Hydroxy-3,5-dimethoxybenzoic acid		
"	p-Hydroxybenzyl alcohol		
NADH/O_2/HRP	Mn^{2+}	2 to 3	Halliwell, 1978
NADH/O_2/HRP	2,4-Dichlorophenol	11 to 12	Halliwell, 1978
"	p-Methylphenol	4	Mader and Amberg-Fisher, 1982
"	p-Hydroxycinnamic acid	7 to 8	
Indoleacetic acid/O_2/HRP	2,4-Dichlorophenol	3	Fox and Purves, 1968

Table 3.5

Enhancement of horseradish peroxidase catalysed non-luminescent reactions proposed to occur by cyclic oxidation and reduction of phenol derivatives

System	Enhancers	Magnitude of enhancement (-fold)	Reference
Ascorbic acid/H_2O_2/HRP	o-Methoxyphenol p-Phenylphenol p-Methoxyphenol	5	Chance, 1949
NADH/H_2O_2/HRP NADH/O_2/Mn^{2+}/HRP	1,3-Dihydroxybenzene 2,4-Dichlorophenol 2-Chloro-4-phenylphenol p-Methylphenol	> 14 > 31 > 21 > 16	Akazawa and Conn, 1958
Epinephrine/H_2O_2/HRP Sodium catechol disulphonate/ H_2O_2/HRP	Thyroxine (or thyroxine analogues eg acetic or propionic acid analogues)	5 18	Klebanoff, 1959
Ascorbic acid/H_2O_2/HRP		2.4	
NADH/H_2O_2/HRP	Thyroxine (or phenolic estrogens)	20	Klebanoff, 1959
NADH/H_2O_2/HRP	2,6-Diiodo-4-aminophenol 2,6-Diiodohydroquinone 2,4,6-Triiodophenol		Takayama and Nakano, 1977
Ascorbic acid/H_2O_2/HRP	1,3-Dihydroxybenzene p-Methylphenol	23 16	Yamazaki and Piette, 1963 Ohnishi et al., 1969 Yamazaki, 1977
Ferrocyanide/H_2O_2/HRP	Benzhydroxamic acid	5	Aviram, 1981
1-Anilinonaphthalene-8-sulphonate/H_2O_2/HRP	Benzhydroxamic acid	> 30	Sasson et al., 1982
2-p-Toluidinylnaphthylene-6-sulphonate/H_2O_2/HRP	Benzhydroxamic acid		
Ferrocyanide/H_2O_2/HRP (NADH/O_2/Mn^{2+}/HRP)	2,4-Dichlorophenol p-Methylphenol Salicylhydroxamate Benzohydroxamate Phenylacetohydroxamate	13 (352) 10 (208) 4 (402) 3 (570) 2 (204)	Brooks, 1983

ammonia, pyridine and imidazole. The enhancement seems to reflect an effect on the reaction rate of HRP compound II with the colorimetric substrate. The unshared pair of electrons on nitrogen appears essential for enhancement and it is proposed the compounds produce enhancement by combining with some site on the peroxidase catalytic surface producing a change in the microenvironment beneficial to the reaction with substrate (Fridovich, 1963). Stimulation is not seen with all HRP substrates and subsequent studies suggest that the mechanism for this acceleration involves the nucleophilic base facilitating the abstraction of a second electron from the substrate radical intermediate (Claiborne and Fridovich, 1979).

The fatty acid palmitic acid increases the rate of HRP peroxidation of low concentrations of colorimetric substrates such as o-dianisidine (Mattoo and Modi, 1975). Similar activation of HRP is observed in the presence of non-ionic detergents including Tween 20 or Triton X-100 (Porstmann et al., 1981a,b). It is proposed enhancement arises by non-ionic detergents slowing down the temperature and time dependent inactivation of HRP, possibly by delaying the formation of, or by increasing the decomposition of HRP compound III, an inactive intermediate (Porstmann et al., 1981a).

HRP activity in the 4-aminoantipyrine-phenol-hydrogen peroxide reaction at high concentrations of hydrogen peroxide is increased on aggregation of the HRP. Similar enhancement is seen with complexes of HRP or HRP labelled alpha-fetoprotein with anti HRP or anti AFP respectively, and it is believed such aggregation or

complex formation suppresses the formation of compound III by the high concentration of hydrogen peroxide (Hoshino et al., 1985). In the milieu of HRP molecules of the aggregates, the concentration of hydrogen peroxide may decrease rapidly through localised enzyme action. The formation of compound III is therefore suppressed and the activity of HRP is controlled by the diffusion of hydrogen peroxide from the outer area.

The HRP catalysed aerobic oxidation of aldehydes is enhanced by enol phosphates of isobutanol (Gallardo et al., 1984). Monoanilinium salts of isobutanol-enolphosphate and its methyl ester (the oxidation of which is not promoted by HRP even in the presence of H_2O_2) greatly enhance the enzymatic oxidation of aldehydes, possibly by catalysing the formation of the reactive enol form of the substrate (Gallardo et al., 1984).

Enhancement of a wide variety of HRP catalysed reactions (generally limited by the hydrogen donors reaction with compound II) by addition of phenolic compounds has been reported. These compounds act as substrates for the enzyme and small amounts of alternative HRP substrates, such as phenols or aromatic compounds containing an hydroxyl group, accelerate the oxidation of hydrogen donors by the HRP-H_2O_2 system. Typical enhancers and the reactions which they stimulate are summarised in Table 3.5. The oxidation of hydrogen donors such as ascorbate can be enhanced by several compounds including guaiacol, p-phenylphenol, hydroquinone, thyroxine and its analogues, or resorcinol. Individual enhancers can also act as enhancers in the oxidation of a number of hydrogen donors, 2,4-dichlorophenol for example,

stimulating the oxidation of NADH, ferrocyanide or dithiothreitol. However, only certain hydrogen donors which are oxidised via one electron processes appear capable of accelerated oxidation, benzhydroxamic acid for instance enhances the oxidation of 1-anilinonaphthalene-8-sulphonate but inhibits the oxidation of dianisidine (Sasson et al., 1982).

Substrates, enhancers, and reaction intermediates and products have been studied in detail during enhanced reactions and a similar mechanism has been proposed for several enhanced reactions (Table 3.5). The enhancers are apparently more readily oxidisable substrates for HRP which form reactive radicals mediating oxidation of other substrates in solution (Sasson et al., 1982). The enhancers are oxidised by HRP compounds I or II to products such as radicals which then proceed to oxidise the hydrogen donor in a rapid non-enzymatic reaction (Chance, 1949; Yamazaki, 1977). During this reaction the radicals are reduced to the original phenolic enhancer which can in turn be re-oxidised by HRP compounds I and II. Provided the rate of formation of enhancer radicals and their subsequent reduction by hydrogen donors is faster than the direct oxidation of the hydrogen donor by compounds I and II, and the disproportionation of enhancer radicals, accelerated or enhanced oxidation of the hydrogen donor occurs. A similar mechanism has been proposed to explain enhancement of the HRP catalysed aerobic oxidation of NADH, in the presence of Mn^{2+}, by phenols such as 2,4-dichlorophenol, 2-chloro-4-phenylphenol or p-methylphenol (Akazawa and Conn, 1958).

Although no mechanistic explanation has been proposed, a series of substituted phenols have been shown to accelerate up to 10-fold the HRP catalysed peroxidation of ABTS (Stout, 1985). Typical enhancers include vanillin (4-hydroxy-3-methoxybenzaldehyde), 4-chloro-3-methyl phenol, p-iodophenol and other halosubstituted phenols. The enhanced colorimetric reactions have been used to quantitate HRP in solution and antibody-HRP conjugate in an immunoassay for choriogonadotrophin.

Phenols have also been shown to enhance a number of plant peroxidase catalysed reactions involving molecular oxygen as the oxidant (Table 3.4). A variety of phenols have been employed either alone, or in combination with Mn^{2+} (Halliwell, 1978; Mader and Amberg-Fisher, 1982). 2,4-Dichlorophenol is widely employed as a promoter of the HRP catalysed aerobic oxidation of indole-3-acetic acid and is proposed to produce enhancement by increasing the concentration or reactivity of free radicals, or by preventing enzyme inactivation (Fox and Purves, 1968). Enhancement of the peroxidase catalysed oxidation of NADH has also been produced by a series of phenols, including p-hydroxycinnamic acid, vanillin alcohol, 4-hydroxy-3-methoxy cinnamic acid and 4-hydroxy-3-methoxy cinnamyl alcohol (Gross et al., 1977). Similar enhancement has been demonstrated using 2,4-dichlorophenol or p-methylphenol (Halliwell, 1978). Oxidation in the presence of these phenols could be increased by H_2O_2 addition, suggesting that phenols enhanced the HRP catalysed reaction in a way that makes it limited by the supply of H_2O_2. In the presence of NADH and under aerobic conditions, peroxidase exists predominantly as compound III, thus enhancement is

proposed to arise by the phenols stimulating the decomposition of compound III to active enzyme. The peroxidase catalysed oxidation of NADH, indole acetic acid or dihydrofumarate is enhanced by Mn^{2+} (Yamazaki and Piette, 1963) however, unlike phenol enhancement, addition of H_2O_2 produced no increase in NADH oxidation in the presence of Mn^{2+} (Halliwell, 1978). Combinations of Mn^{2+} with phenols such as 2,4-dichlorophenol accelerate the peroxidase catalysed oxidation of NADH. The presence of Mn^{2+} accelerated NADH oxidation 2- to 3-fold; phenols alone enhanced the reaction 10 - 100-fold, however together they enhanced the reaction 1000 - 10,000-fold (Mader and Fussl, 1982). Mn^{2+} appears to catalyse a non-enzymatic reaction between NADH and $O_2^{\cdot -}$ to generate catalytic amounts of H_2O_2, whereas the main function of phenols in enhancement of NADH oxidation is proposed to be the breakdown of HRP compound III (Mader and Fussl, 1982).

3.2 EXPERIMENTAL

Materials and Methods

3.2.1 Reagents

7-Dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide was obtained from Boehringer Mannheim GmbH, Germany; N-(6-aminobutyl)-N-ethyl isoluminol from LKB Wallac, Finland. Benzothiazole was purchased from Fluka AG, Switzerland; synthetic firefly D-luciferin and its 6-ethyl ether from Sigma Chemical Company. Dehydroluciferin was kindly supplied by Professor M DeLuca (Chemistry Department, University of California, La Jolla, USA), all other benzothiazole derivatives, synthesised following literature procedures (Bowie, 1978), were kindly supplied by Dr N

Baggett (Chemistry Department, University of Birmingham, UK).

p-Iodophenol, p-phenylphenol, and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone were purchased from Aldrich Chemical Company (Gillingham, Dorset, UK); 2,4-dichlorophenol, haemin and sodium perborate from BDH Chemicals Ltd (Poole, UK) and 3,3',5,5' tetramethylbenzidine and dimethyl sulphoxide (grade 1) from Sigma. Other compounds investigated as potential enhancers of chemiluminescent reactions were obtained from Aldrich Chemical Company, Sigma, BDH Chemicals Ltd or Lancaster Synthesis Ltd (Morecambe, UK). The compounds were of AnalaR or the purest quality available and were used without further purification.

Horseradish peroxidase (RZ 3.1, 245 guaiacol units/mg) was obtained from Miles Laboratories (PTY) Ltd (South Africa). Horseradish peroxidase type VI (RZ approx 3.0, 250-330 purpurogallin units/mg) and horseradish peroxidase isoenzymes type VII (RZ approx 3.0, 129 units/mg), type VIII (RZ approx 3.0, 80 units/mg) and type IX (RZ approx 3.2, 316 units/mg) were purchased from Sigma. Haemoglobin (human, type IV), myoglobin (equine, type I), cytochrome-c (equine, type II-A), and lactoperoxidase (from milk, 5.8 mg protein/ml in 3.2 mol/l ammonium sulphate; 58 purpurogallin units/mg) were also obtained from Sigma. Lactoperoxidase was dialysed against distilled water prior to use. All other compounds were obtained as described in Chapter 2 Section 2.2.1. All buffers and solutions in this and subsequent sections were prepared using distilled water.

3.2.2 Investigation of enhanced chemiluminescent reactions

3.2.2.1 Identification of enhancers

General screening Anti AFP-HRP (10 µl 1:1000 dilution in distilled water) and the compound under investigation (10 µl; 1 mg/ml in DMSO or Tris buffer, 0.1 mol/l, pH 8.5) were placed in separate corners of a cuvette. The luminescent reaction was initiated by manual addition of 900 µl of a luminol (1.25 mmol/l), hydrogen peroxide (2.7 mmol/l) mixture prepared in Tris buffer (0.1 mol/l, pH 8.5). Levels of light emitted 30 seconds after initiation were compared with controls in which DMSO or buffer replaced the candidate enhancer. The influence of potential enhancers on background light emission from the luminol-hydrogen peroxide reaction was determined as previously described, but with anti AFP-HRP replaced by distilled water. Enhancement was deemed to have occurred when the compound under investigation increased the HRP catalysed light emission and had an insignificant effect on (or reduced) background light emission.

6-Hydroxybenzothiazole derivatives A series of 6-hydroxybenzothiazole derivatives (1 mg/ml in DMSO) were examined for enhancer activity as previously described. Light intensity was also determined 30 and 60 seconds after initiation using enhancer (10 µl; 0.5 mg/ml) and 1 ml of a luminol (60 µmol/l), hydrogen peroxide (2 mmol/l) mixture prepared in Tris buffer (0.1 mol/l, pH 8.0).

Halophenols A series of mono-, di-, and polysubstituted halophenols were investigated using the general screening

procedure. Compounds producing enhancement were also examined using 10 µl or 90 µl, of 4.54 mmol/l halophenol solutions in DMSO.

Naphthols and halonaphthols A series of naphthols and halonaphthols were examined using the general screening procedure.

Reported enhancers of luminescent reactions Previously reported enhancers of chemiluminescent or bioluminescent reactions; Tables 3.1 to 3.3 (10 µl of 4.54 mmol/l solutions) were incorporated into both the general screening procedure, and into a modified procedure using 900 µl of a luminol (60 µmol/l), hydrogen peroxide (2 mmol/l) mixture.

Enhancers of colorimetric HRP catalysed reactions and miscellaneous compounds A series of compounds reported to enhance non-luminescent HRP catalysed reactions, known HRP substrates, and other miscellaneous compounds were investigated for enhancer activity using the general screening procedure.

The degree of enhancement produced by different enhancers The general screening reaction was performed using 5, 10 or 90 µl solutions (4.54 mmol/l in DMSO) of compounds identified as enhancers. The maximal increase in light intensity 30 seconds after initiation produced by individual enhancers, and the influence on background light emission at this particular enhancer concentration was determined.

3.2.2.2 The synergistic action of enhancers

Anti AFP-HRP (10 µl of a 1:1000 dilution), luminol (50 µl) and

hydrogen peroxide (50 µl, 40 mmol/l) in Tris buffer (0.1 mol/l) were placed in separate corners of a cuvette. The enhancer under investigation (10 µl) was placed in the remaining corner and the luminescent reaction initiated by manual injection of 900 µl Tris buffer (0.1 mol/l). Light emission against time was recorded and light intensity after 30 seconds or 60 seconds determined. The experiments were repeated with combinations of components replaced by the appropriate solvent.

Reactions were performed at pH 8.0, using luminol (1.2 mmol/l) and 6-hydroxybenzothiazole derivatives (0.5 mg/ml in DMSO); at pH 8.0 using luminol (6 mmol/l) and p-iodophenol or 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (1 mg/ml); and at pH 8.5 using anti AFP-HRP (50 µl of a 1:5000 dilution), luminol (1.2 mmol/l) and 20 µl p-iodophenol (18.2 mmol/l) or p-phenylphenol (0.6 mmol/l).

3.2.2.3 Specificity of enhancement

Chemiluminescent compound

Acyl hydrazide Solutions of luminol (120 µmol/l), isoluminol (135 µmol/l), 7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide (88 µmol/l) and N-(6-aminobutyl)-N-ethyl isoluminol (86 µmol/l) were prepared by dissolving the acyl hydrazide in NaOH (100 µl; 0.1 mol/l) and then dilution of this solution with Tris buffer (25 ml; 0.1 mol/l, pH 8.0).

Acyl hydrazide (50 µl), anti AFP-HRP (10 µl of a 1:1000 dilution) and hydrogen peroxide (50 µl; 40 mmol/l) in Tris buffer (0.1 mol/l, pH 8.0) were placed in separate corners of a cuvette. Enhancer or solvent (10 µl) was placed in the remaining corner,

and the luminescent reaction initiated by manual injection of 900 µl Tris buffer (0.1 mol/l, pH 8.0). Light emission against time was recorded and light intensity 60 seconds after initiation determined. Firefly luciferin, dehydroluciferin or 6-hydroxybenzothiazole (0.5 mg/ml in DMSO) were employed as enhancers. Reactions were also performed using diacyl hydrazides (120 µmol/l) and H_2O_2 (4 mmol/l) at pH 8.5, with p-iodophenol (3.6 mmol/l) or p-phenylphenol (118 µmol/l) as enhancer.

Purpurogallin The purpurogallin-hydrogen peroxide reaction was performed essentially as in Chapter 2, Section 2.2.3.6. Purpurogallin (55 µl, 9.1 mmol/l in methanol) and anti AFP-HRP conjugate (10 µl, of a 1:100 dilution in distilled water) were placed in separate corners of a cuvette. The luminescent reaction was initiated in front of the photomultiplier tube by automatic injection of hydrogen peroxide (900 µl, 0.12 mol/l) in potassium phosphate buffer (0.15 mol/l, pH 6.5) and peak light emission recorded. The reaction was repeated in the presence of either 20 µl p-phenylenediamine, 2,4-dichlorophenol, firefly luciferin (25 mmol/l) or p-iodophenol (13.6 mmol/l) prepared in a 50% ethanolic phosphate buffer solvent.

Oxidant

Anti AFP-HRP (10 µl of a 1:1000 dilution), luminol (50 µl, 1.2 mmol/l) and either hydrogen peroxide or sodium perborate (50 µl, 40 mmol/l), in Tris buffer (0.1 mol/l, pH 8.0) were placed in separate corners of a cuvette. Enhancer or solvent (10 µl) was placed in the remaining corner and the luminescent reaction initiated by manual injection of Tris buffer (0.1 mol/l, pH 8.0).

Light emission after 30 seconds was determined. Firefly luciferin, 6-hydroxybenzothiazole, or dehydroluciferin (0.5 mg/ml in DMSO) were employed as enhancers. Reactions were also performed at pH 8.5 using 20 µl p-iodophenol (18 mmol/l) or p-phenylphenol (590 µmol/l) as enhancer.

3.2.2.4 Influence of pH

Anti AFP-HRP (10 µl of a 1:1000 dilution), luminol (50 µl, 6 mmol/l), and hydrogen peroxide (50 µl, 40 mmol/l) prepared in distilled water were placed in separate corners of a cuvette. Enhancer solution (10 µl; firefly luciferin, 2,4-dichlorophenol or p-iodophenol 1 mg/ml in 10 mmol/l Tris buffer, pH 8.0) was placed in the remaining corner and the luminescent reaction initiated by manual addition of 900 µl Tris buffer (0.1 mol/l, pH 6.5 - 9.0) or glycine/NaOH buffer (0.1 mol/l, pH 9.0 - 12.0). Light intensity after 30 seconds was determined. The p-iodophenol enhanced reaction was also performed with final concentrations of p-iodophenol, 336 µmol/l; luminol 59 µmol/l and H_2O_2 1.87 mmol/l.

3.2.2.5 Influence of enhancer concentration

Anti AFP-HRP (100 µl of a 1:5000 dilution in Tris buffer (0.1 mol/l, pH 8.5)) and p-iodophenol (40 µl, 0-90 mmol/l in DMSO) were placed in separate corners of a cuvette. The luminescent reaction was initiated by manual addition of 2 ml of a luminol (60 µmol/l), hydrogen peroxide (2 mmol/l) mixture prepared in Tris buffer (0.1 mol/l, pH 8.5). Light emission against time was recorded and light intensity after 30 seconds determined. Similar experiments were performed using 6-hydroxybenzothiazole

(0 - 14.3 mmol/l) or p-phenylphenol (0 - 5.9 mmol/l) in place of the p-iodophenol.

3.2.2.6 Influence of catalyst

Horseradish peroxidase isoenzyme: Horseradish peroxidase preparation or specific isoenzyme (10 µl, 1 µg/ml in 0.1 mol/l Tris buffer pH 8.5) was placed in the corner of a cuvette. Enhancer or DMSO solvent (20 µl) was placed in a separate corner and the luminescent reaction initiated by manual addition of 1 ml of a luminol (60 µmol/l), hydrogen peroxide (2 mmol/l) mixture prepared in Tris buffer (0.1 mol/l, pH 8.5). Light intensity up to 90 seconds after initiation was recorded. p-Iodophenol (18.2 mmol/l), p-phenylphenol (0.6 mmol/l) or 6-hydroxybenzothiazole (1.5 mmol/l) prepared in DMSO were employed as enhancers. The p-iodophenol enhanced reaction was repeated using various dilutions of HRP preparations.

Haemproteins: Dilutions of haemproteins (10 µl, in Tris buffer (0.1 mol/l, pH 8.5)) were placed in the corner of a cuvette. p-Iodophenol (20 µl, 18.2 mmol/l in DMSO) was placed in a separate corner and the luminescent reaction initiated by manual addition of 1 ml of a luminol (60 µmol/l), hydrogen peroxide (2 mmol/l) mixture prepared in Tris buffer (0.1 mol/l, pH 8.5). Light intensity after 30 seconds was determined and compared to that obtained with DMSO (20 µl) substituted for p-iodophenol.

3.2.2.7 Luminescent emission spectra of enhanced reactions

Luminescence emission spectra were determined using a model SRF 100 recording spectrofluorimeter (Baird-Atomic Ltd, England). Scans were determined between 350 and 600 nm at 4 nm/second.

Luminol, H_2O_2, HRP:

Unenhanced, firefly luciferin or 2,4-dichlorophenol enhanced reactions

Anti AFP-HRP conjugate (10 µl of a 1:100 dilution in water) and either 2,4-dichlorophenol or firefly luciferin (10 µl, 1 mg/ml (6.1 or 3.6 mmol/l) in Tris buffer 0.1 mmol/l, pH 8.0) were placed in separate corners of a disposable cuvette. The luminescent reaction was initiated by manual injection of 900 µl of a mixture of luminol (1.25 mmol/l) and hydrogen peroxide (2.7 mmol/l) in Tris buffer (0.1 mol/l, pH 8.0). The solution was mixed, the cuvette placed in the spectrofluorimeter and the emission spectrum, 30 seconds after initiation, recorded. Spectra with buffer replacing the 2,4-dichlorophenol, or firefly luciferin were also determined.

Para-halophenol enhanced reaction

The experimental procedure previously described was repeated using a 1:1000 dilution of anti AFP-HRP conjugate and 10 µl of 1 mg/ml solutions of 2,4-dichlorophenol (6.1 mmol/l), p-chlorophenol (7.8 mmol/l) or p-iodophenol (4.5 mmol/l) prepared in DMSO.

6-Hydroxybenzothiazole or dehydroluciferin enhanced reactions

Anti AFP-HRP (10 µl of a 1:1000 dilution in 0.1 mol/l Tris buffer pH 8.0) and either 6-hydroxybenzothiazole or dehydroluciferin (10 µl, 0.5 mg/ml (3.3 or 1.8 mmol/l in DMSO) were placed in separate corners of a cuvette. Luminol (50 µl, 1.2 mmol/l) and hydrogen peroxide (50 µl, 40 mmol/l) in Tris buffer (0.1 mol/l, pH 8.0) were placed in the remaining corners of the cuvette and the

luminescent reaction initiated by manual injection of Tris buffer (900 µl, 0.1 mol/l, pH 8.0). Scans were performed 2 and 4 minutes after initiation.

7-Dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide, H_2O_2, HRP:

Luminol or 7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide (30 µl, 5 and 3.7 mmol/l respectively), hydrogen peroxide (100 µl, 40 mmol/l in water) anti AFP-HRP conjugate (40 µl, 1:100 dilution in water) and p-iodophenol (10 µl, 4.5 mmol/l in DMSO) were placed in corners of a cuvette. The luminescent reaction was initiated by manual injection of 900 µl phosphate buffer (0.1 mol/l, pH 9.0) and the emission spectrum, 30 seconds after initiation recorded. Spectra with DMSO replacing p-iodophenol were also determined.

3.3 RESULTS

3.3.1 Identification of enhancers

Several features of the enhanced luminescent reaction were demonstrated when compounds were screened for the ability to increase light emission from the HRP-conjugate catalysed peroxidation of luminol:

(1) Enhancement of light emission could be produced by molecules other than synthetic firefly D-luciferin. These included certain 6-hydroxybenzothiazole derivatives and certain substituted phenols.

(2) Different increases in light emission were produced by compounds capable of acting as enhancers.

(3) Stringent structural properties were required for a molecule to act as an enhancer. Simple structural modification could dramatically alter enhancement properties.

(4) The degree of enhancement of light emission was dependent on the enhancer concentration. The concentrations of individual enhancers, which produced maximal light emission differed.

(5) Increases in light emission occurred if enhancer was added during, or at the start of luminescent reactions.

(6) Light emission from the luminol-hydrogen peroxide reaction (in the absence of HRP-conjugate) could be decreased by compounds that enhanced light emission from the HRP-conjugate catalysed reaction.

(7) Compounds previously reported to increase light from luminescent reactions, did not produce similar increases to that produced by firefly luciferin.

(8) Several compounds reported to accelerate colorimetric HRP catalysed reactions were also found to increase light emission from the HRP-conjugate catalysed luminol-hydrogen peroxide reaction. Certain other compounds, also reported to accelerate colorimetric reactions involving HRP, produced no corresponding increase in light emission.

3.3.1.1 6-Hydroxybenzothiazole derivatives

6-Hydroxybenzothiazole derivatives screened for enhancer activity are shown in Table 3.6 and Figure 3.1. In addition to firefly D-luciferin, four of the benzothiazole derivatives tested (6-

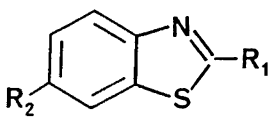
Table 3.6

Benzothiazoles screened for ability to increase light emission from the HRP-conjugate catalysed oxidation of luminol

Enhancers	Non-enhancers
6-Hydroxybenzothiazole (II)	Benzothiazole (I)
2-Cyano-6-hydroxybenzothiazole (VII)	6-Methoxybenzothiazole (III)
Dehydroluciferin (IX)	2-Amino-6-hydroxybenzothiazole (IV)
D-Firefly luciferin (X)	2-Amino-6-methoxybenzothiazole (V)
L-Firefly luciferin	2,6-Dihydroxybenzothiazole (VI)
	2-Cyano-6-ethoxybenzothiazole (VIII)
	6-Ethoxy luciferin (XI)

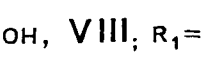
Figure 3.1

6-Hydroxybenzothiazole derivatives screened for enhancer activity



I; $R_1=H, R_2=H$, II; $R_1=H, R_2=OH$, III; $R_1=H, R_2=OCH_3$,

IV; $R_1=NH_2, R_2=OH$, V; $R_1=NH_2, R_2=OCH_3$, VI; $R_1=OH, R_2=OH$,

VII; $R_1=CN, R_2=OH$, VIII; $R_1=CN, R_2=OCH_2CH_3$, IX; $R_1=$  $, R_2=OH$,

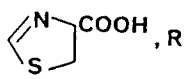
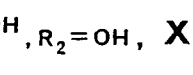
X; $R_1=$  $, R_2=OH$, XI; $R_1=$  $, R_2=OCH_2CH_3$.

Table 3.7

Influence of benzothiazole derivatives on light emission from the luminol, hydrogen peroxide system in the presence and absence of horseradish peroxidase conjugate

Benzothiazole derivative	Relative light emission (mV)		Magnitude of enhancement of light emission		Percentage reduction in background light emission
	30s	60s	30s	60s	(5-180s)
DMSO (solvent control)	16	11	-	-	0
Benzothiazole (I)	10	8	-	-	
6-Hydroxybenzothiazole (II)	5950	7340	372	667	98.9
6-Methoxybenzothiazole (III)	13	10	-	-	94.5
2-Amino-6-hydroxybenzothiazole (IV)	8	8	-	-	99.9
2-Amino-6-methoxybenzothiazole (V)	9	6	-	-	95.0
2,6-Dihydroxybenzothiazole (VI)	5	5	-	-	99.5
2-Cyano-6-hydroxybenzothiazole (VII)	600	750	38	68	95.9
2-Cyano-6-ethoxybenzothiazole (VIII)	3	2	-	-	96.3
Dehydroluciferin (IX)	7240	6990	453	636	96.9
Firefly luciferin (X)	2480	2460	155	224	96.7
6-Ethoxy luciferin (XI)	5	4	-	-	99.7

hydroxybenzothiazole, 2-cyano-6-hydroxybenzothiazole, 2-(6-hydroxy-2-benzothiazolyl)thiazole-4-carboxylic acid (dehydroluciferin) and firefly L-luciferin) produced enhanced light emission from the HRP-conjugate catalysed oxidation of luminol. The influence of benzothiazole derivatives on light emission from the reaction in the presence and absence of HRP-conjugate is shown in Table 3.7. 6-Hydroxybenzothiazole and dehydroluciferin produced greater enhancement than firefly luciferin and several hundred-fold increases of light emission over the unenhanced reaction were achieved. The enhancers also depressed the background light emission from the luminol-oxidant mixture by over 90 %. This produced a further improvement in the signal to background ratio. Reduced background light emission was however also produced by benzothiazole derivatives which did not enhance light emission. Stringent structural requirements, notably a hydroxyl group at the 6-position, were required for a benzothiazole to act as an enhancer. 6-Hydroxybenzothiazoles with a 2-amino or 2-hydroxy group did not act as enhancers and substitution of the 6-hydroxy group (eg 6-methyl ether or 6-ethyl ether derivatives) or its removal (eg benzothiazole) eliminated the enhancement properties of the derivatives.

3.3.1.2 Halophenols

Several halophenols produced increases in light emission from the HRP-conjugate catalysed oxidation of luminol. Table 3.8 summarises the halophenols tested and their ability to act as enhancers. Monosubstituted ortho or meta halophenols produced no increase in light emission. Several monosubstituted para-

Table 3.8

Halophenols screened for ability to increase light emission from the HRP-conjugate catalysed oxidation of luminol

Enhancers	Non-enhancers
p-Chlorophenol	p-Fluorophenol
p-Bromophenol	p-Chloromethoxyphenol
p-Iodophenol	p-Iodomethoxyphenol
2,4-Dichlorophenol	o-Chlorophenol
3,4-Dichlorophenol	m-Chlorophenol
2-Chloro-4-bromophenol	2,3-Dichlorophenol
	2,5-Dichlorophenol
	2,6-Dichlorophenol
	2,3,4-Trichlorophenol
	2,3,5-Trichlorophenol
	3,3,6-Trichlorophenol
	2,4,5-Trichlorophenol
	2,4,6-Trichlorophenol
	3,4,5-Trichlorophenol
	Pentachlorophenol

Table 3.9

Enhancement of light emission from the HRP-conjugate catalysed oxidation of luminol by addition of halophenols

Enhancer	Light emission after 30s (mV) Quantity added 45 nmoles	408 nmoles	% Reduction in background light emission after 30s 45 nmoles	408 nmoles
None (DMSO solvent)	9	15	-	-
p-Iodophenol	903	3056	43	89
p-Bromophenol	261	1379	24	80
p-Chlorophenol	190	1190	13	80
2-Chloro-4-bromophenol	80	555	7	69
2,4-Dichlorophenol	29	165	50	96
3,4-Dichlorophenol	20	82	49	88

halophenols produced pronounced enhancement of light emission. Substitution of the hydroxyl group (eg p-chloromethoxy phenol) prevented halophenols acting as enhancers and additional halogen substitution in para-halophenols reduced or eliminated enhancement. The levels of light emission produced by addition of para-substituted halophenols to the luminol-peroxide-HRP-conjugate reaction are shown in Table 3.9. p-Iodophenol produced particularly effective increases in light emission (up to 200-fold) under the conditions employed. The magnitude of enhancement was dependent on the type of halogen substituent (increases in light emission with p-iodophenol > p-bromophenol > p-chlorophenol) and the concentration of enhancer employed. In the absence of HRP-conjugate, addition of halophenols to luminol-H_2O_2 reduced light emission (Table 3.9). Under the various conditions employed, p-iodophenol reduced background light emission by approximately 43 - 93 % (Tables 3.9 and 3.15).

3.3.1.3 Naphthols and halonaphthols

Although 1-naphthol and 4-chloro-1-naphthol failed to produce increased light emission from the luminol-H_2O_2-HRP reaction, 2-naphthol and several halonaphthols such as 1,6-dibromo-2-naphthol produced effective enhancement of light emission (Table 3.10).

3.3.1.4 Reported enhancers of luminescent reactions

Several compounds reported to increase light emission from luminescent systems (Tables 3.1 to 3.3) were incorporated into the HRP-conjugate catalysed luminol-hydrogen peroxide reaction. Under the conditions employed none produced any increase in light intensity. All the compounds investigated produced some degree

Table 3.10

Naphthols and halonaphthols screened for ability to increase light emission from the HRP-conjugate catalysed oxidation of luminol

Enhancers	Non-enhancers
1-Bromo-2-naphthol	4-Chloro-1-naphthol
6-Bromo-2-naphthol	1-Naphthol
1,6-Dibromo-2-naphthol	6-Bromo-2-methoxynaphthalene
2-Naphthol	2-Methoxynaphthalene

Table 3.11

Compounds exhibiting enhancement of light emission in luminescent reactions, screened for ability to increase light emission from the HRP-conjugate catalysed oxidation of luminol

Enhancers	Non-enhancers	Reduction in light emission (%)	
		A	B
None			
	Potassium bromide	26	52
	Potassium chloride	6	35
	Ascorbic acid	>99	>98
	Pyrogallol	>99	>98
	Sodium azide	11	28
	Mannitol	19	74
	Histidine	32	60
	Thiourea	86	97
	Sodium benzoate	32	38
	p-Phenylenediamine	>99	-
	o-Phenylenediamine	23	-

Compounds under investigation (10 µl; 4.54 mmol/l) were added to luminol-hydrogen peroxide-HRP-conjugate mixtures (900 µl).
A: luminol, 1.25 mmol/l; H_2O_2 2.7 mmol/l.
B: luminol, 60 µmol/l; H_2O_2, 2 mmol/l.

of reduction of light emission (Table 3.11). Pyrogallol, ascorbic acid and p-phenylenediamine (known HRP substrates) produced particularly pronounced decreases (> 99 %) in light emission from the HRP-conjugate catalysed reaction.

3.3.1.5 Enhancers of colorimetric HRP catalysed reactions

Several compounds previously reported to enhance HRP catalysed reactions also produced an enhancement of light emission from the HRP-luminol-H_2O_2 reaction. Not all colorimetric enhancers however increased light emission (Table 3.12); many, such as o-methoxyphenol (guaiacol) and 1,3-dihydroxybenzene (resorcinol), actually decreased light emission. Particularly effective enhancers in both systems were p-iodophenol, p-hydroxycinnamic acid, p-phenylphenol and 2-chloro-4-phenylphenol. p-Iodophenol and vanillin (4-hydroxy-3-methoxybenzaldehyde) which both produced effective enhancement of the oxidation of ABTS did not react similarly in the chemiluminescent system. p-Iodophenol produced effective increases in light emission whereas vanillin decreased light output.

3.3.1.6 Miscellaneous compounds

Incorporation of chemiluminescent polyphenol HRP substrates such as pyrogallol, purpurogallin, gallic acid etc, into the HRP-luminol-H_2O_2 reaction produced no increase in light emission (Table 3.13) and all except umbelliferone produced dramatic decreases in intensity. The influence of colorimetric or fluorometric substrates not previously tested are also included in Table 3.13, although many such compounds, including several para-substituted, produced no enhancement, increased emission was

Table 3.12

Compounds exhibiting enhancement of non-luminescent HRP catalysed reactions, screened for ability to increase light emission from the HRP-conjugate catalysed oxidation of luminol

Enhancers	Non-enhancers
p-Hydroxycinnamic acid	Potassium or sodium chloride
2,4-Dichlorophenol	Sodium sulphate
p-Methylphenol	Imidazole
p-Phenylphenol	Palmitic acid
2-Chloro-4-phenylphenol	4-Hydroxy-3-methoxycinnamic acid (ferulic acid)
p-Iodophenol	4-Hydroxy-3-methoxycinnamyl alcohol (coniferyl alcohol)
p-Bromophenol	o-Methoxyphenol (guaiacol)
p-Chlorophenol	p-Methoxyphenol (hydroquinone monomethyl ether)
4-Chloro-3-methyl-phenol	1,3-Dihydroxybenzene
	Thyroxine
	3,3',5-Tri-iodo-L-thyronine
	Salicylhydroxamic acid
	4-Hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol)
	Benzhydroxamic acid
	Mn^{2+}
	4-Hydroxy-3,5-dimethoxybenzoic acid (syringic acid)
	p-Hydroxybenzyl alcohol
	Vanillin (4-hydroxy-3-methoxybenzaldehyde)

Table 3.13

Additional peroxidase substrates and their derivatives screened for ability to increase light emission from the HRP-conjugate catalysed oxidation of luminol

Enhancers	Non-enhancers
3,5,3',5'Tetramethylbenzidine	Pyrogallol (1,2,3-trihydroxybenzene)*
N,N,N',N'Tetramethylbenzidine	Phloroglucinol (1,3,5-trihydroxybenzene)*
	Purpurogallin*
	Umbelliferone*
	Gallic acid (3,4,5-trihydroxybenzoic acid)*
	Hydroquinone
	4-Hydroxy-3-methoxyphenyl acetic acid
	Catechol (o-hydroxyphenol)
	p-Hydroxyphenyl acetic acid
	Tyramine (4-(2-aminoethyl)phenol)
	p-Aminobenzoic acid
	p-Aminophenol
	Phenol
	m-Phenylenediamine
	p-Methoxybiphenyl
	p-Methoxycinnamic acid

* Chemiluminescent substrates

Table 3.14

The degree of enhancement produced on addition of various enhancers to the HRP-conjugate-luminol-H_2O_2 reaction

Enhancer	Light emission after 30 s	Fold increase in light emission	Improvement in signal/ background ratio
p-Phenylphenol[a]	5830	530	3482
p-Iodophenol[c]	3056	278	2500
1,6-Dibromo-2-naphthol[a]	2401	218	2055
p-Hydroxycinnamic acid[b]	2317	210	2935
6-Hydroxybenzothiazole[b]	297	27	1699
Firefly luciferin[a]	50	5	58

Final enhancer concentration [a] = 25 µmol/l, [b] = 49 µmol/l, [c] = 408 µmol/l

obtained with 3,5,3',5'tetramethylbenzidine and N,N,N',N'tetramethylbenzidine. No enhancement of light emission was achieved by addition of p-nitrophenol and benzoic acid (compounds shown not to produce radicals with the HRP-H_2O_2 system) or dimethyl-p-phenylenediamine (a compound known to breakdown HRP compound III).

3.3.1.7 The degree of enhancement produced by individual enhancers

The quantitative influence of individual enhancers on the chemiluminescent luminol-H_2O_2 reaction is shown in Table 3.14. The increase in light emission from the HRP-conjugate catalysed reaction is different for each enhancer, as is the degree to which they reduce the background light emission. The concentration of enhancer at which the maximal increase in light emission was produced varies for individual enhancers, the concentration of p-iodophenol required being significantly higher than for other substituted phenols, and 6-hydroxybenzothiazole derivatives.

3.3.2. The synergistic action of enhancers

Light emission from components of enhanced and unenhanced reactions involving luminol are shown in Table 3.15. Similar results to those from the firefly luciferin enhanced reaction (Chapter 2, Table 2.3) were obtained using 6-hydroxybenzothiazole, dehydroluciferin, p-iodophenol or p-phenylphenol. Enhancement occurred at several pHs and with various concentrations of luminol, H_2O_2 and enhancer. Very low levels of light emission were obtained in the absence of H_2O_2,

Table 3.15

Light emission from components of enhanced chemiluminescent reactions

REACTION COMPONENTS	ENHANCER	A Light emission after 60s (mV) 6-Hydroxy-benzothiazole	A Dehydro-luciferin	B Light emission after 30s (mV) p-Iodophenol	B p-Phenylphenol	C Light emission after 30s (mV) p-Iodophenol	C 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
Luminol, H_2O_2, HRP-conjugate,	-	22	22	230	230	119	153
Luminol, H_2O_2, HRP-conjugate,	enhancer	11,410	18,350	127,143	16,150	195,500	110,160
Luminol, H_2O_2, - ,	-	10	10	96	96	8	8
- , H_2O_2, - ,	enhancer	< 1	< 1	< 1	< 1	< 8	8
- , H_2O_2, HRP-conjugate,	enhancer	8	5	180	67	85	25
Luminol, - , - ,	enhancer	< 1	< 1	< 4	4	< 8	< 8
Luminol, - , HRP-conjugate,	enhancer	< 1	< 1	< 1	< 1	< 8	< 8
Luminol, H_2O_2, - ,	enhancer	< 1	< 1	< 7	48	< 8	109,480

A. pH 8.0; final concentrations: luminol 59 µmol/l; H_2O_2 1.9 mmol/l; 6-hydroxybenzothiazole 32.3 µmol/l; dehydroluciferin 17.6 µmol/l.
B. pH 8.5; final concentrations: luminol 56 µmol/l; H_2O_2 1.86 mmol/l; p-iodophenol 340 µmol/l; p-phenylphenol 11 µmol/l.
C. pH 8.0; final concentrations: luminol 290 µmol/l; H_2O_2 1.95 mmol/l; p-iodophenol 45 µmol/l; 2,3-dichloro-5,6-dicyano-1,4-benzoquinone 43 µmol/l.

and very intense light emission only in the presence of HRP-conjugate. A dramatic increase in light emission was only observed when 6-hydroxybenzothiazole derivatives or para-substituted phenols were added to a mixture of luminol, hydrogen peroxide and HRP-conjugate. Although some light was emitted with enhancers in the presence of H_2O_2 and HRP-conjugate, the dramatic increase in light emission on addition of enhancer to the luminol-H_2O_2-HRP-conjugate system significantly exceeded that from systems with either luminol or enhancer alone. Increased light emission could not be explained as an additive effect, the enhancers apparently acted synergistically with luminol to increase total light emission. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone also produced dramatic increases in light emission from the luminol-H_2O_2-HRP-conjugate system however, in sharp contrast to other enhancers, this also occurred in the absence of HRP-conjugate.

3.3.3 Specificity of enhancement

3.3.3.1 Chemiluminescent compound

Increases in light emission from the oxidation of a range of cyclic diacyl hydrazides (including luminol, isoluminol, N-(6-aminobutyl)-N-ethyl isoluminol and 7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide) were achieved by addition of a variety of 6-hydroxybenzothiazole derivatives or para-substituted phenols (Tables 3.16 and 3.17). Light emission from the HRP-conjugate catalysed reactions were enhanced at pH 8.0 or pH 8.5, and with various concentrations of cyclic diacyl hydrazide, oxidant and enhancer. Maximum increases in light emission were obtained with luminol (up to 2,500-fold that of the unenhanced

Table 3.16

Increases in light emission from the horseradish peroxidase conjugate catalysed chemiluminescent oxidation of cyclic diacyl hydrazides by 6-hydroxybenzothiazole derivatives

Cyclic diacyl hydrazide	Fold increase in light emission		
	6-Hydroxybenzothiazole	Dehydroluciferin	Firefly luciferin
Luminol (59 µmol/l)	520	544	190
Isoluminol (6.6 µmol/l)	29	30	22
N-(6-Aminobutyl)-N-ethyl isoluminol (4.2 µmol/l)	18	15	35
7-Dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide (4.3 µmol/l)	114	62	54

pH 8.0; Final concentrations: cyclic diacyl hydrazide - as indicated; hydrogen peroxide 1.96 mmol/l; 6-hydroxybenzothiazole derivatives - 5 mg/l

Table 3.17

Increases in light emission from the horseradish peroxidase conjugate catalysed chemiluminescent oxidation of cyclic diacyl hydrazides by the addition of para-substituted phenols

CYCLIC DIACYL HYDRAZIDE	FOLD INCREASE IN LIGHT EMISSION*	
	With p-Iodophenol	With p-Phenylphenol
Luminol	2492	814
Isoluminol	120	37
N-(6-Aminobutyl)-N-ethyl isoluminol	88	32
7-Dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide	107	70

* Light intensity was measured 60 seconds after initiation

pH 8.5; cyclic diacyl hydrazides 6 µmol/l; hydrogen peroxide 0.2 mmol/l; p-iodophenol 34 µmol/l; p-phenylphenol 1.1 µmol/l

Table 3.18

The influence of various compounds on the light emission from the HRP-conjugate catalysed oxidation of purpurogallin at pH 6.5

LUMINESCENT SYSTEM	ADDITIVE	PEAK LIGHT EMISSION (mV)
Purpurogallin/H_2O_2/ HRP-conjugate	none	374
"	p-Phenylenediamine	4352
"	2,4-Dichlorophenol	343
"	Firefly luciferin	200
"	p-Iodophenol	344

Light emission was monitored for 100 seconds.
Final reagent concentrations: Purpurogallin (0.51 mmol/l), H_2O_2 (0.11 mol/l), HRP (2.4 nmol/l); p-iodophenol (0.28 mmol/l), other enhancers (0.5 mmol/l).

In subsequent studies incorporation of p-iodophenol (0.14 mmol/l) into a purpurogallin (0.66 mmol/l)-H_2O_2 (1.3 mmol/l)-HRP (25 nmol/l) mixture produced a 26-fold increase in the peak light emission occurring within 60 seconds of initiation, and a 5-fold increase in total light emission occurring within 1000 seconds.

Incorporation of p-hydroxycinnamic acid (0.37 mmol/l) under these conditions produced a 36-fold increase in peak light emission and a 14-fold increase in total light emission (Williams and Thorpe, 1985, unpublished observations).

reaction), but this had been carefully purified whereas the other compounds were used as supplied. Improvements in the magnitude of enhancement from other cyclic diacyl hydrazides may be possible on purification.

Enhancement of light emission from the HRP-conjugate catalysed purpurogallin-H_2O_2 reaction at pH 6.5 was achieved by addition of p-phenylenediamine. No increase was achieved, under similar conditions, on addition of 2,4-dichlorophenol, firefly luciferin or p-iodophenol (Table 3.18). However, subsequent studies using p-iodophenol or p-hydroxycinnamic acid under slightly different conditions demonstrated increased light emission could be produced by these compounds (Table 3.18).

3.3.3.2 Oxidant

Hydrogen peroxide and sodium perborate acted as oxidants in enhanced luminescent reactions. Similar increases in light emission were obtained with either oxidant in luminol-HRP-conjugate reactions enhanced with 6-hydroxybenzothiazole derivatives (Table 3.19) or para-substituted phenols (Table 3.20).

3.3.4 Influence of pH

Enhancement of light emission from the HRP-conjugate catalysed luminol-hydrogen peroxide reaction exhibited a marked pH dependence. Addition of firefly luciferin or p-iodophenol produced significant increases in light emission between pH 7 and pH 9.5, with maximal intensity at approximately pH 8.6 (Figure 3.2, B and C). Enhancement by 2,4-dichlorophenol showed a

Table 3.19

The influence of oxidant on enhancement of light emission from the luminol-oxidant-HRP-conjugate reaction by 6-hydroxybenzothiazole derivatives

Oxidant	Fold increase in light emission		
	6-Hydroxybenzothiazole	Dehydroluciferin	Firefly luciferin
Hydrogen peroxide (1.96 mmol/l)	372	453	155
Perborate (1.96 mmol/l)	406	451	71

pH 8.0; Final concentrations: luminol 59 µmol/l; 6-hydroxybenzothiazole derivatives 5 mg/ml; oxidant as indicated.

Table 3.20

The influence of oxidant on light emission from reactions involving luminol, in the presence and absence of para-substituted phenols

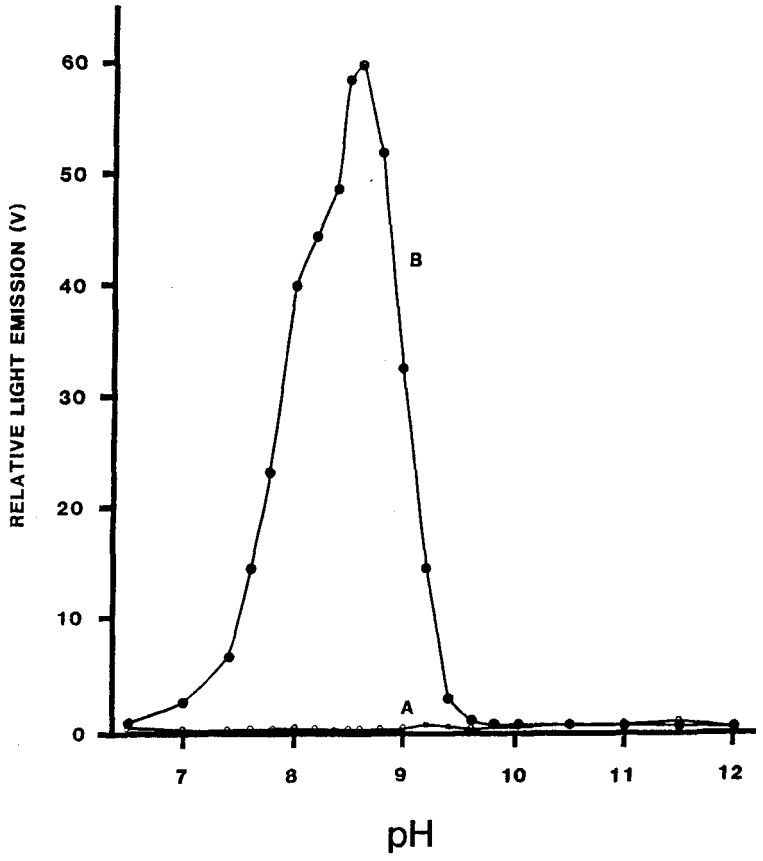
Oxidant	LIGHT EMISSION* (mV)					
	NO HRP-CONJUGATE			WITH HRP-CONJUGATE		
	No enhancer	With p-iodophenol	With p-phenylphenol	No enhancer	With p-iodophenol	With p-phenylphenol
Hydrogen peroxide	96	7	48	230	127,143	16,150
Perborate	100	16	11	263	143,140	26,605

* Light intensity was measured 30 seconds after initiation

pH 8.5; Final concentrations: luminol 59 µmol/l; p-iodophenol 350 µmol/l; p-phenylphenol 12 µmol/l; oxidant 1.96 mmol/l

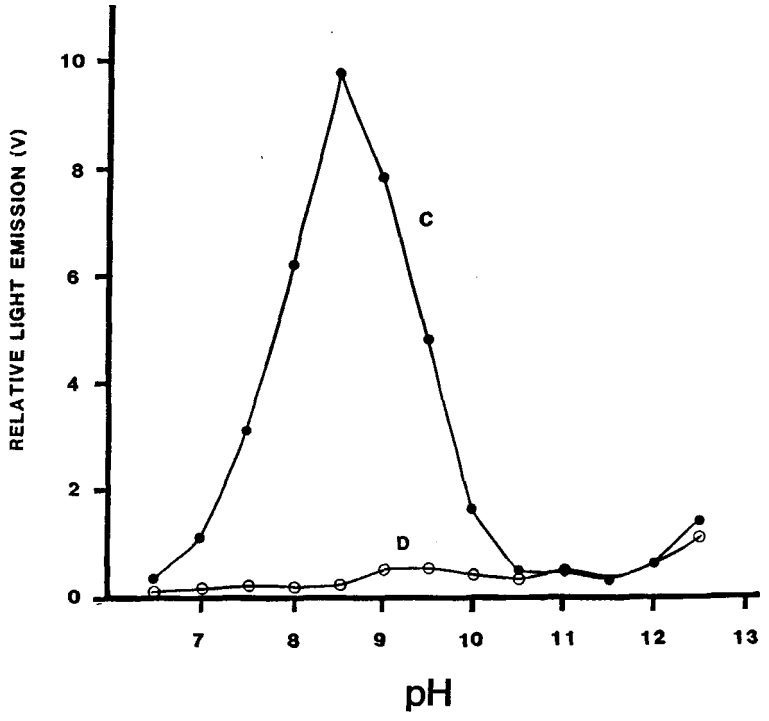
Figure 3.2

Variation with pH of light emission from HRP-conjugate catalysed chemiluminescent reactions with luminol and hydrogen peroxide



A, D, unenhanced; B, p-iodophenol enhanced; C, firefly luciferin enhanced

Light emission after 30 seconds was recorded



Final concentrations: p-Iodophenol 336 µmol/l; Luminol 59 µmol/l; H_2O_2 1.87 mmol/l :firefly luciferin 35 µmol/l; Luminol 294 µmol/l; H_2O_2 1.96 mmol/l

slightly shifted pH profile with maximal light emission at approximately pH 8.0.

3.3.5 Influence of enhancer concentration

The degree of enhancement of light emission was dependent on the enhancer concentration. Figures 3.3A and 3.3B illustrate the influence of various concentrations of 6-hydroxybenzothiazole or p-iodophenol on light emission from the HRP-conjugate catalysed luminol-H_2O_2 reaction. Addition of enhancer increased light emission up to an optimal enhancer concentration above which further addition of enhancer decreased light intensity and the degree of enhancement. The optimal final concentrations for firefly luciferin, 6-hydroxybenzothiazole, p-iodophenol and p-phenylphenol (at the specified concentration of luminol and hydrogen peroxide) differed, and were approximately 55, 24, 340 and 11 µmol/l respectively. Optimal enhancer concentration was also dependent on the concentration of the luminol-peroxide mixture employed. Increases in concentration of luminol and hydrogen peroxide from 56 µmol/l and 1.87 mmol/l to 1.17 mmol/l and 2.55 mmol/l respectively, altered optimal p-iodophenol enhancer concentration from 340 µmol/l to between 600 and 925 umol/l. Variation in the type and concentration of enhancer produced only minor alterations in the kinetics of the prolonged light emission.

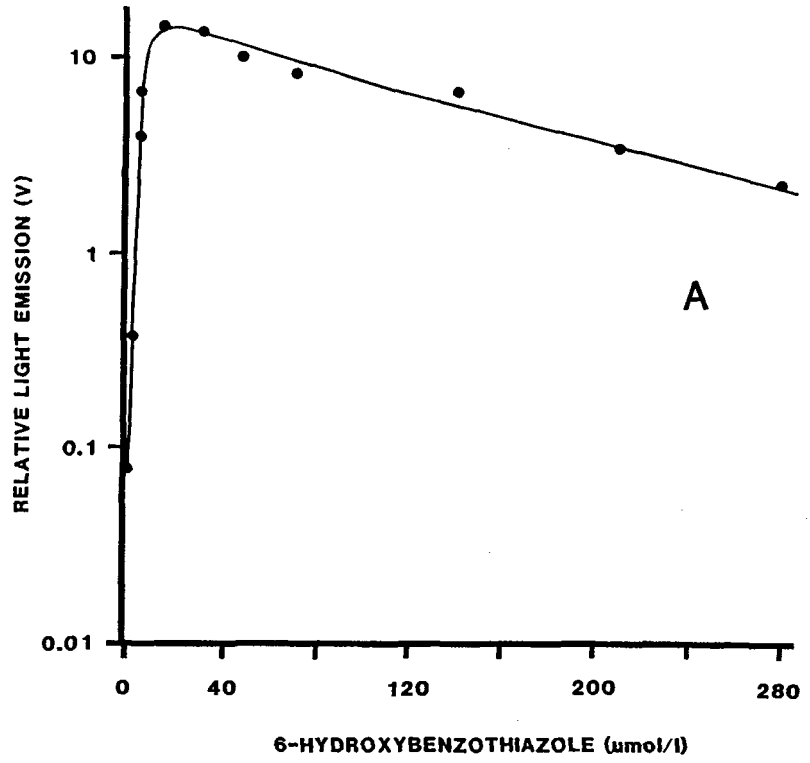
3.3.6 Influence of catalyst

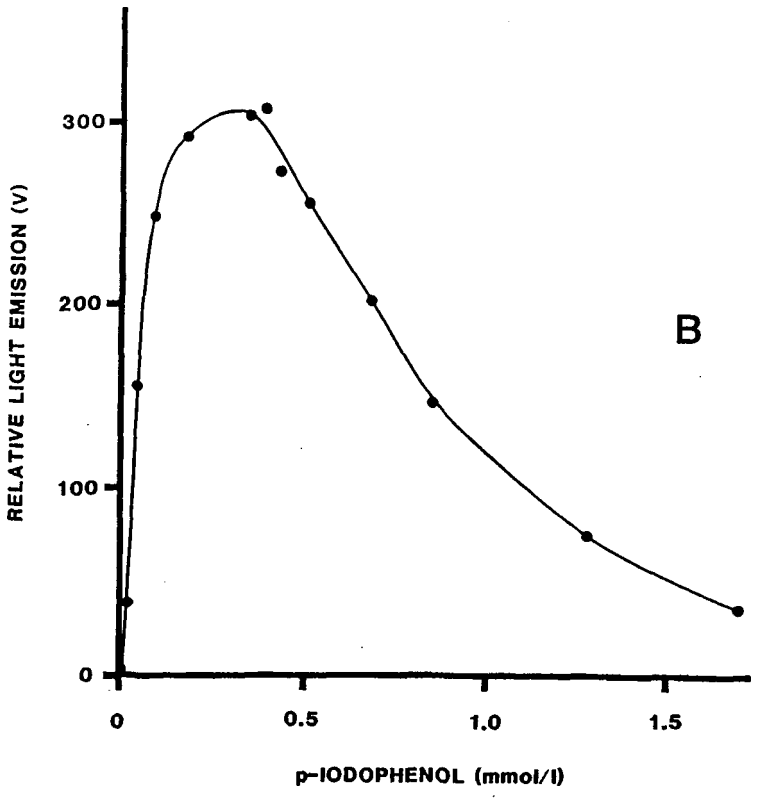
3.3.6.1 Horseradish peroxidase isoenzymes

Basic and acidic horseradish peroxidase isoenzymes catalysed the chemiluminescent oxidation of luminol, however dramatic

Figure 3.3

Influence of 6-hydroxybenzothiazole (A) or p-iodophenol (B) concentration on light emission from the HRP-conjugate catalysed oxidation of luminol





Final concentrations: Luminol 56 µmol/l; H_2O_2 1.87 mmol/l; 6-hydroxybenzothiazole 0 - 267 µmol/l; p-iodophenol 0 - 1.6 mmol/l

enhancement of light emission by 6-hydroxybenzothiazole, p-iodophenol or p-phenylphenol occurred only with the basic isoenzyme (Table 3.21). The influence of p-iodophenol on light emission from the luminol-H_2O_2 reaction catalysed by various quantities of a preparation containing 2 basic HRP isoenzymes or an acidic HRP isoenzyme are shown in Figure 3.4. More than 10,000 percent increases in light emission were achieved with the basic isoenzyme, and this contrasted with increases of less than 100% with the acidic isoenzyme.

The high degree of enhancement achieved with anti AFP-HRP conjugate suggested that it had been prepared using the basic isoenzyme. The presence of predominantly basic isoenzymes was confirmed by isoelectrofocussing of the HRP preparation (Miles 36451) used to prepare the conjugate, on LKB PAG electrofocussing gels pH range 3.5 to 9.5.

3.3.6.2 Haemproteins

The influence of p-iodophenol on light emission from the luminol-H_2O_2 reaction catalysed by various haemproteins is shown in Figure 3.5. Light emission with lactoperoxidase was significantly increased (approximately 3,500 - 20,000%). Light emission from reactions catalysed by 10 µg/ml to 0.1 µg/ml solutions of haemoglobin, myoglobin, cytochrome-c or haemin was reduced by up to approximately 70%.

3.3.7 Luminescent emission spectra of enhanced reactions

The prolonged light emission from the HRP-conjugate catalysed luminescent reactions enabled determination of emission spectra. Light emission from the firefly luciferin enhanced luminol-H_2O_2

Table 3.21

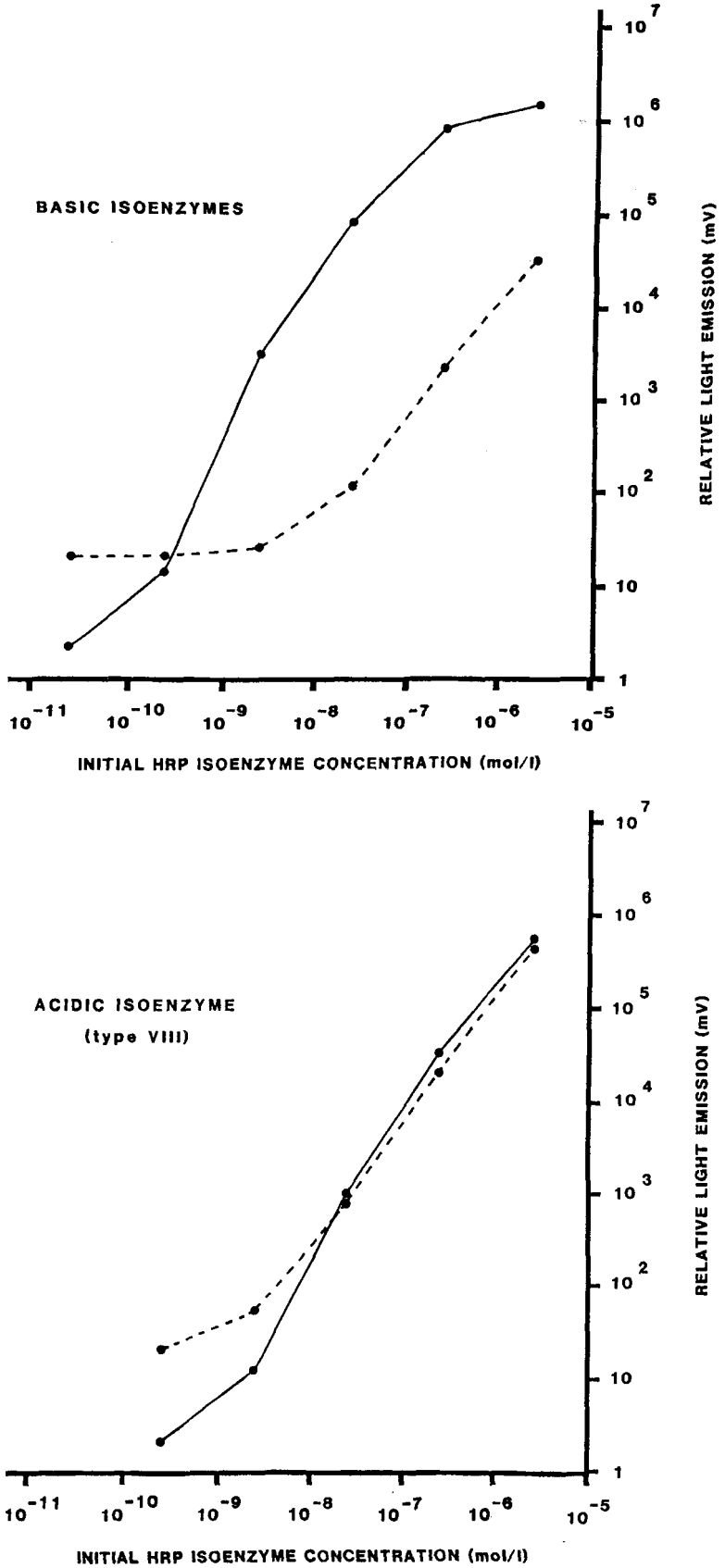
Light emission from the peroxidation of luminol catalysed by HRP isoenzymes in the absence or presence of enhancers

HRP ISOENZYME PREPARATION	LIGHT EMISSION[a] (V)			
	unenhanced	p-iodophenol enhanced	p-phenylphenol enhanced	6-hydroxybenzothiazole enhanced[b]
Type VI; two basic, no acidic	0.04	96.56	47.77	8.24
Type VII; one acidic	1.36	1.96	0.24	0.03
Type VIII; one acidic	0.75	1.29	0.24	0.03
Type IX; one basic	0.02	67.32	36.72	4.84

[a]Light intensity with 1 µg/ml solutions of HRP was measured 60 seconds or [b] 90 seconds after initiation

Figure 3.4

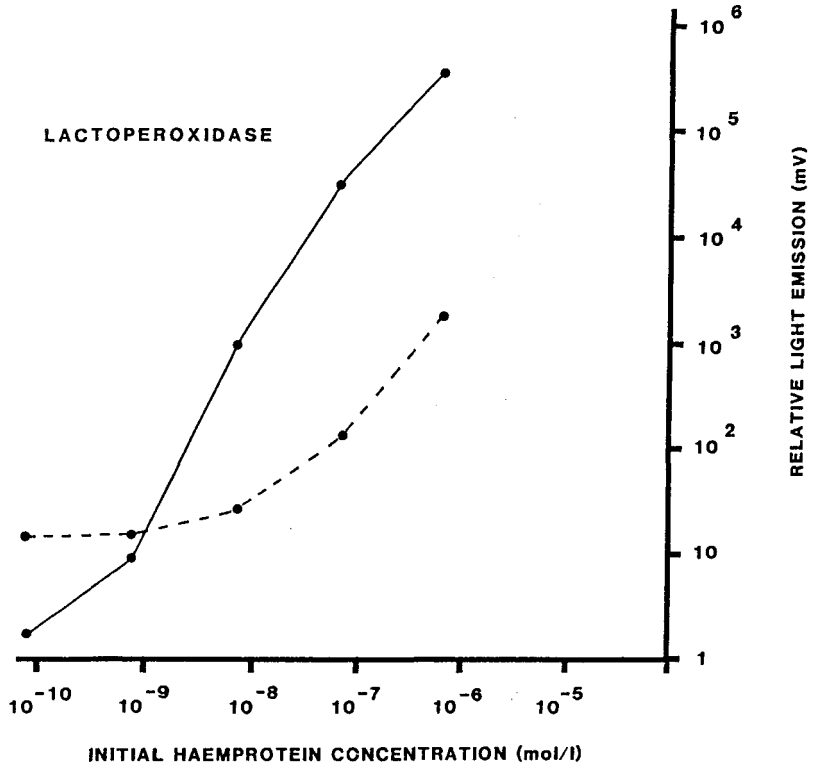
The influence of p-iodophenol on light emission from the luminol-H_2O_2 reaction catalysed by basic HRP isoenzymes or an acidic HRP isoenzyme

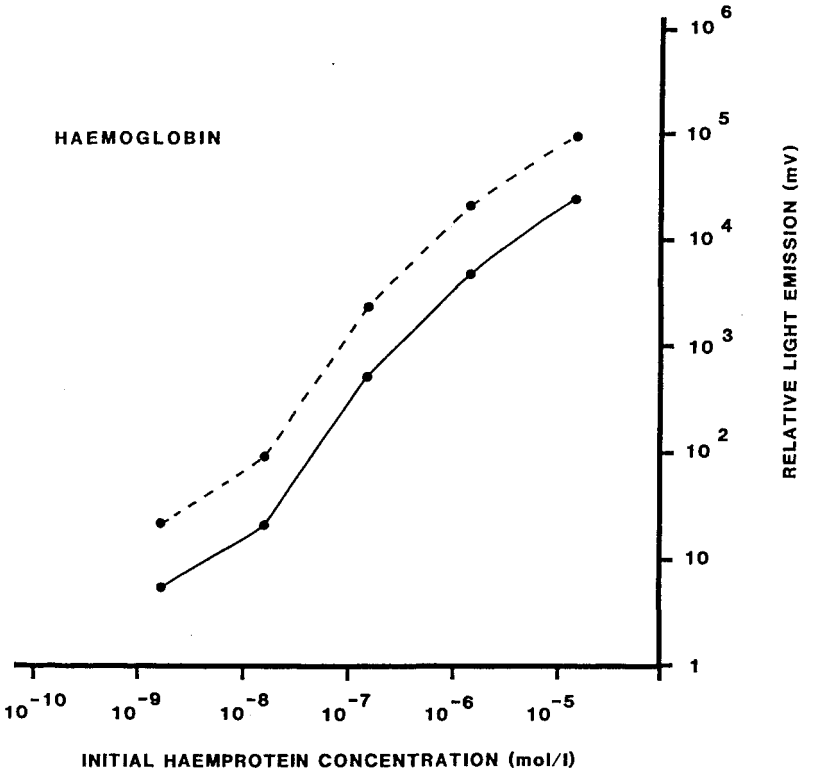


Luminol-H_2O_2 Luminol-H_2O_2 plus p-iodophenol

Figure 3.5

The influence of p-iodophenol on light emission from the luminol-H_2O_2 reaction catalysed by lactoperoxidase or haemoglobin





Luminol-H_2O_2 ——— Luminol-H_2O_2 plus p-iodophenol

reaction exhibited maximal emission around 425 nm (Figure 3.6C). This was similar to that in the unenhanced luminol-H_2O_2-HRP-conjugate reaction (Figure 3.6D). Spectra of enhanced reactions using 6-hydroxybenzothiazole or dehydroluciferin (Figure 3.6A and B) were also similar to that of the unenhanced reaction. Light emission spectra from p-iodophenol, p-chlorophenol and 2,4-dichlorophenol enhanced reactions with luminol were similar and essentially the same as those obtained in the unenhanced and 6-hydroxybenzothiazole derivative enhanced reactions, with peak emission at approximately 425 nm.

The spectrum of light emitted from the HRP-conjugate catalysed oxidation of 7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide (peak emission near 495 nm) was at a longer wavelength than that of luminol; Figure 3.7 illustrates how this difference was maintained in the p-iodophenol enhanced reaction.

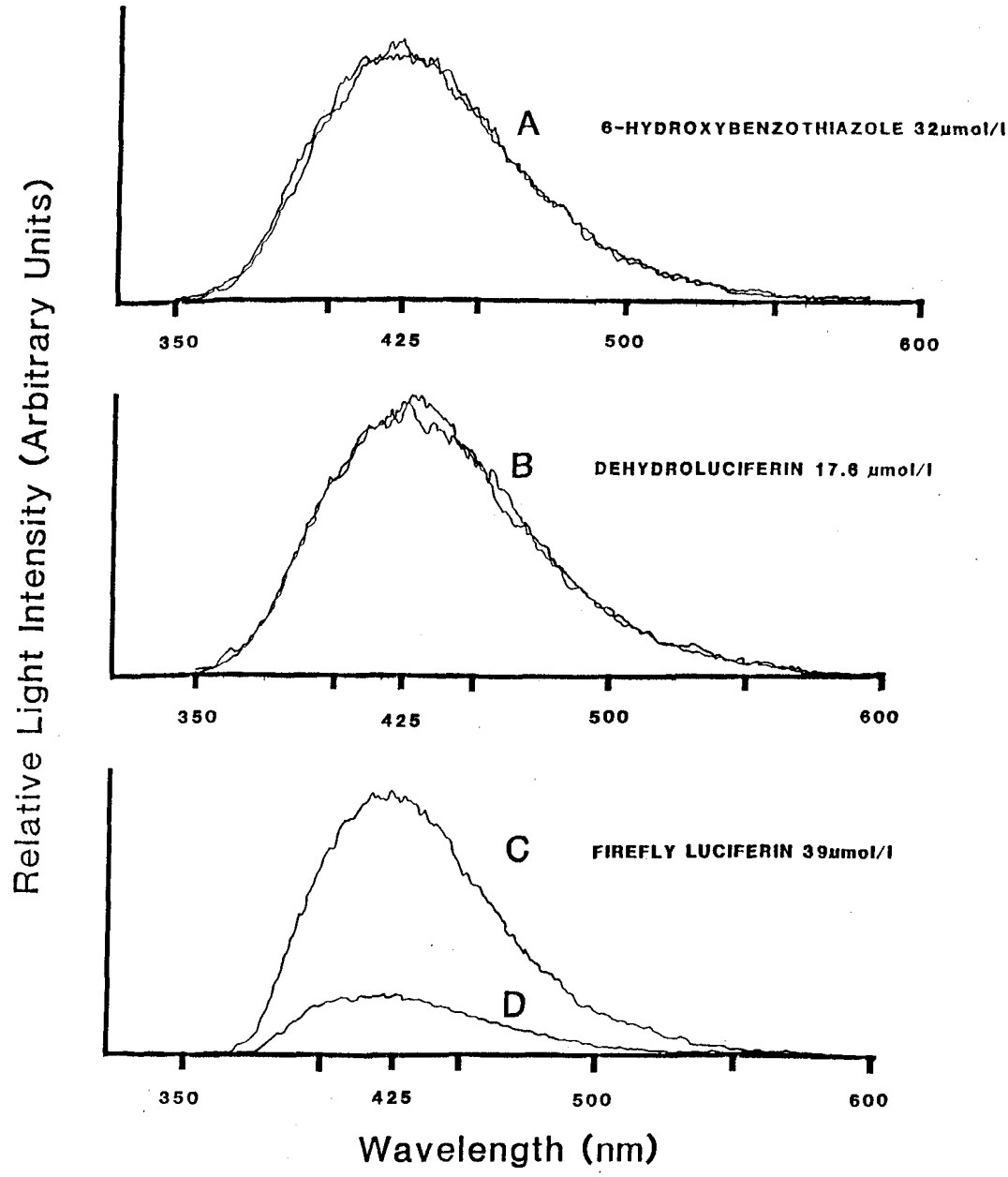
3.4 DISCUSSION

3.4.1 Characteristics and advantages of enhanced chemiluminescent reactions

Certain phenol derivatives and 6-hydroxybenzothiazole derivatives enhance light emission from the horseradish peroxidase catalysed oxidation of cyclic diacyl hydrazides such as luminol. Rapid and sensitive chemiluminescent assays for HRP-conjugates based on such reactions are possible. The light emitted is easily measured, there is no need to initiate individual reactions in front of the photodetector, and the reagents employed are readily available and inexpensive. Light emission from enhanced reactions may be up to 2500-fold that of unenhanced reactions, an

Figure 3.6

Spectral energy distribution of the enhanced and unenhanced HRP-conjugate catalysed oxidation of luminol



A 6-Hydroxybenzothiazole enhanced
B Dehydroluciferin enhanced
C Firefly luciferin enhanced
D Unenhanced

Final concentrations:

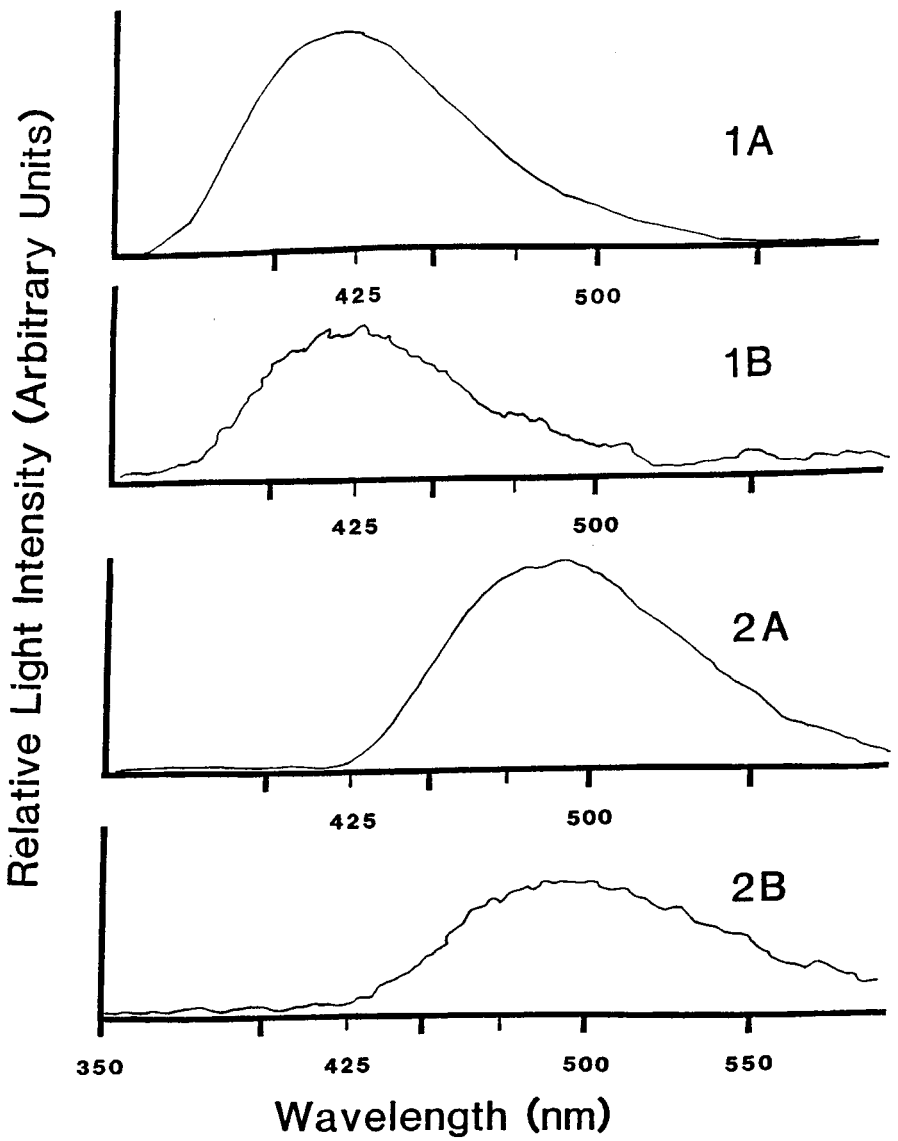
Reactions A and B: Luminol 58.8 µmol/l; H_2O_2 1.96 mmol/l
C and D: Luminol 1.22 mmol/l; H_2O_2 2.64 mmol/l

Enhancers as indicated

Figure 3.7

Spectral energy distribution of the p-iodophenol enhanced and unenhanced HRP-conjugate catalysed oxidation of cyclic diacyl hydrazides

1, luminol: A, enhanced; B, unenhanced
2, 7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide: A, enhanced; B, unenhanced



Final concentrations: Luminol 139 µmol/l;
7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide 103 µmol/l; H_2O_2 3.7 mmol/l;
p-iodophenol 41.7 µmol/l

improvement over the enhancement previously observed using firefly luciferin. Enhancement is not confined to synthetic D(-)luciferin, and a range of diacyl hydrazides and oxidants participate in enhanced reactions, in which the enhancers and chemiluminescent substrate act in a synergistic manner.

Several para-substituted phenols enhance light emission to a greater extent than possible with 6-hydroxybenzothiazole derivatives and the kinetics of the reaction are such that emission occurs as a prolonged high intensity glow. These features overcome limitations of many luminescent assays for labels in which the light emitted is of low intensity and of short duration (a flash). Although prolonged light emission can be produced with cyclic diacyl hydrazides alone, the ability to combine such kinetics with high intensity emission and improved discrimination between peroxidase concentrations is achieved only by adding enhancer. The enhancers also reduced background light emission from the cyclic diacyl hydrazide-oxidant mixture in the absence of HRP, producing a further improvement in the signal to background ratio. The exact analytical utility of the reduced background light emission, also observed with compounds which did not enhance light emission, and its effect on HRP-conjugate quantitation has not yet been fully determined. In studies with solutions of type VI peroxidase, the signal to background ratio (measured as light intensity after 30 seconds) for 2.5 femtomoles of peroxidase was 7.0 with the p-iodophenol enhanced reaction versus 1.1 in the unenhanced reaction with luminol and peroxide. Further improvements in sensitivity and the kinetics of light emission should be possible by careful selection of luminescent

reagents and enhancer, their relative proportions and reaction conditions.

Enhancement of light emission occurred if enhancer was added during, or at the start of the luminescent reaction and was a dose-dependent phenomenon. Addition of enhancer increased light emission up to an optimal concentration above which further addition of enhancer decreased light emission and the degree of enhancement. As the degree of enhancement was dependent on the particular enhancer and its concentration, careful optimisation is therefore necessary to obtain maximal light emission or sensitivity when starting with individual unenhanced systems. The enhanced reactions are pH dependent with optima near pH 8.5 and can thus be performed under relatively mild conditions. Enhancement of light emission appeared specific for basic isoenzymes of HRP, conjugates of these isoenzymes with antibody, and lactoperoxidase. No useful enhancement of light emission was seen with acidic HRP isoenzymes or other commonly encountered haemproteins. Although haemoglobin catalyses reactions of colorimetric peroxidase substrates (Takayanagi and Yashiro, 1984) and catalyses light emission from luminol (Olsson et al., 1981) it shows no increase in light emission upon enhancer addition. This allows improvement in the sensitivity and specificity with which peroxidase conjugates can be quantitated in the presence of haemoglobin. Light emitted in reactions enhanced by phenol or 6-hydroxybenzothiazole derivatives showed remarkable similarity to the emission spectra of unenhanced reactions and the emission spectrum of the chemiluminescent acyl hydrazide used is retained

in the enhanced reactions. Although the emission spectra of the low intensity light emitted with enhancer alone could not be determined, the enhancers apparently do not act as more efficient enhancers but exert their action earlier in the complex reaction between peroxidase, oxidant and luminol (Section 3.4.4).

3.4.2 Relationship between enhancer structure and degree of enhancement

A range of compounds have been screened for their ability to enhance light emission from the HRP-luminol-H_2O_2 reaction. Based on the results they have been divided into two groups: Group 1, compounds which enhance light emission and; Group 2, compounds which have no effect on, or decrease light emission.

Stringent structural requirements are necessary for compounds to act as enhancers and compounds shown to produce enhancement range from simple monosubstituted phenols to more complex annelated derivatives such as naphthols and 6-hydroxybenzothiazoles. Minor structural changes such as substitution of the OH-group or alteration of other substituents can significantly alter enhancer activity. Generally the enhancers are monosubstituted phenols with an unsubstituted OH- group and a para-substituent which acts as an activating group, phenol itself however does not act as an enhancer. Monosubstituted phenols with substituents at the ortho or meta position also do not produce enhancement. Several disubstituted phenols with a para substituent acted as enhancers however if the para substituent was a hydroxyl group, or if more than one hydroxyl group was present, no enhancement was obtained. A range of para-substituted phenols have been investigated.

Phenols with para substituents such as NO_2, NH_2 or OCH_3 groups produced no enhancement but particularly effective increases in emission were obtained with a halogen, a phenyl group or a CH=CH-COOH substituent. Increases in light emission appeared to vary with the substituent, for example in the halophenols the degree of enhancement was in the order I > Br > Cl > F. Further substitution of a halogen into a para-substituted monohalophenol reduced the degree of enhancement and no significant enhancement was seen with various halophenols with three or more halogen substituents.

As in colorimetric enhanced systems the OH group appears very important in a compound's ability to produce enhancement and its activity (ease of ionisation, electron density) may be greatly influenced by either the electron releasing or electron attracting ability of para substituents (Takayama and Nakano, 1977). Correlation between such properties or the substituent constant (Hammett σ value; Job and Dunford, 1976) for para substituents and the degree of enhancement has not been exhaustively investigated. Other electronic characteristics of enhancers which may possibly correlate with enhancer ability include, the net charge at the oxygen atom, the total or frontier electron densities on the oxygen atom, and the lowest unoccupied or the highest occupied orbital energy (Hosoya et al., 1983).

Phenoxy radical formation, implicated in one mechanistic hypothesis (Section 3.4.4.1), occurs independently of the phenol's molecular size but is dependent on redox potential. Several parameters such as critical oxidation potential or half-

wave oxidation potential of enhancers may be related to the relative ease of oxidation of phenols to phenoxy radicals (Job and Dunford, 1976) and therefore possibly to the degree of enhancement. Similar correlation may be possible with enhancer structure and its relationship with the reactivity or stability of enhancer radicals.

The molecular structure of enhancers at present can only be compared with preliminary data on the degree of enhancement as definitive data would require extensive optimisation of systems with individual enhancers. The diversity and structural complexity of enhancers also complicates identification of parameters implicit in determining ability to increase light emission. Many factors may contribute to determining the degree of enhancement, these may include for example electronic characteristics, steric effects, electrostatic effects, oxidation potential, ionisation constants, solvation energy, solubility or bond strength. The degree of contribution of each factor cannot yet be estimated and further physico-chemical investigations are required in order to predict enhancer ability from molecular structure.

3.4.3 Comparison of enhanced chemiluminescence with other enhanced systems

3.4.3.1 Comparison with other chemiluminescent and bioluminescent systems

Compounds capable of increasing light emission from the luminol-H_2O_2 reaction differed from those producing enhancement in other chemi- or bioluminescent systems. Under the conditions employed

for enhanced reactions several compounds previously reported to increase light emission from luminescent systems (for example potassium bromide, potassium chloride, sodium azide, mannitol, histidine, thiourea and sodium benzoate) produced no increase in emission from the HRP-conjugate catalysed peroxidation of luminol (Table 3.11). Others such as pyrogallol, ascorbic acid and p-phenylenediamine produced pronounced decreases (> 99 %) in light emission. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone, which has structural similarities to other chemiluminescent enhancers (Yurow and Sass, 1977), also produced enhancement of light emission from the luminol-H_2O_2-HRP-conjugate reaction, but in sharp contrast to 6-hydroxybenzothiazole and phenol derivative enhancers this increase also occurred in the absence of conjugate.

3.4.3.2 Comparison with enhancement produced by structural modification of HRP, environmental factors or coupling reactions

Enhancement of HRP catalysed reactions can occur by modification of the enzyme structure. Such modifications include the form in which it exists (for instance as monomers or dimers), subtle changes in the tertiary structure produced by KCl or dimethyl sulphoxide (Duran et al., 1981), and gross changes such as removal of haem from the protein under alkaline conditions (Ewetz and Thore, 1976). As enhancers appear to be HRP substrates it is unlikely they would mediate such changes. It is also unlikely they would bind to the enzyme active site (as proposed for certain nitrogenous compounds which are not substrates, Fridovich, 1963) producing changes in the microenvironment beneficial to subsequent oxidation of luminescent substrates or

the production of compound I. Enhancement due to improvement of the reaction of oxidant with HRP, similar to the lowering of K_m for hydroperoxide produced by nitrogen heterocycles with haemoglobin (Selgren and Gantzer, 1982), is unexpected as this does not appear to be rate limiting in luminescent reactions.

Enhancers are not known to form micelles and it is unlikely they could create hydrophobic environments beneficial to luminescent reactions. Although the lifetime of certain reaction intermediates in luminescent reactions may be solvent dependent as yet the effect of enhancer solutions on such parameters is unknown. Radical scavengers can under certain conditions augment luminescence from the luminol-HRP-H_2O_2 system (Misra and Squatrito, 1982), however, hydroxyl radical scavengers such as mannitol or DMSO produce no significant increase in light emission and ascorbic acid produces a dramatic decrease in light emission suggesting luminescent enhancers operate by a different mechanism.

Horseradish peroxidase can participate in a wide range of oxidative coupling reactions (Artiss et al., 1981), generally of an aromatic amine with another aryl compound (Ngo and Lenhoff, 1980). Several compounds employed in oxidative coupling reactions, such as sulphonated 2,4-dichlorophenol and other halophenols, are remarkably similar to luminescent enhancers. Although modification of luminol, such as replacement of the amino group by other substituents, can produce compounds with an improved luminescence quantum yield compared with luminol (Roswell and White, 1978), it appears unlikely enhancement occurs

by the production of more efficient luminescent compounds by coupling diacyl hydrazides to enhancers as at least some alteration in the spectra of the light emitted would be expected. An analysis of reaction products formed in enhanced luminescent reactions may establish if any conjugation between the enhancers and luminescent substrate had occurred. The similarity of the light emission spectra for unenhanced and enhanced reactions also suggests enhancers do not act as efficient emitters in resonance energy transfer reactions. If energy were to be passed from luminol to the enhancers, light emitted would be of a longer wavelength to that from luminol or the original diacyl hydrazide employed.

3.4.3.3 Similarities to other enhanced colorimetric or chemiluminescent reactions

Colorimetric reactions: Although apparently different to enhanced colorimetric HRP reactions which are mediated by microenvironmental changes produced by salts, specific antibodies or hydrophobic reagents, the enhanced chemiluminescent reactions have interesting similarities to several enhanced colorimetric systems. A number of compounds which are efficient chemiluminescent enhancers have also been employed in enhanced colorimetric systems (Table 3.22). 2,4-Dichlorophenol and p-hydroxycinnamic acid are effective enhancers in the aerobic HRP catalysed oxidation of NADH or indole acetic acid, and 2-chloro-4-phenylphenol (in combination with Mn^{2+}) enhances NADH oxidation. A variety of HRP catalysed colorimetric reactions using hydrogen peroxide are also enhanced by compounds which act

Table 3.22

Enhancers common to HRP catalysed enhanced colorimetric and chemiluminescent reactions

System	Enhancer	Reference
NADH/O_2/HRP	p-Hydroxycinnamic acid	Gross et al., 1977
NADH/O_2/HRP	2,4-Dichlorophenol p-Hydroxycinnamic acid p-Methylphenol	Halliwell, 1978 Mader and Amberg-Fisher, 1982
NADH/O_2/(Mn^{2+})/HRP	2,4-Dichlorophenol 2-Chloro-4-phenylphenol p-Methylphenol	Akazawa and Conn, 1958
Indole acetic acid/ O_2/HRP	2,4-Dichlorophenol	Fox and Purves, 1968
Ascorbic acid/ H_2O_2/HRP	p-Phenylphenol	Chance, 1949
Ferrocyanide/H_2O_2/HRP Dithiothreitol/H_2O_2/ HRP	2,4-Dichlorophenol	Brooks, 1983
ABTS/H_2O_2/HRP	p-Iodophenol p-Bromophenol p-Chlorophenol 2,4-Dichlorophenol	Stout, 1985

as luminescent enhancers. 2,4-Dichlorophenol and p-methylphenol enhance the peroxidation of ferrocyanide, and p-phenylphenol the oxidation of ascorbic acid. The accelerated colorimetric peroxidation of ABTS is analogous to the enhanced chemiluminescent system and similar enhancers are effective. These include halophenols such as p-iodophenol, p-bromophenol, p-chlorophenol and 2,4-dichlorophenol; also, the influence of halosubstitution on the degree of enhancement is comparable (I > Br > Cl). There are however also some exceptions such as vanillin, an effective enhancer of the colorimetric reaction which produced no increase in light emission (Table 3.12).

In addition to the structural similarities of enhancers, general characteristics of the enhanced chemiluminescent reaction are also shared by several colorimetric enhanced systems. Unenhanced and enhanced colorimetric reactions for example, are monitored at the same wavelength suggesting there is simple accelerated oxidation of the substrate and not formation of a complex with enhancer. The enhanced chemiluminescent HRP catalysed reactions and enhanced colorimetric systems are also analogous in that although several compounds act as enhancers with one particular hydrogen donor, they do not necessarily produce equivalent changes with other donors. In addition, compounds incorporated into colorimetric reactions may be inactive, inhibitory or active enhancers (Akazawa and Conn, 1958). The influence of enhancer concentration on enhanced colorimetric systems resembles that in the enhanced chemiluminescent reactions. At several enhancer concentrations increasing amounts of enhancer accelerate coloured product formation, above this concentration the degree of

enhancement falls on further addition of enhancer (Gross et al., 1977; Sasson et al., 1982; Brooks, 1983). As in enhanced chemiluminescent reactions, the optimal concentration of individual enhancers in colorimetric systems differ, in some cases the optimal concentration of enhancers differing by up to several orders of magnitude (Gross et al., 1977; Halliwell, 1978; Brooks, 1983). Also, the optimal concentration for a particular enhancer such as 2,4-dichlorophenol may also be HRP isoenzyme dependent (Mader and Amberg-Fisher, 1982).

Structural modification of substrates known to participate in 'cycling' enhanced colorimetric reactions can inhibit their action, for example the methyl ethers of a number of thyroxine analogues are inactive under conditions in which the parent compounds are active (Klebanoff, 1959). This effect has been seen in the HRP catalysed release of fluoride ion from substituted phenols (Siddiqi, 1982), and in the enhanced chemiluminescent reactions. Reduction of background signal on enhancer addition in the absence of HRP, similar to that seen in the enhanced chemiluminescent system, has also been observed in colorimetric systems. Thyroxine for example, decreases the rate of ascorbic acid autoxidation but increases its oxidation catalysed by peroxidase (Klebanoff, 1959).

Previously reported enhanced chemiluminescent reactions: General characteristics of enhanced chemiluminescent reactions involving diacyl hydrazides are also shared by HRP catalysed reactions in which light emission from pyrogallol or purpurogallin is enhanced by addition of o- or p-phenylenediamine. For example, emission

spectra are apparently unchanged, similar effects are produced by changes in enhancer concentration, and depression of background light emission occurs on addition of enhancer.

In the o-phenylenediamine enhanced pyrogallol-H_2O_2-HRP system although total light emission is not increased, the velocity of the luminescent reaction is increased and pyrogallol acts as the emitter and is responsible for light emission (Ahnstrom and Nilsson, 1965). Preliminary results indicate that under certain conditions, several substituted phenols which act as enhancers with luminol, also produce acceleration of light emission with pyrogallol similar to that observed with o-phenylenediamine (Thorpe and Williams, unpublished results, 1985). In the enhanced pyrogallol system the degree of enhancement is influenced by the ratio of chemiluminescent compound and enhancer (Ahnstrom and Nilsson, 1965). At several pyrogallol concentrations increasing amounts of enhancer increase light emission to a maximum, above this concentration the degree of enhancement falls on further addition of enhancer, as in enhanced reactions involving diacyl hydrazides. In addition, o-phenylenediamine lowers background light emission due to non-enzymatic oxidation of pyrogallol or that catalysed by haemoglobin to a minimum, whilst still enhancing HRP catalysed light emission (Ahnstrom and Nilsson, 1965).

3.4.4 Possible mechanisms for enhanced chemiluminescent reactions

The characteristics of the enhanced chemiluminescent reactions, and their similarity to both enhanced colorimetric reactions and

the o- or p-phenylenediamine enhanced chemiluminescent reactions of pyrogallol or purpurogallin suggest they may operate via a common mechanism. Two basic mechanisms can be proposed for such reactions, they involve either (a) rapid HRP catalysed production of enhancer radicals which in turn rapidly oxidise the original substrate or (b), the enhancers reducing the amount of HRP which exists as an inactive intermediate.

It should however be stressed that the exact mechanism of HRP catalysed reactions remains in doubt (Ugarova and Lebedeva, 1978) and peroxidase catalysed reactions can be extremely complex (Childs and Bardsley, 1975). This complicates formulation of mechanistic hypotheses to explain enhanced chemiluminescence.

3.4.4.1 The 'enhancer radical' hypothesis

This assumes light production from the HRP-luminol-H_2O_2 system is limited by the relatively slow reaction of luminol with HRP. Luminol is known to undergo reactions with HRP compounds I and II to produce luminol radicals which can then undergo further reactions leading to light emission, the overall reaction however appears limited by the relatively slow reaction of HRP compound II with luminol (Cormier and Prichard, 1968). It is proposed that enhancers are also substrates for HRP but react much faster than luminol with compounds I and II resulting in rapid regeneration of HRP and rapid production of enhancer radicals. The enhancer radicals, which can exist outside or away from the HRP active site, are reactive, powerful oxidising agents capable of performing at least one electron oxidations. Once formed, enhancer radicals can oxidise luminol to luminol radicals in

Figure 3.8

Schematic illustration of unenhanced and enhanced chemiluminescent reactions according to the 'enhancer radical' hypothesis

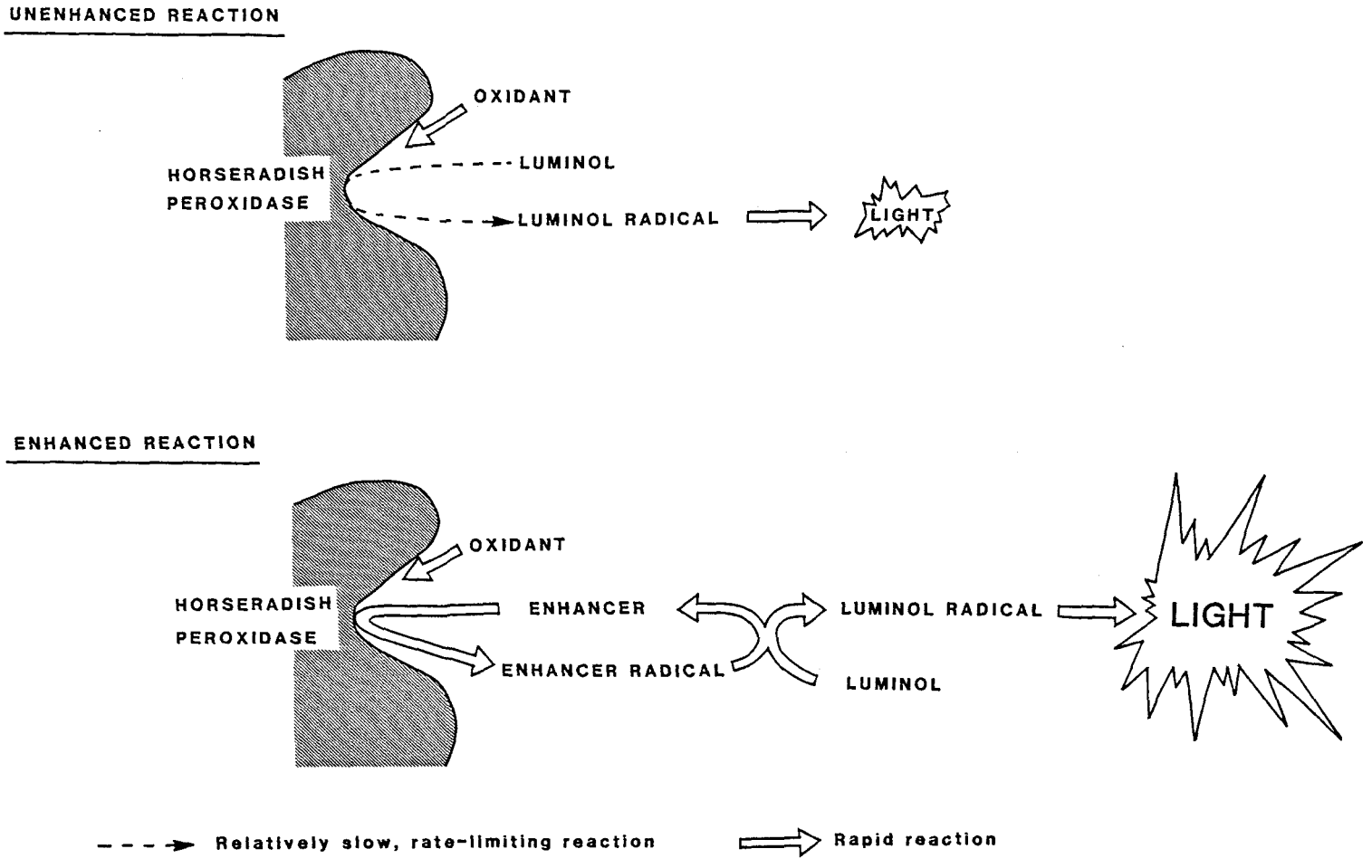
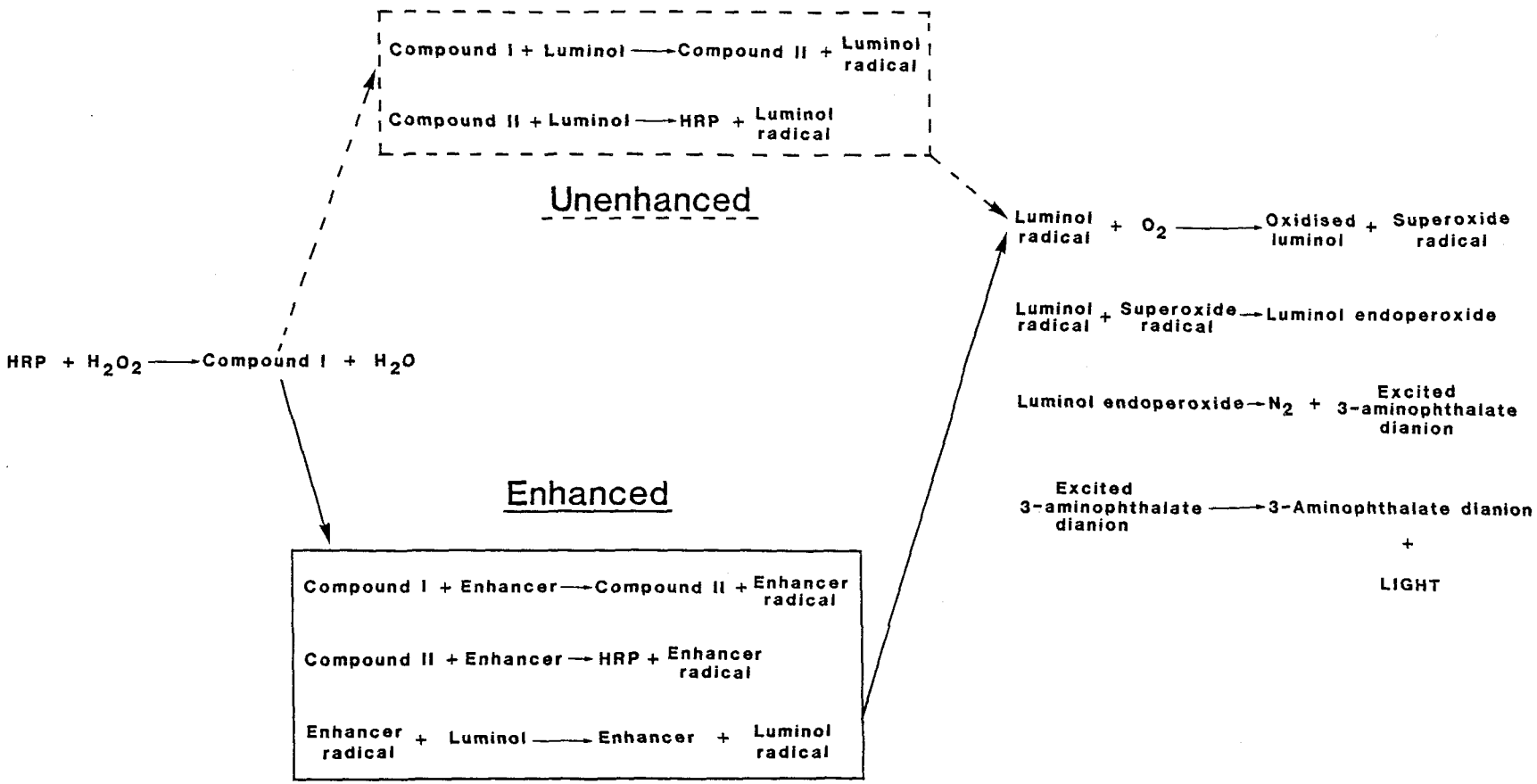


Figure 3.9

Simplified reactions occurring in unenhanced and enhanced chemiluminescent systems according to the 'enhancer radical' hypothesis



rapid non-enzymatic reactions. Luminol radicals produced, identical to those formed directly by compounds I or II in the unenhanced reaction, then proceed through the conventional reactions leading to light emission. In the process of oxidising luminol, enhancer radicals are reduced back to the original enhancer molecule which can then be re-oxidised by HRP compounds I and II. In this manner the enhancer acts in a cyclic fashion carrying an oxidising equivalent from HRP compounts I and II to luminol molecules. The reaction mechanism is schematically illustrated in Figure 3.8 and detailed steps in the overall process in Figure 3.9. Although enhancer radicals could be formed by reaction with either compound I or II, it is most probably the rapid reaction of enhancers with compound II (also regenerating HRP) which produces an accelerated rate of luminol oxidation and higher levels of light emission.

3.4.4.2 The 'inactive intermediate' hypothesis

This assumes that the chemiluminescent HRP-luminol-H_2O_2 reaction is limited mainly by the amount of HRP which exists as an inactive intermediate, compound III. Compound III is formed by reaction of oxidant with compound II (Chance et al., 1984) and its breakdown to active enzyme is slow (Gross et al., 1977). It is proposed that enhancement arises because enhancers either rapidly decompose compound III, thus speeding up the production of active HRP, or possibly by preventing the formation of compound III during the course of the HRP-luminol-H_2O_2 reaction. The process is schematically illustrated in Figure 3.10, and relevant individual reactions are shown in Figure 3.11.

Figure 3.10

Schematic illustration of unenhanced and enhanced chemiluminescent reactions according to the 'inactive intermediate' hypothesis

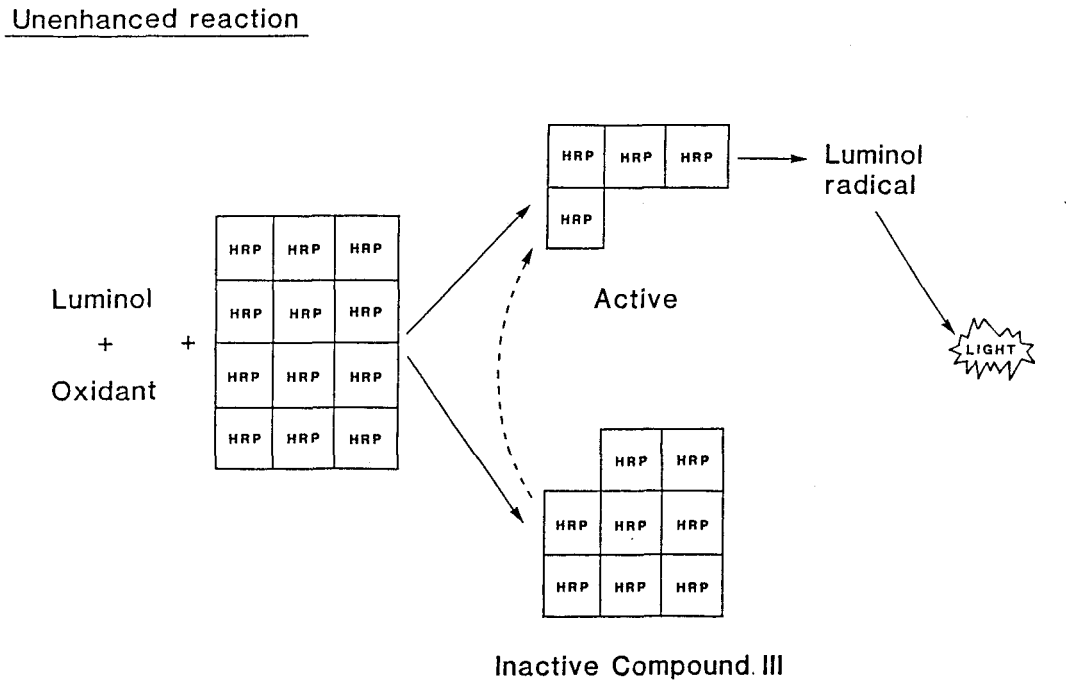
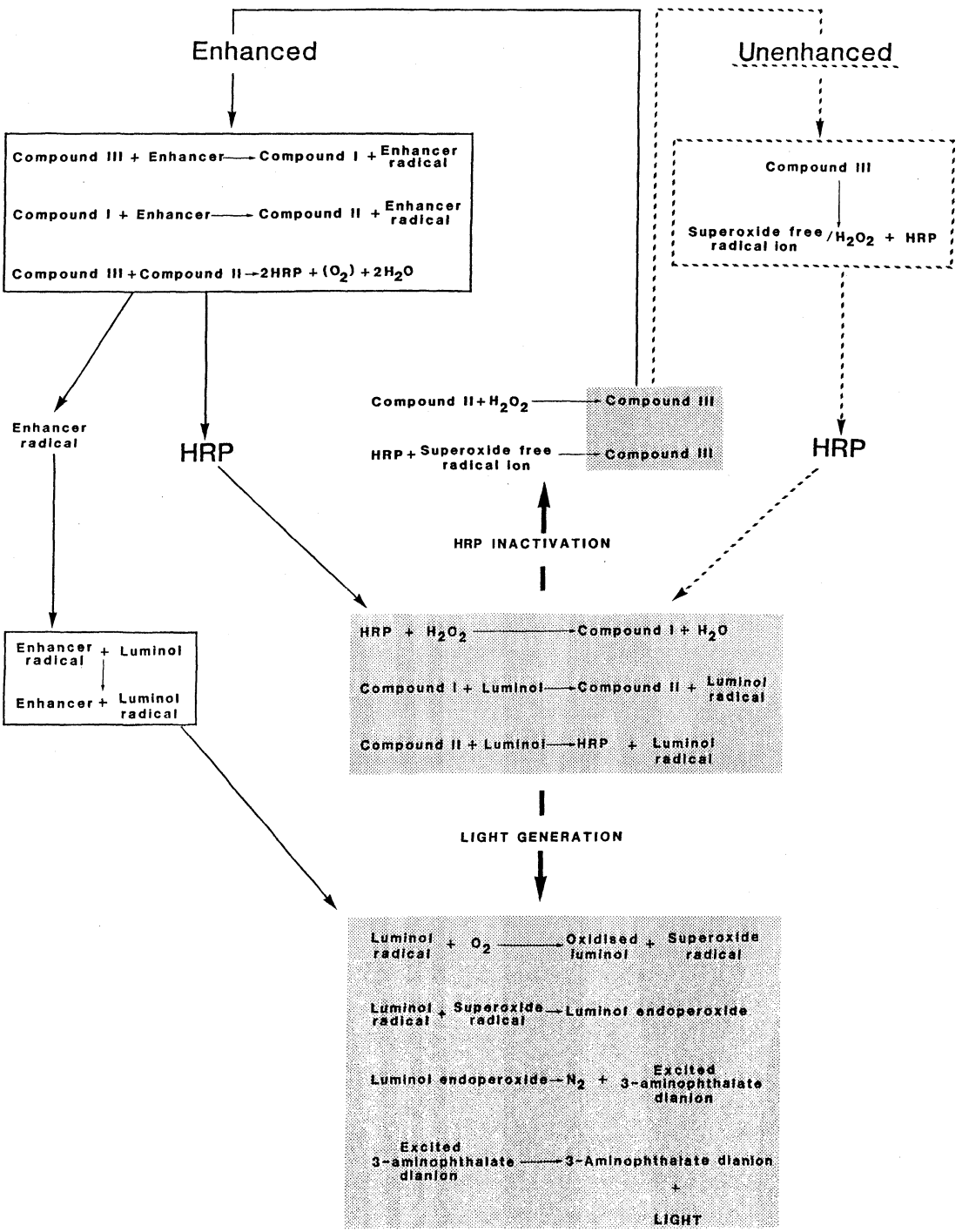


Figure 3.11

Reactions occurring in unenhanced and enhanced chemiluminescent systems according to the 'inactive intermediate' hypothesis



It is however also possible that aspects of the two postulated mechanisms could occur simultaneously, enhancers for example both generating radicals and reducing levels of compound III.

3.4.5 Interpretation of experimental findings in relation to the proposed mechanisms

Involvement of HRP substrates

The two hypotheses would predict characteristics of the enhanced luminescent reactions which agree with the experimental observations, and in both hypotheses enhancers would have the structure of peroxidase substrates. In the 'enhancer radical' hypothesis enhancers would require entry (or partial entry) into the HRP active site in order to react with compounds I and II to produce enhancer radicals. Similar structural requirements would be anticipated for enhancers in the 'inactive intermediate' hypothesis. Interaction of enhancer with compound III appears to require at least partial entry into the active site as electron donors for compounds I and II have been shown capable of compound III breakdown (Tamura and Yamazaki, 1972). Due to HRP's broad substrate specificity the hypotheses would allow enhancement by a range of compounds and would predict different degrees of enhancement. This would arise from either enhancers reacting with HRP to produce radicals at differing rates, or from variations in the efficiency of radical formation and their subsequent oxidation of luminol. In the 'inactive intermediate' hypothesis differing levels of light emission would arise from the differing rates or abilities of enhancers to react with, or reduce levels of compound III (Tamura and Yamazaki, 1972). However, not all HRP substrates which produce radicals or reduce

levels of compound III would meet the kinetic, structural and redox properties necessary to allow enhancement of the luminescent reaction.

Essential components in enhanced reactions

High levels of light emission would require the presence of HRP, oxidant, enhancer and luminol, with enhancer and luminol working in a synergistic manner. The removal of enhancer would re-establish light levels limited either by the reaction of compound II with luminol, or by the level of HRP compound III. Very low levels of light emission occur in the absence of HRP or oxidant as compound I and II, and hence luminol or enhancer radicals, cannot be formed. A range of oxidants could participate in enhanced chemiluminescent reactions as the nature of compound I (and presumably compounds II and III) and its subsequent reactions are independent of the oxidant used to produce it (Adediran and Dunford, 1983), and light emission from luminol radicals is not confined to one particular oxidant. Enhancer addition at the start of, or during, the chemiluminescent reaction would enable cyclic oxidation of luminol by enhancer radicals or reduction of compound III levels, producing increased light emission. In addition, if enhancers also reduced autoxidation of chemiluminescent compounds, a decrease in background emission would occur. Omission of luminol from enhanced reactions results in extremely low levels of light emission, this fits with both hypotheses as luminol itself is responsible for the formation of the excited state intermediates and subsequent light emission. Alternative chemiluminescent HRP

substrates, such as luminol analogues or even polyphenols, could substitute for luminol in both hypotheses which would predict increased light emission and retention of the emission spectrum of the original chemiluminescent substrate employed.

Influence of pH and alternative catalysts

Horseradish peroxidase activity and subsequent chemiluminescent reactions of luminol are pH dependent. As the production of either enhancer radicals and their subsequent reaction with chemiluminescent substrates, or the influence of enhancer on levels of compound III would also show pH dependency, both hypotheses would predict enhanced reactions which were themselves pH dependent. The overall effect probably reflecting a change in one, or a combination of changes in several, of these reactions. The inability of other catalysts of the luminol-hydrogen peroxide reaction to generate rapidly appropriate radicals from enhancers, or if such catalysts are not limited by inactive intermediates closely related to compound III, would explain the absence of enhanced light emission from such systems and the improved discrimination, specificity and sensitivity obtained when enhanced reactions are employed to quantitate HRP luminescently.

HRP isoenzyme dependence of enhancement

Differences in the reactivity and specificity of different HRP isoenzymes to various substrates, and hence the differing rates of radical production, would explain why individual enhancers do not increase light emission from all HRP isoenzymes to the same extent. Such differences have been reported and are consistent with the 'enhancer radical' hypothesis. Certain substrates

interact only weakly with HRP acidic isoenzymes but avidly bind to other isoenzymes with up to 1000 - fold differences in the dissociation constants of peroxidase-substrate complexes (Reimann and Schonbaum, 1978). Many-fold variations in reaction rate (eg the k_4 velocity constant) are seen between different isoenzymes, and with phenolic substrates variations in pH affects k_4 in opposite ways with basic and acidic isoenzymes (Marklund et al., 1974). Basic isoenzymes have a higher k_4 at pH 7.0 than 4.5 whereas acidic isoenzymes show the reverse behaviour. Although this would be consistent with the preferential enhancement of light emission with the basic isoenzyme observed around pH 8.5 by phenol derivatives, as yet k_4 values for substrates with individual isoenzymes under appropriate conditions are not available so correlation of reaction rate with ability to act as luminescent enhancer cannot be made. Information on differences in the formation of inactive intermediates from HRP isoenzymes, and on breakdown by electron donors also does not appear available.

Influence of enhancer concentration

The influence of enhancer concentration on the degree of enhancement would be explained in the 'enhancer radical' hypothesis by differences in the enzyme's affinity for substrates, and by the need to ensure the maximal rate of enhancer oxidation and subsequent reaction with luminol whilst avoiding reactions such as dimerization of enhancer radicals (Sawahata and Neal, 1982) which do not produce light emission. Similar effects of enhancer concentration are seen in enhanced reactions in which an inactive intermediate has been proposed to

be involved (Gross et al., 1977; Halliwell, 1978; Mader and Fussl, 1982). The concentrations of compounds required to breakdown compound III have been shown to differ widely, and an inverse relationship has been proposed between the K_m of compounds such as phenols for HRP and the optimal enhancer concentration required (Mader and Fussl, 1982). Although such enhanced reactions may occur in two steps - initial breakdown of compound III and then enhancement of the reaction itself - no molecular explanation has been put forward for the decrease in enhancement seen at high concentrations of enhancer.

3.4.6 Evidence for possible involvement of enhancer radicals in enhanced chemiluminescent reactions

The 'enhancer radical' hypothesis requires evidence that enhancers react rapidly with HRP and that reactive radicals are formed from them. Evidence is available for both. Several luminescent enhancers are known substrates for HRP or lactoperoxidase, others show structural similarity to known substrates. p-Phenylphenol, a highly reactive HRP substrate, produces dramatic increases in light emission and has a high k_4 value, 8×10^7 mole/l/second (Chance, 1951) in comparison with that of luminol 7.2×10^4 (Cormier and Prichard, 1968). As yet k_4 values are not available for all luminescent enhancers with HRP isoenzymes so correlation between rate of reaction and degree of enhancement cannot be made. p-Methoxyphenol or hydroquinone, substrates with relatively high k_4 values, do not enhance light emission indicating a high k_4 is not the only requirement of a potential enhancer. All HRP substrates would not act as

enhancers as they would either not react as rapidly as luminol, or produce a suitable intermediate such as a radical with appropriate redox and structural properties enabling it to rapidly oxidise luminol. Relatively simple modifications in enhancer structure would produce dramatic changes in the degree of enhancement as they could influence either reaction with HRP, the formation and reactivity of radicals, or subsequent reactions with chemiluminescent substrates.

The HRP-H_2O_2 system has been shown to produce free radicals from many substrates, they exist freely in solution not combined with enzyme and can accumulate at concentrations 5 to 20 times that of HRP (Yamazaki et al., 1960). The intermediate free radicals generated in peroxidase reactions can be studied by electron spin resonance spectroscopy (Shiga and Imaizumi, 1975; Josephy et al., 1982a; Kalyanaraman et al., 1982). Radicals identified include phenoxy and naphthoxy radicals from phenols and naphthols (Shiga and Imaizumi, 1975) and cation radicals from aromatic amines (Damerau et al., 1973). For several compounds which act as luminescent enhancers such radicals have been identified with the HRP or lactoperoxidase-H_2O_2 system, these include p-methylphenol, 2-naphthol and 3,5,3',5'tetramethylbenzidine. Once formed the radicals can undergo a variety of rapid secondary reactions in which the free radicals act as one electron oxidants or reductants (Ohnishi et al., 1969). As luminol oxidation occurs in one electron steps it is feasible such radicals mediate its oxidation. Enhancer radicals have been postulated to account for enhancement in a variety of enhanced reactions (Table 3.5) and free radicals from p-methylphenol have been observed in the

presence of large amounts of HRP. When a colorimetric substrate such as ascorbic acid is added, enhanced oxidation occurs and the electron spin resonance signal of the enhancer radical is completely replaced by that of the colorimetric substrate radical (Ohnishi et al., 1969; Yamazaki, 1977). Modification of substrates which hinder radical formation, such as esterification of phenolic groups also reduces the compounds ability to enhance luminescent reactions. Substances such as p-nitrophenol or benzoic acid, for which no radical formation with the HRP-H_2O_2 system could be demonstrated (Shiga and Imaizumi, 1975), do not act as luminescent enhancers. All substrates producing radicals would not however necessarily act as enhancers as the radicals would still require suitable properties such as an appropriate redox potential and formation at an appropriate rate.

Enhanced light emission from the oxidation of pyrogallol or purpurogallin with HRP when o- or p-phenylenediamine are added may occur by a similar 'enhancer radical' mechanism, providing further indirect evidence in support of radical involvement. Ortho- and p-phenylenediamine are known HRP substrates (Damerau et al., 1973; Fridovich, 1963), o- and m-phenylenediamine having high k_4 values (Chance, 1951; Hosoya et al., 1983). p-Phenylenediamine cation radical formation has also been observed in the HRP-H_2O_2-p-phenylenediamine reaction (Damerau et al., 1973; Shiga and Imaizumi, 1975). In addition, it has been shown that some form of enhancement can be produced by incorporation of p-iodophenol into the pyrogallol-H_2O_2-HRP system (Thorpe and Williams, unpublished observations, 1985).

As yet, there is no direct evidence that luminescent enhancers act in a cyclic manner other than the fact that an amount of enhancer less than the initial amount of luminol or oxidant can sustain enhancement of light emission. This is similar to enhanced colorimetric reactions in which cycling has been proposed (Chance, 1949; Klebanoff, 1959; Sasson et al., 1982; Brooks, 1983). Experimental evidence in support of such a cycling mechanism could be obtained by demonstrating that products from enhancers were not produced as long as luminol was in excess, or by demonstrating that hydrogen peroxide and luminol disappear at equivalent related rates in both unenhanced and enhanced reactions. Additional evidence may arise from measurement of the concentration of enhancer radicals during the course of enhanced luminescent reactions using electron spin resonance, and comparison with similar observations from enhanced colorimetric assays.

3.4.7 Summary

Several compounds have been shown to enhance dramatically light emission from the HRP catalysed oxidation of cyclic diacyl hydrazides, forming the basis of novel enhanced chemiluminescent assays for HRP-conjugates. The enhanced reactions have been partially characterised and although the exact mechanism by which increased light emission is produced remains to be established by further studies, two mechanistic hypotheses consistent with the experimental observations have been proposed. The enhanced chemiluminescent assays have several attractive features which suggest they are well suited to application in

immunoassays. In order to verify this, it was necessary to incorporate them into a typical immunoassay system, and to compare their performance with conventional chemiluminescent or bioluminescent end-points.

CHAPTER 4

DEVELOPMENT OF AN IMMUNOMETRIC ASSAY FOR SERUM ALPHA-FETOPROTEIN EMPLOYING A HORSERADISH PEROXIDASE LABELLED SPECIFIC ANTIBODY

4.1 INTRODUCTION

4.1.1 Objective

The major objective of this chapter was to develop a sensitive immunometric assay, involving a HRP-conjugate, which could be used as a test system for the development and assessment of immunoassays using enhanced chemiluminescent end-points based on the reactions described in Chapter 3.

4.1.2 Choice of assay

Requirements of the assay were that it should be a heterogeneous immunometric assay (to maximise sensitivity and minimise potential interference) and that it should be performed on a solid support compatible with the available luminometer. In addition, the assay had to be reproducible, suitable for analysis of unextracted serum, and for a clinically relevant antigen present at a relatively low concentration.

A partially developed, semi-automated immunometric assay for alpha-fetoprotein (Hill *et al.*, 1981) fulfilled several of the requirements and was used as the basis for the test system. In the assay, polystyrene beads coated with excess anti AFP were successively incubated with diluted serum samples and excess HRP-labelled AFP antibody. After washing, which removed non-specifically bound serum constituents and excess conjugate, residual HRP-conjugate bound to the beads was quantitated colorimetrically using ABTS as chromogen. The assay was performed on a prototype Kone immunoassay module (Figure 4.1) which could accommodate 4 incubation blocks each holding 24 polystyrene tubes. Antibody coated beads, loosely supported in

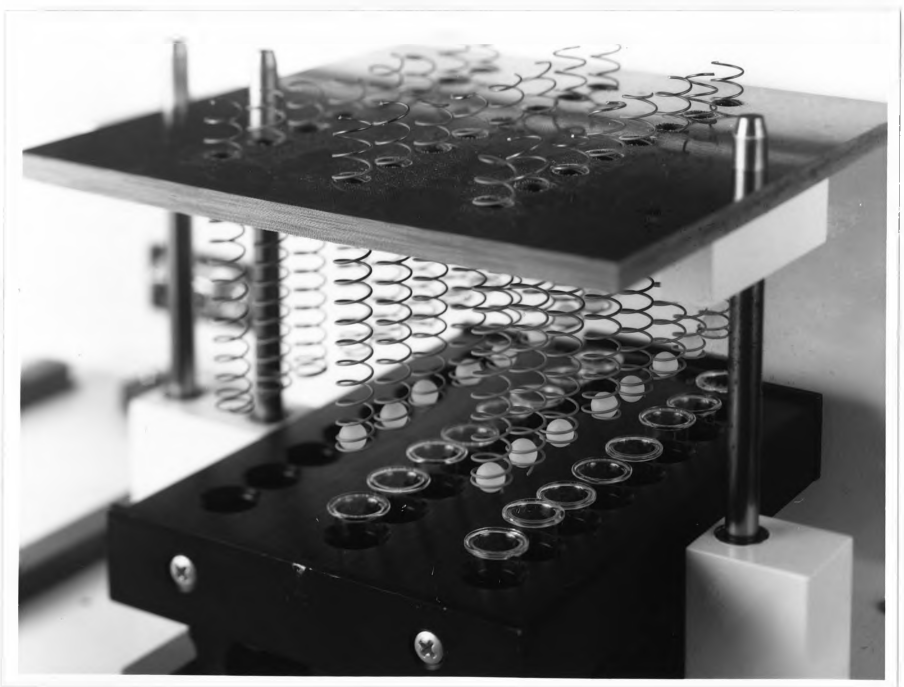
Figure 4.1

Prototype Kone immunoassay timing module employed in the AFP immunometric assay



Figure 4.2

Temperature controlled incubation block, and coils supporting antibody coated beads



PTFE-coated spring coils (Figure 4.2) could be automatically incubated in the tubes for predetermined periods with continuous vertical agitation. Coils could be simultaneously lowered into tubes containing diluted sera, HRP-conjugate, or colorimetric substrate solution, thereby simultaneously starting individual reactions. On completion of temperature controlled incubation periods, coils holding the beads were automatically lifted out of solutions thus simultaneously stopping reactions and producing a ready separation of solid phase bound components. Washing of solid supports was achieved by immersion and agitation of the beads in a single, relatively large container filled with washing solution. After incubation with colorimetric substrate, and removal of beads to terminate reactions, the incubation blocks were transferred to a Kone C multicuvette photometer capable of rapid and automatic absorbance measurements on the solutions.

4.1.3 Assay design

Theoretical and practical aspects of enzyme immunoassay design including - general immunoassay principles, assay configurations, procedures for separation of bound and free fractions, immunological reactions, preparation of HRP-conjugates, practicality, interferences and precision - are the subject of several reviews (Schuurs and van Weemen, 1977; Clark and Engvall, 1980; Oellerich, 1980; Maggio, 1980; Kricka, 1985). Appropriate reaction conditions and reagents were selected to facilitate production of a convenient, sensitive and practical assay. Particular attention was given to those aspects of the assay design which were anticipated to be critical in the eventual chemiluminescent versions of the assay. Design features

incorporated into the assay test system are briefly discussed below:

Choice of solid support: Several separation procedures can be employed to separate bound and free fractions in immunoassays (Kricka, 1985), particularly effective separation being produced using reagents bound to solid phases. The efficiency of various separation procedures previously used in conjunction with conventional chemiluminescent HRP based end-points is discussed in Chapter 5, Section 5.1.4. Etched polystyrene beads appeared particularly well suited for assays employing enhanced chemiluminescent end-points as (a) simple and efficient separation of bound components was possible, (b) they had previously been successfully used with chemiluminescent end-points and (c) they were compatible with the available luminometer. Additional advantages of beads were the large surface area available for immobilisation of antibody, and the range of commercially available assays employing beads (Chapter 6) into which enhanced chemiluminescent end-points could be extended.

Immobilisation of functional antibody: Physical adsorption of proteins to plastic supports, through non-covalent interactions such as hydrophobic bonds, has been successfully employed in a large number of assay systems (Schuurs and van Weemen, 1977; MacDonald and Kelly, 1978). This technique was therefore chosen for immobilising commercially available polyclonal antibody preparations onto the surface of polystyrene beads. Protein adsorption to polystyrene has been studied although the process

of attachment is incompletely understood (Cantarero et al., 1980). It is however known to be affected by the source of antisera, its purity and the conditions employed for binding (Clark and Engvall, 1980; Kricka, 1985). In order to avoid variation in the amounts and properties of adsorbed protein the adsorption process requires standardised, carefully controlled conditions. Any loosely immobilised antibody can be removed by washing with buffered saline solutions containing non-ionic detergent.

Incubations with samples or conjugate: Precise timing of incubations is required to achieve reproducible results in immunometric assays which employ excess reagents and relatively short incubation periods during which equilibrium may not be attained. Precise timing and temperature control of incubations was easily achieved using the Kone timing base. Continuous agitation of beads can improve the rate of antigen:antibody reactions, which are mainly limited in immunometric assays by the diffusion rate of reactants (Yolken and Leister, 1981). However, in order to ensure reproducible diffusion of reactants care is needed to treat beads uniformly with respect to agitation (Maggio, 1980).

Non-specific binding, interferences and washing prodedures: Non-specific binding of serum constituents to solid supports has proved problematical in several assays using conventional chemiluminescent end-points (Chapter 5, Section 5.1.4). Such effects are generally reduced by filling unoccupied binding sites on the surface of antibody coated beads with BSA or detergents

such as Tween, and by diluting samples (Schuurs and van Weemen, 1977) preferably in solutions containing BSA, gelatin, glycine or Tween, (Saunders, 1979; Clark and Engvall, 1980; Tsang et al., 1980). Alternatively these compounds or high concentrations of salts (Olsson et al., 1979a,b) can be incorporated into wash buffers. Non-specific binding of HRP-conjugates, through interaction of hydrophobic regions on the enzyme and solid support surfaces, can also be minimised by similar procedures (MacDonald and Kelly, 1978; Herrmann et al., 1979; Olsson et al., 1979b).

Washing procedures, used in solid phase immunometric assays to remove excess reagents or potential interfering substances, can considerably influence assay performance. Such procedures should produce maximal removal of unwanted constituents with minimal desorption of immune complexes from the solid phase. Buffered saline solutions containing detergent are commonly used for washing, individual washes being generally short in duration (2-3 minutes) and repeated several times (Schuurs and van Weemen, 1977). Many protein coated solid supports display an electric potential at their surfaces which produces a diffuse double layer of ions at the liquid-solid interface (Clark and Engvall, 1980). This double layer, which can contain potential interferents, free antigen or HRP-conjugate, may be difficult to displace except by vigorous or prolonged washing.

Assay validation: In order to demonstrate the overall assay was fully functional and of adequate sensitivity (prior to incorporation of enhanced chemiluminescent end-points), results

from serum samples previously determined using radioimmunoassay were compared to results obtained using the AFP immunometric assay with a conventional colorimetric end-point.

4.2 EXPERIMENTAL

Materials and Methods

4.2.1 Reagents

Etched polystyrene beads (6.5 mm diameter) were purchased from Northumbria Biologicals Ltd, Northumberland, NE23 9HL.

Standard alpha-fetoprotein serum was obtained from Behring (Hoechst) Pharmaceuticals UK Ltd, Houndslow, TW4 6JH. Dilutions were prepared in either PBS containing BSA (1%) and sodium azide (1mg/ml) or, in human plasma obtained from the Regional Blood Transfusion Service, Edgbaston, Birmingham. Alpha-fetoprotein purified from fetal liver (Al-Awqati et al., 1978) was obtained from the Medical College of St Bartholomew's Hospital, London, EC1A 7BE.

Rabbit anti-human AFP, rabbit anti-human ferritin and rabbit anti-human AFP-HRP conjugate were supplied by Dako Products, Mercia Brocades Ltd, Weybridge, Surrey. Sheep anti-human AFP was obtained from Seward Laboratories, UAC House, Blackfriars Road, London, SE1 9UG.

Iodine-125 (100 mCi/ml) was purchased from the Radiochemical Centre, Amersham; Lipsol liquid detergent from LIP (Equipment and Services) Ltd, Shipley, West Yorkshire; chloramine T and sodium metabisulphite (BDH) and all other reagents from Sigma Chemical Company.

4.2.2 Instrumentation

Immunoassays were performed using a prototype Kone Immunoassay module (Figures 4.1 and 4.2), Kone Instruments, Stockport, Cheshire. The analyser is controlled by a Kone OMS-81 microcomputer and employs four, 24-place temperature controlled incubation blocks. Beads are held in four matrix plates, each capable of supporting 24 beads suspended in individual inert PTFE-coated coils. Beads can be incubated for predetermined intervals at regulated temperatures (30-37^{o}C $\pm$ 0.1^{o}C), with continuous vertical agitation ($\pm$ 1 mm; 3 Hz). When incubations were complete, coils were automatically lifted out of the incubation blocks, thus stopping the reaction.

Absorbance measurements were performed automatically using a Kone C Compact Clinical Analyser.

4.2.3 Standardised assay procedure

4.2.3.1 Coating polystyrene beads with anti AFP

Glycine/NaOH buffer (0.1 mol/l pH 8.8; 125 ml) and BSA (0.25 g) were added to a 500 ml glass bottle and the solution mixed for 2 hours on a roller mixer at 20^{o}C. The bottle, and its lid were then rinsed with glycine buffer (3 x 50 ml) to remove any unbound albumin and finally allowed to drain.

Polystyrene beads (1000) were washed with 1% Lipsol (500 ml) for 30 minutes, then, following washing with distilled water, were placed in a Buchner flask with 200 ml glycine buffer, and degassed.

Glycine buffer (400 ml) was transferred to the pre-coated glass

bottle and rabbit anti-human AFP (1.5 ml) added. The degassed beads were then added, the bottle capped, and mixed on the roller mixer for 6 hours at 20°C. Following mixing the glycine buffer-antibody solution was decanted and the beads washed twice with 400 ml PBS (0.015 mol/l phosphate, 0.15 mol/l NaCl, pH 7.2). After the second PBS wash, BSA solution (0.5% in PBS; 400 ml), was added and mixed on the roller mixer at 20°C. After 30 minutes the PBS-albumin solution was removed and the beads washed with PBS buffer containing 0.05% Tween (2 x 400 ml). Finally the PBS was decanted and the beads dried on filter paper at 20°C for 30 minutes. Beads were stored in screw capped bottles at 4°C.

4.2.3.2 Assay procedure

A sample of a specimen containing AFP (50 µl) was added to a 10 x 50 mm acrylic tube (Kone, Helsinki) together with 0.95 ml PBS/BSA/Tween (phosphate 0.015 mol/l; NaCl 0.15 mol/l; BSA 0.2%; Tween 20 0.05%; pH 7.2). The solution was warmed to 37°C and then incubated for 1 hour with the beads supported in the agitated spring supports. Following incubation each set of beads was washed in PBS/Tween (200 ml for 15 seconds then 200 ml for 5 minutes). The beads were then incubated with 0.9 ml of a 1:1000 dilution of rabbit anti-human AFP-HRP conjugate for 1 hour. (The conjugate was diluted in PBS/BSA/Tween and, prior to addition to the beads, was warmed to 37°C). Washing was then repeated with PBS/Tween (200 ml for 15 seconds) and then distilled water (200 ml for 5 minutes).

4.2.3.3 Colorimetric quantitation of bound anti AFP-HRP

This was performed as described in Chapter 2 Section 2.2.4.2.

ABTS-H_2O_2 (0.9 ml) was incubated with each bead at 37°C for 30 minutes with agitation. After incubation and removal of beads, the absorbance at 405 nm was measured against working enzyme substrate solution using a Kone C Compact Clinical Analyzer.

4.2.4 Investigation of factors affecting the assay system

4.2.4.1 Source of antibody immobilised onto beads

1) Preparation of antibody coated beads:

Anti AFP raised in sheep or rabbits was bound to polystyrene beads by physical adsorption. The coating procedure previously described was used except that beads were incubated with antisera diluted 1:1000 with coating buffer (1 ml per bead) for 2 hours at room temperature and then overnight at 0-4°C. Coated beads were employed in assays directly after the final wash procedure and anti-human ferritin coated beads served as appropriate controls. Antibody bound to the bead was investigated for its ability to bind ^{125}I labelled AFP and for its performance in the full colorimetrically monitored immunometric assay.

2) Binding of ^{125}I labelled AFP:

Alpha-fetoprotein (10 µg) purified from fetal liver by the method of Al-Awqati et al., 1978, was iodinated using ^{125}I and chloramine T (Kennedy, 1979). Sodium metabisulphite was used to stop the reaction and bound and free ^{125}I separated by Sephadex G50 gel filtration chromatography. Standards (0, 50, 200 and 400 ng/ml) were prepared from the iodinated AFP by dilution with PBS containing 1% BSA. AFP standard (200 µl) and PBS (400 µl) were mixed in a plastic tube, an antibody coated bead added, and incubation performed for 2 hours at room temperature with no

agitation. Beads were then removed, washed with PBS/Tween (3 x 5 ml) and each bead counted for 60 seconds on an LKB-Wallac gamma counter.

3) Binding of AFP determined with anti AFP-HRP conjugate:
Beads coated with either sheep or rabbit anti-human AFP were processed according to the colorimetric immunometric assay procedure using 300 µl AFP standard (0 or 312 IU/ml prepared in PBS containing 1% BSA) and 600 µl PBS diluent.

4.2.4.2 Influence of temperature and time on coating polystyrene beads with antibody

The standardised coating procedure was employed with minor modification. Batches of 10 beads were coated, in BSA coated 25 ml glass universal containers, with rabbit anti-human AFP diluted 1:1000 with coating buffer (1 ml/bead). Beads were incubated with mixing at either 0-4°C, room temperature or at 37°C for varying periods up to 24 hours. After the appropriate incubation time beads were removed, washed, coated with BSA and washed again at room temperature. Beads were dried and stored at 0-4°C before use. Beads were then processed according to the finalised assay procedure using a PBS-based 100 IU/ml AFP standard.

4.2.4.3 Influence of incubation time of AFP samples with antibody coated beads

AFP standards (PBS-based) were processed according to the assay procedure, varying the incubation time between AFP samples and coated beads from 0 to 3 hours.

4.2.4.4 The influence of spring position on bead agitation

A transparent plastic holder was substituted for the incubation block employed with the immunoassay module. This enabled tubes to be positioned exactly as in the incubation block used in the assay procedure and also allowed both spring position and movement of individual beads to be monitored visually. Springs were adjusted to either touch, or be positioned 1 to 4 mm from the tube bottom, and 1 ml of PBS/BSA/Tween added to each tube. An antibody coated bead was suspended in each spring and the matrix plate agitated (± 1 mm, 3Hz) as in the assay procedure. Bead movement was photographed in a darkroom using strobe lighting (9 flashes/second).

4.2.4.5 The influence of bead storage prior to antibody-HRP conjugate quantitation

AFP standards (PBS-based) were processed according to the assay procedure. However, after the final washing, and prior to colorimetric HRP quantitation, the beads were removed from the springs and transferred either to an empty cuvette or one containing PBS/Tween (1 ml). Beads were stored under these conditions for varying times up to 45 minutes after which they were transferred back to the spring supports and colorimetric label quantitation performed.

4.2.5 Assay performance and validation

AFP standards (PBS or plasma-based) were processed according to the assay procedure. Twenty six serum samples, previously analysed by radioimmunoassay at Birmingham Maternity Hospital and chosen to cover the range of the immunometric assay, were

assayed. Specimens were assayed in duplicate using the plasma-based AFP standards employed in the radioimmunoassay.

4.3 RESULTS

4.3.1 Investigation of factors affecting the assay system

4.3.1.1 Influence of source of antibody for immobilisation onto beads

Antibody immobilised onto polystyrene beads using commercially available polyclonal antibody preparations raised in either sheep or rabbits showed differing binding of ^{125}I labelled AFP (Figure 4.3). Coated beads prepared using the antibody raised in rabbits bound approximately 60 to 80% more AFP than beads coated with the sheep anti-human AFP preparation. Only small quantities of ^{125}I labelled AFP were bound by control beads coated with a similar antibody preparation raised in rabbits against human ferritin. Detailed investigations to establish why the AFP antibody preparations performed differently were not undertaken. Beads coated with the rabbit antibody also produced much clearer differentitation between standards when bound AFP was determined in the full immunometric assay using colorimetric quantitation of bound HRP-labelled anti AFP, also raised in rabbits. In preliminary experiments using 0 and 312 IU/ml AFP standards, absorbance changes of 0.61 and 1.51 respectively were produced using beads coated with the sheep anti AFP preparation, whereas absorbance changes of 0.58 and 4.30 were achieved using beads coated with the antibody preparation raised in rabbits.

Figure 4.3

Binding of ^{125}I labelled AFP to antibody coated polystyrene beads

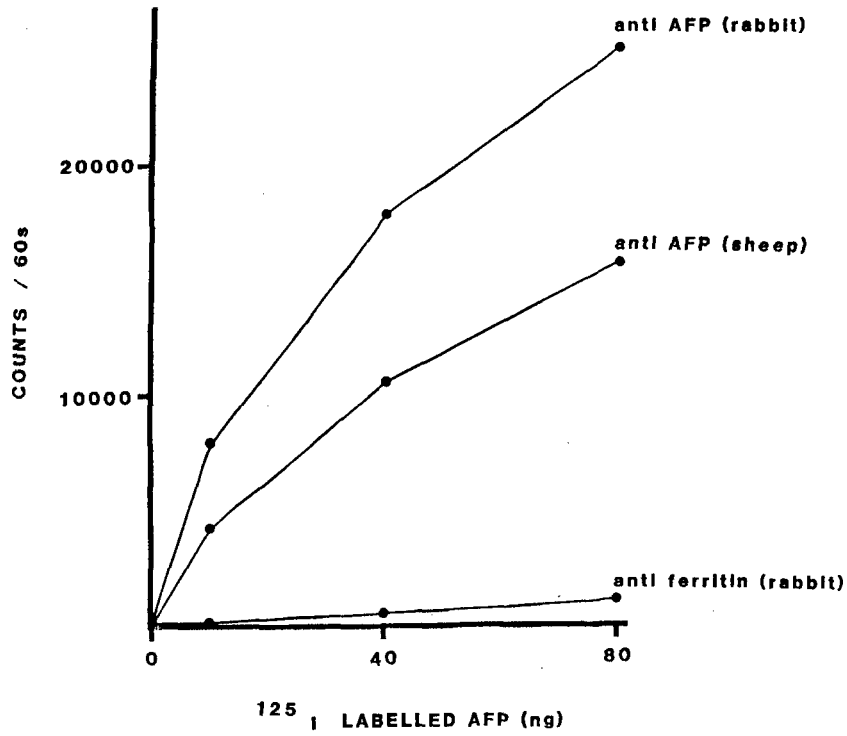
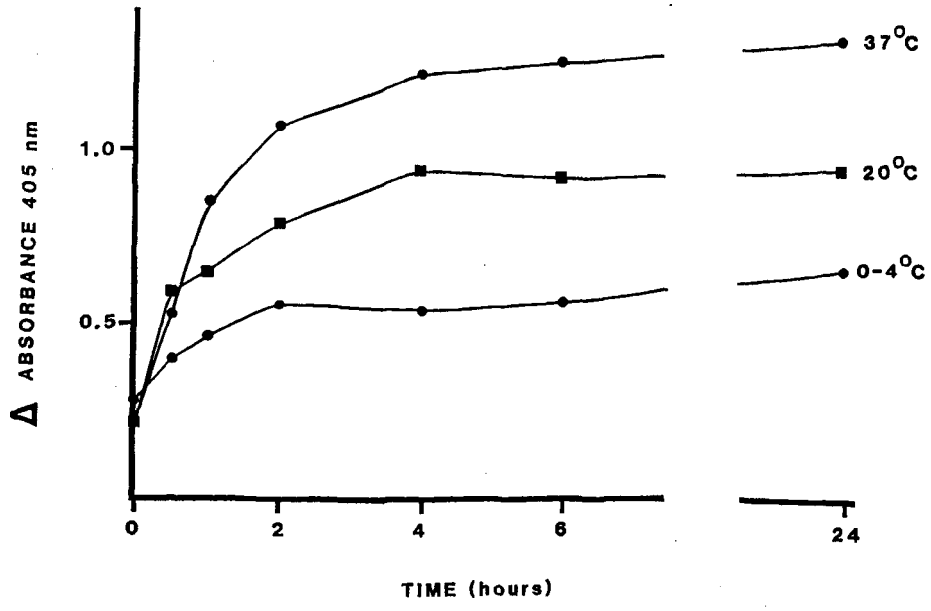


Figure 4.4

The influence of temperature and incubation time on binding of anti AFP to polystyrene beads



4.3.1.2 The influence of temperature and time of incubation on antibody immobilisation

The influence of temperature and time of incubation on binding of functional antibody onto the surface of polystyrene beads is illustrated in Figure 4.4. Increasing quantities of antibody were bound as the incubation temperature was increased from 0 - 4, to 20, to 37^{o}C. At each temperature antibody binding increased with time, however the majority of binding taking place during 24 hour incubations occurred within the first 6 hours.

4.3.1.3 The influence of incubation time on AFP binding to antibody coated beads

Figure 4.5 illustrates the kinetics of binding of AFP to immobilised antibody in the immunometric assay using standards of differing concentration. Binding of AFP increases with time and incubation times of over 3 hours appeared to be required for equilibrium to be reached. Significant binding of AFP and clear differentiation between standards was possible after 1 hour. Although equilibrium had not been reached, the ability to precisely control incubation times enabled a 1 hour incubation to be routinely used in the assay test system.

4.3.1.4 The influence of spring position on bead agitation

The position of springs used to support beads during incubations was shown to influence the movement of beads during the agitation process (Figure 4.6). Efficient agitation, and free movement of beads through solutions, was produced when the spring tip was positioned approximately 1 mm from the bottom of the tube. Increasing the distance to 4 mm resulted in decreased agitation,

Figure 4.5

The influence of AFP concentration and its incubation time with antibody coated beads on antibody-HRP conjugate uptake

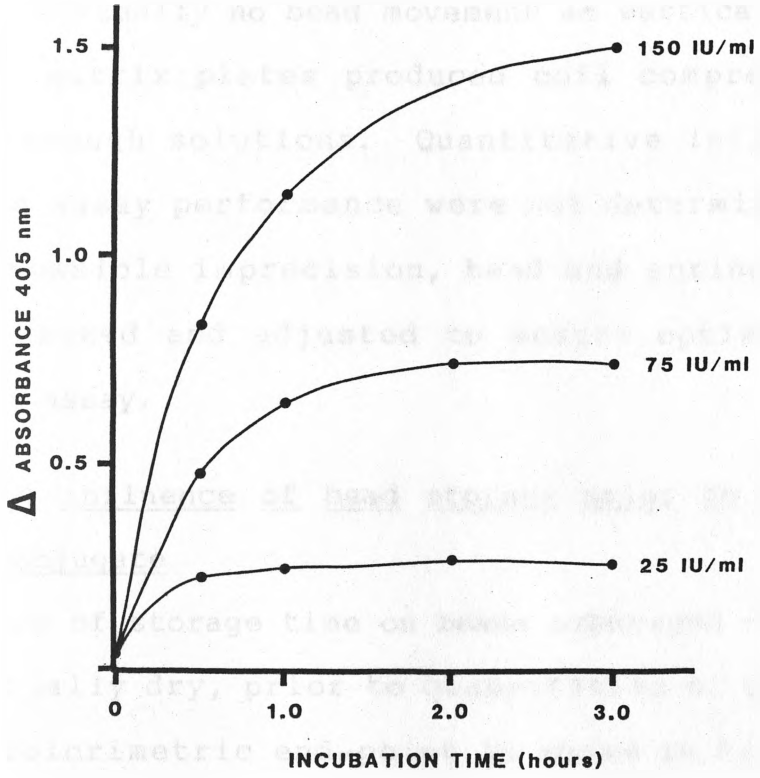
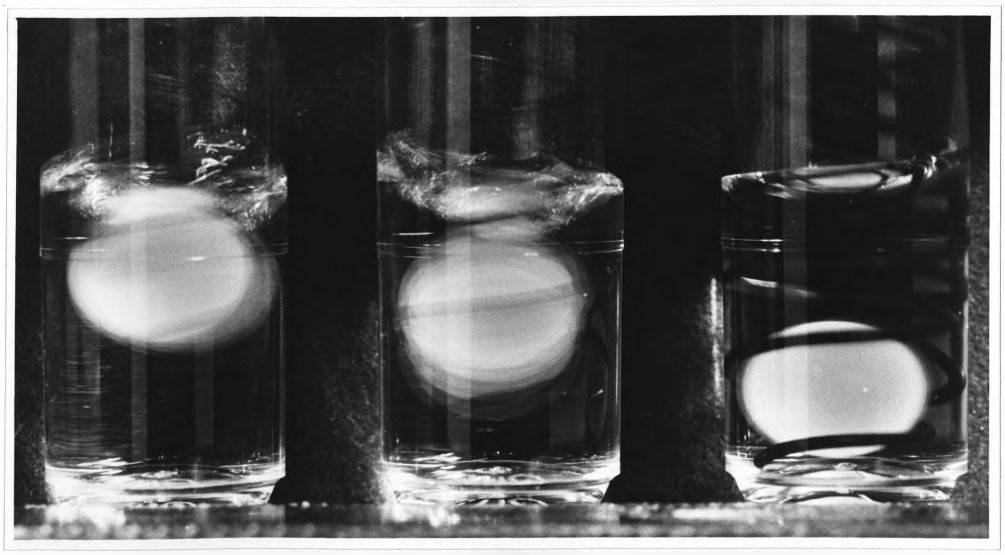


Figure 4.6

The influence of spring support position on bead agitation

Springs were positioned A, 4 mm from the tube base; B 1 mm from the tube base; or C, touching the tube base



A B C

less vertical movement of beads in the solutions, and small sections of the bead surface occasionally becoming incompletely submerged. Positioning spring tips to touch the tube base resulted in virtually no bead movement as vertical oscillation of the spring matrix plates produced coil compression not free movement through solutions. Quantitative influences of bead position on assay performance were not determined, however to avoid any possible imprecision, bead and spring position were visually checked and adjusted to ensure optimal positioning before each assay.

4.3.1.5 The influence of bead storage prior to quantitation of bound HRP-conjugate

The influence of storage time on beads submerged in PBS/Tween, or stored partially dry, prior to quantitation of bound conjugate using the colorimetric end-point is shown in Figure 4.7. Good differentiation between standards and relatively constant responses were achieved using either storage procedure. Submersion of the beads for up to 45 minutes produced no significant dissociation and loss of conjugate from the bead surface. Storage of beads partially dry for such periods produced similar colorimetric responses and did not result in irretrievable inactivation of bound HRP-conjugate.

4.3.2 Assay performance and validation

Dose-response curves for AFP, using PBS or plasma-based standards, in the colorimetrically monitored immunometric assay are shown in Figure 4.8. A sensitive, near linear response was obtained and the assay showed good differentiation between AFP

Figure 4.7

The influence of bead storage prior to quantitation of bound antibody-HRP conjugate

■——■ stored in PBS, ●- - -● stored partially dry

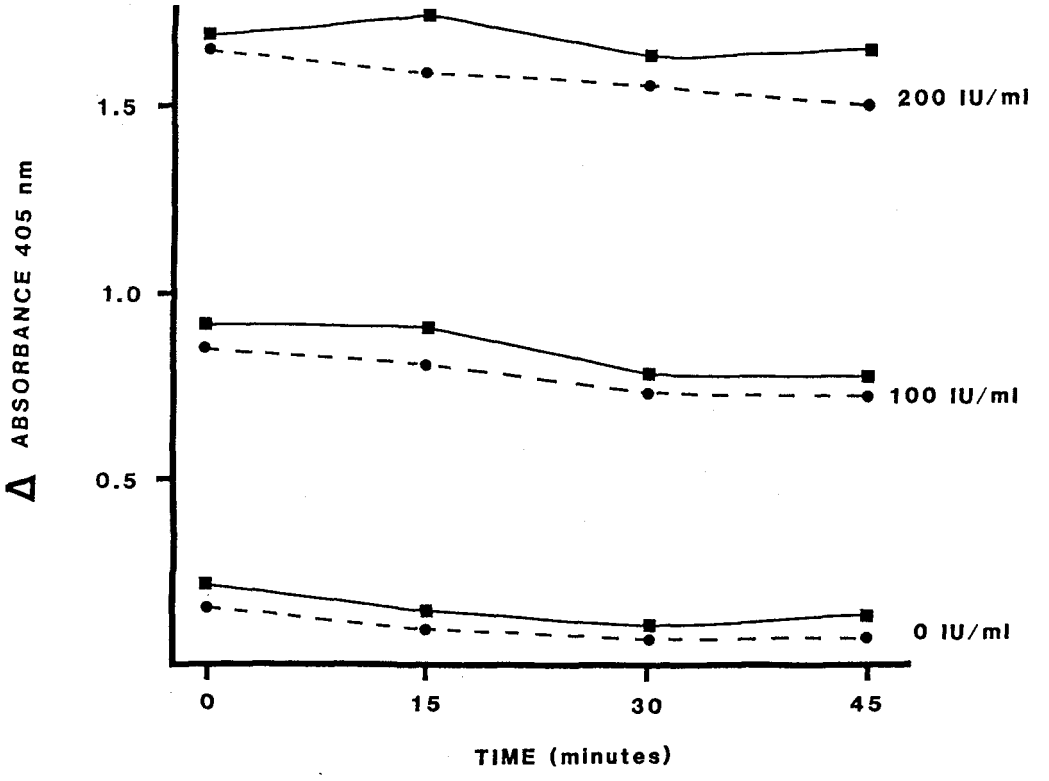
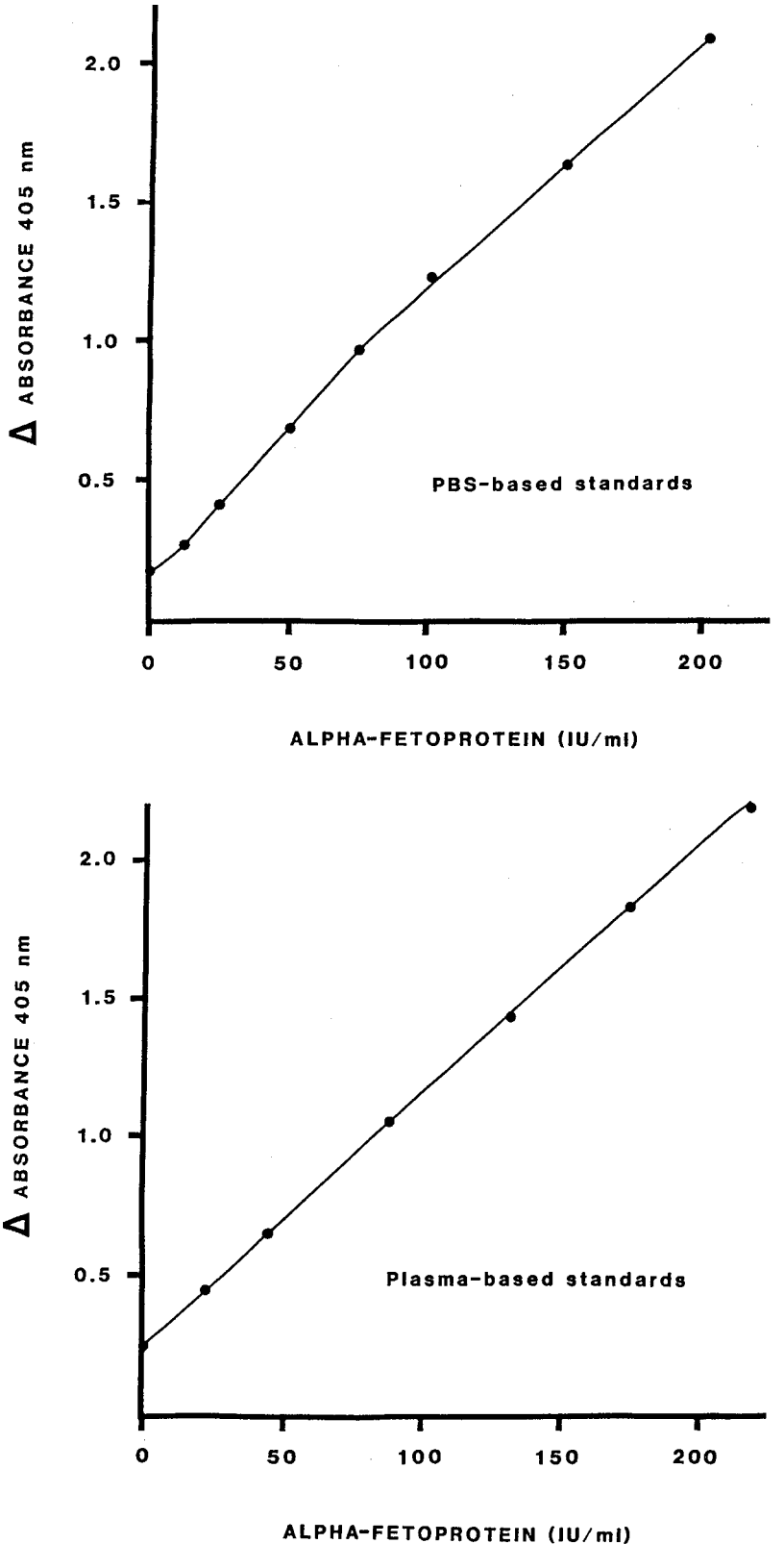


Figure 4.8

Dose-response curves in the immunometric assay using PBS or plasma-based AFP standards



standards in the routinely used radioimmunoassay. Plasma-based standards introduced no significant interference and in a study on 26 patients' serum samples good correlation ($r = 0.98$) was achieved for results obtained using radioimmunoassay (y) or the colorimetric immunometric assay (x) ($y = 0.83x + 12$).

4.4 DISCUSSION

A sensitive, inexpensive, simple, rapid and semi-automated solid phase immunometric assay for AFP was developed. The assay employed HRP labelled specific antibody and appeared suited to subsequent investigations on the incorporation of luminescent techniques for conjugate quantitation. The assay was suitable for AFP measurement, over a clinically useful range, in aqueous or serum-based samples (50 µl) which required no pretreatment. All the reagents used, (including the solid support) were commercially available, and the results obtained were in good agreement with those obtained using radioimmunoassay.

The assay was based on conventional enzyme immunoassay methodology, however the use of the Kone immunoassay module allowed partial automation and involved a novel solid support handling system in which beads were loosely supported in inert PTFE-coated coils. Vertical oscillation of these coils allowed bead agitation during the assay. Antibody binding to the beads, AFP binding and bead agitation were examined to produce a suitable assay system and eliminate major sources of imprecision which could affect comparison of luminescent end-points. Equilibrium was not attained in the immunological reactions, however, good sensitivity and precision were achieved as

incubations with sample, conjugate or colorimetric substrate could be performed with agitation under temperature controlled conditions. The incubations could also be accurately timed, and reactions involving up to 96 individual beads started and terminated simultaneously.

Relatively large batches of approximately 1000 beads could be coated by passive adsorption with functional antibody, and these conveniently stored dry at 0-4°C. Although batch to batch differences in coating were seen, sufficient numbers could be coated in one batch to perform experiments to compare results obtained using the colorimetric immunometric assay with those obtained by radioimmunoassay, and to compare the performance of the immunometric assay using a colorimetric or chemiluminescent end-point. Beads provided an impervious, non-porous solid support allowing both efficient separation of bound and free components, and the use of vigorous washing steps. In order to minimise any interference from components of biological samples or non-specific binding to the solid support, BSA and Tween were used to fill unoccupied binding sites, two washing procedures were employed between each step in the assay, and samples diluted (1 in 20) in buffer specifically chosen to reduce such effects. Conjugate was diluted in similar buffer and used at a concentration producing an adequate dose-response curve without high levels of non-specific binding.

In addition to providing a solid support compatible with the luminometer, the suitability of the assay for incorporation of luminescent quantitation of conjugate was reinforced by findings

that conjugate immobilised via immune complex formation to beads did not become totally dissociated from the surface if stored in a small volume of buffer, or enzyme activity irretrievably lost if stored partially dry for up to 45 minutes. This was important as it was not possible to monitor several chemiluminescent reactions simultaneously on the available equipment and variable storage of solid supports prior to label quantitation would be required to monitor batches of beads.

Further modification of the assay, using techniques to improve the quality of immobilised antibody and conjugates or further reduce non-specific binding (Place and Schroeder, 1982; Imagawa et al., 1984; Ruan et al., 1986), may have improved performance and sensitivity. However, the assay appeared suitable for the inclusion of luminescent quantitation of conjugate, and provided a colorimetric system against which the performance of the enhanced chemiluminescent assays could be compared.

CHAPTER 5

LUMINESCENT QUANTITATION OF PEROXIDASE CONJUGATES IN AN IMMUNOMETRIC ASSAY FOR ALPHA-FETOPROTEIN

5.1 INTRODUCTION

5.1.1 Objectives

The overall objective of this Chapter was to develop a chemiluminescently monitored immunometric assay for AFP by incorporating enhanced chemiluminescent quantitation of HRP-conjugate (described in Chapters 2 and 3) into an immunometric assay for AFP (described in Chapter 4). In order to achieve this it was necessary to:

1 determine any potential problems when initiating or monitoring luminescent reactions in conjunction with the polystyrene bead solid support.

2 evaluate the luminescent assays, this time for HRP-conjugate immobilised via an immune complex onto the surface of polystyrene beads.

3 compare the performance of enhanced chemiluminescently and colorimetrically monitored immunometric assays for AFP using serum samples.

5.1.2 The advantages of luminescently monitored immunoassays

The general advantages of luminescently monitored immunoassays have been discussed in Chapter 1. The incorporation of luminescent assays for HRP-conjugate quantitation into immunoassay systems should produce improvements in the speed and sensitivity in the assays similar to those achievable for HRP-conjugate quantitation in solution. Although reductions in overall assay time and improvements in sensitivity would be the most significant improvements, luminescent techniques should also produce an improved range of linear response and the potential to

employ relatively simple instrumentation. The use of HRP labels circumvents the decreased luminescent efficiency encountered with some luminescent labels after conjugation, and allows a degree of amplification. Rapid extension of enhanced chemiluminescently monitored immunoassay systems should also be possible due to the availability of a wide range of HRP-conjugates and the large number of immunoassay systems employing such conjugates (Chapter 6).

5.1.3 Previously reported luminescently monitored immunoassays employing peroxidase conjugates

Chemiluminescent quantitation of HRP labels has been used in several heterogeneous immunoassay systems, a comprehensive list of these assays is presented in Table 5.1. Techniques using luminol analogues or phenol derivatives have been employed in conjunction with either photon counting or photocurrent determination to measure light emission, and chemiluminescent reactions on the solid supports have been initiated both directly in front of the detector (Velan et al., 1979) or prior to their introduction (Arakawa et al., 1979). The sensitivities of the assays are generally similar to those using radioactive labels, and improvements in the speed of HRP label quantitation are easily achieved when luminescent procedures are incorporated into immunoassay systems.

Improvements in assay sensitivity have been produced by incorporation of conventional unenhanced chemiluminescent end-points into several immunoassays. Ten-fold increases in sensitivity over those monitored colorimetrically using ABTS have

Table 5.1

Heterogeneous immunoassays employing chemiluminescent detection of horseradish peroxidase labels

ANALYTE	DETECTION SYSTEM	HRP LABELLED COMPOUND	SAMPLE EXTRACTION	SOLID SUPPORT OR SEPARATION PROCEDURE	SENSITIVITY	REFERENCE
Cortisol			Yes	Sepharose 4B	10 pg	Tsuji et al., 1978 Arakawa et al., 1979
Dehydroepiandrosterone			Yes	Sepharose 4B or Polystyrene tubes	25 pg/tube	Arakawa et al., 1981
Progesterone	Luminol/H_2O_2 pH 7.0	Ag	Yes		5 pg/tube	
Testosterone			Yes		1 "	
Oestriol			Yes	Polystyrene beads	8 "	Roda et al., 1984a
Cortisol			No		5 "	
Conjugated lithocholate			No		100 "	
Candida albicans protein antigen	Luminol/H_2O_2 pH 9.0	Ab	-	Precipitation or Polyacrylamide microspheres	-	Stevens et al., 1980
Herpes simplex virus					100 PFU/ml	Pronovost et al., 1981
IgG					10 ng/tube	
Human serum albumin	Isoluminol/H_2O_2 pH 10.5	Ab		Polystyrene beads	0.4 ng/ml	Pronovost and Baumgarten, 1982
Alpha fetoprotein					1.9 µg/ml	
Cytomegalovirus					$>10^{0.3}$ $TCID_{50}$/sample	Pronovost et al., 1982
Thyroxine	7-Dimethylamino-naphthalene-1,2-dicarboxylic acid hydrazide/H_2O_2 pH 8.2					
Insulin						
Digoxin		Ag	No	Plastic tubes	-	Gundermann et al., 1982
Venezuelan equine encephalomyelitis virus	Luminol/H_2O_2 pH 12 - 13	Ab	-	Polyacrylamide beads	10^2-10^3 PFU	Miller and Reichard, 1980
Human serum albumin	Luminol/perborate pH 12 - 13	Ab	No	Polystyrene tubes	10 ng	Olsson et al., 1979a
Serratia marcescens	Pyrogallol/H_2O_2 pH 6.5	Ab	Yes	Alcar strips	30-300 cells	Halmann et al., 1977
Staphylococcal enterotoxin B		Ag		Sepharose 4B	1 ng	Velan and Halmann, 1978
Sindbis virus	Pyrogallol or Purpurogallin, p-phenylenediamine/H_2O_2 pH 6.5	Ab	-	Polyvinyl chloride microtitre plates	3 x 10^6 virus PFU/ml	Velan et al., 1979

PFU = plaque-forming units, $TCID_{50}$ = tissue culture infectious dose (50 %), Ag = antigen, Ab = antibody

been reported in assays for thyroxine, insulin and digoxin by the use of the 7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide-H_2O_2 reaction at pH 8.2 to quantitate HRP-labelled antigen bound to antibody coated tubes (Gundermann et al., 1982). Use of the isoluminol-H_2O_2 reaction at pH 10.5 to quantitate HRP-antibody conjugate bound to polystyrene beads has produced 16 to 95-fold increases in sensitivity over immunoassays with a colorimetric o-phenylenediamine end-point. The chemiluminescent end-point has been incorporated into immunoassays for serum albumin, alpha-fetoprotein, IgG (Pronovost and Baumgarten, 1982) cytomegalovirus and herpes simplex virus (Pronovost et al., 1981; Pronovost et al., 1982). Two to five-fold increases in sensitivity have also been produced in a range of competitive immunoassays for steroids using polystyrene beads as solid supports, by replacing a colorimetric o-phenylenediamine end-point with an unenhanced luminol-H_2O_2 chemiluminescent end-point performed at pH 7.0 (Roda et al., 1984a).

Overall immunoassay sensitivity however is dependent on several factors including, the luminescent assay, the instrumentation employed to quantitate light emission and the overall immunoassay design including the degree of conjugate non-specific binding, background, and interference. Comparisons of the absolute sensitivities achievable by the introduction of a particular luminescent assay into such systems is obviously difficult, and comparisons with those determined using colorimetry complicated by the use of various colorimetric assays of differing sensitivity.

5.1.4 The incorporation of luminescent procedures into immunoassays

5.1.4.1 Ideal requirements

Ideally, the luminescent assay for HRP-conjugates would be fast, sensitive and specific (ie, not prone to interference), and would be used in conjunction with a highly efficient separation system which is compatible with the light measuring instrumentation.

Unfortunately, several luminescent procedures are susceptible to interference from substances present in complex biological fluids (Chapter 2) and this may be manifested as variable, high background light emission from solid supports. The successful incorporation of luminescent techniques into immunoassays requires either minimal association of such compounds with the solid supports, their efficient removal by washing procedures, or sample purification prior to HRP-conjugate quantitation. For maximal sensitivity non-specific adsorption of HRP-conjugates to solid supports should obviously be minimal. The solid support employed should enable efficient reproducible initiation of luminescent reactions and allow maximal collection of the light emitted at its surface.

5.1.4.2 Interference problems encountered in luminescently monitored immunoassays

The varying degrees of success achieved in attempts to incorporate luminescent assays for HRP-conjugates into immunoassay systems is probably due in part to differing interference problems encountered with biological fluids. Interference in luminescently monitored assays is particularly

dependent on the separation procedure for bound and free conjugate, and the specificity of the luminescent assay employed. As yet no comparison has been made of all the luminescent assays available for HRP-conjugates in a single immunoassay system, using the same instrument to measure light emission.

Separation procedures:

In order for a separation procedure to be successful in a luminescently monitored immunoassay it should effectively minimise the quantity of interfering substances which remain associated with the fraction in which HRP-conjugate is to be quantitated.

Precipitation procedures may entrap interfering substances in the precipitate. This may explain the problems encountered in an assay for candida protein antigen using polyethylene glycol precipitation to separate bound and free HRP labelled antibody, employing conjugate quantitation with the luminol-hydrogen peroxide reaction at pH 9.0 (Stevens et al., 1980).

When reagents immobilised onto solid supports are used for separation, the avidity of the interfering substances for the solid support and the efficiency of the washing steps are both factors in determining the extent of any interference. Problems have been encountered with serum samples when certain supports such as Sepharose particles or microcrystalline cellulose have been used in conjunction with either luminescent quantitation of HRP or isoluminol labels (Strasburger et al., 1982). The luminol-hydrogen peroxide reaction at pH 7-8 with photon counting has successfully been employed to quantitate HRP activity bound

to Sepharose 4B particles in immunoassays for serum cortisol, dehydroepiandrosterone and its sulphate using HRP-steroid conjugates (Arakawa et al., 1979; Arakawa et al., 1981). Sensitivities comparable to radioimmunoassay were achieved, however to avoid problems from interfering substances samples required purification using solvent extraction. Similar assays for insulin and thyroid stimulating hormone based on Sepharose particles, but without sample purification, could not be monitored luminescently due to interferences (Tsuji et al., 1978). Chemiluminescent reactions using pyrogallol at pH 6.5 have been employed to quantitate HRP labelled antigen bound to Sepharose particles in an immunoassay for staphylococcal enterotoxin B (Velan and Halmann, 1978). Light emission is dependent on both the number and proximity of HRP molecules as a cooperative effect is believed to occur between adjacent HRP-conjugates bound to immobilised antibodies producing a steep calibration curve. However, whether actual biological samples can be analysed, is not stated. Interference problems with Sepharose solid supports may be attributed to inclusion of interfering substances into the porous Sepharose matrix.

Fewer problems due to interference have been encountered using impervious, non-porous solid supports fabricated from plastics. The use of polystyrene tubes allowed elimination of interfering substances from serum whereas extraction of samples was required if Sepharose particles were used as the solid support (Arakawa et al., 1981). Polystyrene beads and tubes have been successfully used in luminescently monitored assays employing HRP-conjugates

in which no sample purification is required. Assays for albumin, alpha-fetoprotein and viral antigens using polystyrene beads (Pronovost and Baumgarten, 1982), and for thyroxine, insulin and digoxin using plastic tubes (Gundermann, 1980; 1981; Gundermann et al., 1982) have been performed luminescently and produced significant increases in sensitivity over those quantitating HRP-conjugates colorimetrically. The use of a conventional unenhanced chemiluminescent end-point in conjunction with polystyrene beads as solid support is however often only applicable to competitive immunoassays for serum steroids after a preliminary sample extraction (Roda et al., 1984a).

Chemiluminescent procedures based on phenol derivatives have been incorporated into an immunometric assay for Sindbis virus carried out on polyvinyl chloride microtitre plates (Velan et al., 1979). The p-phenylenediamine enhanced purpurogallin chemiluminescent reaction (shown to be more sensitive, for HRP quantitation in solution, than pyrogallol chemiluminescence and colorimetric procedures) failed to produce any increase in immunoassay sensitivity with crude virus preparations due to elevated background light emission. The high background was presumably due to interferences attached to, but not removed from the surface of the well.

Bioluminescent quantitation of HRP-conjugate bound to polystyrene tubes has recently been employed in an immunometric assay for serum human choriogonadotrophin with a sensitivity of 0.1 ng/tube (Longeon et al., 1986). The end-point utilised Pholas dactylus luciferin and molecular oxygen in the presence of

diethyldithiocarbamate which induced a decrease in background light emission possibly by complexing metal impurities or inhibiting intermediate radicals.

In addition to the separation system employed, the efficiency of the washing procedures used to remove interfering substances would also influence the extent of any interference problem.

The specificity of luminescent quantitation:

The specificity of luminescent quantitation of HRP-conjugates in immunoassays can influence the overall assay performance. Evaluating a particular end-point in luminescently monitored immunoassays is complicated by the differing specificities of the various luminescent assays and the degree to which interfering substances can be eliminated from the solid supports. A highly specific luminescent assay should perform well even in conjunction with a solid support from which interferences from biological samples cannot be completely eliminated. A non-specific assay would produce high background light emission and poor sensitivity.

The luminol-hydrogen peroxide and luminol-perborate reactions at high pH (> 12) are relatively non-specific (Ewetz and Thore, 1976; Neufeld et al., 1965; Chapter 2). The alkaline conditions release haem from the HRP molecule allowing reactions not limited by steric hindrance at the solid surface and even allow the use of enzymatically inactive HRP labels. However, as the reactions are based on haem content they are particularly prone to interference from serum constituents such as haemoglobin or other haemproteins which may bind to solid supports (Olsson et al.,

1979a,b; Olsson et al., 1981; Imagawa et al., 1984). The non-specific nature of the luminescent luminol-perborate reaction may explain its poor performance compared to colorimetric quantitation of HRP-antibody conjugates in immunoassays for anti albumin using antibody coated polystyrene tubes (Olsson et al., 1979b). Although HRP-conjugates have been quantitated using the luminol-hydrogen peroxide reaction at high pH in an immunoassay for Venezuelan equine encephalomyelitis virus using antibody coated polyacrylamide beads (Miller and Reichard, 1980), it is not stated whether complex biological samples can be analysed. The specificity of enhanced chemiluminescent assays for HRP and the fact that light emission catalysed by haemoglobin is not enhanced (Chapter 3) suggested such end-points were well suited for immunoassay applications.

5.1.4.3 Initiation of luminescent reactions on solid supports

Measurement of the luminescent activity associated with solid supports such as Sepharose particles (Arakawa et al., 1979) microtitre plates (Velan et al., 1979), plastic tubes (Gundermann et al., 1982) and polystyrene beads (Pronovost and Baumgarten, 1982; Roda et al., 1984a,b) has been successfully employed in luminescently monitored immunoassays. Tubes and supports such as beads and Sepharose particles (which can be held in suitable containers) can be conveniently used with most conventional luminometers or liquid scintillation counters. Monitoring light emission from individual wells of intact transparent microtitre plates however requires specialised instrumentation (Schroeder et al., 1981).

When luminescent labels remain associated with the solid support, quantitation of bound conjugate is made in its presence and this may influence light measurements. For conjugate bound to small particles which can produce a homogeneous solution, light is emitted almost as in solution. Large particles however, may move relative to the photodetector, produce diffusion problems and introduce complex support/liquid/air interfaces. The solid support may also influence the efficiency and reproducibility of initiating the luminescent reactions. Precision for quantitating luminescent labels should be such that acceptable overall immunoassay CVs, at least comparable to those employing non-luminescent techniques, are obtained. With luminescent labels such as isoluminol derivatives or luminescent detection of products of enzyme labels, immunoassay CVs are generally under 10% in the assay working range (Wood et al., 1982), and they are comparable to, but sometimes slightly inferior to those using radioisotopes (Pazzagli et al., 1981b; Weeks et al., 1983b). For luminescently monitored immunoassays employing HRP-conjugates, CVs (when quoted) are comparable to, but again often slightly inferior to those using colorimetric end-points (Pronovost et al., 1981; Roda et al., 1984a). An inability to improve assay precision by incorporating luminescent techniques may arise from imprecision in light measurement introduced by the instrumentation, solid support, interferences, or the initiation procedures. Enzyme activity must generally be retained by luminescent HRP labels (unlike ^{125}I or isoluminol labels) and as most luminometers cannot simultaneously monitor multiple samples, the variable storage of bound conjugate prior to analysis may

introduce further imprecision.

A variety of processes have been employed to initiate luminescent reactions with bound conjugate. Luminescent reagents may simply be added to conjugate on the solid support as a premixed cocktail, or the support directly introduced into such reagents (Hara et al., 1983b). Alternatively, various components of the luminescent reaction can be added to bound HRP-conjugate (usually a sufficient volume to cover or suspend the support) and the reaction initiated by addition of a final component (Pronovost et al., 1981). Volumes and mixing procedures employed to initiate such reactions differ widely, eg, initiation directly in front of the photodetector (Weeks et al., 1983a) or manually outside the luminometer (Arakawa et al., 1981). When luminescent conjugates are dissociated from the solid support surface prior to quantitation, the luminescent reaction becomes less influenced by the solid support but an extra step often requiring timing is introduced (Olsson et al., 1979b; Weerasekera et al., 1983a).

5.2 EXPERIMENTAL

5.2.1 Automatic injection of reagents onto antibody coated beads

Replacement of the luminometer photomultiplier tube by a high speed camera was achieved by removal of the PMT and insertion of a modified surrounding wall which allowed an unobstructed view of the reaction chamber and injection tip during the injection process. A modified cuvette holder enabled observation of the automatic injection of 900 µl of a luminol-hydrogen peroxide solution (1.25 and 2.7 mmol/l respectively in Tris pH 8.0 buffer,

10 mmol/l) into a cuvette containing an antibody coated bead positioned in the operating position. The injection of reagent and mixing process were monitored using high speed photography (5 frames per second). The procedure was repeated using white or black polystyrene beads with the reagent injected in either 0.6, or less than 0.2 seconds.

5.2.2 The influence of bead position on light detection

The AFP assay (Chapter 4, Section 4.2.3) was performed using PBS-based AFP standards (0, 25 and 200 IU/ml), and bound HRP-conjugate quantitated using the firefly luciferin enhanced luminol-hydrogen peroxide reaction. Each bead was positioned in the corner of a cuvette, firefly luciferin (10 µl; 3.6 mmol/l in 0.1 mol/l, pH 8.0 phosphate buffer) added in a separate corner and the chemiluminescent reaction initiated by manual injection of 900 µl of luminol-hydrogen peroxide solution (1.25 and 2.7 mmol/l in Tris pH 8.0 buffer, 10 mmol/l). The cuvette was gently shaken to ensure mixing, submersion of the bead and the removal of any bubbles trapped on the bead surface. The bead was then manoeuvred to a particular corner of the cuvette, positioned in front of the PMT and light emission recorded. Bead position relative to the PMT was altered by withdrawing the cuvette, rapidly altering the beads position and reintroducing the cuvette into the luminometer. Alternatively, the bead could be repositioned while in front of the PMT by tilting the luminometer and allowing the bead to roll into the appropriate corner. Variations in light levels produced by changes in the luminescent reaction with time were compensated for by only comparing light

levels immediately prior to, and after changing the bead position.

5.2.3 Incorporation of luminescent assays for HRP-conjugate bound to beads in the AFP immunometric assay

AFP standards (PBS-based) were analysed using the immunometric procedure described previously (Chapter 4, Section 4.2.3). HRP-conjugate bound to beads was assayed either colorimetrically or luminescently using assays described in Chapters 2 and 3. Luminescent assays were performed on slightly damp beads immediately after the final distilled water wash, and all luminescent reactions were initiated by manual addition of the appropriate reagents outside the luminometer. Prior to light measurement, cuvettes into which beads had been transferred from the spring supports, were gently and uniformly shaken to ensure mixing and removal of bubbles from the bead surface. In order to achieve optimal, reproducible light measurements beads were maintained in a constant position in the cuvette relative to the photodetector (position 1, Figure 5.2a). Light emission against time was recorded with minimal delay after initiation of the luminescent reaction. The following luminescent end-points were employed.

1) Pholas dactylus luciferin-O_2: Pholas dactylus luciferin (550 µl; 48 µg/ml; approximately 1 µmol/l) in phosphate buffer (0.125 mol/l, pH 7.8) containing sodium chloride (25 mmol/l) was added to each cuvette containing a bead. Steady state light emission after 60 seconds was recorded.

2) Pyrogallol-hydrogen peroxide: Pyrogallol (55 µl; 0.29 mol/l) in potassium phosphate buffer (0.15 mol/l, pH 6.5) was placed in a corner of a cuvette containing a bead. The reaction was initiated by the addition of 900 µl hydrogen peroxide (0.12 mol/l) in the same buffer, and the highest intensity of light emission within 60 seconds recorded.

3) Purpurogallin-p-phenylenediamine-hydrogen peroxide: Purpurogallin (55 µl; 9.1 mmol/l in methanol) and p-phenylenediamine (20 µl; 25 mmol/l in 0.15 mol/l potassium phosphate buffer, pH 6.5) were placed in separate corners of a cuvette containing a bead. Reactions were initiated by addition of hydrogen peroxide, 900 µl (0.12 mol/l) in the phosphate buffer. Peak light emission was recorded.

4) Luminol-perborate, pH 12.9: Luminescent reactions were initiated by addition of 900 µl of a mixture of luminol (0.11 mmol/l) and sodium perborate (5.6 mmol/l) in sodium hydroxide (0.125 mol/l). Light intensity after 30 seconds was recorded.

5) Luminol-hydrogen peroxide, pH 8.0: Luminescent reactions were initiated by addition of 900 µl of a mixture of luminol (1.25 mmol/l) and hydrogen peroxide (2.7 mmol/l) in Tris buffer (10 mmol/l, pH 8.0). Light intensity after 30 seconds was recorded.

6) Firefly luciferin enhanced luminol-hydrogen peroxide, pH 8.0: Synthetic firefly luciferin, 10 µl (3.6 mmol/l in 0.1 mol/l pH 8.0 phosphate buffer) was added to a corner of each cuvette containing a bead. The luminescent reaction was

initiated by addition of 900 µl of a mixture of luminol (1.25 mmol/l) and hydrogen peroxide (2.7 mmol/l) in Tris buffer (10 mmol/l, pH 8.0). Light intensity after 30 seconds was recorded.

7) 2,4-Dichlorophenol enhanced luminol-hydrogen peroxide, pH 8.0: The reaction was performed as in the preceding enhanced system with 10 µl of 2,4-dichlorophenol (1 mg/ml or 5 mg/ml; 6.1 or 30.7 mmol/l) substituted for the firefly luciferin.

5.2.4 Enhanced chemiluminescent assay of plasma-based AFP standards and patients' specimens

Plasma-based standards and 25 patients' serum specimens (previously assayed by radioimmunoassay and chosen to cover the assay working range) were analysed using the AFP immunometric assay (Chapter 4, Section 4.2.3). Standards and samples were assayed in duplicate, with 24 beads processed on one incubation block. The position of standards and samples in the block, and the spring supports used, were identical for assays in which beads were either transferred to cuvettes and bound conjugate quantitated with the firefly luciferin enhanced luminol-H_2O_2 reaction or analysed using the ABTS colorimetric reaction (Chapter 4, Section 4.2.3).

Plasma-based standards were also analysed with p-iodophenol (10 µl, 6.1 mmol/l in 0.1 mol/l Tris buffer, pH 8.0) substituted for firefly luciferin.

5.3 RESULTS

5.3.1 Automatic injection of reagents onto antibody coated beads

Four examples of automatic injection of reagent onto antibody coated beads, monitored by high speed photography are shown in Figure 5.1. Mixing was irreproducible and pronounced bubble formation occurred both in solution and on the bead surface. Variable bead movement occurred around the container base or by beads 'floating' through the solution. These effects, and 'splashing' of reagent off the bead surface, were extremely pronounced at the higher injection speed.

5.3.2 The influence of bead position on light detection

Four possible positions in the base of a reaction cuvette, which a bead could adopt relative to the photomultiplier cathode surface are shown in Figure 5.2a. The effect on light detection in an enhanced luminescently monitored assay, by beads adopting different positions in relation to the PMT is shown in Figure 5.2b. Under conditions in which high levels of light were emitted (eg, from the 200 IU/ml AFP standard) movement of the bead away from position 1 produced a dramatic decrease in the light recorded by the detector. Simple differences in the position of a bead could account for up to approximately 35% differences in recorded light measurements. A similar, less pronounced effect was seen with lower concentrations of conjugate bound to beads. Under conditions where very low concentrations of conjugate were bound (eg, with the 0 IU/ml AFP standard), movement of the bead away from position 1 produced slight increases in signal, possibly by exposing a larger volume of glowing luminescent

Figure 5.1

High speed photography of automatic injection of reagent into a cuvette containing an antibody coated polystyrene bead

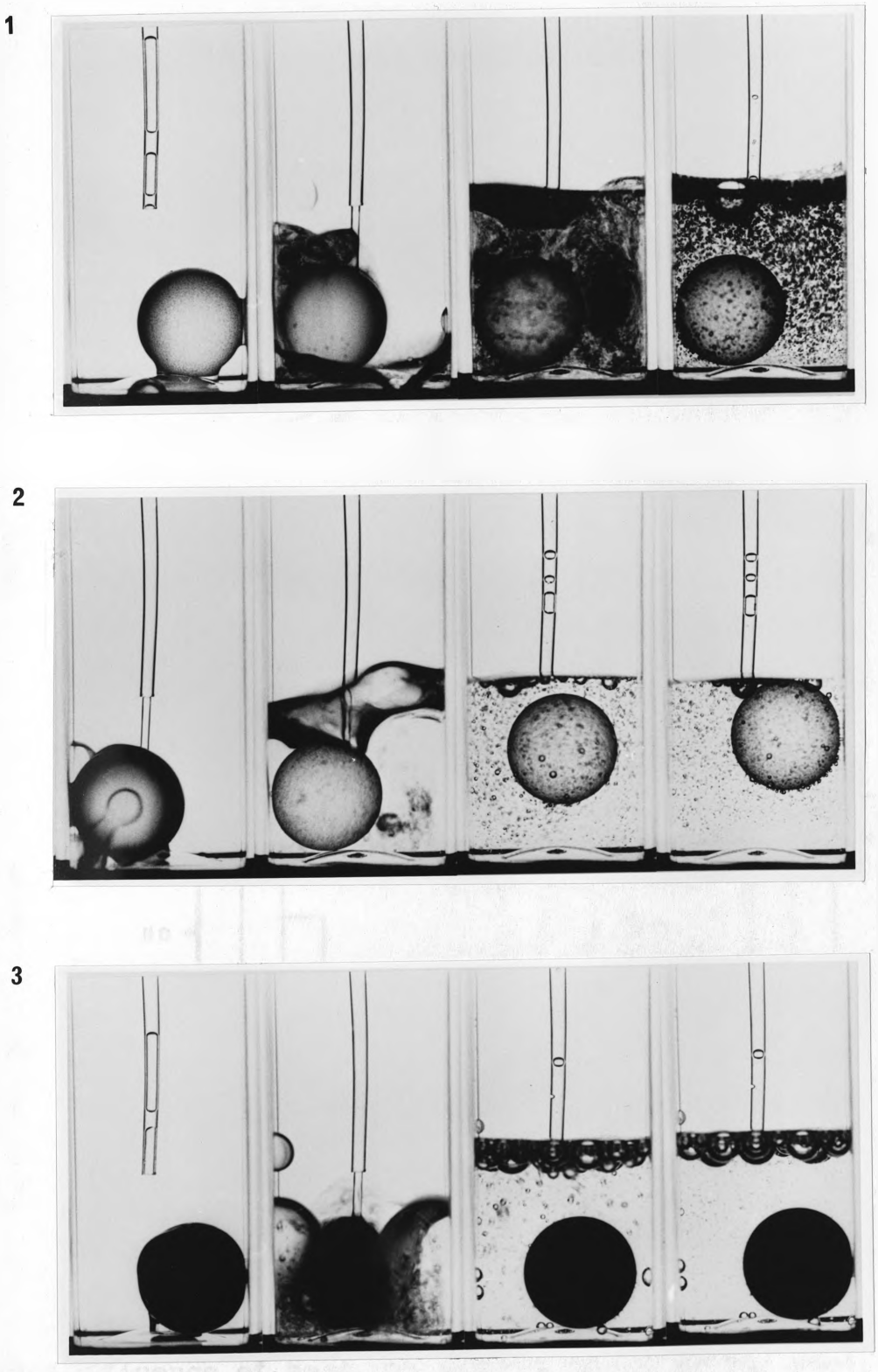
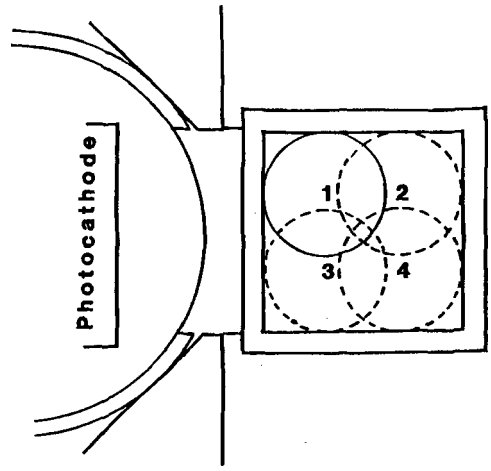
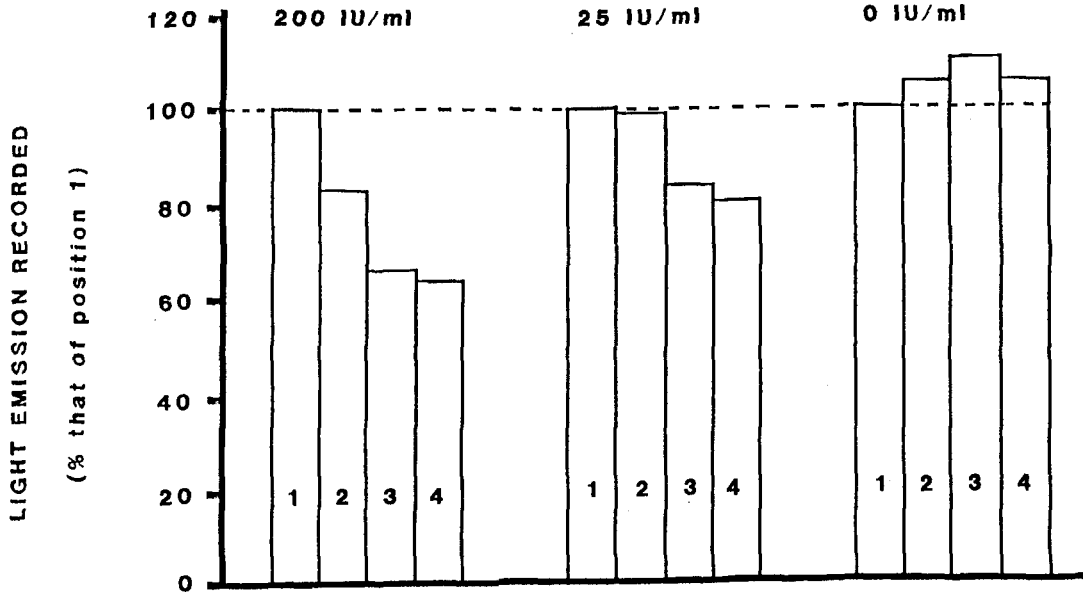


Figure 5.2

The influence of bead position on light detection



a) Positions in a reaction cuvette which beads could adopt relative to the photomultiplier tube



b) The influence of bead position relative to the photomultiplier tube on the light emission detected in an AFP immunometric assay employing a firefly luciferin enhanced chemiluminescent end-point

reagent to the detector.

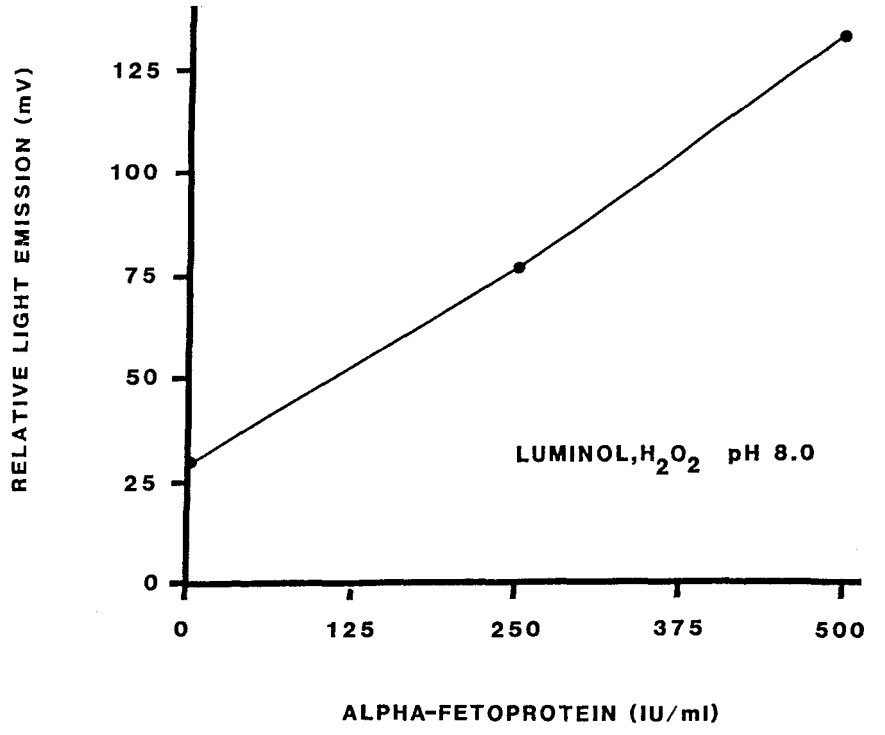
5.3.3 Incorporation of luminescent assays for HRP-conjugate bound to beads in the AFP immunometric assay

Figures 5.3 to 5.6 illustrate levels of light emitted and the dose-response curves obtained by incorporating various luminescent end-points into the AFP immunometric assay using aqueous standards. For comparison, and to ensure steps prior to the end-point functioned correctly, an assay employing an ABTS-H_2O_2 colorimetric end-point was performed alongside each luminescently monitored assay (as in Figure 5.4).

Incorporation of a luminol-H_2O_2 end-point (pH 8) or a luminol-perborate end-point (pH 12.9) produced relatively high levels of light emission but shallow dose-response curves (Figure 5.3). In contrast, the purpurogallin-p-phenylenediamine-H_2O_2 luminescent end-point produced a sensitive standard curve with low background light emission (Figure 5.4); the kinetics of light emission from individual standards are shown in Figure 5.7. Both the pyrogallol-H_2O_2 and the Pholas dactylus luciferin-O_2 luminescent end-points produced extremely low level light emission and no useful discrimination between standards (Figure 5.5). Figure 5.6 illustrates the excellent calibration curve and intense light emission achieved using a 2,4-dichlorophenol enhanced luminol-H_2O_2 reaction, pH 8.0. In contrast to the short lived and rapidly changing light emission with the purpurogallin - p-phenylenediamine end-point, light emitted from individual standards using an enhanced end-point is prolonged and relatively constant (Figure 5.8).

Figure 5.3

Dose-response curves obtained by employing luminol-oxidant chemiluminescent end-points in the AFP immunometric assay



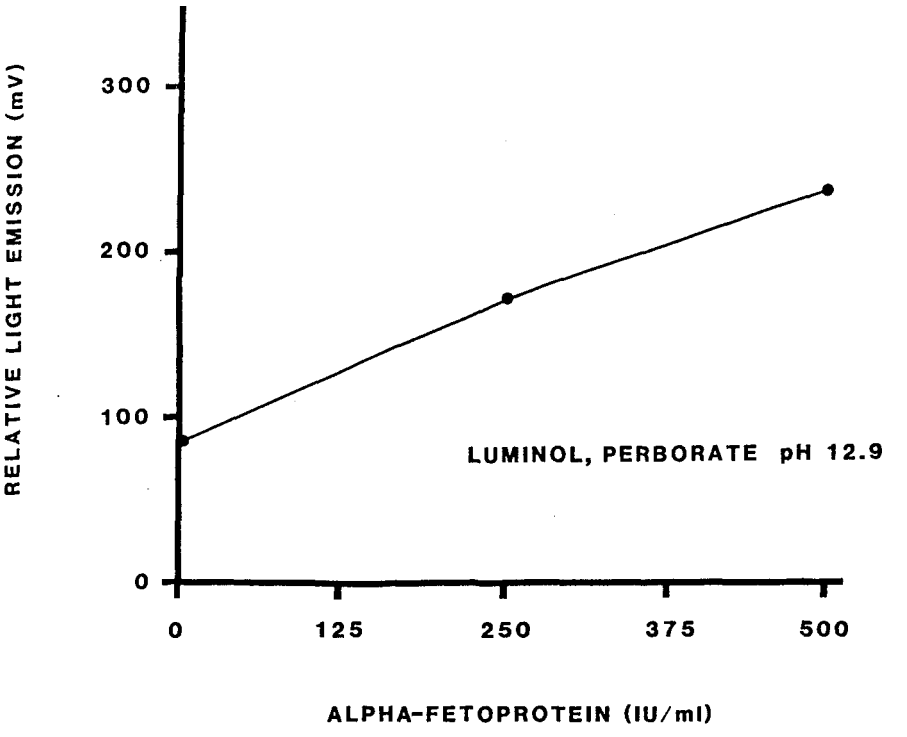


Figure 5.4

Dose-response curves obtained by employing a purpurogallin-p-phenylenediamine-H_2O_2 chemiluminescent or a colorimetric end-point in the AFP immunometric assay

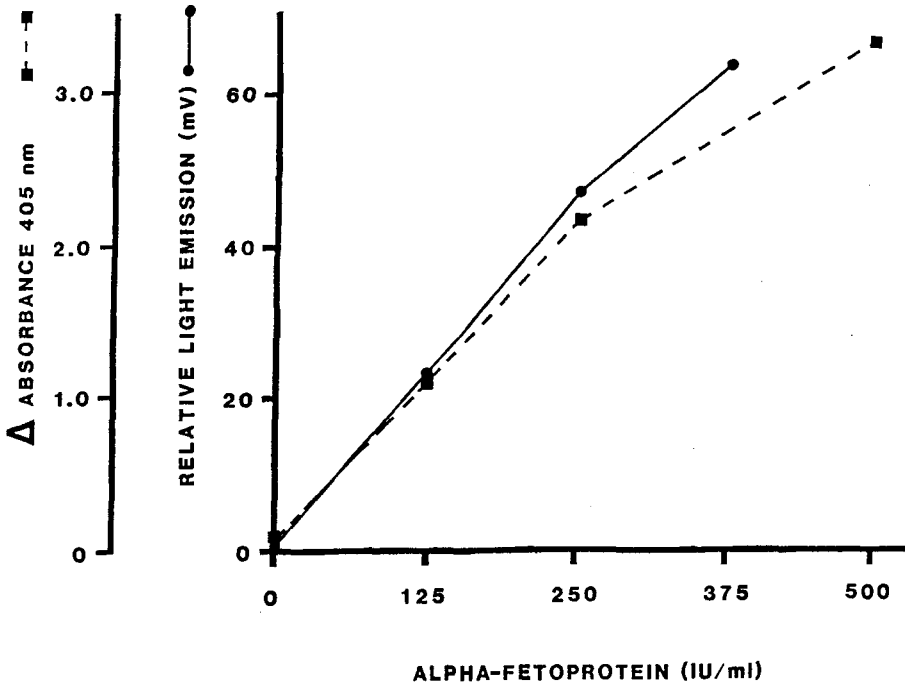


Figure 5.5

Dose-response curves obtained by employing either a pyrogallol-H_2O_2 (A) or a Pholas dactylus luciferin-O_2 (B) chemiluminescent end-point in the AFP immunometric assay

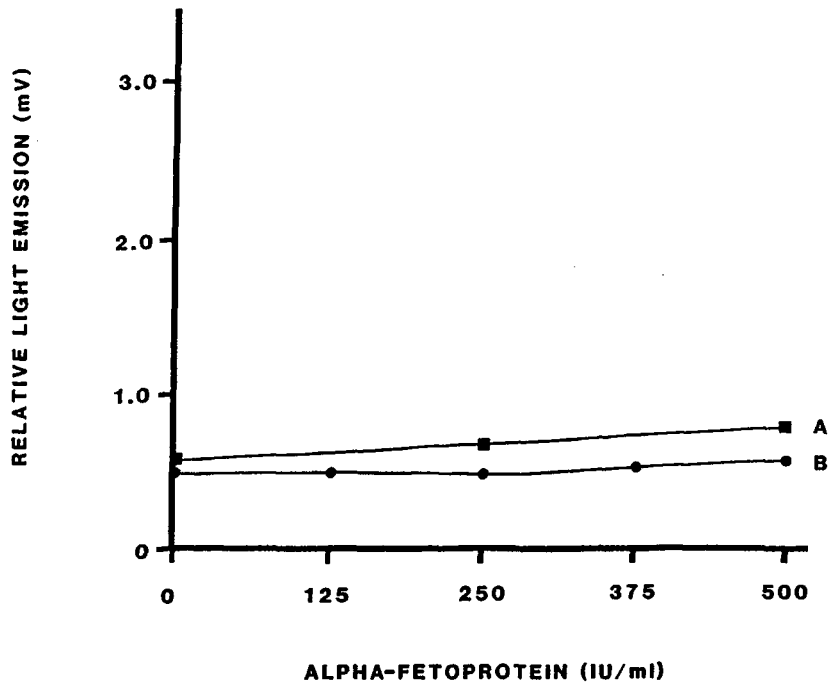
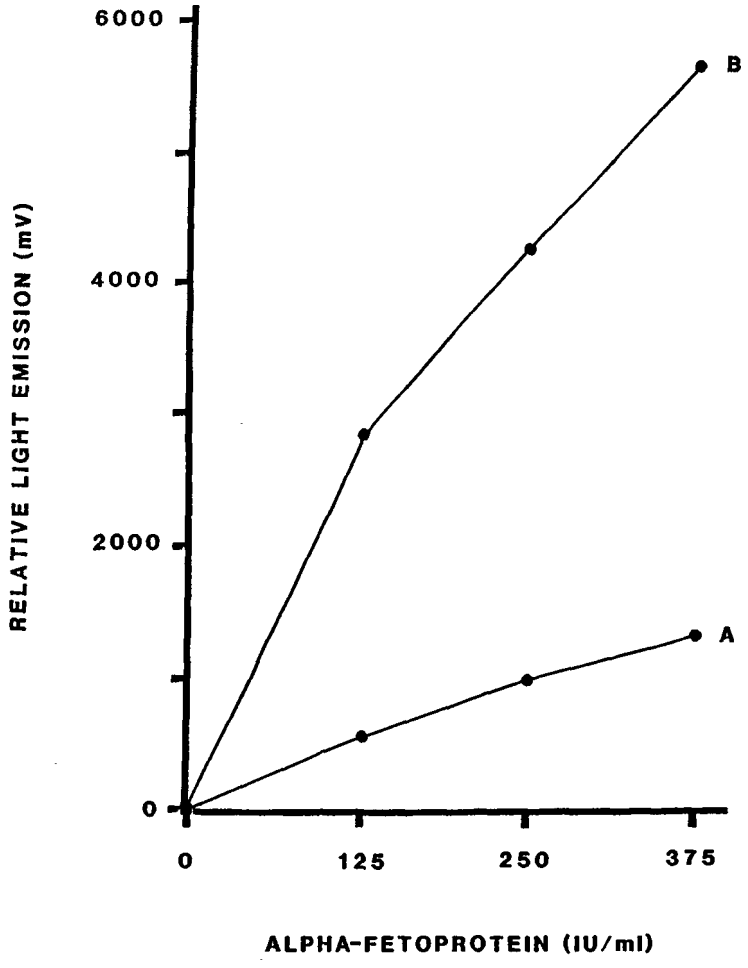


Figure 5.6

Dose-response curves obtained by employing 2,4-dichlorophenol enhanced chemiluminescent luminol-H_2O_2 end-points in the AFP immunometric assay



Initial 2,4-dichlorophenol concentration: A 6.1 mmol/l,
B 30.7 mmol/l

Figure 5.7

Kinetics of the light emitted using a purpurogallin-p-phenylenediamine-H_2O_2 chemiluminescent end-point in the AFP immunometric assay

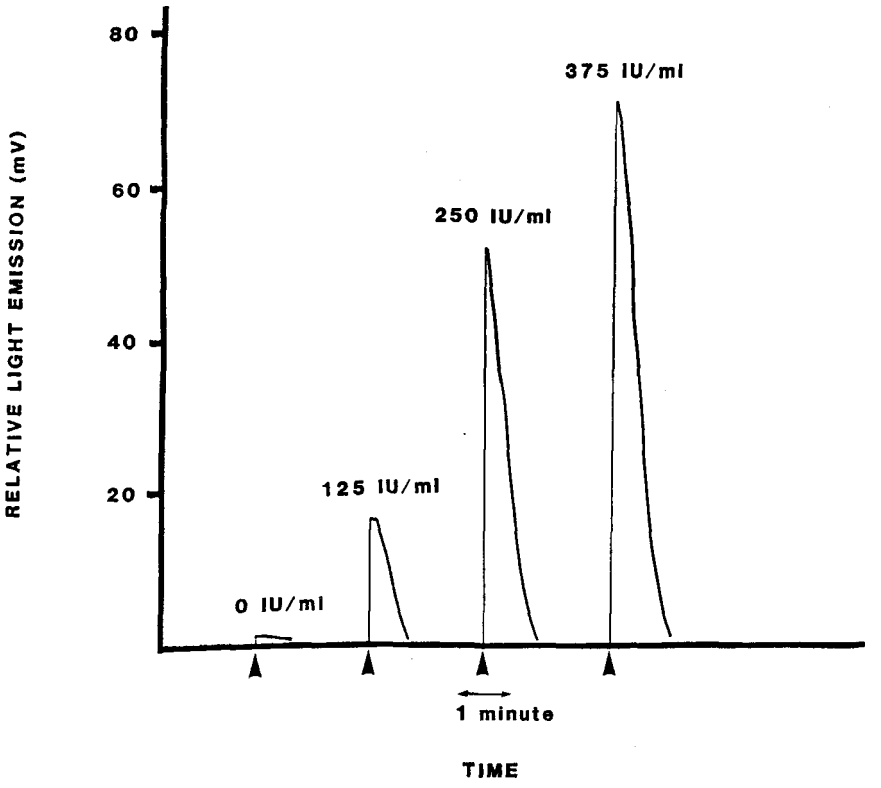
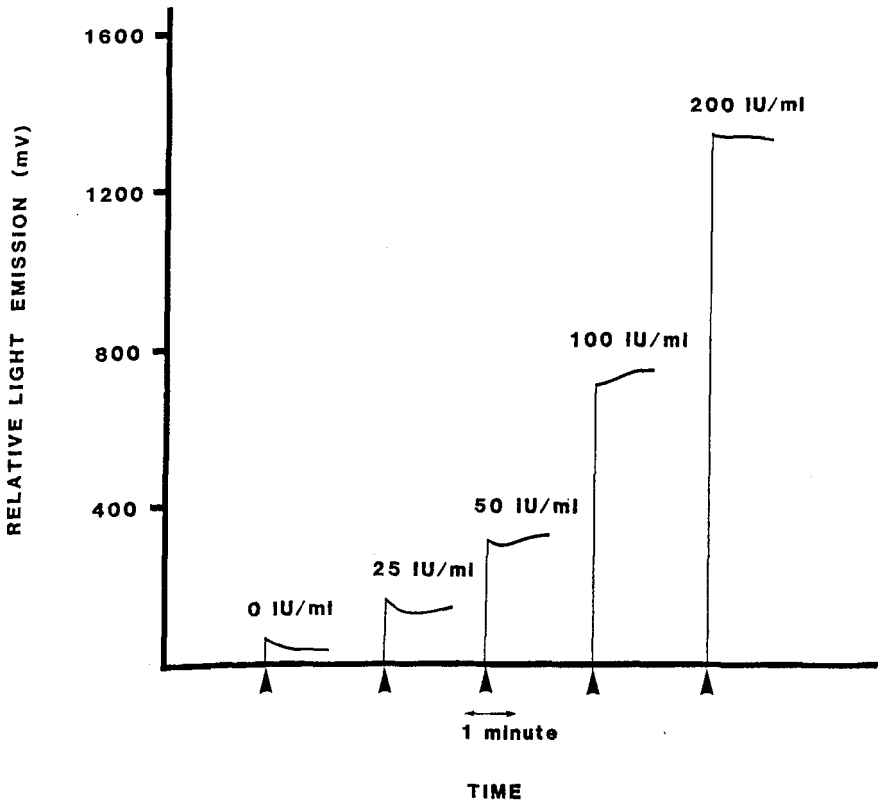


Figure 5.8

Kinetics of the light emitted using a firefly luciferin enhanced luminol-H_2O_2 chemiluminescent end-point in the AFP immunometric assay



5.3.4 Assay of plasma-based AFP standards and patients' specimens using an enhanced end-point

A typical dose-response curve using plasma-based AFP standards and a firefly luciferin enhanced luminol-H_2O_2 end-point is shown in Figure 5.9. For a series of 25 serum specimens, good correlation ($r = 0.99$, $y = 0.94x + 3.2$) was obtained between results determined using the immunometric assay with either a colorimetric (x) or a firefly luciferin enhanced luminescent (y) end-point (Figure 5.10).

Enhanced chemiluminescent end-points also produced improvements in assay sensitivity (expressed as signal to background ratio) over the colorimetrically monitored assay. For example, the ratios of the signal from alpha-fetoprotein plasma standards of 22, 44, 88 and 174 IU/ml, to that of the signal from the 0 IU/ml standard was 2, 3, 5 and 9 respectively using the ABTS colorimetric end-point, and 9, 23, 46 and 100 respectively using a 2,4-dichlorophenol enhanced chemiluminescent end-point (final enhancer concentration 67 umol/l).

5.4 DISCUSSION

5.4.1 Problems identified when initiating or monitoring luminescent reactions on polystyrene beads

Poor precision was encountered in initial attempts to employ luminescent quantitation of peroxidase conjugate bound to polystyrene beads in the AFP assay. Several causes of the imprecision were identified and are discussed in detail in the following sections.

Figure 5.9

Dose-response curve obtained by employing a firefly luciferin enhanced luminol-H_2O_2 chemiluminescent end-point in the AFP immunometric assay

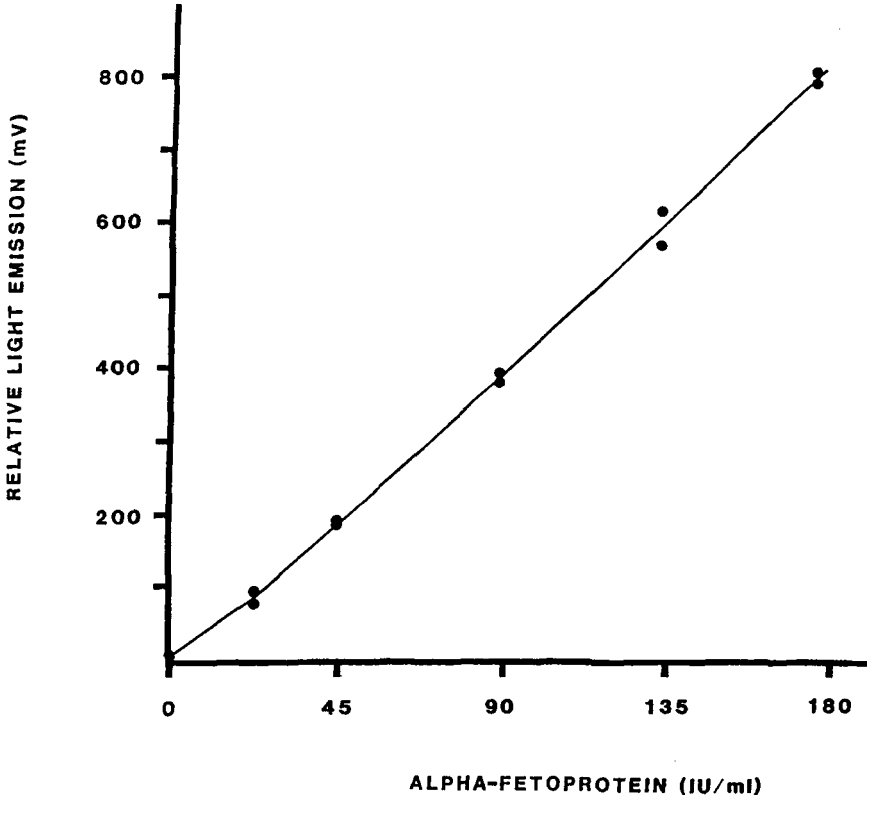
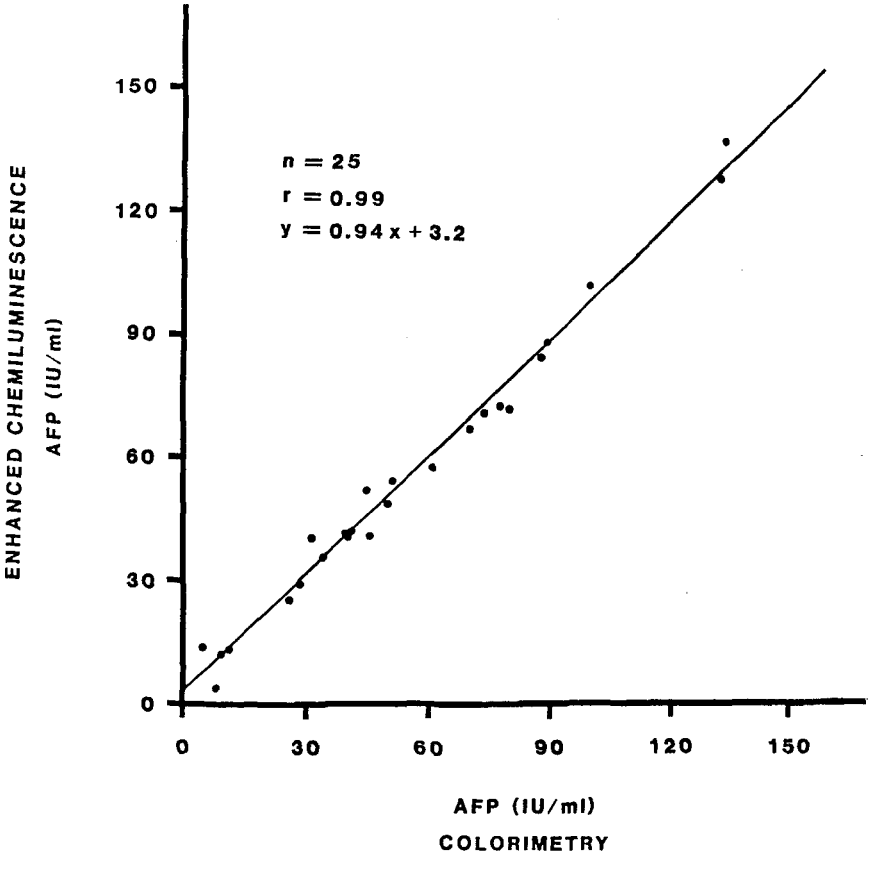


Figure 5.10

Comparison of serum AFP concentrations determined using an immunometric assay with either a firefly luciferin enhanced luminol-H_2O_2 chemiluminescent or a colorimetric end-point



5.4.1.1 Bead position

The size of the bead and the size of the square cuvettes allowed the bead to adopt various positions in front of the photomultiplier tube. The bead could move to various random positions in the cuvettes during and after injection, and these variations were shown to have dramatic effects on the light emission registered by the photomultiplier. In order that beads assumed a constant and optimal position in front of the detector the luminometer was tilted so the bead rolled into one corner of the cuvette and was precisely located by it, close to the photodetector. This could not however guarantee that in conjunction with automatic injection of reagents onto the bead this position would be adopted. Although the use of suitable round bottomed tubes (instead of square cuvettes) would have ensured that beads adopted a constant position, modification of the luminometer to accept such tubes was not possible in these initial experiments. The possibility of beads moving relative to the photodetector, and the effects of such movement, has not previously been discussed for luminescently monitored immunoassays although holders have been employed to ensure tubes adopt constant positions in liquid scintillation counters (Stevens et al., 1980). The problems encountered may have been exaggerated by the use of a side-window photomultiplier tube and a single, large, relatively opaque solid support.

5.4.1.2 Irreproducible initiation of luminescent reactions

Several problems in initiating luminescent reactions were identified particularly when the luminescent reaction was fast. In this case, initiation and mixing with the bead in the

measuring position were required in order to record the entire course of light emission. Automatic injection of approximately 900 - 1000 µl of reagent produced irreproducible mixing, bubble formation and bead movement.

Irreproducible mixing: Automatic injection of reagent onto the bead produced variable mixing and this was clearly demonstrated using high speed photography. The speed of reagent injection and the initial position of the bead could produce dramatic variations in mixing. The bead position influenced the behaviour of the jet of injected reagent. Reagent could strike different parts of the bead (producing variable sample flicker), become pushed to one side of the bead, splash in an erratic manner off its upper surface, rebound up the cuvette walls, and move or spin the bead around in the cuvette.

Bubble formation: Automatic injection of reagent led to variable bubble formation both on the bead surface and in the bulk solution. Variable bubble formation leads to possible imprecision due to differences in aeration of solutions and scattering and internal reflection of emitted light. Removal of bubbles from bead surfaces produced both improvements in precision and the levels of light emitted. The bubbles presumably prevented reagents coming into contact with HRP-conjugate on the bead surface and influenced the light emitted.

Bead movement: Beads were moved in cuvettes relative to the photodetector by the force of reagent injection, in addition bubble formation on the bead surface increased their buoyancy,

and turbulence in the solution led to beads 'floating' through the solution in the cuvette as light emission occurred.

Injection of reagent onto submerged beads in front of the photodetector was not feasible as the injection system was not designed for precise delivery of small volumes, and rapid, efficient bubble-free mixing could not be achieved. More importantly, without instrument modification it was not possible to ensure the bead remained constantly positioned relative to the photomultiplier tube. Manual initiation of reactions outside the luminometer allowed thorough, and with care, reproducible mixing, and eliminated bubble formation on the bead surface or in solution. The technique allowed beads to be precisely positioned in front of the detector, however, in common with measurements using a liquid scintillation counter, the initial peak light emission in extremely rapid reactions and the light emitted between initiation and positioning in front of the photomultiplier tube could not be recorded.

5.4.1.3 Storage of beads prior to luminescent quantitation

Luminescent reactions could not be monitored simultaneously and thus storage of beads was required. Storage for variable periods may diminish peroxidase activity and thus influence rapid light measurements and contribute to imprecision. Similar effects are seen in other luminescent systems where CVs of measurement with dry beads were more than double those where beads were kept covered with physiological saline (Gadow et al., 1984). Keeping beads wet is also reported to prevent 'drift' effects. Such effects were minimised in the luminescent AFP assays by analysing

only small batches of beads and storing them slightly damp, for the minimal possible period prior to luminescent analysis.

5.4.2 The performance of luminescent assays in the AFP immunometric assay using aqueous standards

The luminescent assays previously described in Chapter 2 were incorporated into the immunometric assay using buffer-based standards to minimise any interference which could be encountered with serum. Manual mixing and initiation outside the luminometer were employed to avoid the sources of measurement imprecision previously described. Despite the inability to monitor the initial few seconds of light emission the general characteristics and performance of the luminescent techniques in the AFP assay were determined.

The pyrogallol-H_2O_2 reaction produced extremely low intensity light emission and no dramatic cooperative effect between immobilised HRP-conjugate complexes was demonstrated. The Pholas dactylus luciferin-O_2 reaction, despite the suitability of light emission kinetics and simple one reagent initiation, produced extremely low levels of light and no useful discrimination between standards was possible. The p-phenylenediamine-purpurogallin-H_2O_2 reaction allowed very good quantitation of bound HRP-conjugate and the production of a sensitive AFP calibration curve. The assay did however show 'flash like' kinetics and light emission was of relatively low intensity. This would have necessitated rapid, efficient, reproducible mixing on the solid support in front of a sensitive photodetector, and accurate timing of light measurements to

achieve good assay performance.

Reactions with luminol and oxidant at pH 8.0 or pH 12.9 produced relatively high levels of light emission from bound HRP-conjugate and despite relatively rapidly changing initial kinetics, light emission was prolonged. However, background light emission with low levels of AFP was high and shallow calibration curves of poor sensitivity were obtained. The enhanced luminol-H_2O_2 reaction at pH 8.0 using synthetic firefly luciferin or 2,4-dichlorophenol produced excellent dose-response curves in the AFP assay with levels of light emitted high and relatively constant for many minutes. Low background light emission, excellent sensitivity and the speed with which measurements could easily be taken, made enhanced procedures extremely attractive for monitoring the immunoassay.

5.4.3 The performance of enhanced chemiluminescent assays in the immunometric AFP assay using plasma-based standards

The use of serum samples introduced no significant interference in the enhanced chemiluminescently monitored AFP assay, and samples could be analysed without pre-extraction. The technique reflected the apparent specificity for HRP-conjugate in solution and the firefly luciferin, 2,4-dichlorophenol or p-iodophenol enhanced reactions retained the excellent sensitivity, relatively constant high light emission and the rapid measurements possible with aqueous standards. Improvements in light levels were dependent on both the type and concentration of enhancer employed. The assays exhibited sensitivity (expressed as signal/background) superior to that achieved using the ABTS

colorimetric end-point, and for 25 serum samples with a range of AFP concentrations, good correlation was achieved between results obtained using the enhanced chemiluminescent or colorimetric end-points (correlation coefficient = 0.99). Subsequent studies have shown that alternative enhancers such as other 6-hydroxybenzothiazole derivatives, or para-substituted phenols can be successfully incorporated into enhanced chemiluminescent end-points with the AFP immunometric assay (Williams and Thorpe, 1984; unpublished results). Enhanced chemiluminescent end-points can also be simply initiated by manual addition of a single reagent (a luminol/oxidant/enhancer/buffer cocktail), as is demonstrated in Chapter 6.

5.4.4 A comparison of the enhanced chemiluminescent and colorimetric AFP assay

Rapid label quantitation in 30 seconds or less was possible with the enhanced luminescent technique, enabling a substantial reduction in overall assay time by replacement of the 30 minute colorimetric incubation. The enhanced assay also produced a dramatic improvement in assay sensitivity, expressed as signal to background ratio. The colorimetric assay however involved incubation of the bead supported by the PTFE coated spring in the ABTS substrate, whereas beads were removed from these supports for luminescent analysis. Thus any non-specific binding of HRP-conjugate to the springs would raise the background colorimetric signal with detrimental effects on sensitivity. Sensitivity with the enhanced chemiluminescent end-point may have been underestimated as no account was taken of the fact that only light emission from a fraction of the bead surface was recorded

whereas HRP-conjugate bound to the entire surface was measured with colorimetry. In addition, although changes in absorbance against substrate solution were recorded, no correction for background light emission was made in enhanced chemiluminescent assays.

The enhanced procedure offered the potential to improve the assay range by quantitating high levels of light emission by simply altering the sensitivity of the detector, whereas a dilution step would be required using colorimetry. The colorimetric assay required reagents to be maintained at 37^{o}C for 30 minutes whereas the rapid chemiluminescent assay was performed with reagents at ambient temperature, although the cuvette holder was maintained at 25^{o}C. Timing of the incubation with ABTS was required in the colorimetric assay. A timed measurement of the light emission (30 seconds after initiation) was also required for the enhanced reaction, however the relatively constant nature of light emission made this timing less critical. The chemiluminescent assay employed simpler instrumentation and relatively high levels of light were emitted. The through-put was however less than that achieved with a multiple photometer, and simple to perform, multiple initiation and rapid light measurements would be required to achieve equivalent through-put. The high and relatively constant light emission should, however, facilitate the production of suitable high through-put luminometers, for example, by initiating all reactions together and then sequentially monitoring each for very short periods of time. The use of enhanced chemiluminescent quantitation would reduce

reagent costs. ABTS contributes 12% of reagent costs and although firefly luciferin would contribute approximately 50%, the use of alternative enhancers such as p-iodophenol would reduce reagent cost to less than 0.1% of that of ABTS.

The ability of HRP to act as a high specific activity label with both colorimetric and enhanced chemiluminescent reactions is dependent on retention of enzyme activity. The inability to analyse simultaneously multiple samples with enhanced chemiluminescence, resulted in bead storage prior to analysis assuming greater importance in the chemiluminescent assay. The colorimetric assay measures the cumulative production of stable products in solution formed by bound conjugate, and quantitation can be made without the solid support being present. This is not possible with the enhanced chemiluminescent assay as light emission presumably occurs from the vicinity of the bead surface and it is not possible to 'accumulate' all light emission for presentation to the photodetector. As the solid support must be present during measurement, its opaqueness, size and shape, and any patchy or irregular distribution of conjugate bound to its surface may assume particular importance in the chemiluminescent assay.

5.4.5 Possible improvements in the enhanced chemiluminescent assay for AFP

Improvement of the enhanced chemiluminescent AFP assay may be possible not only by incorporating general immunoassay improvements (Chapter 4) but also by modification of the instrumentation or the chemistry of the enhanced chemiluminescent

end-points.

Instrumentation: Improvements in the sensitivity and efficiency of the photodetector, light collection, or other instrumental factors previously outlined for measurement of HRP-conjugate in solution (Chapter 2), could also be incorporated into the AFP assay. Reflectors to collect light emission from a greater surface area of the opaque bead, and to reduce light losses, should improve assay performance. The exact area from which light emission from conjugate bound to the bead arises, has yet to be determined. By monitoring this area alone, less background light emission from the solution may be detected and sensitivity improved. The proposed enhancement mechanisms predict the existence of enhancer and luminol radicals outside the active site of conjugate on the bead surface, but the reactivity of the radicals would suggest light emission arises close to its surface. Reflection and refraction effects at interfaces between solutions, containers and the surrounding air can dramatically influence light emission (Rich et al., 1981), and study of the physics and geometry of light emission from a bead submerged in a container may improve light collection and hence assay sensitivity. Container shape may also influence the accessibility of HRP-conjugate on the bead surface to reagents in solution and thus influence light emission. Chemiluminescence intensity depends not only on the kinetics of the light producing reaction but also on the efficiency with which mass transfer processes bring the necessary reactants to the interface between the two phases. Improvements in such factors may also improve assay performance.

Enhanced chemiluminescent reactions: Although various features of enhanced chemiluminescent assays have been investigated (Chapter 3), extensive optimisation of reactions when applied to the detection of the levels of HRP-conjugate bound to the bead surface in the AFP assay has not been performed. Variations in the pH at which the reaction is performed, the concentrations of diacyl hydrazide, oxidant and enhancer; their relative proportions and the particular compounds employed, may achieve improved performance and sensitivity. Modification of assay conditions may also improve light emission kinetics in the AFP assay by producing stable light emission more quickly and maintaining such levels for longer periods.

5.4.6 Summary

Enhanced chemiluminescent reactions were successfully incorporated into an immunometric assay for serum alpha-fetoprotein using polystyrene beads as solid support. The advantages offered by enhanced chemiluminescent end-points, previously demonstrated for quantitation of HRP-conjugate in solution (Chapter 3), were directly incorporated into the immunometric assay. Such advantages included the ability to rapidly and sensitively assay HRP labels using reactions from which light emission was relatively intense and prolonged, and which could be performed simply, under mild conditions. The applicability and performance of enhanced chemiluminescent end-points in a wide range of commercially available immunoassays, and the use of simple instrumentation to quantitate light emission from enhanced chemiluminescent immunoassays are outlined in Chapter 6.

CHAPTER 6

IMMUNOASSAY SYSTEMS EMPLOYING ENHANCED CHEMILUMINESCENT END-POINTS TO QUANTITATE HORSERADISH PEROXIDASE CONJUGATES

6.1 INTRODUCTION

6.1.1 Objectives

The previous chapter describes the successful application of enhanced chemiluminescent assays for HRP-conjugates in an immunometric assay for AFP. The objectives of this Chapter were:

a) to incorporate enhanced chemiluminescent quantitation of HRP-conjugates into other immunoassays, particularly those commercially available, or employing different solid supports.

b) to demonstrate the utility of enhanced chemiluminescent end-points employing either 6-hydroxybenzothiazole derivatives or para-substituted phenols as enhancers.

c) to demonstrate the value of enhanced chemiluminescent quantitation of HRP-conjugates in rapid immunoassays.

d) to demonstrate the potential of simple inexpensive instrumentation, based on photographic film, for monitoring enhanced chemiluminescent immunoassays.

6.1.2 Immunoassays employing peroxidase conjugates

A wide range of immunoassays employing enzyme labels have been developed and HRP is now one of the most common enzyme labels and considered by some to be the enzyme of choice for colorimetrically monitored enzyme immunoassay (Porstmann et al., 1985). HRP has been used to label specific antibodies, high molecular weight proteins and smaller molecules such as polypeptides, steroids hormones or drugs, thus enabling assays for both large and small analytes to be developed (Schuurs and van Weemen, 1977). A variety of procedures involving

glutaraldehyde, periodate (Boorsma and Streefkerk, 1979), toluene-2,4-diisocyanate or heterobifunctional reagents such as N-succinimidyl 3-(2-pyridyldithio)propionate (Carlsson et al., 1978) have been employed to chemically couple HRP to other molecules. Immunoassay performance is influenced by the homogeneity, molar composition and purity of conjugates, and their immunological and enzyme activity. Highly efficient and simple methods for purification of HRP prior to conjugation, chemical procedures producing defined conjugate preparations, and efficient purification of conjugates are being developed (Imagawa et al., 1984; Tijssen and Kurstak, 1984) and will facilitate production of more active conjugates and more sensitive immunoassays.

Enhanced chemiluminescent quantitation of HRP-conjugates in immunoassays could be exploited in several clinical disciplines including clinical chemistry, microbiology and serology. The technique is not however confined to these areas and should be applicable in haematology, and the veterinary and agricultural sciences. Many important analytes such as proteins, hormones, tumour antigens, drugs and enzymes can already be assayed using HRP labelled conjugates (Table 6.1). Similar systems are being increasingly used for the detection of viral, fungal, bacterial and parasitic antigens in body fluids (Yolken and Leister, 1981; Pronovost and Baumgarten, 1982) in situations where early diagnosis of certain infections is a significant problem and clinical presentation can be non-specific. Infection related antibodies can also be measured with HRP based immunoassays and

can be employed to identify susceptible individuals with low antibody levels and to diagnose infectious disease.

Exploitation of enhanced chemiluminescent reactions in immunoassays depends upon the availability of appropriate HRP-conjugates or the availability of suitable kits based on HRP-conjugates. A range of such kits is available commercially (Table 6.1) and the market for such diagnostic products is large and expected to increase, stimulated in part by new gene cloning technology (for instance by allowing bulk production of antigenic proteins from bacteria and viruses) and the advent of monoclonal antibodies.

Several immunoassays employing HRP-conjugates in conjunction with a variety of solid supports have been monitored luminescently (Chapter 5, Table 5.1) however only one reference has been made to the direct incorporation of chemiluminescent determination into commercially available kits (Gundermann et al., 1982). The ability to incorporate directly enhanced chemiluminescent determination of HRP into such systems should enable improvements in their speed and sensitivity, and aid the rapid introduction of enhanced chemiluminescently monitored systems into routine use without major modification of existing systems.

6.1.3 Exploitation of enhanced chemiluminescent quantitation of HRP-conjugates

Enhanced chemiluminescent quantitation of HRP-conjugates in immunoassays could be exploited in a variety of ways, these include:

Table 6.1

Commercially available immunoassay kits employing HRP labels

Manufacturer	Solid Support	Analytes
ABBOTT LABORATORIES	BEADS	AFP, TSH, Ferritin, CEA, IgE, HCG, Prostatic acid phosphatase, Hepatitis B surface or core antigens, Rotavirus antigen, Neisseria Gonorrhoeae antigen Antibodies to: Rubella virus (IgG or IgM), HTLV III, Hepatitis B surface or core antigen, Hepatitis A virus
BECKMAN	BEADS	TSH, HCG, IgE
ROCHE	BEADS	HCG, CEA
SIBAR	BEADS	Testosterone
BIONETICS	MAGNETIC BEADS	Antibodies to: Toxoplasma gondii, Rubella virus, CMV, Herpes Simplex virus
BOEHRINGER	TUBES	AFP, TSH, Ferritin, CEA, TBG, T_4, T_3, Digoxin, Insulin
DIAGNOSTIC PRODUCTS CORPORATION	TUBES	Cortisol, T_4, T_3, Digoxin
BEHRING	TUBES	AFP, IgE, Pancreatic lipase, B_2-Microglobulin, Prostatic acid phosphatase, Pregnancy-specific B_1-glycoprotein
	MICROTITRE PLATE	Hepatitis B surface antigen
DAKO	MICROTITRE PLATE	Herpes Simplex virus, Rotavirus
NORTHUMBRIA BIOLOGICALS	MICROWELLS	Chlamydia trachomatis antigen, Toxoplasma gondii. IgG or IgM antibodies to: CMV, Rubella
MELISA, WALKER LABORATORIES	MICROPINS	AFP, B_2-Microglobulin, IgE, CEA, Ferritin Antibodies to: Thyroglobulin, Insulin

Abbreviations AFP - alpha-fetoprotein, TSH - thyroid stimulating hormone, CEA - carcinoembryonic antigen, IgE - immunoglobulin E, HCG - human choriogonadotrophin, HTLV III - human T-lymphotropic virus type III, CMV - cytomegalovirus, T_4 - thyroxine, T_3 - triiodothyronine, TBG - thyroxine binding globulin

a) replacement of colorimetric end-points b) the development of rapid assays c) the development of ultrasensitive assays d) the development of simple assays employing inexpensive instrumentation based on instant photographic film.

Replacement of colorimetric techniques in immunoassays

Enhanced chemiluminescence could be employed as a simple replacement for existing colorimetric procedures. This would eliminate the need for relatively long, temperature controlled incubations (15 minutes - 2 hours) and it could also offer improved sensitivity using relatively simple instrumentation

The development of rapid assays

The ability to quantitate HRP in seconds using enhanced chemiluminescent end-points would facilitate the development of rapid immunoassay systems. Several systems employing colorimetric quantitation of HRP labelled conjugates have been modified by shortening the incubation times with sample and with conjugate to as little as 10 minutes. This is illustrated in a 30 minute assay for Haemophilus influenzae type b polysaccharide (sensitivity 1 ng/ml) which uses 10 minute incubations with sample, HRP-conjugate and colour reagent (Yolken and Leister, 1981). An immunometric assay for human choriogonadotrophin (sensitivity 5 mIU/ml) has also been performed using a single 30 minute incubation of sample and HRP labelled antibody with an antibody coated bead, and a 15 minute colorimetric end-point (Anaokar et al., 1982). In conjunction with enhanced chemiluminescent determination of HRP labels the speed and sensitivity of such assays could be further improved. Clinical

situations benefiting from such assays include the rapid diagnosis of infectious conditions (by the detection of viral or bacterial antigens, or specific antibodies) where a therapeutic decision often needs to be made in less than one hour, and situations where further processing of potentially labile material such as donor blood requires initial screening for certain agents.

The development of ultrasensitive assays

In conjunction with sophisticated instrumentation and extensive optimisation of enhanced chemiluminescent reactions for maximal sensitivity, enhanced end-points should enable extremely sensitive determination of HRP-conjugates and could be used with other improvements in immunoassay design for the development of ultrasensitive assay systems.

Photographic monitoring of enhanced chemiluminescent immunoassays

Immunoassay systems capable of giving a clear-cut response using simple, inexpensive instrumentation to quantitate HRP labelled conjugates could possibly extend the use of immunoassays outside the confines of specialised laboratories into clinics, the physician's office, or even the home. They would also be of use in developing countries or in the field, particularly in simple rapid assays where yes/no, or semi-quantitative answers are required (Chandler and Hurrell, 1982). Visual interpretation of colorimetric reactions catalysed by HRP labels could be used in such situations as no instrumentation is required, it does however involve relatively long incubations and subjective interpretation of a transient response. An alternative approach

involves the use of photographic film to monitor chemiluminescent reactions catalysed by HRP labels. The use of photographic film rather than a photomultiplier tube or silicon photodiode has a number of advantages and offers an extremely simple, rapid and less expensive means of monitoring such reactions. Totally chemical assays are possible in which light produced in a chemical reaction is detected by means of the chemical reactions occurring in the film emulsion, and multiple samples can be analysed simultaneously in a semi-quantitative manner using inexpensive, uncomplicated instrumentation. No electrical power supply is required, and rapid assays are possible particularly using sensitive instant film where developing and processing of the film are carried out automatically. An additional benefit would be the permanent visual record of results obtained without any requirement for complex ancillary equipment. The major limitations of photographic film as a detector are that it is less sensitive than a photomultiplier tube and the photographic results are semi-quantitative.

Photographic film has been used as a detector in a range of bio- and chemiluminescent assays including those for metal ions (Isacsson and Wettermark, 1974; Bunce et al., 1985), ozone (Cavanagh, 1975), glucose (Carter et al., 1982) enzymes (Becvar, 1981) ATP, NADH, cholyl glycine, ethanol (Green et al., 1984) and luminol derivatives (Schroeder and Vogelhut, 1983), results being assessed either visually or quantitated by densitometry, spot size, or using an optical step wedge. Photographic monitoring of certain conventional luminescent immunoassays has been hampered because rapid emission of light necessitated initiation of

individual reactions in front of the film and the intensity of light emission was relatively low. Enhanced chemiluminescent end-points, in which light emission is prolonged and of high intensity, appear much more applicable to photographic based systems and should enable both rapid label detection and initiation of multiple reactions prior to exposure to the film.

6.2 EXPERIMENTAL

Materials and Methods

6.2.1 Reagents

Rubazyme kits (for assay of anti rubella virus IgG), CEA-EIA kits (for assay of carcinoembryonic antigen), and Auszyme II kits (for assay of hepatitis B surface antigen) were supplied by Abbott Laboratories Ltd, Diagnostics Division, Basingstoke, UK. Enzygnost IgE kits (for quantitation of immunoglobulin E) were purchased from Hoechst UK Ltd, Hounslow, Middlesex, UK. Enzymun-Test T_4 kits (for assay of total thyroxine) and Enzymun-Test Digoxin kits (for assay of digoxin and its derivatives) were supplied by The Boehringer Corporation (London) Ltd, East Sussex, UK. The assays were performed essentially as in the manufacturer's instructions; standards and specimens were analysed in duplicate.

Serum specimens with a range of IgE concentrations, and serum specimens with a range of rubella specific IgG concentrations were kindly supplied by the Department of Immunology, University of Birmingham, and the Regional Virology Laboratory, East Birmingham Hospital respectively. Serum-based hepatitis B surface antigen standards and serum specimens with a range of

concentrations of antibody against CMV (previously analysed using a colorimetric enzymoimmunometric assay employing an alkaline phosphatase conjugate, (Voller and Bidwell, 1976)) were kindly supplied by the Regional Blood Transfusion Service, Birmingham UK.

Rabbit anti-human ferritin and rabbit anti-human ferritin - HRP conjugate were obtained from Dakopatts a/s, Denmark. Cytomegalovirus complement fixation antigen was purchased from Flow Laboratories, Irvine, Scotland and goat anti-human IgG - HRP conjugate from Miles Laboratories Ltd, Slough, UK. Transparent, U-bottomed, polyvinyl chloride M24 and black polystyrene MicroFLUOR 'B' microtitre plates were obtained from Dynatech Laboratories Ltd, Sussex UK. The immunoassay solid supports used in conjunction with enhanced chemiluminescent end-points are shown in Figure 6.1.

6.2.2 Incorporation of enhanced chemiluminescent end-points into commercially available immunoassay systems employing beads or tubes

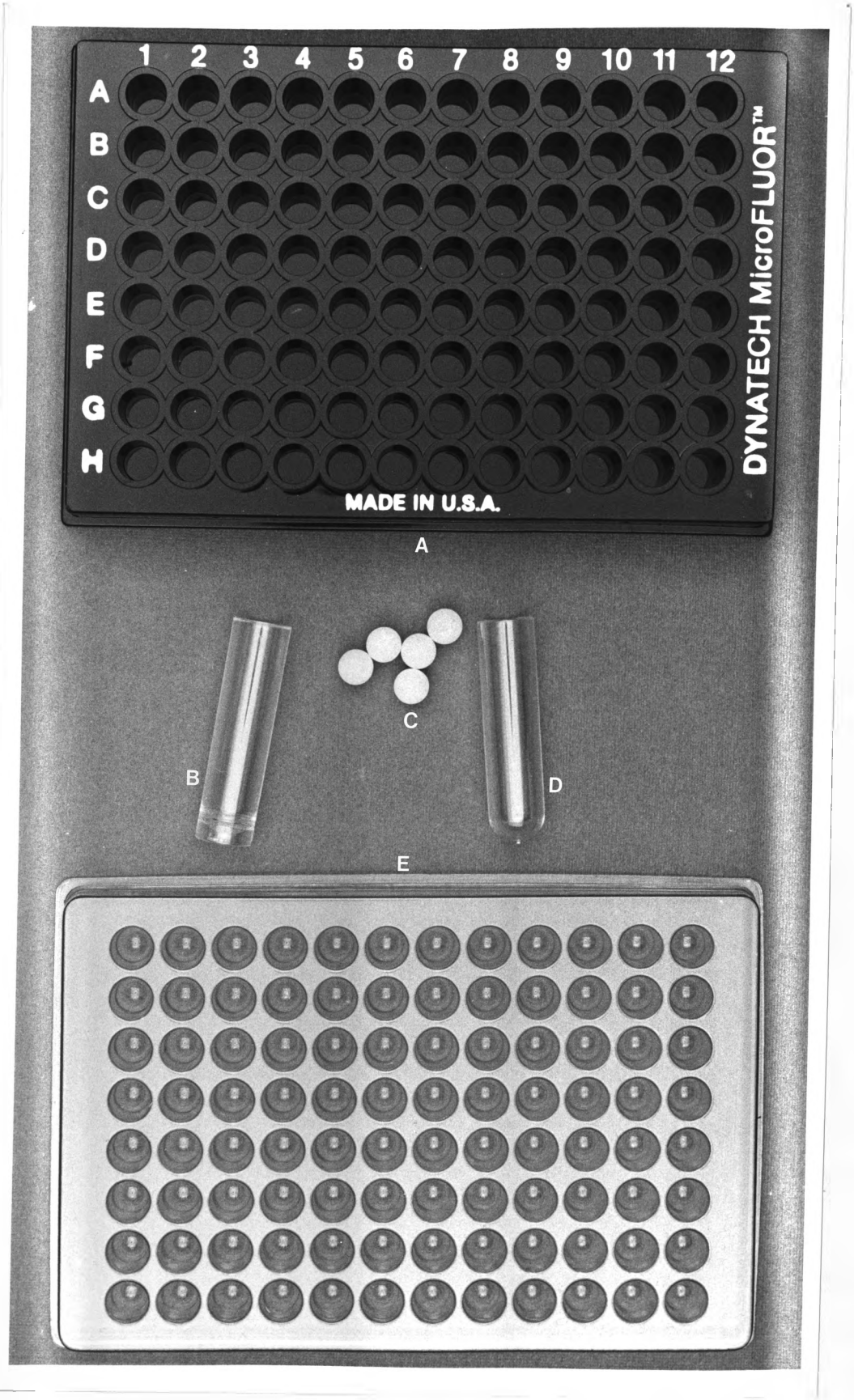
6.2.2.1 Light Measurement

All reagents employed in chemiluminescent assays were obtained as described in previous chapters and stored protected from light.

Manual initiation of enhanced chemiluminescent reactions and light measurement with the WRL luminometer (Chapter 2, Section 2.2.2) were employed in assays using beads (rubella specific IgG, CEA and HB_sAg) or tubes (IgE, thyroxine and digoxin). Beads were contained in disposable 1 cm square cuvettes and precautions were

Figure 6.1

Immunoassay solid supports used in conjunction with enhanced chemiluminescent end-points



A, black opaque microtitre plate; B, Enzymun-test digoxin tube; C, beads; D, Enzygnost IgE tube; E, transparent microtitre plate

taken to ensure constant, optimal positioning of the bead relative to the PMT, reproducible initiation and elimination of bubbles from their surface (Chapter 5, Section 5.4.1). Tubes were directly introduced into the WRL luminometer with similar precautions for those used with beads to ensure reproducible measurements.

Automatic initiation of enhanced chemiluminescent reactions and measurement of light emission from beads in the CEA assay were performed using an Auto-Biolumat LB 950 luminometer supplied by Laboratorium Professor Dr Berthold, Wildbad, Germany. The instrument employed photon counting and was controlled by a 48K Apple II computer with extensively modified software. Reactions were carried out in 55 x 11 mm clear polystyrene round bottomed tubes supplied by LIP (Equipment and Services) Ltd, Shipley, West Yorkshire.

6.2.2.2 Assay of anti rubella virus IgG with a Rubazyme kit

Serum specimen or standard (10 µl) was diluted with 200 µl Tris buffer (containing protein stabilizers and 0.01% thimerosal) and mixed in the well of a plastic reaction tray. One rubella virus coated bead was dispensed into each well and the tray tapped gently to remove any air bubbles attached to the bead surface. The tray was covered and incubated at 37°C for 60 minutes. Trays were removed from the water bath, samples aspirated and each bead washed with distilled water (4 x 2 ml). Goat anti-human IgG-HRP conjugate (200 µl; approximately 2 µg/ml in the Tris buffer) was added to each well containing a bead, the tray tapped as before and incubated at 37°C for a further 60 minutes. After aspirating

the solution, beads were washed with distilled water (4 x 2 ml) as before, the beads transferred to polystyrene tubes and the washing step repeated (2 x 4 ml). HRP-conjugate bound to beads was quantitated chemiluminescently or colorimetrically. An index for each specimen was calculated by dividing its response by that of a low positive control serum.

Colorimetric end-point: o-Phenylenediamine 2HCl (12.8 mg) was dissolved in 5 ml citrate-phosphate buffer, containing 0.02% hydrogen peroxide and 0.01% thiomerosal preservative, 10 minutes before use. Substrate solution 300 µl, was added to each bead at 10 second intervals and incubated for 30 minutes at room temperature in the dark. Reactions were terminated by the timed addition of 2.0 ml hydrochloric acid (1 mol/l) followed by vortex mixing. The solutions were transferred to cuvettes and the absorbance at 492 nm measured using a Gilford 250 spectrophotometer blanked against distilled water.

Enhanced chemiluminescent end-point

Firefly luciferin enhancement: Beads were transferred to cuvettes. Firefly luciferin, 10 µl (3.6 mmol/l) in phosphate buffer (0.1 mol/l, pH 8.0) was added to a separate corner and the luminescent reaction manually initiated by the addition of 900 µl of a mixture of luminol (1.25 mmol/l) and hydrogen peroxide (2.7 mmol/l) in Tris buffer (10 mmol/l, pH 8.0). Light intensity after 30 seconds was recorded.

p-Iodophenol enhancement: Beads were stored in distilled water (1 ml). Prior to luminescent analysis the water was removed by aspiration and the bead transferred to a cuvette. The

chemiluminescent reaction was initiated by manual addition of 910 µl of a luminol-hydrogen peroxide-p-iodophenol solution prepared by addition of 1.1 ml p-iodophenol (13.6 mmol/l in dimethyl sulphoxide) to 100 ml of a luminol (1.25 mmol/l), hydrogen peroxide (2.7 mmol/l) mixture prepared in Tris buffer (0.1 mol/l, pH 8.5). Light intensity after 30 seconds was recorded.

6.2.2.3 Assay of carcinoembryonic antigen with a CEA-EIA kit

Human CEA standards, 200 µl, prepared in sodium acetate buffer (0.2 mol/l, pH 5.0, containing 0.01% thimerosal preservative) were added to wells of a plastic reaction tray. One plastic bead coated with anti CEA raised in guinea-pigs, was rinsed in distilled water and added to each well. The tray was gently tapped to remove any bubbles attached to the bead surface, covered and incubated at 45°C for two hours in a water bath. Samples were then aspirated and each bead washed with distilled water (5 x 2 ml). Goat anti-human CEA-HRP conjugate 200 µl (approximately 0.05 µg/ml in Tris buffer containing protein stabilizer and 0.01% thimerosal) was added to each bead, the tray treated as before and incubated at 45°C for two hours. After aspirating the solution, beads were washed with distilled water (5 x 2 ml), transferred to polystyrene tubes and the washing repeated (2 x 4 ml). Bound HRP-conjugate was then quantitated colorimetrically or using the p-iodophenol enhanced chemiluminescent reaction. Within-assay precision using the enhanced chemiluminescent end-point was determined by analysing batches of beads with the 5 standards assayed in quadruplicate.

Colorimetric end-point: This was performed using the 30 minute

o-phenylenediamine-hydrogen peroxide procedure previously described (6.2.2.2).

Manual enhanced chemiluminescent end-point: Immediately after washing, beads were transferred to cuvettes containing 600 µl Tris buffer (0.05 mol/l, pH 8.0). Individual chemiluminescent reactions were initiated by the manual addition of 500 µl of a luminol-hydrogen peroxide-p-iodophenol solution prepared by the addition of 1.0 ml p-iodophenol (81.7 mmol/l in DMSO) to 100 ml of a luminol (2.5 mmol/l), hydrogen peroxide (5.4 mmol/l) mixture prepared in Tris buffer (0.05 mol/l, pH 8.0). Light intensity after 30 seconds was recorded.

Automatic enhanced chemiluminescent end-point: Immediately after washing, beads were transferred to 55 x 11 mm clear polystyrene round bottomed tubes (LIP (Equipment and Services Ltd), Shipley, West Yorkshire) containing 500 µl Tris buffer (0.05 mol/l, pH 8.0). The shape and dimensions of these tubes ensured beads always adopted a constant position in the base of the tube. Chemiluminescent reactions on batches of beads were automatically initiated on an Auto-Biolumat LB 950 luminometer by injection of 500 µl of a mixture of luminol (2.5 mmol/l), hydrogen peroxide (5.4 mmol/l) and p-iodophenol (809 µmol/l) prepared in Tris buffer (0.05 mol/l, pH 8.0) as in the manual procedure. Integrated light emission between 30 and 60 seconds after initiation was automatically recorded for each bead.

6.2.2.4 Assay of immunoglobulin E with an Enzygnost IgE kit

Serum specimens or standards (50 µl) were diluted with 500 µl of

the incubation medium provided in the kit. After mixing, diluted serum (200 µl) was added to a plastic tube coated with goat anti-human IgE. Tubes were covered and then incubated at room temperature for 2 hours after which the contents were aspirated and discarded. Tubes were washed by filling with phosphate buffer containing Tween, this was left for one minute then aspirated, washing and aspiration were then repeated. Rabbit anti-human IgE-HRP conjugate (200 µl; pre-diluted in buffer provided) was added to each tube which was then covered and incubated for a further 2 hours at room temperature. The tube contents were then aspirated and the washing procedure repeated three times. HRP-conjugate bound to tubes was quantitated colorimetrically or luminescently. Within-assay precision was determined by analysing replicate standards. A standard curve was performed with each batch of replicate standards.

Colorimetric end-point: o-Phenylenediamine was dissolved, 10 minutes before use, in citrate-phosphate buffer (supplied with the test kit) containing hydrogen peroxide (0.3 g/l) and sodium p-ethyl-mercury-mercapto-benzene sulphonate preservative. Chromogen solution (200 µl) was added to each tube at 10 second intervals, the tubes covered and incubated at room temperature in the dark for 30 minutes. Reactions were stopped by the timed addition of 1 ml sulphuric acid (0.5 mol/l). Solutions were mixed, transferred to microcuvettes and the absorbance at 492 nm measured on a Gilford 250 spectrophotometer blanked against distilled water.

Firefly luciferin enhanced chemiluminescent end-point: Firefly

luciferin (10 µl; 3.6 mmol/l) in Tris buffer (10 mmol/l, pH 8.0) was added to the tube and the chemiluminescent reaction initiated by the manual addition of 900 µl of a mixture of luminol (1.25 mmol/l) and hydrogen peroxide (2.7 mmol/l) in Tris buffer (10 mmol/l, pH 8.0). The tube was gently shaken to ensure mixing and the removal of any bubbles on the plastic surface, and then introduced into the WRL luminometer. Reproducible, optimal positioning of the tube was achieved by introducing a reflective packing insert into the cuvette holder to support rigidly the tube close to the PMT. Light intensity after 30 seconds was recorded.

p-Iodophenol enhanced chemiluminescent end-point: Washing solution (1 ml) was added to each tube to prevent the reactive surface drying out. Immediately prior to chemiluminescent analysis washing solution was removed by aspiration and reactions manually initiated by the addition of 910 µl of a luminol (1.25 mmol/l), hydrogen peroxide (2.7 mmol/l), p-iodophenol (150 µmol/l) solution prepared in Tris buffer (0.1 mol/l, pH 8.5). Light intensity after 30 seconds was recorded with tubes positioned as in the preceding section.

6.2.2.5 Assay of total thyroxine with an Enzymun-Test T_4 kit

Serum standards or the control supplied (20 µl) and thyroxine-HRP conjugate in incubation buffer (1 ml; barbitone, 120 mmol/l; phosphate buffer, 17.8 mmol/l, pH 8.6, containing 8-anilino-1-naphthalenesulphonic acid, 1.27 mmol/l; BSA, 0.2%) were added to plastic tubes coated with a limiting quantity of thyroxine antibodies raised in rabbits. Each tube was incubated at room

temperature for 2 hours after which the contents were aspirated and discarded. Tubes were washed by filling to the brim with cold tap-water and aspirating after 2 minutes. Aspiration was repeated after approximately 2 minutes to remove run-off water collected at the bottom of the tube, and bound conjugate then quantitated.

Colorimetric end-point: At timed intervals, 1.0 ml phosphate-citrate buffer (0.1 mol/l, pH 5.0) containing ABTS (9.1 mmol/l) and sodium perborate (1.47 mmol/l), was added to each tube and the tubes incubated at room temperature for 1 hour. At the appropriate time intervals tubes were mixed well, the contents transferred to microcuvettes and the absorbance at 420 nm measured on a Gilford 250 spectrophotometer blanked against substrate solution.

Firefly luciferin enhanced chemiluminescent end-point: This was performed exactly as with Enzygnost IgE tubes (6.2.2.4). The tubes had a recessed base, and hence only part of the active surface was visible to the photomultiplier tube. A section of the cuvette carrier floor was therefore lowered in order that the maximal active surface of each tube was exposed to the PMT. For processing large numbers of tubes, 600 µl Tris buffer (10 mmol/l, pH 8.0) was added to each tube and individual luminescent reactions initiated by addition of 10 µl firefly luciferin (3.6 mmol/l) and 400 µl of a mixture of luminol (3.1 mmol/l) and hydrogen peroxide (7.3 mmol/l) in the Tris buffer. Light emission 30 seconds after initiation was recorded.

6.2.2.6 Rapid assay of digoxin with an Enzymun-Test Digoxin kit

Serum standards or control (100 µl) and digoxin-HRP conjuate (1 ml; in phosphate buffer, 40 mmol/l, pH 6.8 containing BSA 0.25%) were added to plastic tubes coated with a limiting quantity of ovine antibodies raised against digoxin. Each tube was incubated at room temperature for 45 minutes after which the contents were aspirated and discarded. Tubes were washed (6.2.2.5) and bound conjugate quantitated.

Colorimetric end-point: This was performed using the one hour ABTS-sodium perborate reaction previously described (6.2.2.5).

p-Iodophenol enhanced chemiluminescent end-point: Immediately after washing, 600 µl Tris buffer (0.1 mol/l, pH 8.5) was added to each tube. Chemiluminescent reactions were initiated manually by the addition of 400 µl of a mixture of luminol (3.1 mmol/l), hydrogen peroxide (7.3 mmol/l) and p-iodophenol (0.37 mmol/l), prepared by addition of 1.1 ml p-iodophenol (34 mmol/l in DMSO) to 100 ml of a luminol-hydrogen peroxide solution prepared in Tris buffer (0.1 mol/l, pH 8.5). Light intensity after 30 seconds was recorded with the tube positioned as described previously (6.2.2.5).

6.2.2.7 Rapid assay of hepatitis B surface antigen with an Auszyme II kit

Serum HB_sAg standards (0, 0.25, 1.0 and 4.0 ng/ml), 200 ul, were added to wells in a plastic reaction tray. A plastic bead coated with guinea-pig antibody to HB_sAg was added to each well and the tray tapped to release any air bubbles from the surface of the beads. The tray was covered and incubated at 50°C in a water

bath for 30 minutes. The contents of each well were aspirated and each bead washed 4 times with 2 ml distilled water. Goat anti HB_sAg-HRP conjugate (200 µl; approximately 0.2 µg/ml in Tris buffer containing protein stabilizers and 0.01% thimerosal preservative) was added to each well containing a bead, the tray treated as before, and incubated again at 50°C for 30 minutes. After aspirating the solution, beads were washed with distilled water (4 x 2 ml), transferred to polystyrene tubes and washed again (2 x 4 ml). Beads were stored in 1 ml distilled water which was aspirated immediately prior to quantitation of bound conjugate.

Colorimetric end-point: This was performed using the 30 minute o-phenylenediamine-H_2O_2 colorimetric procedure previously described (6.2.2.2).

p-Iodophenol enhanced chemiluminescent end-point: Beads were transferred to cuvettes and individual luminescent reactions initiated by the manual addition of 910 µl of a luminol-hydrogen peroxide-p-iodophenol solution prepared by the addition of 1.1 ml p-iodophenol (20.4 mmol/l in DMSO) to 100 ml of a luminol (1.25 mmol/l), hydrogen peroxide (2.7 mmol/l) mixture prepared in Tris buffer (0.1 mol/l, pH 8.5). Light emission after 30 seconds was recorded.

6.2.3 Enhanced chemiluminescent end-points in immunoassay systems using black microtitre plates

6.2.3.1 Light Measurement

Light emission from individual wells of a microtitre plate was

measured using a photomultiplier based luminescent microtitre plate reader. An end-window photomultiplier tube (9924A, Thorn EMI Electron Tubes Ltd, UK) was fitted with a small, circular aperture and positioned close to the upper surface of a black microtitre plate contained in a light-tight box. Multiple reactions were initiated manually outside the luminometer. The glowing plate, located on a simple carrier, could be manually positioned rapidly and precisely to align individual wells with the aperture. The photomultiplier tube was operated in photocurrent mode, and the results displayed on a digital voltmeter. Measurement of light intensity and positioning of individual wells could be completed in approximately one second allowing very rapid sequential measurements from a series of wells.

6.2.3.2 Assay of cytomegalovirus specific IgG

Cytomegalovirus antigen, 200 µl of a 1:200 dilution in carbonate-bicarbonate buffer (0.05 mol/l, pH 9.6) was added to each well of a black polystyrene microtitre plate. The microtitre plate was covered and incubated at 0 - 4°C overnight. The contents were discarded and the coated plate washed three times with phosphate-buffered saline (pH 7.3) containing 0.1% Tween 20 (PBST). Serum samples or standards were diluted 1:20 in PBST and 200 µl added to individual wells using an 8-channel multiple pipette. The plate was covered and incubated on a microtitre plate vibrator (model 804, Luckham Ltd, UK) for either 30 or 15 minutes at room temperature. The contents of the plate were discarded and the washing procedure repeated. Anti-human IgG-HRP conjugate (200 µl of a 1:25,000 dilution in PBST) was added to each well and

incubated for a further 30 or 15 minutes at room temperature. The contents were emptied from the wells and the plate washed three times with PBST.

Enhanced chemiluminescent end-point:

Immediately after the final wash procedure chemiluminescent reactions in each well were initiated by addition of 200 µl of a luminol-hydrogen peroxide - 6-hydroxybenzothiazole mixture. This mixture was prepared by addition of 200 µl of 6-hydroxybenzothiazole (3.3 mmol/l in DMSO) to 20 ml of a luminol (60 µmol/l), hydrogen peroxide (2 mmol/l) solution prepared in Tris buffer (0.1 mol/l, pH 8.0 or pH 8.5). The plate was introduced into the microtitre plate luminometer, individual wells positioned under the photomultiplier tube and the light intensity recorded at various times after initiation.

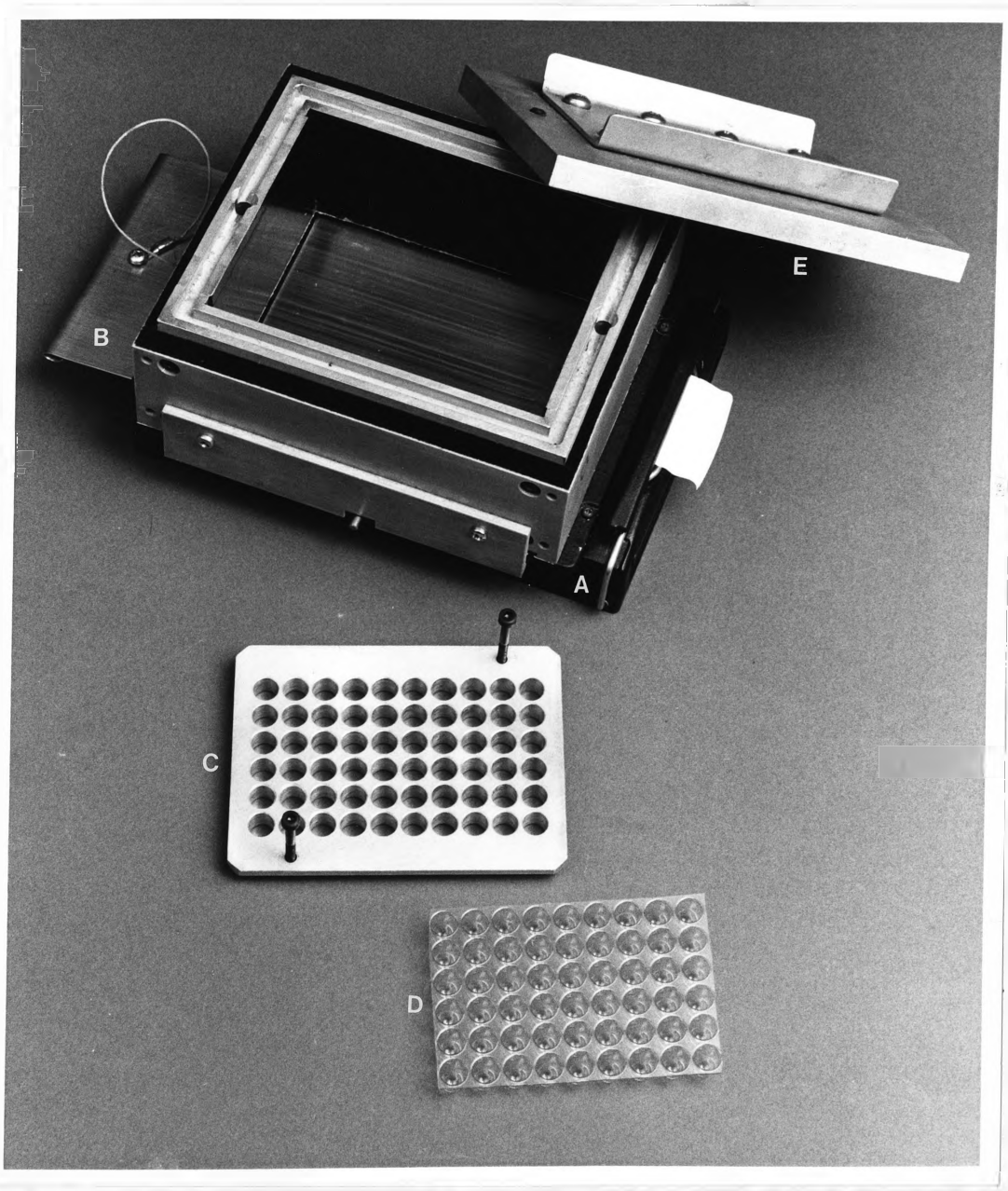
6.2.4 Photographic monitoring of enhanced chemiluminescent immunoassays

6.2.4.1 Light measurement

Light emission from assays in transparent microtitre plates was recorded using a photographic film based camera luminometer (Figure 6.2). This consisted of a light-tight box with a matt black interior, mounted on top of a Polaroid film pack (type CB103). Standard U-bottomed microtitre plates were cut down to 7 x 9 arrays of wells to suit the size of film available. After manual initiation of chemiluminescent reactions the glowing microtitre plate was placed in a metallic mask (made in the form of an array of holes) to isolate light emission from individual wells. In the luminometer the microtitre plate and mask were

Figure 6.2

Components of a camera luminometer



A Polaroid instant film holder

B Sliding shutter

C Metallic mask

D Transparent microtitre plate

E Camera luminometer top

supported by a slidable shutter, removal of which positioned the microtitre plate on top of Polaroid 612 ASA 20,000 instant photographic film. After exposure (typically 15-120 seconds) the shutter was re-inserted and the instant film developed for 35 seconds.

6.2.4.2 Assay of serum ferritin

Transparent polyvinyl chloride microtitre plates were coated overnight at 0 - 4°C with a 1:1000 dilution of rabbit anti-human ferritin antibody in carbonate buffer (0.05 mol/l, pH 9.5). After washing 3 times with phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (PBS Tween) the coated plate was incubated with samples or standards (100 µl) for 3 hours at room temperature. The washing procedure was repeated and the plate incubated for a further 2 hours with 100 µl of a 1:200 dilution of rabbit anti-human ferritin - HRP conjugate in PBS Tween containing 0.5% BSA. The contents were emptied from the wells and the plate washed three times with PBS Tween. Immediately after the final wash procedure chemiluminescent reactions were initiated by addition of 200 µl of a luminol (1.25 mmol/l), hydrogen peroxide (2.7 mmol/l), firefly luciferin (39 µmol/l) mixture prepared in Tris buffer (0.1 mol/l, pH 8.0). The glowing microtitre plate was positioned in the mask and this placed into the camera luminometer. The microtitre plate was exposed to the film for 30 seconds and this then developed for 35 seconds.

6.2.4.3 Assay of cytomegalovirus specific IgG

The assay was performed essentially as in section 6.2.3.2 except transparent polyvinyl chloride U-bottomed microtitre plates were

employed and incubations with diluted sample or HRP-conjugate extended to 90 and 60 minutes respectively. Chemiluminescent reactions were initiated at pH 8.5 using the 6-hydroxybenzothiazole enhanced luminol-hydrogen peroxide system and the plate exposed to the photographic film for 90 seconds.

6.3 RESULTS

6.3.1 Assay of anti rubella virus IgG

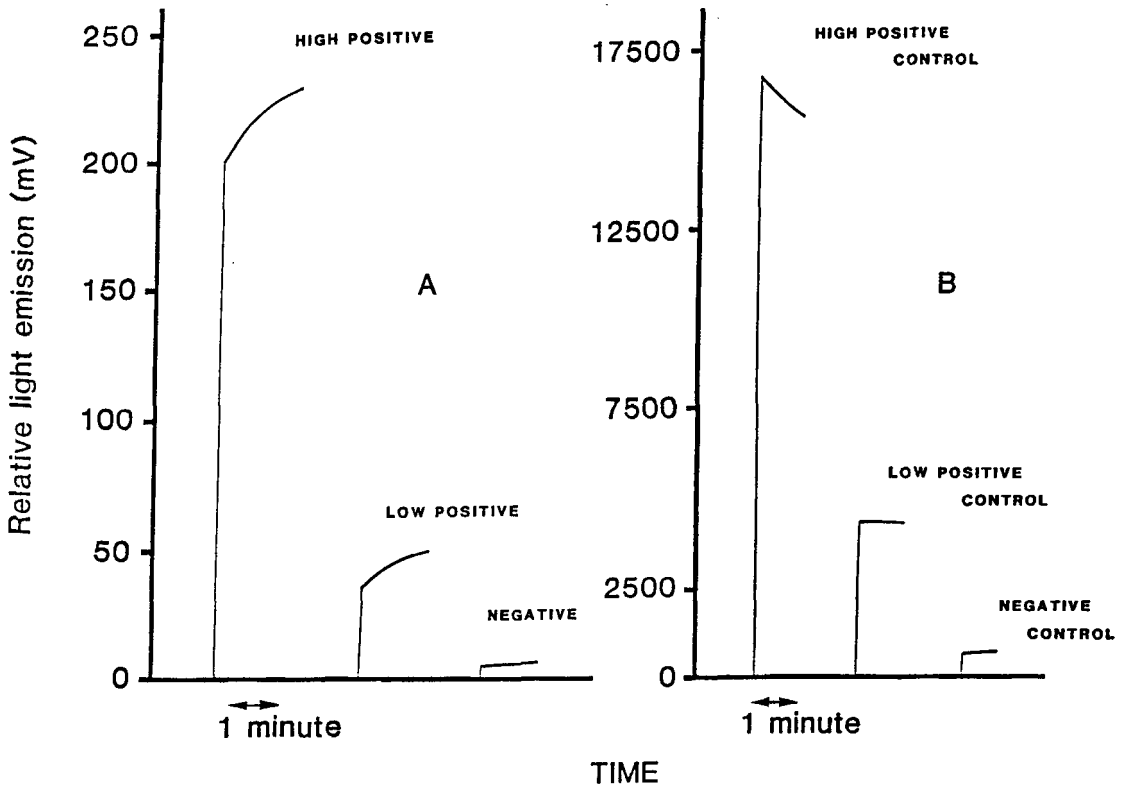
Figure 6.3 illustrates the kinetics of light emission obtained using either a firefly luciferin or p-iodophenol enhanced chemiluminescent end-point to quantitate HRP-conjugate bound to polystyrene beads in the Rubazyme assay. Light intensity was recorded 30 seconds after initiation, however as light emission is prolonged and relatively stable, measurements could be made either earlier or several minutes later. Similar discrimination between the three controls was obtained using assays with a colorimetric, or firefly luciferin or p-iodophenol enhanced chemiluminescent end-points (Table 6.2). For a series of 84 specimens, good correlation ($r = 0.97$, regression line $y = 0.93x + 0.03$) was obtained between results determined using the colorimetric or the p-iodophenol enhanced chemiluminescent end-point (Figure 6.4). The within-batch precision (CV) of light measurements with various standards and serum specimens ranged from 4.7 to 8.1% (Table 6.3).

6.3.2 Assay of carcinoembryonic antigen (CEA)

Incorporation of a rapid enhanced chemiluminescent end-point into the bead based CEA immunometric assay produced a significant reduction in overall assay time without adversely affecting assay

Figure 6.3

Kinetics of light emission using (A) the firefly luciferin enhanced, or (B) the p-iodophenol enhanced chemiluminescent reaction in an immunometric assay for rubella specific IgG employing beads as solid support



Final reagent concentrations: Luminol 1.24 mmol/l, H_2O_2 2.67 mmol/l, firefly luciferin 39.6 µmol/l (pH 8.0) or p-iodophenol 148 µmol/l (pH 8.5)

Table 6.2

Responses from controls in the Rubazyme assay using colorimetric or enhanced chemiluminescent end-points

END-POINT	ANTI RUBELLA VIRUS IgG CONTROL		
	NEGATIVE	LOW POSITIVE	POSITIVE
o-Phenylenediamine-colorimetry (absorbance change at 492 nm after 30 minutes)	0.054	0.461	1.513
Firefly luciferin enhanced chemiluminescence (light intensity 30s after initiation, mV)	8	93	457
o-Phenylenediamine-colorimetry (absorbance change at 492 nm after 30 minutes)	0.115	0.558	1.666
p-Iodophenol enhanced chemiluminescence (light intensity 30s after initiation, mV)	864	6168	15120

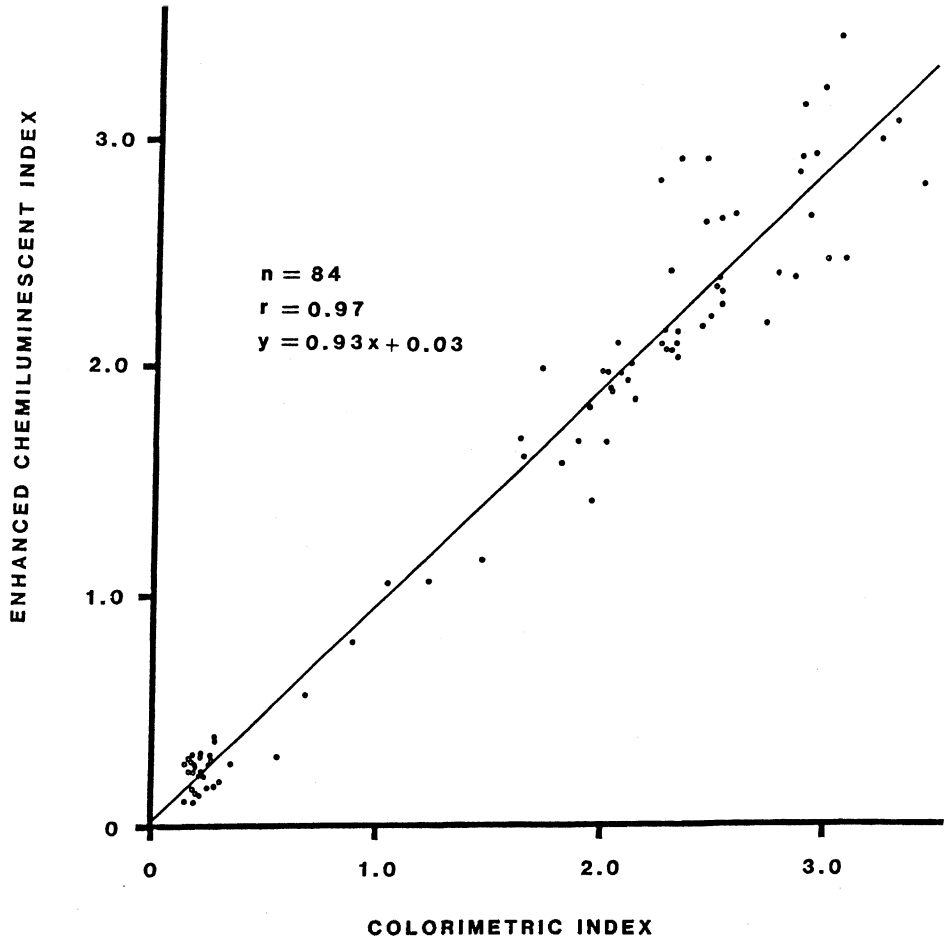
Table 6.3

Within-batch precision of light measurements in an assay for rubella virus specific IgG employing a p-iodophenol enhanced chemiluminescent end-point

Rubella virus specific IgG preparation	Light emission (mV)	Coefficient of variation (%)	Index	Number of determinations
High positive serum sample	15558	6.3	3.44	8
High positive control	15842	8.1	3.50	7
Low positive serum sample	4567	7.0	1.01	8
Low positive control	4531	4.7	1	8
Negative serum sample	867	7.7	0.19	8
Negative control	700	7.6	0.16	8

Figure 6.4

Comparison of serum rubella antibody levels using an immunometric assay with either a p-iodophenol enhanced luminescent or a colorimetric end-point



The index for each specimen was obtained by dividing its response by that of the low positive control

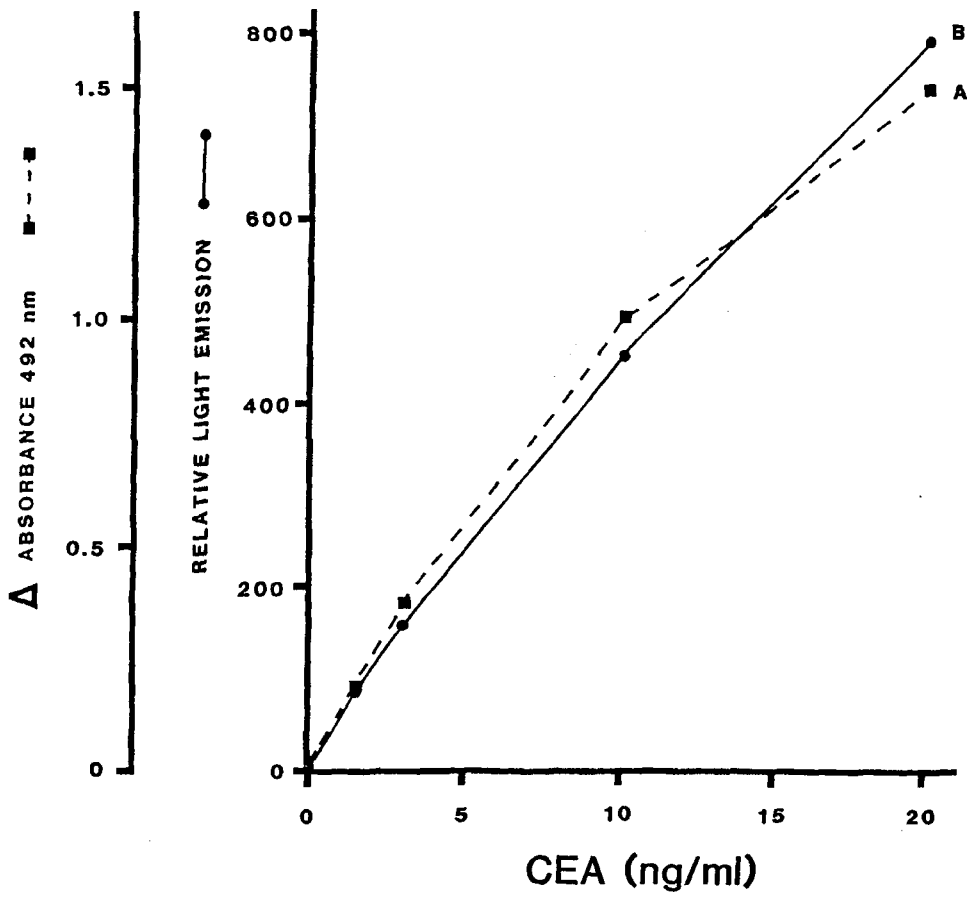
performance. Dose-response curves produced in the CEA assay using a colorimetric or a p-iodophenol enhanced chemiluminescent end-point are shown in Figure 6.5. Within-batch precision of light measurements from different standards was good, particularly using automatic initiation and recording of integrated light emission. The coefficient of variation of measurements obtained using manual initiation of reactions and photocurrent measurement, or automatic initiation of reactions and photon counting are shown in Table 6.4.

6.3.3 Assay of IgE

The shapes of the standard curves of the IgE tube based assay, monitored using a colorimetric or an enhanced chemiluminescent end-point were very similar (Figure 6.6). p-Iodophenol or firefly luciferin enhanced reactions could be employed although the latter produced lower levels of light emission. Figure 6.7 shows the time course of light emission for p-iodophenol enhanced chemiluminescent immunoassays of various IgE standards. Under the conditions employed in the chemiluminescent reaction the kinetics of light emission were dependent on the concentration of bound HRP-conjugate. With IgE standards below 100 IU/ml, the low concentrations of HRP labelled antibody bound produced relatively constant light emission for several minutes. More pronounced decays in light intensity occurred with the higher concentrations of HRP-conjugate bound with IgE standards above 500 IU/ml. Since light emission was prolonged, and provided individual measurements were made at a fixed time after initiation (or rapid sequential measurements taken from small batches of

Figure 6.5

Standard curves for carcinoembryonic antigen using an immunometric assay employing either A) a colorimetric or B) a manually initiated p-iodophenol enhanced chemiluminescent end-point



Final reagent concentrations: Luminol 1.12 mmol/l,
H_2O_2 2.43 mmol/l,
p-iodophenol 367 µmol/l (pH 8.0)

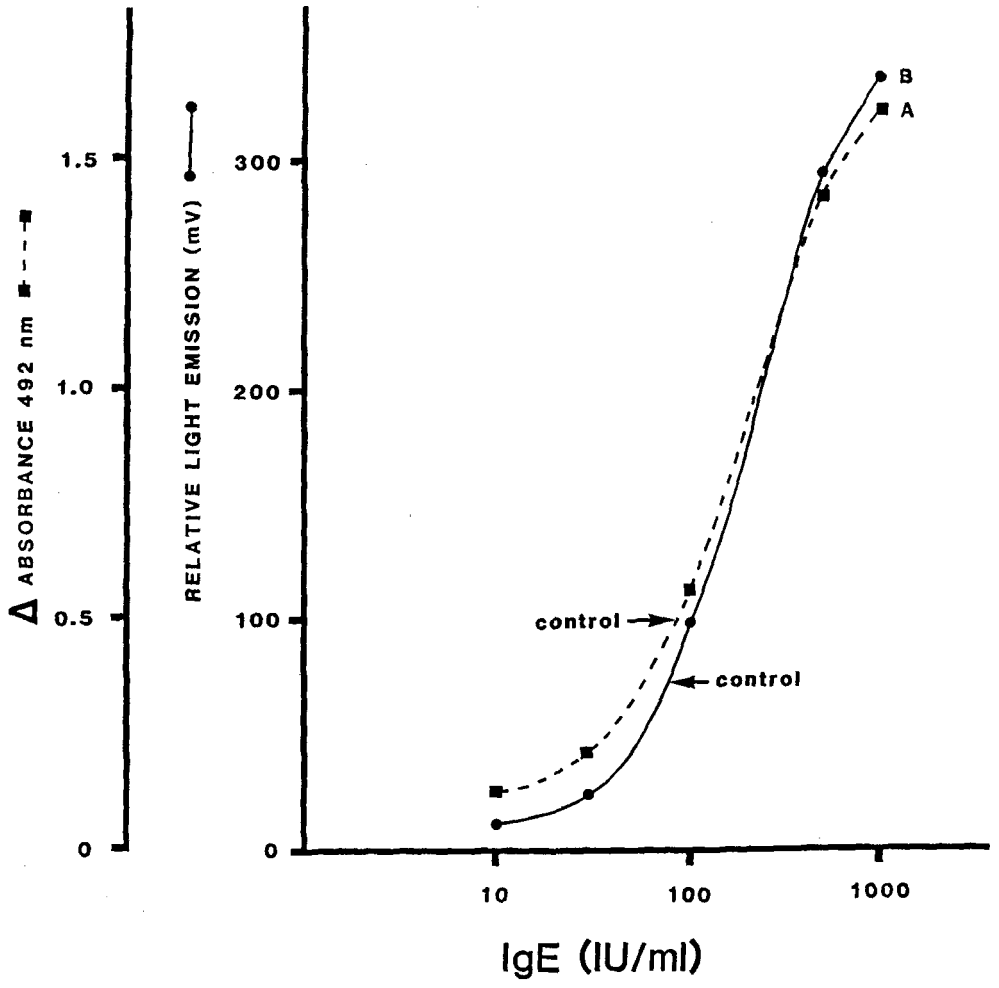
Table 6.4

Within-batch precision of light measurements in a CEA assay employing a p-iodophenol enhanced chemiluminescent end-point

Manual Initiation					
CEA concentration (ng/ml)	0	2.1	4.2	8.4	16.8
Number of determinations	12	12	12	12	12
Coefficient of variation (CV %)	14.1	9.8	4.5	2.4	3.6
Automatic Initiation					
CEA concentration (ng/ml)	0	2.5	5.0	10.0	20.0
Number of determinations	4	4	4	4	4
Coefficient of variation (CV %)	8.4	4.8	2.1	1.0	2.4

Figure 6.6

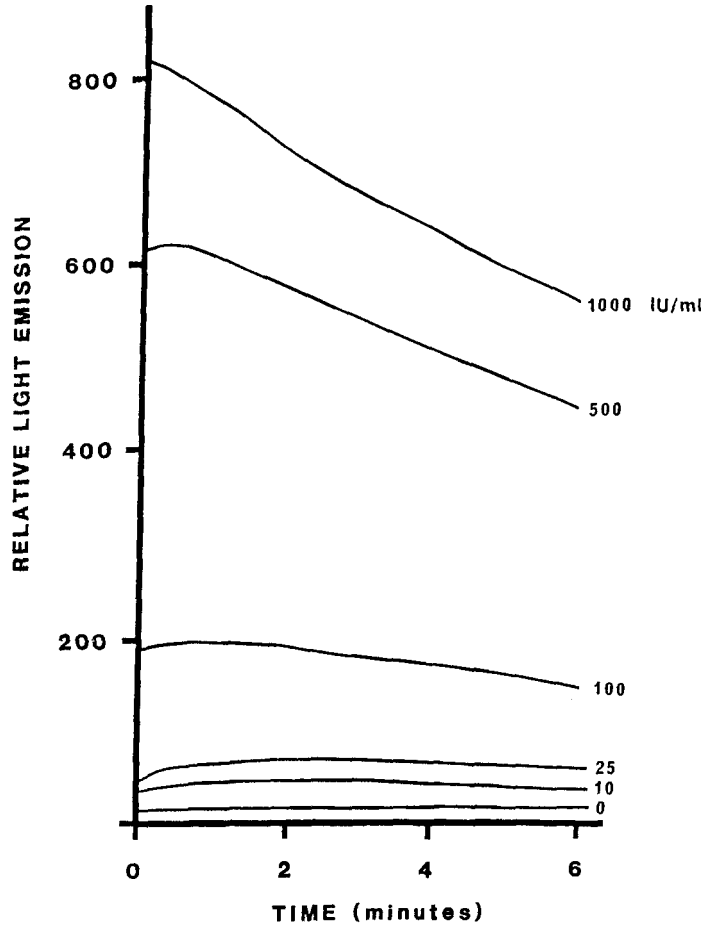
Standard curves of human IgE in an immunometric assay using tubes as solid support and quantitation of peroxidase labels with either A) a colorimetric or B) a firefly luciferin enhanced chemiluminescent end-point



Values for control sera are indicated

Figure 6.7

Kinetics of light emission of various IgE standards assayed using a p-iodophenol enhanced chemiluminescent end-point



Final reagent concentrations: luminol 1.25 mmol/l,
H_2O_2 2.7 mmol/l,
p-iodophenol 150 µmol/l (pH 8.5)

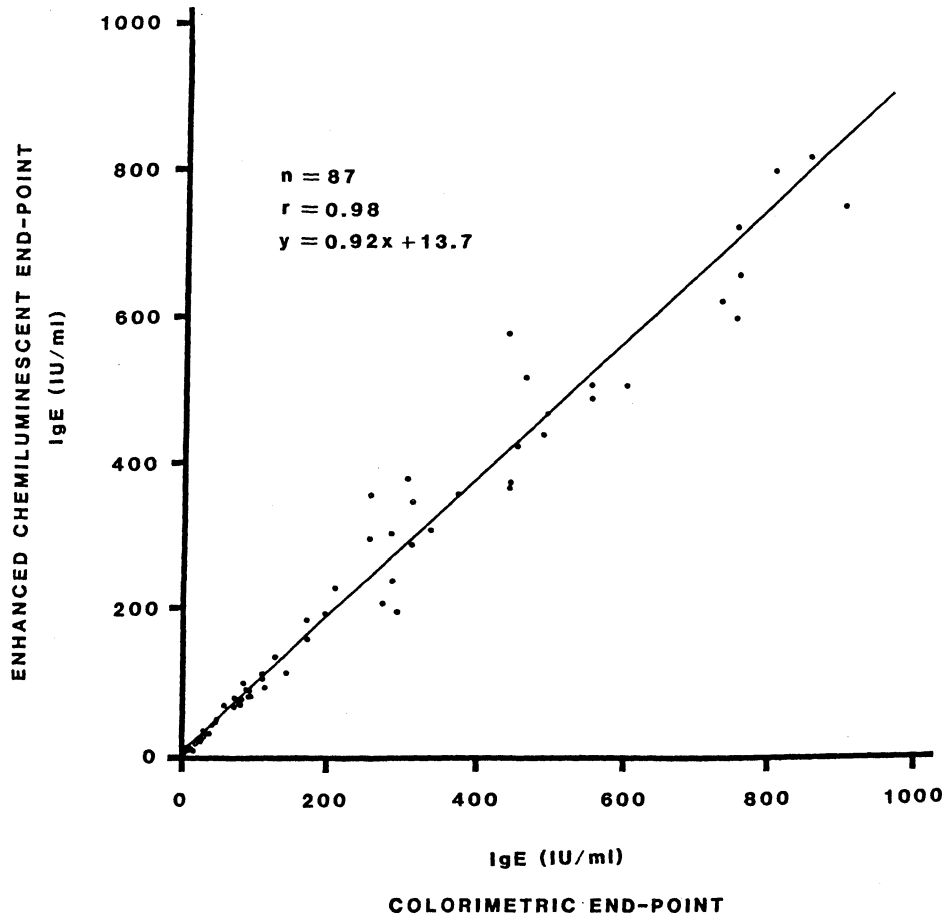
Table 6.5

Within-batch precision of serum IgE measurement using an immunometric assay with a p-iodophenol enhanced chemiluminescent or a colorimetric end-point

IgE Concentration (IU/ml)	CHEMILUMINESCENT ASSAY		COLORIMETRIC ASSAY	
	Coefficient of Variation (%)	Number of Determinations	Coefficient of Variation (%)	Number of Determinations
25	10.4	19	9.8	19
100	7.3	13	5.9	13
370	3.9	12	4.6	12
1000	13.2	13	-	-

Figure 6.8

Correlation between p-iodophenol enhanced chemiluminescent immunometric and colorimetric immunometric assay results for IgE on a series of 87 serum specimens



simultaneously initiated reactions), effects due to changes in kinetics were minimised and measurements could be made several minutes after initiation. In Table 6.5, values obtained for within-batch precision obtained using the enhanced chemiluminescent enzyme immunoassay are compared with those obtained using the same assay monitored colorimetrically. The precision of the IgE immunoassays with either a colorimetric or a chemiluminescent end-point were similar. Figure 6.8 shows the comparison of the colorimetric and enhanced chemiluminescent version of the IgE assay for analysis of a series of 87 serum specimens. A correlation coefficient of 0.98 was obtained ($y = 0.92x + 13.7$).

6.3.4 Assay of total thyroxine

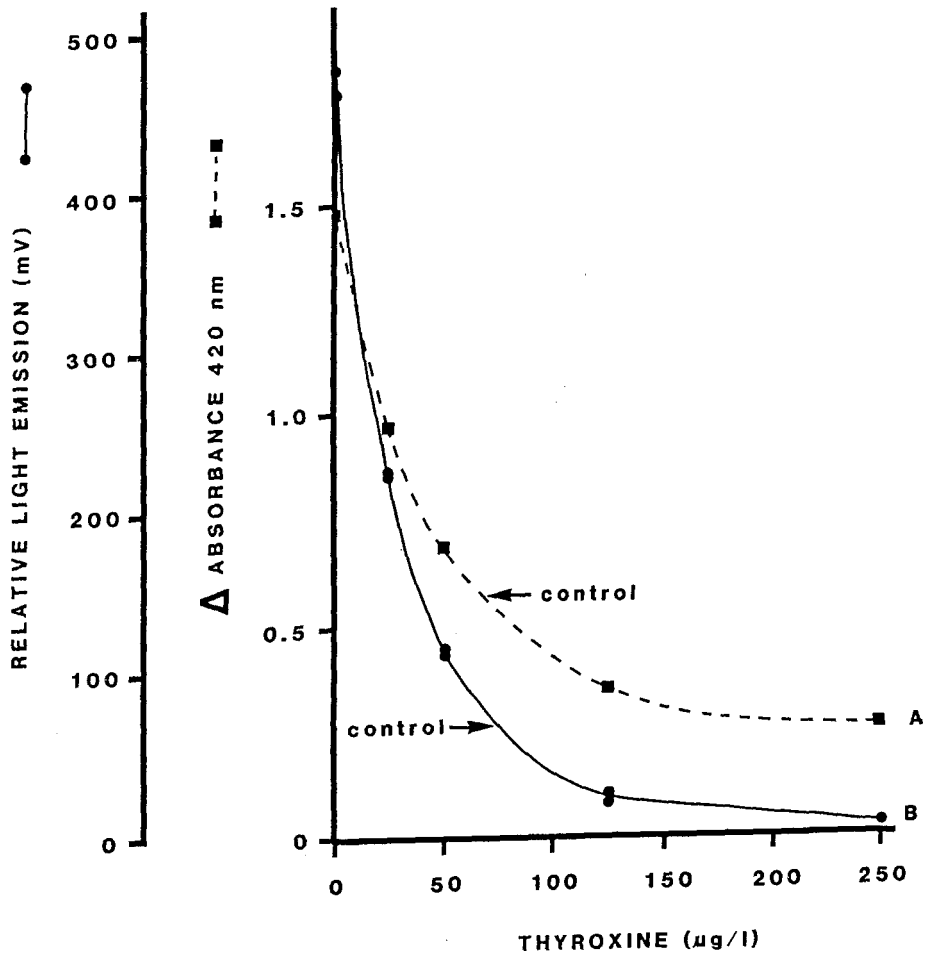
Dose-response curves for thyroxine using a firefly luciferin enhanced chemiluminescent and an ABTS colorimetric end-point in the competitive immunoassay are illustrated in Figure 6.9. Appropriate values for control serum were obtained with both assays. The enhanced chemiluminescent end-point produced the more sensitive assay despite the fact that the light detector only monitored 50% of the active area of the assay tube.

6.3.5 Assay of digoxin

Standard curves for digoxin assayed using competitive enzyme immunoassays with an ABTS colorimetric or a p-iodophenol enhanced chemiluminescent end-point are shown in Figure 6.10. Appropriate values for the control serum were obtained in both assays, and expressing the response from standards as a percentage of the maximum response revealed that the chemiluminescent method was

Figure 6.9

Dose-response curves for serum thyroxine using A) a colorimetric or B) a firefly luciferin enhanced chemiluminescent immunoassay

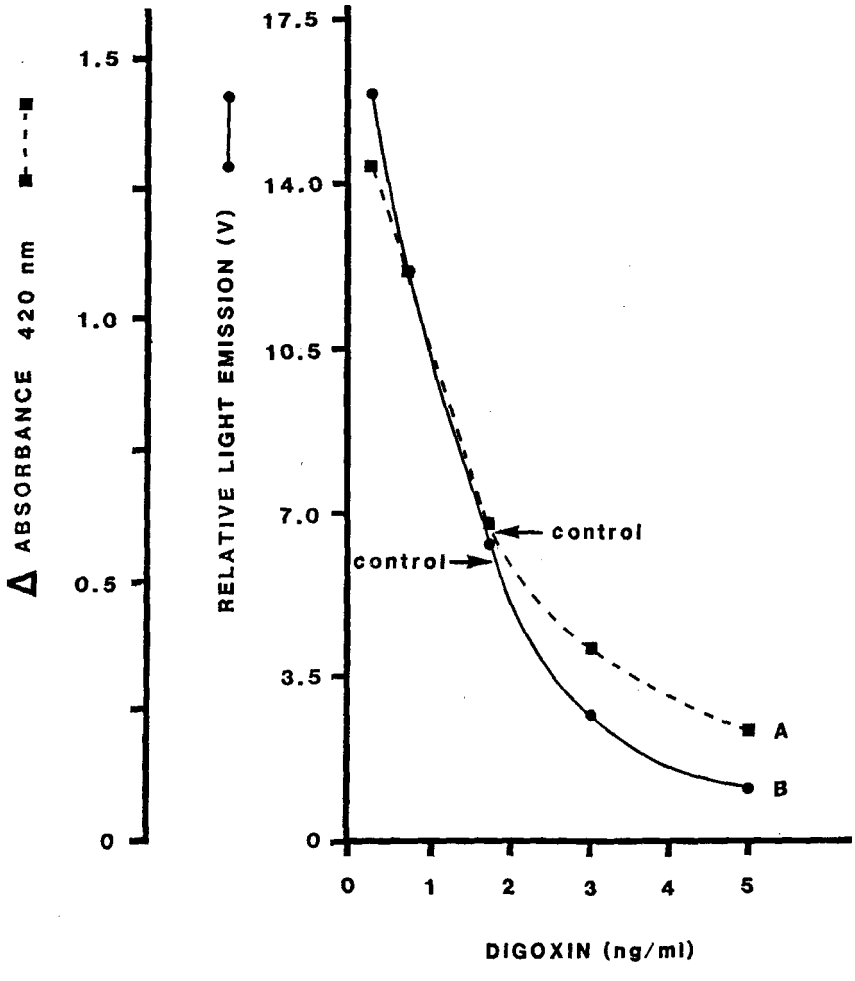


Values for control sera are indicated

Final reagent concentrations: Luminol 1.23 mmol/l,
H_2O_2 2.89 mmol/l,
firefly luciferin 35.6 µmol/l
(pH 8.0)

Figure 6.10

Standard curves of digoxin in an immunoassay using tubes as solid support and quantitation of peroxidase labels with either A) a colorimetric or B) a p-iodophenol enhanced chemiluminescent end-point



Values for control sera are indicated

Final reagent concentrations: Luminol 1.24 mmol/l,
H_2O_2 2.92 mmol/l,
p-iodophenol 148 µmol/l (pH 8.5)

slightly more sensitive. Replacement of the colorimetric end-point by the 30 second enhanced chemiluminescent end-point halved the overall assay time.

6.3.6 Assay of hepatitis B surface antigen

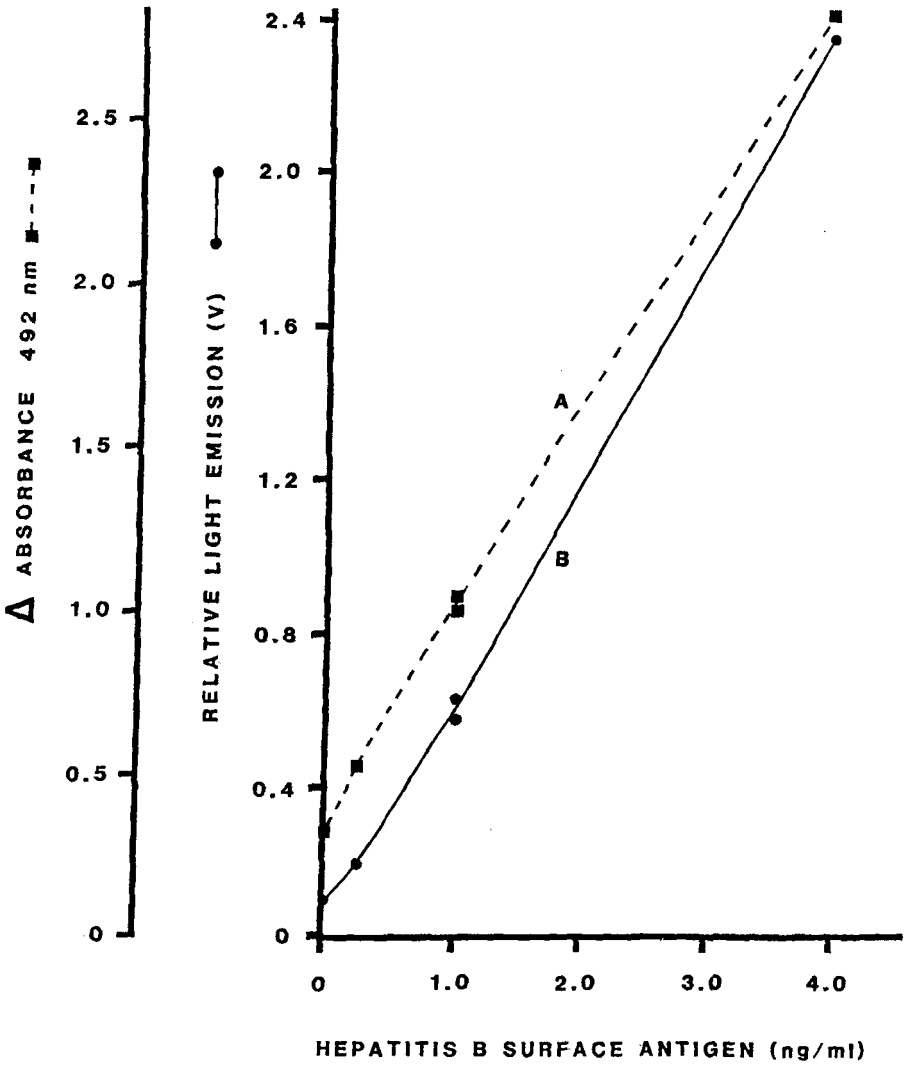
Standard curves for serum hepatitis B surface antigen in a bead based immunometric assay, using a 30 minute o-phenylenediamine colorimetric or a p-iodophenol enhanced chemiluminescent end-point, are illustrated in Figure 6.11. A near linear response, and clear differentiation between the 0 and 0.25 ng/ml standards was obtained. In conjunction with 30 minute incubations with sample and HRP labelled antibody, the 30 second enhanced chemiluminescent end-point produced a useful reduction in overall assay time.

6.3.7 Assay of cytomegalovirus specific IgG

The kinetics of light emission obtained with various standards in an assay for cytomegalovirus specific antibody, performed on black microtitre plates and using a 6-hydroxybenzothiazole enhanced chemiluminescent end-point, are shown in Figure 6.12. Light emission was prolonged and relatively constant. Even with the high concentrations of conjugate bound with the high positive control (where under the conditions employed decay of light emission was more pronounced) light emission continued for over 15 minutes. Simultaneous initiation of multiple reactions was possible as the prolonged light emission eliminated any requirement for initiation of individual reactions in front of the photodetector. Light measurement and positioning of individual wells under the detector could be completed in

Figure 6.11

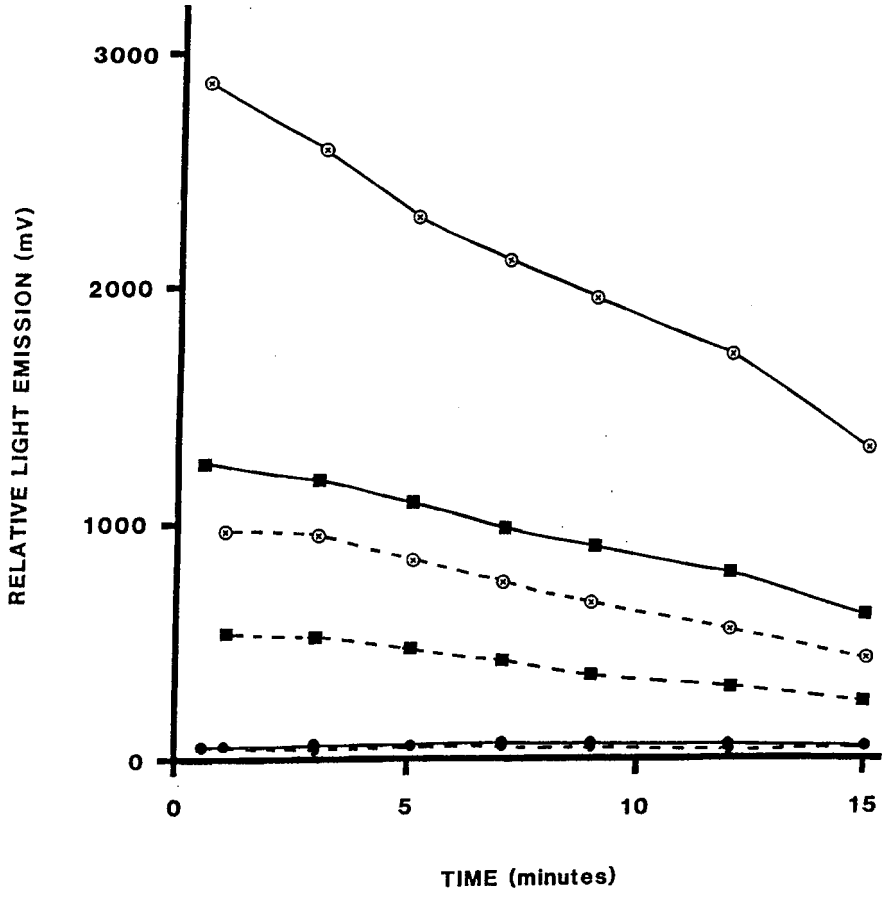
Standard curves for hepatitis B surface antigen in an immunometric assay employing beads as solid support and A) a colorimetric or B) a p-iodophenol enhanced chemiluminescent end-point



Final reagent concentrations: Luminol 1.24 mmol/l,
H_2O_2 2.67 mmol/l,
p-iodophenol 222 µmol/l (pH 8.5)

Figure 6.12

Kinetics of light emission in a 6-hydroxybenzothiazole enhanced chemiluminescent immunometric assay for CMV specific IgG performed on black microtitre plates



——— pH 8.5, ----- pH 8.0

Serum specimens • (A), ■ (C) and ⊙ (D) produced absorbance changes of 0.04, 0.6 and 0.9 respectively in the immunometric assay employing an alkaline phosphatase conjugate

Final concentration of reagents in the enhanced chemiluminescent end-point: Luminol 60 µmol/l, H_2O_2 2 mmol/l,
6-hydroxybenzothiazole 33 µmol/l

Table 6.6

Light emission from bound HRP-conjugate in assays for CMV specific IgG performed on black microtitre plates and using a 6-hydroxybenzothiazole enhanced chemiluminescent end-point

	LIGHT EMISSION (mV)[b]	
Incubation time with sample or conjugate	15 mins	30 mins
Antibody standard[a]		
Negative	19	40
Borderline	20	48
Low Positive	572	1230
High Positive	1404	2815

[a] In an immunometric assay employing an alkaline phosphatase conjugate absorbance changes with the negative, borderline, low positive and high positive control sera were 0.04, 0.07, 0.6 and 0.9 respectively.

[b] The enhanced reaction was performed at pH 8.5 and light intensity after 60 seconds recorded.

approximately one second. Sequential rapid measurements, or initiation of relatively small numbers of reactions at a time, minimised effects due to any changes in the kinetics of light emission. Good differentiation between standards was possible (Table 6.6), even when incubations with sample and conjugate were reduced to 15 minutes. Carryover of light from neighbouring wells was not significant. For wells from which relative light emission in the assay was recorded as 214, 233, 2026 and 2774 mV, the mean signals from the 8 immediately surrounding wells were 40, 42, 42 and 43 mV respectively.

6.3.8 Photographically monitored assay of serum ferritin

The semi-quantitative results obtained using 20,000 ASA Polaroid instant film to monitor a firefly luciferin enhanced chemiluminescent immunometric assay of a series of ferritin standards are shown in Figure 6.13. A graded response was obtained which readily allowed visual discrimination between the ferritin standards and identification of sera with abnormally low ferritin concentrations. The response of the film was such that light emission from the 0 ng/ml standard produced virtually no exposure of the film and its response was indistinguishable from the black unexposed background. Increasing concentrations of ferritin produced higher intensity light emission and increasing partial exposure of the film (ie, larger areas of white exposed film). Almost complete exposure of the film was obtained with the 17.5 ng/ml ferritin standard. Concentrations of 20 ng/ml and above, produced full exposure of the film and were recorded as a white disc. The prolonged light emission from HRP-conjugate bound to the transparent microtitre plate enabled initiation of

Figure 6.13

A firefly luciferin enhanced chemiluminescent immunometric assay for a series of ferritin standards using instant photographic film to monitor light emission.

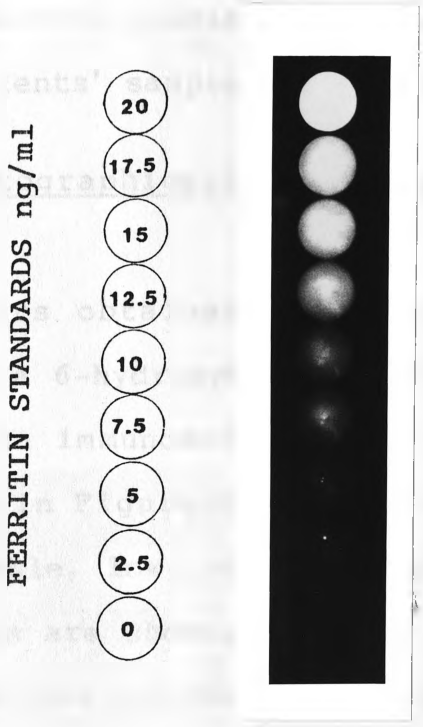
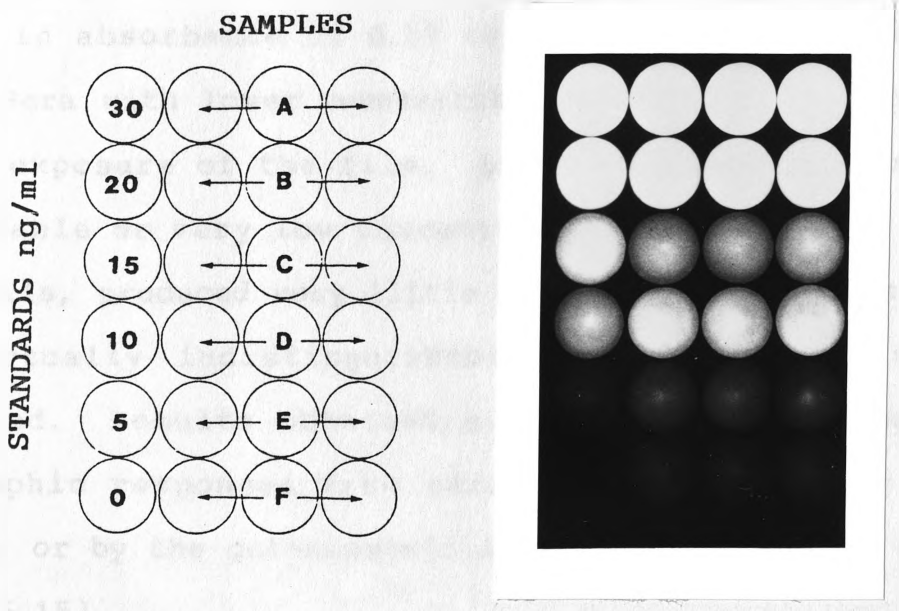


Figure 6.14

Analysis of a series of patients' samples and ferritin standards using a photographically monitored firefly luciferin enhanced chemiluminescent immunometric assay.



Final reagent concentrations: Luminol 1.25 mmol/l, H_2O_2 2.7 mmol/l, firefly luciferin 39 µmol/l (pH 8.0)

Samples were previously analysed by an immunoradiometric assay and assigned the following values: A, 47; B, 29; C, 18; D, 17; E, 9; F, 7 ng/ml

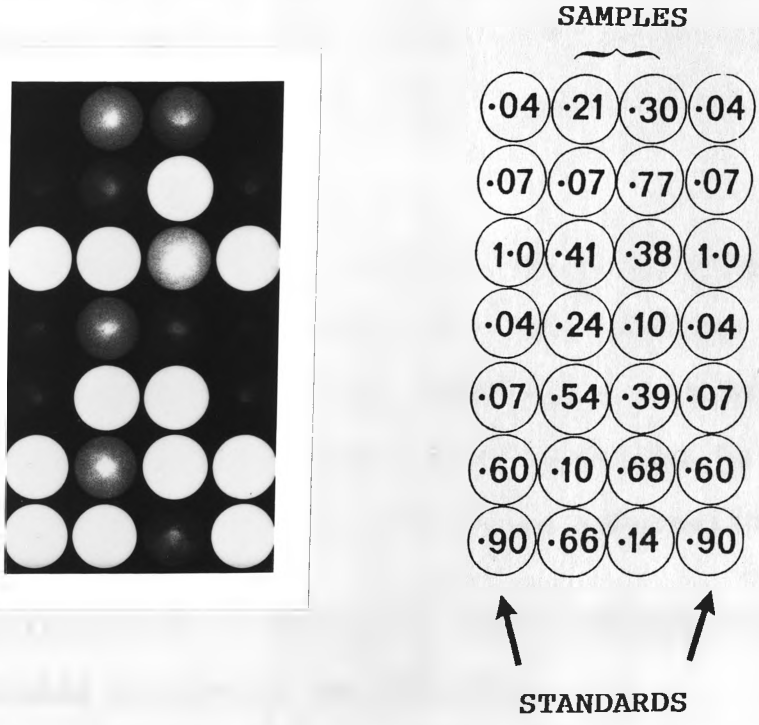
multiple reactions prior to exposure to the film and simultaneous analysis of several specimens. The application of the assay to a series of patients' samples is shown in Figure 6.14.

6.3.9 Photographically monitored assay of cytomegalovirus specific IgG

Typical results obtained using instant film to detect light emission from a 6-hydroxybenzothiazole enhanced chemiluminescent end-point in an immunometric assay for cytomegalovirus specific IgG are shown in Figure 6.15. The responses from control sera with undetectable, low, and high concentrations of antibodies to cytomegalovirus are shown, together with those from a series of patients' samples previously analysed with a colorimetric immunometric assay using an alkaline phosphatase label. Light emission from sera with high concentrations of antibody produced complete exposure of the film and were recorded as a white disc. Similar responses were obtained with all sera which produced changes in absorbance of 0.39 or greater in the colorimetric assay. Sera with lower concentrations of antibody produced only partial exposure of the film. Light emission from sera with undetectable or very low concentrations of antibody, and from blank wells, produced very little or no exposure of the film and were virtually indistinguishable from the black unexposed background. Results obtained either by visual comparison of photographic responses from sera and simultaneously analysed controls, or by the colorimetric assay were in general agreement (Figure 6.15).

Figure 6.15

A 6-hydroxybenzothiazole enhanced chemiluminescent immunometric assay for CMV specific IgG monitored by use of instant photographic film



Absorbance changes for standards and specimens in a colorimetric immunometric assay employing an alkaline phosphatase label are indicated.

Enhanced chemiluminescent end-point final reagent concentrations: Luminol 60 µmol/l, H_2O_2 2 mmol/l, 6-hydroxybenzothiazole 33 µmol/l (pH 8.5)

6.4 DISCUSSION

The four objectives detailed in section 6.1.1 were achieved. The novel enhanced chemiluminescent end-point has been shown to be a reliable replacement for conventional colorimetric assays in immunoassay. The enhanced end-points have been tested in immunometric and competitive immunoassays using beads, tubes or microtitre plates as solid support, and in rapid assays using short incubations with sample or HRP-conjugate. The enhanced chemiluminescent assays were successfully monitored using either luminometers based on photomultiplier tubes or instant photographic film.

Applications of enhanced chemiluminescence described here and in Chapter 5, and the identification and properties of enhancers described in Chapter 3, have formed the basis of patents and patent applications which have been assigned to the British Technology Group and licensed to Amersham International plc.

6.4.1 Incorporation of enhanced chemiluminescent end-points into commercially available immunoassays

Enhanced chemiluminescent assays for HRP-conjugates using 6-hydroxybenzothiazole derivatives (firefly luciferin, 6-hydroxybenzothiazole) or p-iodophenol were incorporated directly into a range of commercially available immunometric or competitive immunoassays. The assays employed either plastic beads or tubes as solid support and were for analytes of importance in clinical chemistry, microbiology or serology. The rapid enhanced chemiluminescent end-points, which could be simply initiated by addition of a single luminol-H_2O_2-enhancer mixture

and which could be performed in approximately 30 seconds, successfully replaced conventional colorimetric end-points using o-phenylenediamine or ABTS which involved 30 or 60 minute incubations respectively. Light emission catalysed by HRP-conjugate bound to the solid supports was prolonged, relatively stable and intense, and rapid, reproducible initiation of individual reactions directly in front of the photodetector was unnecessary.

Dose-response curves and correlation of results with those obtained using colorimetric end-points: Excellent dose-response curves were obtained in assays for IgE, rubella specific IgG, carcinoembryonic antigen, thyroxine, digoxin and hepatitis B surface antigen. Improvements in sensitivity, expressed as signal to background ratio, were achieved over those assays using colorimetric end-points. No attempt was made to further optimise enhanced chemiluminescent end-points for maximal sensitivity in individual systems. Good correlation was achieved between results determined using a colorimetric or an enhanced chemiluminescent end-point. No interference from components of clinical specimens was obtained in this limited study which involved analysis of over 150 clinical specimens. Subsequently, the enhanced chemiluminescent immunoassays for thyroxine and digoxin (Thorpe et al., 1984; Coyle et al., 1986) and an immunometric assay for human choriogonadotrophin (Thorpe et al., 1985) have successfully been used to analyse a series of unextracted clinical specimens. Again no interferences were encountered and excellent correlation with colorimetric results achieved ($r = 0.97$ ($n = 28$); 0.98 ($n = 34$); and 0.98 ($n = 38$),

respectively). Further extensive studies with clinical samples and comparison with results obtained using other techniques, would obviously be required for any enhanced chemiluminescently monitored assay prior to routine introduction into clinical laboratories. This is particularly so for competitive immunoassays where components of samples and the HRP labelled conjugate come into direct contact.

Kinetics of light emission and assay precision: Light emission using enhanced end-points to detect HRP-conjugate bound to either tubes or beads in assays for serum IgE or rubella specific IgG was prolonged and relatively constant. Under the reaction conditions employed, the kinetics of light emission appeared dependent on the concentration of conjugate, and the decay in light intensity was more pronounced at high concentrations of bound conjugate. Provided individual measurements were made at a fixed time after initiation, or rapid sequential measurements taken from small batches of simultaneously initiated reactions, effects due to changes in kinetics were minimised, and measurements could be made several minutes after initiation. Provided simple precautions were taken to ensure solid supports adopted a constant position relative to the photodetector, good within-batch precision of light intensity measurements from manually initiated reactions was achieved in assays for rubella specific IgG and carcinoembryonic antigen using beads, and in an assay for IgE performed using tubes, as solid support. Good within-batch precision was also achieved when enhanced chemiluminescent assays were initiated automatically in an

immunometric assay for carcinoembryonic antigen, even though several small bubbles could be seen on the bead surfaces.

Compatibility of solid supports and luminometers: Beads were compatible with both the available luminometers although it was only possible to monitor light emission from a fraction of their surface. Constant positioning of beads was achieved by the walls of square cuvettes in the WRL luminometer (Chapter 5, Section 5.2.3) and in the Auto-Biolumat LB 950 by the base of round bottomed tubes, the shape and dimensions of which ensured beads adopted and maintained a constant position in their base. Enhanced chemiluminescent assays for conjugate bound to tubes were easily initiated manually with minimal bubble formation. In conjunction with a reflective metal insert positioned in the cuvette holder, immunoassay tubes could be precisely located in the WRL luminometer. Tubes used in the IgE assay were well suited for chemiluminescent measurements, they were cylindrical, regular shaped, round bottomed with an optically smooth surface (Figure 6.1) and their entire active surface could be monitored by the photodetector. Tubes employed in thyroxine and digoxin immunoassays were less compatible with the WRL luminometer. Tubes appeared optically imperfect and possessed a recessed and complex shaped base (Figure 6.1). Despite modification of the support holder the base of the tube remained partially in front of the photomultiplier tube thus potentially influencing light detection. In addition, the tube dimensions were such that it was only possible to monitor light emission from 50% of the active surface. Despite these limitations, dose-response curves of excellent sensitivity and appropriate control values were

obtained, and subsequently acceptable within-batch precision of measurements has been demonstrated (Thorpe et al., 1984; Coyle et al., 1986). The tubes used in the immunoassays examined, could not be monitored with the Auto-Biolumat LB 950, as due to their size it was not possible to ensure they adopted a constant position relative to the photodetector. This, and other problems could easily be overcome by purpose designed immunoassay tubes.

Storage of solid supports prior to initiation of enhanced chemiluminescent end-points: Very rapid sequential measurement of light emission from large numbers of tubes or beads was not possible and although the available system could be used for relatively small batches of approximately 20 to 40 samples, it was not suitable for larger batches. This was due to a combination of a) the time taken to position supports in front of the detector and b) the decay in light emission from simultaneously initiated reactions during this period, under the end-point conditions used. Storage of the solid supports prior to label quantitation was therefore required. In order to prevent the surface of supports drying out and possible loss of enzyme activity during storage, it was necessary to keep active surfaces covered with buffer. Whether this involves addition of wash buffer, which is eventually removed, or addition of buffer to which enhanced chemiluminescent reagents can be directly added, loss of conjugate from surfaces during storage must be avoided. Dissociation of conjugate from the surface can result in either total loss of conjugate, or increasing quantities free in solution, which could produce drift in the measured signal.

Verification that appropriate storage conditions could be employed in commercial immunoassays using beads or tubes and that storage had no influence on light emission, would be required prior to routine introduction of enhanced chemiluminescent versions of such assays monitored with conventional luminometers.

6.4.2 Incorporation of enhanced chemiluminescent end-points into assays performed on microtitre plates

The feasibility of employing an enhanced chemiluminescent end-point to monitor immunoassays performed on microtitre plates was demonstrated in an assay for CMV specific IgG using black, opaque microtitre plates. A 6-hydroxybenzothiazole enhanced chemiluminescent end-point was employed and measurements made with a prototype microtitre plate luminometer. Microtitre plates are a widely used solid support which provide a convenient way of handling 96 samples simultaneously. Previous instrumentation for monitoring chemiluminescent immunoassays performed on transparent microtitre plates was complex and required sophisticated injection systems to rapidly initiate reactions directly in front of the photodetector (Schroeder et al., 1981; Schroeder and Vogelhut, 1984). Problems were also encountered due to transmission of light from partly spent reactions in adjacent wells. Such problems are easily overcome by the use of opaque microtitre plates and enhanced chemiluminescent reactions to quantitate bound HRP-conjugate. The prolonged and intense light emission achievable with enhanced reactions simplifies the design of manual or automatic microtitre plate luminometers which would be suitable for routine applications. Rapid and reproducible initiation of reactions directly in front of the photodetector is

unnecessary, therefore obviating the need for expensive injection systems or sophisticated detectors. Enhanced chemiluminescent end-points enabled reactions to be initiated outside the luminometer and, after several minutes, groups of measurements could be conveniently made with a single detector. The use of opaque microtitre plates eliminates carryover of light from neighbouring wells, and as positioning of individual wells under the detector and measurement of light intensity could be completed in approximately one second, rapid sequential measurements from a series of wells was possible. Reactions could be initiated simultaneously in 8 wells, and light intensity measured before significant decay of light emission had occurred. This procedure could then be rapidly repeated for remaining wells of the microtitre plate. Wells analysed last do have potentially longer to dry out, however the same initiation procedure has been successfully employed in a p-iodophenol enhanced immunometric assay for factor VIII related antigen (Wang et al., 1985a) and in a competitive immunoassay for progesterone in serum extracts (Wang et al., 1985b). Acceptable within-batch precision was obtained in both assays and the storage problems encountered with beads or tubes was eliminated.

Using 6-hydroxybenzothiazole as enhancer and under the conditions employed, light emission in the CMV specific IgG assay was prolonged, however, the intensity of light emission decayed to approximately 50% of its original value 15 minutes after initiation. The stability of this signal would have to be improved if sequential measurements were to be made from

reactions initiated simultaneously in all 96 wells of a microtitre plate. This has proved possible in the commercially available 'Amerlite' system which employs an enhanced chemiluminescent end-point based on reactions described in Chapter 3. Assays are performed in opaque microtitre plate wells which can be assembled in a 96 plate holder. Enhanced chemiluminescent reactions in all wells are initiated manually by addition of a single reagent mixture containing acyl hydrazide, oxidant and enhancer. Light emission stabilizes within 2 minutes and is emitted for a minimum of 20 minutes. Light intensity can be measured at any time during this period using an automated microtitre plate luminometer which will take measurements from 96 wells, automatically fit a dose-response curve to standards, interpolate unknowns and print out the assay result within 2 minutes (Edwards, 1985). The technique has been used in assays for thyroid stimulating hormone (with a range of 0.04 to 200 uIU/ml) and alpha-fetoprotein (0.5 to 750 IU/ml), which have within-batch precision of less than 5%.

Assays for total or free thyroxine, unconjugated oestriol, and ferritin have also been described, in which enhanced chemiluminescent end-points have been used to quantitate HRP-conjugate bound to antibody coated magnetisable latex particles contained in opaque microtitre plates (Holian et al., 1985).

6.4.3 Enhanced chemiluminescent quantitation of conjugates in rapid immunoassays

Enhanced chemiluminescent assays for HRP-conjugates which can be completed in under a minute have been successfully exploited in

several rapid, sensitive immunoassays. Simple replacement of colorimetric assays can save 30 to 60 minutes in overall assay time. Such reductions become extremely significant when the enhanced end-point is used in immunoassays which use short incubations with sample and conjugate. The ability of enhanced chemiluminescent end-points to allow rapid label quantitation whilst still achieving excellent sensitivity, has been demonstrated in several such assays. These include immunometric assays for hepatitis B surface antigen (2 x 30 minute incubations with beads as solid support), antibodies against cytomegalovirus (2 x 15 minutes with a microtitre plate as solid support), and a competitive immunoassay for digoxin (a single 45 minute incubation using tubes). Such rapid assays have tremendous potential for quantitation of microbiological agents, specific antibodies and other analytes.

In conjunction with luminometers capable of very rapid sequential measurements, and general improvements in immunoassay reagents and even shorter incubations with sample and conjugate, the enhanced chemiluminescent end-points should eventually enable development of assays for clinically important analytes which could be completed in 5 to 10 minutes. Small numbers of samples could easily be processed in such assays, however, large batches of samples would require some means of simultaneously starting or terminating incubations with sample and conjugate in order to avoid significant errors in the timing of incubation periods, introduced simply by the time taken to dispense samples or reagents.

6.4.4 Presentation of reagents employed in enhanced chemiluminescent end-points

As the reagents employed in enhanced assays are stable, low cost and not obviously hazardous they appear suitable for inclusion in commercial kits. The oxidants hydrogen peroxide and perborate are already used in numerous colorimetric kits. Although enhancers such as p-iodophenol have the general properties of phenol and are irritants, they are employed in extremely low concentrations and similar compounds such as phenol and 2,4-dichlorophenol have been widely used in coupled assays for quantitating hydrogen peroxide (Barham and Trinder, 1972; Gallati, 1977). Although mixtures of luminol, hydrogen peroxide and enhancer were generally prepared several hours before use, if stored in the dark at 4°C they were usable for several days, albeit with reduced peroxidase catalysed and background emission. A detailed study of the influence of the age of solutions, or the use of more stable oxidants such as perborate, on assay sensitivity has not been made. The readily soluble sodium salts of luminol and p-iodophenol can be used in enhanced reactions, facilitating the production of suitable reagents for kits. Reagents can be lyophilised or made as a simple tablet containing appropriate quantities, requiring only buffer addition.

6.4.5 Photographic monitoring of enhanced chemiluminescent immunoassays

Several enhanced chemiluminescent immunoassays performed on transparent microtitre plates were successfully monitored with simple, inexpensive instrumentation based on instant photographic film. The dramatic improvements in the levels of light emission

with enhanced reactions enabled low concentrations of HRP-conjugate to produce light intensities sufficiently high to easily allow the use of high speed (20,000 ASA) instant photographic film to monitor immunoassays. Light emission was also relatively constant for several minutes and therefore chemiluminescent reactions could be initiated prior to exposure to the film.

Instant film provides a rapid means of detecting light emission, generally requiring approximately 30 to 90 seconds exposure and 30 seconds development. Multiple samples can be assayed simultaneously and a permanent visual record of results is obtained. In addition, the equipment employed is portable, cheap, uncomplicated with few moving parts, and requires no external power source. Results of photographically monitored enhanced chemiluminescent immunoassays were interpreted visually by comparison of the degree of exposure from samples with that of controls or standards analysed at the same time. Assay conditions can be adjusted so that exposure of the film occurs only above a certain analyte concentration, thus making it ideal for screening purposes. Although the photographic record is a mirror image of samples on the microtitre plate, sample identification with multiple samples is possible with a simple pre-marked transparent overlay.

The feasibility of employing instant film to monitor enhanced chemiluminescent assays has been demonstrated in immunometric assays designed to identify sera with low ferritin concentrations, and an assay for antibody against

cytomegalovirus. In the ferritin assay the response of the film was such that it was completely exposed by samples with ferritin concentrations greater than 20 ng/ml. Below this level intermediate degrees of exposure were observed enabling samples with low levels of ferritin to be immediately identified. The ability to perform photographically monitored specific antibody assays was demonstrated using a 6-hydroxybenzothiazole enhanced end-point. Samples with no, or low levels of antibody produced partial exposure, whereas those with higher levels produced full exposure of the film. Ideally, in such assays, an antibody concentration below which sera are classified as negative should produce nearly complete exposure of the film. In this manner, samples with antibody levels below this concentration produce no or partial exposure of the film, and concentrations above the threshold produce full exposure. This has subsequently been achieved by incorporation of a p-iodophenol enhanced end-point which produces much more intense light emission and enables incubation times with sample and conjugate to be reduced to 30 minutes (Nickless et al., 1985). The practicality of the technique was also demonstrated in a blood transfusion service laboratory for identification of blood which was CMV antibody negative for transfusion into immunosuppressed patients. Photographically monitored p-iodophenol enhanced chemiluminescent end-points have also been incorporated into assays for factor VIII related antigen (Thorpe et al., 1985) and specific antibodies against rubella virus or malarial parasites (Kricka and Thorpe, 1986).

Enhanced chemiluminescent end-points and high speed film enable very short exposure times (often 30 seconds or less) to be used in immunoassays. This contrasts with the 30 minutes required to quantitate bound HRP-conjugate in a recently described assay for total IgE and allergen-specific IgE in which light emission from an unenhanced reaction, involving a patented cyclic diacyl hydrazide, is detected using Polaroid Type 57 film (Brown et al., 1985). The assay is peformed in a test-chamber which contains a parallel array of allergen-sensitised cellulose threads. After overnight and 4 hour incubations with serum and anti IgE-HRP conjugate respectively, bound conjugate is detected by addition of H_2O_2, cyclic diacyl hydrazide and a dye mixture which acts as a chemical mask. Glowing threads are exposed to 3000 ASA instant film for 30 minutes, and the amount of bound allergen-specific IgE determined from densitometric measurements on the developed print. Incorporation of enhancers into such systems should increase the intensity of light emission enabling significant reductions in both exposure time and incubation periods.

Unlike quantitation of light emission from luminescent immunoassays with photomultiplier tubes, photographic monitoring does not produce numerical results. Semi-quantitative or yes/no results are possible by visual interpretation of photographs. A further degree of quantitation could be achieved using neutral density filters, densitometry or by slightly separating the glowing solid support from the film surface so spot size is proportional to light intensity. This however complicates an essentially simple technique which appears best suited to applications, particularly outside specialised laboratories where

numerical results are not essential. Photographically monitored enhanced immunoassays could for example be used to determine the presence or absence of a specific analyte, to identify specimens with high or low analyte concentrations, or differentiate between levels in a limited concentration range.

Simple modifications could improve the enhanced photographic system. Camera luminometers could be constructed from lightweight plastic with the light-tight section manufactured as an integral part of the film holder. Such instruments could be used in conjunction with holders capable of accommodating individual microtitre plate wells or purpose constructed microtitre plates exactly fitting the film and designed to eliminate any possibility of interference from 'light piping' to neighbouring wells. Improvements in the sensitivity and dynamic range of the film may also be possible, and the availability of different film sizes would enable either analysis of larger numbers of samples or more economical use with small batches of samples.

Extension of photographically monitored enhanced chemiluminescent reactions into the detection of DNA probes or immunoassays performed on tubes, microparticles or dipsticks may eventually be possible.

6.4.6 Summary

The utility of 6-hydroxybenzothiazole derivative or para-substituted phenol enhanced chemiluminescent end-points has been demonstrated in a range of immunoassays, both immunoextraction

and competitive, and in conjunction with a variety of solid supports (beads, tubes and microtitre plates). Replacement of conventional colorimetric end-points with the simple enhanced procedure was not difficult. No interferences were detected with clinical samples and correlation between results obtained using an enhanced chemiluminescent or a colorimetric end-point was excellent in this limited study. The rapidity of an enhanced chemiluminescent end-point is a major advantage in immunoassay. Lengthy colorimetric end-points can be replaced as in the digoxin assay, and the sensitivity of the enhanced end-point allows the use of short incubation times with sample or peroxidase conjugate.

The prolonged and intense light emission achievable with enhanced reactions simplifies the design of manual or automatic instrumentation required for routine applications. Rapid and reproducible initiation of reactions directly in front of photodetectors is unnecessary, therefore obviating the need for expensive injection systems or sophisticated detectors. This is particuarly relevant for chemiluminescent immunoassays performed on microtitre plates. Previous instrumentation for this type of assay was complex but with enhanced chemiluminescence multiple reactions can be initiated manually outside the luminometer and after several minutes, groups of measurements can be conveniently made on batches of samples, with a single detector. The use of opaque microtitre plates eliminates carryover of light from neighbouring wells and enables rapid sequential measurements from a series of wells to be made. Similar assays employing the patented enhanced chemiluminescent end-point form the basis of

the commercially available 'Amerlite' system manufactured by Amersham International plc.

Although instant photographic film has been used previously to detect light emitted in a range of luminescent reactions so far its application in immunoassays has been limited. The intense and prolonged light emission from reactions enhanced by para-substituted phenols or 6-hydroxybenzothiazole derivatives is however particularly attractive for use in such immunoassays and extends the applicability of this simple and convenient approach to analysis. Multiple samples can be assayed rapidly and simultaneously with a camera luminometer which is inexpensive, small and portable and requires no power source. Reactions can be initiated manually before exposing the reaction mixture to the film and a permanent visual record of the semi-quantitative results is obtained. Although no numerical results are obtained, the response is acceptable for many clinical situations, particularly for an assay performed outside of specialised laboratories, when it is only necessary to know whether the analyte concentration lies outside a certain range.

The use of an enhancer facilitates rapid and sensitive chemiluminescent quantification of peroxidase conjugates and the enhancement technique should facilitate the introduction of chemiluminescently monitored immunoassays into clinical laboratories.

REFERENCES

Adediran, S.A. and Dunford, H.B. (1983) Structure of horseradish peroxidase compound I. Kinetic evidence for the incorporation of one oxygen atom from the oxidising substrate into the enzyme. Eur. J. Biochem., 132, 147-150.

Ahnstrom, G. and Nilsson, R. (1965) Activation of chemiluminescent oxidations catalysed by peroxidase and the differentiation of peroxidase agents. Acta Chemica Scand., 19, 313-316.

Akazawa, T. and Conn, E.E. (1958) The oxidation of reduced pyridine nucleotides by peroxidase. J. Biol. Chem., 232, 403-415.

Al-Awqati, M.A., Gordon, Y.B. and Chard, T. (1978) A simple and reliable method for the purification of human alphafetoprotein (AFP) from amniotic fluid and fetal livers. Clin. Chim. Acta, 89, 173-182.

Alder, F.L. and Lau, C.T. (1971) Detection of morphine by hemagglutination-inhibition. J. Immunol., 106, 1684-1685.

Alexander, P.W. and Maitra, C. (1982) Enzyme-linked immunoassay of human immunoglobulin G with the fluoride ion selective electrode. Anal. Chem., 54, 68-71.

Anaokar, S., Korom, G. and Fries, P. (1982) Two rapid enzyme immunoassays for human chorionic gonadotropin (HCG). Clin. Chem., 28, 1623.

Arakawa, H., Maeda, M. and Tsuji, A. (1985) Chemiluminescence enzyme immunoassay for thyroxin with use of glucose oxidase and a bis(2,4,6-trichlorophenyl)oxalate-fluorescent dye system. Clin. Chem., 31, 430-434.

Arakawa, H., Maeda, M. and Tsuji, A. (1982) Chemiluminescence enzyme immunoassay of 17α-hydroxyprogesterone using glucose oxidase and bis(2,4,6-trichlorophenyl) oxalate-fluorescent dye system. Chem. Pharm. Bull. Tokyo, 30, 3036-3039.

Arakawa, H., Maeda, M., Tsuji, A. and Kambegawa, A. (1981) Chemiluminescence enzyme immunoassay of dehydroepiandrosterone and its sulfate using peroxidase as label. Steroids, 38, 453-464.

Arakawa, H., Maeda, M. and Tsuji, A. (1979) Chemiluminescence enzyme immunoassay of cortisol using peroxidase as label. Anal. Biochem., 97, 248-254.

Arrio, B., Lecuyer, B., Dupaix, A., Volfin, P., Jousset, M. and Carrette, A. (1980) Design of a chemiluminescent and bioluminescent photometer. Biochimie, 62, 445-453.

Artiss, J.D., Thibert, R.J., McIntosh, J.M. and Zak, B. (1981) Study of various substrates for peroxidase-coupled peroxide oxidations. Microchem. J., 26, 487-505.

Augusto, O. and Bechara, E.J.H. (1980) Hemin-catalysed generation of triplet acetone. Biochim. Biophys. Acta, 631, 203-209.

Aviram, I. (1981) The interaction of benzohydroxamic acid with horseradish peroxidase and its fluorescent analogs. Arch. Biochem. Biophys., 212, 483-490.

Barham, D. and Trinder, P. (1972) An improved colour reagent for the determination of blood glucose by the oxidase system. Analyst, 97, 142-145.

Barnard, G.J.R., Kim, J.B. and Williams, J.L. (1985) Chemiluminescence immunoassays and immunochemiluminometric assays. In Alternative Immunoassays (Collins, W.P., Ed.) John Wiley and Sons Ltd., pp 123-152.

Barnard, G.J., Kim, J.B., Brockelbank, J.L., Collins, W.P., Gaier, B. and Kohen, F. (1984) Measurement of chorio-gonadotropin by chemiluminescence immunoassay and immuno-chemiluminometric assay: 1. Use of isoluminol derivatives. Clin. Chem., 30, 538-541.

Barnard, G., Collins, W.P., Kohen, F. and Lindner, H.R. (1981a) A preliminary study of the measurement of urinary pregnanediol-3α-glucuronide by a solid phase chemi-luminescence immunoassay. In Bioluminescence and Chemiluminescence. Basic Chemistry and Analytical Applications (DeLuca, M.A. and McElroy, W.D., Eds.) Academic Press, New York, pp. 311-317.

Barnard, G., Collins, W.P., Kohen, F. and Lindner, H.R. (1981b) The measurement of urinary estriol-16α-glucuronide by a solid phase chemiluminescence immunoassay. J. Steroid Biochem., 14, 941-948.

Bause, D.E. and Patterson, H.H. (1979) Enhancement of luminol chemiluminescence with halide ions. Anal. Chem., 51, 2288-2289.

Becvar, J.E. (1981) Autolumography: using bioluminescence to 'stain' for enzyme activities resolved on polyacrylamide gels. In Bioluminescence and Chemiluminescence. Basic Chemistry and Analytical Applications (DeLuca, M.A. and McElroy, W.D., Eds.) Academic Press, New York, pp. 583-589.

Berkowitz, D.B. and Webert, D.W. (1981) The inactivation of horseradish peroxidase by a polystyrene surface. J. Immunol. Methods, 47, 121-124.

Berthold, F. and Kubisiak, H. (1982) Automatic instrumentation for chemi- and bioluminescence assays. In Luminescent Assays: Perspectives in Endocrinology and Clinical Chemistry (Serio, M. and Pazzagli, M., Eds.) Raven Press, New York, pp. 23-28.

Boguslaski, R.C., Carrico, R.J. and Christner, J.E. (1983) Heterogenous chemiluminescent specific binding assay. U.S. Patent 4380580.

Boguslaski, R.C. and Schroeder, H.R. (1981) Immunoassays monitored by chemilumigenic labels. In Bioluminescence and Chemiluminescence. Basic Chemistry and Analytical Applications (DeLuca, M.A. and McElroy, W.D., Eds.) Academic Press, New York, pp. 283-286.

Boguslaski, R.C. and Carrico, R.J. (1980) Fluorescor-labelled specific binding assay. U.K. Patent Applic. 2044449.

Boitieux, J.-L., Desmet, G. and Thomas, D. (1979) An 'antibody electrode', preliminary report on a new approach in enzyme immunoassay. Clin. Chem., 25, 318-321.

Boitieux, J.L., Lemay, C., Desmet, G. and Thomas, D. (1981) Use of solid phase biochemistry for potentiometric enzyme immunoassay of oestradiol-17β-preliminary report. Clin. Chim. Acta, 113, 175-182.

Boorsma, D.M. and Streefkerk, J.G. (1979) Periodate or glutaraldehyde for preparing peroxidase conjugates. J. Immunol. Methods, 30, 245-255.

Botti, R., Leoncini, P., Tarli, P. and Neri, P. (1982) A chemiluminescent system used to increase the sensitivity in enzyme immunoassay. In Luminescent Assays: Perspectives in Endocrinology and Clinical Chemistry (Serio, M.and Pazzagli, M., Eds.) Raven Press, New York, pp. 163-168.

Botti, R., Leoncini, P., Neri, P. and Tarli, P. (1981) Chemiluminescence enzyme-immunoassay of human chorionic somatomammotropin. J. Clin. Chem. Clin. Biochem., 19, 623.

Bowie, L.J. (1978) Synthesis of firefly luciferin and structural analogs. In Methods in Enzymology Vol. LVII, Bioluminescence and Chemiluminescence (DeLuca, M.A., Ed.) Academic Press, New York, pp. 15-28.

Brockelbank, J.L., Kim, J.B., Barnard, G.J., Collins, W.P., Gaier, B. and Kohen, F. (1984) The measurement of urinary LH, by a solid phase chemiluminescence immunoassay. Ann. Clin. Biochem., 21, 284-289.

Brolin, S.E., Berggren, P.-O. and Naeser, P. (1984) Application of light guides for enhancement of signal to noise ratios at low levels of luminescence detectability. In Analytical Applications of Bioluminescence and Chemiluminescence (Kricka, L.J., Stanley, P.E., Thorpe, G.H.G. and Whitehead, T.P., Eds.) Academic Press, New York, pp. 479-482.

Brooks, J.L. (1983) Stimulation of peroxidase reactions by hydroxamates. Biochem. Biophys. Res. Comm., 116, 916-921.

Brown, C.R., Higgins, K.W., Frazer, K., Schoelz, L.K., Dyminski, J.W., Marinkovich, V.A., Miller, S.P. and Burd, J.F. (1985) Simultaneous determination of total IgE and allergen-specific IgE in serum by the MAST chemiluminescent assay system. Clin. Chem., 31, 1500-1505.

Bunce, R.A., Thorpe, G.H.G., Gibbons, J.E.C., Killeen, P.R., Ogden, G., Kricka, L.J. and Whitehead, T.P. (1985) Camera luminometer for use with luminescent assays. Analyst, 110, 657-663.

Bunce, R.A., Carter, T.J.N., Kricka, L.J. and Whitehead, T.P. (1980) Determination of concentration of an analyte in a sample. U.K. Patent 2025609.

Burdo, T.G. and Seitz, W.R. (1975) Mechanism of cobalt catalysis of luminol chemiluminescence. Anal. Chem., 47, 1639-1643.

Cais, M., Dani, S., Eden, Y., Gandolfi, O., Horn, M., Isaacs, E.E., Josephy, Y., Saar, Y., Slivin, E. and Snarsky, L. (1977) Metalloimmunoassay. Nature, 270, 534-535.

Caldini, A.L., Orlando, C., Barni, T., Messeri, G., Pazzagli, M., Baldi, E. and Serio, M. (1986) Measurement of transferrin in human seminal plasma by a chemiluminescent method. Clin. Chem., 32, 153-156.

Campbell, A.K. and Patel, A. (1983) A homogeneous immunoassay for cyclic nucleotides based on chemiluminescence energy transfer. Biochem. J., 216, 185-194.

Campbell, A.K. and Simpson, J.S.A. (1979) Chemi- and bioluminescence as an analytical tool in biology. Techniques in Metabolic Research, B213, 1-56.

Cantarero, L.A., Butler, J.E. and Osborne, J.W. (1980) The adsorptive characteristics of proteins for polystyrene and their significance in solid-phase immunoassays. Anal. Biochem., 105, 375-382.

Carrico, R.J. and Boguslaski, R.C. (1984) An immunoassay for sisomicin monitored by peroxyoxalate-induced chemiluminescence. In Clinical Immunochemistry. Principles of Methods and Applications (Boguslaski, R.C., Maggio, E.T. and Nakamura, R.M.N., Eds.) Little Brown and Company, Boston/Toronto, pp. 169-180.

Carrico, R.J., Johnson, R.D. and Boguslaski, R.C. (1978) ATP-labelled ligands and firefly luciferase for monitoring specific protein-binding reactions. In Methods in Enzymology Vol. LVII, Bioluminescence and Chemiluminescence (DeLuca, M.A., Ed.) Academic Press, New York, pp. 113-122.

Carrico, R.J., Christner, J.E., Boguslaski, R.C. and Yeung, K.K. (1976) A method for monitoring specific binding reactions with cofactor labelled ligands. Anal. Biochem., 72, 271-282.

Carlsson, J., Drevin, H. and Axen, R. (1978) Protein thiolation and reversible protein-protein conjugation. N-succinimidyl 3-(2-pyridyldithio)propionate, a new heterobifunctional reagent. Biochem. J., 173, 723-737.

Carter, T.J.N., Whitehead, T.P. and Kricka, L.J. (1982) Investigation of a novel solid-phase chemiluminescent analytical system, incorporating photographic detection for the measurement of glucose. Talanta, 29, 529-531.

Cavanagh, L.A. (1975) Photographic detection and integration of light emitted from luminescent reactions. U.S. Patent 3923462.

Chance, B., Powers, L., Ching, Y., Poulos, T., Schonbaum, G.R., Yamazaki, I. and Paul, K.G. (1984) X-ray absorption studies of intermediates in peroxidase activity. Arch. Biochem. Biophys., 235, 596-611.

Chance, B. (1951) The iron-containing enzymes. C. The enzyme-substrate compounds and mechanism of action of the hydroperoxidases. In The Enzymes, Chemistry and mechanism of action, Vol II (Sumner, J.B. and Myrback, K., Eds.) Academic Press, New York, pp. 428-453.

Chance, B. (1949) The properties of the enzyme-substrate compounds of horseradish peroxidase. III. The reaction of complex II with ascorbic acid. Arch. Biochem., 24, 389-409.

Chandler, H.M. and Hurrell, J.G.R. (1982) A new enzyme immunoassay system suitable for field use and its application in a snake venom detection kit. Clin. Chim. Acta, 121, 225-230.

Childs, R.E. and Bardsley, W.G. (1975) The steady-state kinetics of peroxidase with 2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. Biochem. J., 145, 93-103.

Claiborne, A. and Fridovich, I. (1979) Chemical and enzymatic intermediates in the peroxidation of o-dianisidine by horseradish peroxidase. 2. Evidence for a substrate radical-enzyme complex and its reaction with nucleophiles. Biochemistry, 18, 2329-2335.

Clark, B.R. and Engvall, E. (1980) Enzyme-linked immunosorbent assay (ELISA): Theoretical and practical aspects. In Enzyme-immunoassay (Maggio, E.T., Ed.) CRC Press, Florida, pp. 167-179.

Collins, W.P., Barnard, G.J., Kim, J.B., Weerasekera, D.A., Kohen, F., Eshhar, Z. and Lindner, H.R. (1983) Chemi-luminescence immunoassays for plasma steroids and urinary steroid metabolites. In Immunoassays for Clinical Chemistry (Hunter, W.M. and Corrie, J.E.T., Eds.) Churchill Livingstone, Edinburgh, pp. 373-397.

Cormier, M.J., Lee, J. and Wampler, J. (1975) Bioluminescence: Recent advances. An. Rev. Biochem., 44, 255-272.

Cormier, M.J., Wampler, J.E. and Hori, K. (1973) Bio-luminescence:chemical aspects. Progr. Chem. Org. Nat. Prob., 30, 1-53.

Cormier, M.J. and Prichard, P.M. (1968) An investigation of the mechanism of the luminescent peroxidation of luminol by stopped flow techniques. J. Biol. Chem., 243, 4706-4714.

Coyle, P.M., Thorpe, G.H.G., Kricka, L.J. and Whitehead, T.P. (1986) Enhanced luminescent quantitation of horseradish peroxidase conjugates: application in an enzyme immunoassay for digoxin. Ann. Clin. Biochem., 23, 42-46.

Damerau, W., Lassmann, G. and Flemming, C. (1973) Nachiveis radikalischer reaktionen an tragergebundener peroxidase. Studia Biophysica, 35, 39-44.

De Boever, J., Kohen, F., Vandekerckhove, D. and van Maele, G. (1984) Solid phase chemiluminescence immunoassay for progesterone in unextracted serum. Clin. Chem., 30, 1637-1641.

De Boever, J., Kohen, F. and Vandekerckhove, D. (1983) Solid-phase chemiluminescence immunoassay for plasma estradiol -17βduring gonadotropin therapy compared with two radio-immunoassays. Clin. Chem., 29, 2068-2072.

Dunford, H.B. (1982) Peroxidases. In Advances in Inorganic Biochemistry (Eichhorn, G.L. and Marzilli, L.G., Eds.) Elsevier Biomedical, New York, pp. 41-68.

Duran, N., Baeza, J., Freer, J., Brunet, J.E., Gonzalez, G.A., Sotomayor, C.P. and Faljoni-Alario, A. (1981) Dimethyl sulfoxide as chemical and biological probe: conformational effect on peroxidase systems. Biochem. Biophys. Res. Comm., 103, 131-138.

Dure, L.S. and Cormier, M.J. (1963) Studies on the bio-luminescence of Balanoglossus biminiensis extracts. II Evidence for the peroxidase nature of balanoglossid luciferase. J. Biol. Chem., 238, 790-793.

Edwards, J. (1985) The light touch. Med. Lab. World, 35-37.

Ekins, R.P. (1985) Current concepts and future developments. In Alternative Immunoassays (Collins, W.P., Ed.) John Wiley and Sons Ltd., pp 219-237.

Ekins, R. (1981a) Towards immunoassays of greater sensitivity, specificity and speed: An overview. In Monoclonal antibodies and developments in immunoassay (Albertini, A. and Ekins, R., Eds.) Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 3-21.

Ekins, R. (1981b) Merits and disadvantages of different labels and methods of immunoassay. In Immunoassays for the 80's (Voller, A., Bartlett, A. and Bidwell, D., Eds.) MTP Press, England, pp. 5-16.

Eshhar, Z., Kim, J.B., Barnard, G., Collins, W.P., Gilad, S., Lindner, H.R. and Kohen, F. (1981) Use of monoclonal antibodies to pregnanediol-3α-glucuronide for the development of a solid phase chemiluminescence immuno-assay. Steroids, 38, 89-109.

Ewetz, L. and Thore, A. (1976) Factors affecting the specificity of the luminol reaction with hematin compounds. Anal. Biochem., 71, 564-570.

Forgione, P.S. and Henderson, W.A. (1983) Heterogeneous chemiluminescent immunoassays utilising metallo porphyrin tag. U.S. Patent 4375972.

Fox, L.R. and Purves, W.K. (1968) Mechanism of enhancement of IAA oxidation by 2,4-dichlorophenol. Plant Physiol., 43, 454-456.

Fricke, H., Strasburger, C.J. and Wood, W.G. (1982) Enzyme enhanced luminescence immunoassay for the determination of transferrin concentrations in serum. J. Clin. Chem. Clin. Biochem., 20, 91-94.

Fridovich, I. (1963) The stimulation of horseradish peroxidase by nitrogenous ligands. J. Biol. Chem., 238, 3921-3927.

Gadow, A., Strasburger, C.J. and Wood, W.G. (1984) Optimisation of macro-solid-phase luminescence immunoassays using covalently immobilised antigens and antibodies. In Analytical Applications of Bioluminescence and Chemiluminescence (Kricka, L.J., Stanley, P.E., Thorpe, G.H.G. and Whitehead, T.P., Eds.) Academic Press, New York, pp. 205-208.

Gallardo, H., Guillo, L.A., Duran, N. and Cilento, G. (1984) Catalysis of the peroxidase-mediated oxidation of aldehydes by enolphosphates. Biochim. Biophys. Acta, 789, 57-62.

Gallati, von H. and Brodbeck, H. (1982) Peroxidase aus meerrettich:Kinetische studien und optimierung der aktivitatsbestimmung mit den substraten H_2O_2 und o-phenylenediamin. J. Clin. Chem. Clin. Biochem., 20, 221-225.

Gallati, von H. (1977) Aktivitatsbestimmung von peroxidase mit hilfe des 'Trinder Reagens'. J. Clin. Chem. Clin. Biochem., 15, 699-703.

Gorus, F. and Schram, E. (1979) Applications of bio- and chemiluminescence in the clinical laboratory. Clin. Chem., 25, 512-519.

Grachev, M.A., Dobrikov, M.I., Knorre, V.D., Pressman, E.K., Roschke, V.V. and Shishkin, G.V. (1983) A new system for ATP-metric immunoanalysis. FEBS Lett., 162, 266-269.

Grayeski, M.L. and Woolf, E. (1984) Enhancement of lucigenin chemiluminescence with cyclodextrin. In Analytical Applications of Bioluminescence and Chemiluminescence (Kricka, L.J., Stanley, P.E., Thorpe, G.H.G. and Whitehead, T.P., Eds.) Academic Press, New York, pp. 565-568.

Green, K., Kricka, L.J., Thorpe, G.H.G. and Whitehead, T.P. (1984) Rapid assays based on immobilized bioluminescent enzymes and photographic detection of light emission. Talanta, 31, 173-176.

Groome, N.P. (1980) Superiority of ABTS over Trinder reagent as chromogen in highly sensitive peroxidase assays for enzyme linked immunoadsorbent assay. J. Clin. Chem. Clin. Biochem., 18, 345-349.

Gross, G.G., Janse, C. and Elstner, E.F. (1977) Involvement of malate, monophenols, and the superoxide radical in hydrogen peroxide formation by isolated cells walls from horseradish (Armoracia lapathifolia Gilib.) Planta, 136, 271-276.

Gundermann, K.-D., Wulff, K., Linke, R. and Stahler, F. (1982) Enzyme immunoassays using horseradish peroxidase as a label assayed by chemiluminescence with luminol derivatives as substrates. In Luminescent Assays: Perspectives in Endocrinology and Clinical Chemistry (Serio, M. and Pazzagli, M., Eds.) Raven Press, New York, pp. 157-161.

Gundermann, K.-D. (1981) Chemiluminescence. In Bioluminescence and Chemiluminescence. Basic Chemistry and Analytical Applications (DeLuca, M.A. and McElroy, W.D., Eds.) Academic Press, New York, pp. 17-19.

Gundermann, K. (1980) Verfahren und reagenz zur bestimmung der peroxidase. Deutsches Patentant, 2906732.

Gupta, D., Moxter, C. Seibold, J., Frick, H.-J., Blum, W., Attanasio, A., Christner, J. and Jung, G. (1985) Chemiluminescence immunoassay of the pineal hormone melatonin in child serum. Neuroendocrinol. Lett., 7, 1-10.

Halliwell, B. (1978) Lignin synthesis: The generation of hydrogen peroxide and superoxide by horseradish peroxidase and its stimulation by manganese (II) and phenols. Planta, 140, 81-88.

Halmann, M., Gabor, G. and Velan, B. (1981) Peroxidase induced chemiluminescence in the oxidation of pyrogallol and purpurogallin. Influence of azide. Photochem. Photobiol., 34, 95-99.

Halmann, M., Velan, B., Sery, T. and Schupper, H. (1979) Peroxidase mediated chemiluminescence with phenol derivatives. Physicochemical parameters and uses in biological assays. Photochem. Photobiol., 30, 165-167.

Halmann, M., Velan, B. and Sery, T. (1977) Rapid identification and quantitation of small numbers of microorganisms by a chemiluminescent immunoreaction. Appl. Environ. Microbiol., 34, 473-477.

Hara, T., Toriyama, M. and Imaki, M. (1985) Immunoassay using a metal-complex compound as a chemiluminescent catalyst. V. Continuous immunoassay by the use of CLCCIA. Bull. Chem. Soc. Japan, 58, 1299-1303.

Hara, T., Toriyama, M. and Tsukagoshi, K. (1983a) Immunoassay using a metal-complex compound as a chemiluminescent catalyst. II. An improvement of the analytical method for practical use. Bull. Chem. Soc. Japan, 56, 2965-2968.

Hara, T., Toriyama, M. and Tsukagoshi, K. (1983b) Immunoassay using a metal-complex compound as a chemiluminescent catalyst. I. Iron(III)phthalocyanine as a labeling reagent. Bull. Chem. Soc. Japan, 56, 2267-2271.

Herrmann, J.E., Hendry, R.M. and Collins, M.F. (1979) Factors involved in enzyme-linked immunoassay of viruses and evaluation of the method for identification of enteroviruses. J. Clin. Microbiol., 10, 210-217.

Hill, P.G., O'Toole, A., Kricka, L.J., Carter, T.J.N. and Whitehead, T.P. (1981) Optimisation of an automated enzyme immunoassay for α-fetoprotein using polystyrene beads as solid phase. J. Clin. Chem. Clin. Biochem., 19, 699-700.

Holian, J., Edwards, J.C., Martin, J.K. and Charles, S.A. (1985) Assay method. Eur. Patent Applic. 0149565.

Hoshino, N., Hama, M., Suzuki, R., Kataoka, Y. and Soe, G. (1985) A new homogeneous enzyme immunoassay. Its application to measurement of α-fetoprotein. J. Biochem., 97, 113-118.

Hosoya, T., Fujii, T. and Ogawa, S. (1983) A molecular orbital study on the oxidation of hydrogen donor molecules by peroxidase compound II., J. Theor. Biol., 100, 283-292.

Hughes, J., Short, F. and James, V.H.T. (1984) Synthesis of a novel bioluminescent conjugate of progesterone for immunoassay. In Analytical Applications of Bioluminescence and Chemiluminescence (Kricka, L.J., Stanley, P.E., Thorpe, G.H.G. and Whitehead, T.P., Eds.) Academic Press, New York, pp. 269-272.

Ikariyama, Y., Suzuki, S. and Aizawa, M. (1984) Solid phase luminescent catalyst immunoassay for human serum albumin with hemin as labeling catalyst. Anal. Chim. Acta, 156, 245-252.

Ikariyama, Y., Suzuki, S. and Aizawa, M. (1983) Luminescence catalyst immunoassay of β_2-microglobulin with haemin as a chemically amplifiable label. Enzym. Microb. Technol., 5, 215-218.

Ikariyama, Y., Suzuki, S. and Aizawa, M. (1982) Luminescence immunoassay of human serum albumin with hemin as labeling catalyst. Anal. Chem., 54, 1126-1129.

Imagawa, M., Hashida, S., Ohta, Y. and Ishikawa, E. (1984) Evaluation of β-D-galactosidase from Escherichia coli and horseradish peroxidase as labels by sandwich enzyme immunoassay technique. Ann. Clin. Biochem., 21, 310-317.

Isacsson, U. and Wettermark, G. (1974) Chemiluminescence in analytical chemistry. Anal. Chim. Acta, 68, 339-362.

Ishikawa, E. (1980) Enzyme-labelling of antigens and antibodies for quantitative enzyme immunoassay. In Immunoassay:Supplement 1, University of Sheffield Biomedical Information Service.

Jablonski, E. (1985) The preparation of bacterial luciferase conjugates for immunoassay and application to rubella antibody detection. Anal. Biochem., 148, 199-206.

Jansen, E.H.J.M., Laan, C.A., van den Berg, R.H., Stephany, R.W. and Zomer, G. (1985a) A solid-phase chemiluminescence immunoassay for 17 α-methyltestosterone. Anal. Chim. Acta, 170 29-33.

Jansen, E.H.J.M., van den Berg, R.H., Zomer, G. and Stephany, R.W. (1985b) A chemiluminescent immunoassay for 17 α-methyltestosterone. Food Add. Contamin., 2, 47-53.

Job, D. and Dunford, H.B. (1976) Substituent effect on the oxidation of phenols and aromatic amines by horseradish peroxidase compound I. Eur. J. Biochem., 66, 607-614.

Josephy, P.D., Eling, T. and Mason, R.P. (1982a) The horseradish peroxidase-catalysed oxidation of 3,5,3',5'-tetramethylbenzidine. Free radical and charge-transfer complex intermediates. J. Biol. Chem., 257, 3669-3675.

Josephy, P.D., Mason, R.P. and Eling, T. (1982b) Cooxidation of the clinical reagent 3,5,3',5'-tetramethylbenzidine by prostaglandin synthase. Cancer Res., 42, 2567-2570.

Kalyanaraman, B., Mason, R.P. and Sivarajah, K. (1982) An electron spin resonance study of a novel radical cation produced during the horseradish peroxidase-catalysed oxidation of tetramethylhydrazine. Biochem. Biophys. Res. Comm., 105, 217-224.

Kelly, T.A. and Christian, G.D. (1982) Homogeneous enzymatic fluorescence immunoassay of serum IgG by continuous flow-injection analysis. Talanta, 29, 1109-1112.

Kennedy, J.H. (1979) Preparation and use of enzyme conjugates in clinical chemistry. Thesis submitted for the degree of Doctor of Philosophy at the University of Birmingham (August 1979).

Kim, J.B., Barnard, G.J., Collins, W.P., Kohen, F., Lindner, H.R. and Eshhar, Z. (1982a) Measurement of plasma estradiol-17β by solid phase chemiluminescence immunoassay. Clin. Chem., 28, 1120-1124.

Kim, J.B., Kohen, F., Lindner, H.R. and Collins, W.P. (1982b) Measurement of plasma progesterone by a solid-phase chemiluminescence immunoassay method. In Luminescent Assays: Perspectives in Endocrinology and Clinical Chemistry (Serio, M. and Pazzagli, M., Eds.) Raven Press, New York, pp. 201-206.

Klebanoff, S.J. (1959) An effect of tyroxine and related compounds on the oxidation of certain hydrogen donors by the peroxidase system. J. Biol. Chem., 234, 2437-2442.

Klinger, W., Postel, G. von, Haupt, O. and Knuppen, R. (1982) Chemiluminescence immunoassay (CIA) of unconjugated oestriol in serum of pregnant women. Fresenius Z. Anal. Chem., 311, 352-353.

Kobayashi, T., Saga, K., Shimizu, S. and Goto, T. (1981) The application of chemiluminescence of a Cypridina luciferin analog to immobilized enzyme sensors. Agric. Biol. Chem., 45, 1403-1408.

Kohen, F., Pazzagli, M., Serio, M., DeBoever, J. and Vandekerckhove, D. (1985) Chemiluminescence and bioluminescence immunoassay. In Alternative Immunoassays (Collins, W.P., Ed.) John Wiley and Sons Ltd., pp 103-121.

Kohen, F., Kim, J.B., Lindner, H.R. and Collins, W.P. (1981) Development of a solid phase chemiluminescence immunoassay for plasma progesterone. Steroids, 38, 73-88.

Kohen, F., Kim, J.B., Barnard, G. and Lindner, H.R. (1980a) An assay for urinary estriol-16α-glucuronide based on antibody-enhanced chemiluminescence. Steroids, 36, 405-419.

Kohen, F., Pazzagli, M., Kim, J.B. and Lindner, H.R. (1980b) An immunoassay for plasma cortisol based on chemiluminescence. Steroids, 36, 421-437.

Kohen, F., Pazzagli, M., Kim, J.B., Lindner, H.R. and Boguslaski, R.C. (1979) An assay procedure for plasma progesterone based on antibody enhanced chemiluminescence. FEBS Lett., 104, 201-205.

Konishi, E., Iwasa, S., Kondo, K. and Hori, M. (1980) Chemiluminescence-linked immunoassay for detection of mumps virus antibodies. J. Clin. Microbiol., 12, 140-143.

Kricka, L.J. and Thorpe, G.H.G. (1986) Enhanced chemiluminescent enzyme immunoassays. Parasitology Today, 2, 123-125.

Kricka, L.J. Ligand-binder assays. Labels and analytical strategies. New York, Marcel Dekker Inc., 1985.

Kricka, L.J. and Thorpe, G.H.G. (1983) Chemiluminescent and bioluminescent methods in analytical chemistry. Analyst, 108, 1274-1296.

Kricka, L.J. and Carter, T.J.N. (1982) Luminescent immunoassays. In Clinical and Biochemical Luminescence (Kricka, L.J. and Carter, T.J.N., Eds.) Marcel Dekker Inc., New York, pp. 153-178.

Kricka, L.J. and DeLuca, M. (1982) Effects of solvents on the catalytic activity of firefly luciferase. Arch. Biochem. Biophys., 217, 674-681.

Leute, R.L., Ullman, E.F., Goldstein, A. and Herzenberg, L.A. (1972) Spin immunoassay technique for determination of morphine. Nature New Biology, 236, 93-94.

Leuvering, J.H.W., Thal, P.J.H.M., van der Waart, M. and Schuurs, A.H.W.M. (1980) Sol particle immunoassay (SPIA). J. Immunoassay, 1, 77-91.

Lindstrom, L., Meurling, L. and Lovgren, T. (1982) The measurement of serum cortisol by a solid phase chemiluminescence immunoassay. J. Steroid Biochem., 16, 577-580.

Longeon, A., Henry, J.P. and Henry, R. (1986) A solid-phase luminescent immunoassay for human chorionic gonadotropin using Pholas dactylus bioluminescence. J. Immunol. Methods, 89, 103-109.

MacDonald, D.J. and Kelly, A.M. (1978) The rapid quantitation of serum alpha-fetoprotein by two-site micro enzyme immunoassay. Clin. Chim. Acta, 87, 367-372.

Mader, M. and Amberg-Fisher, V. (1982) Role of peroxidase in lignification of tobacco I. Oxidation of nicotinamide adenine dinucleotide and formation of hydrogen peroxide by cell wall peroxidases. Plant Physiol., 70, 1128-1131.

Mader, M. and Fussl, R. (1982) Role of peroxidase in lignification of tobacco cells II. Regulation by phenol compounds. Plant Physiol., 70, 1132-1134.

Maggio, E.T. (1980) Enzymes as immunochemical labels. In Enzyme-immunoassay (Maggio, E.T., Ed.) CRC Press, Florida, pp. 53-70.

Maier, C.L. Jr. (1977) A method for the detection and analysis of small quantities of organic materials in body fluids. Belgian Patent 856182.

Makela, O. (1966) Assay of anti-hapten antibody with the aid of hapten-coupled bacteriophage. Immunology, 10, 81-86.

Marino, D.F. and Ingle, J.D. (1981) Microprocessor-based data acquisition system for chemiluminescence measurements. Anal. Chem., 53, 1175-1179.

Marklund, S. (1978) Determination of plasma or serum haemoglobin by peroxidase activity employing 2,2'-azino-di-(3-ethyl-benzthiazolinsulphonate-6) as chromogen. Scand. J. Clin. Lab. Invest., 38, 543-547.

Marklund, S., Ohlsson, P.-I., Opara, A. and Paul, K.-G. (1974) The substrate profiles of the acidic and slightly basic horseradish peroxidases. Biochim. Biophys. Acta, 350, 304-313.

Mattiasson, B. and Borrebaeck, C. (1980) Novel approaches to enzyme-immunoassay. In Enzyme-Immunoassay (Maggio, E.T., Ed.) CRC Press, Florida, pp. 213-248.

Mattoo, A.K. and Modi, V.V. (1975) Palmitic acid activation of peroxidase and its possible significance in mango ripening. Biochim. Biophys. Acta, 397, 318-330.

McCapra, F. and Leeson, P.D. (1979) Possible mechanism for bacterial bioluminescence and chemiluminescence. J. Chem. Soc. Chem. Comm., 3, 114-117.

McCapra, F. (1976) Chemical mechanisms in bioluminescence. Accounts of Chemical Research, 9, 201-208.

Merenyi, G. and Lind, J.S. (1980) Role of a peroxide intermediate in the chemiluminescence of luminol. A mechanistic study. J. Am. Chem. Soc., 102, 5830-5835.

Messeri, G., Caldini, A.L., Bolelli, G.F., Pazzagli, M., Tommasi, A., Vannucchi, P.L. and Serio, M. (1984) Homogeneous luminescence immunoassay for total estrogens in urine. Clin. Chem., 30, 653-657.

Messeri, G., Pazzagli, M., Kim, J.B., Martinazzo, G. and Serio, M. (1981) Luminescence immunoassay of cortisol in plasma and urine. J. Clin. Chem. Clin. Biochem., 19, 771.

Michelson, A.M. (1978) Purification and properties of Pholas dactylus luciferin and luciferase. In Methods in Enzymology Vol. LVII, Bioluminescence and Chemiluminescence (DeLuca, M.A., Ed.) Academic Press, New York, pp. 385-406.

Michelson, A.M. and Isambert, M.F. (1973) Studies in bioluminescence. XI. Further studies on the Pholas dactylus system. Mechanism of luciferase. Biochimie, 55, 619-634.

Miles, L.E.M. and Hales, C.N. (1968) Labelled antibodies and immunological assay systems. Nature, 219, 186-189.

Miller, T.E., Odstrchel, G. and Takeguchi, M. (1985) A 'sandwich' chemiluminescent assay for serum ferritin with paramagnetic particles as the solid phase. Clin. Chem., 31, 911.

Miller, Jr. R.J. and Reichard, D.W. (1980) Chemiluminescent immunoreactive assay (CLIA) for rapid detection of Venezuelan equine encephalomyelitis (VEE) virus. Fed. Proc., 39, 919.

Misra, H.P. and Squatrito, P.M. (1982) The role of superoxide anion in peroxidase-catalysed chemiluminescence of luminol. Arch. Biochem. Biophys., 215, 59-65.

Naveh, D., Mizrahi, S. and Kopelman, I.J. (1981) Quantitative determination of peroxidase in sweet corn by chemiluminescence. J. Agric. Food Chem., 29, 36-38.

Neufeld, H.A., Conklin, C.J. and Towner, R.D. (1965) Chemiluminescence of luminol in the presence of hematin compounds. Anal. Biochem., 12, 303-309.

Ngo, T.T. and Lenhoff, H.M. (1981) Recent advances in homogeneous and separation-free enzyme immunoassays. Appl. Biochem. Biotech., 6, 53-64.

Ngo, T.T. and Lenhoff, H.M. (1980) A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. Anal. Biochem., 105, 386-397.

Nickless, G.G., Thorpe, G.H.G., Kricka, L.J., Whitehead, T.P., Wells, L.J. and Ala, F.A. (1985) A rapid chemiluminescent enzyme-linked immunosorbent assay for cytomegalovirus immunoglobulin G antibodies using instant photographic film. J. Virol. Methods, 12, 313-321.

Ohnishi, T., Yamazaki, H., Iyanagi, T., Nakamura, T. and Yamazaki, I. (1969) One-electron-transfer reactions in biochemical systems II. The reaction of free radicals formed in the enzymic oxidation. Biochim. Biophys. Acta, 172, 357-369.

Olsson, T., Bergstrom, K. and Thore, A. (1982) A sensitive method for determination of serum hemoglobin based on iso-luminol chemiluminescence. Clin. Chim. Acta, 122, 125-133.

Olsson, T., Bergstrom, K. and Thore, A. (1981) Determination of hemoglobin and other hemoproteins in serum by luminol chemiluminescence. In Bioluminescence and Chemiluminescence: Basic Chemistry and Analytical Applications (De Luca, M.A. and McElroy, W.D., Eds.) Academic Press, New York, pp. 659-666.

Olsson, T. and Thore, A. (1981) Chemiluminescence and its use in immunoassay. In Immunoassays for the 80s (Voller, A., Bartlett, A. and Bidwell, D., Eds.) MTP Press, Lancaster, pp. 113-125.

Olsson, T., Thore, A., Carlsson, H.E., Brunius, G. and Eriksson, G. (1979a) Quantitation of immunological reactions by luminescence. In Analytical Applications of Bioluminescence and Chemiluminescence (Schram, E. and Stanley, P., Eds.) State Printing and Publishing Inc., California, pp. 421-430.

Olsson, T., Brunius, G., Carlsson, H.E. and Thore, A. (1979b) Luminescence immunoassay (LIA): A solid phase immunoassay monitored by chemiluminescence. J. Immunol. Methods, 25, 127-135.

Paleos, C.M., Vassilopoulos, G. and Nikokavouras, J. (1981) Chemiluminescence of lucigenin in micellar systems. In Bioluminescence and Chemiluminescence. Basic Chemistry and Analytical Applications (DeLuca, M.A. and McElroy, W.D., Eds.) Academic Press, New York, pp. 729-734.

Patel, A. and Campbell, A.K. (1983) Homogeneous immunoassay based on chemiluminescence energy transfer. Clin. Chem., 29, 1604-1608.

Pazzagli, M., Bolelli, G.F., Messeri, G., Martinazzo, G., Tommasi, A., Salerno, R. and Serio, M. (1982) Homogeneous luminescent immunoassay for progesterone: A study on the antibody-enhanced chemiluminescence. In Luminescent Assays: Perspectives in Endocrinology and Clinical Chemistry) Serio, M. and Pazzagli, M., Eds.) Raven Press, New York, pp. 191-200.

Pazzagli, M., Kim, J.B., Messeri, G., Kohen, F., Bolelli, G.F., Tommasi, A., Salerno, R., Moneti, G. and Serio, M. (1981a) Luminescent immunoassay (LIA) of cortisol-1. Synthesis and evaluation of two chemiluminescent labels of cortisol. J. Steroid Biochem., 14, 1005-1012.

Pazzagli, M., Kim, J.B., Messeri, G., Kohen, F., Bolelli, G.F., Tommasi, A., Salerno, R. and Serio, M. (1981b) Luminescent immunoassay (LIA) of cortisol-2. Development and validation of the immunoassay monitored by chemiluminescence. J. Steroid Biochem., 14, 1181-1187.

Pazzagli, M., Kim, J.B., Messeri, G., Martinazzo, G., Kohen, F., Franceschetti, F., Moneti, G., Salerno, R., Tommasi, A. and Serio, M. (1981c) Evaluation of different progesterone-isoluminol conjugates for chemiluminescence immunoassay. Clin. Chim. Acta, 115, 277-286.

Pazzagli, M., Kim, J.B., Messeri, G., Martinazzo, G., Kohen, F., Franceschetti, F., Tommasi, A., Salerno, R. and Serio, M. (1981d) Luminescent immunoassay (LIA) for progesterone in a heterogeneous system. Clin. Chim. Acta, 115, 287-296.

Peng, C.T. (1976) Chemiluminescence. In Liquid Scintillation: Science and Technology (Noujaim, A.A., Ediss, C. and Weibe, L.I., Eds.) Academic Press, London, pp. 315.

Place, J.D. and Schroeder, H.R. (1982) The fixation of anti-HB_sAg on plastic surfaces. J. Immunol. Methods, 48, 251-260.

Porstmann, B., Porstmann, T., Nugel, E. and Evers, U. (1985) Which of the commonly used marker enzymes gives the best results in colorimetric and fluorimetric enzyme immunoassays. Horseradish peroxidase, alkaline phosphatase or β-galactosidase. J. Immunol. Methods, 79, 27-37.

Porstmann, B., Porstmann, T. and Nugel, E. (1981a) Comparison of chromogens for the determination of horseradish peroxidase as a marker in enzyme immunoassay. J. Clin. Chem. Clin. Biochem., 19, 435-439.

Porstmann, B., Porstmann, T., Gaede, D., Nugel, E. and Egger, E. (1981b) Temperature dependent rise in activity of horseradish peroxidase caused by non-ionic detergents and its use in enzyme-immunoassay. Clin. Chim. Acta, 109, 175-181.

Pratt, J.J., Woldring, M.G. and Villerius, L. (1978) Chemi-luminescence-linked immunoassay. J. Immunol. Methods, 21, 179-184.

Pronovost, A.D. and Baumgarten, A. (1982) Comparison of chemi-luminescence and absorptiometry in enzyme immunoassays for protein quantitation. Experientia, 38, 304-306.

Pronovost, A.D., Baumgarten, A. and Andiman, W.A. (1982) Chemiluminescent immunoenzymatic assay for rapid diagnosis of viral infections. J. Clin. Microbiol., 16, 345-349.

Pronovost, A.D., Baumgarten, A. and Hsiung, G.D. (1981) Sensitive chemiluminescent enzyme-linked immunosorbent assay for quantification of human immunoglobulin G and detection of herpes simplex virus. J. Clin. Microbiol., 13, 97-101.

Puget, K., Michelson, A.M. and Avrameas, S. (1977) Light emission techniques for the microestimation of femtogram levels of peroxidase. Anal. Biochem., 79, 447-456.

Rauhut, M.M. (1979) Chemiluminescence. In Kirk-othmer Encycl. Chem. Technol. 3rd Ed. Vol. 5 (Graysen, M. and Echroth, D., Eds.) Wiley, New York, pp. 416-450.

Rauhut, M.M., Semsel, A.M. and Roberts, B.G. (1966) Reaction rates, quantum yields and partial mechanism for the chemiluminescent reaction of 3-aminophthalhydrazide with aqueous alkaline hydrogen peroxide and persulfate. J. Org. Chem., 31, 2431-2436.

Rawlins, T. and Peacock, R. (1982) Design principles for a modern luminometer. Internat. Lab., 49-56.

Reichard, D.W. and Miller, R.J. Jr. (1981) Bioluminescent immunoassay: A new enzyme-linked analytical method for the quantitation of antigens. In Bioluminescence and Chemiluminescence. Basic Chemistry and Analytical Applications (DeLuca, M.A. and McElroy, W.D., Eds.) Academic Press, New York, pp. 667-672.

Reimann, L. and Schonbaum, G.R. (1978) Purification of plant peroxidases by affinity chromatography. In Methods in Enzymology Vol. LII, Biomembranes (Fleischer, S. and Packer, L., Eds.) Academic Press, New York, pp. 514-521.

Riad-Fahmy, D., Read, G.F. and Turkes, A. (1983) Enzyme immunoassays for synthetic contraceptive steroids in plasma and saliva. In Immunoassays for Clinical Chemistry (Hunter, W.M. and Corrie, J.E.T., Eds.) Churchill Livingstone, Edinburgh pp. 358-372.

Rich, Jr. E.S., Groover, C.H. and Wampler, J.E. (1981) The spatial distribution of light emission from liquid phase bio- and chemiluminescence: variations with container types, turbidity and container frosting. Photochem. Photobiol., 33, 727-736.

Richardson, A.P., Kim, J.B., Barnard, G.J., Collins, W.P. and McCapra, F. (1985) Chemiluminescence immunoassay for plasma progesterone with progesterone-acridinium ester used as the labeled antigen. Clin. Chem., 31, 1664-1668.

Roda, A., Girotti, S., Lodi, S. and Preti, S. (1984a) Development of a sensitive enzyme immunoassay for plasma and salivary steroids. Talanta, 31, 895-900.

Roda, A., Girotti, S., Lodi, S., Preti, S. and Piancentini, A. (1984b) Development of a sensitive direct solid-phase competitive enzyme luminescent assay for serum and salivary cortisol. In Analytical Applications of Bioluminescence and Chemiluminescence (Kricka, L.J., Stanley, P.E., Thorpe, G.H.G. and Whitehead, T.P., Eds.) Academic Press, New York, pp. 227-231.

Roswell, D.F. and White, E.H. (1978) The chemiluminescence of luminol and related hydrazides. In Methods in Enzymology Vol. LVII, Bioluminescence and Chemiluminescence (DeLuca, M.A., Ed.) Academic Press, New York, pp. 409-423.

Ruan, K., Hashida, S., Yoshitake, S., Ishikawa, E., Wakisaka, O., Yamamoto, Y., Ichioka, T. and Nakajima, K. (1986) A more sensitive and less time-consuming sandwich enzyme immunoassay for insulin in human serum with less serum interference. Ann. Clin. Biochem., 23, 54-58.

Sasson, L., Sharabani, M. and Aviram, I. (1982) The oxidation of naphthalene sulphonate dyes by horse radish peroxidase. Arch. Biochem. Biophys., 217, 529-535.

Saunders, G.C. (1979) The art of solid-phase enzyme immunoassay including selected protocols. In Immunoassays in the clinical laboratory (Nakamura, R.M., Dito, W.R. and Tucker III, E.S., Eds.) Alan R. Liss Inc., New York, pp. 99-118.

Sawahata, T. and Neal, R.A. (1982) Horseradish peroxidase-mediated oxidation of phenol. Biochem. Biophys. Res. Comm., 109, 988-994.

Schall, Jr. R.F. and Tenoso, H.J. (1981) Alternatives to radioimmunoassay: Labels and methods. Clin. Chem., 27, 1157-1164.

Schram, E. (1981) Present and future applications of luminescence assays in clinical chemistry. In Monoclonal antibodies and developments in immunoassay (Albertini, A. and Ekins, R., Eds.) Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 123-134.

Schroeder, H.R. and Vogelhut, P.O. (1984) Instrumentation for measurement of low level luminescence. In Clinical Immunochemistry. Principles of Methods and Applications (Boguslaski, R.C., Maggio, E.T. and Nakamura, R.M.N., Eds.) Little Brown and Company, Boston/Toronto, pp. 181-197.

Schroeder, H.R. and Vogelhut, P.O. (1983) Luminescence detection device. Eur. Patent Applic. 0071859.

Schroeder, H.R., Hines, C.M., Osborn, D.D., Moore, R.P., Hurtle, R.L., Wogoman, F.F., Rogers, R.W. and Vogelhut, P.O. (1981) Immunochemiluminometric assay for hepatitis B surface antigen. Clin. Chem., 27, 1378-1384.

Schroeder, H.R., Yeager, F.M., Boguslaski, R.C. and Vogelhut, P.O. (1979) Immunoassay for serum thyroxine monitored by chemiluminescence. J. Immunol. Methods, 25, 275-282.

Schroeder, H.R., Boguslaski, R.C., Carrico, R.J. and Buckler, R.T. (1978) Monitoring specific protein-binding reactions with chemiluminescence. In Methods in Enzymology Vol. LVII, Bioluminescence and Chemiluminescence (DeLuca, M.A., Ed.) Academic Press, New York, pp. 424-445.

Schroeder, H.R. and Yeager, F.M. (1978) Chemiluminescence yields and detection limits of some isoluminol derivatives in various oxidation systems. Anal. Chem., 50, 1114-1120.

Schroeder, H.R., Carrico, R.J., Boguslaski, R.C. and Christner, J. (1976a) Specific binding reactions monitored with ligand-cofactor conjugates and bacterial luciferase. Anal. Biochem., 72, 283-292.

Schroeder, H.R., Vogelhut, P.O., Carrico, R.J., Boguslaski, R.C and Buckler, R.T. (1976b) Competitive protein binding assay for biotin monitored by chemiluminescence. Anal. Chem., 48, 1933-1937.

Schuurs, A.H.W.M. and van Weemen B.K. (1977) Enzyme-immunoassay. Clin. Chim. Acta, 81, 1-40.

Seitz, W.R. (1984) Immunoassay labels based on chemiluminescence and bioluminescence. Clin. Biochem., 17, 120-125.

Seitz, W.R. (1981) Chemiluminescence and bioluminescence analysis: Fundamentals and biomedical applications. CRC Crit. Rev. Anal. Chem., 13, 1-58.

Seitz, W.R. and Neary, M.P. (1977) Chemiluminescence and bioluminescence analysis. Contemp. Topics Anal. Clin. Chem., 1, 49-125.

Selgren, S.F. and Gantzer, M.L. (1982) Stimulation of the peroxidative reaction of hemoglobin by nitrogen heterocycles. Clin. Chem., 28, 1600.

Shiga, T. and Imaizumi, K. (1975) Electron spin resonance study on peroxidase - and oxidase - reactions of horse radish peroxidase and methemoglobin. Arch. Biochem. Biophys., 167, 469-479.

Siddiqi, I.W. (1982) An electrochemical assay system for peroxidase and peroxidase-coupled reactions based on a fluoride ion-selective electrode. Clin. Chem., 28, 1962-1967.

Simpson, J.S.A., Campbell, A.K., Ryall, M.E.T. and Woodhead, J.S. (1979) A stable chemiluminescent-labelled antibody for immunological assays. Nature, 279, 646-647.

Slawinska, D., Slawinski, J., Pukacki, W. and Polewski, K. (1979) Chemiluminescent method for determination of peroxidase. In Analytical Applications of Bioluminescence and Chemiluminescence (Schram, E. and Stanley, P., Eds.) State Printing and Publishing Inc., California, pp. 239-257.

Stanley, P.E. (1982) Instrumentation. In Clinical and Biochemical Luminescence (Kricka, L.J. and Carter, T.J.N., Eds.) Marcel Dekker Inc., New York, pp. 219-260.

Stanley, P.E. (1979) Luminescence in clinical analysis. In Analytical Applications of Bioluminescence and Chemiluminescence (Schram, E. and Stanley, P., Eds.) State Printing and Publishing Inc., California, pp. 89-108.

Stanley, P.E. (1974) Analytical assays using the liquid scintillation spectrometer: A review. In Liquid Scintillation Counting (Crook, M.A. and Johnson, P., Eds.) Heyden, London, pp. 253-271.

Stevens, P., Berdischewsky, M. and Huang, N. (1980) Chemiluminescence enzyme immunoassay for the detection of cytoplasmic protein antigens of candida albicans. In Immunoassays. Clinical Laboratory Techniques for the 1980s (Nakamura, R.M., Dito, W.R. and Tucker III, E.S., Eds.) Alan R. Liss Inc., New York, pp. 205-212.

Stout, R.L. (1985) Catalysed colorimetric and fluorometric substrates for peroxidase enzyme determinations. U.S. Patent 4521511.

Strasburger, C.J., Fricke, H. and Wood, W.G. (1982) Development and optimisation of a chemiluminescent immunoassay (CLIA) for serum and urinary cortisol. Fresenius Z. Anal. Chem., 311, 351-352.

Straus, W. (1982) Imidazole increases the sensitivity of the cytochemical reaction for peroxidase with diaminobenzidine at a neutral pH. J. Histochem. Cytochem., 30, 491-493.

Takayama, K. and Nakano, M. (1977) Mechanism of thyroxine-mediated oxidation of reduced nicotinamide adenine dinucleotide in peroxidase-H_2O_2 system. Biochemistry, 16, 1921-1926.

Takayanagi, M. and Yashiro, T. (1984) Colorimetry of hemoglobin in plasma with 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Clin. Chem., 30, 357-359.

Takayasu, S., Maeda, M. and Tsuji, A. (1985) Chemiluminescent enzyme immunoassay using β-D-galactosidase as the label and the bis(2,4,6-trichlorophenyl)oxalate-fluorescent dye system. J. Immunol. Methods, 83, 317-325.

Tamura, M. and Yamazaki, I. (1972) Reactions of the oxyform of horseradish peroxidase. J. Biochem., 71, 311-319.

Terouanne, B. Nicolas, J.C. and Crastes de Paulet, A. (1986) Bioluminescent immunoassay for α-fetoprotein. Anal. Biochem., 154, 132-137.

Thorpe, G.H.G., Kricka, L.J., Moseley, S.B. and Whitehead, T.P. (1985) Phenols as enhancers of the chemiluminescent horseradish peroxidase-luminol-hydrogen peroxide reaction: Application in luminescence-monitored enzyme immunoassays. Clin. Chem., 31, 1335-1341.

Thorpe, G.H.G, Gillespie, E., Haggart, R., Kricka, L.J. and Whitehead, T.P. (1984) An immunoassay for serum thyroxine employing enhanced luminescent quantitation of horseradish peroxidase conjugates. In Analytical Applications of Bioluminescence and Chemiluminescence (Kricka, L.J., Stanley, P.E., Thorpe, G.H.G. and Whitehead, T.P., Eds.) Academic Press, pp. 243-248.

Tijssen, P. and Kurstak, E. (1984) Highly efficient and simple methods for the preparation of peroxidase and active peroxidase-antibody conjugates for enzyme immunoassays. Anal. Biochem., 136, 451-457.

Tommasi, A., Pazzagli, M., Damiani, M., Salerno, R., Messeri, G., Magini, A. and Serio, M. (1984) On-line computer analysis of chemiluminescent reactions with application to a luminescent immunoassay for free cortisol in urine. Clin. Chem., 30, 1597-1602.

Tsang, V.C.W., Wilson, B.C. and Maddison, S.E. (1980) Kinetic studies of a quantitative single-tube enzyme-linked immunosorbent assay. Clin. Chem., 26, 1255-1260.

Tsuji, A., Maeda, M. and Arakawa, H. (1984) Enzyme immunoassay monitored by chemiluminescence reaction using bis(2,4,6-trichlorophenyl)oxalate-fluorescent dye. In Analytical Applications of Bioluminescence and Chemiluminescence (Kricka, L.J., Stanley, P.E., Thorpe, G.H.G. and Whitehead, T.P., Eds.) Academic Press, New York, pp. 253-256.

Tsuji, A., Maeda, M., Arakawa, H., Matsuoka, K., Kato, N., Naruse, H. and Irie, M. (1978) Enzyme immunoassay of hormones and drugs by using fluorescence and chemiluminescence reaction. In International Symposium on Enzyme-Labeled Immunoassay of Hormones and Drugs (Pal, S.B., Ed.) Walter de Gruyter, Berlin, pp. 327-339.

Ugarova, N.N. and Lebedeva, O.V. (1978) Structure and functions of horseradish peroxidase. Biokhimiya, 43, 1731-1742.

Ullman, E.F., Schwarzberg, M. and Rubenstein, K.E. (1976) Fluorescent excitation transfer immunoassay. J. Biol. Chem., 251, 4172-4178.

Vannucchi, P.L., Messeri, G., Bolelli, G.F., Pazzagli, M., Masala, A. and Serio, M. (1983) A solid phase chemiluminescent immunoassay (LIA) for testosterone glicuronide in diluted urines. J. Steroid Biochem., 18, 625-629.

Velan, B., Schupper, H., Sery, T. and Halmann, M. (1979) Solid phase chemiluminescent immunoassay. In Analytical Applications of Bioluminescence and Chemiluminescence (Schram, E. and Stanley, P., Eds.) State Printing and Publishing Inc., California, pp. 431-437.

Velan, B. and Halmann, M. (1978) Chemiluminescence immunoassay; A new sensitive method for determination of antigens. Immunochemistry, 15, 331-333.

Voller, A. and Bidwell, D.E. (1976) Enzyme-immunoassays for antibodies in measles, cytomegalovirus infections and after rubella vaccination. Br. J. Exp. Pathol., 57, 243-247.

Wang, H.X., George, J., Thorpe, G.H.G., Stott, R.A., Kricka, L.J. and Whitehead, T.P. (1985a) Enhanced luminescence enzyme immunoassay for factor VIII related antigen. J. Clin. Pathol., 38, 317-319.

Wang, H.X., Stott, R.A., Thorpe, G.H.G., Kricka, L.J., Holder, G. and Rudd, B.T. (1985b) An enhanced chemiluminescence enzyme immunoassay for serum progesterone. Steroids, 44, 317-328.

Wannlund, J. and DeLuca, M. (1982) A sensitive bioluminescent immunoassay for dinitrophenol and trinitrotoluene. Anal. Biochem., 122, 385-393.

Wannlund, J., Egghart, H. and DeLuca, M. (1982) Bioluminescent immunoassays: A model system for detection of compounds at the attomole level. In Luminescent Assays: Perspectives in Endocrinology and Clinical Chemistry (Serio, M. and Pazzagli, M., Eds.) Raven Press, New York, pp. 125-128.

Wannlund, J and DeLuca, M. (1981) Bioluminescent Immunoassays: Use of luciferase-antigen conjugates for determination of methotrexate and DNP. In Bioluminescence and Chemiluminescence. Basic Chemistry and Analytical Applications (DeLuca, M.A. and McElroy, W.D., Eds.) Academic Press, New York, pp. 693-696.

Wannlund, J., Azari, J., Levine, L. and DeLuca, M. (1980) A bioluminescent immunoassay for methotrexate at the subpicomole level. Biochem. Biophys. Res. Comm., 96, 440-446.

Ward, W.W. and Cormier, M.J. (1978) Protein-protein interactions as measured by bioluminescence energy transfer in Renilla. In Methods in Enzymology Vol. LVII, Bioluminescence and Chemiluminescence (DeLuca, M.A., Ed.) Academic Press, New York, pp. 257-267.

Watson, R.A.A., Landon, J., Shaw, E.J. and Smith, D.S. (1976) Polarization fluoroimmunoassay of gentamicin. Clin. Chim. Acta, 73, 51-55.

Webster, J.J., Taylor, P.B. and Leach, F.R. (1981) Studies on the sensitivity of ATP determination using commercial firefly luciferase. In Bioluminescence and Chemiluminescence. Basic Chemistry and Analytical Applications (DeLuca, M.A. and McElroy, W.D., Eds.) Academic Press, New York, pp. 497-504.

Weeks, I., Beheshti, I., McCapra, F., Campbell, A.K. and Woodhead, J.S. (1983a) Acridinium esters as high-specific-activity labels in immunoassay. Clin. Chem., 29, 1474-1479.

Weeks, I., Campbell, A.K. and Woodhead, J.S. (1983b) Two-site immunochemiluminometric assay for human α_1-fetoprotein. Clin. Chem., 29, 1480-1483.

Weeks, I., McCapra, F., Campbell, A.K. and Woodhead, J.S. (1983c) Immunoassays using chemiluminescent labelled antibodies. In Immunoassays for Clinical Chemistry (Hunter, W.M. and Corrie, J.E.T., Eds.) Churchill Livingstone, Edinburgh, pp. 525-530.

Weerasekera, D.A., Kim, J.B., Barnard, G.J., Collins, W.P., Kohen, F. and Lindner, H.R. (1982) Monitoring ovarian function by a solid phase chemiluminescence immunoassay. Acta Endocrinologica, 101, 254-263.

Weerasekera, D.A., Kim, J.B., Barnard, G.J. and Collins, W.P. (1983a) Measurement of serum thyroxine by solid-phase chemiluminescence immunoassay. Ann. Clin. Biochem., 20, 100-104.

Weerasekera, D.A., Koullapis, E.M., Kim, J.B., Barnard, G.J., Collins, W.P., Kohen, F. and Lindner, H.R. (1983b) Chemiluminescence immunoassay of thromboxane B_2. In Advances in Prostaglandin, Thromboxane, and Leukotriene Research, Vol. 11 (Samuelsson, B., Paoletti, R. and Ramwell, P., Eds.) Raven Press, New York, pp. 185-190.

Whitaker, J.R. and Tappel, A.L. (1962) Modification of enzymic activity. II. Effect of salts on α-amylase, alcohol dehydrogenase, peroxidase and hematin catalysis. Biochim. Biophys. Acta, 62, 310-317.

White, E.H. and Roswell, D.F. (1985) Luminol chemiluminescence. In Chemi- and Bioluminescence (Burr, J.G., Ed.) Marcel Dekker Inc., New York, pp. 215-244.

Whitehead, T.P., Kricka, L.J., Carter, T.J.N. and Thorpe, G.H.G. (1979) Analytical luminescence: Its potential in the clinical laboratory. Clin. Chem., 25, 1531-1546.

Wisdom, G.B. (1976) Enzyme-immunoassay. Clin. Chem., 22, 1243-1255.

Wong, J.K. and Salin, M.L. (1981) Quenching of peroxidized luminol chemiluminescence by reduced pyridine nucleotides. Photochem. Photobiol., 33, 737-740.

Wood, W.G., Hantke, U. and Gross, A.J. (1985) Initiation of luminol-based luminescence by injection of a single reagent, coupled with enhancement of light output. J. Clin. Chem. Clin. Biochem., 23, 47-49.

Wood, W.G. (1984) Luminescence immunoassays: Problems and possibilities. J. Clin. Chem. Clin. Biochem., 23, 47-49.

Wood, W.G., Fricke, H., Haritz, J., Gadow, A., Krausz, H.S., Tode, B., Strasburger, C.J. and Scriba, P.C. (1984) An evaluation of four different luminescence immunoassay systems: CELIA (chemiluminescent immunoassay), SPALT (solid-phase antigen luminescence technique), ILMA (immunoluminometric assay) and ILSA (immunoluminometric labelled second antibody). J. Clin. Chem. Clin. Biochem., 22, 349-356.

Wood, W.G., Fricke, H., Klitzing, L. von., Strasburger, C.J. and Scriba, P.C. (1982) Solid phase antigen luminescent immunoassays (SPALT) for the determination of insulin, insulin antibodies and gentamicin levels in human serum. J. Clin. Chem. Clin. Biochem., 20, 825-831.

Woodhead, J.S. and Weeks, I. (1985) Chemiluminescence immunoassay. Pure and Appl. Chem., 57, 523-529.

Woodhead, J.S., Simpson, J.S.A. and Campbell, A.K. (1979) Detecting or quantifying substances using labelling techniques. U.K. Patent Applic. GB 2008247A.

Wynberg, H., Meijer, E.W. and Hummelen, J.C. (1981) 1,2-Dioxetanes as chemiluminescent probes and labels. In Bioluminescence and Chemiluminescence. Basic Chemistry and Analytical Applications (DeLuca, M.A. and McElroy, W.D., Eds.) Academic Press, New York, pp. 687-689.

Yamada, M., Nakada, T. and Suzuki, S. (1983) The determination of sulfite in a flow injection system with chemiluminescence detection. Anal. Chim. Acta, 147, 401-404.

Yamazaki, I. (1977) Free radicals in enzyme-substrate reactions. In Free Radicals in Biology Vol III (Pryor, W.A., Ed.) Academic Press, New York, pp. 183-218.

Yamazaki, I. and Piette, L.H. (1963) The mechanism of aerobic oxidase reaction catalysed by peroxidase. Biochim. Biophys. Acta, 77, 47-64.

Yamazaki, I., Mason, H.S. and Piette, L. (1960) Identification, by electron paramagnetic resonance spectroscopy, of free radicals generated from substrates by peroxidase. J. Biol. Chem., 235, 2444-2449.

Yalow, R.S. and Berson, S.A. (1959) Assay of plasma insulin in human subjects by immunological methods. Nature, 184, 1648-1649.

Yolken, R.H. and Leister, F.J. (1981) Investigaton of enzyme immunoassay time courses: Development of rapid assay systems. J. Clin. Microbiol., 13, 738-741.

Yurow, H.W. and Sass, S. (1977) Structure-chemiluminescence correlations for various organic compounds with luminol-peroxide. Anal. Chim. Acta, 88, 389-394.

Zaitsu, K. and Ohkura, Y. (1980) New fluorogenic substrates for horseradish peroxidase: Rapid and sensitive assays for hydrogen peroxide and peroxidase. Anal. Biochem., 109, 109-113.

Zaitsu, K., Nakashima, K., Akiyama, S. and Ohkura, Y. (1982a) Rapid enzyme immunoassay for human choriogonadotropin in serum using horseradish peroxidase as a label. Chem. Pharm. Bull. Tokyo, 30, 4114-4119.

Zaitsu, K., Nakashima, K., Akiyama, S. and Ohkura, Y. (1982b) Sensitive fluorogenic substrates for peroxidase and its application to enzyme-immunoassays. J. Pharmacobio-dynamics, 5, s-20.