

DEVELOPMENT AND CHARACTERISATION OF ANALYTICAL
SYSTEMS BASED ON BACTERIAL BIOLUMINESCENCE

By David Cameron MANUEL

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Centre for Biotechnology
Imperial College of Science and Technology
London SW7 2AZ

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ABSTRACT

The bacterial bioluminescence assay for NADH using NADH:FMN oxidoreductase and luciferase provides the basis for the development of a wide range of dehydrogenase linked assays suitable for use in a biosensor. Previously many dehydrogenase linked assays had been developed in solution, however the operation of the assays in a sensor configuration is frustrated by the complexity of the biological components. In order to develop a convenient sensor package the six components of the assay, the three enzymes, FMN, NAD⁺ and a long chain aldehyde (ie. decanal) must be made into a stable package despite conflicting storage requirements.

The work described in this thesis investigates the development of a flow injection sensor using a packed bed of immobilised enzymes with an HPLC type flow injection facility for introducing samples. A novel method for the immobilisation and storage of the aldehyde, despite incompatibility with aqueous solution was developed. The packed bed system was found to be capable of analysing 30 samples an hour and had a sensitivity upto 1000 times that of solution assays with the same enzyme concentrations. Both malate and lactate were consistently and reproducibly detected using the packed bed.

Immobilisation of luciferase showed the enzyme to be particularly delicate with regard to chemical modification, as such this proved to be a limiting factor in the development and improvement of the sensors specification.

Characterisation of both assay and flow injection analysis system revealed ways of optimising operational accuracy and reducing sources of error. A model of the assay kinetics was developed and tested against observed kinetics this led to further understanding and refining of the sensor development process. A significant improvement in our understanding of the kinetics and in particular its controlling features will allow the design of other dehydrogenase based sensors to have an engineered rather than semi-empirical approach.

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CHAPTER ONE

INTRODUCTION

1 BIOLUMINESCENCE AND ITS ANALYTICAL APPLICATIONS

The objective of this project was to utilise the high sensitivity and large range of analytes detectable with a bioluminescence assay linked to NADH (Wienhausen and DeLuca 1982, McElroy and DeLuca 1983). The combination of appropriate enzymes and simple instrumentation to measure the intensity of the emitted light leads towards a biosensor of great generality. Although such a biosensor is conceptually simple to construct there are a number of pre-requisites that must be achieved; in particular a suitable method of immobilizing the enzymes involved is necessary. While this is relatively straightforward for a single enzyme it is much more complicated for the multi-component bioluminescence system. As well as the two bacterial bioluminescence enzymes and an appropriate dehydrogenase to produce NAD(P)H from the analyte, two co-factors, FMN and NAD(P)⁺, a long chain aldehyde and oxygen must all be present at appropriate concentrations.

The work described here deals with possible methods for solving the problem of immobilising and packaging the system in several different ways, depending on the configuration of the sensor. It is also necessary to characterise the changes that occur when moving from a solution assay to a solid state assay characteristic of the biosensor.

Changes in the assay configuration affect both the active components individually and their interactions. Without an understanding of these changes it is not possible to produce a biosensor whose performance is reliable.

1.1 BIOSENSORS

The function that biosensors are designed to perform is that of providing analytical information without the capital cost, overheads and trained staff normally associated with an analytical laboratory. The targets of biosensor research are devices which can be used outside a laboratory environment and without requiring the skills of an analytical chemist.

The growth and importance of biosensor research can be appreciated by considering two facts. Firstly the biosphere contains a vast number of substances which influence various aspects of life for good or bad. Secondly the desire to control and monitor environments whether natural or artificial is becoming a subject of growing importance; public and government concern are creating a need to monitor many substances in the environment, and in the agricultural, food and chemical industries. It is anticipated that biosensors will enable these substances to be measured easily, quickly and possibly continuously.

The construction of such devices combines the recognition capabilities of biological molecules with the signal handling capacity of microelectronics, the two being linked by a transducer which translates a physical or chemical response into an electrical signal. This concept of a biosensor is shown schematically in figure 1.1.

1.1.1 Construction of a Biosensor

There are five components to a biosensor as shown in the diagram.

1.1.1.1 The analyte

Although the biocomponent can react with the analyte very specifically the nature of the environment will also affect the sensor's performance ie. it is no use trying to analyse glucose in a strongly acidic solution, where the biocomponent may well be destroyed, or in solid form where mass transfer of molecules is not possible. In some cases pre-treatment of the sample may be required.

1.1.1.2 Bioactive matrix

This is the biocomponent, it responds specifically to the analyte and produces a response which varies, preferably in a linear fashion, with analyte concentration.

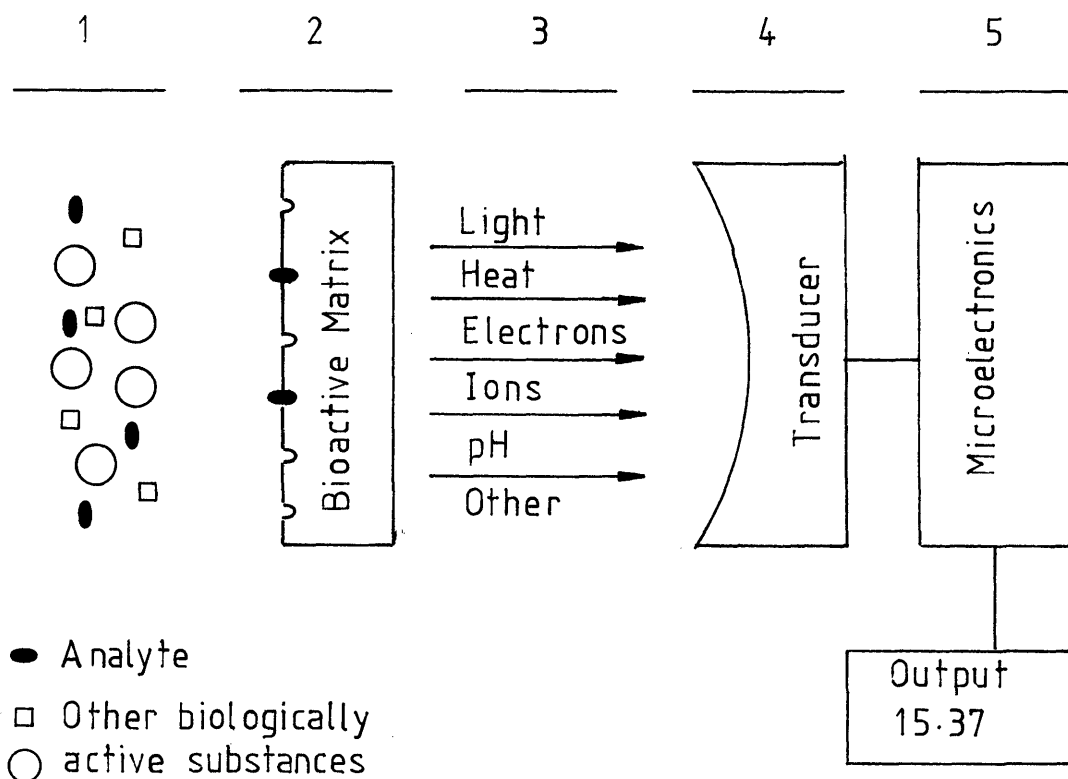


FIGURE 1.1

1.1.1.3 Signal

This is the physico-chemical response of the bioactive matrix to the analyte. Sometimes a given reaction will result in several different responses, the one chosen is that most compatible with the particular analytical requirements, ie sensitivity, response time or exclusion of some particular source of interference.

1.1.1.4 Transducer

This translates the physico-chemical response into an electrical signal which is measured by the micro-electronics. The signal and transducer usually give rise to the generic name of the biosensor. Some of the combinations of signal and transducer are shown in table 1.1.

The different technologies which can be used to interface the biological and microelectronic components are myriad. Bioluminescence devices involve light generated by a luciferase being detected by either a photomultiplier or a photodiode. Many of the other technologies can be found reviewed by Lowe (1985).

1.1.1.5 Microelectronics

These handle the electrical signal from the transducer and display it as a numerical result after interpretation based on a standard calibration. In some instances this conversion may require a microprocessor based algorithm.

1.1.2 Specifications of a biosensor

The precise specification for any individual device is dictated by the operating conditions and detection requirements for the sensor. However several commonly desired properties of a biosensor have been collated in table 1.2.

Some of these properties are mutually exclusive. It is the role of the biosensor designer to optimise them for the particular use that he has to satisfy.

1.1.3 Development of a Biosensor

Many regard the ultimate biosensor as a small device or probe which can be used in a wide range of environments remote from a laboratory and which may work continuously without the need for technical maintenance. For instance in the medical field this might take the form of a sensor for a diagnostically important metabolite, i.e. glucose, if required the sensor being implanted in a patient's body to control treatment of a disease i.e.

TABLE 1.1

Combinations of Signal and Transducer

TRANSDUCER	SIGNAL	APPLICATIONS
Enzyme Electrodes	Electrical current	Enzyme substrates
Field Effect Transistors (FET)	Changes in electrical potential	Ions, gases and enzyme substrates
Ion sensitive Electrodes	Changes in electrical potential	Ions and in enzyme electrodes
Gas sensing Electrodes	Changes in electrical potential	Gases, from enzyme, organs or tissues
Optoelectronic	Optical changes	Fluorescence and spectrophotometric linked assays
Thermistors	Small changes in temperature	Enzyme reactions or changes in enthalpy
Conductimeter	Changes in conductance	Enzyme substrates
Piezoelectric crystals	Mass change	Volatile gases, vapours binding reactions

TABLE 1.2

Desirable Attributes of biosensors

-
1. Provide convenient and straightforward measurement methods for substances of biological, industrial or clinical importance.
 2. Pretreatment of samples is at a minimum.
 3. Devices are easy to calibrate.
 4. Sensors are often very sensitive.
 5. Probes can be made cheaply.
 6. Probes are reusable.
 7. Rapid response time.

NB Probe refers to the bioactive matrix and transducer if the two are physically coupled.

diabetes. Currently the analytical procedures are likely to be available only in a laboratory and often involve time consuming procedures. The development towards a biosensor from an assay occurs by a series of steps. These steps must overcome a variety of problems which will vary with both analyte and operational conditions for the assay/biosensor.

The problems to be overcome fall into two categories, biochemical and physical. Most often it is the biochemical problems which control the rate of biosensor development.

1.1.3.1 Biochemical Problems

These arise from the inherent delicacy and sensitivity of many substances to their environment. The three stages of development where they have a major effect are.

- * The packaging of the biological reagents in a stable form capable of both extended shelf and operational life times requires overcoming the natural processes which cause often rapid degradation of the reagents. The severity of these bio-degradative processes will vary from reagent to reagent.

- * The qualitative use of a biochemical reaction for a specific substance in a complex mixture of potentially interfering biological materials which comprise the solution to be analysed. In addition to any biochemical interference from the assay solution the effects of pH changes, buffering capacities and coloured solutions upon signal detection and transduction have to be considered in some circumstances.

- * For reuse or continuous use a sensor requires the regeneration of the assay system in situ. While this is not difficult for a simple system consisting of one enzyme i.e. a glucose oxidase sensor for glucose, for a complicated system with co-factors, secondary substrates and several enzymes this can be a major problem.

1.1.3.2 Physical Problems

These are usually far easier to solve than the problems involving biological materials directly since the design of the electronics, optics and other physical components does not have to contend with the effects of biodegradation on the components of the sensor. Often the problems are little more than the adaptation of existing technologies, the major difficulty is often miniaturisation.

Biosensor research over the last decade has shown the great potential offered by harnessing the sensitivity and

specificity of enzymes as detecting elements in sensors. However the problems of producing commercially reliable instruments based on biological components should not be underestimated. Scheller et al (1985) reviews the progress of biosensors towards commercialisation and current developments show the importance of simplicity in the design of the biological 'nose' for such sensors

1.1.4. Bioluminescence as a basis for a biosensor

Bioluminescence offers several advantages as a biosensor detection system.

- 1) It has already been shown to produce reliable assays for a range of analytes in solution (McElroy and DeLuca 1983).
- 2) The signal produced, light, can be easily and sensitively measured by a photodiode or photomultiplier tube.
- 3) The response time for the assays is rapid.
- 4) It can be used for many different analytes.

The complexity of the bacterial bioluminescence system involving six components gives rise to the bulk of design and development problems.

For instance the conflicting storage requirements of the substrate decanal and the other components.

* Decanal is autoxidised to the fatty acid decanoic acid in aqueous solutions with a typical half life of less than two hours (Hastings et al 1963).

* Luciferase the principle enzyme in the assay, is susceptible to thermal denaturation at room temperature, proteolysis and oxidation of an active site thiol group (Hastings et al 1978).

* Excess decanal in the presence of luciferase can produce a pronounced inhibition of bioluminescence which is only slowly reversed on dilution of decanal concentration.

* The two co-factors NAD^+ and FMN are required by the assay system and further complicate the packaging problems, not least due to their pH and photo-sensitivity.

* Still more problems arise if the sensor is to work continuously as both co-factors and decanal will need to be regenerated.

1.2 BIOLUMINESCENCE

'....some things, though they are not
in their nature fire, nor any species of
fire, yet seem to produce light.'

Aristotle

(DeLuca et al 1982)

Bioluminescence is one of the most intriguing of natural phenomena. The eerie glow of bioluminescence has been marveled at since ancient times. While Aristotle and Pliny marvelled in ignorance we know many of the whys and hows that beset the early philosophers, and are now able to employ the phenomenon for our own uses.

Bioluminescence is a phenomena that originates as the name implies from living organisms. It may be defined as a particular type of chemiluminescence involving the generation of a chemical species in an electronically excited state which reverts to its ground state by the emission of a photon in the visible spectrum. The production of this excited state in bioluminescence is mediated by components produced by living organisms.

It is apparent from the vast diversity of organisms which have developed a luminous function that there is no one clear evolutionary origin of bioluminescence. In fact although bioluminescence has been observed in comparatively few species some 30-50% of all known Phyla and Classes of organism contain a luminous species (Harvey 1952). The diversity of luminous organisms is reflected in the size of the accumulated literature Harvey's book (Harvey 1952) and McCapra's review (McCapra 1982) provide extensive backgrounds to the biological and chemical aspects of the subject. Both publications illustrate the variety amongst the systems that have evolved. However, in addition to the phenomenon of light emission there are a few common features which unite the systems despite their diversity.

The need for molecular oxygen is believed to be universal amongst bioluminescent systems (McCapra 1982). A requirement noted as long ago as the seventeenth century when Robert Boyle experimented with his bell jar on bioluminescent bacteria and fungi. Another common feature although not as strictly adhered to in nature is the pattern of requiring a luciferin, or specific substrate which carries the chemiluminescent element needed for bioluminescence, and a luciferase, the enzyme which carries out the bioluminescent reaction. These were first observed by Dubois in the nineteenth century as his heat stable and heat labile extracts from the luminous beetle Pyrophorus. While not applicable to all systems, other substrates and co-factors may also be necessary,

the concept of a luciferin and luciferase have remained central to the study of bioluminescence.

Of the many bioluminescence chemistries several may be suitable for analytical use in biosensors. Indeed the system from the jelly fish Aequorea aequorea is used as a sensitive assay for Ca^{2+} , the uses of this bioluminescence system have been reviewed by Blinks et al (1978). The firefly luminescence system is also widely used, its requirement for the co-substrate ATP is the basis for an assay of ATP and ATP producing reactions in particular the measurement of bacterial contamination by ATP production (Lundin et al 1985). Commercially it is commonly used to quantitate bacterial biomass from the amount of ATP present in samples of material (DeLuca et al 1982).

It was decided to use the bacterial system because it offers the widest range of potential analytes. Based on the reactions catalysed by luciferase and an FMN:NADH oxidoreductase NADH can be measured very sensitively. Using secondary enzymes a vast array of NADH linked assays of the form shown in figure 1.2 can be constructed (table 1.3).

Other more complicated assay systems using bioluminescence can and have been designed making use of the sensitivity of the assay to either of the two secondary substrates, oxygen and aldehyde. Sensitive assays for oxygen based on bioluminescence, using whole bacterial cells, have been designed by Chance and Oshino (1978). The sensitivity of luciferase to aldehyde concentration was used by Watanabe et al (1982) to produce an extra sensitive assay with the aid of a substrate cycling step, both NADH and NADPH were used, NADPH to drive the luminescence reaction through a NADPH specific oxidoreductase, NAD is cycled in the assay leading to the synthesis of aldehyde from acid hence incorporating an amplification step into the assay. Such complicated assays are not an ideal basis for a biosensor employing the principle of simple biochemistry, further these assays can exhibit a lag time of tens of minutes before a stable light emission is achieved a far from ideal response time.

The development effort for bioluminescence biosensors is hence concentrated on simple NADH linked assays, with some 240 dehydrogenases known there is ample scope for a large 'family' of sensors with high substrate sensitivity - table 1.3 lists some of the existing substrates assayed by bioluminescence.

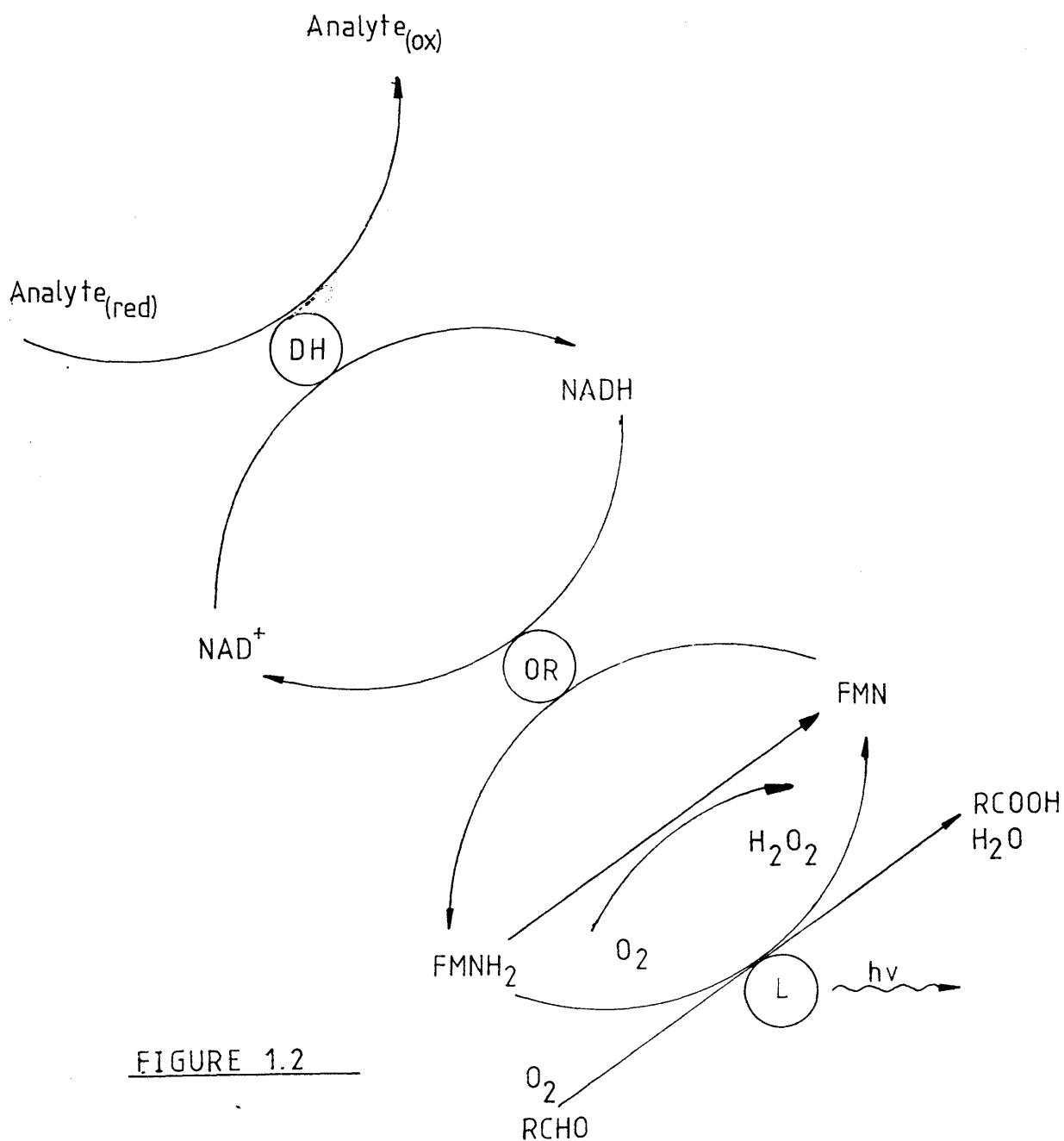


FIGURE 1.2

FIGURE 1.2

NADH linked bioluminescent assays

The simple scheme shows the basic reaction sequence for the 'family' of dehydrogenase linked bioluminescence assays. The three enzymes, the dehydrogenase (DH), FMN:NADH Oxidoreductase (OR), and Luciferase (L) link together to quantitatively translate analyte concentrations into a light signal.

TABLE 1,3

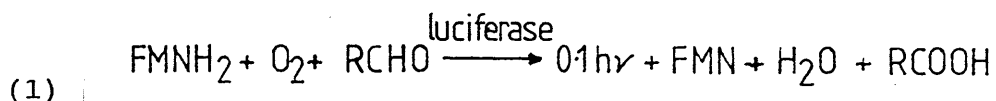
SUBSTANCES DETECTED BY BACTERIAL BIOLUMINESCENCE SYSTEM

Analyte assayed for	Detection Range
Alcohol dehydrogenase	0.01 - 10 pmoles
AENADH - biotin	25 - 200 pmoles
Ethanol	0.003 - 12 % v/v
FMN	10 fmoles - 100 nmoles
Glucose	0.5 - 6 pmoles
Glucose -6- phosphate dehydrogenase	0.001 - 1 pmoles
Glucose -6- phosphate	2 - 1000 pmoles
Hexokinase	1 - 10 pmoles
3-hydroxy butyrate	50 - 1000 pmoles
Lactate dehydrogenase	0.001 - 1 pmole
L-Glycerol-3-phosphate	300 - 2000 pmoles
Malate	250 - 1500 pmoles
Myristic acid	10 pmoles
NAD +	1 pmole
NADH	0.1 fmoles - 100 nmoles
NADPH	10 pmoles - 200 nmoles
O ₂	0.1 - 100 nmoles
Oxaloacetate	5 - 60 pmoles
Pyruvate	0.5 - 4 pmoles
Testosterone/Androsterone	0.2 - 5 nmoles
TNT	10 attomoles

1.2.1 BACTERIAL BIOLUMINESCENCE

There are many species of bacteria which display bioluminescence. They are virtually all marine dwelling and are either free living or associated with fish or squid (Harvey 1952; Nealson and Hastings 1979). The bacteria fall into a few main genera: Photobacterium, Beneckea, Alteromonas, Vibrio and the terrestrial Xenorhabdus (Nealson and Hastings 1979).

All bacterial luciferases so far investigated catalyse the same overall reaction in vivo (Eq 1.)



Luciferases differ in their exact substrate specificities and kinetic behaviour.

Vibrio harveyi was chosen as the source of enzyme for this work for several reasons. The available literature on the luciferases suggested that it was the most robust of the enzymes (Hastings et al 1978), and the most thoroughly studied. This information helps in the choice of appropriate immobilisation chemistries and operating conditions.

It is worth noting that the nomenclature of many bioluminescent bacteria has changed several times over recent years. Within this context the bacteria known as Vibrio harveyi started life in the literature as the MAV strain of Photobacterium fisheri, itself reclassified as Vibrio fisheri, the MAV strain was then reclassified as Beneckea harveyi before its current classification as Vibrio harveyi.

1.2.2 The Biochemistry of Bacterial Bioluminescence

Luciferase is a mixed function oxidase, catalysing the con-comitant oxidation by molecular oxygen of FMNH₂ to FMN and of a long chain aldehyde to the corresponding fatty acid. This enzyme is particularly notable for the slowness of its catalytic cycle; it turns over approximately once every 10 seconds. This means that the enzyme catalysed turnover of FMNH₂ is many times slower than the auto-oxidation of FMNH₂ in aqueous solution by oxygen. This has far reaching implications for the interpretation and functioning of the coupled assay as will be seen later.

Luciferase is further unique among flavoproteins in having a dissociating co-factor, the reduced flavin is bound to the protein for oxidation to FMN, the FMN then dissociates and is reduced once again by a second enzyme or chemical reaction. Normally the flavin moiety is associated permanently with the flavoprotein.

The basis of the reaction scheme in Figure 1.3 was postulated by Hastings and Gibson (1963) in their early work on Achromobacter fisheri. Since then the characterisation of both intermediates and mechanisms within the scheme has almost reached completion for the Vibrio harveyi scheme. Both intermediates II and III are long lived and typify the slowness of the reaction which is a remarkable characteristic of the enzyme. Reduced flavin mononucleotide (FMNH₂) reacts rapidly in the first instance with the luciferase to form intermediate I. The flavin-enzyme complex reacts rapidly with molecular oxygen to form intermediate II. This is sufficiently stable to be isolated at low temperatures (Presswood and Hastings 1978). In the absence of a long chain aldehyde the complex breaks down slowly to give enzyme flavin mononucleotide (FMN) and H₂O₂ in equimolar amounts (Ziegler and Baldwin 1981), a common feature of monooxygenases. The intermediates in the reaction sequence have been extensively studied by absorbance and fluorescence spectroscopies. The properties of intermediate II are consistent with it being a peroxyhydroflavin, and 13-C NMR studies with the flavin labeled at the 4a position indicate that the molecular oxygen reaction forms a 4a flavin adduct (Ghisla et al 1978, Veroort et al 1986). McCapra has also shown that an active peroxide is present by the catalytic oxidation of dialkyl sulphides by intermediate II (McCapra and Hart 1978).

The mechanism proposed by Kurfust et al (1984) for the steps after intermediate II including the light emission step are shown in figure 1.3. By absorbance and luminescence studies they have identified the primary emitter species as a 4a flavin hydroxide although they do not completely rule out a secondary emitter.

1.2.2.1 Quantum yield

This is an important concept in chemiluminescence, it refers to the number of photons produced for each molecule of reactant (substrate in bioluminescence) used. The quantum yield can be expressed as a function of several other yields (equation 2).

$$Q_b = Q_c \times Q_{ex} \times Q_f \quad (2)$$

Q_b is the bioluminescent quantum yield

Q_c is the chemical yield of product. It accounts for non-luminescent side reactions.

Q_{ex} is the efficiency of formation of the excited state as opposed to the formation of the ground state.

Q_f is the fluorescence quantum yield of the emitter species.

There is disagreement on how to standardise the absolute measurement of bioluminescence and quantum yield and hence enzyme specific activities. Several methods of calibration have been used (Zeigler and Baldwin 1981) and the probable maximum quantum yield is 0.1. However because of the efficiency of light collection and detection by different luminometers the simplest approach is to express activities in terms of relative light units (RLU) for a particular instrument.

1.2.2.2. Subunit structure of Luciferase

Vibrio harveyi luciferase like all other known bacterial luciferases is a heterodimeric protein. The two subunits α and β may be separated by ion-exchange chromatography (Tu 1978) in the presence of urea. The α subunit is slightly larger (40K) than the β subunit (38K) the definition of α and β subunits from the different species of photobacterium was made on the basis of structural and functional analogies (Ruby and Hastings 1980). The α and β subunits were so named after the order in which they first came off the column.

The luciferase heterodimer is thought to be of an elongated shape from electron spin resonance (ESR) labelling experiments. The binding sites for spin labelled maleamide, the essential sulphhydryl group on the α subunit, and for FMN were found to be 21-37 Å and 30-58 Å distant from the binding site of 8-anilino-1-naphthalenesulphonate (ANS) respectively (Tu et al 1978). ANS binds competitively with FMN₂. Results from ESR with spin labelled maleamides showed the essential sulphhydryl to be in a 17 Å cleft with a hydrophobic environment similar to that of propan-2-ol (Merritt and Baldwin 1980).

The allocation of catalytic activity to one or other of the subunits was originally done on the basis of hybridisation studies of the subunits. The subunits alone are inactive but the native activity is recovered by mixing the subunits and removing the urea. Early hybridisation studies with chemically (Cousineau and Meighen 1976) and mutationally generated variants (Nealson and Markovitz 1970) of the subunits indicate that the catalytic activity resides on the α subunit,

the beta subunit having no specific role. However Meighen and Bartlet (1980) in hybridisation studies with subunits from V. harveyi and Photobacterium phoshoreum suggested a role for both the alpha and beta subunits in the binding of FMNH₂. Chemical modification of amino groups on both subunits by Welches and Baldwin (1981), and Watanabe et al (1982) further suggested that both subunits are involved in the initial binding of FMNH₂ but thereafter the alpha subunit is solely involved in catalytic activity.

1.2.2.3 Essential Residues:

Other chemical modification work has shown that modification of a single sulphhydryl group (Nicoli et al 1974) or a histidyl group (Cousineau and Meighen 1976) results in inactivation of the enzyme. Affinity labelling of the alpha subunit with the aldehyde analogue 2-bromo [C-14]1-decanal has identified the -SH residue as being proximal to the aldehyde subsite (Fried and Tu 1984) while Nicoli's work (Nicoli et al 1974) shows that the bulkier N-ethyl maleimides block both the FMN and aldehyde subsites. Tu and Henkin (1983) used a photoaffinity label 1-diazo-2-oxoundecane to label the enzyme in the region of the aldehyde binding site, both alpha and beta subunits were labeled stoichiometrically with the loss of activity indicating that the active site may be close to the subunit interface. It has been shown that the reactive sulphhydryl is Cys 106 by C-14 labelling the thiol and identifying the radioactive peptide fragment (Baldwin et al 1981). This thiol occurs as part of a protease labile region between 100 and 125 residues from the NH₂ terminus of the alpha subunit (Holzman et al 1980) explaining the enzymes extreme sensitivity to proteases.

1.2.2.4. Stability

The enzyme is stable at -20°C and for limited periods at 4°C. Stability can be markedly improved by storage with 0.1 mM dithiothreitol (DDT) and the susceptibility to denaturation by proteases, temperature and urea can be improved by high anion concentrations (reviewed in Ziegler and Baldwin 1981).

1.2.3. In vivo synthesis of the luciferase operon

The induction of bioluminescence was observed to coincide with the appearance of several membrane bound polypeptides by Ne'eman et al (1977). The identity of these membrane bound polypeptides is not known, however the genes encoding enzymes sufficient for the expression of bioluminescence have been located and mapped. In vivo the requirement for long chain aldehydes suggests a

cellular location associated with the lipids of the membrane.

The genetic control and expression of luciferase has been studied in both Vibrio fischeri and Vibrio harveyi, the complete operon function for V. harveyi is not yet fully described (Belas et al 1982, Miyamoto et al 1985), luminescence of the cloned operon has not yet been achieved in E. coli without the addition of exogenous aldehyde. The operon codes for two polypeptides downstream of the lux genes, only one is coded for in the V. fischeri operon.

The function of the bioluminescence operon in V. fischeri has been largely solved. The control of bioluminescence in vivo is achieved at the genetic level through the synthesis of the luciferase subunits from genes LUX A and LUX B, and through the aldehyde synthetase genes, LUX C LUX D and LUX E (Engebrecht and Silverman 1983, Ultzur 1989). The primary level of control is exercised by means of a positive feed back mechanism involving two operons R and L (figure 1.4). The gene product of LUX R regulates the positive feedback by the auto-inducer molecule on LUX I the first gene on operon R whose product is necessary for autoinducer production. The autoinducer is secreted in to the medium and when it reaches a critical concentration, reflecting bacterial cell density, the rate of transcription of operon R is dramatically increased. The other enzymes required for a complete bioluminescent system, ie those involved in fatty acid synthesis and FMNH₂ synthesis are not known to be encoded for on operons. It is thought that other biosynthetic pathways in the bacteria supply the substrates. This is consistent with the observation that when the operon is cloned into E. coli luminescence ensues when the recombinant cells are exposed to either long chain fatty acid or aldehyde. Recently two acyltransferases have been isolated, the enzymes appear to be induced in the mid-exponential growth phase characteristic of luminescence induction. Further work on the cloned luminescence operons in E. coli has revealed two other levels of control. The left operon coding for the regulator protein requires cAMP-CAP protein and the level of this and the sigma 32 protein affect the expression of the left operon. The right operon coding for the luminescence proteins themselves is influenced by SOS inducing agents. Since the LexA protein binds to the start of the operon inhibiting expression, heat shock or other damage to the bacteria will thus increase the level of expression of this operon (Durlap and Greenburg 1985, Ultzur 1989).

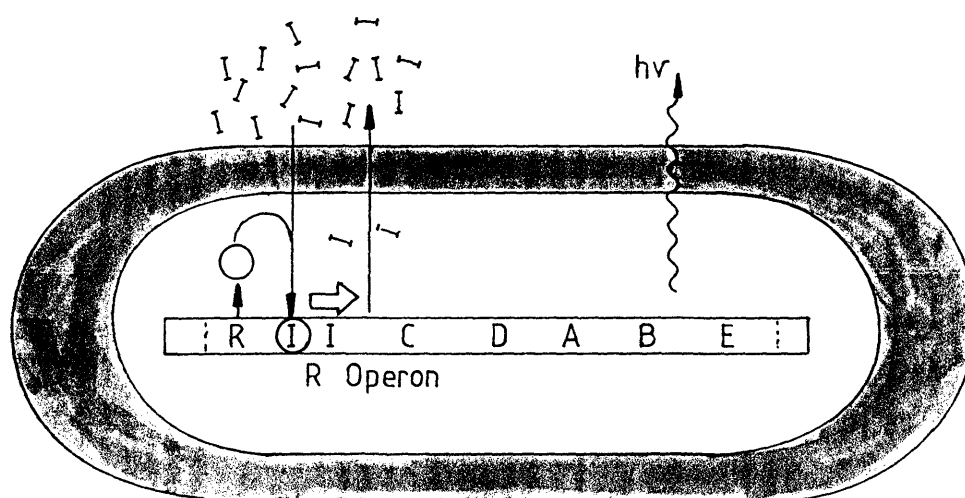
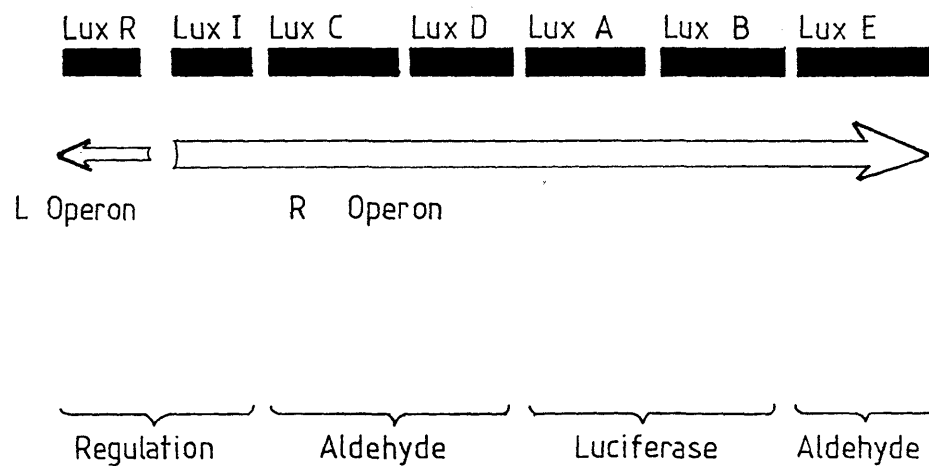


FIGURE 1.4

FIGURE 1.4
Genetic control and expression of the bioluminescence system in *Vibrio fischeri*

The L operon codes for a regulatory protein which can interact with the autoinducer to dramatically increase the rate of transcription of the R operon from the low constitutive level when a certain concentration of autoinducer has built up in the media.

1.2.4 FMN:NAD(P)H oxidoreductase

Both the NADH and NADPH specific oxidoreductases have been purified from V. harveyi (Gerlo and Charlier 1975). The study of bioluminescence throughout this work is of assays involving the NADH specific FMN Oxidoreductase. A highly purified preparation of the NADH specific enzyme has been prepared on 5'AMP sepharose 4B (Jablonski and DeLuca 1977). There is considerable discrepancy in the reported molecular weights of the protein Gerlo and Charlier quote 19,000 whilst Jablonski and DeLuca give a value of 30,000.

Kinetic data indicates that the enzyme functions by the sequential binding of the two substrates NADH and FMN; but it has not been possible to discriminate between an ordered or a random binding mechanism (Gerlo and Charlier 1975, and Jablonski and DeLuca 1977).

1.3 IMMOBILISATION

Immobilisation of enzymes aims at producing insoluble, and potentially reusable biocatalysts. The use of an insoluble enzyme as a biocatalyst elegantly overcomes several of the problems inherent in using a soluble enzyme (Table 1.4).

Since the first published report of a water insoluble enzyme preparation based on charcoal in 1916 the range of enzyme supports has grown phenomenally so that there are now very few organic or inorganic materials to which enzymes cannot be attached (Chibata 1978). Today immobilisation techniques are widely used, however while they have solved many problems of process design and operation they have introduced additional complications to understanding the structure and kinetics of the catalyst.

An analogy between immobilisation and imprisonment can be made, an immobilised enzyme is no longer able to move about freely as a result of physical restraints. Which activities it can still perform and how well depends on the nature of the restrictions, these in turn depend upon three main factors.

- 1) How much physical freedom it retains. An enzyme often needs to undergo conformational changes during catalysis.
- 2) How much damage has the enzyme received during immobilisation. The active site may have been chemically or conformationally altered by immobilisation, changing or destroying the catalytic activity.
- 3) Whether the active site still has access to the substrate, and whether the product can be dispersed from the active site after a catalytic turnover. The orientation of the enzyme in the matrix can physically impede the approach of the substrate, causing steric hindrance in a confined space, or the substrate and product may have difficulty diffusing through the matrix.

1.3.1 Immobilisation Techniques

The techniques used to immobilise enzymes can be divided into two main groups entrapment and carrier binding and these groups, with broad subdivisions, are outlined in figure 1.5. and illustrated in figure 1.6.

1.3.1.1 Encapsulation and Entrapment

This approach involves a physical rather than chemical immobilisation of enzymes. Generally the enzyme is

TABLE 1.4

Advantages of immobilised enzyme over soluble enzyme

1. Permits the reuse of the enzyme.
 2. Ideal for continuous operation.
 3. Product is enzyme free.
 4. Permits accurate control of the catalytic process.
 5. May improve enzyme stability.
 6. Allows easy use of multienzyme systems.
 7. Reduces effluent disposal problems
 8. Can be used to localise the enzyme in space.
-

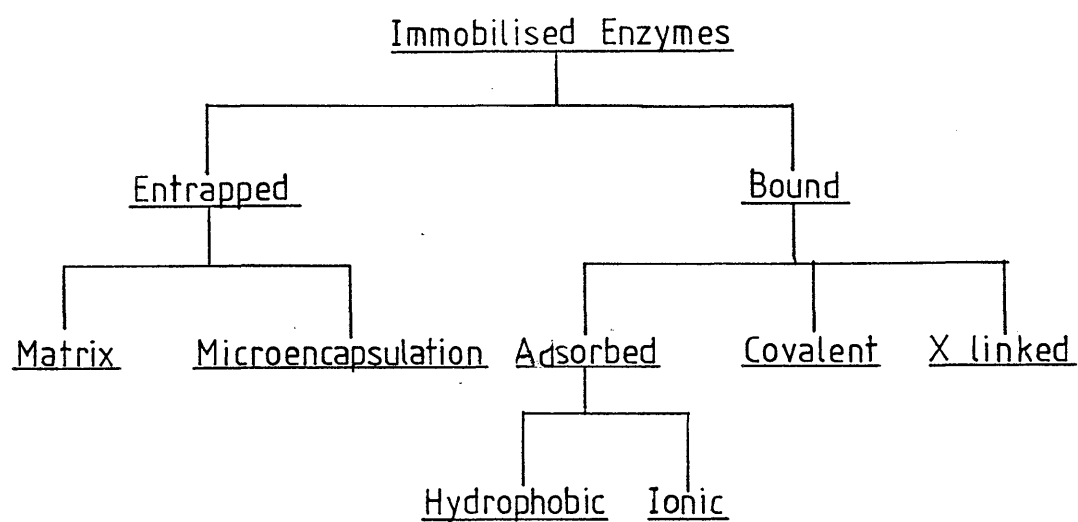


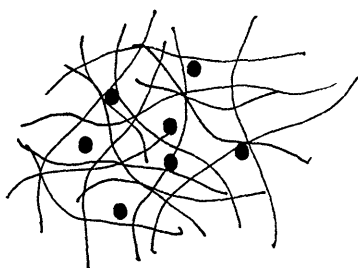
FIGURE 1.5

FIGURE 1.5

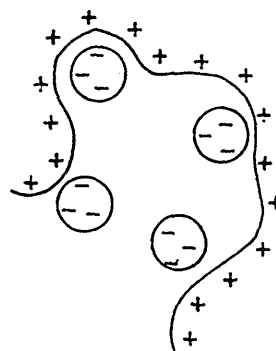
Types of commonly used immobilisation technique.

The diagram shows the immobilisation techniques grouped in families and related in a 'family' tree fashion.

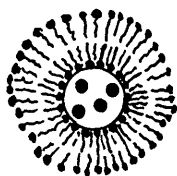
Matrix (gel) Entrapped



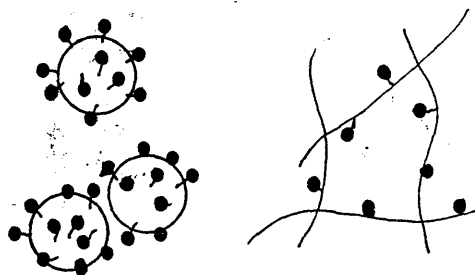
Adsorption



Microencapsulation



Covalent



X-linked

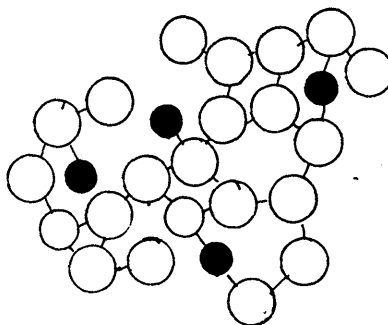


FIGURE 1.6

FIGURE 1.6

Illustration of the basic types of immobilisation.

Shows an idealised picture of the interaction between enzyme and matrix. Entrapped among a dense mesh of fibres, encapsulated in an artificial liposome surrounded by a lipid bilayer, attached to a carrier bead or a matrix of fibres by a chemical bond, or forming part of the matrix structure together with a 'feeder' protein such as BSA.

immobilized by the formation of some physical structure around it, either a matrix or a capsule. The enzyme becomes entrapped in the newly formed structure which prevents the loss of enzyme by producing a physical barrier. This technique usually leads to greater problems with diffusion, and partitioning than are experienced in other techniques.

For example entrapment of enzymes in carrageenan (a polysaccharide derived from Irish peat moss) is achieved by mixing the enzyme with a carrageenan solution and precipitating the carrageenan gel with calcium ions, a high concentration of phosphate or a temperature drop (Chibata 1978, Tosa et al 1979). Encapsulation involves the creation of a complete envelope of selectively permeable material around the enzymes. Examples of encapsulation are the entrapment behind an artificial membrane such as cellulose acetate, or a dialysis membrane forming a dialysis bag, or as illustrated in figure 1.6 encapsulation in an artificial liposome.

1.3.1.2 Adsorption

Enzyme is retained on a surface by a non-covalent association with the matrix or carrier. The interaction may be hydrophobic or ionic and is generally the result of a large number of individually weak bonds. As a result the method usually suffers from problems of displacement and leaching of the enzyme from the support. Fouling and poisoning of the biocatalyst as a result of adsorption of other substances can also be a particular problem with this method, especially if reusability is required.

For instance anionic enzymes can be readily adsorbed to anion exchange resins such as DE-52 at low ionic strength and a suitable pH. The ionic interaction holds the enzyme on to the matrix. However it must not be forgotten that if the pH or ionic strength of the local environment changes the enzymes can be released.

A matrix itself may be modified to improve adsorption. For instance polystyrene normally adsorbs via hydrophobic interactions, but by first adsorbing poly(phenylalanine-lysine) through these hydrophobic forces the surface can be made positively charged and so act as an anion exchanger binding proteins via salt bridges.

1.3.1.3 Covalent binding to a carrier

This method produces the most stable immobilisations, a chemical bond is formed between carrier and enzyme effectively eliminating both leaching and displacement problems. However a covalent link is more likely to produce changes to the enzymes inherent conformation and

catalytic activity than the other methods described here.

The chemistry of immobilisation must be tailored to the stability and chemical susceptibility of the enzyme to avoid either denaturing reaction conditions or modification of an essential group on the protein.

One common technique is to use a cyanogen bromide (CNBr) activated carrier. The technique is particularly popular as pre-activated carriers are commercially available. For instance CNBr activated sepharose easily reacts with enzymes through $-NH_2$ groups at pH 8.0 the chemistry is illustrated in figure 1.7(a).

Enzymes can also be covalently attached to amino or carboxyl groups by synthetic peptide bonds created with the aid of a carbodiimide figure 1.7(b).

Another way of covalently immobilizing an enzyme is to cross link it with a bifunctional reagent, such as glutaraldehyde, to other proteins of either the same type or to a "feeder" protein such as bovine serum albumin (BSA) to produce enzyme pellets or membranes.

The ease with which enzymes can become damaged during immobilisation by covalent coupling means it is worth following a few guidelines in order to minimise damage to enzyme activity.

- * Use immobilization chemistry specific for non-essential residues.

- * If necessary protect the active site with substrates, products or reversible blocking reagents.

- * Choose a large coupling agent to provide some steric hindrance to inactivation of buried essential residues.

- * Choose a type of residue where a high number of easily accessible non-essential residues are available in addition to the essential residue.

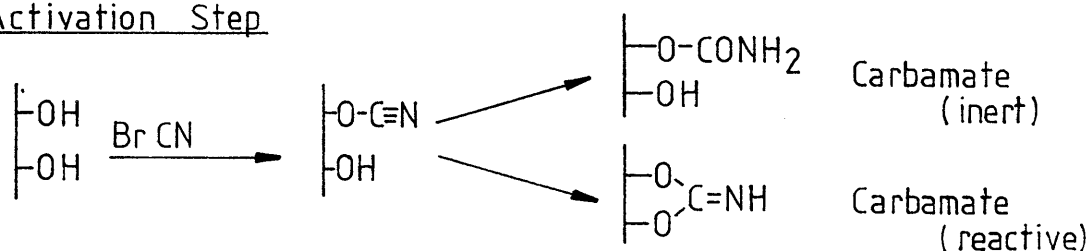
- * In addition to these chemical considerations, physical restrictions on the enzyme may be reduced by introducing a long flexible 'spacer' arm.

1.3.2 Effects of immobilisation on enzyme activity

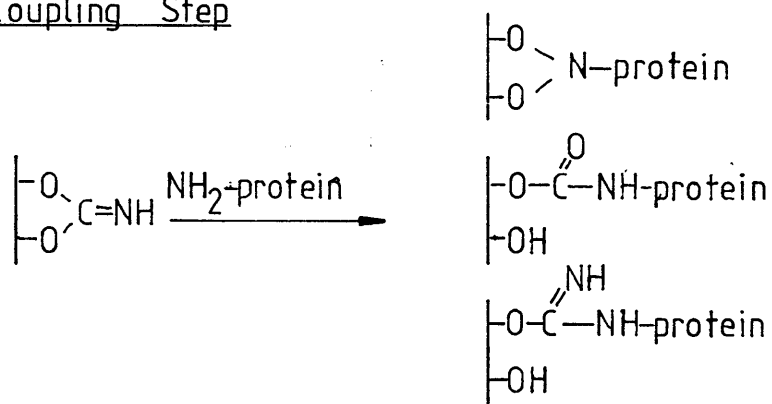
The immobilisation of an enzyme on a solid support can have a number of consequences some of which are a result of immobilisation per se whilst others arise from the specific features of the immobilisation technique employed. Changes in the enzymes immediate environment, its microenvironment, or physico-chemical changes to the enzyme are common and can cause real and apparent changes

CNBr Immobilisation (a)

Activation Step



Coupling Step



Carbodiimide Coupling (b)

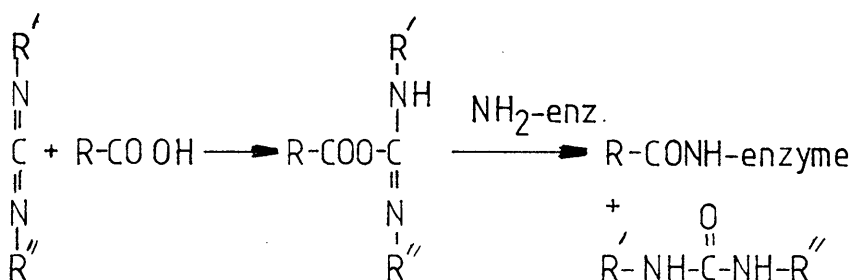


FIGURE 1.7

FIGURE 1.7

Examples of chemical coupling reactions

a) CNBr activation of a matrix and the coupling of an enzyme through NH₂ groups.

b) Carbodiimide mediated peptide bond formation.

in catalytic activity.

Changes to the microenvironment have two main effects. Firstly partitioning effects between the bulk phase and the matrix phase due to the chemical nature of the support may attract or repel, substrate, product, inhibitor or other molecules which affect the enzyme reaction. Secondly a matrix can exert an effect by restricting diffusion of molecules to and from the enzyme.

1.3.2.1 Partitioning

The most obvious example of this is when the support/matrix is charged and concentrates ions of the opposite charge. This will alter the concentration of the charged species in the matrix. This effect also has affects on the pH of the microenvironment.

1.3.2.2 Diffusion

If the matrix restricts the movement of molecules it may control the rate of substrate consumption through the rate of substrate supply. This case is known as diffusion limited kinetics and has important consequences for the behaviour of a biocatalyst. When enzyme and substrate come into contact the reaction is initiated, and if diffusion is significant a local depletion of substrate and an increase in the product concentration will develop. If the system is diffusion limited the apparent rate of enzymatic reaction will be controlled by the rate of substrate repleishment and the rate of product removal. Concentration profiles will as a result be set up through out the matrix for all species involved in the reaction whether consumed or created. Figure 1.8 illustrates the kind of profiles obtained (Trevan 1980). In addition to these enzyme generated concentration gradients a second effect can be observed in stirred or flow systems. At the surface of the matrix a thin layer of solution surrounding the matrix is found to be stationary. This layer is known as the Nernst layer it always exists but its size varies with flow conditions. Figure 1.8 illustrates the effects of all three of these phenomena. The extent to which these phenomena occur depends on the physico-chemical properties of the matrix. In particular the degree of hydrophobicity and the charge distribution of the matrix are responsible for determing the partitioning effects, while the physical distribution of the matrix material also plays a role in determining the diffusion effects.

1.3.2.3 Conformational changes

Physical flexing of the enzyme structure may be important in both binding of substrates and enabling the catalytic

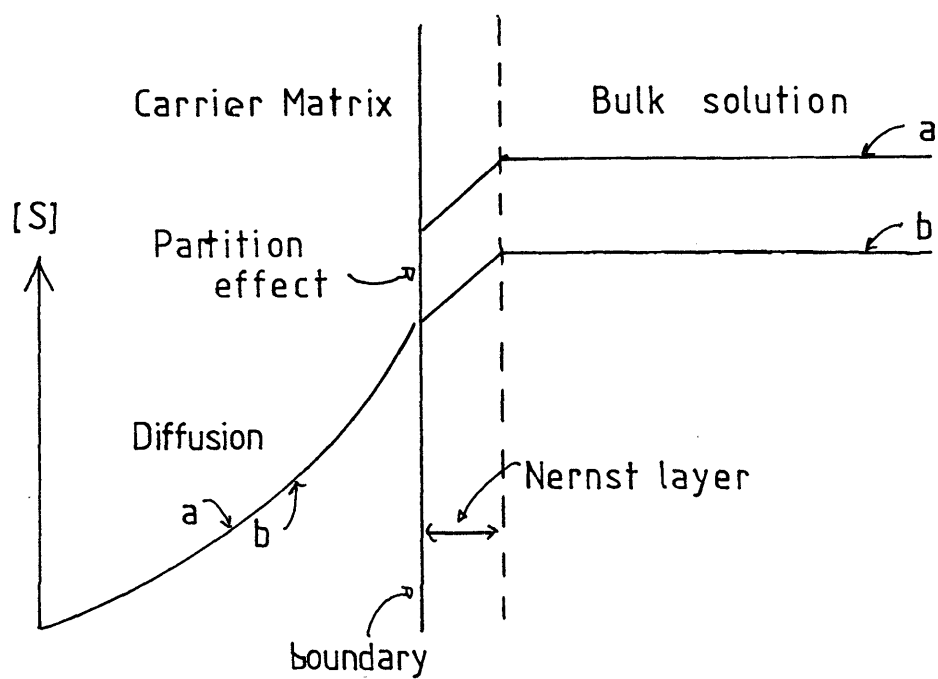


FIGURE 1,8 Carrier induced effects
on concentration gradients
a - with partition
b - without "

reaction to occur. Immobilisation particularly by covalent coupling or adsorption where the enzyme is attached to the matrix surface through multiple connections can easily cause a restriction on conformational changes. Ionic binding can cause changes in charge distribution, net charge, and the local electrostatic field altering the intrinsic kinetic parameters of the enzyme as well as changing the microenvironment. Covalent binding can have similar effects if it is carried out through charged groups or introduces charged groups. The normal conformational state of the enzyme may be modified changing the activity, or the enzyme may be inactivated by direct coupling to the active site or essential residues as mentioned earlier, similarly allosteric properties may be lost. Covalent binding may cause steric hindrance to enzymatic activity by obscuring the active site. These problems are summarised and illustrated in figure 1.9.

The effect of these physical and chemical disruptions to the enzyme are to transform the simple homogeneous environment associated with solution biochemistry into a heterogeneous environment; the enzyme population will consist of inactive, partially active and active molecules and a different environment pertains in the matrix compared to that in the bulk solution. The environment within the matrix may also be heterogeneous, like the distribution of enzyme, as a result of partition and diffusion effects as well as the direct consequences of a heterogeneous immobilisation.

The properties of such systems are therefore described by effective parameters for the insoluble system, K_m , V_m , k_{cat} as measured from the bulk solution substrate and product concentrations, pH, ionic strength. K_m , V_m , and k_{cat} can all differ from the case of a soluble enzyme system as a result of the effects discussed above. The changed kinetic parameters are referred to as the new intrinsic parameters of the enzyme after immobilisation effects, inherent parameters are taken to include partitioning effects, and finally the effective parameters after considering the diffusional effects.

1.3.2.4 Operational parameters

1.3.2.4.1 Stability

There has been much made of the potential for increasing the stability of enzymes by immobilisation. However sometimes the observed improvements may be apparent rather than real, as a result of measuring the effective parameters.

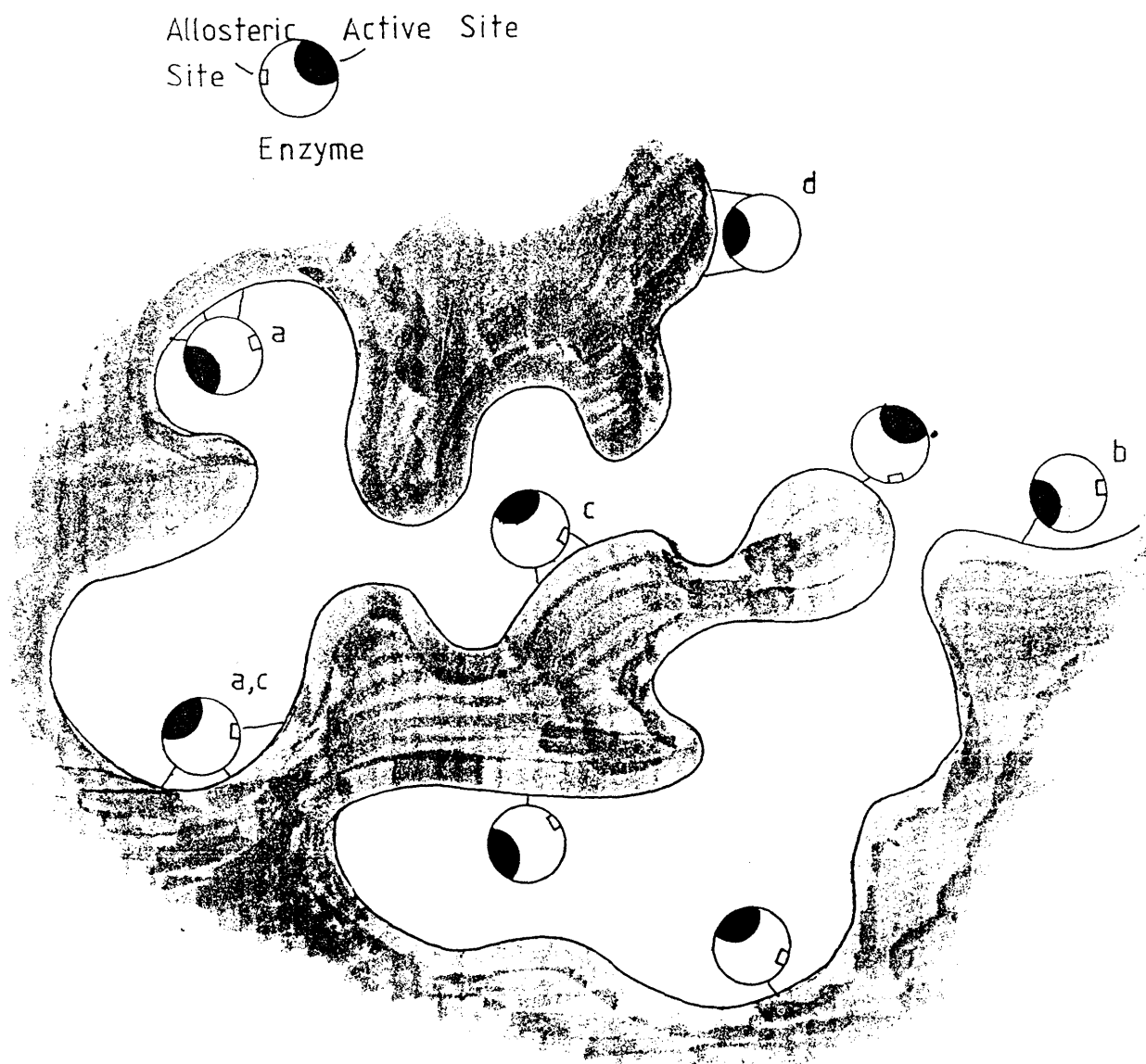


FIGURE 1.9

FIGURE 1.9

The effects of covalent coupling on enzyme activity.

- a) Conformational restriction on enzyme movements by multiple point attachment.
- b) Modification of residues at the active site or essential to the activity of the enzyme.
- c) Modification of an allosteric site.
- d) Steric obstruction of the active site.

For example in diffusion limited cases because the enzyme reaction in a matrix sets up a substrate concentration gradient, buried enzymes will not be fully utilized, activity being limited by the diffusion of substrate to the enzyme and its local concentration. In this case when enzyme activity is lost unutilised enzyme becomes utilised maintaining the same total observed activity despite loss of active enzyme. This kind of effect is also seen where transport of substrate to the enzyme is rate limiting, again some loss of enzyme can be sustained before any change in the catalytic activity of the matrix is observed. However to the biotechnologist an effective increase in stability is as good as a real one.

1.3.2.4.2 pH Optimum

Both partitioning and diffusion effects can alter the pH of the matrix with respect to the bulk solution. Partitioning effects in ion exchange resins will shift the pH, up for a cationic exchanger and down for an anion exchanger. Gradients which are produced by consumption or production of H⁺ ions will also cause changes to the internal pH in a manner dependent on the external pH.

$$J_i = \frac{A \cdot D \cdot (H_e^+ - H_i^+)}{d}$$

Where:

H_i⁺ is the internal proton concentration

H_e⁺ is the external proton concentration

A is the average pore size

D is the diffusivity coefficient

d is the distance between the internal and external readings.

J_i is the proton current (rate of flow)

As the absolute value of H_i⁺ declines the difference in p_H_i - p_H_e must increase to maintain the same value of (H_i⁺ - H_e⁺). The effect of diffusion limitation is to cause alkaline or acid limbs to the pH profile. Proton diffusion can be apparent under conditions where substrate diffusion is not observed since [H⁺] < 10⁻⁷ mol l⁻¹. Increasing buffer capacity and ionic strength can dissipate the effects of partitioning by neutralising the charge on the matrix with counterions and dissipating the proton gradient by buffer species diffusion [buffer] >> [H⁺].

1.4 ANALYTICAL USES

The bacterial bioluminescence system, consisting of luciferase and oxidoreductase, clearly offers a potentially useful method of measuring NADH or NADPH levels. Combining these two enzymes with various dehydrogenases provides the basis for a wide range of possible assays as discussed in section 1.2, the list of assays in table 1.3 have all been taken from published papers.

It is not difficult for someone familiar with the system to add new analytes to the range which can be detected, however as is often the case with new techniques the routine use of bioluminescence as an analytical technique is still in its formative stages. Commercialization of the general use of bioluminescence has been led by the equipment manufacturers with reagent suppliers now starting to supply very basic analysis kits of reagents and freeze dried enzyme preparations.

The published literature on individual analytical uses concentrates on showing a straightforward relationship between bioluminescence and analyte concentrations. While this establishes the method a more detailed analysis of how the assay works is not available; the mechanism of luciferase itself is well studied but the larger assay system of dehydrogenase linked bioluminescence assays is not well studied.

The work presented in this thesis goes some way to elucidate the mechanism and regulation of assays in a way that has implications for all possible analytes not just the model analytes chosen to illustrate the system.

1.4.1 Wider uses of bacterial bioluminescence

The discussion in this introduction has concentrated on the use of bioluminescence in the detection of analytes through a biochemical reaction which resembles a straightforward enzyme assay. It is however worth noting that bioluminescence has wider applications which are not explored in this work but will be important to the future development of bioluminescence as an analytical tool. Other potential uses for luciferase would use the ease with which an unambiguous signal can be detected and the very high sensitivity of detection available with suitable equipment.

1.4.2 Immobilized enzyme studies

The potential of using immobilised bioluminescence enzymes in assays was realised some time ago when DeLuca first immobilised luciferase on glass (Jablonski and

DeLuca 1976). Since then many groups have explored ways of immobilising the enzymes, these were reviewed in section 1.3.

In terms of analytical uses of immobilised enzymes in bioluminescence applications several groups have recently been publishing methods for using immobilised enzyme systems for many of the assays previously detailed for solution assays. As with published articles on the solution assays the target has been a functional assay, the work on flow injection analysis (f.i.a.) presented in chapter five attempts to understand some of the behavioural characteristics of the system to provide a basis for designing assays and systems appropriate to the particular needs of analytical biochemists. The theoretical work on the assay kinetics shown in chapter six compliments the experimental studies described in the preceeding chapters. In the concluding chapter the results are brought together to show how bioluminescence can be best utilised in practice as an analytical tool.

CHAPTER 2

MATERIALS AND METHODS

2.1 REAGENTS

Laboratory chemicals were of analar grade or better where available. Where specific grades were used these are listed individually with supplier below.

Water, purified by reverse osmosis, ion-exchange and carbon filtration was equal to at least double distilled water and was used throughout unless otherwise stated.

2.1.1 Polyacrylamide gel electrophoresis (PAGE)

Electran grade chemicals from British Drug House (BDH), Poole were used for all PAGE work. Acrylamide, ammonium persulphate (APS), sodium dodecyl sulphate (SDS), and PAGE blue were all of Electran grade. N N'Methylene bisacrylamide was of a specially purified electrophoresis grade (BDH).

2.1.2. Microbiological Media

Tryptone and Yeast extract were both supplied by Oxoid. Agar and marine mix were from Difco.

2.1.3. Enzymes

Luciferase (E.C. 1.14.14.3) and NADH:FMN Oxidoreductase (E.C. 1.6.8.1) were purified from Vibrio harveyi strain B-392 or MB-20. Which were a gift from J.W. Hastings.

A luciferase, oxidoreductase mix was also supplied by Sigma Chemical Co., preparations from both V. harveyi and V. fisheri were supplied.

Diaphorase (E.C. 1.6.4.3) was supplied by Genzyme

Glucose Oxidase (E.C. 1.1.3.4) was supplied by Genetics International

Catalase (E.C. 1.11.1.6) was obtained from Boehringer Mannheim (BCL)

Lactate dehydrogenase (LDH) (E.C. 1.1.1.27), from hog muscle, and Malate Dehydrogenase (MDH) (E.C. 1.1.1.37) were also from BCL.

Lysozyme chloride (E.C. 3.2.1.17) from chicken egg white grade VI was supplied by Sigma.

Bovine Serum Albumin (BSA) was of radio-immuno assay grade (R.I.A.) and also from Sigma.

Trypsin (E.C. 3.4.21.4) and Deoxyribonuclease I (E.C. 3.1.21.1) were also from Sigma.

2.1.4. Co-factors

Oxidized nicotinamide adenine dinucleotide (NAD⁺), 100% grade and Reduced nicotinamide adenine dinucleotide (NADH), grade III were purchased from BCL.

Flavin mononucleotide (FMN) was a gift from Sigma.

2.1.5. Purification materials

DE-52	-	Whatman
DEAE-Sephacel	-	Pharmacia
Sephacel Q	-	Pharmacia
ACA 34 Ultrogel	-	LKB
Mono-S, FPLC column	-	Pharmacia
Mono-Q, FPLC column	-	Pharmacia
Mono-P, FPLC column	-	Pharmacia
5'AMP Sepharose 4B	-	Pharmacia

Ammonium Sulphate (enzyme grade) was supplied by BDH

2.1.6 Immobilisation reagents

Hydroxymethacrylate gel reagents: were supplied by BDH.

Latex beads: LB-1 and LB-16 came from Sigma, beads of diameter 72µm were supplied by Polysciences.

CNBr activated sepharose 4B beads were supplied in a dry powder form by Pharmacia.

Aminopropyl controlled pore glass, pour size 500 Å and bead size mesh 100 were obtained from Pierce.

Cellulose acetate powder was from Polysciences.

Carrageenan Type 1, 20% Lambda and 80% kappa, was supplied by Sigma. Agar was obtained from Difco.

Both DE-52 and CM-52 were from Whatman

Coupling agents: Poly phenyl:lysine HBr (1:1), M.W. c40,000, Poly L-lysine HBr, M.W. 14,000, glutaraldehyde, 1,6 diamino hexane, and 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide toluene sulphonate were all from Sigma.

2.1.7. Other reagents

Ethylenediaminetetraacetic acid disodium salt (EDTA)	- Fisons
Dithiothreitol (DTT)	- BCL and BDH
Cyclohexanone	- Fisons
Propan-2-ol	- Fisons
Acetone	- May and Baker
Decyl aldehyde, decanal	- Sigma
Myristic acid	- Sigma
2-mercaptoethanol	- BDH
L-Malic acid	- Aldrich
L-Lactic acid	- Aldrich
Amino ethane sulphonic acid, Taurine	- Sigma
U-14-C Taurine	- Amersham
Amino methane sulphonic acid	- Sigma
Glycine methyl ester	- Sigma
2,5 Diphenyloxazole, (PPO)	- Fisons (Sintilation grade)
1,4-Di-2-(4-methyl-5 phenyloxazole, (POPOP)	- BDH (Sintilation grade)
Triton X-100	- BDH (Sintilation grade)
Norleucine, Type II amino acid standard	- Sigma
Polyvinylpyrrolidone, (PVP)	- Aldrich
Charcoal, activated powder	- BDH
Charcoal, activated granuales 10-18 mesh	- BDH
Charcoal, acticated cloth	- Charcoal Cloth Co.

2.2 GENERAL TECHNIQUES

2.2.1 Spectrophotometry

All measurements were made using a Perkin Elmer lambda 3 spectrophotometer. For work at wavelengths greater than 340nm disposable polystyrene cuvettes (BCL or Elkay) were used, below 340nm quartz cuvettes (HELMA) were used.

For kinetic work and wavelength scans a lambda 3 was linked to a Perkin Elmer R100 recorder or a Varian DMS 2000 was used. Continuous 280nm absorbance readings for column chromatography were made using a Pharmacia UV-1 single path monitor connected to a chart recorder.

2.2.2 pH Measurements

All pH measurements were carried out using a Data Scientific PT-15 pH meter linked to either a standard Corning series 500 electrode or a Corning micro combination electrode. Merck pH strips or Whatman pH paper was used where a pH meter was not practical.

2.2.3 Conductivity

All conductivity measurements were carried out on a Data Scientific PTI-18 fitted with a conductivity probe.

2.2.4 Temperature control

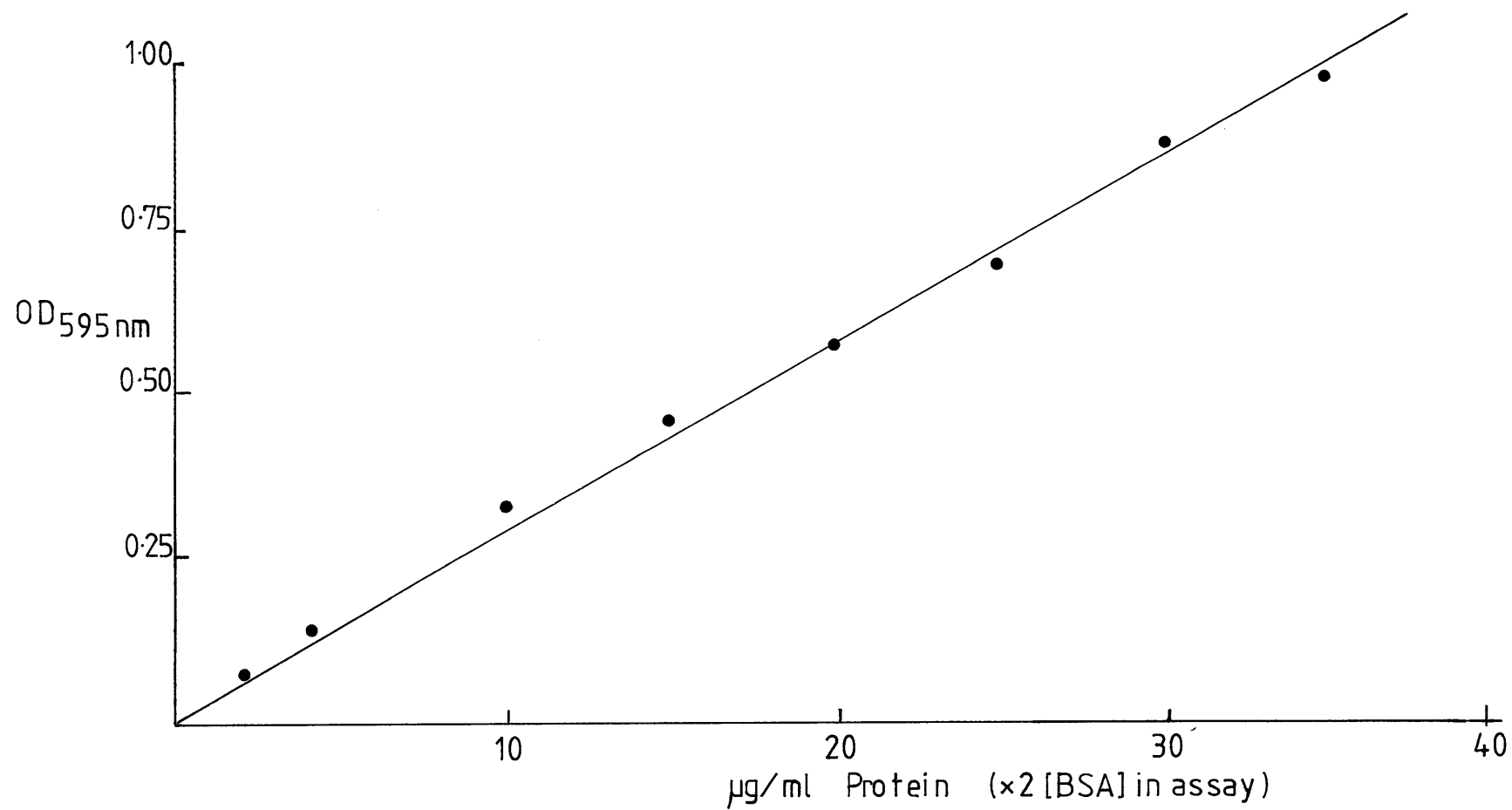
Unless otherwise stated the experiments were carried out at room temperature (21°C). Controlled temperature environments were provided by either incubation in a controlled temperature Grant water bath, a jacketed vessel fed with water from a Grant FH-15 circulator or by incubation in a controlled temperature room 4°C or 28°C. Temperatures were measured with a standard -10°C to + 110°C thermometer or with a thermocouple probe attached to a Kane-May 7002 portable pH/Temperature meter.

2.2.5 Protein estimation

Estimates of protein were obtained by measuring the absorbance at 280nm and using the approximation of 1 Absorbance unit = 1.0 mg/ml or more accurately for luciferase, 1 Absorbance unit = 0.94 mg/ml (Yien et al 1985).

More accurate protein estimations were done using the BioRad assay system based on the method of Bradford (1976). Standard calibration curves based on a series of standard BSA solutions were used to calibrate the protein assays (figure 2.1).

FIGURE 2.1 BIORAD Standard curve



2.2.6 Polyacrylamide Gel Electrophoresis (P.A.G.E.)

Both non-denaturing and SDS gels were prepared using the Pharmacia gel casting system. Slab gels were of dimensions 8.2 X 0.27 X 13.9 cm. Stock solutions were made as described by Hames and Rickwood (1981).

Solution A: 30% w/v acrylamide, 0.8% w/v bisacrylamide.

Solution B: 3.0M Tris-HCl pH 8.8

Solution C: 10% w/v SDS

Solution D: 0.5M Tris-HCl pH 6.8

All solutions were made up with double distilled water and filtered through Whatman No. 1 filter paper then stored at 4°C except for solution C which was stored at room temperature.

The following compositions were used to make the gels:

10% Resolving gel

40.0 mls solution A
60.8 mls ddH₂O
15.0 mls solution B
3.00 mls 3.0% ammonium persulphate (APS)

The above four components were mixed and degassed.
The following components were then added.

1.20 mls solution C
60.0 µls TEMED

4.5% Stacking gel

5.00 mls solution A
10.0 mls solution D
23.6 mls ddH₂O
1.00 mls 3.0% APS

The above four components were mixed and degassed.
The following components were then added.

0.40 mls solution C
30.0 µls TEMED

Running Buffer

A 10 X stock running buffer (30.3g Tris, 144g glycine in 1000 mls) was made up and stored at 4°C. The stock was diluted and 1g of SDS added per litre of running buffer.

Sample Buffer

1.25 mls solution D
2.00 mls solution C
0.50 mls 2-mercaptoethanol
1.00 mls glycerol
0.20 mls 0.1% Bromophenol blue
0.05 mls ddH₂O

Stored in aliquots at -20°C

Samples were mixed in a 1:1 ratio with sample buffer and placed in a water bath at 100°C for 5 minutes to complete denaturation. Approximately 10 µg of protein was loaded per track

Staining was done using 0.1% Coomassie blue in a water methanol glacial acetic acid mixture in the ratio of 5:5:2, staining took between 4 and 12 hours. Destaining was by incubation with a water methanol glacial acetic acid mixture in the ratio 7:2:1, after two washes with the above solution some MB-1 amberlite mixed bed resin can be added to speed up the destaining process.

2.3 BIOLUMINESCENCE ASSAYS

2.3.1 The turnover assay for luciferase, the dithionite assay

Of the two single turnover assays commonly used to estimate luciferase activity (Hastings et al 1978), the one using chemically reduced FMN was chosen. This assay uses sodium dithionite to reduce FMN and provide an anaerobic environment for the saturation of luciferase molecules with FMNH₂. Then an air equilibrated aldehyde emulsion is rapidly mixed and the light output monitored. The reaction follows the path described in the introduction.

Protocol for dithionite solution:

10 µl	10 mM FMN
x µl	Enzyme sample
480 - x µl	50 mM potassium phosphate pH 7.0, 0.2% BSA
10 µl	15 mg/ml N ₂ saturated solution of sodium dithionite

Mixed gently to give a homogeneous decolourised solution

Then the following solution was rapidly injected into the above solution already placed in the luminometer reaction chamber the emulsion is injected using a 500µl SGE syringe via the injection port through a light tight rubber septum.

500 μ l Air equilibrated aldehyde emulsion

Made as follows:

100 μ l	1.0 M Decanal in Ethanol
9900 μ l	50 mM potassium phosphate pH 7.0, 0.2% BSA

Were mixed in a 30ml glass sample bottle, the mixture was then sonicated for 30 second periods with 2 minutes cooling time until a homogeneous emulsion has been obtained.

The output of light was measured using both a chart recorder and the BBC computer (Appendix A). Peak light intensity is taken as a measure of luciferase activity.

2.3.2 Coupled assay

NADH is assayed using a defined quantity of luciferase and FMN:NADH oxidoreductase, and bioluminescence enzymes present using a standard concentration of NADH.

Protocol:

10 μ l	0.1 mg/ml Decanal in isopropanol
10 μ l	0.5 mM FMN
960 μ l	50 mM potassium phosphate pH7.0, 0.2% BSA
10 μ l	Enzyme solution
10 μ l	NADH stock, or unknown sample

If different amounts of enzyme solution or NADH are added the quantity of buffer is altered to maintain the final volume at 1.0 ml.

When activity of an enzyme solution is being tested a final concentration of 0.1 mM NADH in the cuvette was used.

2.3.3 Bioluminescent assay for FMN:NADH oxidoreductase

Reductase was assayed by this method after affinity purification. The method of Hastings *et al* (1975) was modified and developed as described below.

Protocol

10 μ l	0.1 mg/ml Decanal in isopropanol
10 μ l	0.5 mM FMN
950 μ l	50 mM potassium phosphate pH 7.0, 0.2% BSA
10 μ l	FPLC purified luciferase
10 μ l	10 mM NADH

*** Take background reading ***

10 μ l Reductase sample

2.3.4 Malate and lactate bioluminescence assays

Both malate and lactate were assayed using enzyme solutions containing the two bioluminescence enzymes and the appropriate dehydrogenase.

2.3.4.1 Protocol for malate assay:

- 10 μ l 0.1 mg/ml Decanal in isopropanol
- 10 μ l 0.5 mM FMN
- 10 μ l 100 mM NAD⁺
- 950 μ l 50 mM potassium phosphate pH 7.0, 0.2% BSA
- 10 μ l Enzyme solution
- 10 μ l Malate sample

2.3.4.2 Protocol for lactate assay:

- 10 μ l 0.1 mg/ml Decanal in isopropanol
- 10 μ l 0.5 mM FMN
- 10 μ l 1.0 mM NAD⁺
- 950 μ l 50 mM potassium phosphate pH 7.0, 0.2% BSA
- 10 μ l Enzyme solution
- 10 μ l Lactate sample

Both assays are made up to a final volume of 1.00 ml by adjusting the volumes of buffer so that different volumes for any reagents are compensated for by the adjusted volume of buffer added.

2.3.4.3 Computer simulation of assay kinetics

A series of computer simulations of the bioluminescence assays were run using a program based on the model developed in chapter six. The program was written in Fortran and run on the Imperial College Cyber 635 mainframe, results were downloaded onto an IBM P.C. using VTERM software the data was edited using WORDSTAR and then displayed using a specially written program in BASIC. The results were displayed graphically on a colour screen, the figures included in chapter six were based on the down loaded data points from the simulations.

Details of the computer simulation programs and how they were used are given in appendix B.

2.3.5 Assay of in vivo luciferase content

2.3.5.1 Enzyme assay

The method of Rossen and Nealson (1981) was used

Protocol:

0.1 - 5.0 mls of cell suspension was filtered through a Millipore 0.47 μ m filter and the filter frozen

at -20°C before assaying. The filter paper was incubated at 4°C with 5 mls of the following lysis buffer: 10 mM EDTA pH 7.0, 1 mM DTT, 0.1% BSA, 50 µg/ml lysozyme for 16 hours. An aliquot of this solution was then used in a dithionite assay.

2.3.5.2 In vivo assay of a cell suspension

Freshly harvested cells, sampled from either a shake flask aseptically or from the fermenter were immediately diluted into sterile sodium chloride complete media to give a measurable signal on the LKB 1250 (i.e. a non overload signal). During a growth it is convenient to start with a 1:100 fold dilution of the sample and add subsequent dilutions as necessary. The initial light reading is taken, since rapid oxygen depletion and cell growth cause substantial variation of light signal in the non stirred cuvette.

2.3.6 Immobilised enzyme bioluminescence assays

2.3.6.1 Suspension assays

All the above bioluminescence assays except the reductase specific assay were also carried out using suspensions of enzymes immobilised on the following carriers:

- Sepharose 4B beads
- CPG beads
- Latex beads
- DE-52
- CM-53

The beads were added as a 10 µl (or larger where necessary) aliquot of the bead suspension with the aid of a pipette tip modified by removing the first 3-5 mm of the tip with a scalpel to allow free flow of the bead suspension during pipetting.

When enzyme is immobilised on the cuvette surface, the cuvette itself provides the enzyme and the 10 µl enzyme aliquot is omitted altogether. In addition since excess decanal is inhibitory to the luciferase activity the assay solution is pre-mixed then added to the assay cuvette, final volume 1.00 ml, in the case of coupled assays.

2.3.6.2 Assays with gel entrapped enzymes

Where the enzyme preparation is other than a suspension of beads or the side of the cuvette, i.e. slices of carrageenan gel, the slice is added to the 1.00 ml assay solution in the cuvette.

2.3.6.3 Flow injection analysis assays

Using the apparatus shown in figure 2.2 it was possible to analyse sequentially samples of NADH, malate or lactate with the appropriate preparation of immobilised enzymes on beads. The beads are loaded into the flow cell Figure 2.3 by removing the top plunger and aliquoting in a suspension and allowing the beads to settle up to the top of the column then replacing the top plunger without allowing any air to become trapped.

The injection loop allows samples of 100, 200, or 500 μ l to be assayed as a plug of solution within the constant flow of buffer, 50 mM potassium phosphate pH 7.0, 1.0 mM DTT. The flow rate is controlled by a motor driving a storage syringe. Samples were made up as for the bioluminescence assays listed under section 2.3.2 in volumes of 1.00 ml but with no enzyme and the injection loop loaded.

Data was recorded on a chart recorder and using the BBC computer and BIOPACK (Appendix A).

2.3.7 Measurement of Response time

Defining the response time of an assay is not as straight forward as might at first appear; since subtle differences in the profile of the bioluminescence assays can occur depending on the precise conditions of the assay. Several different measurements of response time are therefore used in this work, the type used is discussed at the appropriate point in the text.

* Time to reach a peak value ie. 100% of maximum light intensity, is used where the kinetics make this a convenient and meaningful measure of the assay response time. The 100% measure was used for dithionite assays in particular, and in some cases of coupled assay where there was significant decay of signal after the peak light level was reached, the peak was found useful in immobilised assays where an initial hump was observed under saturating substrate conditions prior to attainment of a plateau level

* Time to reach 95% of the measured maximum light level was routinely used in solution coupled assays, and with some immobilisations where response was slow and the time to final, near plateau, light intensity highly variable with assay conditions.

* A further measure the $t_{1/2}$ of the response time was used for more specific correlation of assay response time to substrate concentrations. The $t_{1/2}$ of the signal rise was taken as the time difference between the time taken to reach 50% and 75% of maximum observed light output.

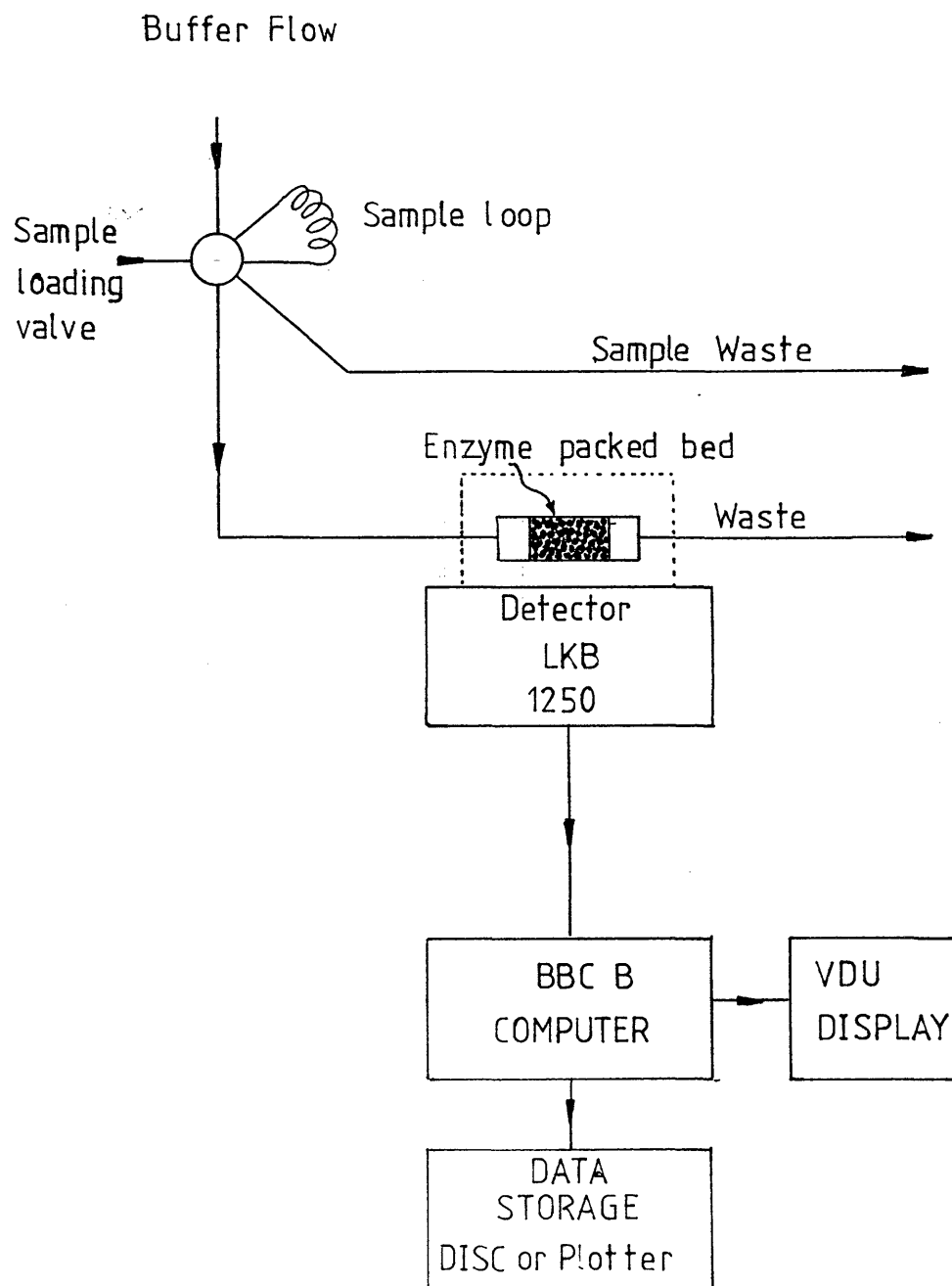


FIGURE 2.2
Arrangement for flow injection analysis

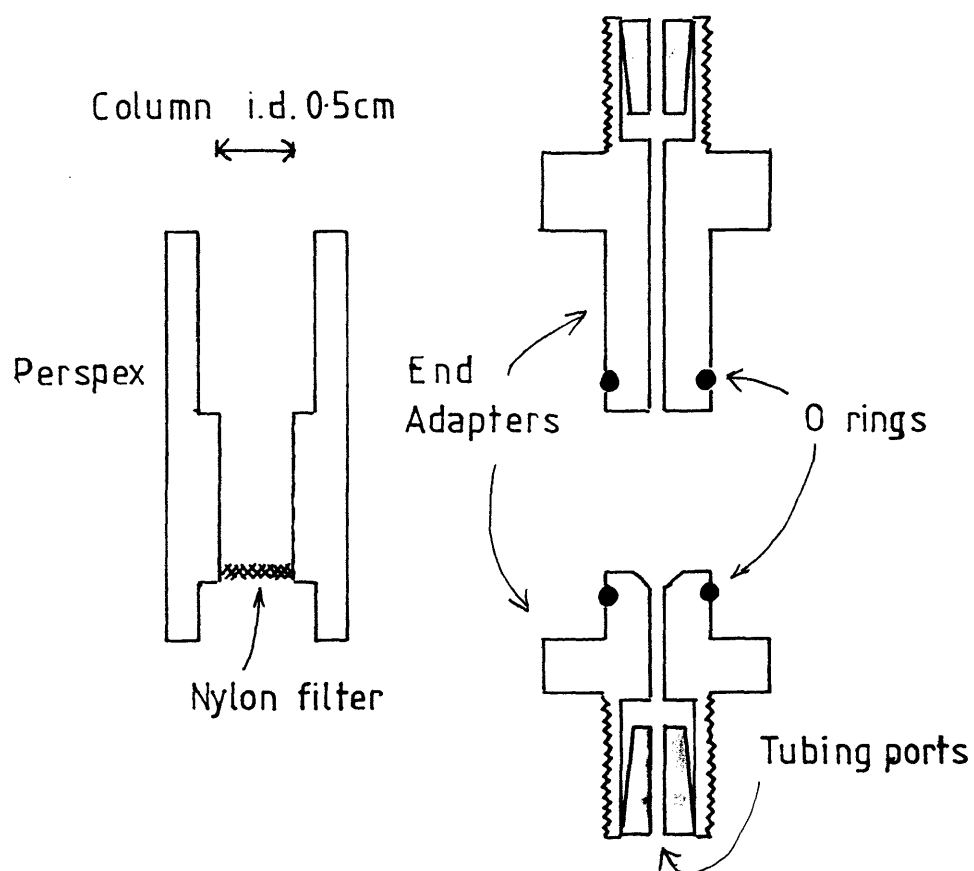


FIGURE 2.3
Flow cell design for packed bed work

Using this measure the worst distortions at either the start and finish of the assay were removed. The origin of these distortions is discussed in Chapter 6.

2.3.8 Determination of the enzyme dependence of the response time

The assay protocols for malate and NADH were altered to allow assay with various concentrations of Malate Dehydrogenase ([MDH]) and Oxidoreductase respectively. Response times were measured by the third method described above. In some of the experiments diaphorase was substituted in the malate assays for the bacterial oxidoreductase to eliminate background activity due to contamination of the reductase.

2.3.9 NADH coupled assay profile at controlled O₂ tensions.

Dissolved oxygen in the standard assay was removed enzymatically by use of excess glucose oxidase and catalase acting upon a specified aliquot of a D-glucose solution. Catalase (E.C. 1.11.1.6) at 20 mg/ml suspension with an activity of 40-50K units/mg and Glucose oxidase (E.C. 1.1.3.4) (GOD) at 270 mg/ml with an activity of 180 units/mg were in a ratio of 1:1 by volume to produce an enzyme mixture capable of converting all the glucose in under 2 minutes.

The standard bioluminescence assay was altered as follows to allow control of the oxygen concentration by use of the catalase/GOD mixture and glucose solution.

10 μ l 0.5 mM FMN
10 μ l 0.1 mg/ml Decanal in iso-propanol
10 μ l Catalase/GOD mixture as above
850-x μ l 50 mM Phosphate buffer solution at pH 7.0
10 μ l Luciferase/Reductase mixture
x μ l Glucose mixture 2mM concentration
100 μ l 1 mM NADH solution

The response time to reach 50% and 75% of the peak (plateau) light levels were recorded and $t_{1/2}$ calculated as described in section 2.3.5. A plot of $\ln|2|/t_{1/2}$ against $[O_2]$ was used to estimate k_a and k_l as described in chapter six.

2.4 SPECTROPHOTOMETRIC ASSAYS

The following extinction coefficients and wavelengths were used for the determination of the following substances

Substance	Wavelength/nm	/ $M^{-1} \text{ cm}^{-1}$
NADH	340	6220
FMN	445	12200
Protein	280	1.0(mg/ml)
BIO-RAD Protein - dye complex	595	Determined by BSA standards
Cell density	660	N/A
DNTB	410	13,600

2.4.1 FMN:NADH oxidoreductase assay

This assay follows the oxidation of NADH by the change in 340 nm absorbance. The method was adapted from that of Jablonski and Deluca (1977).

Protocol:

968-x μl 50 mM potassium phosphate pH 7.0
14 μl 10 mM FMN Final concentration 130 μM

Mix and read at 445 nm to check FMN concentration on first assay

x μl enzyme solution to be assayed

** Mix and zero at 340 nm before adding the NADH **

17 μl 10 mM NADH Final concentration 160 μM

2.4.2 Determination of - SH groups

This method was adapted from that of Talgoy et al (1979).

Protocol:

A stock solution containing Ellman's reagent, 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was prepared: 1 mM DTNB in 0.2 M KCl, 0.1 M Tris-HCl and 0.002 M EDTA pH 7.5.

In a disposable 1 ml cuvette 500 μl of enzyme solution previously dialysed into 0.1 M Tris-HCl pH 7.5 was mixed with, 50 μl of the above DTNB stock in 450 μl of 0.2 M

KCl, 0.1 M Tris-HCl 0.002 M EDTA pH 7.5. The increase in absorbance at 410 nm due to release of the 5-mercapto-2-nitrobenzoate dianion (figure 2.4) is followed and the concentration of -SH groups per mole of enzyme worked out using $\epsilon = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$.

2.4.3 Malate assay

A BCL L-malic acid assay kit was used for estimation of malate in samples. Standard BCL instructions provided with the kit were followed.

2.4.4 Lactate assay

As with the malate samples a BCL L-lactic acid assay kit was used to determine the concentration of lactate in samples. With the lactate samples a pretreatment step was used to reduce the turbidity of the solution. The method used Carrez reagents to precipitate excess proteins, details were as described in the assay kit instructions.

Carrez clarification technique:-

1g of sample was placed into a 100ml volumetric flask and 60 mls of distilled water added, the mixture was then left at 70°C for 15 minutes with occasional shaking before adding the following:

5 ml (3.6g Potassium ferrocyanide trihydrate in 100 ml)
5 ml (7.2g Zinc sulphate septahydrate in 100 ml ddH₂O)
10 ml 0.1 M NaOH

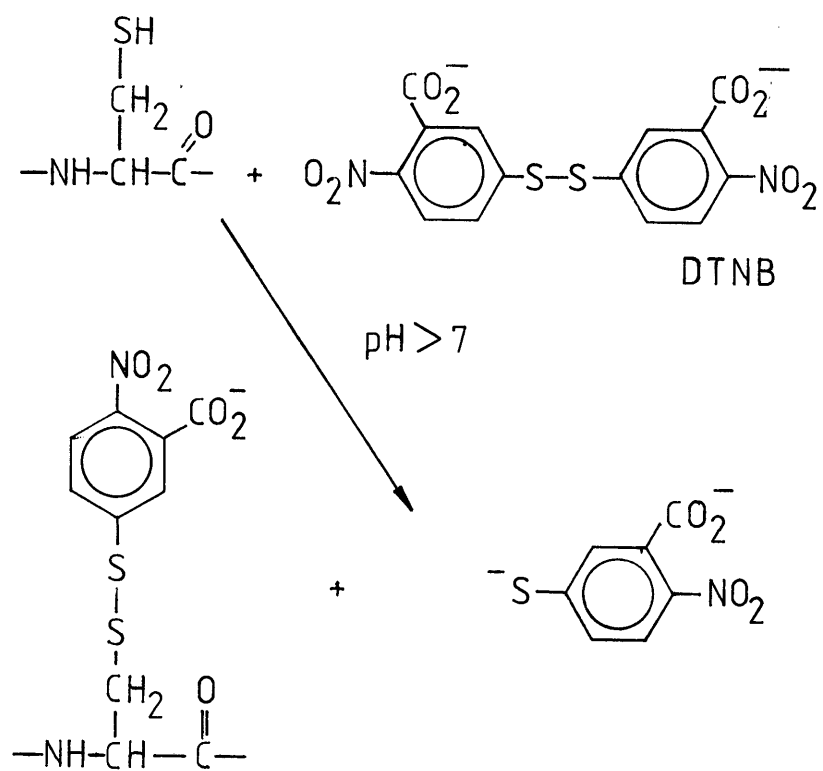


FIGURE 2.4
DTNB assay for free thiol groups

2.5 RADIOLABELING

2.5.1 Scintillation Counting

C-14 labelled samples were counted in an Intertechnique SL30 liquid scintillation counter after at least 30 min incubation in 5 mls of a Toluene based scintillant:

660 mls Toluene A.R.
330 mls Triton X-100
5.0 g PPO
0.25 g POPOP

PPO is 2,5-Diphenyloxazole

POPOP is 1,4-Di-2(5-phenyloxoly)benzene

Counting was done for four minutes on a window setting to count only the C-14 channel.

2.5.2 Preparation of protein samples for radioactive assay of taurine content.

100 ul aliquots protein were mixed with 0.9 ml of 12% TCA solution on ice and then left for 30 minutes to allow the protein precipitate to form. The TCA/protein solution was then filtered on 0.22 μ m Glass fibre filters (Whatman) and the filters washed with 12% TCA, 0.5 M NaCl and absolute alcohol in that order to ensure that non-specifically bound radioactivity was all removed. The filter papers were then air dried before assay in the scintillation fluid as described above.

Controls of unlabelled protein were used to measure the level of background radioactivity. The specific activity of the label was calculated from the assay of a 5 μ l aliquot of the modification mixture, containing a known specific activity of 14-C taurine or a background unlabelled taurine stock, added directly to a scintillation vial with 5ml of scintillant.

2.6 AMINO ACID ANALYSIS

2.6.1 Sample preparation

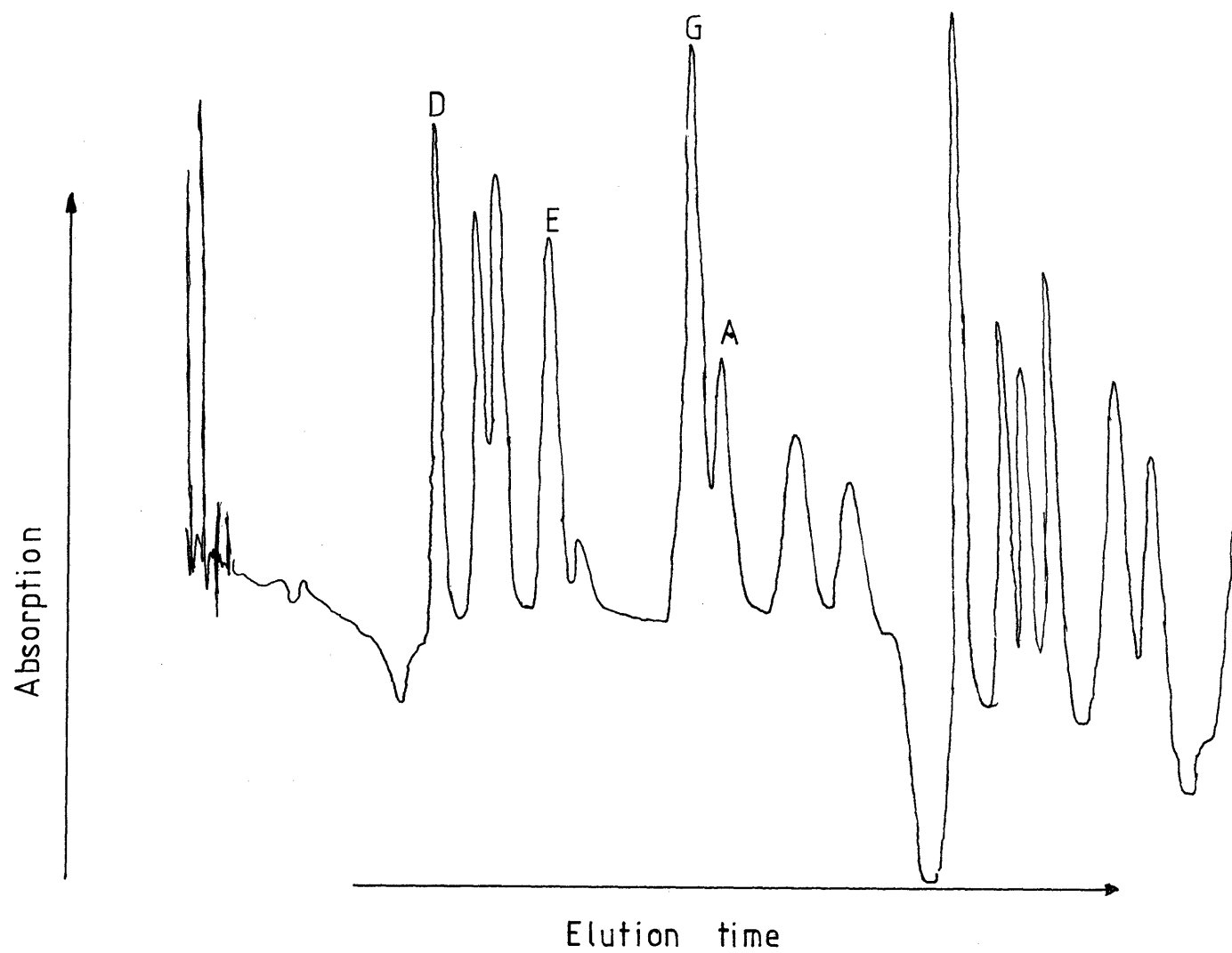
Samples were prepared by drying down aliquots of material in hydrolysis tubes in a rotovap centrifuge. The samples were then incubated in the same tubes with 50 μ l of 6 M HCl and 2 nmoles/50 μ l norleucine as a standard marker. The incubation was in the hydrolysis tube, sealed under vacuum, for 24 hrs at 100°C.

After breaking the seal the sample was dried in the rotovap to a powder and then given to the departmental amino acid analysis service, who provided amino acid profiles and a standard trace for identification of peaks using a Beckman 121 MB machine (Figure 2.5).

2.6.2 Interpretation of Amino acid analysis for Taurine.

Calculation of the number of moles of taurine incorporated per mole of protein was done by reference to the ratio of taurine, after correction for the ninhydrin colour strength against nor-leucine (0.33x), to the three indigenous amino acids leucine arginine and glycine present in the protein in previously determined quantities.

FIGURE 2.5 amino acid standard trace



2.7 MICROBIOLOGICAL TECHNIQUES

2.7.1 Growth Media

Vibrio harveyi strains B 392, MB 20 and MB 17 were the kind gift of J.W. Hastings. The strains were revived from the stab cultures on two media: marine mix and sodium chloride complete mix. Both marine broth and marine agar were supplied as a powder by Difco. The sodium chloride complete media were made up as follows:

Growth Media

7.0g Na₂HPO₄·7H₂O
1.0g KH₂PO₄
0.5g (NH₄)₂HPO₄
0.1g MgSO₄
30 g NaCl
5.0g Tryptone
3.0g Yeast extract
2.0 ml Glycerol

Add 1 litre ddH₂O and adjust the pH to 7.0 for the sodium complete broth

For solid media add 12g agar (Difco)

2.7.2 Storage and Maintenance

The strains were all plated out onto petri-dishes, fresh slope and stab cultures were also set up. All strains were successfully revived. Long term storage was in the dark at room temperature.

B 392 and MB 20 were plated out and the brightest colonies picked out after 16 hrs incubation at 29°C prior to each experiment for which they were needed. Primary cultures were grown in shake flasks at 28°C.

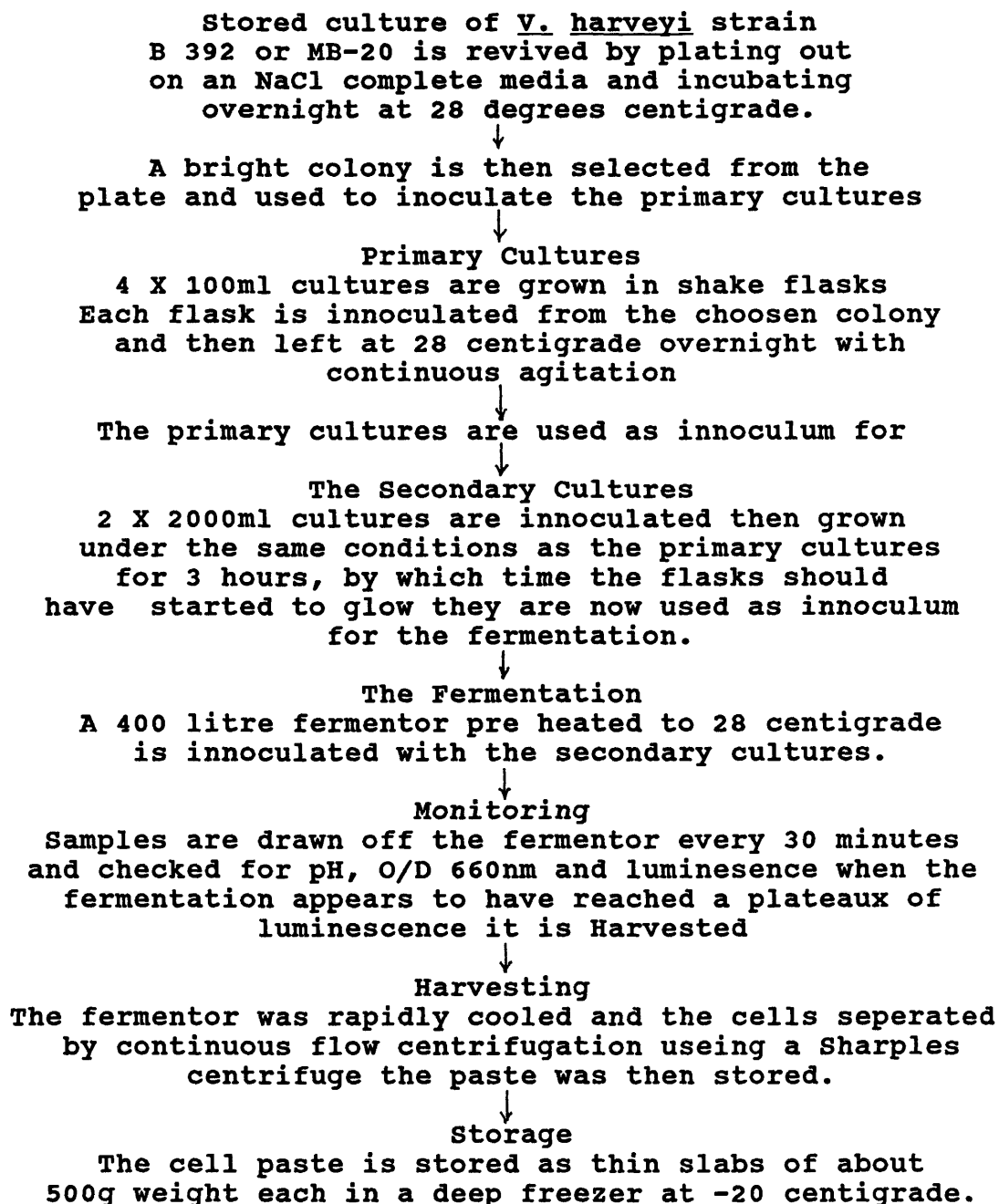
2.7.3 Fermentation protocol

The 60 litre and 400 litre fermentation protocols of V. harveyi were developed from the method described by Hastings et al (1978), figure 2.6. A single bright colony was picked off a plate and used to inoculate 4 X 100 ml primary cultures. These were then grown for 14 hrs and used to inoculate 2 litre secondary cultures. The secondary cultures were then grown for 3 hrs and added to the fermenter as an inoculum. The cells were harvested when the luminescence from the culture reached a plateau, harvesting involved cooling the fermenter rapidly and harvesting through a continuous flow Sharples centrifuge. The cell paste was then frozen in thin sheets of about 500g each for future use.

FIGURE 2.6

Flow chart for a Vibrio harveyi fermentation

The chart shows the development of a 400 litre fermentation of bacteria from a stored colony. Growth times are approximate, the final harvesting is determined on the basis of rapid in vivo assays as described in section 3.1.



2.8 PROTEIN PURIFICATION

2.8.1 Luciferase

The following method was developed from the original method of Hastings et al (1978). Changes were made to improve the speed and ease with which the purification could be carried out without sacrificing yield or purity (figure 2.7).

Lysis of cells

500g blocks of cells were broken up, as they thawed small amounts of DNase and magnesium chloride were added to break down nucleic acids which otherwise give the cell paste a sticky and less managable consistency. A thin cell suspension is made by adding 100 mls of 3% NaCl solution to the cells and gently mixing in the paste. Lysis is then achieved by osmotic shock. The cell suspension is slowly added to 4.5 litres of lysis buffer, 5 mM potassium phosphate, 0.1 mM dithiothreitol (DTT), 10 mM EDTA pH ~7.0, and continuously stirred with an overhead stirrer at 4°C.

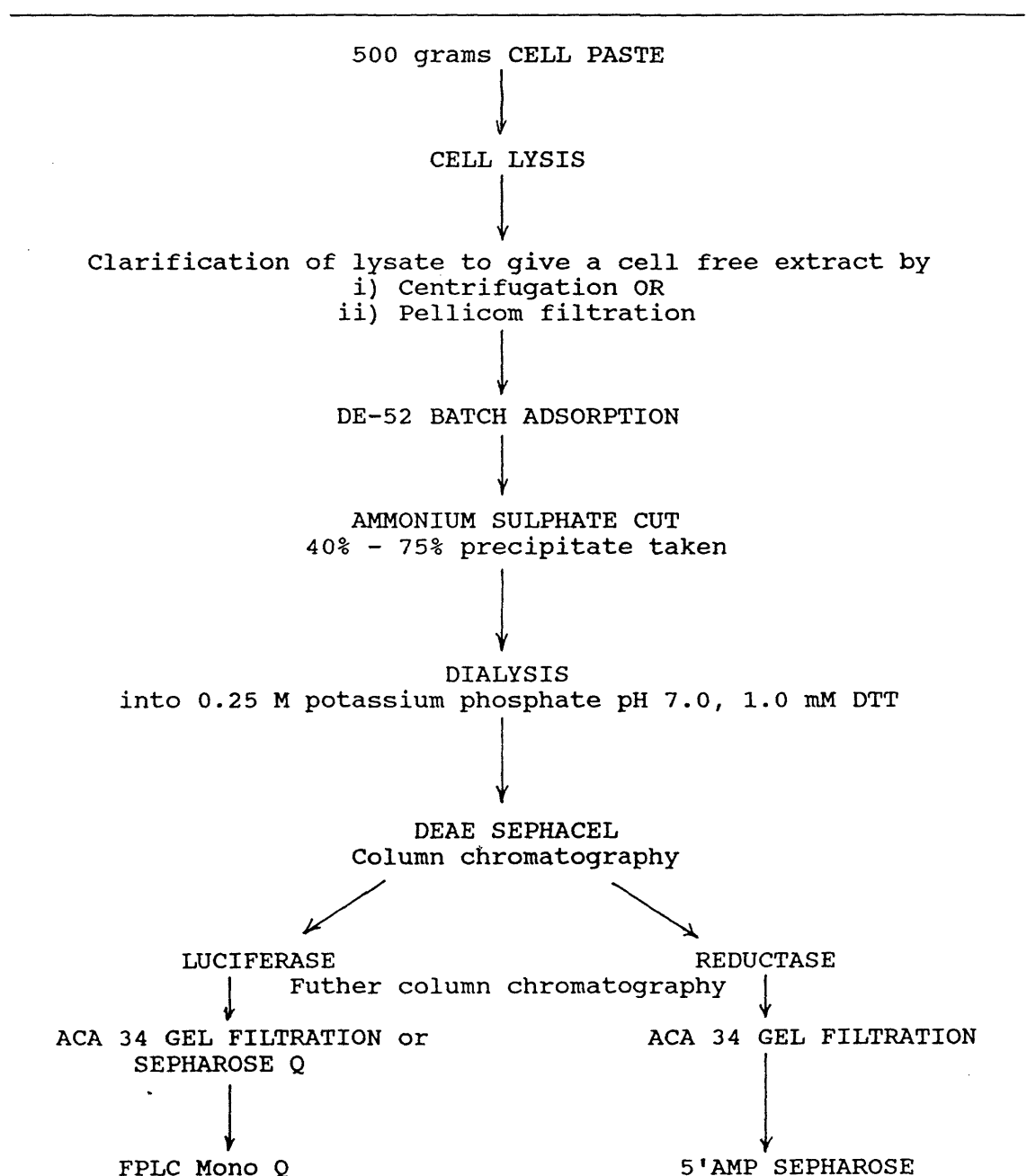
Lysis was monitored by withdrawing 0.5 - 1.0 ml aliquots. These samples were then spun down in a bench centrifuge, the supernatant and pellet are assayed for luciferase by the dithionite assay. Samples were taken every 5 - 10 minutes until the activity in the supernatant represented more than 90% of the total and had reached a plateau level.

Clarification of the lysate

The cell debris was removed from the lysate by one of two methods. In early work the lysate was clarified by centrifugation at 10,000 rpm in a Beckman J2-21 centrifuge using a JA 20 rotor for 30 minutes. This method was superceded by using a Millipore Pellicon apparatus to filter the lysate. When pellicon filtration was used the thickening slurry was diluted with fresh lysis buffer, up to three additional litres of buffer were added. Fractions, of one litre or more were collected and added to a bucket containing 200 mls of regenerated DE-52 resin. The DE-52 adsorption step was carried out at pH ~6.8, the pH being maintained by addition of solid Tris base when necessary, the mixture was continuously stirred with an over head stirrer and maintained at 4°C. Adsorption to the resin was monitored in the same way as for lysis. When the activity in the supernatant was reduced to less than 10% of the original the batch elution process was started.

FIGURE 2.7

Purification scheme for luciferase and FMN:NADH oxidoreductase from *Vibrio harveyi*



Batch Elution

DE-52 batch elution was carried out at three different ionic strengths. All washes were carried out by mixing the resin with an aliquot of wash solution for 5-10 minutes then draining the resin under vacuum in a buchner funnel lined with muslin before adding the next aliquot. The following washes were used to extract the enzymes.

- 1) 5 X 100 ml aliquots of ddH₂O at 4°C
- 2) 2 X 200 ml aliquots of 0.15 M potassium phosphate pH 7.0, 0.1 mM DTT at 4 C
- 3) 5 - 10 X 200 ml aliquots of 0.35 M potassium phosphate pH 7.0, 0.1 mM DTT at 4 C

The fractions were assayed for activity and protein content. The luciferase is found to be concentrated in the first 5 -10 0.35 M phosphate washes. The fractions containing the bulk of the luciferase are pooled and then precipitated with ammonium sulphate. Reductase activity is normally found in both the 0.15 M washes and the 0.35 M washes. Sufficient reductase is found in the 0.35 M washes pooled for luciferase purification. Two proteins are separated later on the DEAE column.

Ammonium sulphate precipitation

A first cut to 40% was made and after checking for activity the precipitate was discarded. The second cut to 75% precipitates both the reductase and luciferase. After resuspension into a minimum volume of 0.25 M potassium phosphate pH 7.0, 0.1 mM DTT the solution was dialysed against 4 litres of the same buffer overnight then against 2 X 4 litres the next day before loading onto the DEAE sephacel column.

2.8.1.1 Low Pressure Chromatography

DEAE sephacel chromatography

Approximately 440 mls of DEAE sephacel were loaded into a 30 cm x 5 cm column and equilibrated with 0.25 M potassium phosphate pH 7.0, 0.1 mM DTT. The dialysate was loaded at a flow rate of 60 mls/hr Then one of two elution profiles were used. Either a step elution of 600 mls 0.25 M potassium phosphate, 0.1 mM DTT followed by 1000 mls of 0.35 M potassium phosphate, 0.1 mM DTT, or a gradient elution of 2000 mls volume from 0.25 M potassium phosphate, 0.1mM DTT to 0.5 M potassium phosphate, 0.1mM DTT. Conservative pools of both reductase and luciferase were made for further column chromatography.

ACA 34 chromatography

The pooled fractions from the DEAE sephacel were concentrated by ultrafiltration to ~100 mg/ml before loading on the ACA 34 column in volumes up to 10 mls. The ACA 34 column was 90 cm x 5 cm and had a volume of ~2000 mls a flow rate of 50 mls/hr was used. After loading the sample an equal volume of 10% sucrose solution was loaded to reduce diffusion of the protein band.

2.8.1.2 FPLC Purification

FPLC Mono Q

Separation of the luciferase from remaining impurities was done using a Pharmacia FPLC apparatus fitted with a Mono Q anion exchange column. The method used is listed below and was found to be the optimal gradient profile for separation. Between batches the gradient profile had to be slightly adjusted. A similar gradient profile also proved successful on Mono S anion exchanger.

Buffers: A, 50 mM Tris-HCl, 1.00 mM DTT pH 8.0
B, 50 mM Tris-HCl, 1.00 mM DTT, 1.00 M NaCl pH 8.0

Alternative buffer combinations used were: as above at a pH of 7.0 or a solution based on a system of potassium phosphate buffers at pH 7.0 giving a gradient from 50 mM phosphate to 1.00 M phosphate. The above Tris buffers at pH 8.0 and the below gradient profile gave the best separation and was routinely used as a final stage in purifying the luciferase to >95% purity, a single protein by SDS/PAGE.

Method

TIME	PARAMETER	VALUE
0.00	CONC %B	30.0
0.00	ML/MIN	0.50
0.00	CM/MIN	0.50
0.00	VALVE.POS	1.2
0.00	PORT.SET	6.1
0.00	CLEAR DATA	
0.00	MONITOR	1
0.00	LEVEL %	5
0.00	MIN/MARK	5
10.0	CONC %B	30.0
20.0	CONC %B	40.0
30.0	CONC %B	40.0
30.0	CONC %B	30.0
40.0	CONC %B	30.0
40.0	INTEGRATE	0
END		

2.6.1.3 Sepharose Q

Samples of DEAE Sephacel purified luciferase were applied to a Sepharose Q fast flow anion exchange column. The column was pre-equilibrated with 0.05 M Tris pH 8.0, 0.1 mM DTT and after sample loading a gradient of 0.00 M to 1.00 M NaCl in the above Tris based buffer was applied. A flow rate of 400 mls/hr was used with a column size of 2.5 cm x 30 cm.

2.8.2 FMN:NADH Oxidoreductase

As mentioned above the purification of the reductase is simultaneous with the luciferase, although some reductase is separated from the luciferase at the batch elution stage sufficient enzyme is carried through to the DEAE Sephacel stage for research purposes. After the DEAE sephacel the reductase fractions are separated and concentrated by ultrafiltration as for the luciferase fractions. The pool was then purified on the same ACA 34 column as the luciferase. The resultant protein fractions however still contain many impurities.

2.8.2.1 FPLC Purifications

FPLC anion exchangers Mono Q and Mono S with gradient profiles similar to those for luciferase were tried.

Buffer A: 50 mM potassium phosphate pH 7.0, 1.0 mM DTT
Buffer B: 0.5 M potassium phosphate pH 7.0, 1.0 mM DTT

Method

TIME	PARAMETER	VALUE
0.00	CONC %B	0.00
0.00	ML/MIN	1.00
0.00	CM/MIN	1.00
0.00	VALVE.POS	1.2
0.00	PORT.POS	6.1
0.00	CLEAR DATA	
0.00	MONITOR	1
0.00	LEVEL %	5
0.00	MIN/MARK	5
5.00	CONC %B	0.00
20.0	CONC %B	50.0
25.0	CONC %B	50.0
25.0	CONC %B	0.00
30.0	CONC %B	0.00
30.0	INTEGRATE	0
END		

2.8.2.2 Affinity Chromatography

However the best results were obtained using the method of Jablonski and Deluca (1977). A 5'AMP sepharose 4B (Pharmacia) column (0.6 cm x 4.0 cm) was poured and pre-equilibrated with 0.05 M potassium phosphate pH 7.0, 0.5 mM DTT followed by sample loading then step elution with 5 mls of:

- 1) 0.05 M phosphate pH 7.0, 0.5 mM DTT
- 2) 0.05 M phosphate pH 7.0, 0.5 mM DTT, 0.5 mM NAD⁺
- 3) 0.05 M phosphate pH 7.0, 0.5 mM DTT, 1.0 mM NAD⁺
- 4) 0.05 M phosphate pH 7.0, 0.5 mM DTT, 2.5 mM NAD⁺
- 5) 0.05 M phosphate pH 7.0, 0.5 mM DTT, 5.0 mM NAD⁺

The fractions were collected manually in 1 ml aliquots and then assayed for reductase activity by the coupled bioluminescence method described in the previous section. Protein assays were by the Bio-Rad assay method.

2.8.3 FPLC Subunit separation

The two subunits were separated by FPLC anion exchange chromatography on Mono Q resin. The subunits were dialysed into 8.0 M urea, 40 mM potassium phosphate buffer pH 7.00, 1.0 mM DTT, 0.1 mM EDTA

Buffer A: 8.0 M urea, 40 mM potassium phosphate buffer pH 7.00, 1.0 mM DTT, 0.1 mM EDTA

Buffer B: 8.0 M urea, 0.5 M potassium phosphate buffer pH 7.00, 1.0 mM DTT, 0.1 mM EDTA

Method

TIME	PARAMETER	VALUE
0.00	CONC %B	0.00
0.00	ML/MIN	0.50
0.00	CM/MIN	0.50
0.00	VALVE.POS	1.2
0.00	PORT.POS	6.1
0.00	CLEAR DATA	
0.00	MONITOR	1
0.00	LEVEL %	5
0.00	MIN/MARK	5
10.0	CONC %B	0.00
30.0	CONC %B	50.0
32.0	CONC %B	100.0
42.0	CONC %B	100.0
42.0	CONC %B	0.00
50.0	CONC %B	0.00
40.0	INTEGRATE	0
	END	

Subunits were then dialysed against 0.1 M potassium phosphate, 10% glycerol, 1 mM EDTA, 1 mM DTT pH 7.0. The subunits were recombined by mixing in a recovery solution of 0.1 M potassium phosphate, 1 mM DTT, 1 mM EDTA pH 7.0 and incubating for > 48 hrs.

2.9 IMMOBILIZATION TECHNIQUES

2.9.1 Protein membrane: Glutaraldehyde - protein cross-linked membrane

The method was adapted from that of Thomas as described by Broun (1976). A solution of 1 mg/ml luciferase (or luciferase/reductase mixture), 99 mg/ml BSA was made up in 0.1 M potassium phosphate buffer pH 7.0. 5 mls of this solution was then stirred slowly using an end over end mixer and 2 mls of 2.5% glutaraldehyde solution was added dropwise. The mixture was left for an hour to react. Protein membranes were then cast in one of three ways.

1) The solution was spread over the base of two petri dishes to form a thin layer or on the surface of a glass plate etched with a square to define the membrane boundaries.

2) Droplets of the solution were placed on the surface of the petri dishes. 50 μ l was chosen as the volume of the droplets.

These two preparations were then left at 4°C overnight to set into a membrane or small enzyme pellets

3) Using semi-disposable innoculating loops thin films of enzyme membrane were formed. The loops were dipped into the solution, removed and allowed to dry/set for 3 hrs the loops were then immersed in a solution of 50 mM potassium phosphate buffer pH 7.0 containing 0.1 M lysine for 16 hrs to remove excess glutaraldehyde.

The above methods were varied in several ways in the light of experiments. Concentrations of glutaraldehyde were altered and a spacer molecule 1-6 diaminohehexane was added to a final concentration of 3 mM.

2.9.2 Entrapment in Agar gel

Agar solutions were made up at concentrations w/v of 1% to 6% in 50 mM potassium phosphate pH 7.0 solution by heating to 100°C. The solutions were allowed to cool to 45°C in a water bath. The agar is maintained at this temperature in 1.0 ml aliquots in eppendorf tubes. 0.1 ml of a 10 mg/ml luciferase preparation is rapidly mixed with a 1.0 ml aliquot of agar and put on ice to set. Agar blocks were then sliced and washed with 50 mM potassium phosphate buffer pH 7.0 until the O/D 280 nm of the washes became insignificant.

2.9.3 Carrageenan "Irish peat moss" entrapment

20 mls of carrageenan solution was prepared at concentrations of between 1.0% and 0.25% w/v in 50 mM potassium phosphate pH 7.0 by heating to 100°C and allowing to cool to 40°C. To the cooling carragenan 4 mls of enzyme solution at room temperature 21°C is added and rapidly mixed. The resulting solution was immediately poured onto a setting plate, this is a flat aluminium plate resting on ice. A thin layer of solution is deposited and after a few minutes as the layer sets it is washed with 0.5 M potassium phosphate pH 7.0 which enhances the membrane setting and rigidity. The membrane is then cut into 1 cm wide strips and stored in the same buffer with 1 mM DTT at 4°C

2.9.4 Cellulose acetate entrapment

Cellulose acetate solutions were made up in acetone and in cyclohexanone according to the method of Taylor et al (1977) with slight modifications.

From the original methad of, 1 part cyclohexanone, 1/24th part cellulose acetate resin, 1/23rd part isopropanol the following mixtures were devised:

solution 1

1g cellulose acetate
1g isopropanol
24g cyclohexanone

solution 2

1g cellulose acetate
25g cyclohexanone

solution 3

1g cellulose acetate
25g acetone

To the above solutions preparations of both lyophilised enzyme and enzyme on liquid anion exchanger LA-2 (BDH) were added. Enzyme was adsorbed to LA-2 by making an emulsion of LA-2 and luciferase solution, then separating the LA-2 after loss of luciferase activity from the solution phase.

2.9.5 Adsorption on to DE-52

1 g of DE-52 thoroughly washed with 50 mM potassium phosphate buffer pH 7.0, then suspended in 20 mls of the

same buffer. The enzyme solution to be adsorbed is then added and gently mixed by non magnetic stirring using a Denley Spiramix for one hour at 4°C.

The degree of adsorption to the DE-52 was estimated from the residual activity in the supernatant.

2.9.6 Adsorption to CM-52

Was used to adsorb the cationic enzyme malate dehydrogenase in the same manner as DE-52 was used in section 5.5 for luciferase and reductase.

2.9.7 Adsorption to LA-2

100 µl of LA-2 was mixed to an emulsion with 100 µl of 5 mg/ml enzyme solution.

2.9.8 Immobilisation on CN-Br activated sepharose

Basically the method was that used by Haggerty *et al* (1978) with a few variations to optimise the procedure and find the effect of changing conditions on the immobilization.

The powdered gel is pre-swollen and washed with 500 mls of 1 mM HCl per gram of gel and washing takes about 15 minutes. The washed gel is then quickly rinsed with the coupling buffer, 0.1 M NaHCO₃ pH 8.3, 0.5 M NaCl, about 20 mls/g gel and then added to the enzyme mixture in coupling buffer. The final volume is about 5-10 mls/g of gel.

The mixture is incubated on an end-over-end stirrer (Jencons) at either 4°C or 21°C for various periods of time.

The enzyme mixture, incubation temperature and time settled upon are given below.

0.5 mls Luciferase ~10 mg/ml, 1 x 10⁸ mV/mg
0.4 mls Reductase 5.2mg/ml with an activity of 0.45 U/ml
0.5 ml other enzyme if needed or else coupling buffer as below
3.0 ml 9.0 mg/ml BSA in coupling buffer to give a final protein concentration of 8 mg/ml

4.0 mls of the above is used in the immobilization the other 0.4 mls are retained as a control preparation. The 4.0 mls is mixed with 1.0g CN-Br activated sepharose 4B swollen volume ~3.5 mls

After 2 hrs incubation with continuous end over end mixing at 4°C the beads were washed with the following solutions.

- 1) 500 mls/g 0.1 M Tris-HCl pH 8.0
- 2) 500 mls/g 0.1 M potassium phosphate pH 7.0, 0.5 M NaCl
- 3) 500 mls/g 0.1 M potassium phosphate pH 7.0, 1.0 mM DTT

The beads are then stored in a suspension of the last buffer with 0.05% sodium azide as an anti-microbial agent.

2.9.9 Immobilisation on controlled pore aminopropyl glass (CPG)

The method was based on the procedures described by Lappi *et al* (1976) and conditions compatible with maintaining enzyme activity. 0.2 g of aminopropyl CPG beads were suspended in 1.0 ml of 0.1 M potassium phosphate pH 7.0 containing 30 mg/ml (70 mM) 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-p-toluene sulphonate. This solution was mixed with 0.7 mls of a luciferase and reductase mixture (1ml luciferase 10mg/ml, 0.5 ml reductase 5.2 mg/ml) and incubated at 4°C for 3 hrs. The beads after incubation were exhaustively washed with 500 mls 0.1 M potassium phosphate pH 7.0, 1 M NaCl then 500 mls 0.1 M potassium phosphate pH 7.0, 1.0 mM DTT and then suspended in this final wash buffer with 0.05% azide.

The above method was varied by altering the concentration of carbodiimide and the incubation time. The effect of substrate protection was tested by adding 75 µl of dithionite solution and 15µl of 10 mM FMN to the enzyme solution.

2.9.10 Immobilisation on polystyrene.

2.9.10.1 Immobilisation on cuvettes

Standard polystyrene cuvettes were used and enzymes immobilised by an adaptation of the method of Wood and Gadow (1983) used for coating latex beads with antibodies.

Adaptations of the method were used to coat the insides of cuvettes and small latex beads. A 1 mg/ml stock solution of poly (Phenylalanine Lysine HBr 1:1) MW ~40,000 D was made in ddH₂O. Cuvettes were coated with the polymer by incubation of the cuvettes with 2 mls of a 0.025 mg/ml polymer solution for 24-48 hrs with gentle shaking at room temperature a Luckhams shaking table was used. The cuvettes were then drained and rinsed out with 2 x 2mls of 50 mM potassium phosphate pH 7.0. The cuvettes were then incubated at 4°C for 24 hrs with enzyme solution of 50 ug/ml containing between 0 and 5 mg/ml carbodiimide.

Assaying the cuvettes was done by the following procedure:

- 1) The cuvettes were rinsed with 3 x 1ml of 50 mM potassium phosphate pH 7.0.
- 2) The cuvettes were then assayed for enzyme activity by the standard coupled and dithionite assays. After the coupled assay the solution was decanted and the activity in the solution measured to ascertain the degree of activity due to tube immobilised enzyme and enzyme released into the solution.
- 3) The cuvettes were then rinsed for reuse to assess the reusability of the cuvettes.
- 4) To achieve a stirred solution and prevent local substrate depletion some assays were aspirated with compressed air by the arrangement shown in figure 2.8.

2.9.10.2 Immobilisation on Latex beads

The above method was used except that a 0.1 mg/ml Poly(Phe:Lys) solution was used with a variety of latex beads LB-1 (0.1 μ m) LB-16, (15.8 μ m) and Latex beads from polysciences (78 μ m) were used. 0.5 mls of a 20% suspension of beads was used in a reaction volume of 1.0 mls. All active components were added as dilutions from stock solutions, stock carbodiimide when used was made fresh immediately prior to use and not re-used. After reaction the beads were washed on a millipore filter (0.22 μ m), with 50 mM potassium phosphate, 0.1 mM DTT pH 7.0.

2.9.10.3 Control Experiments for Latex immobilisation

A luciferase/reductase mixture in solution was exposed to concentrations of PPL and PL of 1.0, 10, and 100 μ g/ml in order to study the effect of their interaction with luciferase activity.

Activity was measured by the assay of a 10 μ l aliquot of the sample in a standard dithionite assay or in a coupled assay. Where samples were incubated with the PPL or PL the temperature was kept at 0 - 4 $^{\circ}$.

2.9.11 Immobilisation of decanal in polystyrene

Decanal was applied to the side of polystyrene cuvettes as a preparation in solvents. 5 - 10 μ l of the mixture was either smeared on the side of the cuvette or deposited as a single blob. The variety of mixtures used are shown below:

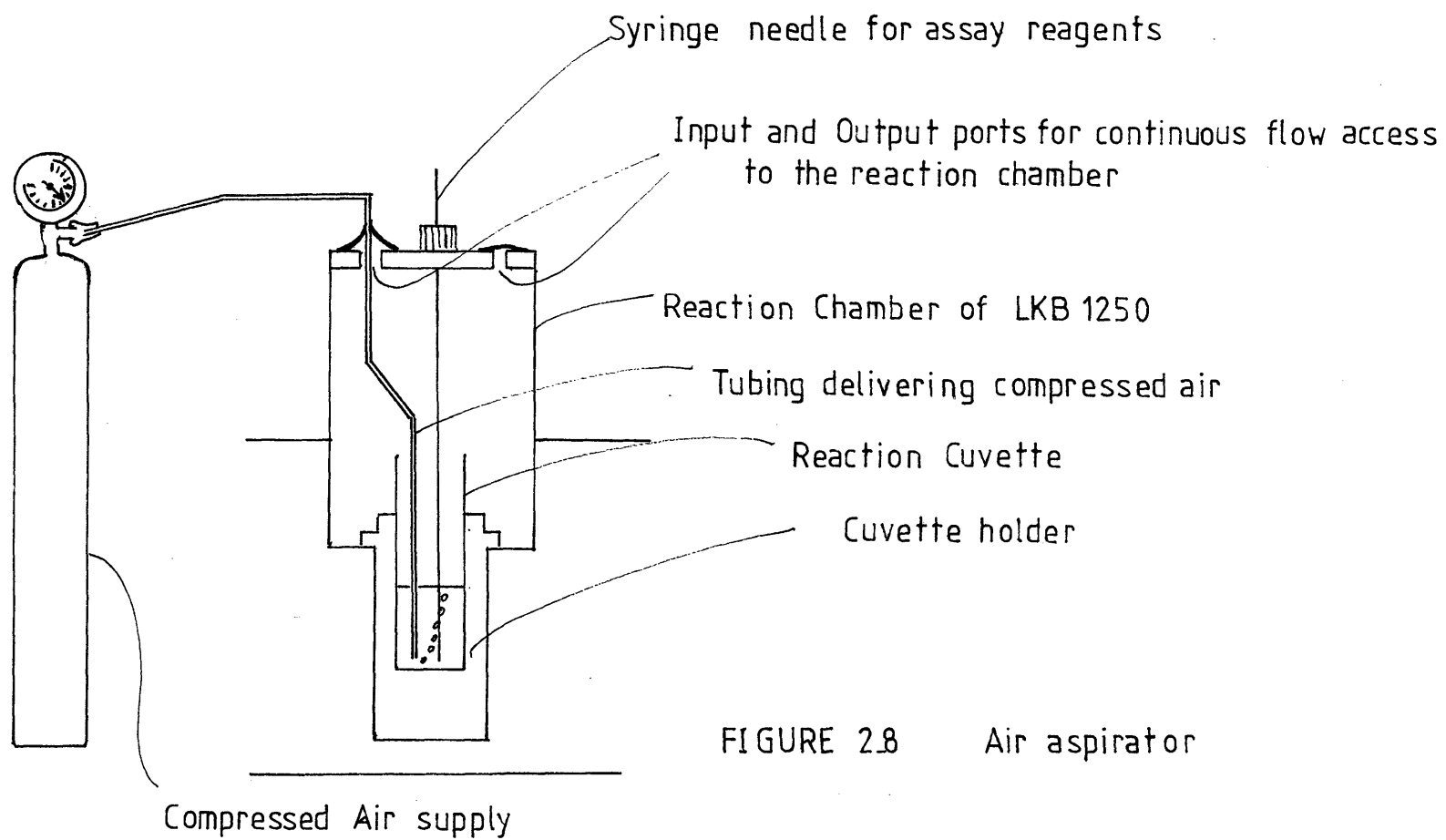


FIGURE 2.8 Air aspirator

Acetone based:

90 - 99% acetone
0.01% - 1.0% decanal
0.0% - 10% ethanol
0.0% - 1% isopropanol

Cyclohexanone based:

50% - 99% cyclohexanone
0.01% - 50% decanal
0.0% - 10% ethanol
0.0% - 1% isopropanol

The ethanol and isopropanol were used as carriers for the decanal and were never used together in the same assay.

2.10 CONTROL EXPERIMENTS FOR IMMOBILISATIONS

2.10.1 Chemical modification of carboxyl groups

The carboxyl groups of luciferase were modified by an adaptation of the method of Hoare and Koshland (1969). The pH of the reaction was found to be optimum at 4.75 by Lin and Koshland (1969), however since luciferase is unstable below pH 6.5 and in our experience precipitates below pH 6.0; the experiments were all carried out in the range 6.5-7.5. The general protocol is shown below, however concentrations, pH and temperature were varied to investigate the kinetics of modification.

A solution of 0.2 M nucleophile (taurine, aminomethylsulphonic acid or glycine methylester) in 0.4 M potassium phosphate buffer pH 7.0 (actual pH's were varied between 6.5 and 8.0), 1.0 M NaCl was made up. To 500ul of this 5 µl of C-14 nucleophile (to provide the label for taurine detection) were added and carbodiimide to make the concentration up to the desired level (10 mM to 0.25 M) to this was immediately added 500 ul of enzyme solution to initiate the reaction. The progress was monitored by taking 10 µl samples at frequent intervals to assay for activity. The samples were immediately diluted 1:100 into 50 mM potassium phosphate pH 7.0, 0.2% BSA to stop the reaction. Samples were then assayed for luciferase activity by the dithionite assay. In the radiolabeling experiments 30 µl samples were also taken and diluted into 1.0 mls of 12% TCA to precipitate the radiolabelled protein.

2.10.2 Solvent inhibition of enzyme activity

The inhibition of the luciferase based coupled reaction by a variety of solvents was investigated. Decanal, the long chain fatty aldehyde substrate for the enzymatic reaction is normally delivered in an iso-propanol or ethanol solution. In the investigation of the inhibitory effects of the solvents iso-propanol, ethanol, acetone and cyclohexanone, the decanal was prepared as a 0.1mg/ml solution with each of the above solvents. A 10 μ l aliquot of these mixtures was then used in the standard assay to supply the decanal for luminescence. The % of solvent was further increased where necessary by the addition of subsequent aliquots of neat solvent. In the case of cyclohexanone the decanal was delivered as a 5 μ l aliquot of 0.2 mg/ml decanal in cyclohexanone.

2.10.3 Reversible blocking of thiol groups on luciferase

The procedure of Cousineau and Meighen (1976) was followed. Luciferase was first dialysed extensively against 50 mM phosphate buffer pH 7.0 to remove all dithiothreitol (DTT). A desalting column of Sepharose G-25 was used to remove excess blocking reagent before reactivation.

A modification of the above technique using DTNB in place of TET as the reversible blocker was developed. DTNB was used at the same final concentration as for thiol group determination (Section 2.4.3). Inactivation was as for the TET method, although initial dialysis was against 0.1M Tris pH 7.5, 0.002M EDTA to remove excess DTT. Reactivation for this method was much longer than with TET, however a higher % of activity was eventually recovered.

2.10.4 Chemical Modification with p-azidophenacylbromide.

Inactivation with Bromoacetophenone (30 mM) in 50 mM phosphate buffer at pH's 7.0 and 8.0 was carried out at 0°C in an ice bucket. The corresponding reaction with p-azidophenacylbromide is carried out in a light sealed vial, covered in aluminium foil. Following the inactivation reaction the mixture was exposed to u.v. light for 10 minutes as in the method of Hixson and Hixson (1975) and the resultant modified enzyme was assayed and prepared for PAGE and SDS/PAGE analysis following desalting on a G-25 column.

CHAPTER THREE

ENZYME PREPARATION AND PURIFICATION TECHNIQUES

3.1 MICROBIOLOGY

Two light producing strains of Vibrio harveyi, B 392 and MB 20, were used as sources of the luciferase and reductase enzymes. Small scale growth of the strains on either of two media, sodium chloride complete or marine broth, revealed only marginal differences in luciferase production as measured by in vivo luminescence levels and culture density by absorbance at 660nm (figures 3.1 and 3.2.).

However, the following observations were made:

- (1) Strain MB 20 showed a strong tendency to aggregate in late log phase and become attached to the shake flask surface.
- (2) Both strains when grown in the sodium chloride complete medium produced substantial quantities of pigments.

60 and 400 litre fermentations

The cell density and luciferase induction profiles for all the 400 litre fermentations closely matched those observed both in the 100 ml shake flasks (see figure 3.2) and also 60 litre fermentations (figure 3.3). Figure 3.3 also shows a comparison of the two methods for measuring luciferase in vivo. The comparison shows that while there are quantitative differences in the results, the rapid in vivo method accurately detects the phases in luciferase induction and the plateau region, signalling the optimal time for harvesting the cells. The rapid method is thus reliable indicator of the optimum harvest time.

Scale up to 400 litre fermentation in the sodium chloride complete medium was attempted for both strains. Cell yield and crude luciferase yield per gram were found to vary between batches but approximately 3.5 Kg of cell paste was harvested from each fermentation and a 500 g sheet of cells could be expected to give around 10^{11} RLU of total activity after lysis. Variation between batches was found to be 3 -4 X and no significant difference in enzyme yield was observed between the two strains.

FIGURE 3.1
Growth on NaCl Media

- ● MB 20
- × ◇ B 392
- OD 660nm
- - - RLU's

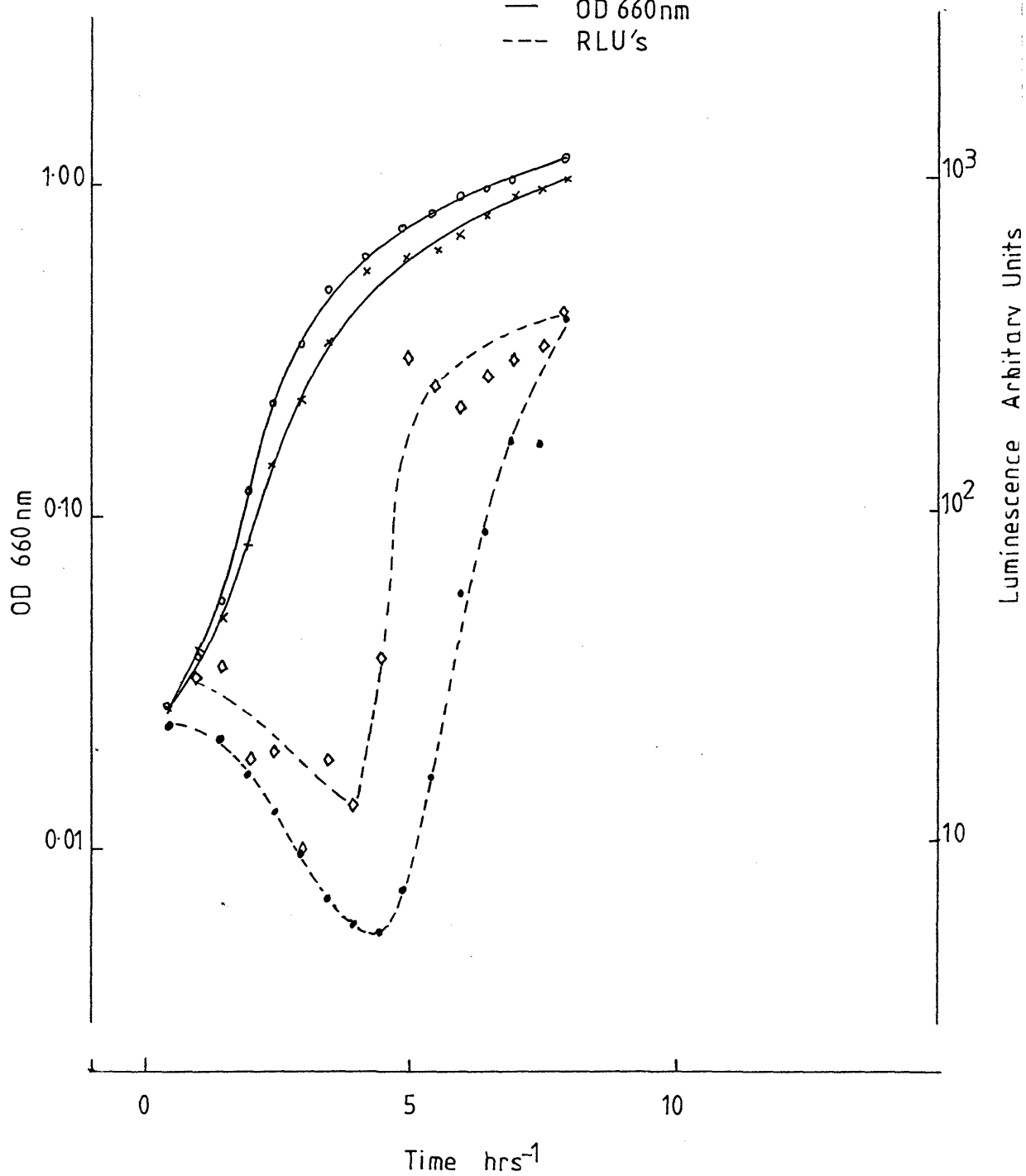


FIGURE 3.2 Growth on Marine Mix
 ● MB 20 ×◇ B 392 — OD 660nm --- RLU's

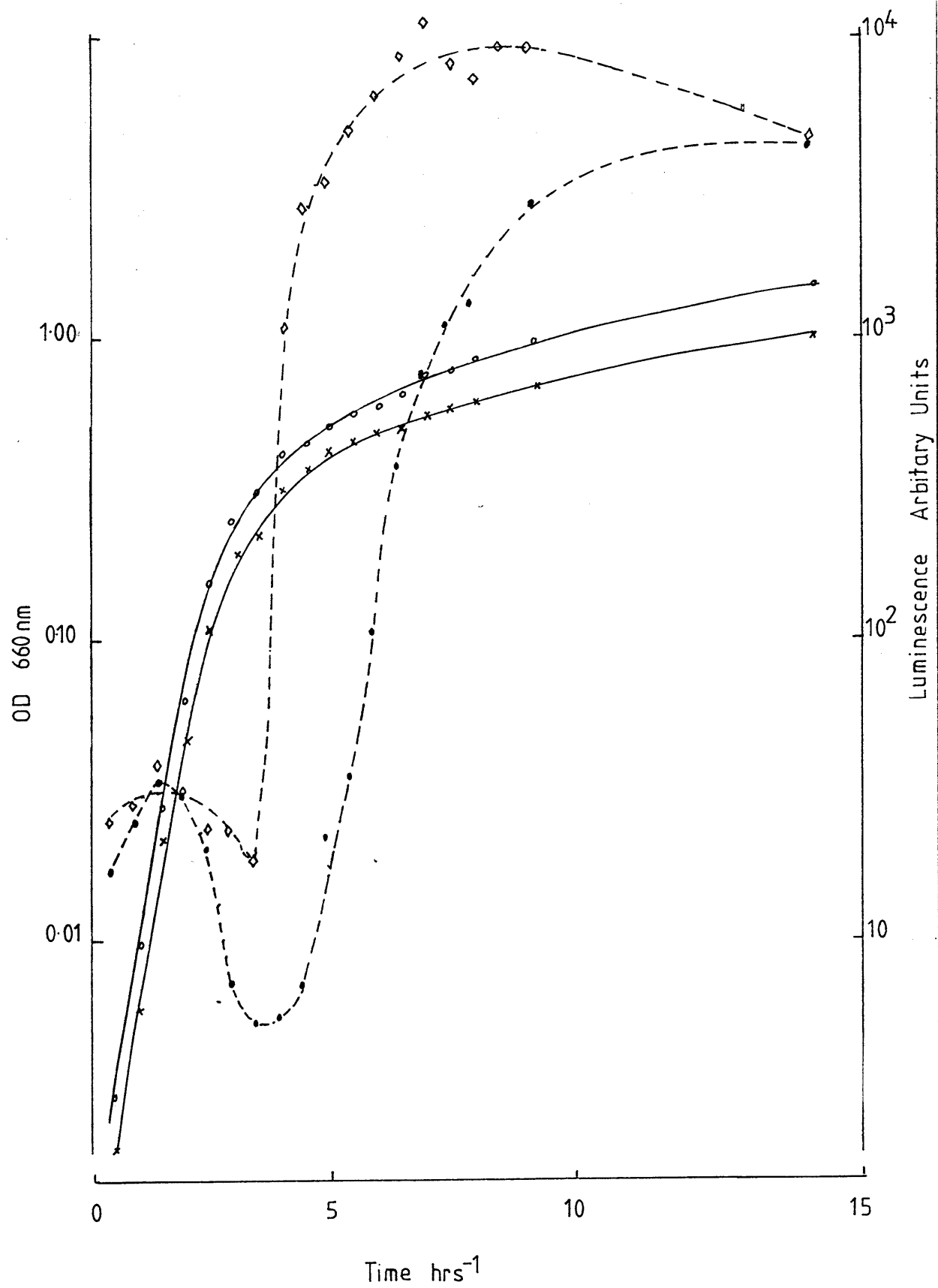
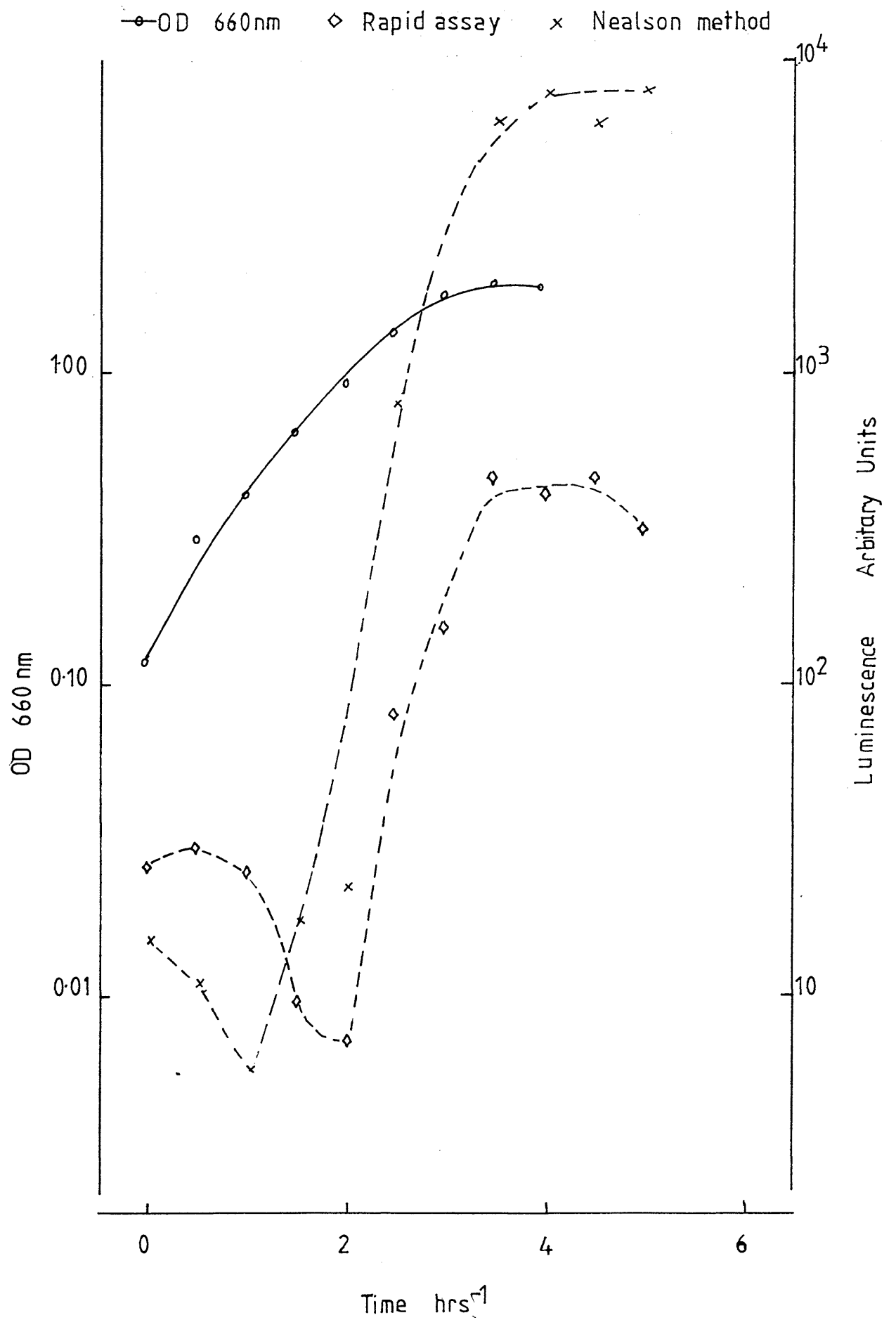


FIGURE 3.3 40 l fermentation



3.2 PROTEIN PURIFICATION

3.2.1 Luciferase

The protocol of Hastings et al (1978) developed by Gunsalus et al (1972) and Baldwin et al (1975) was further modified in several respects to improve the yield of luciferase and to speed up the purification process. Table 3.1 compares a typical luciferase purification to the original protocol of Hastings.

3.2.1.1 Purification and Yield

The purification table shows a final yield of 20% and a purification factor of 190, these compare to the DEAE pool figures of 18% and 75 for Hastings' method. At the same stage in the new protocol developed the yield was 31% with a purification factor of 81. The major difference in the table appears to be at the points where fractions are chosen and pooled. The DE-52 elution pool is made more conservative while the pooling of fractions from the DEAE sephacel column is less conservative with a net result of doubling the protein yield.

3.2.1.2 Modifications to the original protocol

The effects of the different modifications to the protocol are discussed below:

1) Membrane Filtration There was no significant change in yield using this technique. However high pressure filtration proved very useful in clarifying the lysate of cell debris. This facilitated a cleaner DE-52 batch adsorption step and easier handling.

2) DEAE Sephacel Replaced the sephadex anion exchange column and allows slightly faster flow rates. Both step elution and gradient elution gave well resolved reductase and luciferase peaks the specific activities and yields of the two methods were the same within experimental variation. However the gradient elution gave better resolution of the individual peaks of luciferase and reductase (figure 3.4).

3) Ultrafiltration The pooled fractions from the DEAE sephacel were concentrated prior to gel filtration by an ammonium sulphate precipitation as in the original protocol. As this had no effect on purification or yield but was time consuming owing to the subsequent dialysis step, this stage was replaced by Amicon ultrafiltration as a method of sample concentration. The change in protocol saved about 16 hrs.

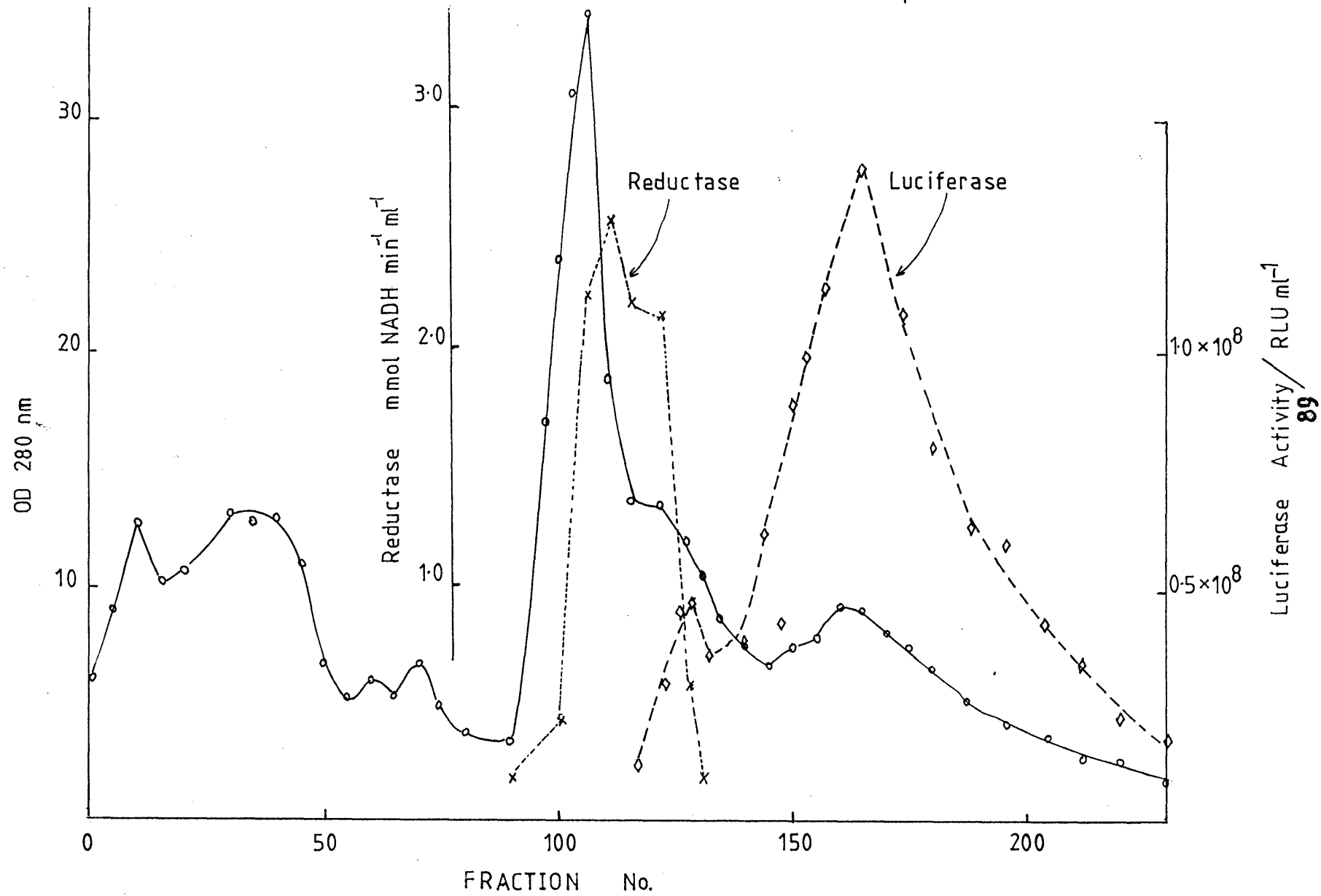
TABLE 3.1

PURIFICATION OF LUCIFERASE FROM *Vibrio harveyi*

Purification Stage	Total Activity	Specific Activity	Yield of Luciferase	Purification Factor
	RLU	RLU/mg	%	
Cell Lysate	1.08×10^{11}	3.94×10^5	100	1.0
DE-52 Pooled Elutions	6.90×10^{10}	3.00×10^6	63.9 (87.5)*	7.62 (8)*
NH ₄ SO ₄ 40% - 75% cut	5.06×10^{10}	2.20×10^7	46.9 (56.3)*	20.95
Dialysate	5.45×10^{10}	2.55×10^7	50.5 (56.3)*	24.3 (10.5)*
DEAE Sepharose Pool	3.30×10^{10}	8.46×10^7	30.6 (17.5)*	80.6 (75)*
ACA 34 Pooled Fractions	2.20×10^{10}	1.40×10^8	20.4	113.3
FPLC Pooled Fractions	-	2.35×10^8	-	190

* figures taken from Hastings et al (1978)

FIGURE 3.4 DEAE Sephacel



4) ACA 34 or Sepharose Q Following the DEAE sephacel column a second column is used to further purify the enzyme. Initially a gel filtration ACA 34 column was used. However this limited the amount of protein which could be purified at one time (10-20 mls on a 2 litre column) and took 24 - 48 hrs to run. Sepharose Q was used as an alternative. It has a much higher limit on sample size in terms of g of protein per ml of gel, upto 12 g of human serum albumin (HSA) may be loaded per 100 mls of gel with no limit on the volume of the sample, and the column can be run in about 4-6 hrs. Figures 3.5 and 3.6 show the elution profiles of the two columns. In these cases the purification by sepharose Q is slightly superior and far more rapid. Essentially this stage is a rapid version of Hastings's use of a second pass down a DEAE sephadex column (Hastings et al 1978).

3.2.1.3 FPLC Purification

Luciferase was well resolved from the final few contaminants by purification on a FPLC MonoQ column see Figure 3.7 (a) & (b). The difference between the two methods is small and represents fine tuning of the purification protocol. Figure 3.7b shows the standard method which is easily adapted to give optimum separation by adjusting the lower and upper percentage concentrations of B for a particular batch of enzyme.

3.2.2 FMN:NADH Oxidoreductase

Following the DEAE Sephacel column the reductase fractions were pooled separately to the luciferase fractions and then purified by gel filtration on ACA 34 the protein profile is shown in figure 3.8. The reductase is still relatively impure. Further purification was carried out by FPLC Mono Q (figure 3.9) or on a 5'AMP Sepharose column (figure 3.10). The complexity of the monoQ elution profile illustrates the very low percentage of reductase in the preparation used as a standard source of the enzyme.

The usefulness of a final purification step for the purposes of this project is doubtful. Purity of the final enzyme was enormously improved, however trace impurities remained. The purified enzyme still showed background activities in NADH linked assays. As explained latter in this work the reason for the background is most likely to be traces of alcohol dehydrogenase in the LDH enzyme preparation.

The results of modifications to the published purification protocol are summarised below:

- * Purification of the reductase is unlikely to remove

FIGURE 3.5 ACA 34 Luciferase elution profile

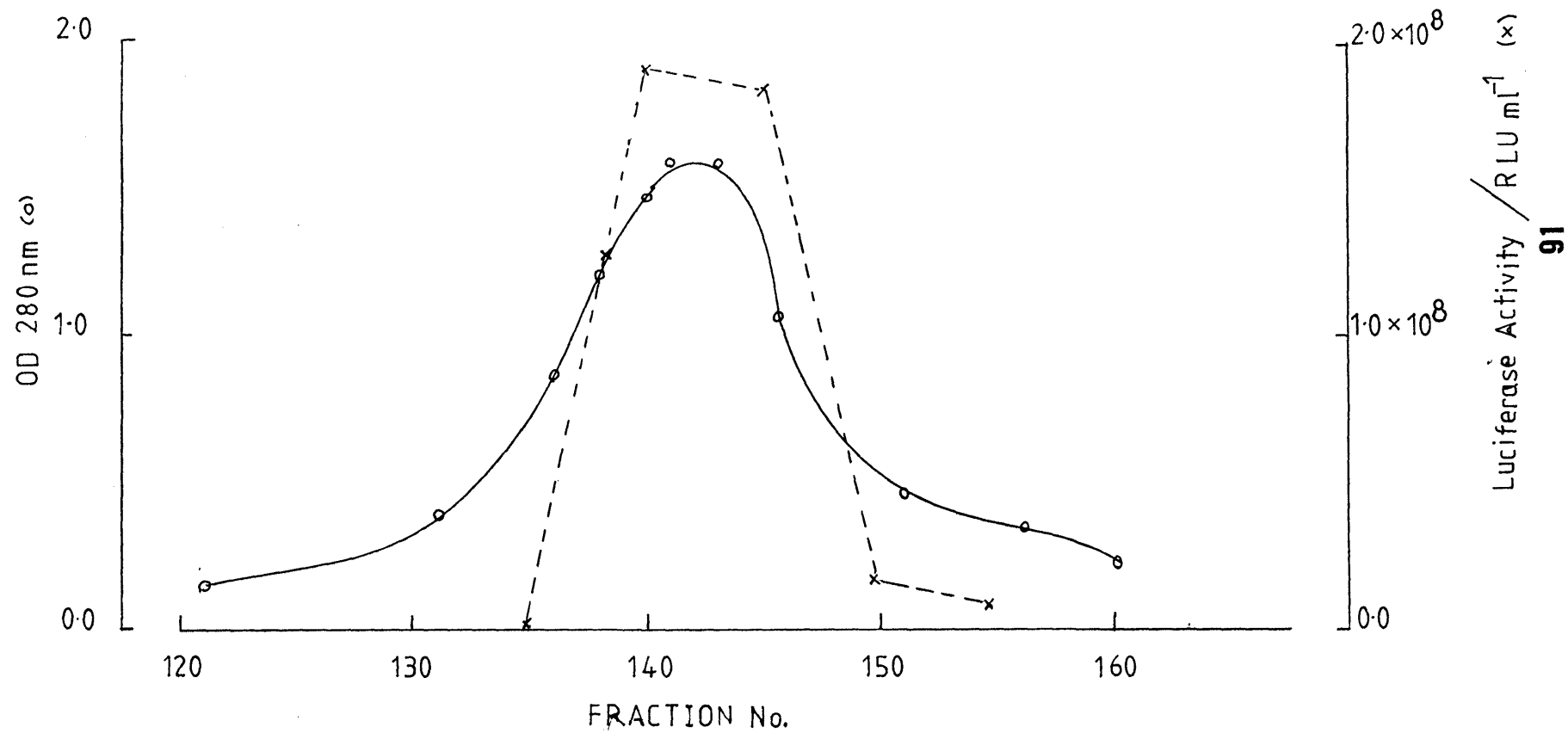


FIGURE 3.6 Sepharose Q Luciferase elution

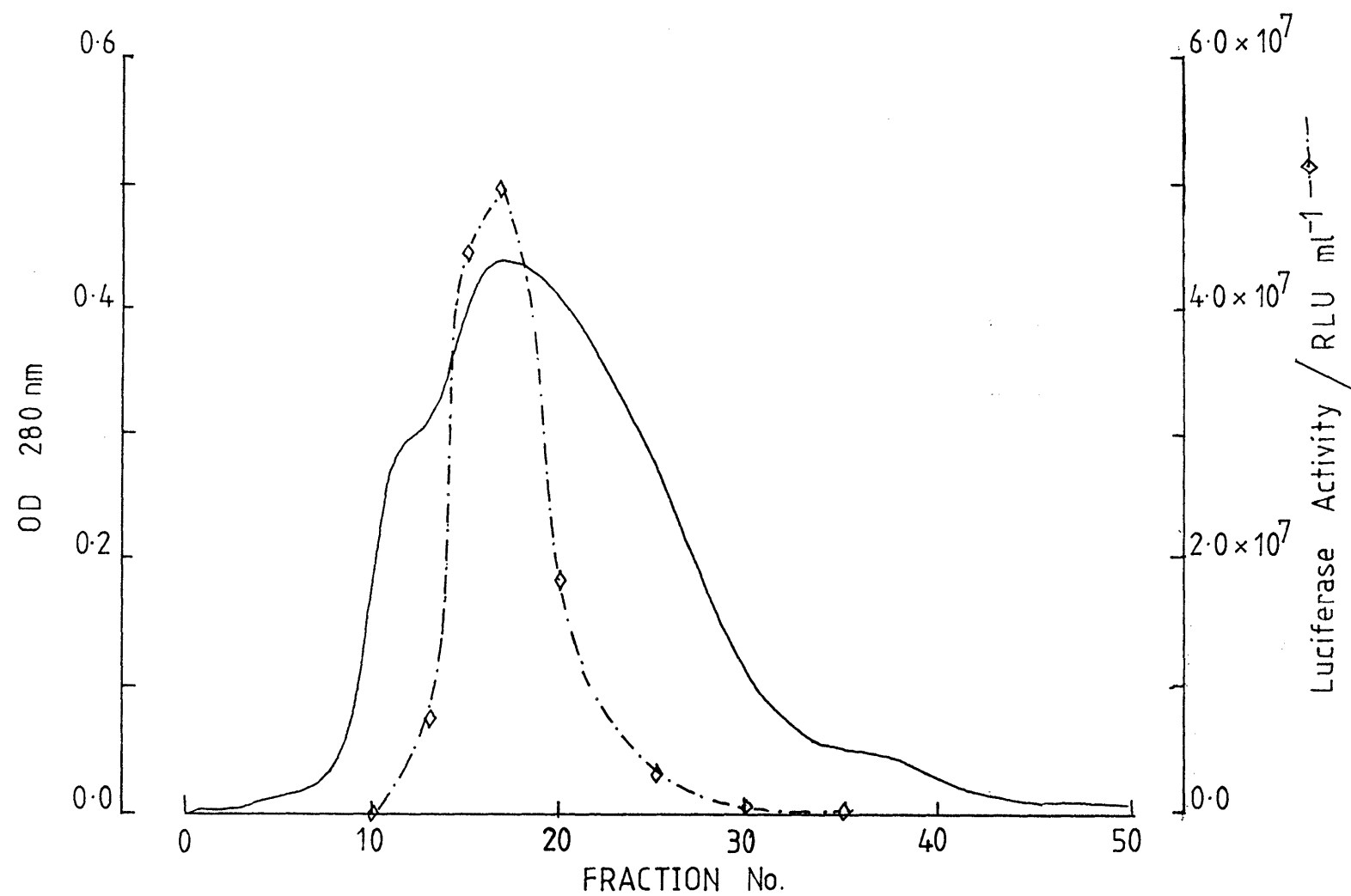


FIGURE 3.7a
Mono Q FPLC
Luciferase purification

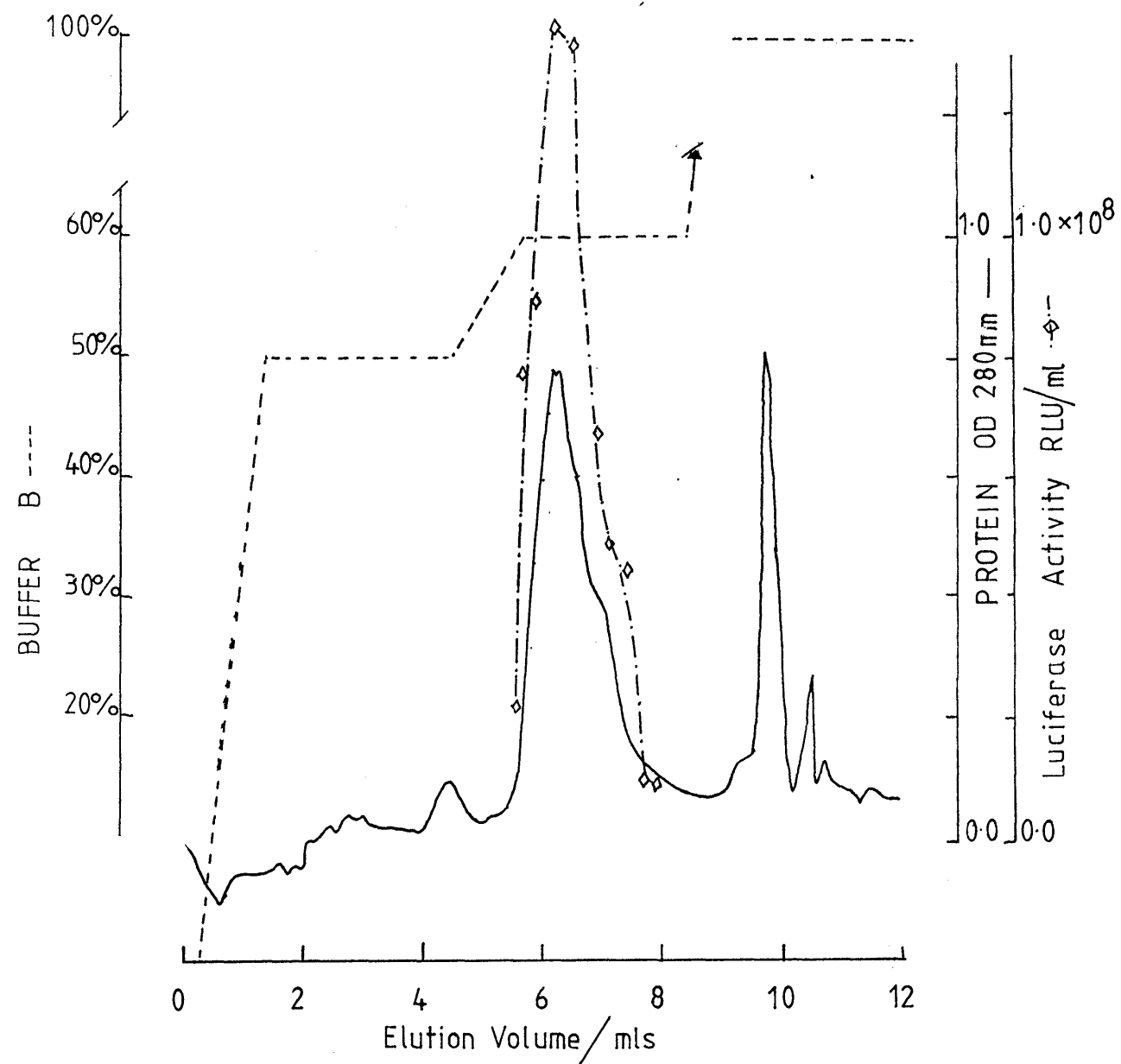


FIGURE 3.7 b Mono Q FPLC Luciferase purification

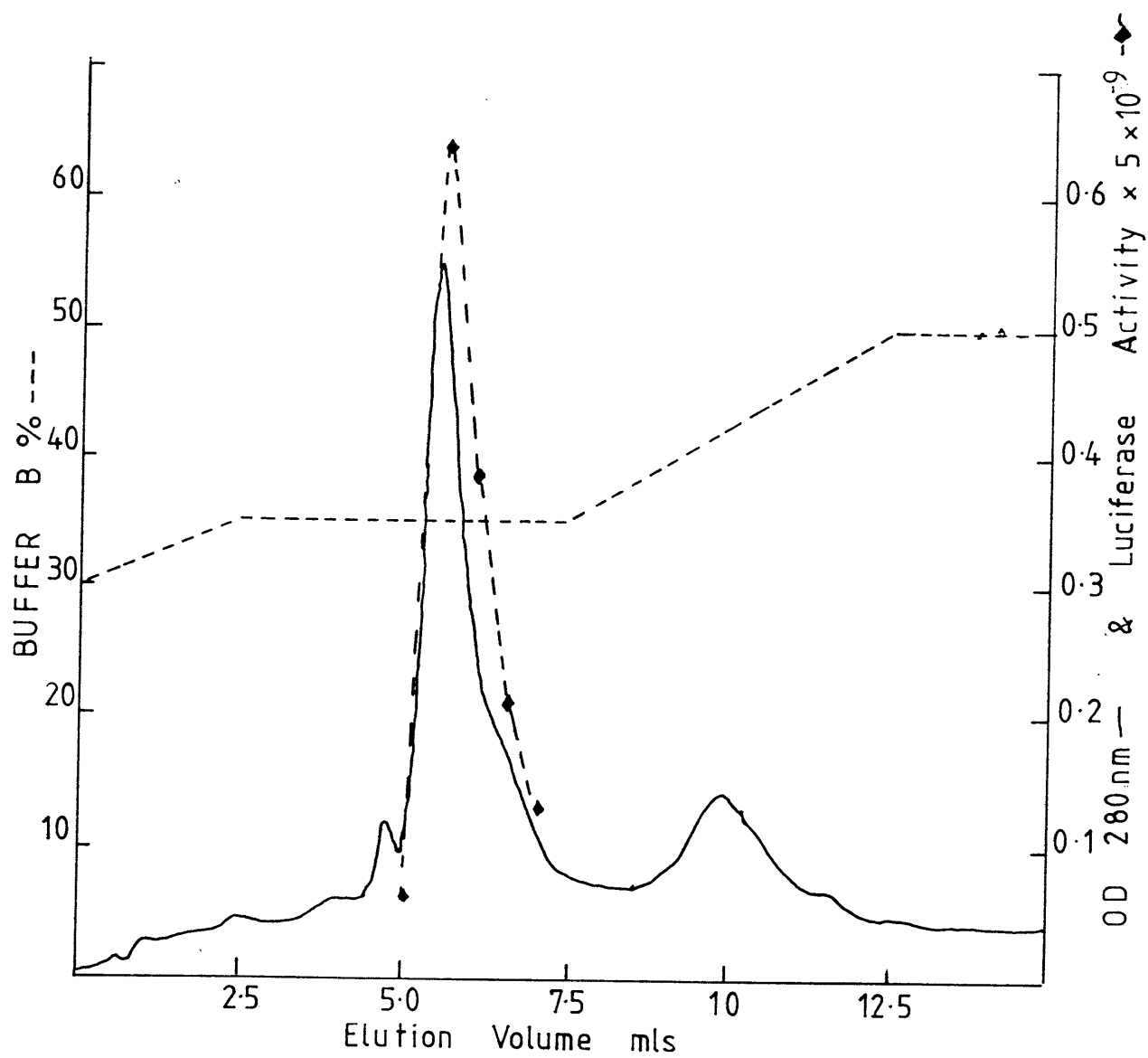
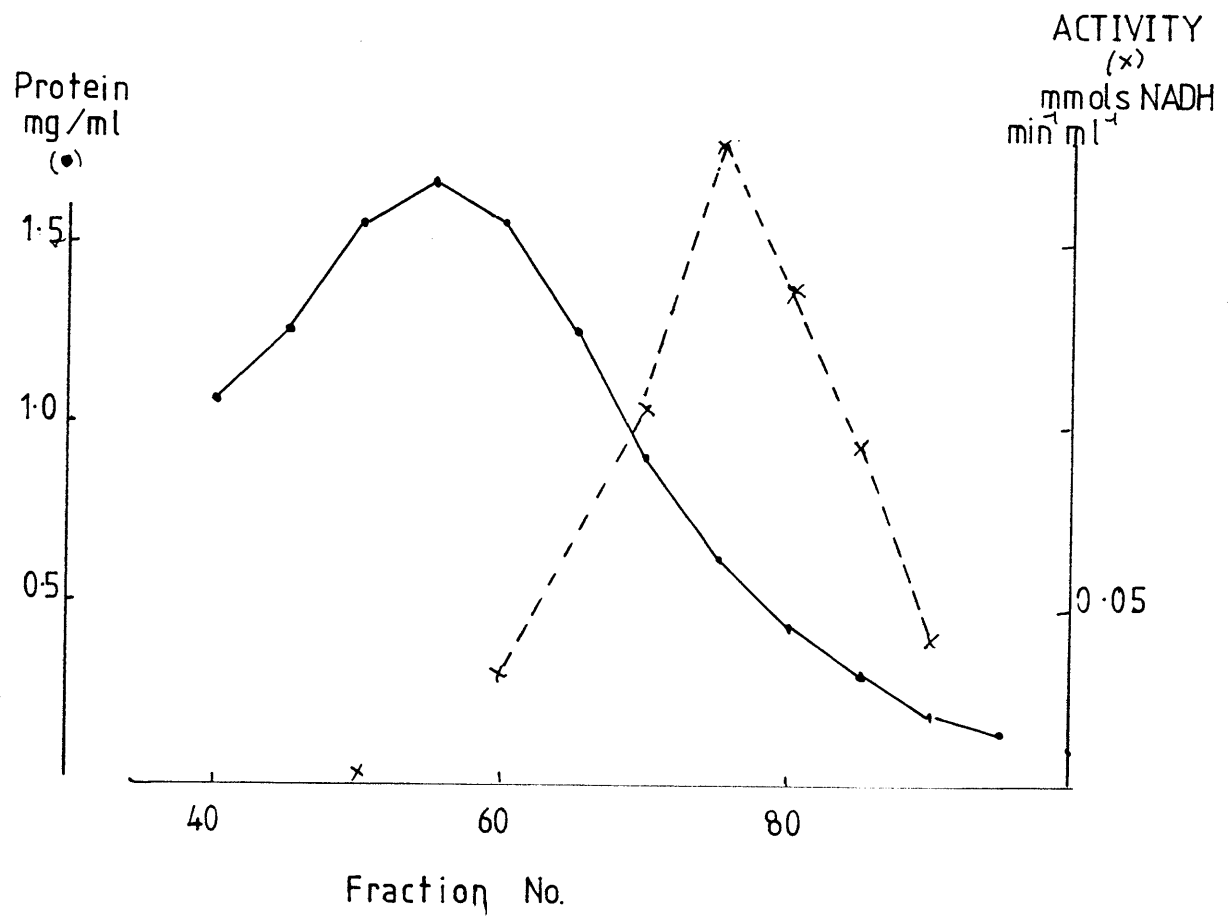


FIGURE 3.8

ACA 34 Reductase Elution



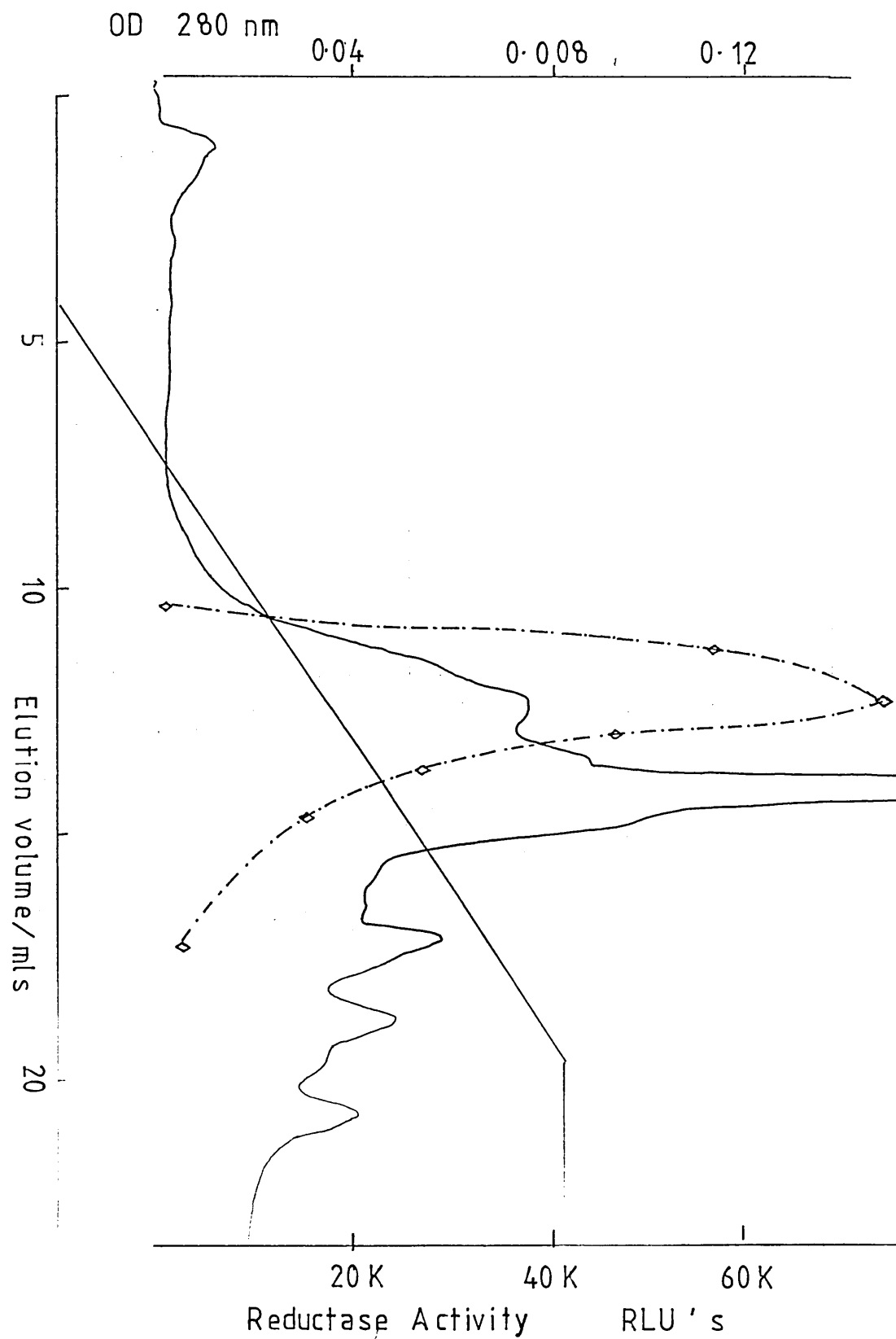


FIGURE 3.9
FPLC Purification of FMN: NADH oxidoreductase

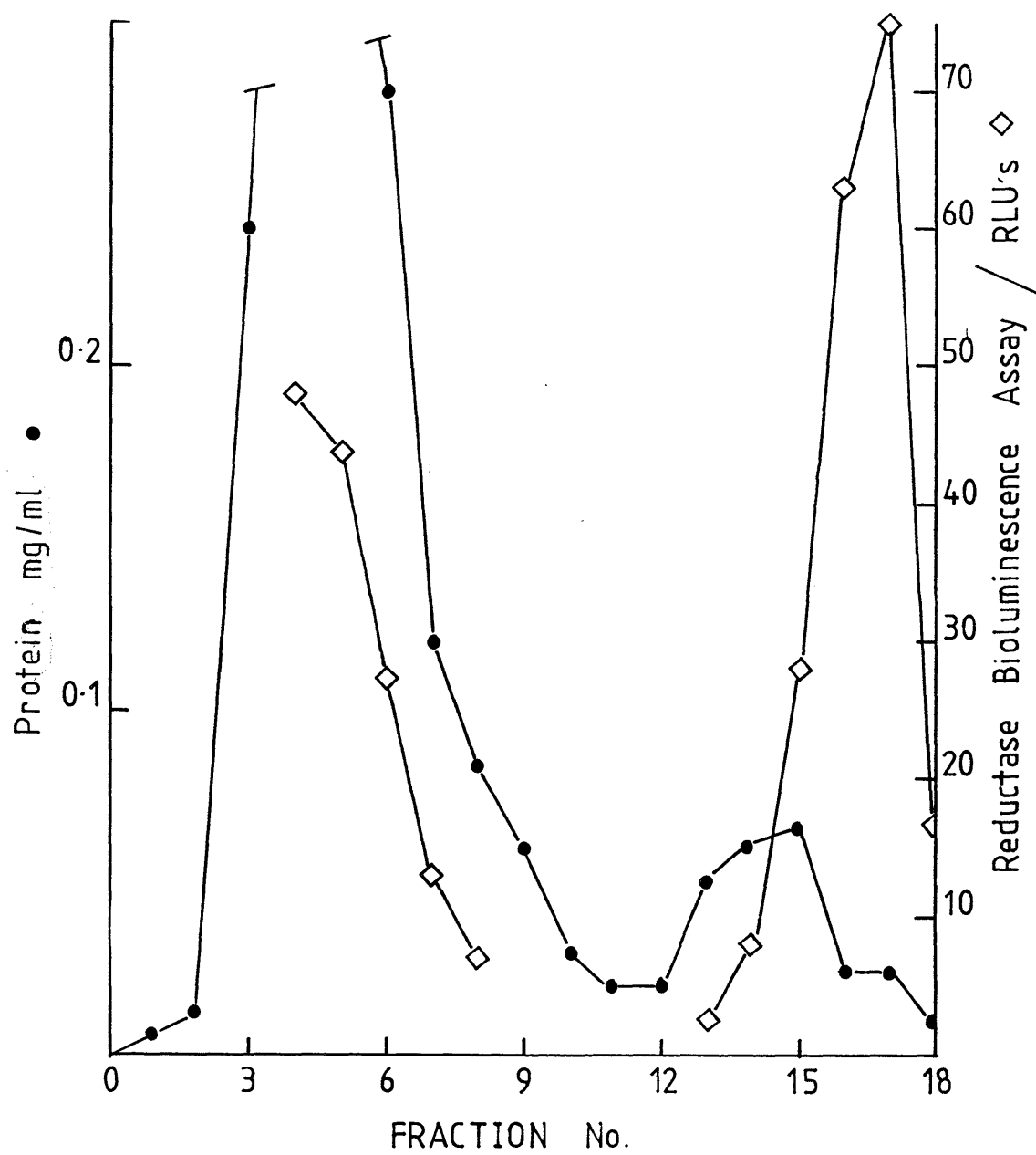


FIGURE 3.10
5'AMP affinity purification of reductase

contaminating dehydrogenase enzymes effectively. Levels of light emission from an assay are determined by the level of analyte only, the response time is determined by level of dehydrogenase, this is shown in the model set out in chapter six. Hence even very low levels of contaminating enzyme will cause a background reading if a suitable contaminating analyte is present, ie. propan-2-ol.

* There is no need for an increase in the concentration of reductase activity for the efficient operation of the bioluminescence assay. Infact as is explained in chapter 6 low concentrations of enzyme are vital for the accurate behaviour of the assay.

Hence purification of the reductase prior to its use would be time consuming and largely unnecessary.

3.2.3 Luciferase Subunit Purification

The two subunits were clearly resolved by anion exchange chromatography on an FPLC apparatus figure 3.11. The discrepancy in the 280 nm absorbance of the two subunits is a result of the different amino acid composition of the subunits.

The two fractions were tested individually for activity following an exhaustive dialysis against assay buffer to remove all Urea. A mixture of the two fractions was also prepared and treated similarly. The results summarised in Table 3.2 show the successful separation and recombination of the two subunits.

FIGURE 3.11 FPLC separation of luciferase subunits

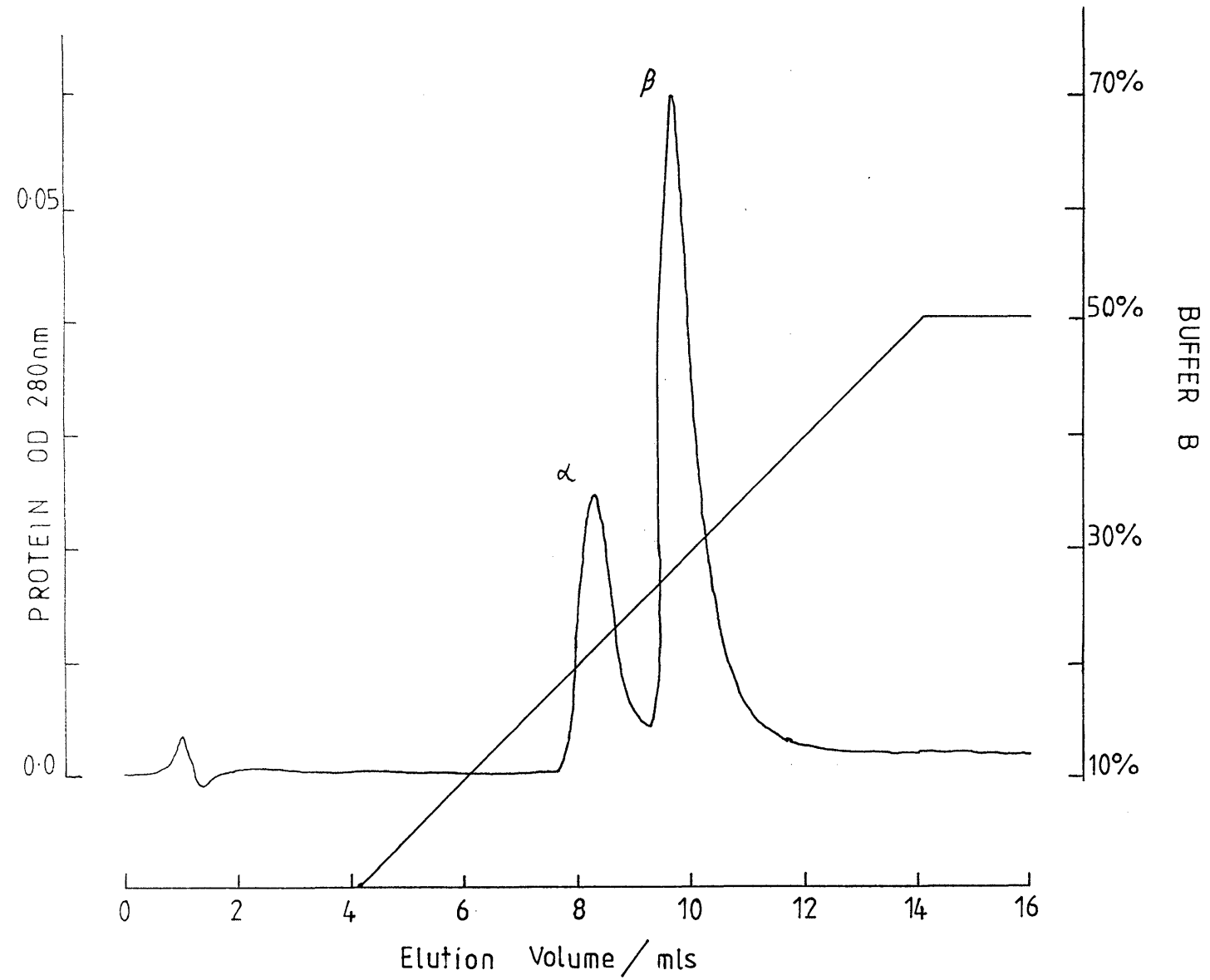


TABLE 3.2

Subunit recombination following separation by FPLC on Mono Q

*						
Dialysis Time	alpha	beta	mV light intensity		% initial	initial sample
	(a)	(b)	alpha	beta	(d/e)	(e)
			(c)	(d)		
			(c - 0.5b)			
1 hr	5.0	675	480	(150)	1.25	12000
24 hrs	4.2	650	540	(215)	2.24	9600
+14 days	6.3	1930	2800	(1815)	6.05	30000

* Dialysis was carried out after a dilution of the FPLC samples into dialysis buffer of 1:25, the dialysis buffer was: 0.1 M phosphate, 1 mM DTT, 1 mM EDTA and 10% Glycerol at pH 7.0.

**The figure in brackets has been adjusted using the beta activity as a blank since the second peak is clearly contaminated with alpha subunits, the corrected activity is expressed as a percentage of the initial sample. Given that the effective dilution of running the column is 5 - 10 fold this is good recovery of activity.

CHAPTER FOUR

IMMOBILISATION OF ENZYMES AND SUBSTRATES INVOLVED IN BIOLUMINESCENCE

Several immobilisation techniques have been used in the past in an attempt to immobilise the bacterial bioluminescence system. The first published attempts were by coupling the bioluminescence enzymes to arylamine porous glass beads (Jablonski and Deluca 1976). Although the enzymes were both active the luciferase activity was only 0.05% of the original activity, reductase activity was reduced to 10.3% and coupled activity 3%. This recovery has been considerably improved on by using activated CNBr sepharose beads as a support for the enzymes, yet the degree of activity recovery is still low. While these more recent systems of immobilised bioluminescence enzymes all give workable activities, luciferase is typically 90% or more inactivated by this method of immobilisation (McElroy & Deluca 1983). Immobilisation by CNBr activated sepharose is through amino groups on the enzymes. As at least one of the luciferase amino groups has been implicated in the inactivation of the enzyme it seems reasonable to conclude that this is a possible cause of low activity recovery. The coupling of the enzymes to collagen film was reported in 1984 (Blum and Coulet 1984), however there is no mention of activity recovery and hence the method can not be critically assessed for immobilisation efficiency.

Due to the comparative failure of previous immobilisation methods to produce high activity recovery that following investigation into various immobilisation methods was undertaken. Additionally the focus of the work on immobilisations is widened to include a careful assessment of the impact of immobilisation on the analytical usefulness of the immobilised enzymes.

The results in this section have been grouped under the headings of the main immobilisation techniques. In the assesment of these methods several criteria have been selected which are explained below.

i) The success of an immobilisation is in part measured by the quantity of active enzyme immobilised. Within this criterion there are two aspects, firstly the loading of the active enzyme and secondly how much of the enzyme is destroyed by the immobilisation process. These are important characteristics of any immobilisation technique; enzyme loading is important to the application the immobilised enzyme is put to. The quantity of enzyme inactivated is important in the costing of production.

ii) Quality of an immobilised preparation may be defined by the kinetic and catalytic characteristics of the immobilised enzyme. The presence of both enzyme with altered substrate affinities and of totally inactivated enzyme complicate analysis of the real kinetic and catalytic characteristics of the preparation.

Differentiation of active and inactive enzyme on the beads is often not possible. The fact that the immobilised enzyme preparation is necessarily a heterogeneous mixture compared to the original enzyme in solution means that usually it is the apparent properties of the preparation which are measured and then compared with the intrinsic properties exhibited in solution studies.

iii) With a multienzyme system gains in efficiency may be obtained on immobilisation through a substrate channelling effect. This is an effect commonly observed with immobilised enzymes, normally the effect arises from two factors; the high local concentration of enzyme leads to high local concentration of intermediates and the proximity of enzymes catalysing sequential reactions means reduced diffusion path lengths between enzymes, these two factors lead to an increased flow of reactants through the reaction pathway. The effect is further enhanced for the bioluminescence reaction pathway since the FMNH₂ intermediate is subject to a competing chemical autocatalytic oxidation step, hence the reduced pathway between enzymes will bring additional benefits by reducing the time for chemical oxidation to occur.

The degree of substrate channelling achieved in the bioluminescence assays is conveniently expressed by the ratio of the light generated in the coupled assay (at 0.1 mM (saturating) NADH or 1mM Malate unless otherwise stated) and the light generated in the dithionite assay for a given enzyme preparation. This ratio, ξ , is used to denote the efficiency of the substrate channelling between the enzymes. The closer the figure to 1.0 the more efficient; it should be noted that the value of ξ may in some cases be distorted, some of these reasons are discussed in this and subsequent chapters.

4.1 ENTRAPMENT METHODS

A variety of techniques were investigated which come under this classification of immobilisation. The hope was to find a satisfactory method of physically localising the enzymes without appreciably destroying their activity.

4.1.1 Entrapment within Polyacrylamide (PA) and Hydroxyethylmethacrylate (HEMA) gels

These two gels offered the potential of a rigid matrix amenable to mechanical processing to give a bio-matrix in a convenient form, both can be constituted to control pore size giving only very limited protein leaching. Although the two polymers differ in their monomer building blocks, they share the same method of polymerisation. Polymerisation is via the formation of free radicals in the reaction between ammonium persulphate (APS) and TEMED in a degassed solution.

All attempts to form an enzymatically active gel failed. The HEMA gels formed extremely hard blocks both these and ground preparations of them failed to give any activity. Similarly the gelatinous PA gel had negligible activity. Monitoring the enzyme activity during the polymerisation process suggested that most of the activity was lost prior to the formation of a gel. A second experiment in which a dilute mixture of 2.5% PA was used showed the inactivation pattern in figure 4.1. This shows that inactivation coincides with the presence of APS and TEMED in the absence of oxygen. While in the resulting gel further loss of activity could be due to small pore size the above investigation of the polymerisation chemistry suggests that at least 99% of the activity is lost by chemical destruction of the protein activity most probably by free radicals.

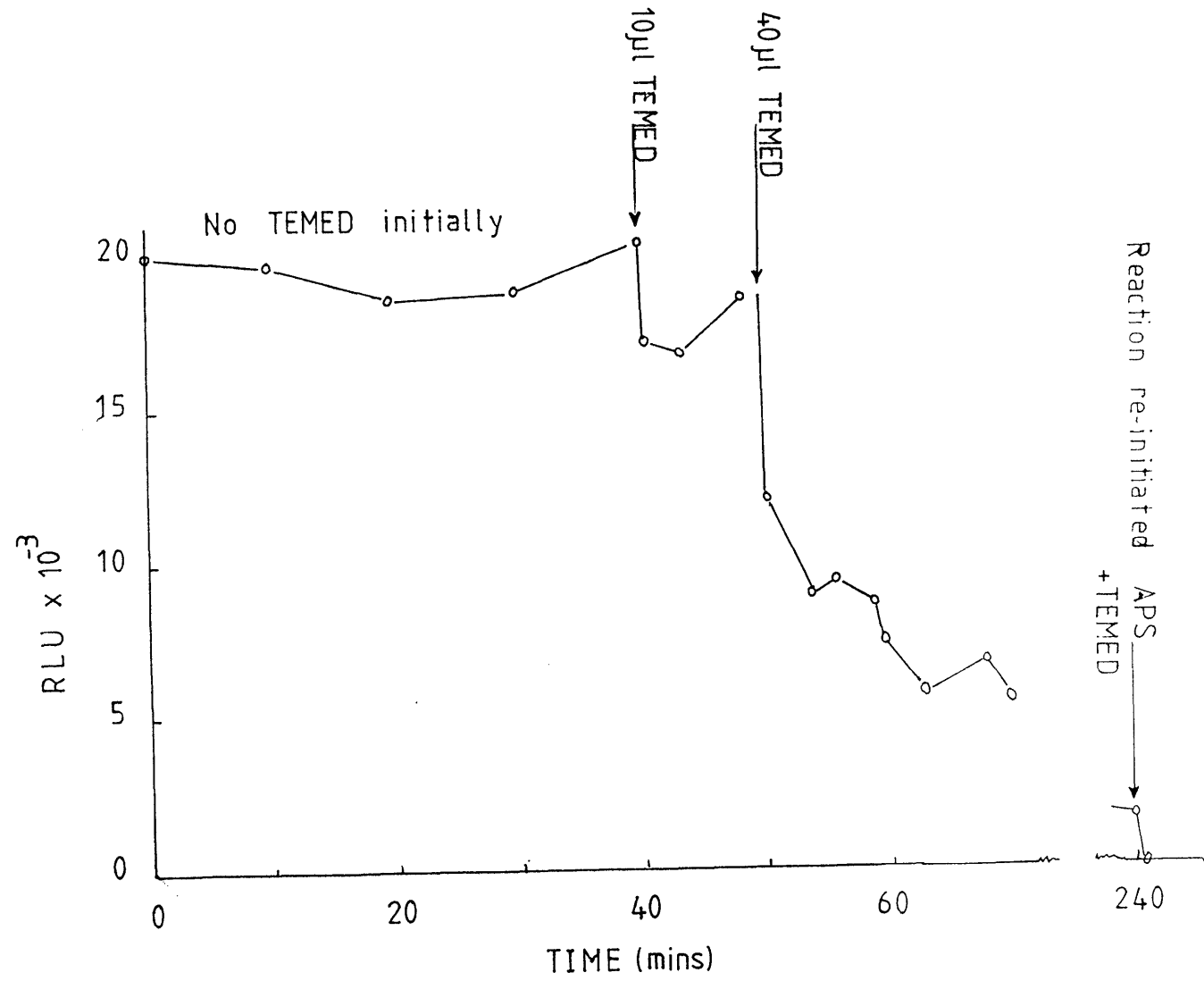
4.1.2 Entrapment in alginate based gels

The problems of an inactivating polymerisation chemistry were circumvented by using Agar or carrageenan, two chemically inert polymers, as gelling agents.

Agar Although in this case it is a crude method the activity of luciferase was monitored by using the dithionite assay. Several concentrations of agar were used to entrap the enzymes. Protein was leached out during the washing as monitored at 280 nm until the apparent absorbance of the wash was zero.

6% Agar: Produced no response to the luciferase assay

FIGURE 4.1 Inactivation during PA gel formation



3% Agar: Produced a small amount of activity
1% Agar: Produced 6% of the original activity

However the residual activity was found to be reduced with successive dithionite assays suggesting either poisoning or further leaching of the enzyme.

The loss of 94% of activity on entrapment cannot be explained by thermal denaturation after the short time the enzyme is exposed to a temperature of 45°C.

While some protein is lost through leaching, other evidence from coupled assays on agar entrapped enzyme showed higher levels of retained activity; this indicates that another factor is causing the loss of activity in dithionite assays. It is likely that the resistance to rapid mass transfer is preventing the assay of buried enzyme by the turnover assay. Reduced mass transfer between the bulk solution and the gel matrix is strongly indicated by the kinetic response of gel slices to the coupled assay. The rise in light emission is almost constant for 5 to 10 minutes before a progressive slowing of the rate to a plateau is observed. This behaviour is consistent with diffusion controlled kinetics.

Initially the constant rate of rise in light emission is a result of substrate transport down the developing concentration gradient. As the gradient is formed the rate of increase in light emitted will fall off, the rate of substrate consumption and product removal will rise until equilibrium is reached with the transport from bulk solution to the gel. The rate of increase in light emission was observed to depend on the percentage of agar in the gel. Equilibration time was also observed to be a function of gel slice thickness, increasing with thickness. Both observations are consistent with the hypothesis that enzyme activity when entrapped exhibits substrate limitation.

Since the objective of producing a solid state assay with a measurable and rapid response to substrate by the entrapment of free enzyme proved unsuccessful with agar, another matrix was investigated with several alterations to the method.

4.1.3 Carrageenan entrapment of DE-52 immobilised luciferase and reductase

To overcome the problems associated with gel entrapment and the problems of handling the DE-52 preparation (section 4.2.1) a combination of the entrapment and adsorption techniques was investigated.

Luciferase and reductase adsorbed onto DE-52 were entrapped in a carrageenan matrix.

* A 0.5% w/v gel mixture was found to set too rapidly, the setting conditions used producing an uneven thickness of gel on the setting slab.

* 0.1% w/v gel mixture produced a gel of insufficient tensile strength to be managable.

* A 0.25% w/v gel mixture was found to produce a conveniently handled uniform sheet of gel for the experiments. Less than 1% of coupled activity was observed to leach out of the preparation.

Table 4.1 shows the activity and time response for different preparations. The concentration of enzymes on the DE-52 was varied to increase the peak response. There was however no change in the response time with these different enzyme loading. The maximum activity of the beads in the gel was consistently less than on beads in solution.

Although the slices of carrageenan were able to discriminate different concentrations of NADH as shown in the calibration experiment (figure 4.2), the response time of upto 20 minutes was once again unsatisfactory.

The assay development was taken one stage further using a mixture of DE-52 (Lux,Red) and CM-52 (MDH) preparations to measure malate levels. The response time of the system was increased to about 40 minutes but it was possible to construct a calibration curve (figure 4.3).

4.1.3.1 Co-entrapment of co-factors

An attempt was made to improve the response time by preparing the gel with FMN, Decanal and NAD+ in situ. This improved the initial level of light intensity but the time to plateau was still about 20 minutes. The gel prepared with the co-factors showed a lower final plateau of light emission, addition of fresh co-factors resulted in a recovery of absolute activity, replacement of FMN alone recovered most of this activity. The FMN was evidently the element sensitive to storage in the gel environment, this is consistent with observations on the co-storage of co-factors with other preparations of the enzymes. Since the effect on response time of these alterations to the gel preparation and assay is small while the effects on magnitude are pronounced we can conclude that the diffusion rate of malate through the gel dominates the response time.

4.1.4 Cellulose acetate entrapment

Cellulose acetate films have been used to create membrane

TABLE 4.1

Response time for assays of 100 μ M NADH to reach plateau light level. Showing effect of enzyme loading and carageenan density.

Enzyme loading μ g/g μ g/ml DE_52 Assay		Enzyme Prep	Activity mV mV/ μ g Enzyme		Response time minutes
-	0.75	Free Solution	620	827	0.4
300	1.5	DE_52 beads	1150	767	< 0.2
300	0.38	DE_52 in gel*	34	89	c20
75	0.075	DE_52 beads	43	573	< 0.2
375	0.38	DE_52 beads	758	1995	< 0.2
750	0.75	DE_52 beads	1625	2167	< 0.2
75	0.23	DE_52 in gel**	80	349	c20
750	2.25	DE_52 in gel**	4000	1778	c26

gel* : is 0.1% w/v carageenan

gel** : is 0.05% w/v carageenan

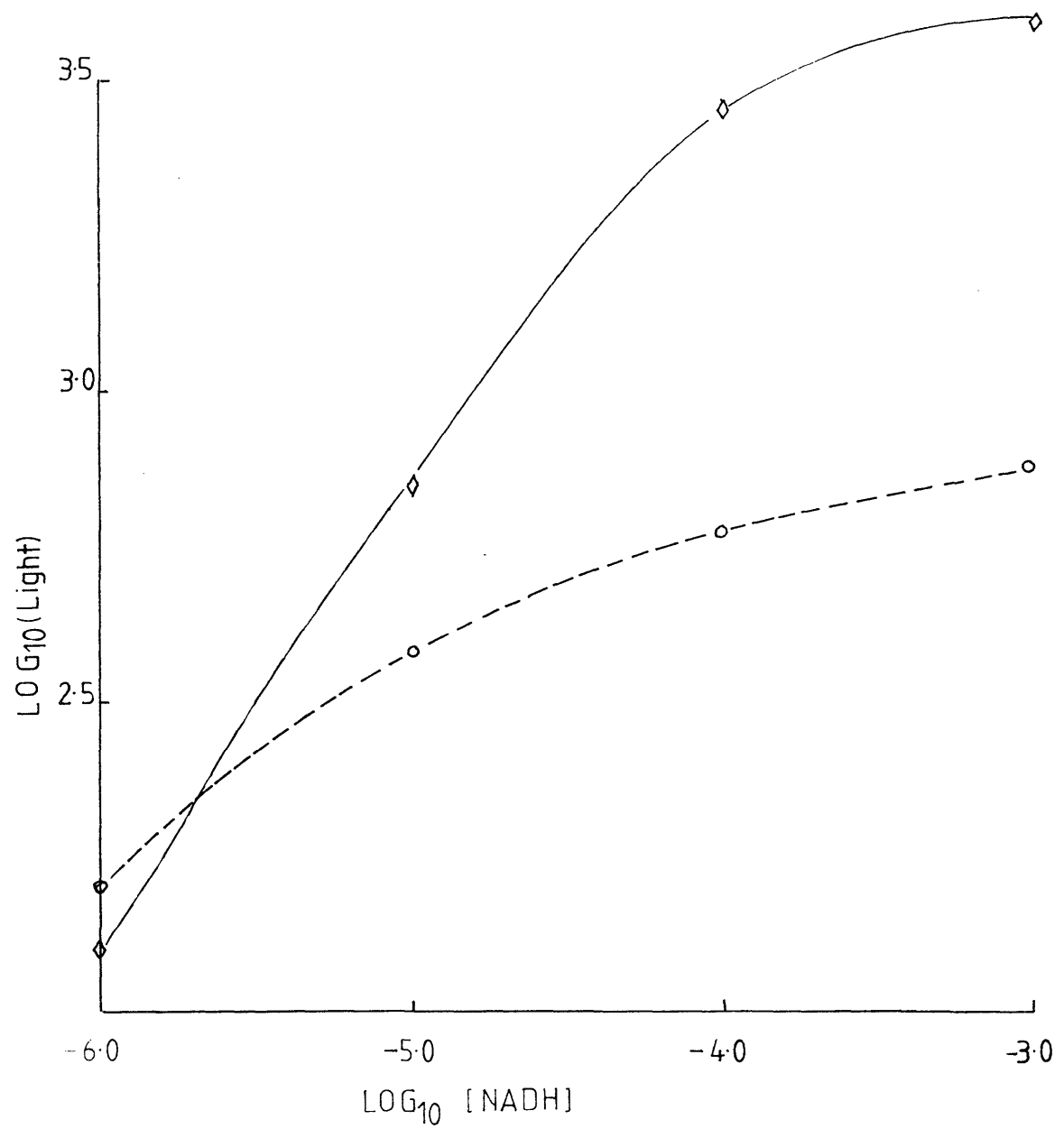


FIGURE 4.2 DE 52 entrapped in
 ◇ 0.05% Carageenan
 ○ 0.1% Carageenan

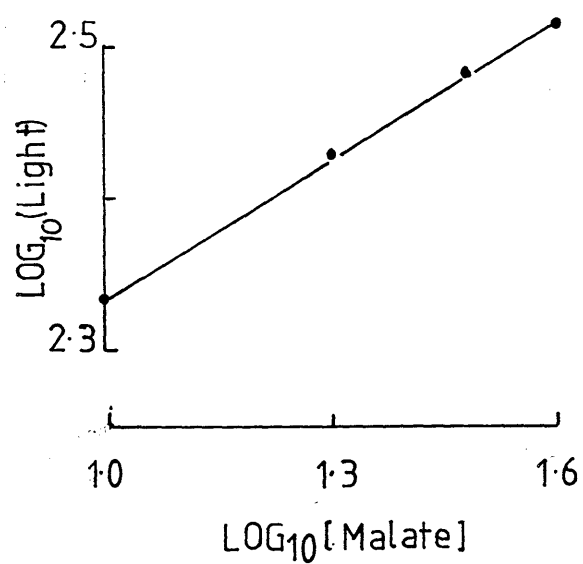


FIGURE 4.3

Malate calibration of DE 52 CM 52

Immobilisations in 0.05 % carageenan

entrapped enzyme preparations for sensors, particularly for enzyme electrode type sensors (Taylor et al 1977, Sim 1986).

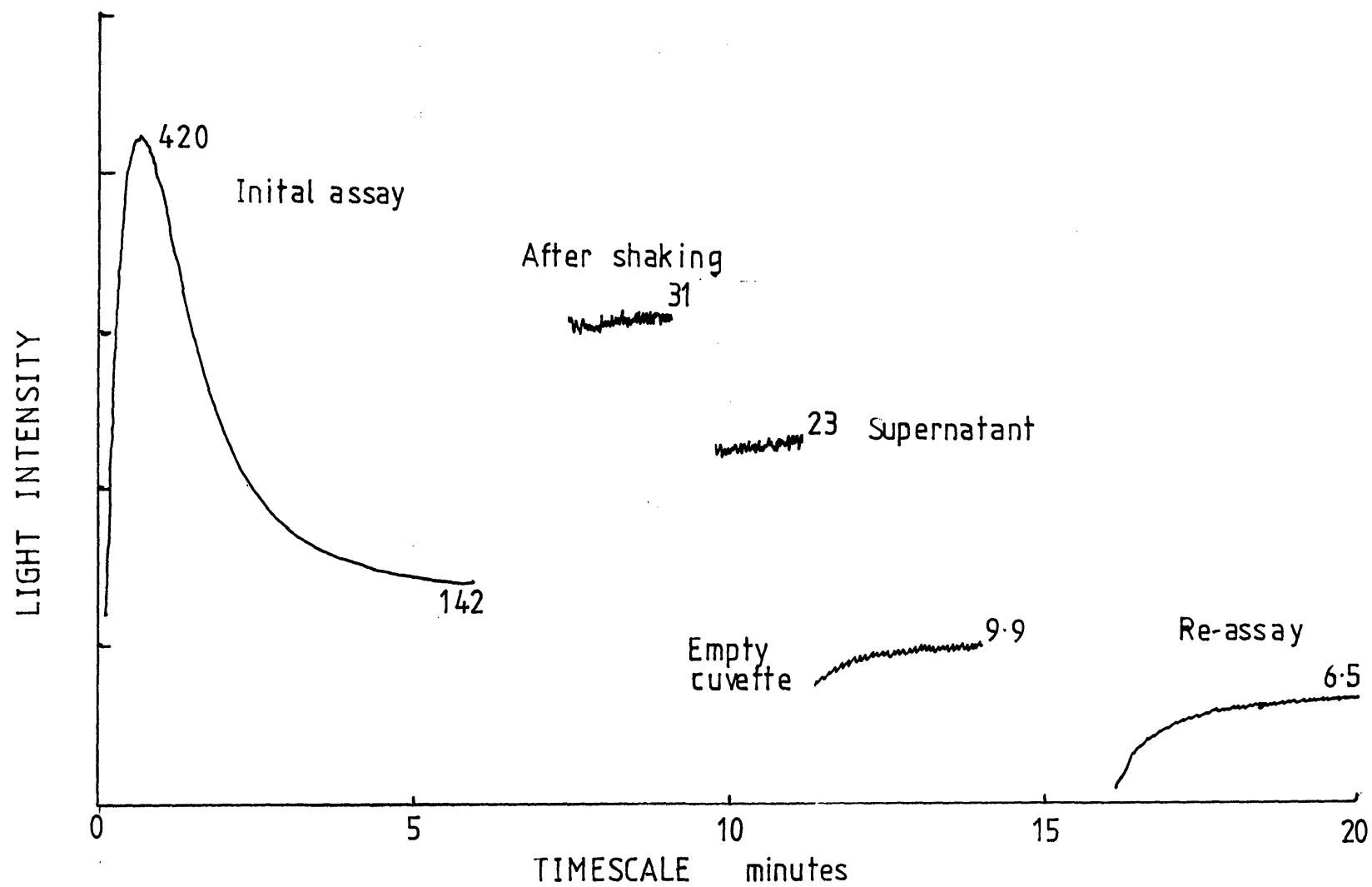
It was found that luciferase and reductase were inactivated by the cellulose acetate forming solution. Investigation of the inhibitory effects of the solvents used in the cellulose acetate film formation showed them to be powerful inhibitors of luciferase activity (Section 4.5.2). However when a lyophilised preparation of the enzyme was used with the cellulose acetate solution to deposit enzyme on the cuvette surface some immobilised activity was observed. The film itself was observed to have incorporated small granules of the lyophilised enzyme onto the cuvette surface. Unpredictable and unusual emission profiles were observed when the cuvettes were first used. Figure 4.4 shows the light emission profile for the first and subsequent uses of a cuvette. The bulk of activity is lost between first and second assay, with a substantial loss of activity during the initial stages of the first assay. Decanting the assay supernatant shows that the bulk of the first activity is associated with the solution phase. The activity in the second, re-assay, of the cuvette represents activity truly immobilised.

The method showed low levels of retained activity (1-2%) compared to the initial activity observed in the first assay, and only about 5% of the activity observed in a control coupled assay with an equal amount of unimmobilised enzyme in solution. The response time of some two minutes as judged by the re-assay is comparable to that of a solution assay, it is however slow in comparison with the response time observed for other immobilised enzyme preparations discussed in the next section.

The profile obtained in the first assay may be explained as follows: at the start of the assay the luciferase is localised in small pockets on the side of the cuvette. On contact with the assay solution the response is characteristic of a high local concentration, as if immobilized. However the enzyme becomes rapidly solubilized and dissipates in the solution with time, lessening the co-operative substrate channelling effects of luciferase and reductase in high local concentration. This accounts for the loss of activity during the first assay. The dissociation of enzyme from the cuvette was confirmed by re-assay of the assay supernatant in a fresh untreated cuvette.

Re-assay showed an intensity profile comparable to the solution assay, however the efficiency of the immobilisation and the response time are poor compared to other techniques described later. The immobilisation

FIGURE 4.4 Cellulose acetate immobilisation



also causes disruption of the polystyrene surface resulting in a kind of frosting of the optical surface. This causes an additional problem in the manufacture of cuvettes producing reproducible levels of transmitted light.

Liquid and solid ion exchange resins were investigated as possible means of improving the efficiency of this entrapment technique by maintaining high local enzyme concentrations. Neither technique worked, the immobilisation of solid ion exchange resin led to highly variable distribution of activity between cuvettes, while the liquid ion-exchange resin was immiscible with aqueous solution and failed to produce a viable immobilised enzyme preparation, most probably due to partition effects between the two phases.

4.2 ADSORPTION METHODS

Adsorption methods of immobilisation were investigated as they offer the potential of high recovery of activity through a non-destructive immobilisation technique.

4.2.1 Adsorption of the bacterial bioluminescence enzymes to DE-52

DE-52 is known to bind both FMN:NADH oxio-reductase and luciferase from its use in the purification of the two enzymes. In a low ionic strength solution at neutral pH the enzymes are known to be tightly bound and the enzyme activity is recoverable with little loss. The use of DE-52 as a carrier matrix for the two enzymes in quantitative assays was investigated.

Table 4.2 shows the activities of the bioluminescence enzymes before and after immobilisation. From these figures it can be seen that ξ is greatly increased on immobilisation. The value of ξ is dependent on the ratio of luciferase to reductase in the preparation. However the experiments with different luciferase to reductase ratios indicate that the geometric increase in ξ is approximately uniform regardless of the ratio of enzymes within the range investigated. This observation is consistent with the theory that ξ is related to the spatial separation of the luciferase and reductase enzymes ie. their local concentration.

Luciferase activity measured by the dithionite turnover assay indicates that as little as 15% of the activity

TABLE 4.2

Immobilisation data for adsorption on to DE-52

Sample	Dithionite assay /RLU	Coupled assay 0.1 mM NADH /RLU	Efficiency No. ξ
Enz mix a)	8.3×10^8	9.0×10^5	0.00108
Adsorbed	1.3×10^7	2.08×10^6	0.16
Lux:Red mixture			
1:5	1.15×10^7	40	0.0000035
	7500	495	0.066
1:10	8.9×10^6	100	0.0000112
	7900	1120	0.14
1:20	8.2×10^6	220	0.0000268
	8000	2220	0.28

recoverable from the DE-52 is detected whilst the enzyme is attached to the matrix. This leads to the conclusion that either the turnover assay is not detecting all active luciferase molecules on the beads or the environment of the beads is inhibiting the enzyme. It is difficult to conclusively prove one or other of these explanations. The evidence below summarises the findings from this immobilisation and is consistent with the first of these explanations.

(1) We know from protein purification data that more than 70% of the luciferase activity is recovered by desorption.

(2) The ξ values observed are very high, greater than 0.50. While these are not impossible they are significantly better than other techniques have produced.

(3) The k_{cat} of luciferase as obtained from the turnover assay is essentially unaltered by the immobilisation.

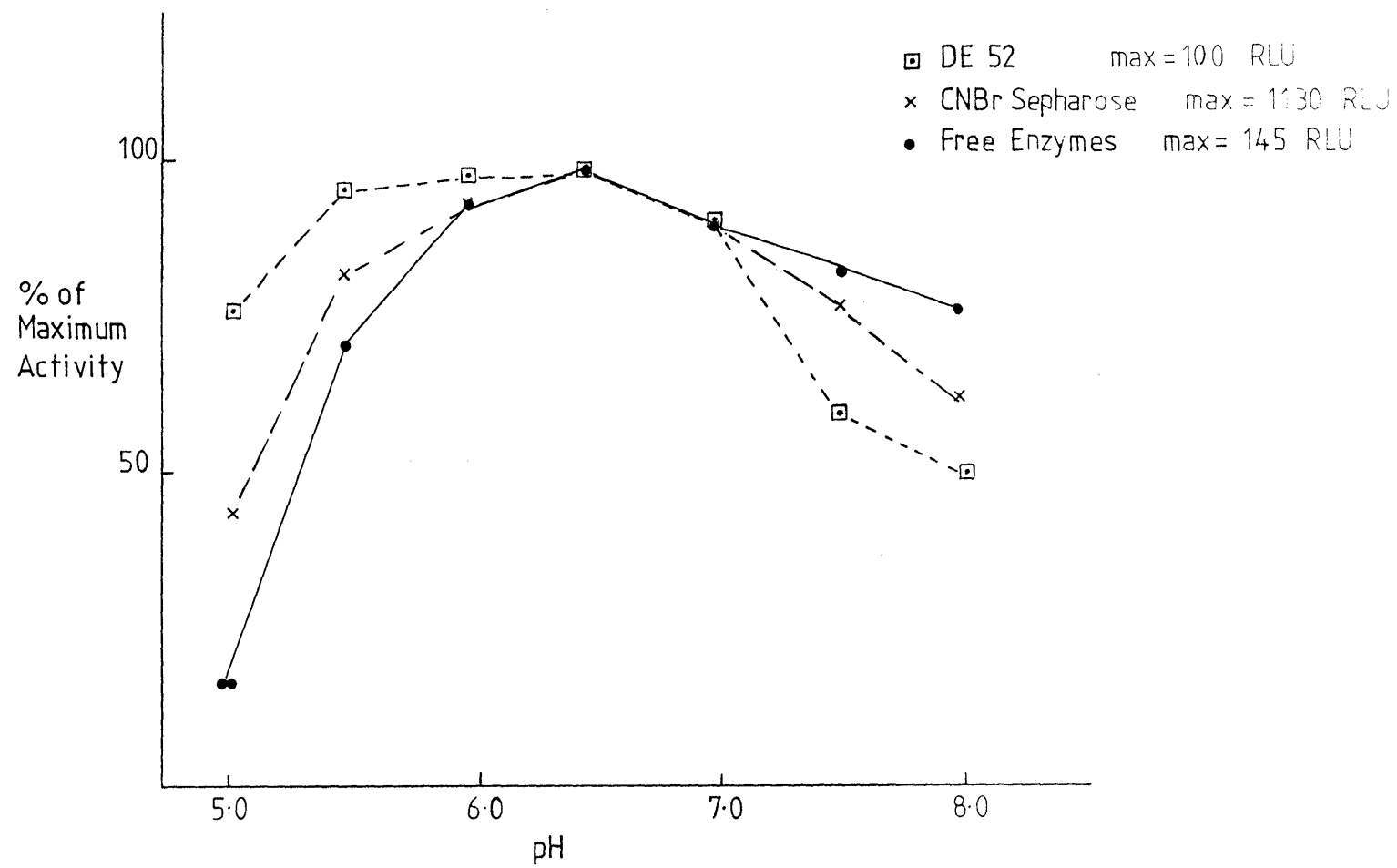
(4) From the kinetics of the dithionite assay we can only expect the enzyme to be detected where there is little inhibition to the mass transfer of decanal. Otherwise oxygen will oxidise the FMNH₂ prior to decanal interacting with the enzyme.

From these observations one explanation appears to be that the enzyme immobilized within the porous structure of the beads is able to participate in the coupled assay but not the dithionite assay. This situation occurs as a result of the differential diffusion of oxygen and decanal to the buried enzyme molecules. The effect of a lower apparent luciferase activity is translated into an increase in the apparent value of ξ as defined in the introduction to this chapter, the value of the denominator in the expression for ξ is reduced as buried enzyme is prevented from participating in the dithionite assay by this differential diffusion.

4.2.1.1 pH profile

The DE-52 preparation shows a pH profile slightly broader than that for the free enzyme and shifted by about 0.5 pH units to acid pH (figure 4.5). This is consistent with the general observation that a positively charged matrix will shift the pH optimum of an enzymic reaction to a more acidic pH. This occurs as a result of the ionic repulsion of protons from the immediate matrix environment, producing a higher local pH than that in the bulk solution. As the pH of the bulk solution is measured for the profile, the apparent shift in the profile to the acidic side reflects the real shift in the pH of the matrix environment compared to the bulk

FIGURE 4.5 pH profiles of activity



solution.

4.2.1.2 Substrate affinitys

The affinity of luciferase for decanal and FMN appears to be slightly altered although not drastically. Figure 4.6 shows the light production against $\ln [\text{decanal}]$ for the free enzyme and the DE-52 bound enzyme. K_s and K_i for the two binding sites for the aldehyde can be calculated by the method of Holzman and Baldwin (1983). The K_s is slightly altered from $3.6 \mu\text{M}$ to $4.4 \mu\text{M}$ while the inhibition constant K_i is substantially altered from $25 \mu\text{M}$ to $58 \mu\text{M}$. Holzman and Baldwin comment in their paper that altered values of K_s and K_i in the presence of BSA may be due to the decanal binding to BSA (which is known to possess an affinity for long chain fatty acids). In the case of this immobilisation the matrix itself may be interacting with the decanal. While decanal is neutral so making significant ionic interaction unlikely there may be some partitioning effect between the matrix and bulk phases.

4.2.2 Adsorption of the bacterial bioluminescence system onto polystyrene

The simplest and ideal way of obtaining a simple assay in a cuvette would be to directly immobilise the assay enzymes onto the side of the cuvette.

However the surface area of the cuvette is small and a maximal enzyme loading is required to obtain a reasonable sensitivity. Theoretically it is possible to estimate a maximum activity obtainable per cuvette

Surface area of the cuvette: approx. 4 cm^2

Number of enzyme molecules in a monolayer $10^9\text{--}10^{10} \text{ cm}^{-2}$

(MW 80,000; Specific activity $1.4 \times 10^8 \text{ RLU/mg}$)

Maximum theoretical activity/cuvette: 600 RLU

Coupled activity assuming = 0.2 : 120 RLU

Direct adsorption to polystyrene was not expected to produce high enough loading through adsorption by hydrophobic interaction alone. Washing a used cuvette left no detectable activity, a result consistent with the above theory. Despite attempts to improve adsorption levels problems of low loading were experienced with the following techniques.

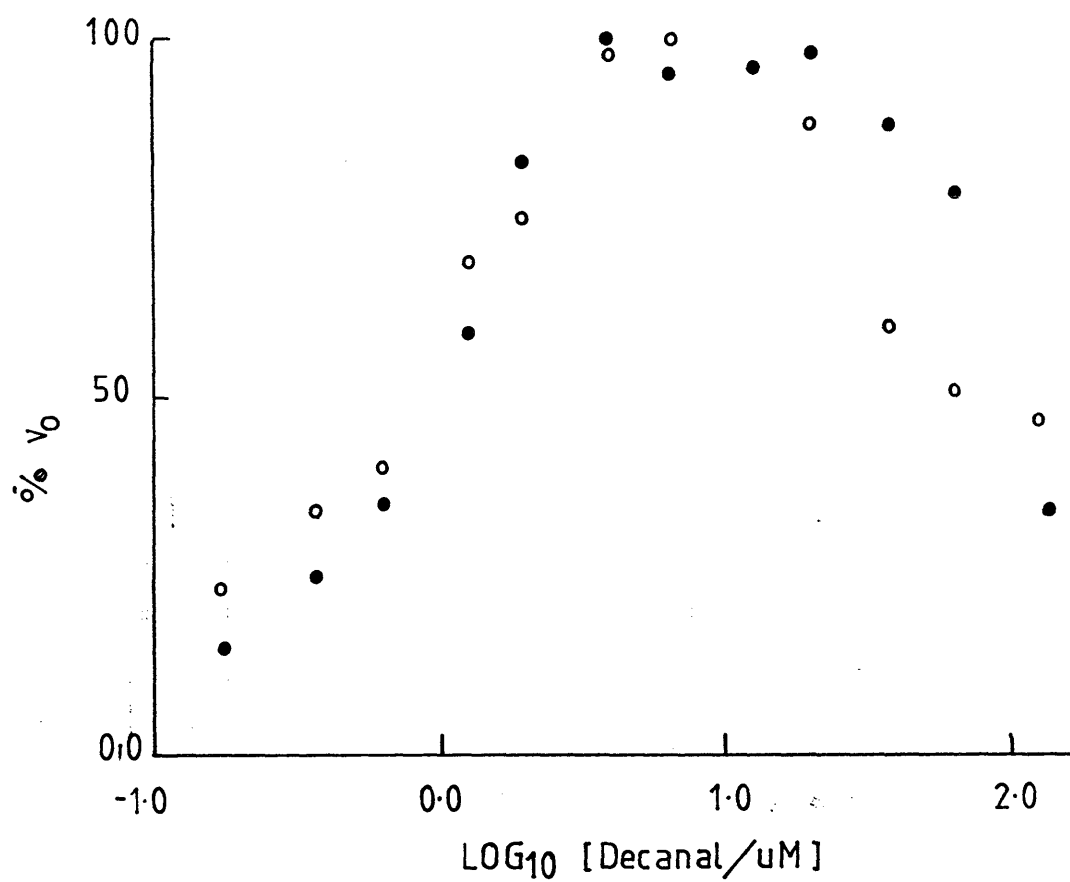


FIGURE 4,6

Activity profile of luciferase against [Decanal]

- Free enzyme
- DE 52 bound

4.2.2.1 Immobilisation onto cuvettes

Immobilisation to the polystyrene surface via an intermediary molecule of poly(Phe:Lys) was investigated. Results shown in table 4.3 demonstrate the results of the method, it can be seen that although an active preparation was made in all cases there was no benefit from using the water soluble carbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulphonate to covalently link the enzyme and the poly(phe-lys). The covalent linkage appears to have a negligible effect on leaching of the enzyme from the surface of the cuvette, suggesting that the poly(phe-lys)/enzyme adsorption is stronger than the polystyrene/poly(phe-lys) interaction. The reaction with carbodiimide also reduces the recovered activity after washing. This is as one would expect in the light of the chemical modification results discussed in section 4.6. A second direct chemical attachment to an acrylic cuvette resulted in very little recovered activity.

The changes in activity following washing and reassay are shown in Table 4.4. It can be seen that while initially activity is lost to the solution phase a constant residual activity remains attached to the cuvette surface. Further the activity measured decreases with time in an unstirred solution, however if the solution is aspirated the activity rises gradually to a new maximum. Initial activity attached to the cuvette wall was increased in the case of 0.5 mg/ml carbodiimide, however after thorough washing and storing at -20°C these preparations showed a reduced level of retained activity.

While the method gave a reusable immobilised preparation the activity per cuvette was too low to produce a satisfactorily sensitive assay system. Increasing the concentration of PPL and/or enzymes in the preparation protocol had no significant effect on the activity recovered per cuvette, so the surface of the polystyrene can be assumed to have been saturated.

4.2.2.2 Effects of adsorption reagents on activity

Poly phenyl-lysine (PPL)

A series of experimental controls showed how PPL affects the activity of the bacterial bioluminescence system were carried out. The results summarised in table 4.5 indicates that some interaction between PPL and luciferase occurs resulting in up to 92% inhibition depending on the relative concentrations of PPL and enzyme, and the incubation time: with 100 $\mu\text{g/ml}$ PPL in solution there is a rapid inactivation while 10 $\mu\text{g/ml}$ PPL shows a slower inactivation rate (Figure 4.7). It is reasonable to assume that the interaction between the two

TABLE 4.4

Typical series of assays and bioluminescence readings to examine a PPL immobilised luciferase/reductase cuvette.

Source	mV
-----	-----
cuvette background	0.20
assay background	0.22
assay added to cuvette	25.35
assay supernatant	2.40
empty cuvette	17.82
recombined	17.5
cuvette is now washed with buffer several times	
cuvette background	5.27
assay background	0.19
assay added to cuvette	11.36
assay supernatant	0.43
empty cuvette	10.2
recombined	8.62
+ use of aspirator	14.0
-----	-----

TABLE 4.3

Immobilisation of luciferase and reductase on polystyrene

Immobilisation sample	Activity Coupled mV (@ 1mM NADH)	Dithionite mV	Inter Enzyme Efficiency Σ
<u>Cuvettes</u>			
Free Enz	9.0	20000	0.00045
Cuvette *	7.0	1850	0.00378
Cuvette **	1.5	430	0.00349
Cuvette ***	0.12	35	0.00343
Free Enz	3.8	10000	0.00038
Cuvette *	0.25	130	0.00192
<u>LB-16</u>			
Free Enz mix	6.5×10^4	1.33×10^8	0.00049
LB-16 beads	32	19000	0.00169
Free Enz mix	1.08×10^5	2.2×10^8	0.00049
LB-16 beads	6.7	2210	0.00303

cuvette * : cuvette prepared with no carbodiimide

cuvette ** : cuvette prepared with 5mg/ml carbodiimide in immobilisation solution.

cuvette ***: cuvette prepared with 0.5mg/ml carbodiimide in immobilisation solution.

TABLE 4.5

Effect of incubation with poly phenylalanine:lysine on coupled bioluminescence activity.

Sample	Activity		Coupling efficiency Σ
	Coupled mV (@ 1mM NADH)	Dithionite mV	
Free Enz	126	5588	0.0225
Incubation with 10ug/ml	62.5	3765	0.0166
Incubation with 100ug/ml	8.9	600	0.0148

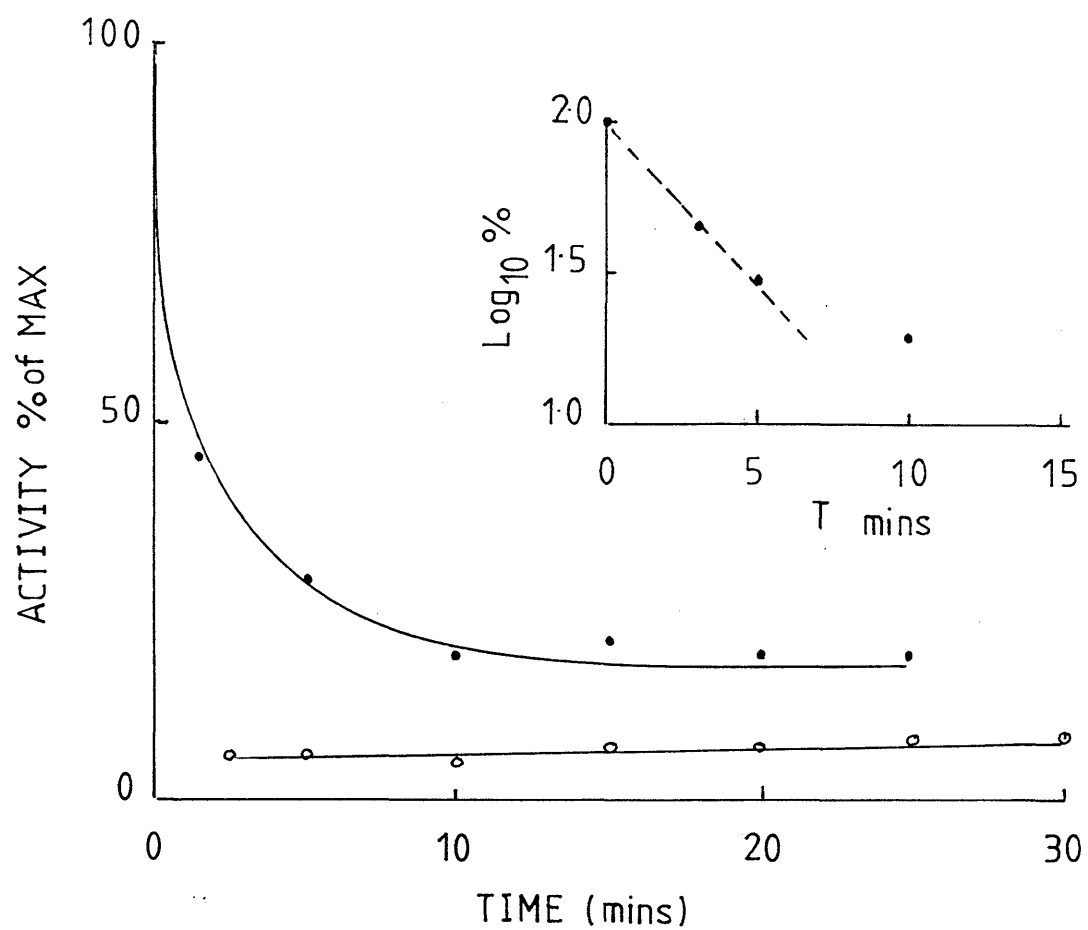


FIGURE 4,7 Effect of PPL on bioluminescence
 ○ 100 mg/ml PPL
 • 10 mg/ml PPL

molecules also occurs in the solid phase attached to polystyrene resulting in the retention of luciferase with perhaps only 8% residual activity. The similarity of the solid and solution forms of luciferase preparation is further supported by the observed decrease of k_{cat} for the two from 0.125 to 0.05 s⁻¹ in the turnover assay.

Poly-lysine

The effect of poly lysine in solution with luciferase and reductase was negligible with deviations from the mean attributable to experimental error over the same range of concentrations used for PPL, indicating that the hydrophobic effect of Phenylalanine in PPL and is responsible for the effect described above.

4.2.2.3 Immobilisation on to Latex (Polystyrene) beads

In order to increase the maximum activity of an assay an increase in the polystyrene surface available to enzyme is needed. This was achieved by using Latex beads.

Unfortunately LB-1 (1 µm) beads proved to small to handle in the preparation stage. They would not easily spin down in a microfuge and washing on a filter resulted in clogging, aggregation and some bead loss. LB-16 proved the most satisfactory sized bead and reproducible results were obtained for the co-immobilisation of luciferase and reductase (table 4.3) and a calibration curve for NADH is obtained (figure 4.8) showing good correlation over two orders of magnitude despite poor absolute levels of activity. The regression analysis of the results showed a slope of 0.45 +/- 0.013 with a correlation of 0.997, theory would predict 0.5 (Chapter 6).

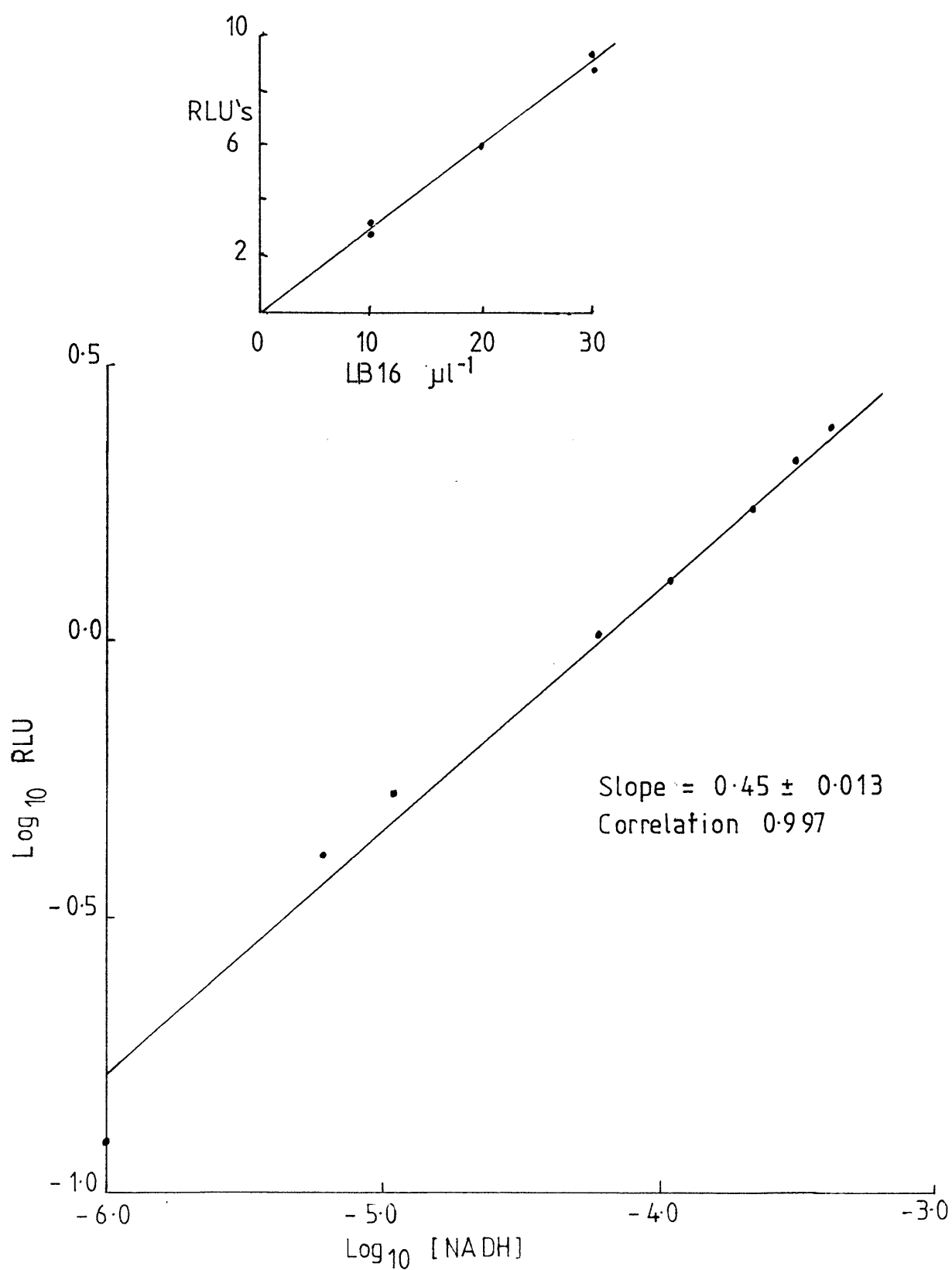


FIGURE 4.8 NADH Calibration of LB 16 immobilisation
Inset shows beads act independently

4.3 COVALENT IMMOBILISATION METHODS

With covalent immobilisation enzymes are irreversibly attached to the support, as a result leaching effects are eliminated and this simplifies interpretation of events occurring to the bio-matrix during operation. Luciferase has previously been covalently immobilised on glass beads and CNBr activated sepharose. Neither of these approaches were very successful; the immobilisation on glass by a diazo reaction produced about 0.01% recovery of activity (Jablonski and Deluca 1976), on CNBr sepharose 5% is a typical recovery. The sepharose method gives satisfactory activity for experimental work but economically a 95% loss of enzyme activity represents a high loss of the most expensive assay component.

4.3.1 Immobilisation onto CN-Br activated sepharose 4B beads

Further work on the CNBr sepharose has been done to characterise the immobilisation and provide a standard by which to gauge other immobilisation techniques.

Immobilisation of luciferase and reductase on activated beads resulted in a startling increase in the value of ϕ , the kinetic coupling between the enzymes. The coupled assay was observed to give more light with immobilised enzyme than with the same amount of enzyme in solution despite 95% loss of luciferase activity. Table 4.6 shows the activities of immobilised preparations prepared under slightly different conditions.

4.3.1.1 Study of Immobilised assay responses

The following general points became clear from these studies:

- * ϕ is increased to ~ 0.3 an increase of about 100 fold.
- * k_{cat} of the luciferase remains unchanged by this immobilisation.
- * K_m for FMNH₂ (figure 4.9) is not significantly changed.
- * Light generated from NADH is found to follow the theoretical linear relationship described by equation 16 in chapter 6, the calibration log vs log plot (figure 4.10) having a slope very close to 1.0.
- * Coupled assays with saturating NADH (0.1 mM) showed a light emission profile significantly changed from that obtained with free enzymes. With solution assays the coupled assay gives a rapidly rising level of light

TABLE 4.6

ENZYMIC ACTIVITIES AFTER IMMOBILISATION

Immobilisation Discription	0.1M NADH	Light RLU Dithionite Assay	Σ	Activity %
(a) Low [Luciferase]				
Enzymes in solution	1.25×10^4	4.3×10^6	0.0029	
1 hr incubation	5.7×10^4	1.68×10^5	0.34	4.45
15 hr incubation	1.44×10^4	1.02×10^5	0.14	2.7
(b) Low [Reductase]				
Enzymes in solution	4.2×10^4	1.5×10^8	0.00028	
2 hr incubation	1.22×10^6	6.25×10^6	0.20	2.1
(c) No BSA				
Enzymes in solution	9.0×10^5	8.3×10^8	0.0011	
2 hr incubation	7.0×10^6	1.2×10^7	0.58	14.0
(d)				
Enzymes in solution	6.0×10^5	6.2×10^8	0.00097	
2 hr incubation	6.66×10^6	2.1×10^7	0.32	4.0
Above + 2 hr with poly-lysine	1.0×10^7	2.5×10^7	0.40	4.76
(e)				
Enzymes in solution	1.4×10^5	1.2×10^8	0.0012	
2 hr incubation	1.3×10^6	3.9×10^6	0.33	4.0

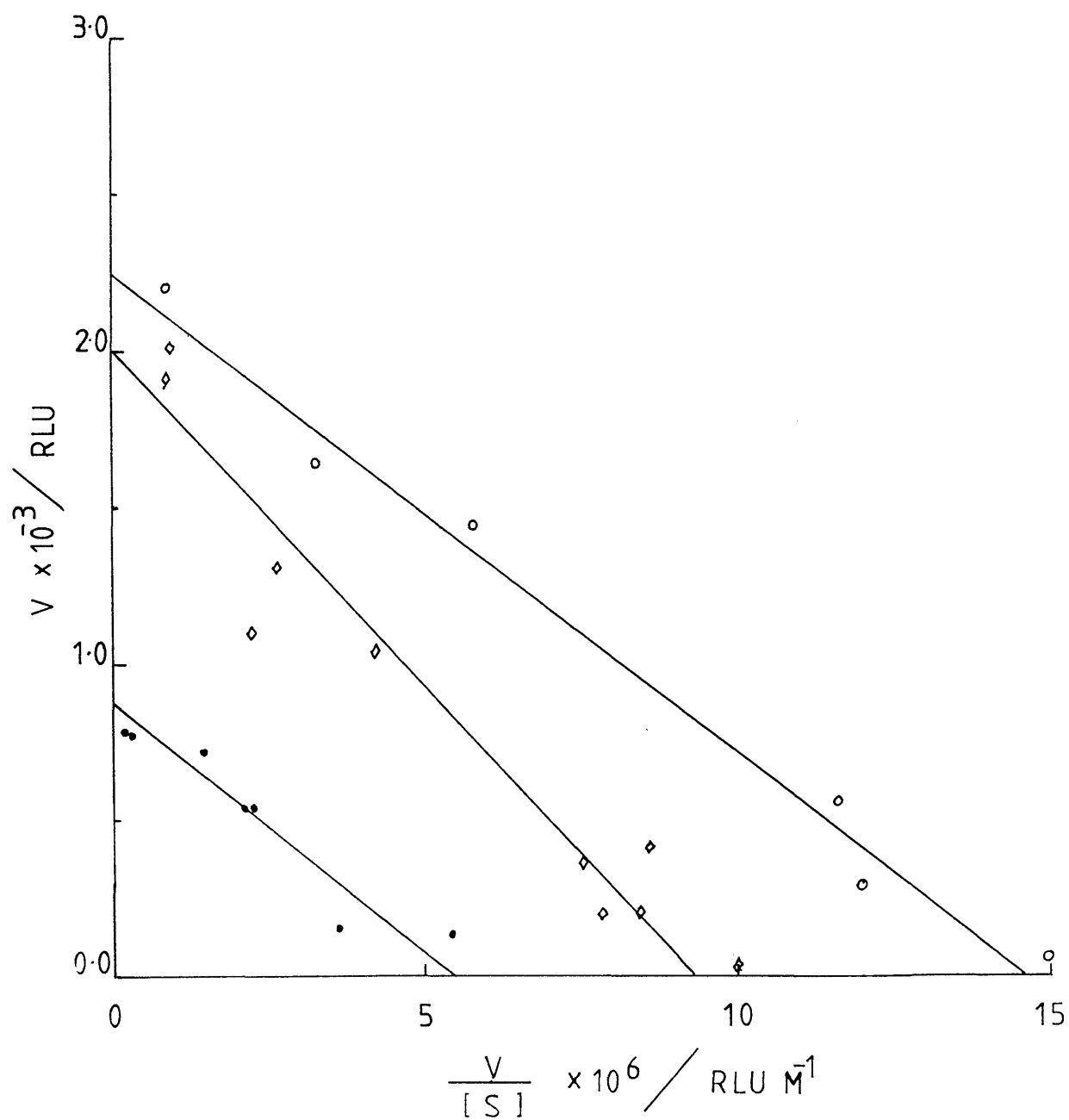


FIGURE 4.9

K_m for FMNH_2

$\diamond$ Enzyme in solution ($K = 0.214 \mu\text{M}$)

$\bullet$ Sepharose beads ($K = 0.154 \mu\text{M}$)

$\circ$ " " treated with Polylysine ($K = 0.179 \mu\text{M}$)

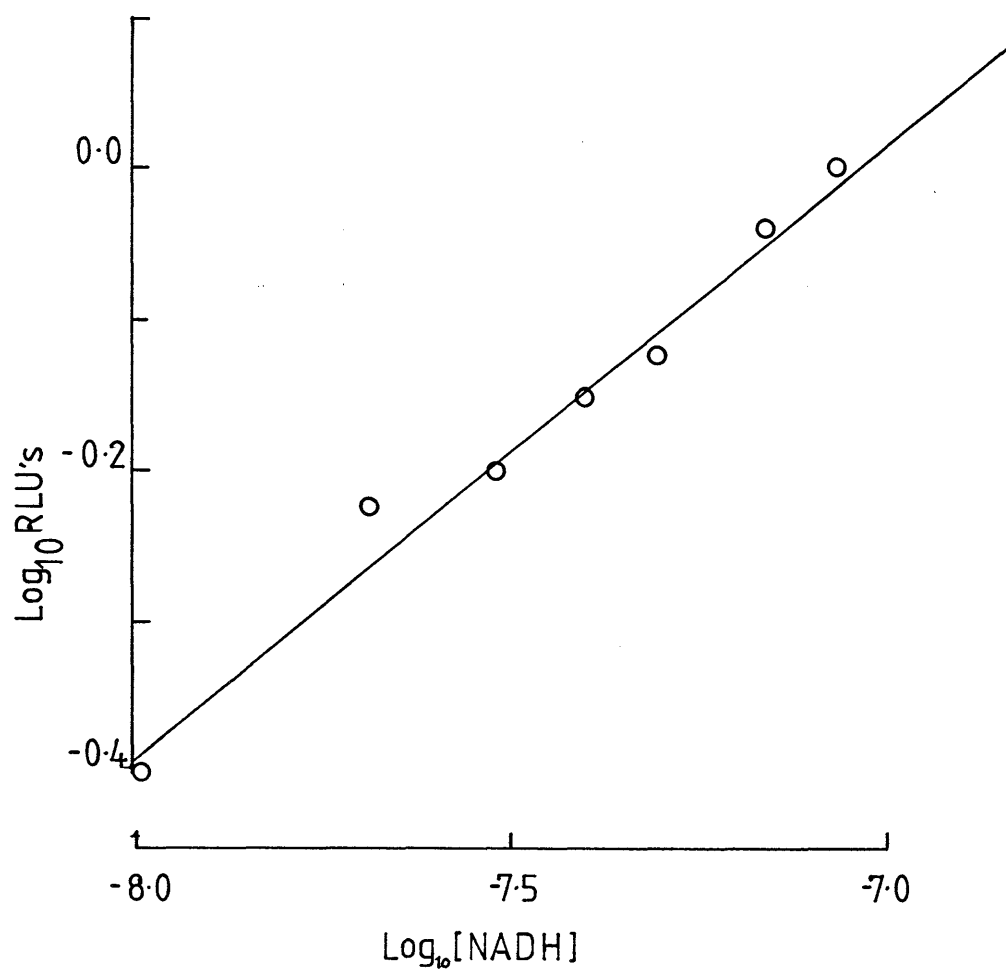


FIGURE 4.10 NADH assay using sepharose 4B enzyme preparation in solution

emission before reaching a plateau level Fig 4.11a. Bead assays in contrast show a rapid rise to a peak then a drop off to a plateau value as illustrated in figure 4.11 (c). However bead assays with lower concentrations of NADH ($> 10 \mu\text{M}$) show a similar profile to that of the solution assay at all concentrations albeit over a shorter time scale. The general kinetic behaviour of the bioluminescence assay is discussed in detail in section 6.3. However the proposed explanation and investigation of this phenomenon are dealt with here. This initial overshoot or hump was investigated by Wigley (1984) it was then suggested that the phenomena was due to the co-existence of two different micro-environments probably of different local pH's. In the original work trypsin was used to digest the first of these two populations the portion bound to the surface and with a microenvironment similar to the bulk solution; this would then leave the buried population with its different microenvironment. However the protocol left traces of trypsin from the tryptic digestion in the assay cuvette, and the samples of protease treated enzyme-matrix showed decaying light emission in the coupled assay figure 4.11 (b).

The problem was re-examined, especially with regard to the effect of proteolytic digestion on the enzyme-sepharose matrix. Experiments on the protease treatment of luciferase during a bioluminescence assay with trace quantities of trypsin had a significant effect on the intensity profile during the assay, causing the profile to resemble trace (b) figure 4.11. The interpretation proposed by Wigley (1984) was that removal of surface enzyme resulted in an internal population of enzyme showing an assay profile similar to the acid pH assay. The pH dependence and characterisation of the hump was re-examined following a repeat of the trypsin treatment of luciferase. Exposure of luciferase to small quantities of trypsin in solution showed light emission profiles of type (b) figure 4.11. Proteolysis of the beads must be controlled to allow a clear interpretation of the results. Exposure of the beads to the trypsin was controlled by packing the beads in the flow cell/mini-column described by (figure 2.3) then flowing trypsin slowly over the beads before washing with assay buffer. Beads were treated for 30 seconds and 60 seconds with a solution of buffer containing $86 \mu\text{g/ml}$ trypsin. Flow injection analysis (F.i.a.) of the beads with a standard concentration of NADH showed losses of 40% and 85% of the original activity respectively. The beads were then removed from the column and assayed in suspension.

The beads treated for one minute to study the effect of limited proteolysis on light emission at different pH's.

* At neutral pH the hump was only observed with the untreated beads.

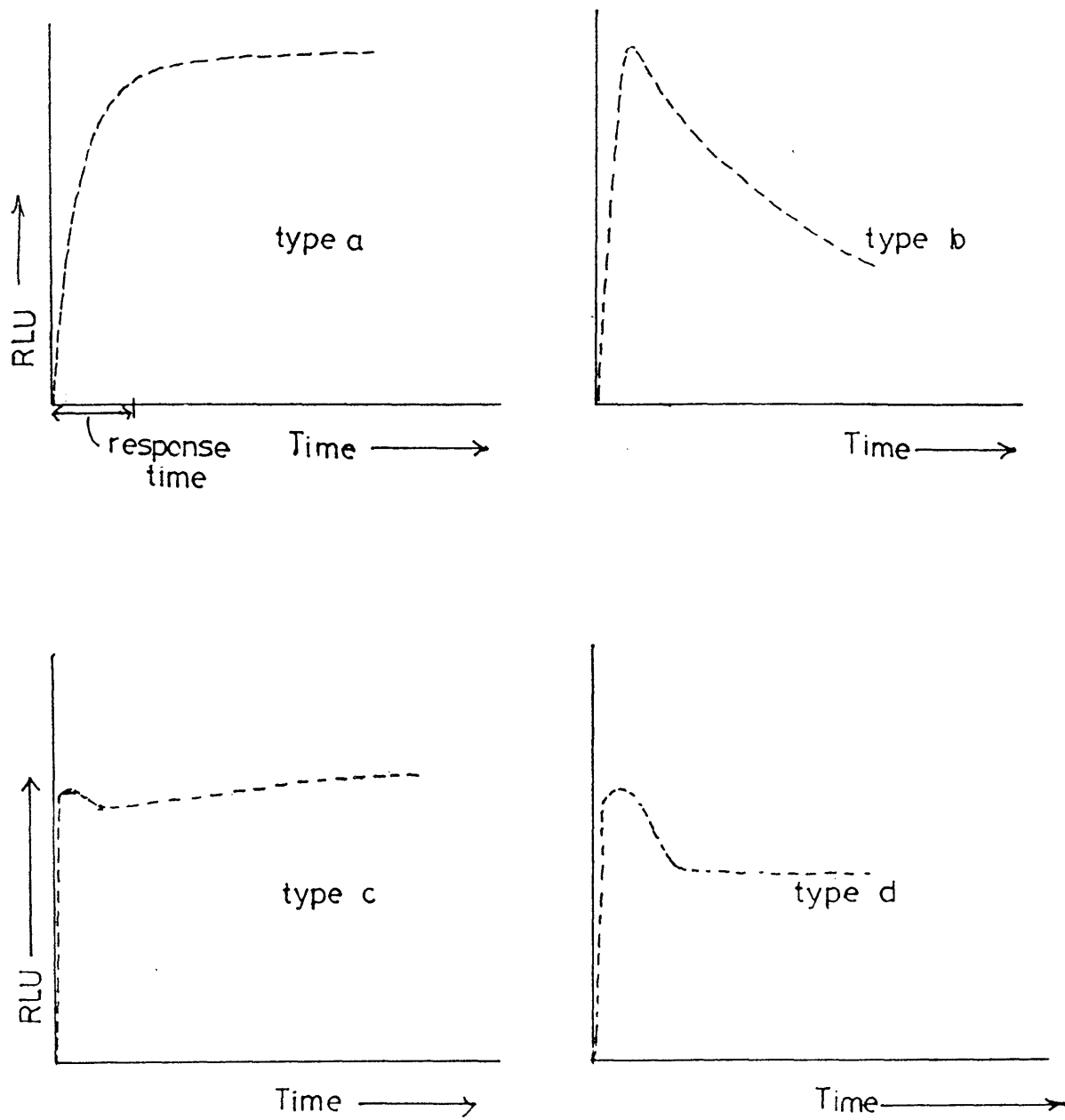


FIGURE 4-11 Four illustrative emission profiles

* At acid pH, 5.8 the decay kinetics after an initial peak dominate in all cases.

* At alkali pH 8.3 there appears to be a mini plateau on the way up to the final plateau level of light emission, an alkali equivalent of a hump perhaps.

To summarise the trypsin treatment has had two effects on the bead assay. i) To lower the level of light emission

ii) To alter the emission profile depending on assay conditions: it eliminates the hump at neutral pH, produces the mini-plateau at alkali pH and almost eliminates the hump at acid pH.

One explanation is that one enzyme population on the bead is responsible for emitting the hump and is largely eliminated by the trypsin. The evidence of the acid and alkali pH assays shows however the effect is still present to some extent at these pH's.

An alternative explanation of the results arises when the relative rates of reaction within the assay scheme are considered. If the rate of FMNH₂ synthesis from a saturating NADH concentration is such that enough FMNH₂ is generated in a few seconds to saturate the luciferase molecules the initial light emission profile will have a pseudo turnover character. This situation is only made possible since luciferase experiences a significant 'dead' time between the binding of FMNH₂ and the final release of FMN. This time is at least of the same order as the decay time for the dithionite assay, there is evidence for believing the dead time is longer than this with a conformational relaxation step being required after the release of FMN (Aboukhair *et al* 1985).

To summarise, the 'hump' can be explained as follows; since the binding of FMNH₂ to luciferase is very rapid, a rapid production of FMNH₂ arising from a high local reductase concentration will result in the bulk of the luciferase reacting with FMNH₂ faster than luciferase is released from the reaction sequence. Hence a semi-synchronous turnover of the enzyme is observed. This 'hump' peters out as the luciferase becomes more evenly spread between the different stages of its reaction cycle. The effect of the trypsin digestion is to reduce the concentration of both the reductase and the luciferase. Most significantly the decrease in reductase activity reduces the rate of FMNH₂ production and the final level of FMNH₂ at steady state according to equation 14 (section 6.1.3). At acidic pH the reductase is closer to its pH optimum of 6.5, hence reducing the relative effects of enzyme depletion, while at alkali pH the dead time of the luciferase is significantly

increased. It can be seen from figure 4.12 how the kcat of luciferase changes with pH.

A more mathematical treatment of this is given in section 6.3. An important consequence of the above argument is that the actual concentration of FMNH₂ produced is critical. If the levels and rate of production of this co-factor were significantly lower the 'tying up' of luciferase would not occur due to the excess luciferase present. The light profiles for the coupled assay using beads with NADH concentrations from 0.1 mM to 1.0 μ M are shown in figure 4.13 and it can be seen that the hump is progressively eliminated. These observations are consistent with and indeed predicted by the explanation given above and the mathematical model in chapter 6.

4.3.1.2 Co-immobilisation of Malate and Lactate dehydrogenases with the bacterial bioluminescent system

Table 4.7 shows an extended table of information on the co-immobilisation of MDH and LDH with the bioluminescence enzymes. ξ_p lactate and ξ_m malate are introduced to give a measure of channelling from the dehydrogenase to luciferase. The light emission profile for the two substrates shows a typical coupled assay pattern with a rapid initial rise followed by a steady intensity. As with the NADH assay, using immobilised enzymes greatly increases both ξ_l, ξ_m values and the response time. Table 4.8 gives a comparison of the response times for different immobilisations and substrates.

4.3.1.3 Effects of Co-immobilisation of poly-lysine to the sepharose beads

The effect of poly-lysine immobilisation on the sepharose beads appears to be to halve the response time for malate while the response time for NADH measurement was doubled. In general there is an order of magnitude decrease in the response times for immobilised assays of substrates. A detailed consideration of the reasons for these changes is left until chapter six dealing with analysis of the kinetics of these systems and how substrate channelling effects arise in the systems.

The pH dependence of a MDH, luciferase and reductase bead preparation was examined for malate, NADH and dithionite assays (note also pH profiles in figure 4.6). Figures 4.14a,b,c,d show the profiles for the plain bead and the polylysine treated bead preparations in NADH, Malate and FMNH (dithionite) assays and the variation in the light decay constant (1/kcat) with pH for single turnover assays. The incorporation of poly - phe lys into the sepharose 4B matrix was attempted with the intention of shifting the pH optimum of the assay by introducing

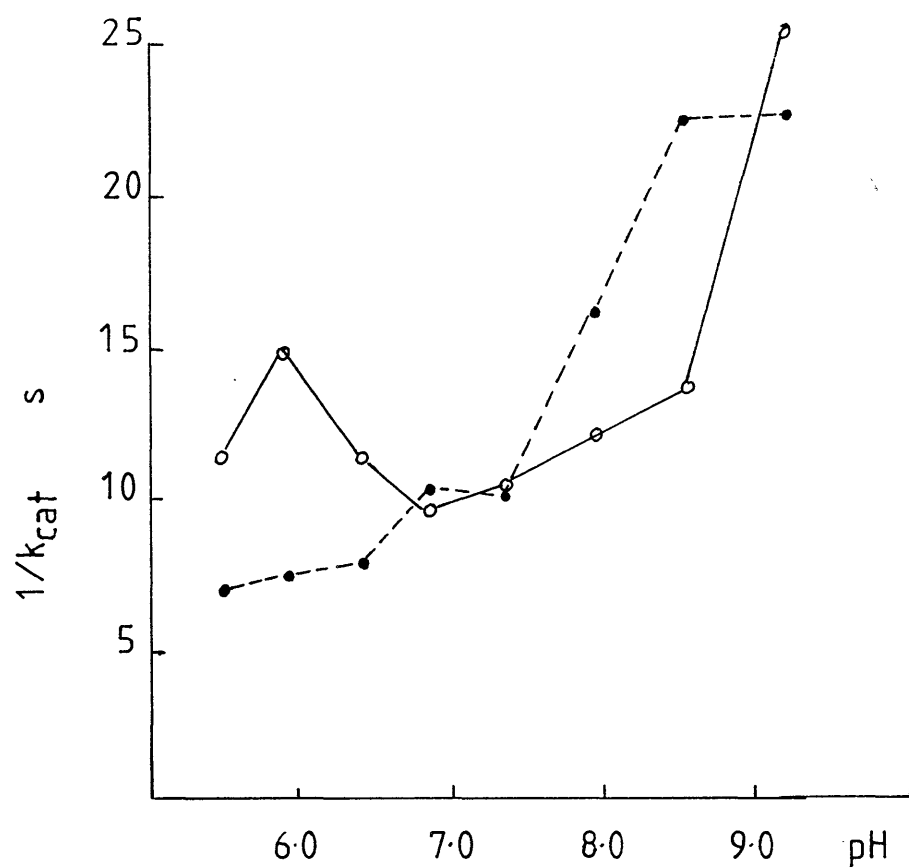


FIGURE 4,12 Variation of luminescence decay, $1 / k_{cat}$, with pH

○ Sepharose 4B immobilisation
 • " " treated with poly lysine

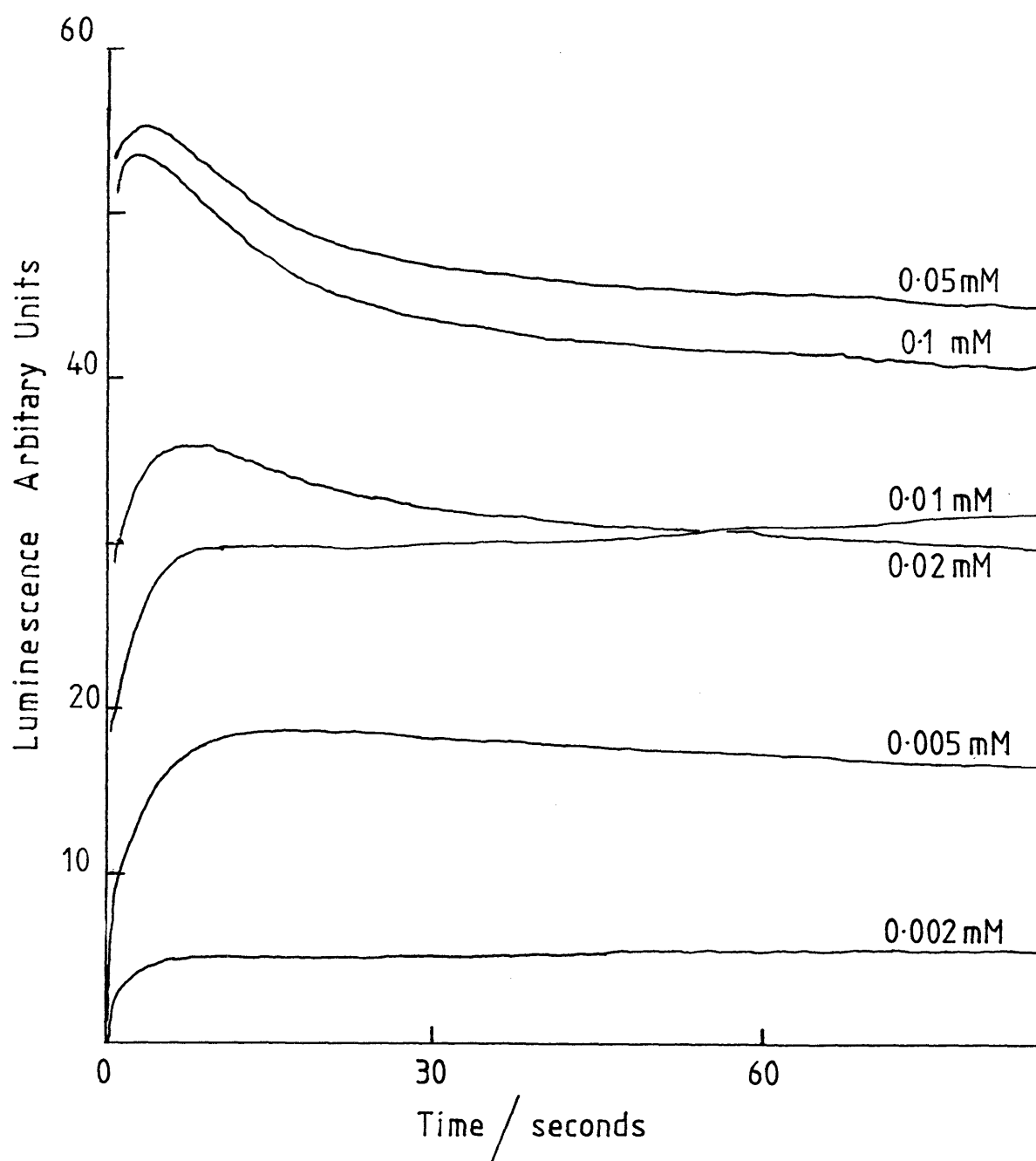


FIGURE 4.13
Effect of [NADH] on emission profile of immobilised enzyme

TABLE 4.7

ENZYMIC ACTIVITIES AFTER IMMOBILISATION

Malate and Lactate Dehydrogenase Immobilisations

Immobilisation Discription	0.1M NADH	Light RLU 1.0 mM Malate or Lactate	Dithionite Assay	ϵ_{NADH}	ΣMalate Lactate
(a) MDH					
[Low [Reductase]					
Enzymes in solution	4.2×10^4	7.0×10^3	1.5×10^8	0.00028	0.000047
2 hr incubation	1.22×10^6	6.0×10^5	6.25×10^6	0.20	0.096
(b) MDH					
Enzymes in solution	6.0×10^5	1.0×10^5	6.2×10^8	0.00097	0.00016
2 hr incubation	6.66×10^6	1.12×10^6	2.1×10^7	0.32	0.053
Above + 2 hr with poly-lysine	1.0×10^7	3.54×10^6	2.5×10^7	0.40	0.14
(c) LDH					
Enzymes in solution	1.4×10^5	6.0×10^2	1.2×10^8	0.0012	0.000005
2 hr incubation	1.3×10^6	8.5×10^5	3.9×10^6	0.33	0.22

TABLE 4.8

Response times and sensitivity ranges for CNBr immobilisations.

Analyte	Response time		Efficiency		Range Gain on immob.
	Beads	Soln. minutes	Beads	Soln.	
			Σ	Σ	
NADH	< 0.1	> 0.5	0.33	0.001	330
Malate	< 0.2	> 1.0	0.14	0.0001	1400
Lactate	< 0.2	> 1.0	0.22	0.00005	4400

The above are guide values as there is considerable variability between immobilisations for the above values. However the improvement both in sensitivity and time response is very clear.

FIGURE 4,14 Variation in assay characteristics of sepharose
immobilisation $\pm$ polylysine treatment

(●) - " " " } used throughout
(○) + " " "

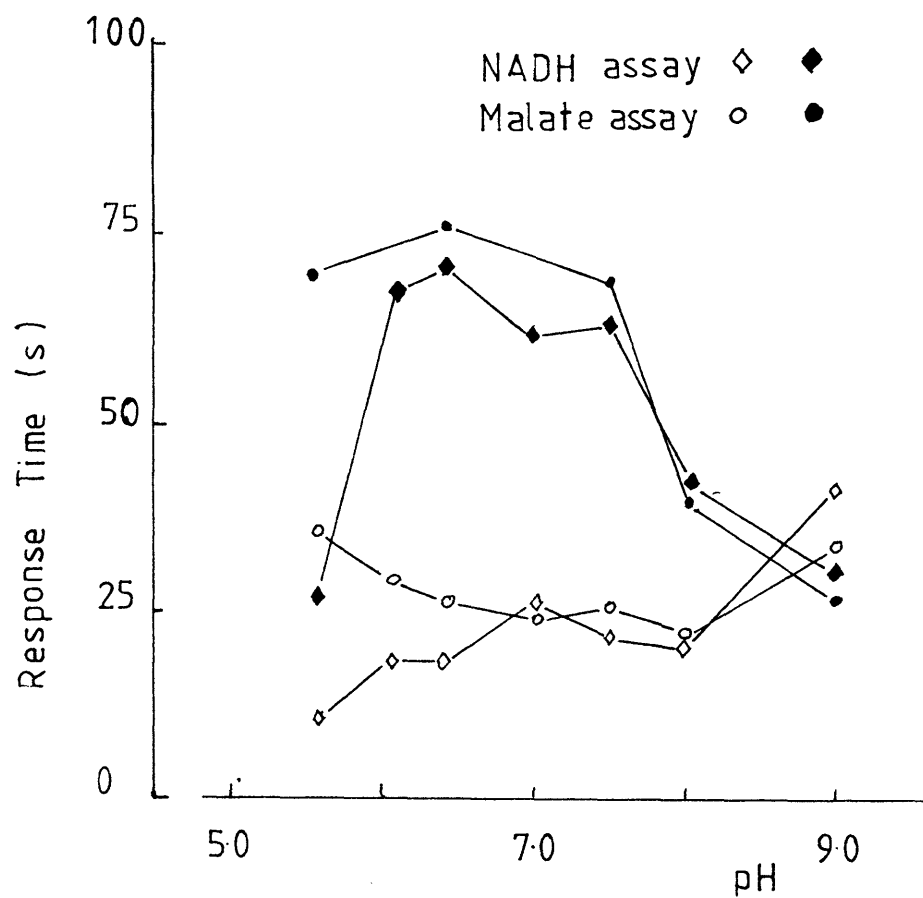
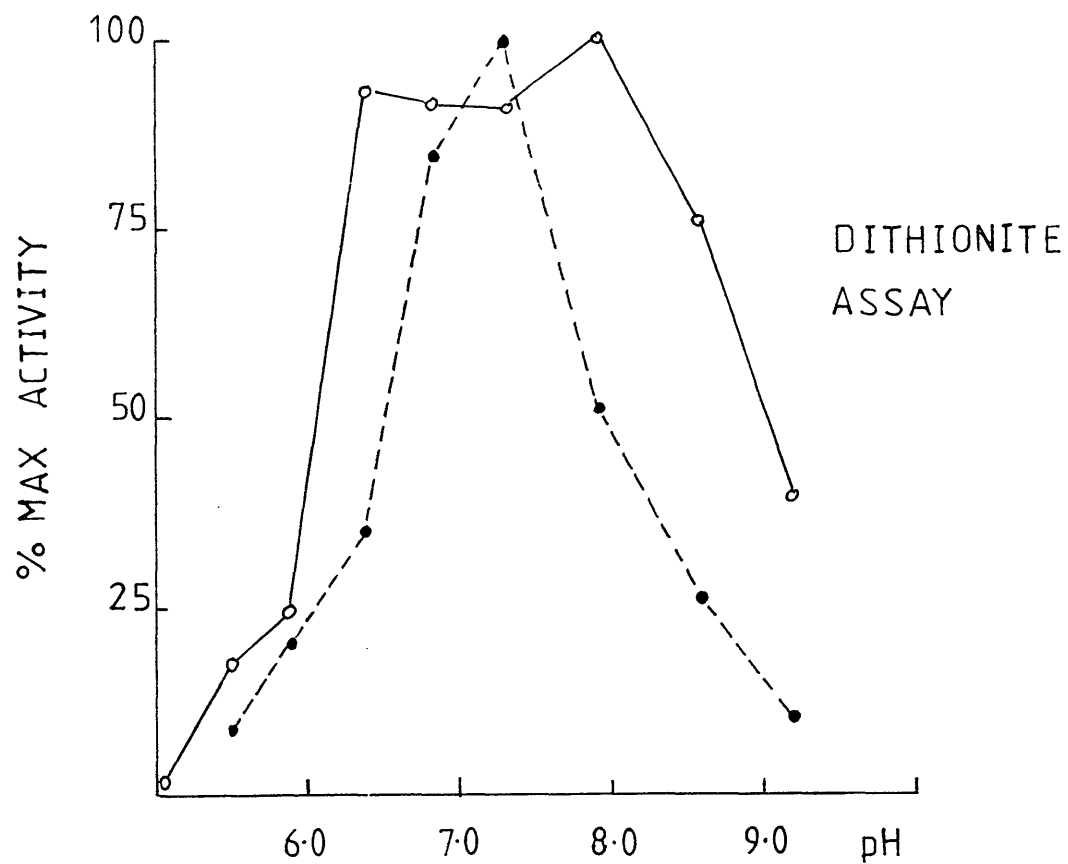
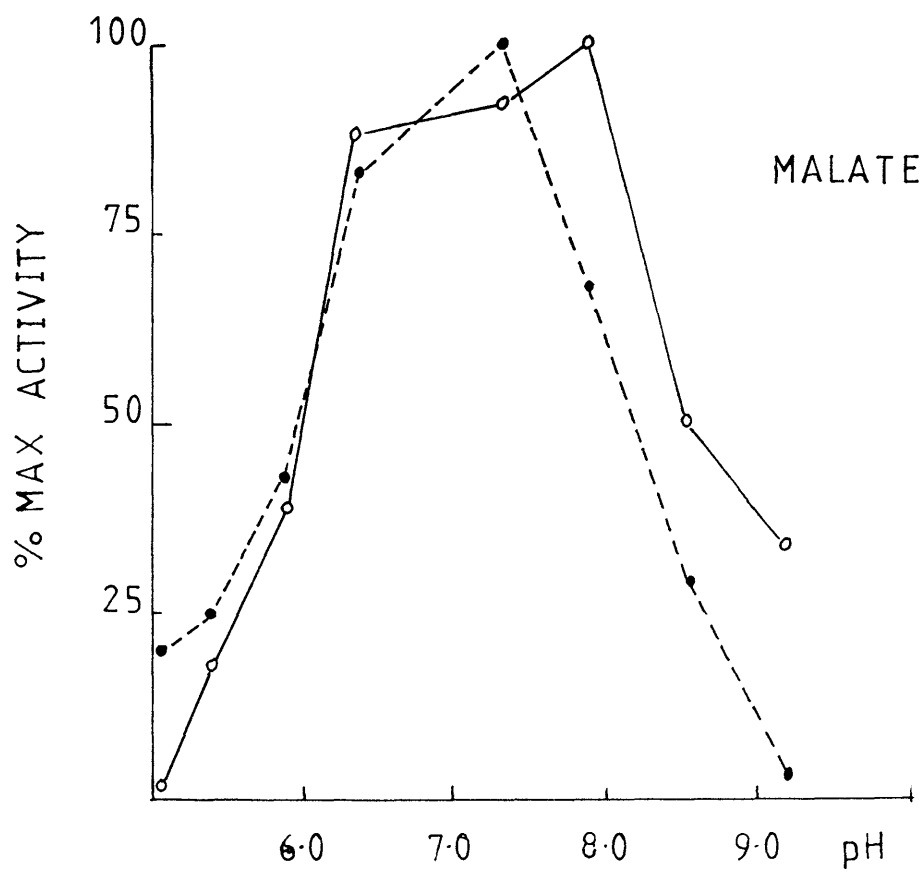
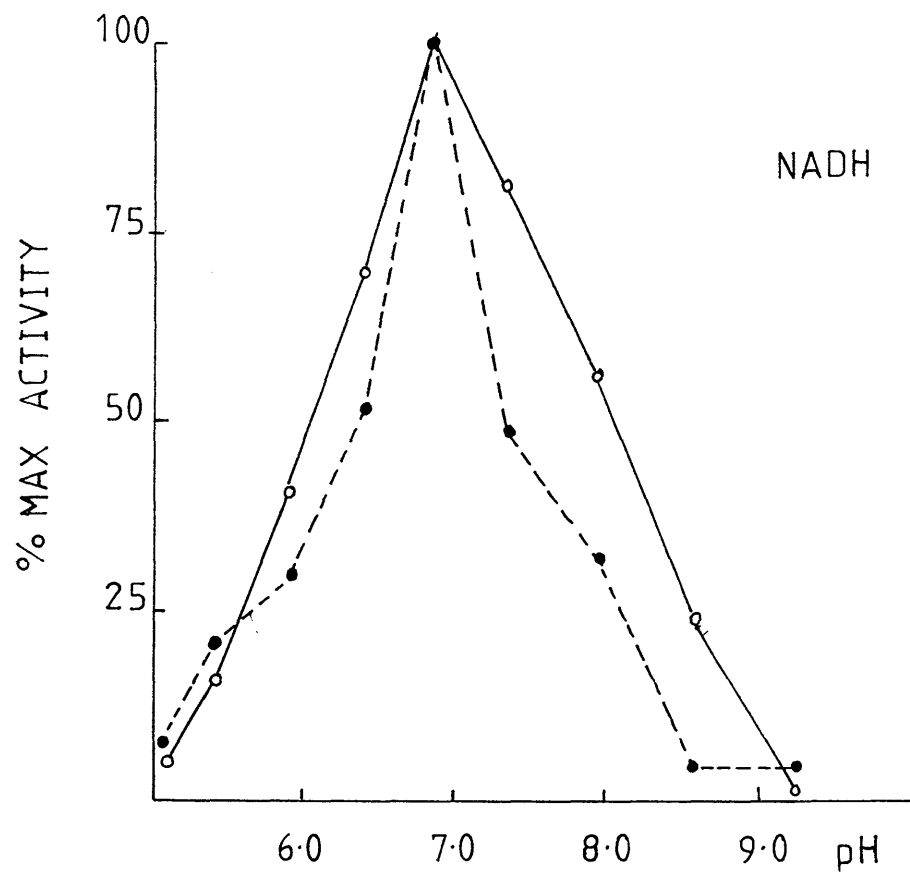


FIGURE 4.14 continued



positive charges into the matrix. While this has been achieved to a small extent, a shift ~0.5 pH units to the acid side was observed, the more marked effect was however to narrow the pH range for a light response of more than 25 percent of the response at optimum pH.

4.3.2 Co-immobilisation on Controlled Pore Glass beads (CPG).

Porous glass was one of the first materials to be extensively studied as a support for immobilised enzymes. It offers a robust physically and chemically stable support with a large surface area. The inert glass surface can be treated to facilitate a range of immobilisation chemistries.

Since chemical modification studies of luciferase (Chapter 1) indicate the existence of an essential reactive SH- group, a histidyl group and that immobilisation through NH₂- groups results in loss of ~95% of enzymic activity. An attempt to immobilise the enzymes through their carboxyl groups to aminopropyl CPG was tried.

Luciferase and reductase enzymes were coupled to aminopropyl CPG beads using a water soluble carbodiimide. Results varied with reaction conditions. The method of Weetall and Mason (1973) was adapted since luciferase precipitates below pH 5.5 and losses in activity occur progressively below pH 6.5, the immobilisation was carried out at pH 7.0 instead of pH 4.0. Table 4.9 shows the effect of immobilisation by this method. It is apparent that the presence of excess FMN during the immobilisation does not protect the enzyme against inactivation. If anything FMN has decreased the activity of the final product. There is some evidence that a proportion of the FMN has itself become immobilised and this may be quenching the bioluminescence emission. The washed FMN treated beads give a higher background level of light emission in the absence of FMN, the FMN could become adsorbed to the glass, further circumstantial evidence of adsorption is the discolouration of the beads after modification.

4.3.2.1 Effects of immobilisation on assay efficiency

Deducing how efficient the immobilisation has been is difficult because of the effects on activity of the coupling agent. In the first immobilisation with 0.1 M carbodiimide there was a significant residual activity in the immobilisation supernatant corresponding to about 14 percent of the original activity. In the light of taurine labeling experiments discussed in section 4.4

TABLE 4.9

Immobilisation on controlled pore glass

Sample	Dithionite assay RLU	Coupled assay 0.1mM NADH RLU	Efficiency No. ξ
Immobilised + FMN	19	9.3	0.49
	23	12.5	0.54
Immobilised -FMN	62	17	0.37
	39	14.5	0.27
Enzyme mix	2000	2.2	0.0011

this could represent almost all the original enzyme. The low value of ξ is also consistent with a low active enzyme loading on the beads.

4.3.2.2 Effects of immobilisation conditions on activity recovery

The coupling of luciferase and reductase to aminopropylCPG with different concentrations of carbodiimide revealed that the degree of immobilisation and efficiency of the coupled assay is dependent upon the concentration of carbodiimide used in the immobilisation. The results are shown in table 4.10, it can be seen that while the level of activity in the coupled assay increases with carbodiimide the activity recorded in the dithionite assay and in the supernatant is reduced on addition of carbodiimide. This is consistent with the inactivation of luciferase by carbodiimide described in the following section 4.4.

The results can be explained by three coincident trends, firstly as the level of carbodiimide is increased the amount of luciferase active in the immobilisation solution is reduced, secondly the amount of enzyme immobilised increases, finally the results are consistent with an increasing ratio of active reductase to active luciferase and a consequent increase in ξ .

It should be borne in mind that the precise interpretation of these results was complicated by a high level of error in duplicate assays in solution from the immobilisation mixture principally as a result of difficulties in pipetting small amounts of the beads reproducibly. The low activity of reductase used in the immobilisation also means that the assessment of effects on reductase activity cannot be independently checked using the spectrophotometric assay for reductase, the luciferase based method developed and described in section 2.3.3. is compromised by the sample contents of carbodiimide and luciferase.

TABLE 4.10

Immobilisation of reductase and luciferase on aminopropyl CPG under different conditions.

[Carbodiimide] /Moles	Immobilisation activity		Coupling efficiencies	
	Dithionite /mV/100ul	Coupled /mV/100ul	Beads x 10 ⁻³	Supernat. x 10 ⁻³
0.000	200	0.1	0.55	3.65
0.0125	18.8	0.05	3.3	0.25
0.05	40	0.05	2.1	0.75
0.10	220	2.25	14.8	0.71

NB the assays on the immobilised beads were carried out after extensive washing and storing in buffer at 0°C for 11 days. The assays on immobilisation supernatant were done following the immobilisation.

4.4 CHEMICAL MODIFICATION OF LUCIFERASE CARBOXYL GROUPS

Previously the chemical coupling of luciferase to a support has only been possible with a typical loss of at least 90% of the luciferase activity. While the remaining 10% activity still enables assays of great sensitivity to be carried out with suitable detection equipment, the poor recovery of luciferase activity frustrates the design of a cheap solid state assay system. The presence of 90% inactive enzyme on the support limits the total enzyme loading achievable, and hence reduces coupling efficiency and sensitivity. It also represents the loss of 90% of the most expensive raw material in the assay. To improve on this low level of activity recovery it is useful to consider the character of the enzyme, then devise a suitable immobilisation technique to which the enzyme will be stable. The presence of essential sulphhydryl, histidyl and amino groups discussed in the introduction, make most of the simple immobilisation coupling techniques unlikely to prove efficient in providing an active immobilised enzyme. There are two alternative strategies:

(1) Reversible blocking of the essential groups, immobilisation followed by unblocking the groups. This is technically more involved and likely to prove expensive at later stages as development progresses to a manufacturing process.

(2) Immobilisation through some non-essential residue.

The literature on luciferase appears to have neglected the role of the numerous carboxyl groups on the protein. It was seen that there might be a simple and convenient way of immobilising the enzyme. These residues seem to offer a potential solution using the tried and tested peptide coupling methods of immobilisation either using a water soluble carbodiimide or Woodward's reagent K.

As there has been no previously published work on the role of carboxyl groups in luciferase activity it was decided to study their reactivity and importance to activity as part of the investigation for a suitable immobilisation protocol.

Specific modification of carboxyl groups was carried out using the water soluble carbodiimide 1-cyclohexyl-3-(2-morpholinomethyl) carbodiimide metho-p-toluene sulphonate to couple the protein to nucleophilic amino residues through a peptide bond with the carboxyl groups. The effects of coupling were observed first with small amino group containing molecules and then with attachment to immobilisation matrices.

4.4.1 Choice of nucleophilic molecules

Three small nucleophilic molecules were tried as possible candidates for modification of the surface carboxyl groups of luciferase. The three were briefly investigated as suitable reagents the results are discussed below.

Amino methane sulphonic acid (AMS)

Incubation using 0.1 M carbodiimide and a 0.25 M concentration of AMS at 0°C resulted in 90% loss of luciferase activity in two hours.

While the inactivation profile for this reaction was promising for further study, the quantity of AMS incorporated into the protein was difficult to measure.

Amino acid analysis failed to detect the AMS residue either as a control or following hydrolysis. Independent ninhydrin staining of the control samples after paper chromatography indicated a very low specific colour development for AMS.

Amino ethane sulphonic acid (Taurine)

Initially inactivation profiles closely resembling the AMS profiles were obtained with this slightly larger nucleophile. Taurine was found unlike AMS to be detectable using an amino acid analyser. The taurine peak runs ahead of the first natural amino acid in a standard mixture, (Figure 4.15 shows the taurine peak in an analysis of some taurine labeled luciferase), the colour development was found to be 0.33 times that for norleucine the amino acid analysis internal standard. In addition a C-14 enriched isotope of taurine is commercially available and provides a second convenient method of assaying the taurine incorporated into the modified protein.

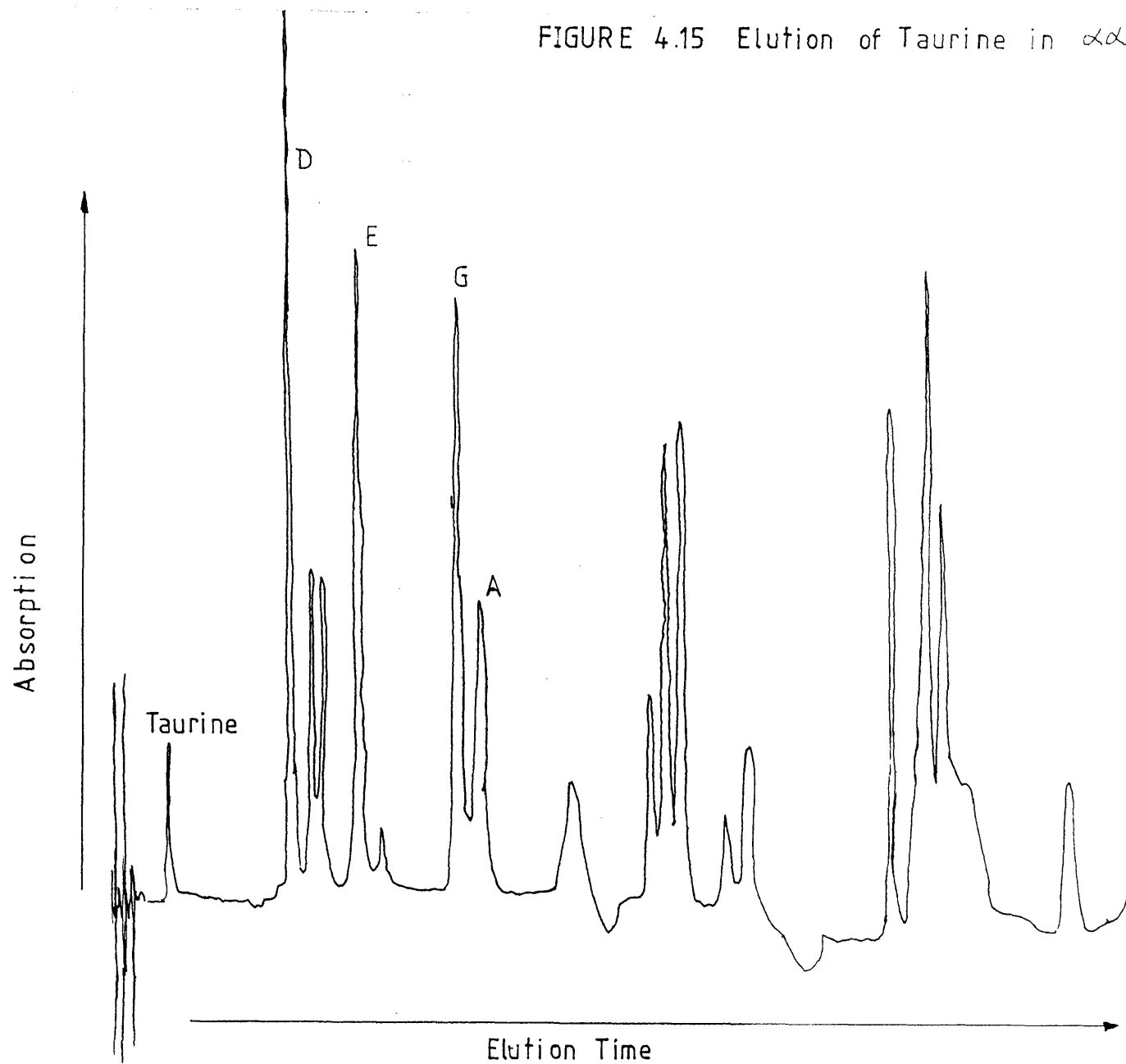
Methyl glycine ester

Inactivation was much less marked with this nucleophile with only about 30% loss of activity in 2 hours, the slow loss of activity and the experimental error in assays made resolution of distinct inactivation phases difficult.

Taurine was chosen as the most suitable nucleophile for three reasons:

- (1) The nucleophile was easiest to accurately measure.
- (2) Coupling to the carboxyl groups on the protein does

FIGURE 4.15 Elution of Taurine in $\alpha\alpha$ analysis



not alter the overall charge density on the enzyme, changing an existing carboxylic acid residue for a sulphonic acid residue. Lin and Koshland (1969) reported that carbodiimide modification of lysozyme with AMS and methyl glycine ester resulted in the labelling of two additional residues with AMS not detected with the ester. They suggest that the protein becomes more positively charged in the course of modification with methyl glycine ester influencing the reactivity and accessibility of the other two carboxyl residues.

(3) A C-14 labelled taurine is readily available commercially and provides another quick and simple assay for measuring incorporation of the nucleophile.

4.4.2 Chemical modification of luciferase with taurine.

Reagent concentrations, temperature and pH all had significant effects on the modification of luciferase by the peptide coupling of taurine. The modification was followed by loss of activity in the dithionite assay for luciferase and showed several phases of inactivation to be occurring. These phases were sensitive to the reaction conditions. The effect of temperature is to alter the rate of inactivation, although the final degree of inactivation is little changed for high concentrations of carbodiimide (figure 4.16a and 4.16b). With conditions leading to a high degree of inactivation ie. >90% requiring carbodiimide > 50mM, there are two inactivation phases; these are discussed in detail later. The profiles for milder modification conditions indicate that there is an activation phase prior to the two inactivation phases. However this effect is rapidly swamped by the later two phases. The effect was also technically difficult to study because of its transient nature and the problems of working at low concentrations of carbodiimide; at low concentrations the carbodiimide concentration is likely to be rapidly reduced by the competing reaction with water molecules. Loss of activity in the control with no carbodiimide shows no inactivation phase and all loss of activity is attributable to thermal denaturation of luciferase. The principle two phases of inactivation were observed to lead to > 90% inactivation, and with [carbodiimide] > 50 mM to the formation of a precipitate on leaving at 0°C overnight.

Taurine appears to have very little effect on the rate of inactivation at high carbodiimide concentration although the initial inactivation is slowed over about 5 minutes, an effect consistent with the transient activation by taurine coupling already mentioned (figure 4.17) Carbodiimide alone was found to inactivate the enzyme (figure 4.18) at high concentrations however the inactivation is slower in both phases.

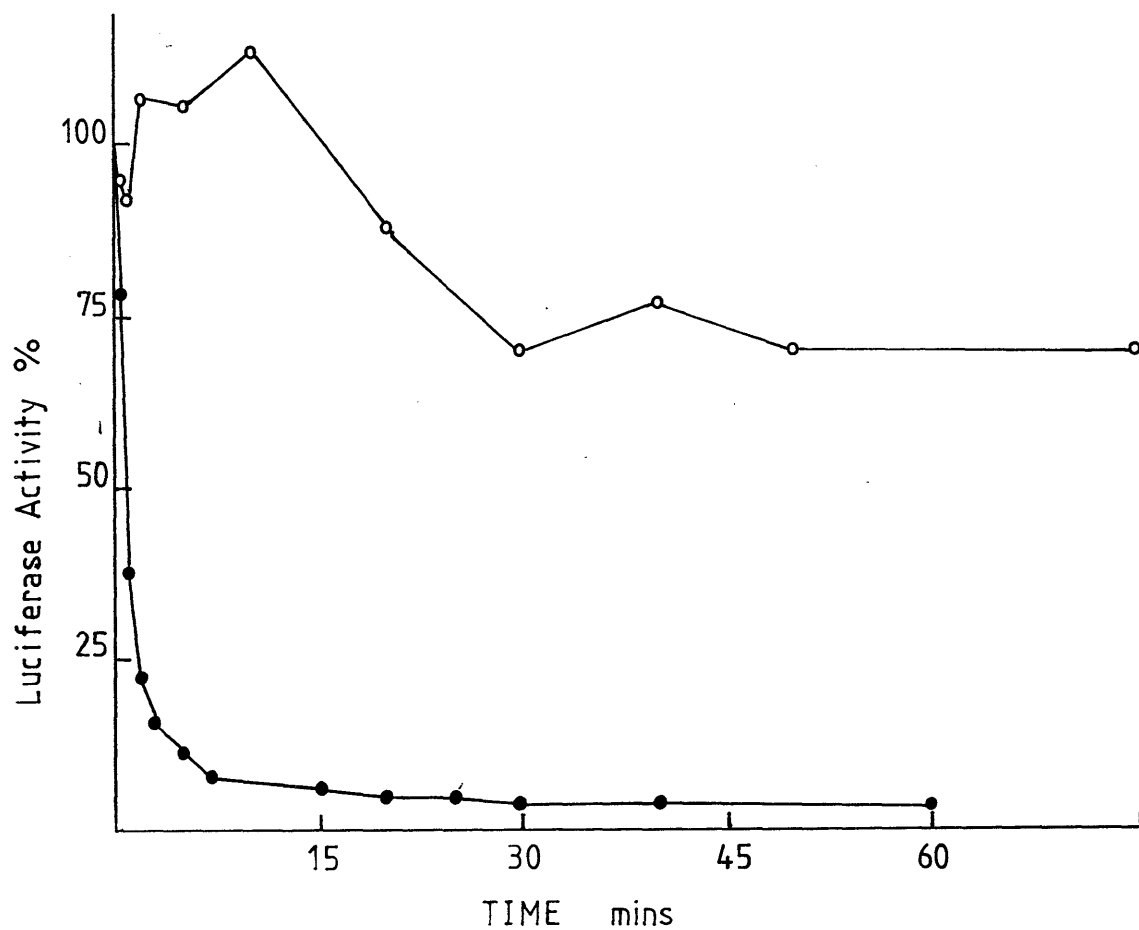
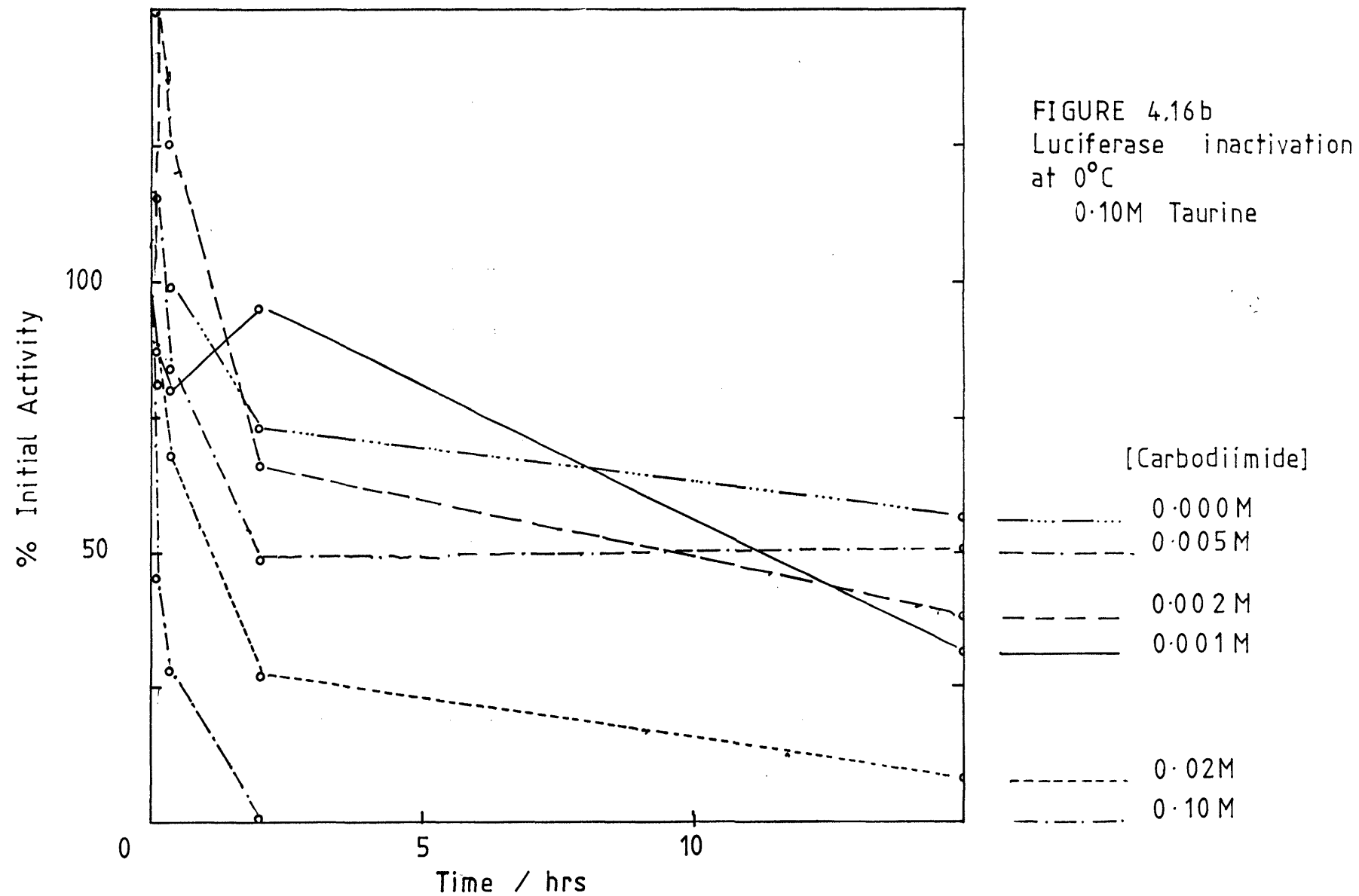


FIGURE 4.16a

Inactivation of luciferase
at 21°C

0.25 M Taurine
○ 0.001 M Carbodiimide
● 0.10 M Carbodiimide



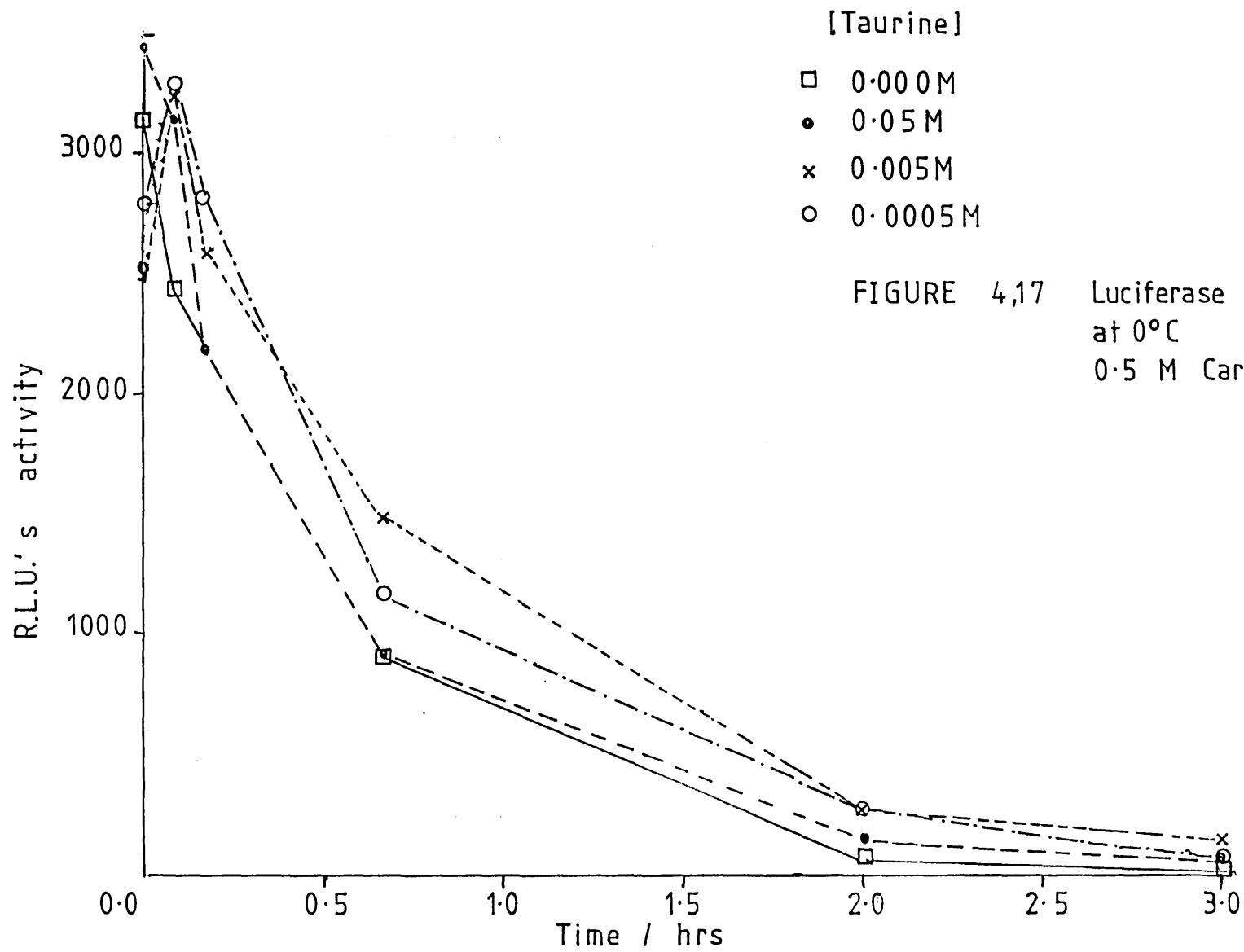


FIGURE 4.18 Substrate protection during chemical modification

0.1M Carbodiimide

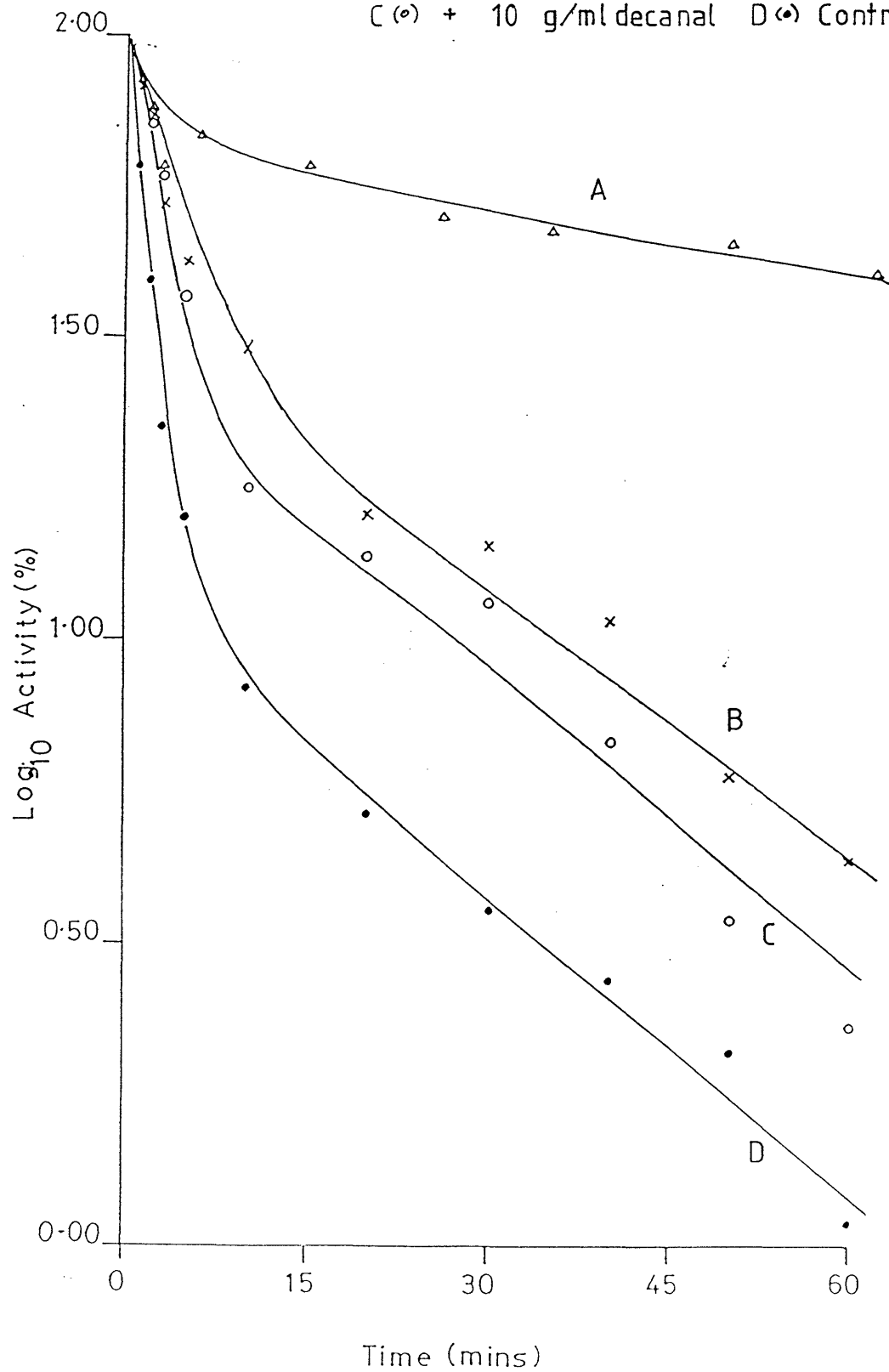
25mM Taurine

A(Δ) No Taurine

B(x) + 10 mM FMN

C(○) + 10 g/ml decanal

D(●) Control



Substrate protection of luciferase by either FMN or decanal has frequently proved effective in other chemical modification work where active site modifications are responsible for inactivation. FMN and decanal had only a limited effect on the rate of inactivation (figure 4.18, table 4.11), Manuel et al (1986). All four experiments showed a biphasic character to the inactivation.

- * The control with no taurine shows significantly slower inactivation in both phases.

- * The extent of the initial inactivation phase is much reduced.

- * Free thiol group (-SH) titration with DTNB after the inactivation without substrate protection showed about 90% of the free thiol groups had been lost, while with FMN only 70% had been modified. Assay of the modified protein was complicated by the formation of a precipitate during overnight dialysis to remove the excess reagents. The resultant low protein concentration in the solution made attribution of -SH groups involvement in the inactivation of the original material difficult, further the effective partitioning of the heterogeneous modified protein mixture into precipitate and supernatant fractions complicates interpretation of the data.

- * The pH dependence of the reaction was examined with 30 mM carbodiimide over the range 6.6 -7.3, the inactivation profiles shown in figure 4.19 show a progressive slowing of inactivation rate with increasingly alkali pH. The pH 7.3 experiment showed only one phase of inactivation during the time the reaction was followed. Table 4.11 shows the rates of inactivation corresponding to the figures.

- * Substrate protection appears to act upon the first and not the second phase of inactivation.

- * The effect of pH and carbodiimide concentration on the inactivation profile were found to be particularly marked. The threefold increase in carbodiimide is responsible for a 20 and 5 fold increase in the two reaction rates. Small increases in the pH of 0.4 units almost double the inactivation rate. A change in pH of 0.4 corresponds to a change in $[H^+]$ of 2.5 fold indicating that the rate of inactivation is proportional to $[H^+]$. These two observations are consistent with the findings of other workers using carbodiimide as a coupling agent.

It is clear from this data that at least two residues are involved in the loss of enzyme activity; one for each of the two inactivation phases.

FIGURE 4.19 Effect of pH on inactivation profile

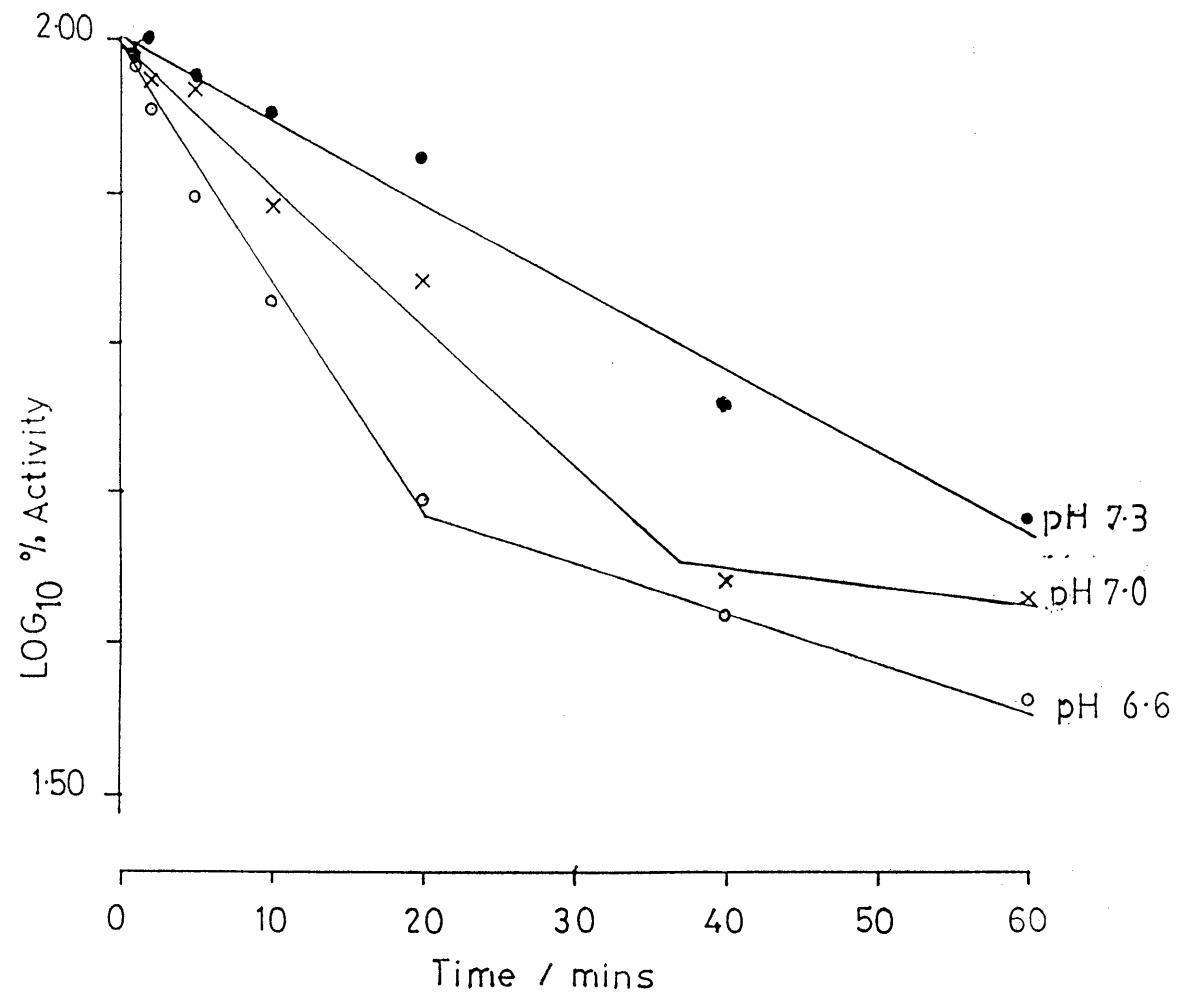


TABLE 4.11

Time constants for the inactivation profiles described in figures 4.18 and 4.19 for various substrate protection strategies and pH variations.

Experiment Profile Fig.	Activity decay constants			
	First Phase		Second Phase	
	Time const./s	Correlation	Time const./s	Correlation
Curve A Fig. 4.18	4.695 +/- 0.150	0.9989	57.04 +/- 2.10	0.9971
Curve B Fig. 4.18	12.58 +/- 1.203	0.9835	65.79 +/- 7.53	0.9758
Curve C Fig. 4.18	13.16 +/- 0.265	0.9996	53.76 +/- 4.81	0.9812
Curve D Fig. 4.18	15.55 +/- 1.223	0.9963	64.26 +/- 40	0.6019
pH 6.6 Fig. 4.19	66.67 +/- 2.567	0.9968	235.3 +/- 50.1	0.9650
pH 7.0 Fig. 4.19	110.1 +/- 4.815	0.9947	549.5 +/- 194.9	0.8753
pH 7.3 Fig. 4.19	There appears to be only one observable phase			
	191.8 +/- 8.817	0.9943		

4.4.2.1 Reversible blocking of the Active Site thiol and its affect on inactivation.

The -SH group titration experiments do not rule out the loss of the active site cysteinyl group which would explain one of the inactivation phases, the second is consistent with the extent of inactivation associated with the modification of the active site thiol. Carbodiimide is specific for carboxyl and amino groups, however the reagent does have some reactivity towards -SH groups modifying them irreversibly without the incorporation of a nucleophile. The assays with DTNB show that modification of the free -SH groups has taken place.

Modification of the active site sulphydryl is known to cause loss of > 95% of the catalytic activity, since the active site SH group is one of three reactive -SH groups on the protein surface and of 15 groups, (Cohen *et al* 1985) all together this loss in activity could be accounted for by loss of a thiol group during the second phase.

Reversible blocking of the essential -SH group has been used to protect the enzyme from irreversible thiol modifying reagents (Meighen and Cousienseau 1976). Following the method of Meighen and Cousienseau to block the active site thiol with sodium tetrathionite (TET) only 10% of the original activity was recovered after either extensive dialysis against reactivation buffer or by dilution into it. The method was altered, DTNB being used as the reversible blocking reagent. Inactivation was very rapid and subsequent reactivation recovered about 50% of the original activity.

DTNB-luciferase was used as a control in the modification of luciferase by taurine with carbodiimide. The inactivation profiles in figure 4.20 show the close similarity of the two reactions when corrected for DTNB protection. The experiment shows that although -SH groups are modified by the coupling reaction this is not the primary cause of inactivation in the reaction.

4.4.2.2 Incorporation of C-14 Taurine into luciferase

14-C labelled taurine was used to trace the molar inclusion of taurine into luciferase (Figure 4.21). There appears to be a low initial rate of taurine incorporation, however when 70% inactivation has taken place there is a large increase in the level of taurine incorporated into the protein. The final level of incorporation at high and low taurine concentration, after exhaustive dialysis overnight to remove salt and excess reagents, was markedly different; 140 moles/mole of luciferase with 0.1 M taurine and 33 moles/mole with

FIGURE 4, 20
Thiol group protection

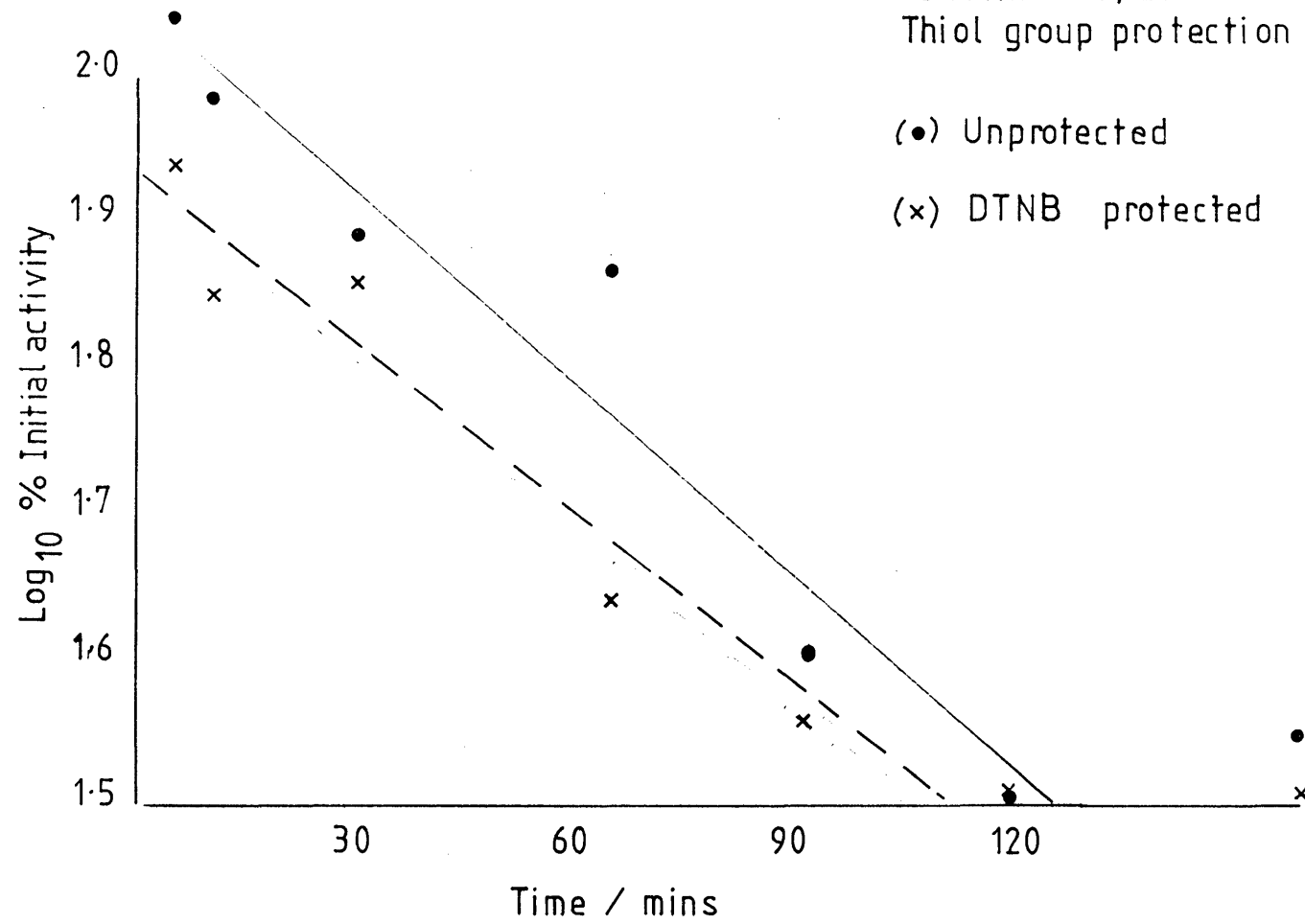
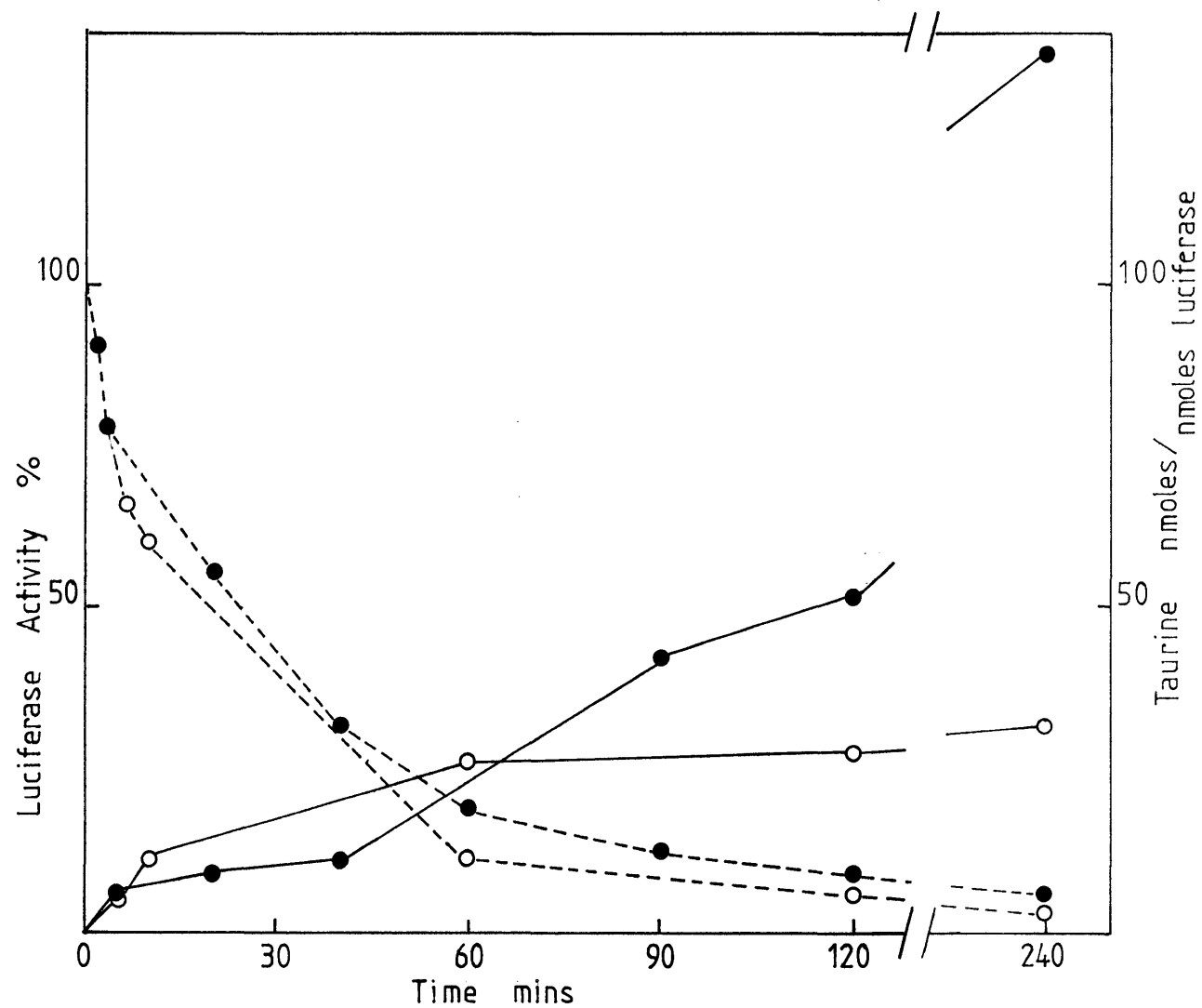


FIGURE 4.21 Taurine incorporation
 ○ 10mM Taurine ● 0.1M Taurine
 --- Activity — Incorporation



10 mM taurine. Amino acid analysis of a taurine modification with 0.1M taurine, after exhaustive dialysis overnight to remove excess reagents confirmed the high level of modification, with 110 moles of taurine per mole of luciferase. This compares with 128 carboxyl groups in the enzyme - from the amino acid sequence there are 63 glutamic and 63 aspartic acid residues per molecule and a residue at the C terminus of each subunit. The observation that residual activity is < 5 % is consistent with this extensive modification and a consequent denaturation of the protein.

4.4.2.3 Effect of inactivation on kcat.

The dithionite assay provides a direct method for the determination of the enzyme's kcat. The effect of taurine coupling to luciferase upon the kcat was small and varied within the narrow range 0.17 - 0.12 s⁻¹. Although the changes (figure 4.22) show a slight increase in kcat followed by a gradual lowering of the kcat to 0.12 s⁻¹ the variations are not statistically significant compared to the normal figure of 0.15 s⁻¹ found for native luciferase. The small changes observed in the kcat also mean that the changes in peak light obtained in the dithionite assays reflect a change in the number of active molecules in the luciferase population rather than a change in catalytic turnover time. This is similar to the changes seen with falling peak light and increasing decay times in the pH dependence of the dithionite assay.

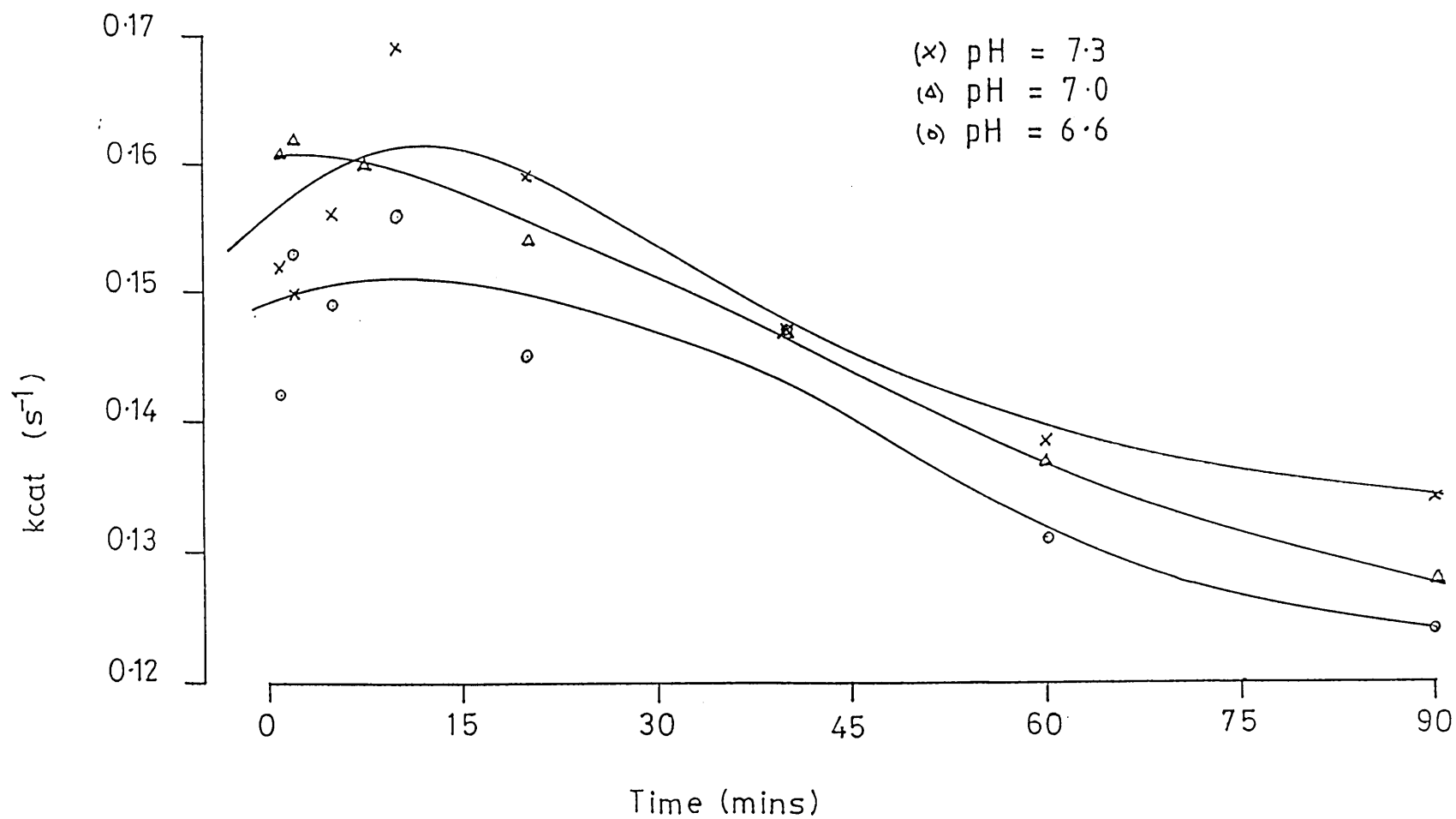
4.5 PACKAGING DECANAL FOR USE IN A BIOLUMINESCENCE KIT OR BIOSENSOR

There are two serious problems to be overcome in creating a biosensor with prepackaged decanal.

(1) The enzyme luciferase is very sensitive to the concentration of decanal, figure 4.6. This effect is also illustrated by the effect of adding decanal to luciferase before FMN in the standard dithionite assay, described in section 2.3.1. Luciferase has two binding sites for decanal and the dead-end complex of luciferase with two aldehyde molecules bound is easily formed in the absence of FMNH. Occupation of the second site prevents the binding of FMNH₂ and catalytic activity. A target concentration of decanal in the centre of the activity plateau, figure 4.6, was chosen to minimise the degree of error from this source.

(2) The actual quantity of decanal required for a single assay is small, approximately 1 nl, equivalent to approximately 6 nmoles.

FIGURE 4.22 Variation of k_{cat} with inactivation



(3) Decanal is unstable in an aqueous environment, it undergoes oxidation to decanoic acid in the presence of H_2O (Hastings et al 1963). Luciferase is inhibited by aldehydes, ketones, alcohols and long chain acids. This restricts the choice of solvents for the stable application of such a small quantity of aldehyde in a solvent cocktail.

4.5.1. Disruption of the polystyrene surface

From work on the use of cellulose acetate membranes to entrap the enzyme on the cuvette wall (Section 4.1.4) the physical disruption of the cuvette by associated solvents had been observed. This disruption was investigated as a possible aid to depositing the decanal on the cuvette wall.

It was found that the degree of disruption could be controlled by altering either solvent or drying conditions and time. The two solvents used were acetone and cyclohexanone.

Acetone was found to be far more effective in disrupting and solubilising the polystyrene. In addition its greater volatility means little residual solvent and no further disruption of the cuvette was observed on storage. A rapid frosting of the cuvette surface is typical of an acetone treatment; if evaporation is prevented or excess acetone applied the plastic will completely dissolve turning an opaque white.

Cyclohexanone with a boiling point of $202^{\circ}C$ and is both less reactive and less volatile. The cyclohexanone continues to etch into the polystyrene for some time while it evaporates. If it is enclosed the process continues slowly, the cuvette is however not completely dissolved as would be the case with acetone.

It was found that cyclohexanone is the easier of the two to use as a medium to deposit decanal reproducibly on a polystyrene surface.

4.5.2 Effect of solvents on the bioluminescence coupled assay for luciferase.

The inhibitory effects of the four solvents propan-2-ol, ethanol, cyclohexanone and acetone on luciferase are illustrated in figure 4.23. The degree of inhibition resulting from a 1% (v/v) solution of each solvent shows the following order of inhibitory effect:

cyclohexanone >> ethanol > acetone > propan-2-ol

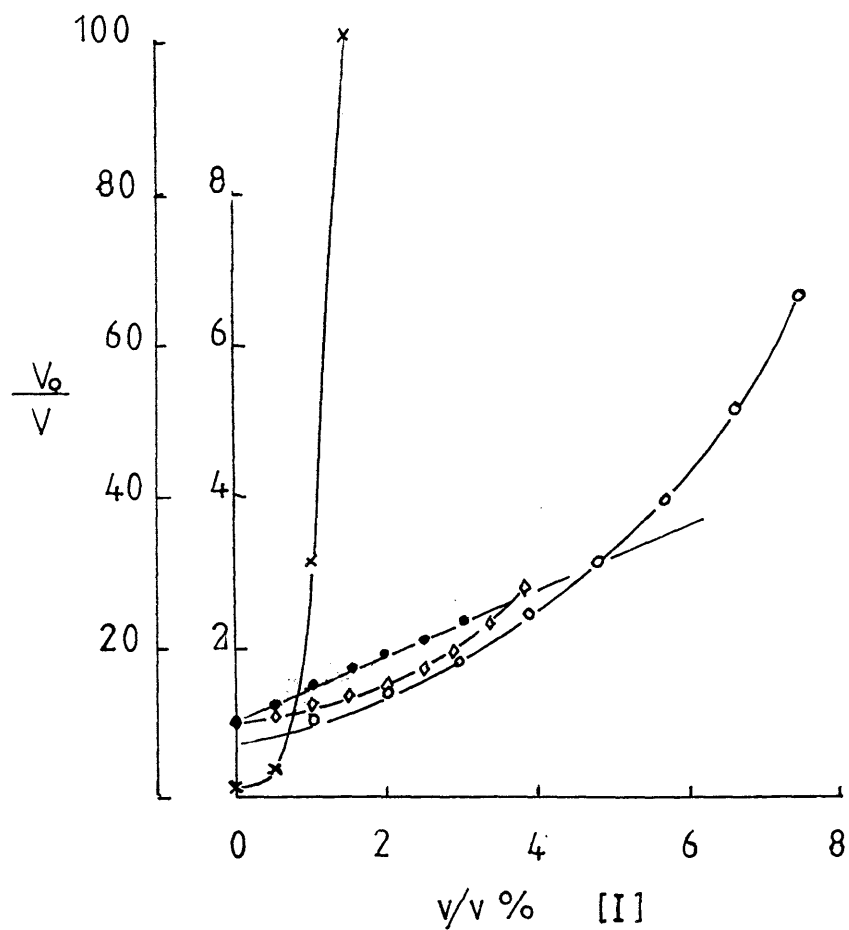


FIGURE 4.23
 Dixon plots of solvent inhibition
 × Cyclohexanone 0 – 100 scale
 ◇ Acetone
 ○ Propan-2-ol
 ● Ethanol

Dixon plots also show that the type of inhibition for the solvents is different. Ethanol exhibits a straight line relationship consistent with simple competitive inhibition, while propan-2-ol, acetone and cyclohexanone show a more complicated relationship. The parabolic curve seen on the plot is consistent with there being multiple inhibitor sites, in particular parabolic competitive inhibition may be related to two binding sites the second binding site becomes accessible once the first is occupied (Roberts 1975). This would be consistent with the two aldehyde sites reported by Holzman and Baldwin (1983), which have a sequential binding relationship.

4.5.3 Deposition of Decanal in solvent

Initial experiments showed great variability in the activity obtained from duplicate cuvettes. This was due to the technical difficulties of reproducibly treating the cuvettes. Obtaining reproducible smears was particularly difficult with the decanal doped acetone; getting a reproducible degree of disruption to the cuvette was more difficult with cyclohexanone. These problems were resolved by depositing the solvent/decanal mixture above the first centimeter of the cuvette wall so that the "frosting" of the polystyrene does not effect the collection of light from the assay. The cyclohexanone mixture could be conveniently deposited as a single drop on the side about 2.0 cm from the base of the cuvette.

A series of assays carried out over two weeks on identically prepared cuvettes produced the following conclusions.

(1) Cyclohexanone produced the most stable assay kinetics, even more stable than control assays.

(2) Compared to the control set of cuvettes the cyclohexanone cuvettes progressively lost activity with time, although for the first five days the level of activity is constant at about 28%.

(3) All the acetone cuvettes showed very low light emission levels not significantly above the background level.

These experiments allowed the same drying time of 5 minutes for all cuvettes. Evaporation of both solvent and substrate could be causing the low levels of light emission since:

* Addition of a control 10 μ l aliquot of 0.1 mg/ml decanal in propan-2-ol produced light at a similar level

to that of the control cuvette.

* If inhibition had been responsible for the reduced activity, inhibition would still be expected after the additional decanal had been injected.

* A 1% v/v concentration of acetone in aqueous solution is expected to produce only 25% inhibition not 99% + inhibition as observed.

Assuming that evaporation of the decanal was causing the problem a mixed solvent deposition system was tried using 1% or 10% ethanol in the mixtures. The results of these experiments, table 4.12, are consistent with the evaporation model.

(1) As successively higher quantities of decanal are included in the deposition aliquot the light emission for the same assay increases. This is despite the fact that the actual quantities of decanal are in vast excess to those which would be inhibitory in the solution assay. Normally 0.001 ul of decanal is added, this optimal amount produces 1000 RLUs well above those observed in the assays observed with the treated cuvettes.

(2) The acetone prepared cuvettes produced significant levels of light emission. The amount of ethanol also appears to have an effect on the level of light with 0.01 ul decanal deposited. The lowering of [ethanol] might be expected to increase light levels by reducing inhibition, instead there is a drop possibly due to increased evaporation of decanal.

Duplicates of the cyclohexanone treated cuvettes gave near identical results when assayed after three days storage at 22°C.

The preparation of cyclohexanone treated cuvettes with much higher loadings of decanal confirmed that the light levels in the previous cuvettes were well below optimum. Experimentation with these cuvettes also showed that the assay mixture did not have to come in contact with the treated area of the cuvette to demonstrate bioluminescence. Either sufficient vapour phase decanal is present to stimulate light production or the decanal vapour is adsorbed onto the whole surface of the cuvette during storage. To differentiate between which of these explanations may be true it is worth noting that:

(1) Cyclohexanone is immiscible with water and the area of the cuvette treated with cyclohexanone / decanal preparation is observed to behave hydrophobically. This may result in a slow release of decanal from the treated area when the assay solution is washed over the cuvette surface, accounting for the large quantity of decanal

TABLE 4.12

Effect of different solvents on the side of cuvettes in immobilising decanal

Solvent	Decanal sample	light intensity RLU	After 3 days/RLU
-	0.1 mg/ml in isopropanol	1000	700
-	1 ul of sol B	475	
cyclo	1% B	7.5	17
cyclo	10% B	36	50
cyclo	1% A	50	44
cyclo	10% A	180	210
aceto	1% B	3.75	
aceto	10% B	5.0	
aceto	1% A	8.25	
aceto	10% A	42.5	
cyclo	10% decanal	1170	1050
cyclo	50% decanal	1800	1980

A is 1mg/ml decanal in iso-propanol

B is 1mg/ml decanal in ethanol

TABLE 4.12b

5 µl of solvent	containing decanal	coupled assay RLU	+ 5µl of 1.0 mg/ml decanal in isopro
isopro	1.0 mg/ml	110	-
cyclo	1.0 mg/ml	24	95
aceto	1.0 mg/ml	2.2	100
cyclo*	-	-	25
aceto*	-	-	90

required for each cuvette.

(2) Decanal has strong hydrophobic characteristics and might be expected to have some natural affinity for an untreated polystyrene surface, leading to the transfer of decanal from the treated area to the cuvette surface as a whole. Such an event would explain the observed activity of an assay even if the solution had had no direct contact with the treated area.

(3) Further to point two it was notable that any cuvettes whose seals became broken during storage lost all activity, either due to evaporation or the effect of water vapour entering the cuvette.

CHAPTER 5

PRACTICAL APPLICATIONS OF THE IMMOBILISED BIOLUMINESCENCE SYSTEM

5.1 PACKED ENZYME BEDS IN FLOW CELL ANALYSIS

A flow injection system was developed to study the behaviour of the immobilised enzyme preparations and to assess their suitability for flow injection analysis (f.i.a.). Initially, as with previous analysis systems, a pump was used to drive the fluid flow through the column. It was found however that the pulsing produced by the pump caused significant perturbations in the light intensity profile produced. Similar problems were observed by Vellom and Kricka (1986).

The pulsing effect was eliminated by using syringes driven by a stepping motor to force buffer through the system. Two arrangements of syringes were used; in the first the motor drove a manifold capable of holding up to five different volume syringes; by introducing a section of bent glass tubing to produce turbulent flow after the manifold substrates could be mixed automatically (figure 5.1a).

A simpler system was later used, based on a single syringe driven by a stepper motor forcing a continuous flow of buffer through the system. The introduction of the samples and reagents was achieved through an HPLC injection port (figure 5.1b). The syringe could be reloaded with buffer without disconnection from the system hence avoiding the introduction of air pockets which would damage the flow characteristics of the enzyme column. The second system was found to be more practical and easier to operate for long periods reproducibly. It eliminated problems of mixing and storage of reagents in the first system. Allowing the experiments to concentrate on the behaviour of the reaction catalysed by immobilised enzymes in a packed bed in a flow system. The problems of storing decanal in a non-aqueous environment and the need for dark storage of the co-factors could be studied in isolation from the enzyme packed bed.

5.1.1 Assay performance - reproducibility and ease of use.

The reproducibility of the f.i.a. system was good, variation between assays was low, being observed as always under 5%. Examples of a typical set of traces can be seen in figure 5.2. The variation is typically much better than 5%. This compares with an estimated limit of $\pm 5\%$ for the solution assay with a practiced technique. The use of the same enzyme population for each assay is undoubtedly the principle reason for this improvement.

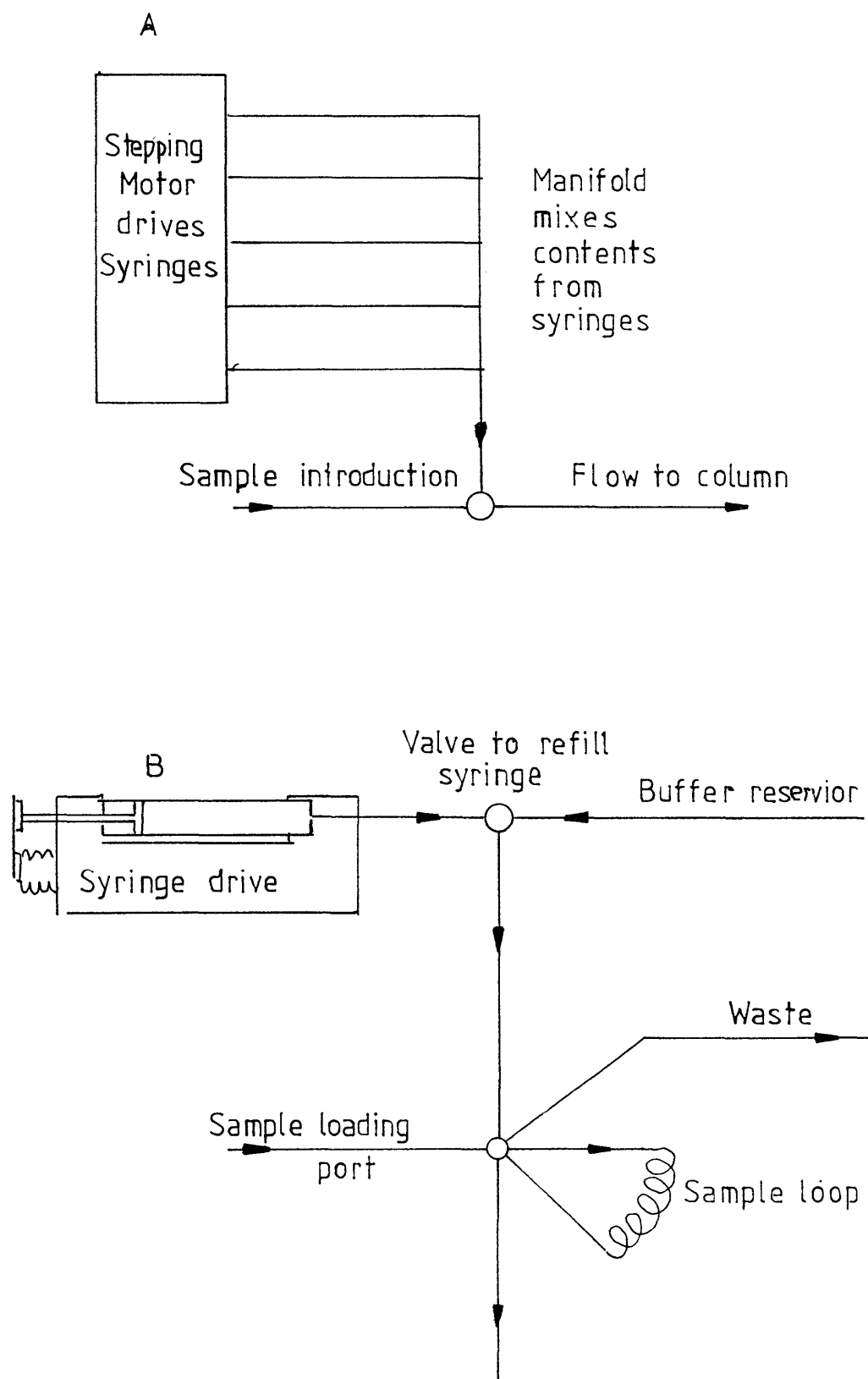


FIGURE 5.1 Flow injection analysis systems

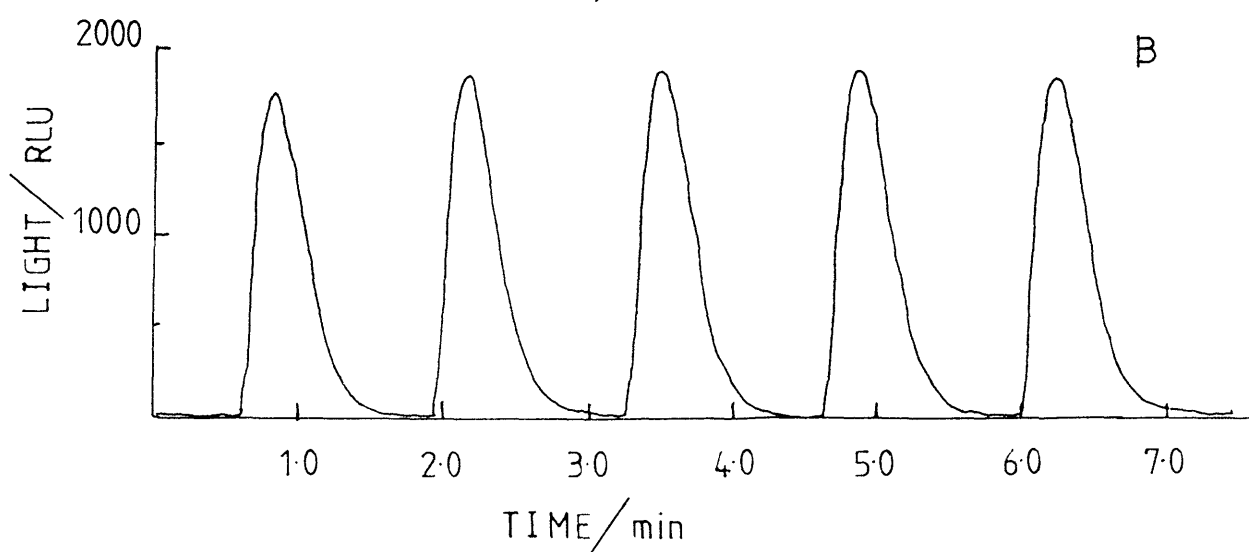
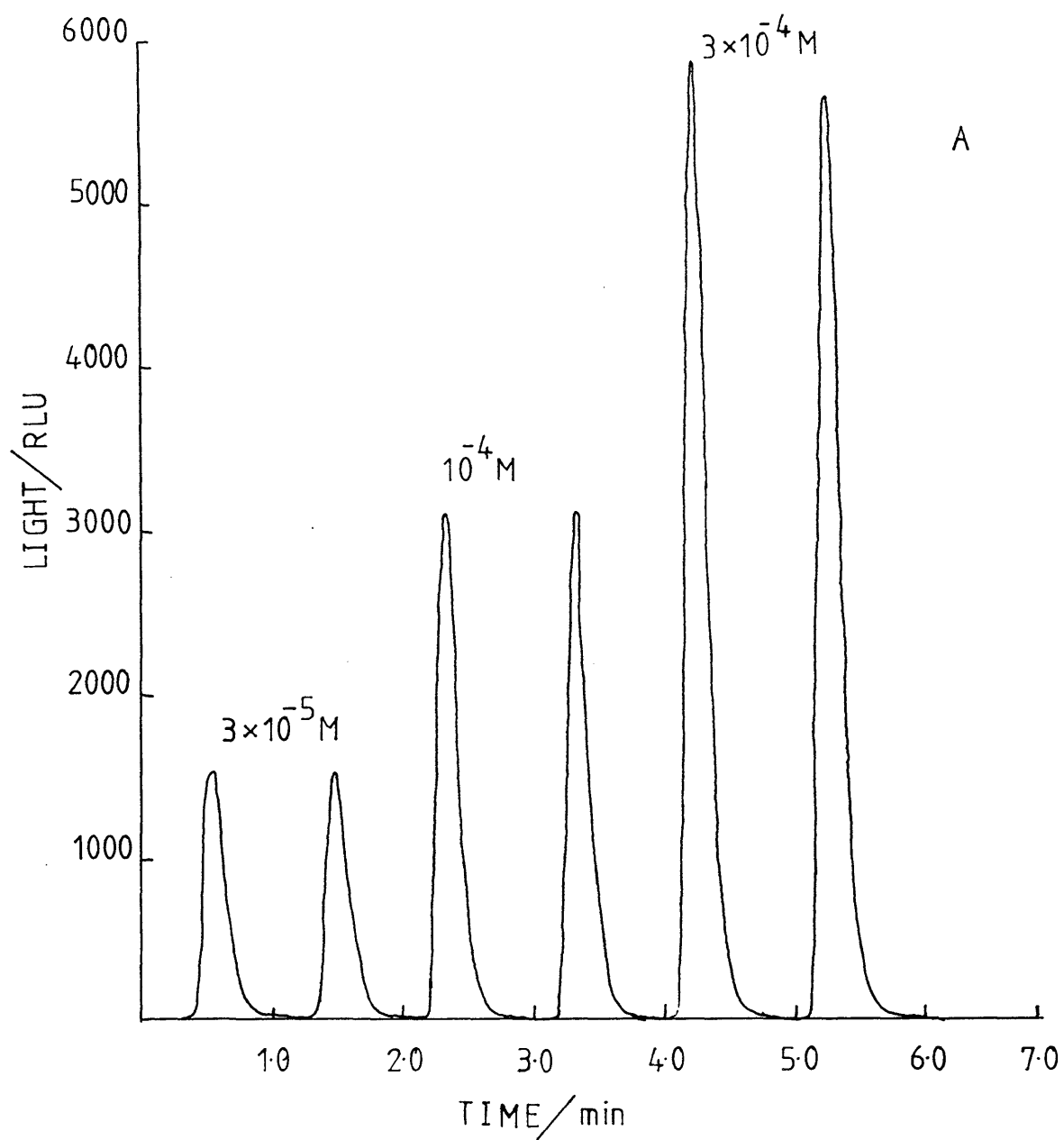


FIGURE 5.2 Sample traces of f.i.a. A sepharose 4B with malate
B CPG with $1.0 \mu\text{M}$ NADH

TABLE 5.1

Sensitivity of flow injection assays

Analyte	Enzyme bed	Analyte range /M	Plot slope
NADH	Sepharose 4B	10^{-6} - 10^{-9}	1.0
NADH	DE-52	10^{-5} - 10^{-8}	1.1*
NADH	CPG Beads	10^{-5} - 10^{-8}	1.1
Malate	Sepharose	10^{-3} - 10^{-7}	0.5
Lactate	Sepharose	10^{-3} - 10^{-7}	0.6

* Because enzyme leached off the column this value is less accurate than the others.

Analyte concentrations are the range observed by modifying the immobilisation enzyme concentrations this range can be changed.

The absolute sensitivity of the assay is considerably greater than that of the solution assay.

The sensitivity to a variety of analytes is compared in table 5.1. The NADH assay appears to be limited by the detecting instrument's sensitivity, while the assays for lactate and malate are limited by levels of background activity. This activity, present with zero analyte, is probably due to minute traces of contaminating alcohol dehydrogenase acting on the propan-2-ol or ethanol used in stock decanal solutions - this activity is discussed in detail later.

Comparison of this flow injection system with that of Vellom and Kricka (1986) shows similar specification for both sensitivity and reproducibility. The use of a non pulsing syringe drive in place of the pump reduces the noise level of the experiments. Besides the change in method of driving the buffer through the system, changes in the sample size, flow rate and column design mean that the f.i.a. described in this work is capable of a faster sample through put and potentially higher sensitivity. Vellom and Kricka's system uses a sample size of 180 μ l; this represents a substrate exposure time to the column of 120 seconds (at a typical flow rate of 90 μ l/min) equivalent to 30 column volumes.

The system described here uses 200 μ l samples at a flow rate of 800 μ l/min, an exposure time of only 15 seconds or 6 column volumes. Experiments on the behaviour of the column under different flow rates show that increased sensitivity arises from increased exposure of the sample to the column rather than reduction of the flow rate. The result is that the system described here is working close to the theoretical maximum for sample throughput for a bioluminescence flow analysis system given the requirement for column cleaning after each assay and the time needed for enzyme turnover. The sensitivity while optimised for the sample size could be improved by using a larger sample loop at some expense to sample processing time. It is also likely that the f.i.a. system could be considerably improved by using shorter lengths of tubing between the injection port and the packed enzyme bed reducing the effect of sample dispersion. This would improve both sensitivity and sample throughput as response profiles would be made sharper as sample dilution would be limited to a minimum between injection and analysis.

5.1.2 Assay performance- reproducibility and calibration.

The immobilised preparations described earlier were used in a flow cell configuration to investigate the possible use of such a system as the basis of a flow injection analysis sensor. The packed beds were made with enzyme immobilised on DE-52, CNBr sepharose or CPG beads. The reproducibility

of sequential assays, and the adherence of the assay calibration to the theory of bioluminescence kinetics were examined. The operational stability of the system and its response to different hydrodynamic conditions was also examined. Figure 5.3 shows the calibration curves for NADH, Malate and Lactate for a sepharose bead preparation. Table 5.1 compares the experimental results of calibration curves obtained from different immobilisations. In all cases the slope K obtained from the relationship:

$$K \log (\text{Light intensity}) = \log [\text{analyte}] + c$$

closely resembles the 0.5 for malate and lactate and the 1.0 for NADH, expected from theory.

The operational stability of the different preparations was generally very good. The exception being DE-52 immobilised enzymes, where activity was steadily lost in successive assays. Either fouling of the matrix with substrates products or co-factors was causing this effect or, one or both of the enzymes was being leached off the gel despite the use of a low salt buffer. Although assays of the wash solution failed to show detectable quantities of protein, a slow leaching of enzyme can not be ruled out completely. Figures 5.1a and 5.1b show a sample traces of assays with 1 μ M NADH, and various Malate concentrations for CPG and Sepharose beads. A flow rate of 1.0 ml/min was used as standard for these assays.

5.1.3 Hydrodynamic flow effects on the assay profile and results

The effect of varying the flow rate on the assay response time, peak and profile was examined. Flow rates were altered between 0.1 and 2.0 mls per minute. The peak light, total light, response time and the decay constant for the fall off of emission were recorded for successive assays of 1.0mM malate. Figures 5.4 and 5.5 show the variation of these quantities with flow rate.

For slow flow rates long response times, low total light output from the assay and a large decay constant indicate that appreciable dispersion of the assay plug has taken place, elongating the assay profile and reducing the concentrations of all assay components producing hence the low total light yield. As the flow rate increases the peak light reaches a peak value and a plateau region followed by a gradual falling off of intensity. This results from three conflicting influences at work;

- * An increase in flow rate reduces the dispersion of the assay plug increasing the peak light.

- * The increased flow rate means that each sample is given

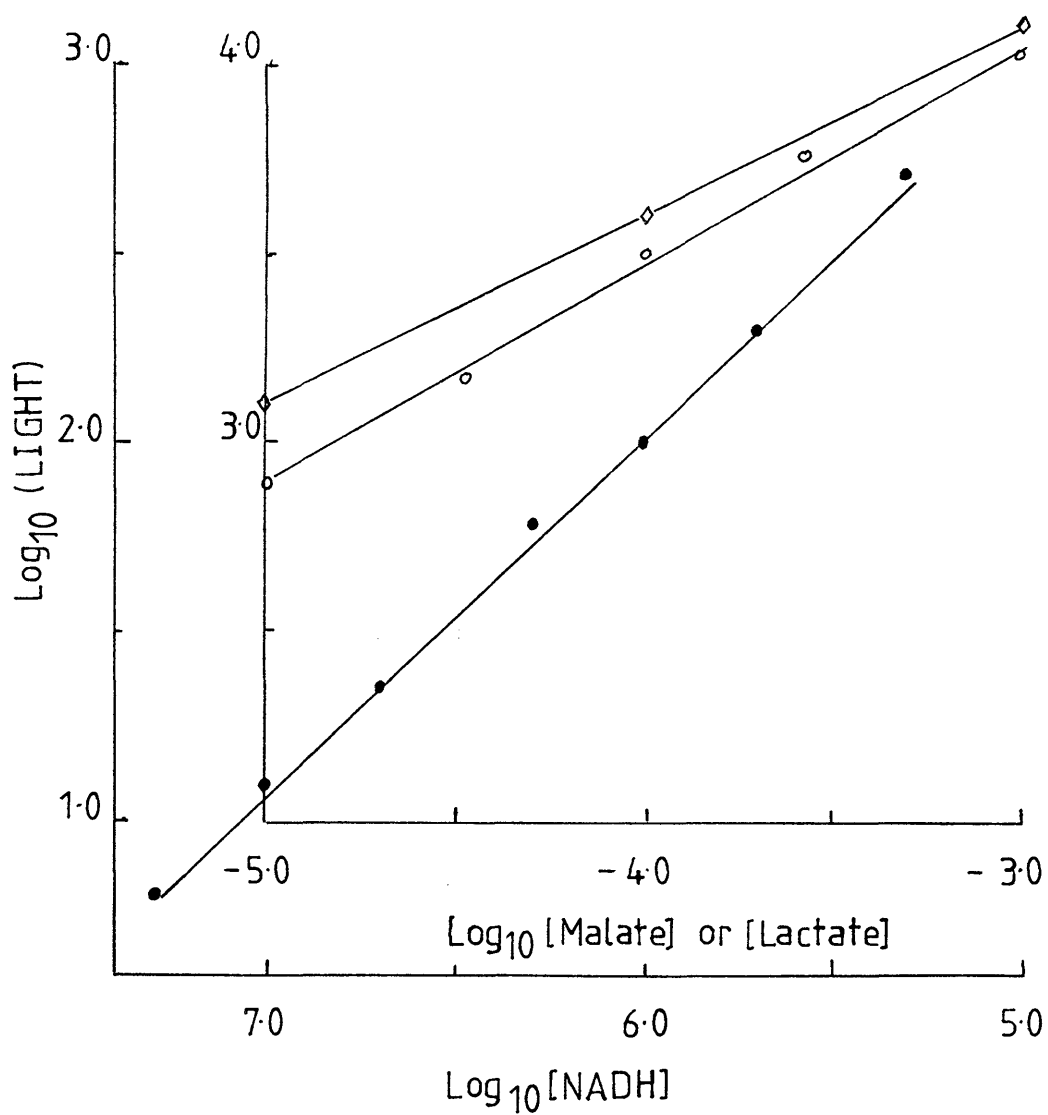


FIGURE 5.3 Calibration plots for sepharose packed bed

- NADH
- ◇ Malate
- Lactate

FIGURE 5.4 Effects of flow rates, I

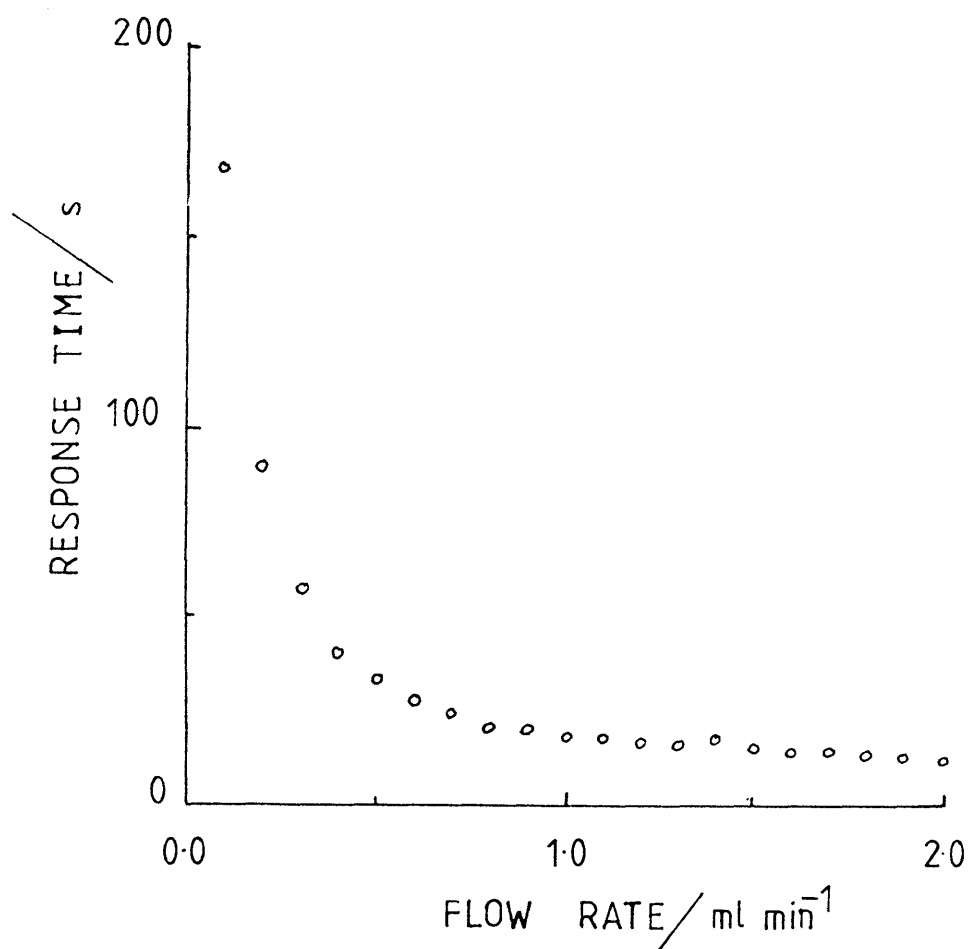
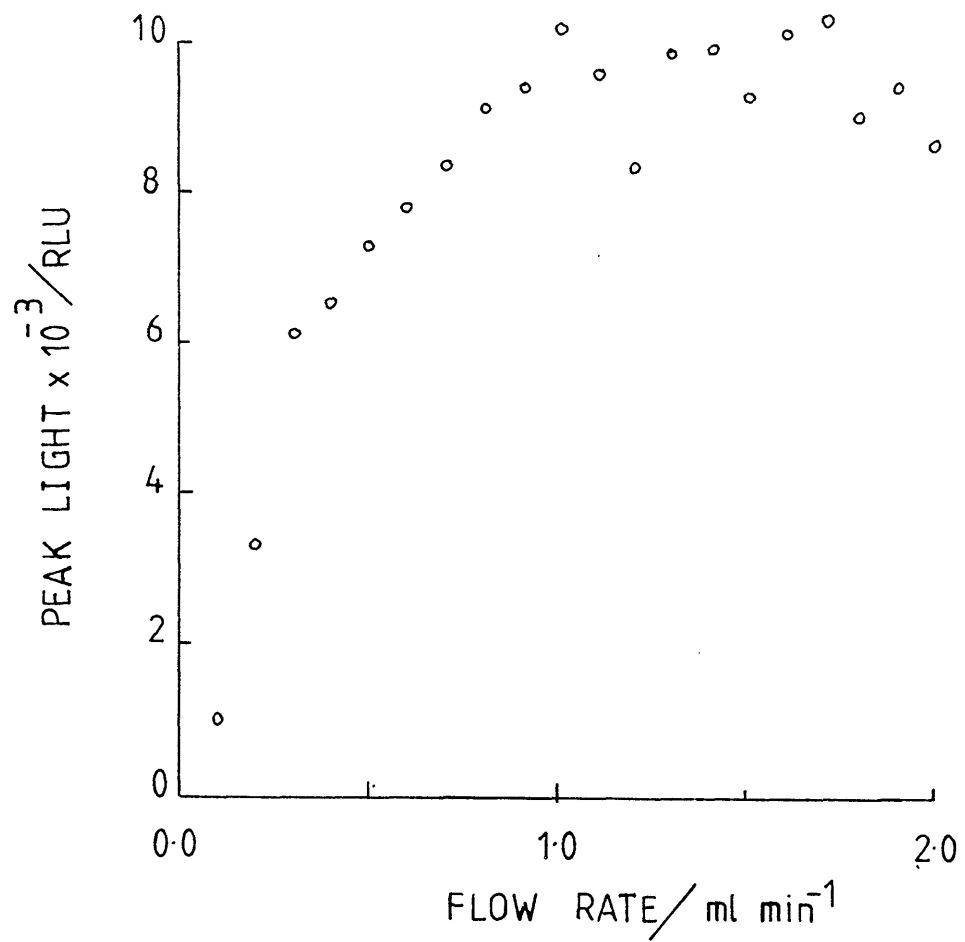
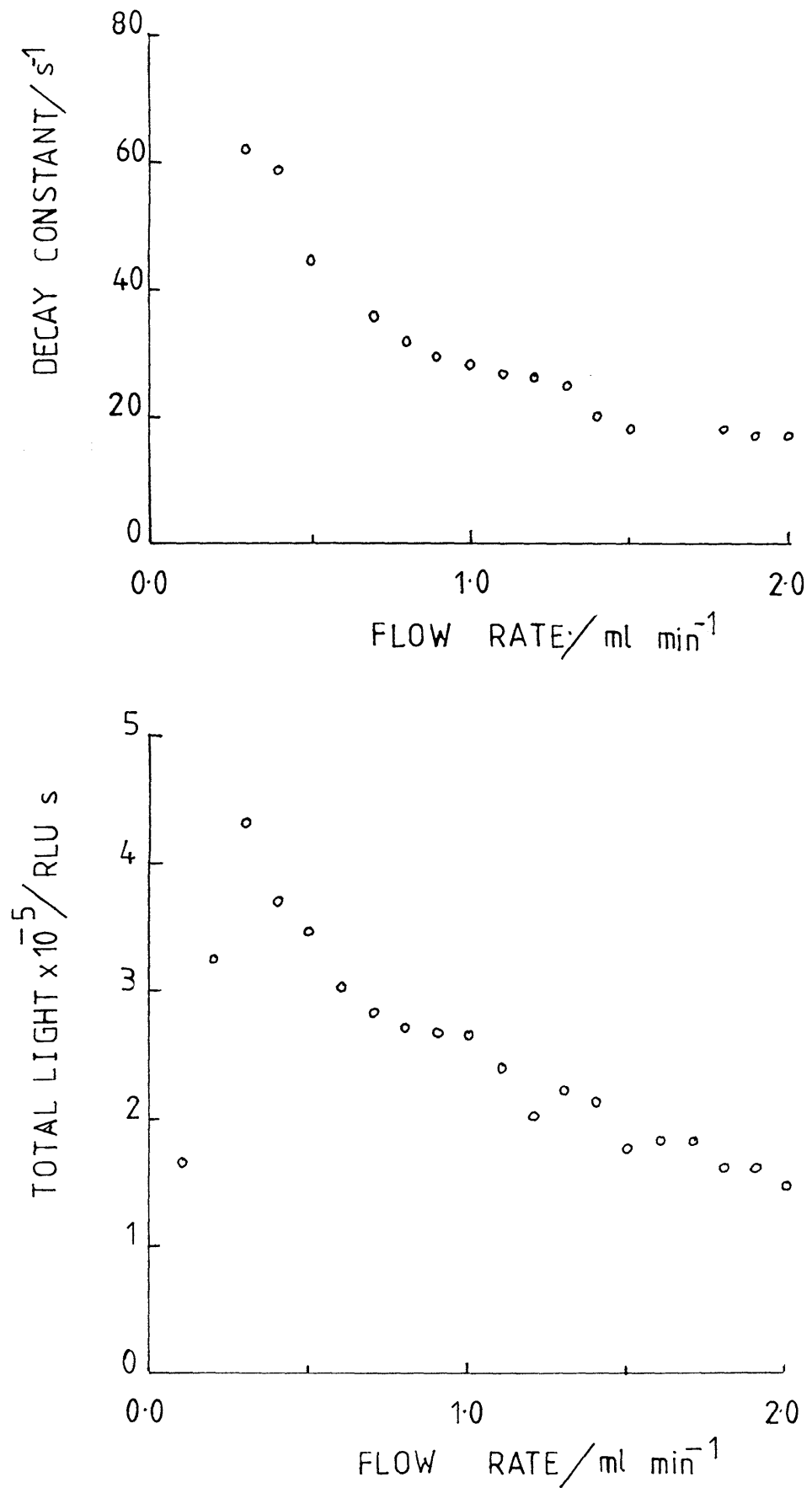


FIGURE 5.5 Effects of flow rates II



a reduced length of time in contact with the enzyme bed. The reduced contact time means penetration of the beads is reduced and hence usage of buried enzyme.

* Finally altering the flow rate affects the thickness of stationary layer around the beads known as the Nernst layer. An increased flow rate reduces the layer and hence improves the rate of external mass transport to the beads.

Total light released follows a similar pattern only the maximum in the figure occurs at a lower flow rate and total light output falls off as penetration of the beads drops, a source of additional light output and extension of the decay time. Both response time and decay constant fall rapidly until a flow rate of 0.5 ml/min is reached; then the fall is more linear with time.

As flow rate is increased one might expect the limiting situation to be a decay constant of the order of the turnover assay plus the residence time of the substrate within the column. The response time will depend on the residence time of the assay plug, i.e. the time to fill the column with assay mixture plus an approximately one second rise time associated with the turnover assay.

Since the analytical reading is taken as the peak of light emission, the flow rate can have an appreciable affect on the recorded analyte concentration. However the experiments have shown that very precise control of flow rate does not appear critical for reasonably accurate assay of analyte concentrations so long as the flow rate is about 1.0 ml/min. At this rate the assay and wash time for a single assay is about one minute. This has economic implications for the cost of flow control equipment for a low cost F.I.A. sensor. The dependence of peak light intensity on flow rate indicates that between 0.6 and 1.5 ml/min there is a fairly broad plateau region, so for a simple device where only crude values of analyte concentration only a crude method of flow control is needed it would even be possible to use a hand operated syringe, resulting in a very low capital cost assay system (figure 5.6).

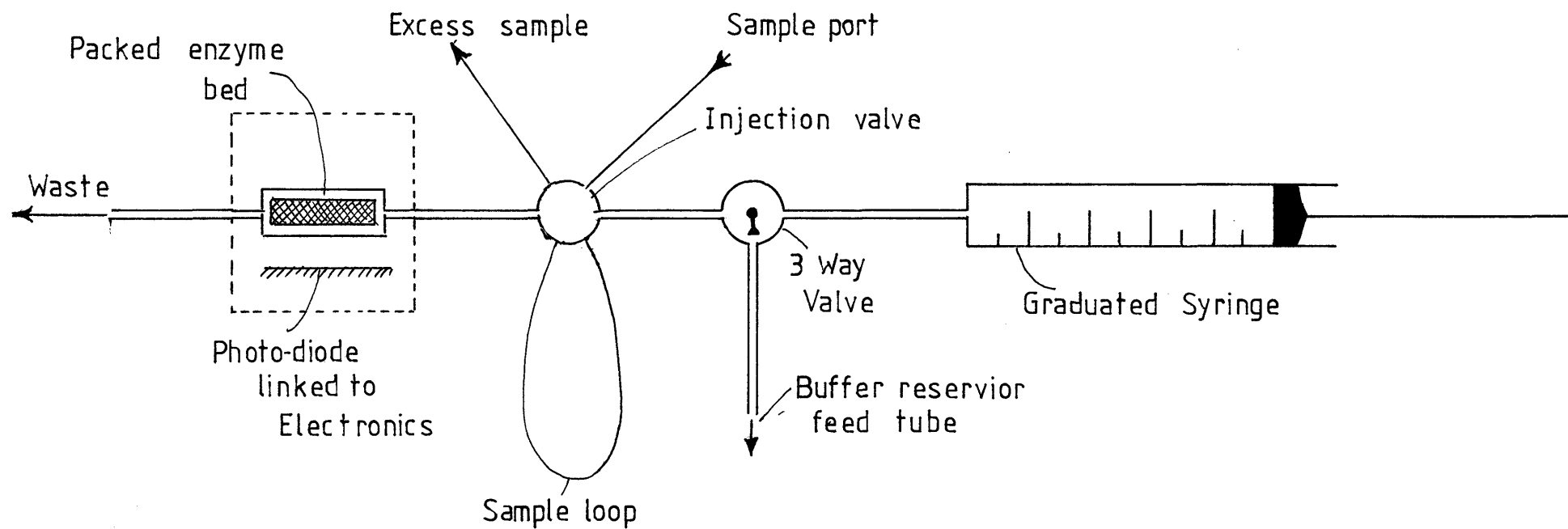


FIGURE 5.6 Proposed low cost f.i.a. system

5.2 FLOW INJECTION ANALYSIS OF MALATE IN WINE

The determination of unknown malate levels in wine samples using the F.I.A. sensor worked very well once a protocol for the pre-treatment of the wine samples was finalised.

5.2.1 Pre-treatment protocol development

Various methods were tried. There are essentially three problems to overcome.

(i) The pH of wines are typically 2.0-5.0. The pH at which the assay is to be carried out is 7.0.

(ii) Wine contains many different organic chemicals. It is probable that several will be potential inhibitors of luciferase, or the other enzymes. Any inhibitory effect will result in low readings for the malate level and must be removed.

(iii) Wine is a coloured solution nominally white, rose or red. Pigmentation in the assay solution will adversely effect the detection of emitted light. The absorption of light will obviously be most marked with red wine (in fact grey/black after adjusting the pH to 7.0).

The pH adjustment is achieved by dilution into a pH 7.0 buffer. A total dilution factor of 400 is used. Any remaining inhibitors or chromophores must be removed. In pre-treatments both polyvinylpyrrolidone (PVP) and activated charcoal both gave promising results. It quickly became obvious that a large absorbant surface area was needed. Granulated or coarse activated charcoal were found to be inefficient both in terms of time and volume needed to decolourise the wine but the powdered variety produced almost instant decolourisation. PVP in a powdered suspension decolourised the wine although a considerable quantity was required.

Both PVP and charcoal worked in suspension. To improve the efficiency a small column of 1 x 2 cm dimensions was packed with powder. The PVP column decolourised the wine sample but only had a useful life for a couple of millilitres of untreated wine. Unfortunately the charcoal powder clogged the filter but a column packed with circles of charcoal cloth decolourised wine well.

Although both materials successfully decolourised the wine, assays indicated that the PVP was not removing all the inhibitors. The charcoal treated samples did not show experimentally detectable levels of inhibitor in the assays.

To decolourise 1 ml of neat wine 100 mg of activated charcoal was found to be necessary.

5.2.2 Accuracy of the assay

Figure 5.7 shows the results of assays on unknown wine samples and the calibration curves taken before and after the assays. The correlation between the results obtained using the Boehringer Mannheim test (carried out at International Distillers and Vintners laboratory) and those obtained using the F.I.A. sensor was very satisfactory.

The time taken to carry out an assay using the F.I.A. system is about 2 minutes per assay including washing the packed bed after the assay in preparation for the next. This would allow for about 30 assays an hour to be processed. The current system uses a 200 μ l loop if this were reduced to say 50 μ l the speed of assays could easily be doubled; much beyond this the unusually long turnover time for the luciferase catalytic cycle would become a problem.

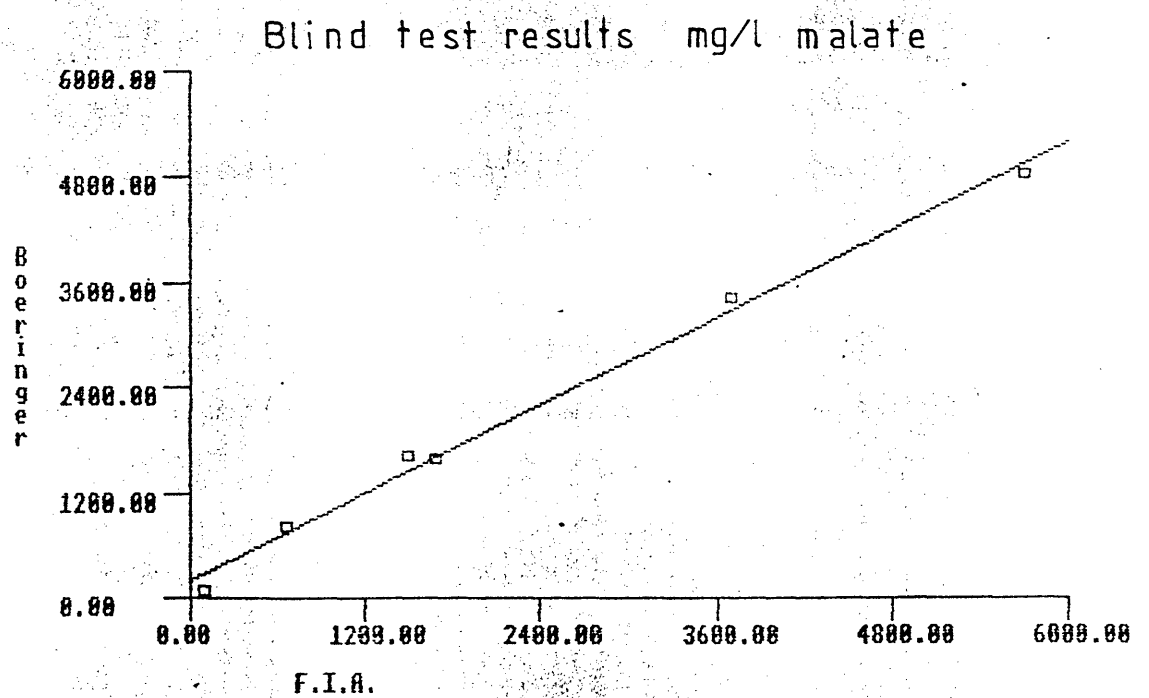
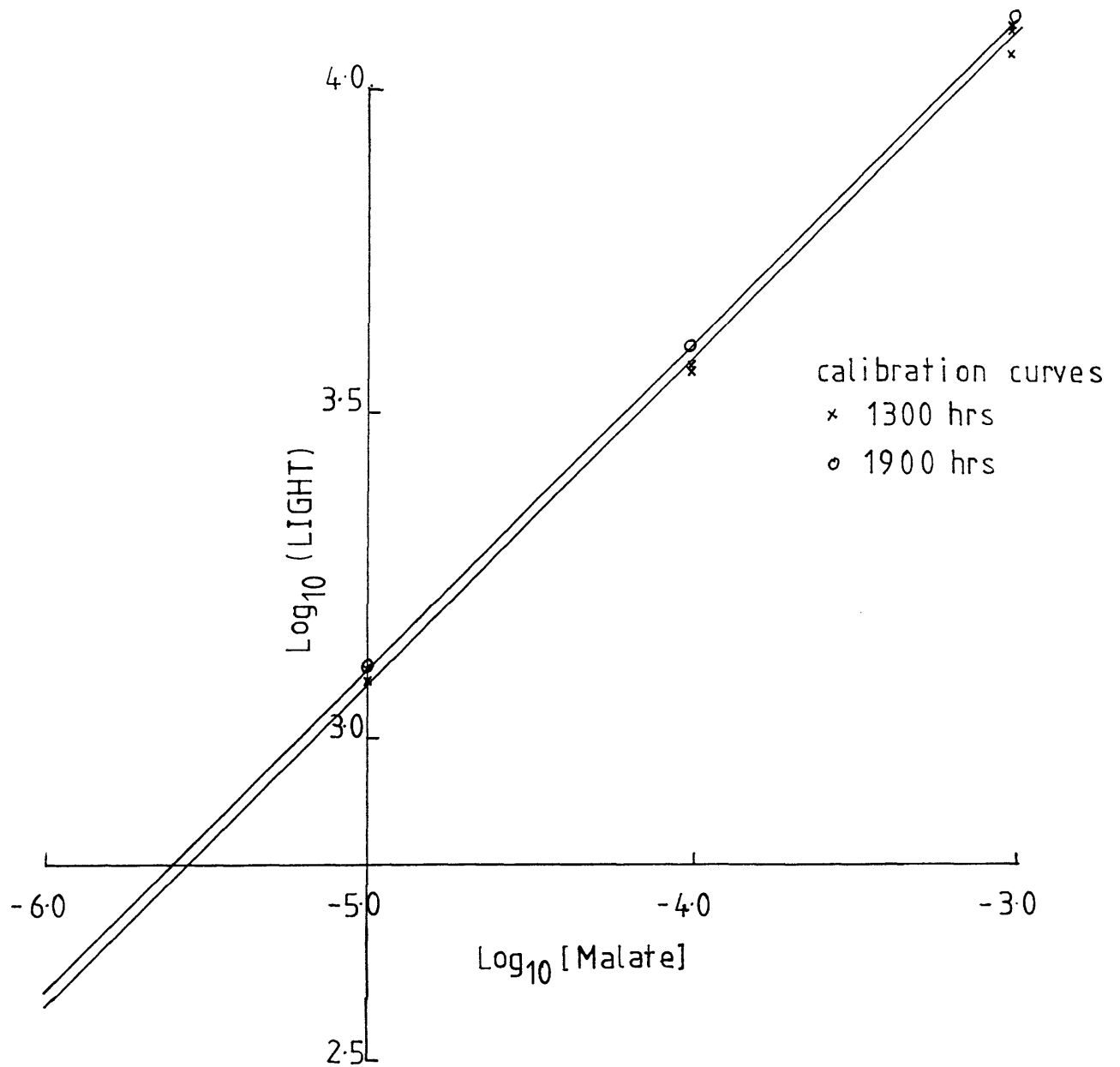
5.2.3 Operational stability of the packed bed.

A mini-column was packed with sepharose beads (MDH, luciferase and reductase) and used for a week, the column was stored in situ in the apparatus overnight. The column was calibrated each day and used for assays of known and unknown malate concentrations in samples. The decline in activity as measured by the assay of 1 mM malate was ~25 percent after five days operation (figure 5.8). An enzyme mixture in solution lost all activity at room temperature over the same period, luciferase is known to be unstable to prolonged exposure to room temperature (21°C).

Successive assays showed no cumulative poisoning of the packed bed with either substrates, products or inhibitors. Calibration at the start and finish of the day showed only small changes in activity accounted for by temperature drift and experimental error.

DTT was found to be important in maintaining the activity of the column. In particular at the start of day four a fresh stock of buffer was made up and the column was observed to revive after washing with the fresh phosphate/DTT buffer for fifty minutes (one syringe full of buffer then leaving for a few hours before the next assay (figure 5.8).

FIGURE 5.7 Blind assays for malate in wine



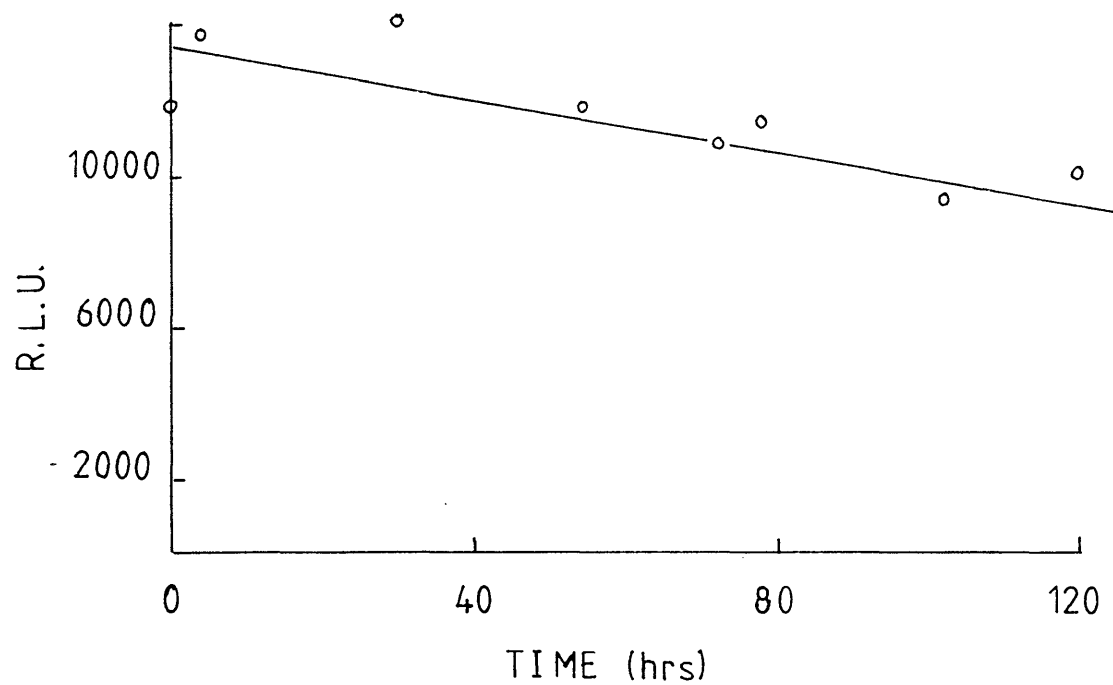


FIGURE 5.8 Operational stability of enzyme at 21°C

5.3 FLOW INJECTION ANALYSIS OF LACTATE

This assay proved to be more problematic than malate, all preparations of sepharose beads with lactate dehydrogenase (LDH), luciferase and reductase co-immobilised showed a significant level of background luminescence in the absence of lactate. This meant that determination of lactate levels below 10^{-6} M was not possible.

With the packed bed a reduction in the level of background light was achieved by altering the concentration of NAD^+ from the 100 mM used in the malate assay to 1 mM. Table 5.2 shows the effect of this change in reducing the resultant background level of light emission thus increasing the accuracy of the assay. The calibration curve obtained produced a slope of 0.6 (figure 5.3).

5.3.1 Assay of lactate in milk products

A selection of dried, powdered milk products were obtained from Grand Metropolitan PLC. Initially an assay with no pretreatment of the samples, apart from dissolving in distilled water, was attempted.

The samples were dissolved at a concentration of 100 mg/ml giving a concentration of 1mg/ml in the assay stream. It was quickly apparent that severe and varied amounts of inhibition of light production were being observed.

For instance Full Cream Milk Powder (FCMP) exhibited light emission below background and even when spiked with 1mM lactate gave a peak light reading well below that for 1 mM lactate in distilled water (table 5.2). These and similar observations for the other samples indicated that a simple single assay determination was not possible.

The composition of the milk products, as is also true of wine to some extent, is so varied with many different contaminating molecules that a simple pretreatment method is not obvious. Dialysis against water, or ultrafiltration appear the most promising methods only one is slow, and the other expensive and awkward for small samples. In order to circumvent the need for time consuming and/or expensive pre-treatment a multiple assay determination for unknown lactate was attempted. The principle behind this relies on the kinetics laid out in the kinetic model for a dehydrogenase linked assay being obeyed despite the presence of inhibitor. The model, chapter six, assumes an equilibrium position for the dehydrogenase catalysed stage so the assay is immune to (limited) inhibition of the dehydrogenase. Inhibition of luciferase will lead to changes in absolute intensity levels although the slope of the calibration curve will be unaltered.

TABLE 5.2

Flow injection Assays for lactate using Sepharose 4B (luciferase, redutase lactate dehydrogenase) in a packed bed of volume 30 μ l.

Sample or [lactate] in moles/litre	Activity / mV		as % of Background (a), (b)
	@ [NAD+] 1mM (a)	@ [NAD+] 0.01mM (b)	
0.001	6000	10200	300 , 7555
0.0001	-	3085	- , 2285
0.00001	4000	770	200 , 570
0.0000001	2000	-	100 , -
0.00	2000	135	100 , 100
10 μ l Full Cream *		76.5	57
10 μ l 90% Demin Whey		450	333
+ 0.00001		550	407
+ 0.00003		1072	794
10 μ l Export Grade Whey		1490	1104
+ 0.00001		1780	1319
+ 0.00003		1810	1341
+ 0.0001		2200	1630
10 μ l S.A Yogurt Powder		2970	2200
+ 0.0001		3000	2222

* sample mixed in 1ml as assay solution, 200 μ l injected for f.i.a.

Mathematically the determination of the lactate concentration relies on fitting the results of three assays to a calibration line. The only fact known about the calibration line is that it has a slope of 0.6 (from the experimental calibration for lactate rather than theory). The three assays are:

- i) Sample with unknown [lactate]
- ii) Above spiked with a known [lactate]
- iii) Sample spiked with a second known [lactate]

Ideally one of the two spikes should be chosen to be as close to the concentration of the unknown sample as possible and the other about an order of magnitude higher. If the first spike increases the light response of the assay by 50-100% it is of the right magnitude.

Using the relationship derived from the theory we draw up a series of three simultaneous equations. These can be used to solve the unknown in groups of three pairs, then taking the mean of these three values produces an approximate value for the lactate in the sample. The range of the three answers gives an idea of the accuracy.

Table 5.3 shows the results for the samples tested. The results are mean values a typical spread is given in figure 5.9 showing the computer printout for a lactate determination. As can be seen the figures are + or - 25%. The computer program is listed below figure 5.9. However the fact that determination of the lactate in 100 mg/ml and 10 mg/ml preparations of the S.A. Yogurt and Full Cream Milk powders gave such close values of mg lactate per gram of original powder indicates that the mean may be more than +/- 25% accurate as implied by the three assays used to locate the calibration line.

In order to compare the accuracy of the lactate sensor with currently available kits, the samples of powder were assayed using the Boehringer-Mannheim lactic acid assay kit. Samples of the four dairy product powders were assayed both with and without pretreatment. Table 5.4 shows the results obtained. It is apparent that the FCMP gave very low lactate readings it also showed some reduction in the level of response to lactate in experiments with samples spiked with known quantities of lactate.

The straight forward assays of FCMP show a slow but definite fall in absorbance at 340nm: this indicates that some interfering reaction is occurring which is consuming either lactate or NADH.

TABLE 5.3

Lactate content of dairy products as assayed using spiked assay method and then compared with BCL test kit results.

LACTATE ASSAYS BY F.I.A.

Sample source	$\mu\text{g}/\text{mg}$ of powder		
	1.0 mg/ml stock	0.1 mg/ml stock	
Spray Dried Whey Export grade:	1.0		
Whey powder 90% Demin :	0.15		
S.A. Yougurt powder :	6.5	7.0	
Full Cream powder :	2.0	2.0	

Sample	fia (i) $\mu\text{g}/\text{mg}$ of powder	BCL untreated (ii) $\mu\text{g}/\text{mg}$ of powder	BCL treated (iii) $\mu\text{g}/\text{mg}$ of powder
SDWEG	1.0	6.24	8.7
WP 90% D	0.15	0.5	0.9
SAYP	6.5	19.7	24.4
FCMP	2.0	--	0.14

(i) Calculated using a packed enzyme bed and spiked samples

(ii) Using BCL kit with no pre-treatment of samples

(iii) Using BCL kit with pre-treatment of samples as suggested by BCL protocol sheet.

SAMPLE	LIGHT RLU	(LACTATE)
TEST	1490	ZERO
SPIKE 1	1810	3E-5
SPIKE 2	2200	1E-4
ASSUMED GRADIENT		0.6

Concentration in sample is 7.83300692E-5M
Concentration in sample is 1.09346569E-4M
Concentration in sample is 1.52134319E-4M

EXPORT GRADE SPRAY DRIED WHEY

SAMPLE	LIGHT RLU	(LACTATE)
TEST	1490	ZERO
SPIKE 1	1810	3E-5
SPIKE 2	2200	1E-4
ASSUMED GRADIENT		0.5

Concentration in sample is 6.3071023E-5M
Concentration in sample is 8.47398757E-5M
Concentration in sample is 1.16637892E-4M

90% DEMIN WHEY POWDER

SAMPLE	LIGHT RLU	(LACTATE)
TEST	550	ZERO
SPIKE 1	700	1E-5
SPIKE 2	1072	3E-5
ASSUMED GRADIENT		0.6

Concentration in sample is 2.02135847E-5M
Concentration in sample is 1.46968079E-5M
Concentration in sample is 9.3297828E-6M

FIGURE 5.9 Determination of lactate using spiked assays

```

10 INPUT "NAME OF SAMPLE",NAME$
20 INPUT "I for sample",It,"I for spiked sample",Ia,"Concentration of "
30 INPUT "I for spiked sample",Ib,"Concentration of spike",B,"Expected slope is "
40 AA=((LOG(a))-(LOGIt))/K:AA=10^AA
40 BB=((LOGIb)-(LOGIa))/K:BB=10^BB
45 CC=((LOG(a))-(LOGIb))/K:CC=10^CC
46 VDU2:PRINT NAME$
50 PRINT "Concentration in sample is",A/(AA-1)
60 PRINT "Concentration in sample is",B/(BB-1)
65 PRINT "Concentration in sample is",(A-CC*B)/(CC-1)
66 VDU3
70 END

```

The clarification method recommended in the BCL kit for liquid foodstuffs, using carrez reagents, was found to clarify the other three powders: however the FCMP solution was still slightly cloudy. Treatment with the clarification agents had little effect on the level of lactate detected using the kit: the treated FCMP did show some trace of activity; this was however observed to once more fall away during the course of the assay.

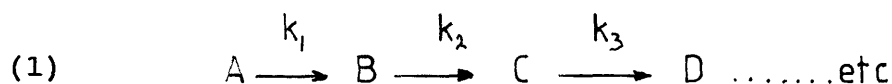
Apart from the FCMP sample the three samples rank in the same order for f.i.a. method although the commercial kit indicates higher levels of lactate overall. It must be concluded that the FCMP sample contained some substance that was interfering with the assay of 0.2g/l lactate alone. This suggests that the interfering reaction is independent of lactate and not of the same nature as that observed in the f.i.a. assay. This observation is consistent with the f.i.a. assay inhibition occurring to the bioluminescence enzymes in the assay. One might suggest that some component of the milk solution is interfering with the GTP enzyme in the commercial kit assay.

CHAPTER SIX

KINETICS OF THE NADH AND DEHYDROGENASE LINKED BIOLUMINESCENCE ASSAYS

6.1 CONSTRUCTION OF A KINETIC MODEL

The kinetics of most multi-enzyme assays can be conveniently modelled by the following scheme:



Where A,B,C,Dare substrates/products within the reaction sequence, and k_1 , k_2 , k_3 are the apparent rate constants for each enzyme catalysed step. The overall rate of flow or flux of substrates is then controlled by the rate determining enzyme in the sequence.

The bacterial luciferase based assays differ in several important respects from this conventional type of assay.

(1) One of the enzymic steps in the assay sequence, the interconversion of FMN and FMNH₂, is affected by the competing chemical reaction oxidising FMNH₂ to FMN using molecular oxygen. This non-enzymatic process as we shall see has a significant effect on the kinetic behaviour of the system.

(2) The luciferase enzyme has a very low k_{cat} , of the order of 0.15 s⁻¹. This is many orders of magnitude smaller than a "typical" k_{cat} for an enzyme, and affects the flux of substrates through the complete system.

6.1.1 Modelling the coupled assay

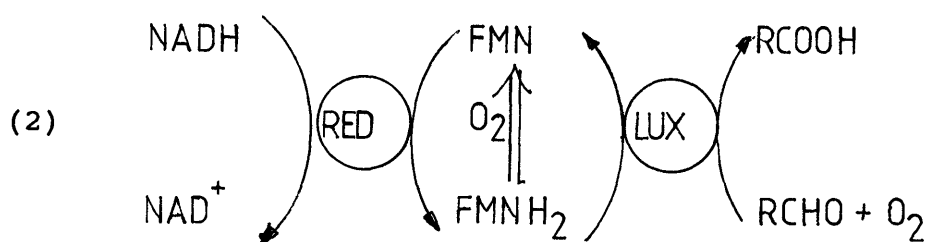
A kinetic model of the assay can be built for both the NADH assay and dehydrogenase linked assays. Such a model provides a framework for discussion of the observed kinetic data and assay behaviour. A theoretical model also allows the consequences of changing the assay conditions to be predicted.

We begin by creating a model for the NADH assay. This is then extended to dehydrogenase linked assays. In both cases the model is for a solution assay. The special cases of immobilised enzyme assays are then considered at the end of this chapter.

In constructing the model a series of assumptions have been made, and for the NADH assay these are:

- (1) The activity of the oxidoreductase is unaffected by the presence of the luciferase enzyme.
- (2) The enzymes, luciferase and oxidoreductase are assumed to show first order kinetics with respect to enzyme, NADH, FMN, and FMNH₂ concentrations i.e. they exhibit non-saturating behaviour. This is consistent with the range of concentrations used and the known K_m values for the different enzymes (Table 6.1).
- (3) The NADH concentration is assumed to remain constant throughout the reaction. The concentration of oxidoreductase is very low, of the order of 10⁻¹² M, as a result the net consumption of NADH per second is several orders of magnitude lower than the actual NADH concentration.

The complete reaction scheme for the NADH assay is described by figure 1.2 in the introduction (Chapter 1). However under normal assay conditions a simplified scheme applies.



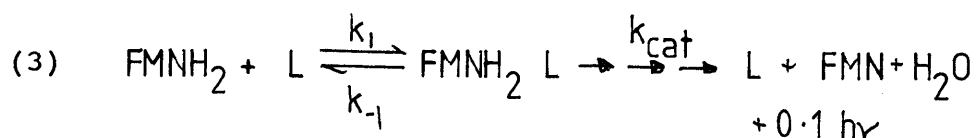
Luciferase is assumed to be saturated with decanal and oxygen and the concentration of these do not appreciably change during the assay. Decanal is present at about 6 μ M and oxygen at 250 μ M. These are both orders of magnitude above the experimentally determined K_m's for luciferase. For decanal 6 μ M is on the plateau of maximum bioluminescent activity as defined by the values of K_m and K_i for the two decanal binding sites (Section 4.2.1.2)

TABLE 6.1

Summary of approximate K_m 's for substrates in the bioluminescence assay system.

Constant	Value	Substrate	Enzyme
K_m	0.2 μM	FMNH ₂	Luciferase
K_m	6.0 μM	O ₂	Luciferase
K_m	4.0 μM	Decanal	Luciferase
K_i	30.0 μM	Decanal	Luciferase
K_m	14.2 μM	NADH	Reductase
k_a	25 ms	rate of autoxidation of FMN	
		see section 6.4	

The luciferase mediated reaction can be described by the equation.



Since $k_{\text{cat}} \ll k_{-1}$ the conditions for Michaelis-Menten kinetics are satisfied. Assumption (2) in which the reaction is assumed to be first order corresponds to the case of $[\text{FMNH}_2] \ll K_m$.

6.1.2 Bioluminescence and kinetic rates

The light intensity recorded is an instantaneous measure of the rate of photon production in the reaction. As such the assay is giving an instantaneous measure of the rate of production of products rather than in the case of a more conventional assay where rates are measured from the increase in product concentration over a period of time.

Light intensity is related to v , the luciferase reaction rate, by the bioluminescence yield quotient Q_b as discussed in the introduction (Section 1.2.2.1).

$$(4) \quad I = Q_b v$$

and under the first order conditions of the assay:

$$(5) \quad v = \frac{k_{\text{cat}}[\text{L}][\text{FMNH}]}{K_m}$$

Therefore we have

$$(6) \quad I = Q_b k_l [\text{FMNH}_2]$$

Where $k_l = k_{\text{cat}} [\text{L}] / K_m$

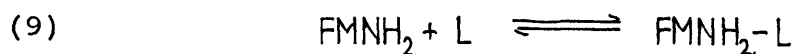
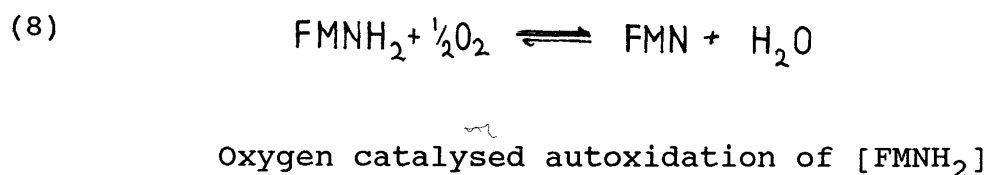
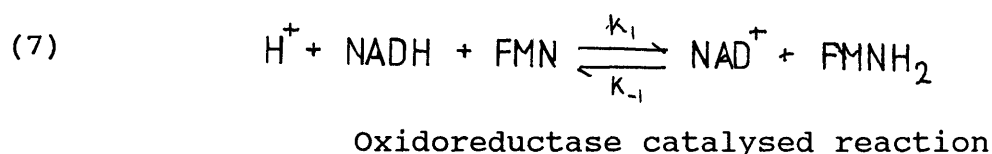
I, the light intensity is hence a measure of the luciferase activity and in this case where $[FMNH_2] \ll K_m$, it is directly proportional to $[FMNH_2]$.

In both the NADH and the dehydrogenase linked assays the light intensity traces the changing level of $FMNH_2$ in solution. As will be seen, when the rate of increase in light intensity is measured, what is being observed is the approach of the coupled system to steady state. In a typical coupled bioluminescence reaction the point closest to steady state approximates to the time when the light intensity reaches a constant level, I_{plat} . Subsequently this intensity decays with time for a number of reasons which are discussed later.

Examining the kinetic scheme of the assay enables a framework of equations and initial conditions to be drawn up which describe this approach to steady state for a particular assay. It also provides a useful model and predictive tool for studying the kinetic behaviour of the system.

6.1.3 Calculation of $[FMNH_2]$ in the NADH assay

In writing an expression for $[FMNH_2]$ as a function of time we need to know all the possible sources and fates for this substrate and to combine these representative equations. There are three principle reactions which affect the concentration of $[FMNH_2]$



Luciferase binding with $FMNH_2$

We are attempting to describe the concentration of FMNH₂ as a function of time, f(t). An expression for f(t) may be obtained by solution of the differential equation $df(t)/dt$, ($d[FMNH_2]/dt$). This differential may be written as the sum of the three expressions representing equations 7, 8, and 9 above. To write these expressions in terms of $d[FMNH_2]/dt$ and solve for f(t) we must recall our assumptions and conditions. The assumptions were defined earlier in this chapter and are reviewed below. They can be supported by experimental evidence this will enable us to define boundary conditions for the reactions and simplify the model.

* [FMN] is approximately constant under the conditions of the assay. - The initial [FMN] in a standard assay is 5×10^{-6} M, the net change to this is small, and [FMNH₂] is small compared to K_m (first order assumption) since the rate of FMNH₂ production is counterbalanced by the much faster autooxidation reaction (equation 8) this maintains a high [FMN]/[FMNH₂] ratio ensuring a [FMN] close to that of the initial assay conditions.

* The [NADH] is assumed to remain relatively constant due to a low net flux of substrate through the reductase enzyme, assumed to be present in only trace quantities. Once more assuming that first order kinetics apply for the oxidoreductase we can estimate a rough maximum value for k_r.

From the expression for the oxidoreductase reaction rate:

$$(10) \quad V_r = \frac{k_{cat}[R][NADH]}{K_m}$$

describing k_r as $k_{cat} [R]/K_m$ we can obtain the expression:

$$(11) \quad V_r = k_r[NADH]$$

following from this we can estimate a maximum value of k_r, bearing in mind assumption 2 that $V_r \ll [NADH]$, as $k_r < 10^{-3}$.

We are now in a position to combine all three reactions to obtain an expression for $d[\text{FMNH}_2]/dt$.

$$(12) \quad \frac{d[\text{FMNH}_2]}{dt} = k_r[\text{NADH}] - (k_a + k_l)[\text{FMNH}_2]$$

Where

k_l is the overall luciferase rate constant described in equation 6.

k_r is the overall oxidoreductase rate constant described in equation 10.

k_a is the overall rate constant for the autoxidation of FMNH_2 back to FMN by molecular oxygen; k_a is defined by the equation:

$$(13) \quad V_a = k_a [\text{FMNH}_2]$$

Where

$k_a = k_{\text{acat}}[\text{O}_2]$ the k_{acat} refers to the autocatalytic rate constant for the autoxidation reaction.

In this analysis the values of k_a and k_l are assumed to be constant. These assumptions are reasonable on consideration of the following argument. If $k_r < 10^{-3}$ and $[\text{NADH}] < 10^{-5}$ as shown above V_r becomes necessarily $< 10^{-9}$ which compares to an $[\text{O}_2]$ of 2.5×10^{-4} , several orders of magnitude higher than the rate of consumption by luciferase. Note also that $d[\text{O}_2]/dt$ by autoxidation is $< k_{\text{rd}}[\text{NADH}]/dt$.

Given the assumptions outlined above we can solve the equation 12 using standard integrals and obtain a precise analytical expression for the system.

Initial conditions are defined at $t=0$ $[\text{NADH}] = N_0$, $[\text{FMNH}_2] = 0$

The following expression is obtained

$$(14) \quad [\text{FMNH}_2]_t = \frac{k_r}{k_a + k_l} [\text{NADH}]_t (1 - \exp(-(k_a + k_l)t))$$

with the aid of the standard integral

$$\int \frac{1}{A - Bx} dx = \int dt$$

$$\Rightarrow -\frac{1}{B} \ln \frac{A - Bx}{A} = t$$

$$\Rightarrow x = \frac{A}{B} (1 - e^{-Bt})$$

For the solution of equation 14

$$A = k_r [\text{NADH}]$$

$$B = k_a + k_l$$

From both the solution of equation 14 at $t = 0$ and the solution of the equation 12 at steady state, $d[\text{FMNH}_2]/dt=0$, we find that a plateau value can be obtained for $[\text{FMNH}_2]$.

$$(15) \quad [\text{FMNH}_2] = \frac{k_r}{k_a + k_l} [\text{NADH}]$$

Hence using equations 6 & 15 we can express I_{plat} in terms of $[\text{NADH}]$

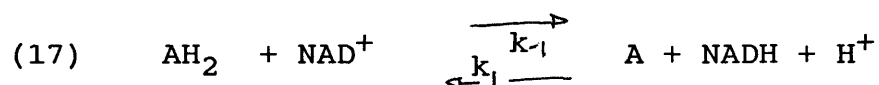
$$(16) \quad I_{\text{plat}} = Q_p \frac{k_l k_r}{k_a + k_l} [\text{NADH}]$$

It may be further noted that the time constant for the rate of approach to steady state is given by $(k_a + k_l)$ in equation 13, remember the k_a and k_l here are reaction rates involving reagent or enzyme concentrations as defined earlier.

6.1.4 Dehydrogenase linked Bioluminescent assay

Analytes with a NADH utilising dehydrogenase can be assayed with a variation of the above assay. An analysis of these dehydrogenase linked assays is more complicated, and the general solution can only be arrived at numerically.

Firstly we construct a model for a generalised dehydrogenase catalysed reaction:



and set up the assumptions and conditions as before so that:

(1) All enzymes are behaving in a pseudo first order manner with respect to their substrates. For the dehydrogenase enzyme this must hold for both forward and reverse reactions.

(2) Initially concentrations of AH_2 , NAD^+ , A and NADH are as follows:

$$\text{At } t=0 \quad \text{AH}_2 = a_{h_0}$$

$$\text{NAD}^+ = n^+_0$$

$$\text{A} = 0$$

$$\text{NADH} = 0$$

(3) The dehydrogenase reaction equation 17 is simplified by assuming that the reaction is occurring in a buffered solution and that the $[\text{H}^+]$ remains constant. The kinetic constants used henceforth assume this constant pH condition. The pH is taken as 7.0.

As with the NADH assay the concentration of the products of the reaction, A and NADH are functions of time:

$$f_1(t) = [\text{NADH}]$$

$$f_2(t) = [\text{A}]$$

For brevity the values of $[\text{NADH}]$ and $[\text{A}]$ will henceforth be expressed as the time dependent variables n and a respectively.

We can now write equations to express the derivatives of $f_1(t)$ and $f_2(t)$ with respect to time in terms of a set of kinetic constants; initial substrate concentrations are set and the variables n and a for subsequent values of t can be calculated by considering all the relevant reactions.

(i) The dehydrogenase mediated reaction of equation 17 duly adapted for condition (3) of constant pH.

(ii) The bioluminescence reaction discussed in the previous section on the NADH assay. This reaction is assumed to be irreversible under the conditions of the assay.

Since we have established that light intensity is proportional to $[NADH]$ we are principally interested in the solution of $f_1(t)$ and the value of n , the predicted time dependence of $[NADH]$. However as can be seen from the expressions below, the derivatives of $f_1(t)$ and $f_2(t)$ contain both $f_1(t)$ and $f_2(t)$ and are interdependent; they must both be solved.

$$(18) \quad \frac{d f_1(t)}{dt} = k_1(n_0^+ - n)(ah_0 - a) - k_{-1}nxa - k_r n$$

While n is not equal to a , ie. $f_1'(t)$ is not equal to $f_2'(t)$ this is not a simple differential equation to solve and the pair $f_1'(t)$ and $f_2'(t)$ have to be solved together.

$$(19) \quad \frac{d f_2(t)}{dt} = k_1(n_0^+ - n)(ah_0 - a) - k_{-1}nxa$$

There is no analytical solution to these as they stand with the initial conditions defined by assumptions 1, 2 and 3 above. The equations only become soluble when they become equal ie. when the initial identity $f_1(0) = f_2(0)$ is held so that $f_1(t) = f_2(t)$ for all values of t . This occurs only if $k_r = 0$.

As the value of k_r is small from assumption (3) in section 6.1.1, it is useful to consider the limiting case of $k_r \rightarrow 0$. The analytical solution is obtained as before with the aid of a standard integral.

The special case of $kr = 0$ and $n = a$ gives the far simpler differential equation:

$$(20) \quad \frac{df_1(t)}{dt} = \frac{dn}{dt} = k_1 n \quad a h - k_{-1} n^2$$

Using the standard integral

$$\frac{1}{b} \int \frac{1}{a^2 - x^2} dx = \frac{1}{2ab} \log \left| \frac{a+x}{a-x} \right| = \int dt$$

$$\Rightarrow \quad x = a \frac{1 - e^{-2abt}}{1 + e^{-2abt}}$$

for (20)

$$\begin{aligned} \frac{dn}{dt} &= B(A^2 - n^2) & B &= k_{-1} \\ A &= (k_1/k_{-1})^{1/2} (n^+ ah)^{1/2} \end{aligned}$$

$\Rightarrow$

$$n = [\text{NADH}]_t = A \frac{1 - e^{-2ABt}}{1 + e^{-2ABt}}$$

As $t \rightarrow \infty$

$$n = [\text{NADH}] = A$$

$$(21) \quad I_{\text{plat}} = Q_p \frac{k_l k_r}{k_a + k_l} (n^+ \text{ ah } k_1/k_{-1})^{1/2}$$

The solution states that as the value of $k_r \rightarrow 0$ the steady state light intensity will be proportional to the square root of the analyte concentration. For a $\log I$ vs $\log [\text{analyte}]$ plot it is expected that a slope of 0.5 will be obtained. The experimental data obtained for dehydrogenase linked assay gives data with slopes close to this theoretical value and is discussed in chapters 4 and 5.

The analytical solution also makes a statement about the dependency of the response time on the conditions. The relationship is more complicated than for the simple exponential equation obtained in the NADH assay. However from the preceding solution it can be seen that the response time depends upon the value of $2AB$

$$(22) \quad 2AB = 2(k_1/k_{-1} n^+ \text{ ah})^{1/2}$$

This simplified treatment is complicated in two ways. Firstly the analytical solution above is an extreme case which is only approached in practice, the solution for real cases must have a finite value assigned to k_r . Equations 18 & 19 then have to be solved numerically with the aid of a computer. Secondly the relationship between $[\text{NADH}]$ and light intensity is not precise except in the case of steady state conditions. In the approach to this state we must consider the equation derived for the NADH assay and the fact that the level of NADH is changing constantly according to the two equations 7 and 17. This new situation can be defined by adding a third differential equation to the two describing n and a , the third equation allows for the concentration of FMNH_2 to be a variable with time $f_3(t)$.

We define $f_3(t)$ as the concentration of FMNH_2 at time t . The differential with respect to time is a slight modification of equation 12:

$$\frac{d[\text{FMNH}_2]}{dt} = k_r [\text{NADH}] - (k_a + k_l) [\text{FMNH}_2]$$

In that $[\text{NADH}]$ is now no longer regarded as a constant but a variable of time, $f_1(t)$ as described above. We now have the three dependent differential equations $f_1'(t)$,

$f_2'(t)$, and $f_3'(t)$ which can be written in terms of $[NADH]$, $[A]$, and $[FMNH_2]$ or $f_1(t)$, $f_2(t)$ and $f_3(t)$.

The expressions for these three equations and the initial conditions are detailed below.

$$\begin{aligned} f_1(t) &= [NADH] ; f_1(0)=0 & A &= k_1 n_{ah} \\ f_2(t) &= [A] ; f_2(0)=0 & B &= - (k_1 a_h + k_r) \\ f_3(t) &= [FMNH_2] ; f_3(0)=0 & B' &= - k_1 a_h \\ & & C &= - k_1 n \\ & & D &= k_1 - k_{-1} \\ & & E &= - (k_a + k_1) \end{aligned}$$

$$(23) \quad \frac{d f_1(t)}{dt} = A + B f_1(t) + C f_2(t) + D f_1(t) f_2(t)$$

$$(24) \quad \frac{d f_2(t)}{dt} = A + B' f_1(t) + C f_2(t) + D f_1(t) f_2(t)$$

$$(25) \quad \frac{d f_3(t)}{dt} = (B - B') f_1(t) + f_3(t)$$

Where the constants A , B , B' , C , D , and E represent combinations of the kinetic constants defining the individual reactions in the assay system and are defined above.

6.1.5 Computer model of Dehydrogenase linked assays

The above set of conditions and equations were used as the data and basis for the computer generated solution, generating sets of data for the $[NADH]$, $[A]$ and $[FMNH_2]$ with respect to time. Solutions were obtained using the programs listed in appendix B and estimates for the kinetic constants k_1 , k_2 , k_r , k_a+k_1 , and the initial concentrations of NAD^+ , AH_2 .

6.1.5.1 Selection of the input values

The numerical analysis was carried out for a variety of conditions and with a variety of kinetic parameters. At the start of each analysis or simulation the program requires the definition of all these parameters.

1) [Dehydrogenase], k_1 and k_{-1}

The values of these two rate constants describe the behaviour of the dehydrogenase enzyme. k_1 and k_{-1} are defined by the equilibrium constant K' for the enzyme at constant pH 7.0.

$$(26) \quad K' = \frac{[A][NADH]}{[AH][NAD]} = \frac{k_1}{k_{-1}}$$

Diffusion limitation of either k_1 or k_{-1} was taken as to be 10^8 moles s^{-1} setting maximum estimates for these kinetic constants.

For instance the values used to give a figure of the same kind of order as malate dehydrogenase were estimated as follows. Since the enzyme is well to the malate and NAD^+ side k_{-1} is taken as 10^8 moles s^{-1} (Estimate if the reaction in this direction is assumed to be diffusion limited), the K' at pH 7.0 is about 10^{-5} (Bergmayer 1978) giving a k_1 value of 10^3 . The concentration of dehydrogenase was estimated from practical experience to be typically 0.1 mg/ml ($10^{-5}M$).

2) [Oxidoreductase] and k_r

The concentration of oxidoreductase per se is not known since impure preparations were generally used, these contain only a fraction by mass of the reductase enzyme as described in section describing the purification of the reductase. The value of k_r was estimated by consideration of the boundary conditions and assumptions already outlined in this section. k_r was given values of less than $10^{-3} s^{-1}$

3) k_l and k_a

These constants represent the luciferase reaction and the O_2 mediated autoxidation of FMNH₂. The constants were estimated from the NADH assay model for the NADH coupled assay and the observed $t_{1/2}$ for the coupled assay, defined as the time taken to reach half the I_{plat} light intensity. The relationship with k_a and k_l is derived from equation 16 and is:

$$(27) \quad \frac{\ln 2}{t_{1/2}} = k_a + k_l$$

A typical value for the half time is 30 seconds which means $k_a + k_l$ has a value of $0.023 s^{-1}$ the actual values of k_a and k_l were determined under different conditions of O_2 tension and luciferase concentration and this estimate is typical of the usual assay conditions.

6.2 COMPUTER SIMULATED ASSAY KINETICS

The programs designed to simulate the kinetics of an assay were run several times in order to examine the behaviour of the model in a variety of situations. These simulations are run as series in which one or other of the biochemical constants is varied progressively. A summary of the computed values for the constants k_1 .. k_6 in the program used to describe the differential equations are shown in table 6.2.

6.2.1 Effects of the dehydrogenase kinetics

The central point to understanding how the dehydrogenase linked assay works is that the enzyme reaches a position approximating to its equilibrium value during the assay. The assay itself requires that the reaction starts with 100% substrates, (NAD^+ and reduced analyte), on the left hand side of equation 15. Individual dehydrogenases have equilibrium positions which determine whether $k_{-1} \gg k_1$, $k_{-1} = k_1$ or $k_{-1} \ll k_1$. The effect of lactate and malate dehydrogenases has been observed in this laboratory their kinetics are similar in that they both have equilibrium positions well to the left hand side of the equation.

A computer simulation with various k_1 and k_{-1} values was run to predict the effect of these different equilibrium positions. All cases result in the production of a light intensity profile, $Q_b[\text{FMNH}_2]$ which exhibits a stable plateau, equilibrium position. However the simulation demonstrates a potential problem in using the plateau level as a measure of analyte in some cases. For the cases where $k_1 \gg k_{-1}$ the final level of NADH is $>99.5\%$ of the total nicotinamide pool (Table 6.3). As a result small inaccuracies in the initial NAD^+ levels will cause large errors in the final light intensity. For these cases a I_{plat} may be an unsuitable measure of [analyte].

The time the concentration profiles take to reach equilibrium appears to be independent of the dehydrogenase kinetics for a given level of FMNH_2 (Table 6.3). This is because the time response of the bioluminescence enzymes to a given NADH level is slow compared to the response time of the dehydrogenase kinetics to the analyte level, the variation in the response time of the luciferase/reductase system is very pronounced as can be seen from the time to reach peak [NADH] in table 6.3. The case where $k_1 = 10^8 \text{ moles s}^{-1}$ has a time of < 5 seconds to peak and as seen is close to the FMNH_2 profile associated with an NADH assay, $t_{1/2}$ for the generated data is approximately 30 seconds for this case ($k_a + k_1$ of 0.023 s^{-1} corresponding to the $t_{1/2}$ of 30s that was assumed).

While the relative values of k_1 and k_{-1} are important the

TABLE 6.2

Summary of constants $k_1 \dots k_6$ generated for the four series of simulations shown in the tables and plates in this chapter.

Filename & Discription	CONSTANTS					
	k_1 ($\times 10^{-8}$)	k_2 ($\times 10^{-4}$)	k_3 ($\times 10^{-6}$)	k_4 ($\times 10^{-5}$)	k_5 ($\times 10^{+3}$)	k_6 ($\times 10^{-2}$)

Dataadh1: The forward and backward rate constants of the dehydrogena modified.						
	1.0000	-0.2000	-10.000	-0.1000	-0.1000	-0.2300
	2.0000	-0.3000	-20.000	-0.2000	-0.2000	-0.2300
	4.0000	-0.5000	-40.000	-0.4000	-0.4000	-0.2300
	8.0000	-0.9000	-80.000	-0.8000	-0.8000	-0.2300
	16.000	-1.7000	-160.00	-1.6000	-1.6000	-0.2300

DataAna: The nominal level of analyte in the assay was progressively increased.						
	0.1000	-1.0000	-0.1000	-10.000	-1.0000	-0.2300
	0.1778	-1.0018	-0.1778	-10.000	-1.0000	-0.2300
	0.3162	-1.0032	-0.3162	-10.000	-1.0000	-0.2300
	0.5623	-1.0060	-0.5623	-10.000	-1.0000	-0.2300
	1.0000	-1.0100	-1.0000	-10.000	-1.0000	-0.2300
	1.778	-1.0177	-1.778	-10.000	-1.0000	-0.2300
	3.162	-1.0316	-3.162	-10.000	-1.0000	-0.2300
	5.623	-1.0562	-5.623	-10.000	-1.0000	-0.2300
	10.00	-1.1000	-10.00	-10.000	-1.0000	-0.2300

Dataaf2:						
	10.000	-1.0316	-100.00	-1.0000	-1.0000	-0.2300
	10.000	-1.1000	-100.00	-1.0000	-1.0000	-0.2300
	10.000	-1.3162	-100.00	-1.0000	-1.0000	-0.2300
	10.000	-2.0000	-100.00	-1.0000	-1.0000	-0.2300
	10.000	-4.162	-100.00	-1.0000	-1.0000	-0.2300
	10.000	-11.00	-100.00	-1.0000	-1.0000	-0.2300
	10.000	-32.62	-100.00	-1.0000	-1.0000	-0.2300
	10.000	-101.0	-100.00	-1.0000	-1.0000	-0.2300

DataK1:						
	($\times 10^{-6}$)	($\times 10^{-3}$)	($\times 10^{-3}$)	($\times 10^{-4}$)	($\times 10^{+2}$)	($\times 10^{-2}$)
	1.0000	-1.0100	-1.0000	-1.0000	-9.9999	-0.2300
	10.000	-10.010	-10.000	-10.000	-0.9999	-0.2300
	100.00	-100.10	-100.00	-100.00	0.0000	-0.2300
	1000.0	-1000.0	-1000.0	-1000.0	0.9999	-0.2300
	10000	-10000	-10000	-10000	9.9999	-0.2300

TABLE 6.3

The effects of varying k_1 and k_{-1} for the dehydrogenase in kinetic model developed for the bioluminescent assay.

k_1 /moles/s	k_{-1} /moles/s	Peak [FMNH ₂] /μM	Peak [NADH] /μM	Time to reach peaks FMNH ₂ /s	peaks NADH /s
$\times 10^4$	$\times 10^8$	0.0135	0.0311	340	150
$\times 10^5$	$\times 10^7$	0.1159	0.2669	365	155
$\times 10^6$	$\times 10^6$	0.3954	0.9089	350	105
$\times 10^7$	$\times 10^5$	0.4370	0.9989	410	15
$\times 10^8$	$\times 10^4$	0.4355	0.9999	310	> 5

concentration of dehydrogenase can be critical, especially when $k_1 \gg k_{-1}$ and the time constants for the dehydrogenase equilibrium and the bioluminescence response to NADH are of the same magnitude. The relative values of k_1 [dehydrogenase] and k_r determine the dominant response time in this case.

A simulation of the cases for dehydrogenase concentrations between 10^{-5} and 1.6×10^{-4} M was run. The results, figure 6.1, show a clear dependence of $t_{1/2}$ upon dehydrogenase concentration. The values of $t_{1/2}$ obtained in the simulation and for real concentrations of malate dehydrogenase are shown in tables 6.4.

Experimental data was obtained using varying quantities of MDH and either luciferase and reductase immobilised on beads or the free enzymes in solution. In solution it was found necessary to replace the bacterial reductase with diaphorase.

As noted by other researchers (Vellom and Kricka 1986) we found that contamination of the bioluminescence enzymes with dehydrogenases causes background levels of light emission which become a significant problem in certain circumstances. It was found that MDH contamination of the reductase was a particular problem at the low sensitivities necessary in order for the response time of the malate assay to be determined by the rate at which the MDH catalysed reaction approaches equilibrium since very high luciferase concentrations were used to minimise the effect of the response time of the bioluminescence enzymes to a given NADH concentration.

The immobilised enzyme, with high local enzyme concentrations, had a response profile with a $t_{1/2}$ of about 2 seconds, however the background level of luminescence activity without addition of MDH was 50 % of that with MDH, the $t_{1/2}$ about 45 seconds.

Solution assays with high concentrations of the two bacterial enzymes in solution also gave a high background with a slow $t_{1/2}$ (of 10+ minutes). Use of 5'AMP purified reductase was little better and so for solution assays the reductase was replaced in these experiments by diaphorase in very low concentrations. These low concentrations are needed to avoid loss of signal strength which results if k_r becomes significant compared to $k_1[\text{MDH}]$ and/or $k_{-1}[\text{MDH}]$. This effect was noticed both with higher concentrations of diaphorase and MDH present and in the effect of trace amounts of MDH in the bacterial reductase mentioned above, ie. 50% response level with a $t_{1/2}$ of 45 seconds. With diaphorase virtually no background was observed. However the solution assays gave much lower light intensities than the immobilised enzymes, 4 mV compared to about 2000 mV

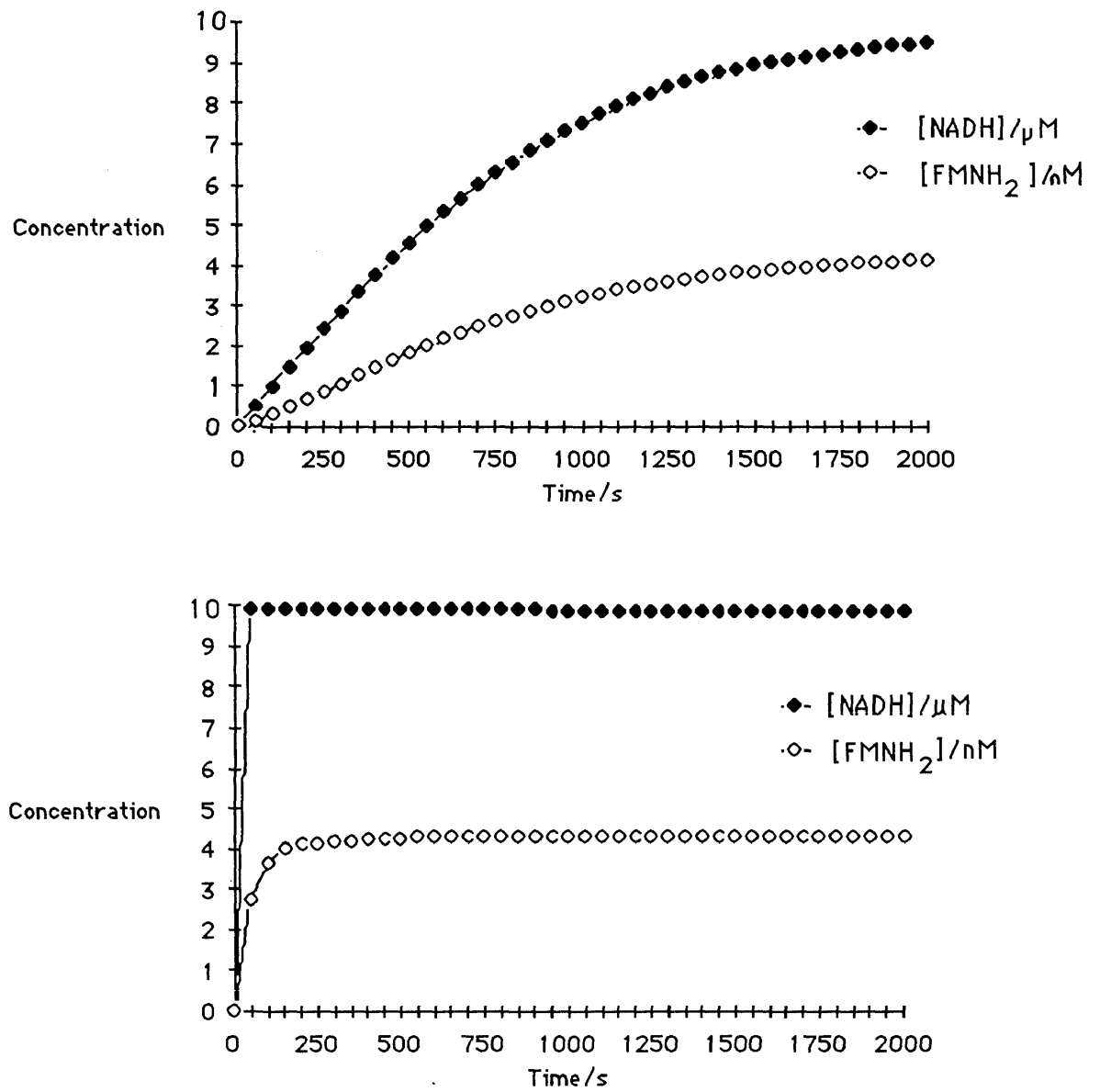


Figure 6.1: The effect of increasing the dehydrogenase concentration in simulations of the coupled dehydrogenase/luciferase system.

The results from 2 data sets (1&4) in the DATADH simulation are shown and the parameters are equal in both, except that the concentration of dehydrogenase is increased 16 fold in the lower plot.

TABLE 6.4

Effect of dehydrogenase concentration on response time in computer simulations and in experimental circumstances (data from the later part of this chapter, section 6.3).

[dh] Theoretical moles/l	$t_{1/2}$ (a) /s	[MDH] $\mu\text{g/ml}$	$t_{1/2}$ (b) /s	a/b^*	$t_{1/2}$ (c) /s	a/c
-	-	1.0	10		13	
1×10^{-4}	550	10.0	8.5	80	7	80
2×10^{-4}	300	-	-	-	-	-
4×10^{-4}	180	50.0	5	55	4	45
8×10^{-4}	130	100	4	65	5	32
1.6×10^{-3}	90	-	-	-	-	-
CONTROL FOR EXPERIMENTS:						
	1 mM NADH		2	-	-	-

with the beads. This meant that the $t_{1/2}$ figures for the luciferase/ diaphorase suffered in accuracy principally from a significant noise level of the luminometer's detector rather than the enzyme contamination problems with the bacterial reductase measurements.

The effect upon the level of bioluminescence emissions of [MDH] was negligible and the addition of extra [MDH], either doubling or increasing the concentration by 10 fold had no significant effect on light level at steady state in individual assays.

6.2.2 Effect of analyte concentration

Analyte concentration was varied over two orders of magnitude, the computer generated data for FMNH₂ (light intensity) for three of the analyte concentrations is shown in figure 6.2. It should be noted that the simulated solutions for analyte $< 10^{3.5}$ M do not reach their peak light intensities within the time limitation of the simulation. It is clear though that the values obtained have a close agreement with the theoretical solution of $kr \rightarrow 0$ and hence as defined in equation 21.

$$I_{plat} = Q_b \frac{k_l k_r}{k_a + k_l} (n^+ \text{ ah } k_1/k_{-1})^{1/2}$$

The three simulations for [Analyte] between $10^{-3.5}$ and 10^{-3} can be plotted as in figure 6.3 to show a log log slope of 0.52 close to the theoretical value of 0.5

Deviations from the value of 0.5 arise from the effect of a finite value for kr . The effect of a real flow of NADH through the bioluminescence enzymes is accentuated at lower analyte concentration. The effect depends upon the relative values of A, B and B' in equations (23) and (24) meaning that at low analyte concentration the value of kr is relatively higher compared to k_1 [DH] [AH] and k_{-1} [DH] [AH].

In the lactate assays with the immobilised enzyme system it was noted that the relative activity in response to lactate was low, high background and low sensitivity. This and the observation that the log log calibration of the light intensity against lactate concentration had a slope close to 0.6 are consistent with the theoretical model and the numerical analysis for the effects of a finite value of kr . Figure 6.3 shows, using simulated data, a plot of $\log I_{plat}$ against $\log [\text{analyte}]$; as [analyte] decreases the slope can be seen to increase.

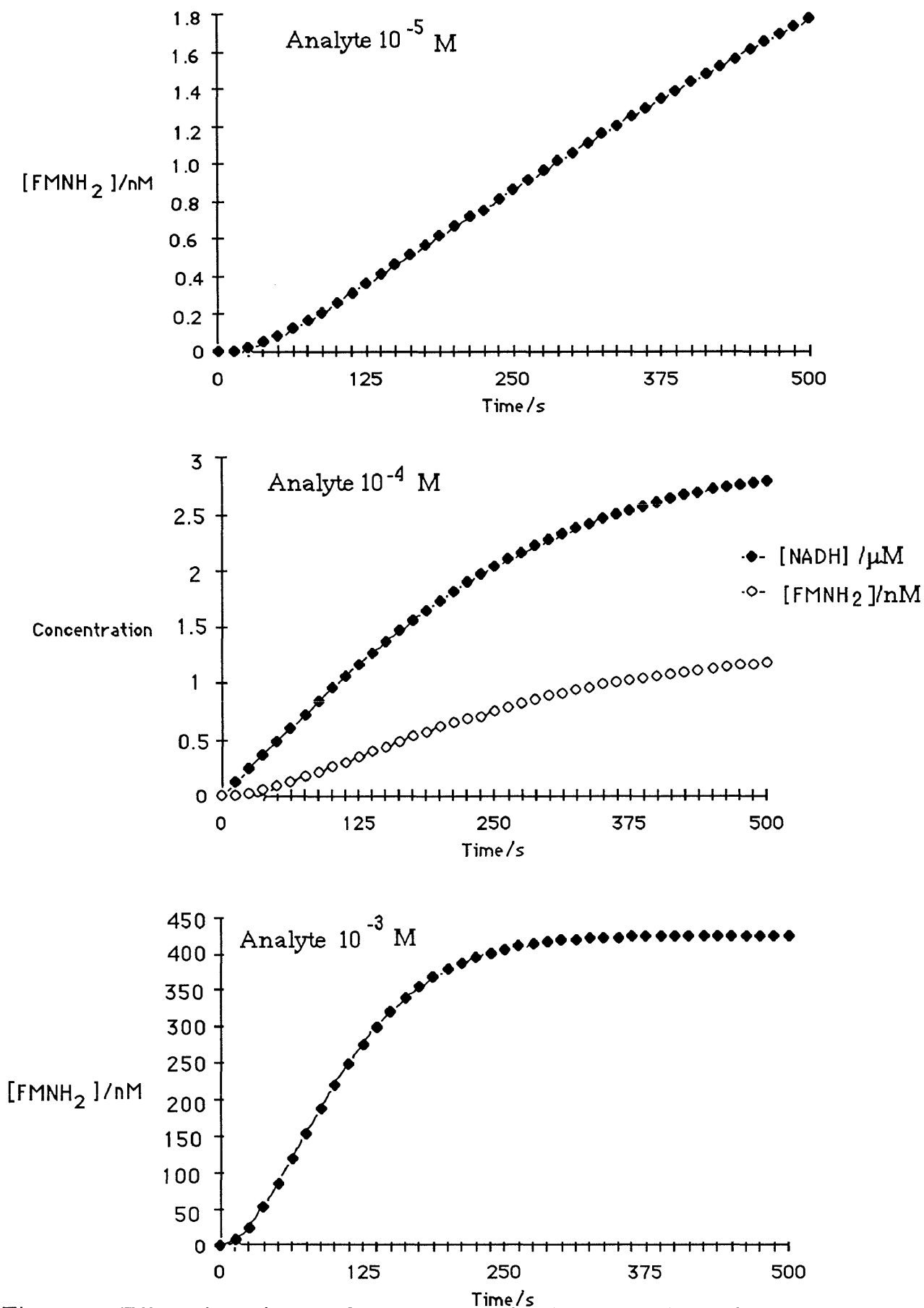
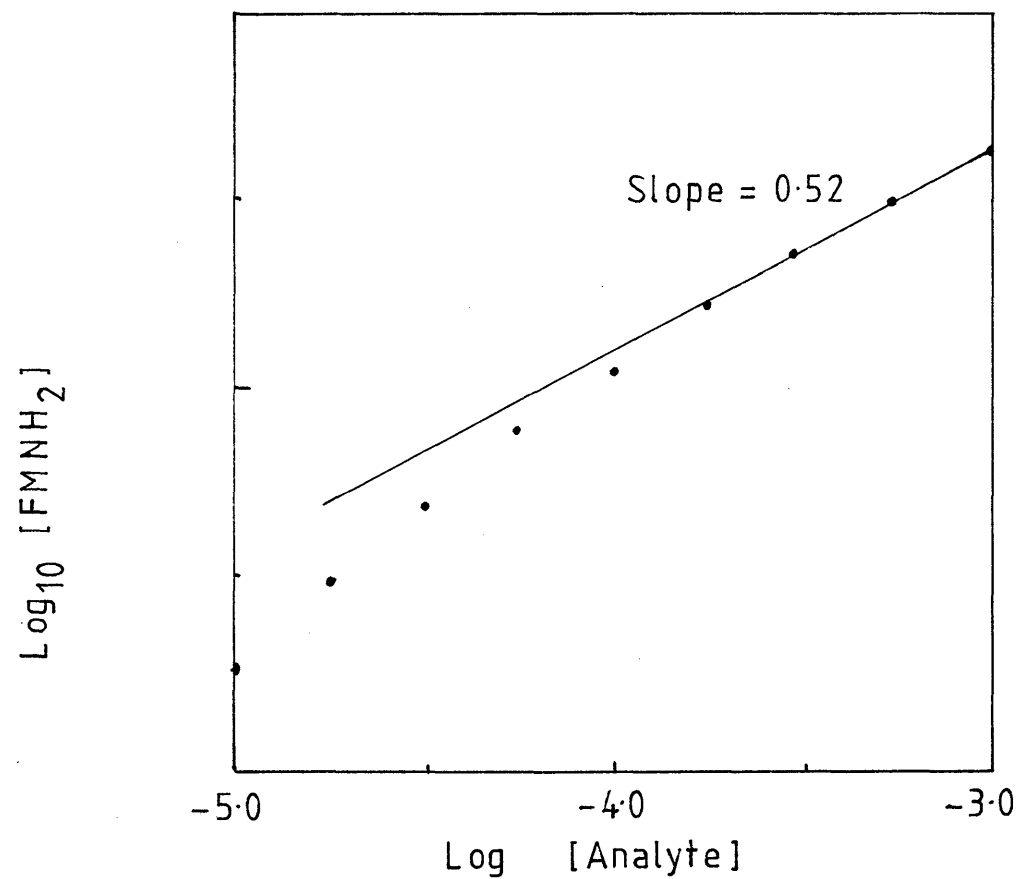


Figure 6.2 Effect of varying analyte concentration in simulations of the dehydrogenase luciferase reaction

FIGURE 6.3 Theory generated
calibration curve



The analytical solution approached for $k_r \rightarrow 0$ also predicts a dependency for the time constant governing the response upon the value of $2AB$. The full expression for $2AB$ as defined by the expression for A and B in solving equation 20 is:

$$(28) \quad 2AB = 2(k_1/k_{-1}[\text{NAD}^+][\text{Analyte}])^{1/2}$$

This also affects the response time of the assay although it is not a simple exponential time constant. As the analyte concentration increases the rate at which equilibrium is reached also increases; this is reflected in a reduction in the $t_{1/2}$ for dehydrogenase equilibration which dominates the response time at low analyte concentration.

Hence one can predict that the response time will decrease with increasing analyte concentration. From the examples plotted in figure 6.2 this indeed appears to be the case.

6.2.3 The effect of k_r 's relative value

It is the very fact that k_r has a finite value which causes the light intensity profile to differ from the analytical solution to the equation 21 when $k_r = 0$. This simulation varies the value of k_r while holding all other kinetic constants constant between simulations. Figure 6.4 shows the computer graphics for a simulation where the value of k_r has been varied over four orders of magnitude. The changes of assay profile with increasing values of k_r provide a "family" of profiles to compare with those experimentally observed profiles. As already commented on the experimentally observed response is that the intensity peaks and then falls off slowly with time. The rate at which the intensity falls off varies from experiment to experiment and in particular with the reductase activity. The simulated profiles model the experimentally observed profiles very closely in character.

The linear relationship proposed in equation (14) between I_{plat} and k_r holds when k_r is small enough to be negligible. However k_r 's of finite value mean that deviation from the analytical solution of equation (18) should be observed. The deviation from a linear relationship between I_{plat} and k_r can be seen from the values obtained in the simulation (figure 6.5). The deviation from the straight line, analytical solution, becomes progressively greater as k_r exceeds the value of the forward reaction rate (left to right) catalysed by

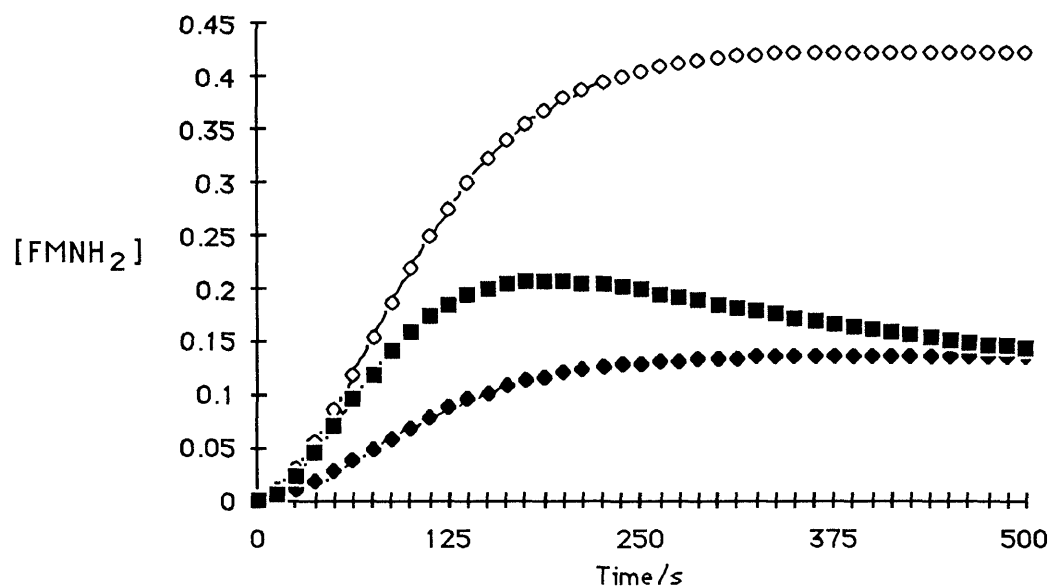
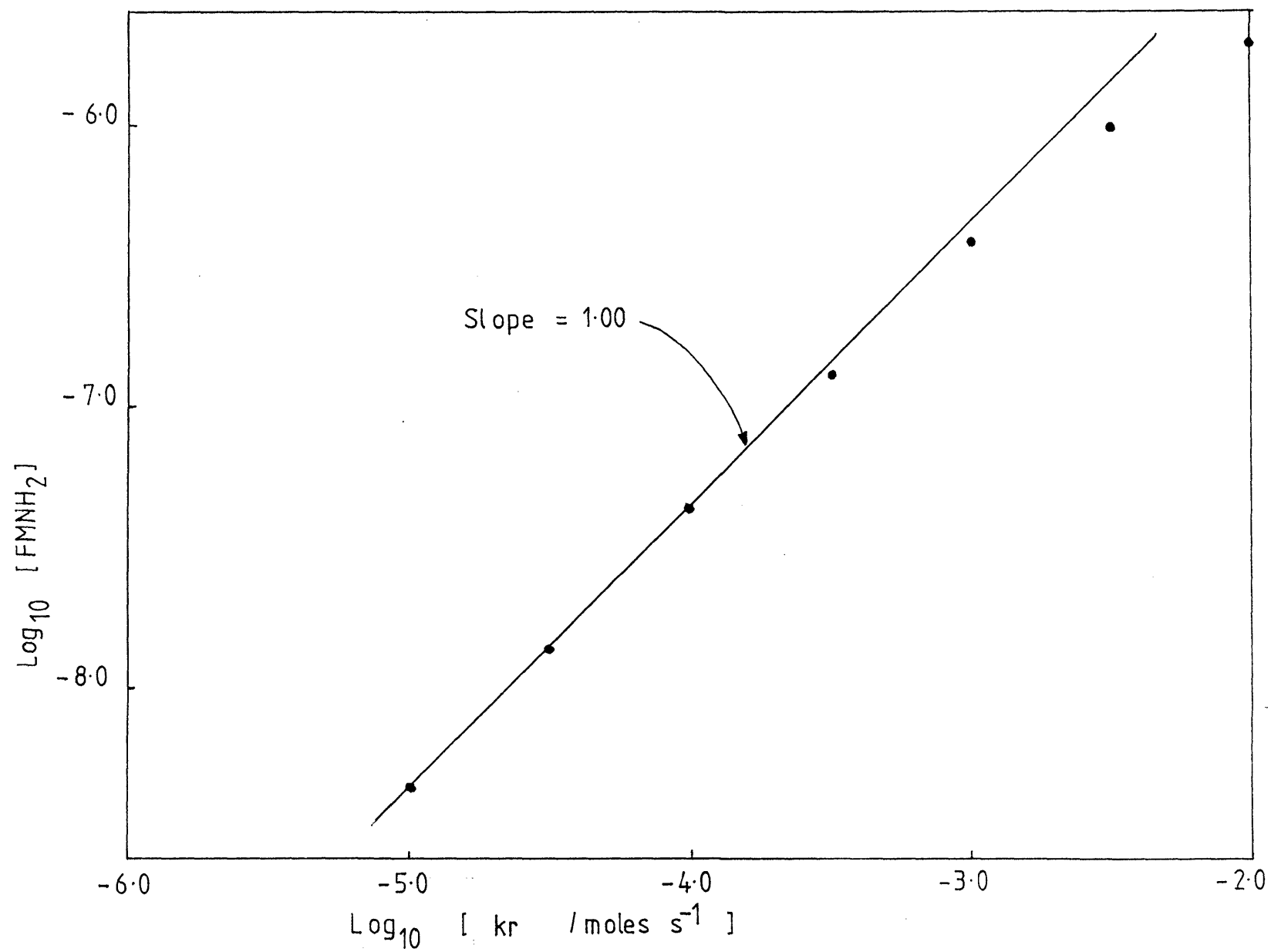


Figure 6.4: The effect of varying k_r on the simulations of the luciferase reaction. 3 data sets from the DATAF2 simulation are shown. The 3 data sets corresponding to the values for k_r shown below:

- ◆- $k_r = 3.16 \times 10^{-6} \text{ s}^{-1}$ Maximum Value 1.3 nM
- $k_r = 1 \times 10^{-4} \text{ s}^{-1}$ Maximum Value 42 nM
- $k_r = 1 \times 10^{-2} \text{ s}^{-1}$ Maximum Value 2 μM

FIGURE 6,5 Effect of altering k_r on $[\text{FMNH}_2]$



the dehydrogenase (equation (15)). In the simulation k_1 [DH] [AH] = 10^{-4} . As mentioned in the introduction the value of k_r was assumed to be $< 10^{-3}$. While this plot suggests a good agreement for this condition a stricter kinetic criteria would be to make k_r always less than k_1 [DH] [AH] for a close adherence to the analytical solution.

6.3 Kinetics of the immobilised enzyme assay

The two main changes observed throughout the immobilisations are that:

(1) There is often a marked increase in the ratio of coupled assay light intensity to that produced in the dithionite assay with an duplicate sample. The extent of this increase varies with immobilisation technique; but in the case of immobilisation on CNBr activated sepharose an increase of up to 1000 times is common. The reason for this can be seen by looking again at equation 21 and noting that while $k_a > k_l$ the level of light intensity will vary with $k_r \times k_l$.

(2) The coupled assay profile is altered. Firstly the response time is altered, increased or decreased depending on the immobilisation used. In the case of the bead immobilisations the response time is greatly reduced $t_{1/2}$ becomes a mere 2 seconds. This is consistent with the local concentration of the enzymes having been greatly increased; increased [luciferase] leads to a reduced response time (equation 6 & 14).

The profile for the sepharose preparation in assays with high NADH concentrations is also markedly altered. In particular a hump is observed (chapter 4): this effect arises from the initial loading of luciferase molecules more rapidly than they are released from the catalytic cycle so creating a temporary synchronous turnover prior to the system reaching a position of steady state; with asynchronous turnover of the luciferase enzyme (AbouKhair et al 1985).

Put into mathematical terms a brief demonstration of how the hump arises can be made. The quantity of luciferase at time t is represented by L_t . If to the first approximation 10% of the luciferase undergoing a catalytic cycle emits light and is then released two seconds later from its conformational changes we can draw up the following model. L_0 , the initial concentration of luciferase is taken as 1.0 and the concentration of NADH, FMNH₂ are so high that 50% of the available luciferase is bound each second.

$$L_{n+1} = L_n \times 0.5 + (1 - L_{n-2}) \times 0.1 \quad \text{Free Luciferase}$$

$$I_{n+1} = (1 - L_n) \times 0.1 \quad \text{Light emission}$$

Time t	Free lucif. Lt	Bound lucif. 1-Lt	Light It
0	1	0	0
1	0.5	0.5	0
2	0.25	0.75	0.05
3	0.175	0.825	0.075
4	0.153	0.847	0.0825
5	0.159	0.841	0.0847 Peak the hump
6	0.165	0.835	0.0841
7	0.167	0.833	0.0835
8	0.167	0.833	0.0833 Plateau level
9	0.167	0.833	0.0833

The above illustrates the principle of how the peak of light output from the assay forms quickly before falling to a plateau level.

The observed increases in the value of ξ show an enhanced co-operativity between the two enzymes. The value of ξ is known to depend on the ratio of luciferase to reductase (figure 6.6) and the absolute concentration of the individual enzymes.

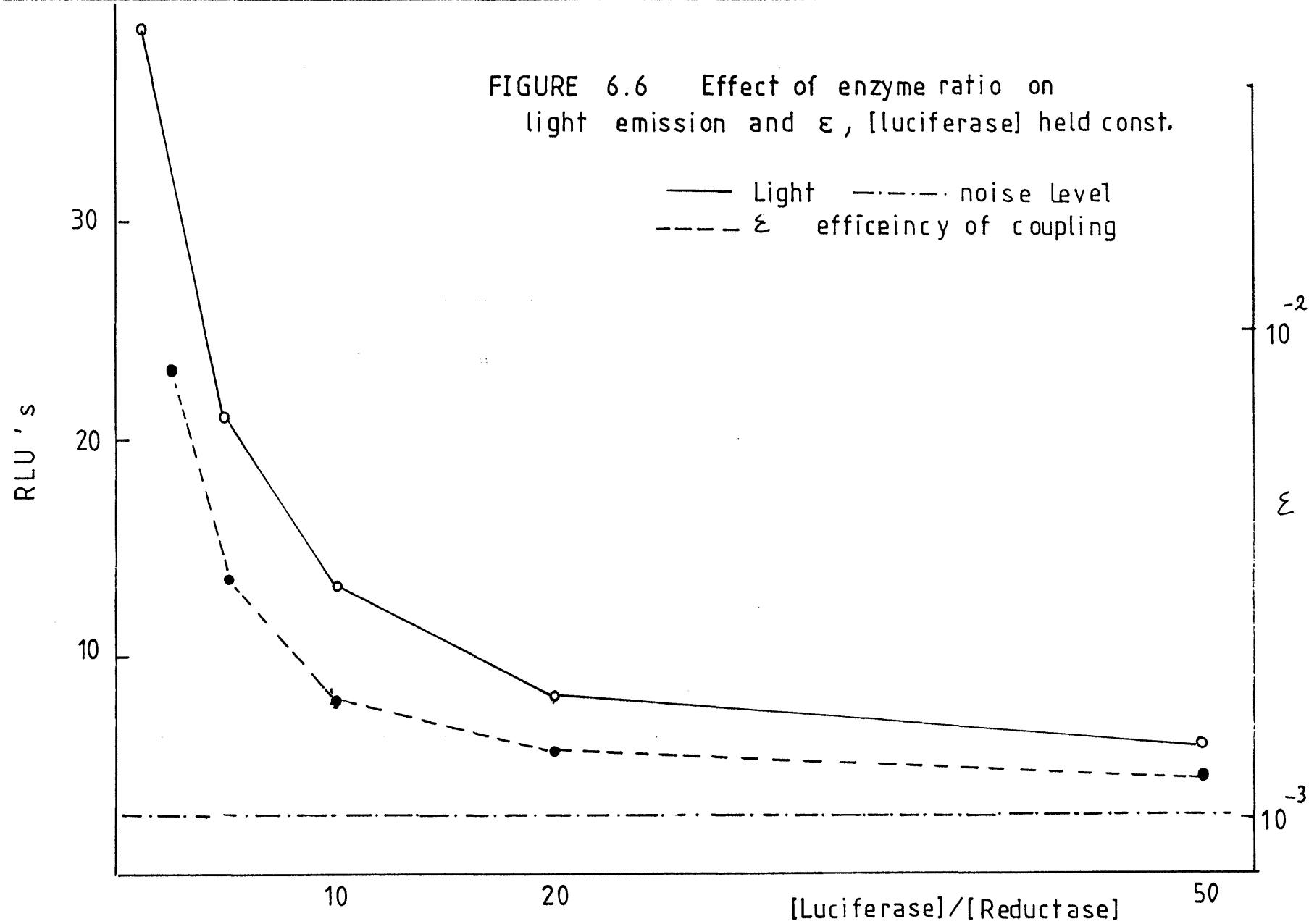
6.3.1 Substrate channeling effects on immobilisation

The kinetic model laid out in this chapter shows that the NADH coupled assay produces a light intensity which is dependent on the kinetic constants and enzyme concentrations as described by equation (16).

$$I_{\text{plat}} = Q_b \frac{k_l k_r [NADH]}{k_a + k_l}$$

In solution assays where k_l is small compared to k_a and the effect of doubling the concentration of both enzymes is a quadrupling of the light intensity and a doubling of ξ .

On immobilisation both enzymes are greatly concentrated. Typically 5 mg of enzyme is immobilised onto a settled volume of 3.5 mls, given the physical volume unavailable to the enzymes by either the presence of bead matrix or void space between beads the local concentration may



easily be many times that in the immobilisation mixture. Equation (15) describes the kind of effects which this high concentration will have in solution, these effects have been discussed above. In the case of immobilised enzymes however other effects may become significant. Goldman and Katchalski (1971) provide a model for the kinetics of a two-enzyme membrane which is applicable to any surface immobilised two enzyme system. Their findings indicate the critical importance of the relative values of $k_1 l$, $k_2 l$ and D

where:

k_1 is the rate constant for enzyme one

k_2 is the rate constant for enzyme two

l is the thickness of the unstirred layer at the surface. Ginzburg and Kalchalski (1963) have shown this to be between 10 and 100 μm .

D is the diffusion coefficient for the substrate.

They show that if $k_1 l \gg D$ and $k_2 l \gg D$ the enzyme reactions will be diffusion limited, however if $k_1 l \ll D$ and $k_2 l \ll D$ the system behaves as a homogeneous solution type assay.

Values of k for the three enzymes, dehydrogenase luciferase and the reductase can be estimated from the known experimental data.

k_1 [DH] 10^{-5}M
forward reaction rate 10^3

k_2 [oxidoreductase] $k_r \sim 10^{-2} \times k_1$
reaction rate

k_3 [luciferase] 10^{-6}M
 $k_{\text{cat}} 0.1\text{ s}^{-1}$
 $K_m 0.1 \times 10^{-6}\text{M}$

$k_1 l = 10^{-5}$

$k_2 l = 10^{-7}$

$k_3 l = 10^{-3}$

$D = 5 \times 10^{-6}$

The implication of these figures is that both the dehydrogenase and the luciferase will demonstrate some

diffusional limitation while the reductase will not. These figures are however only rough and are all within two orders of magnitude of the value for D . Hence some apparent substrate channeling effect might be expected to occur through local build up of products in the unstirred layer. This is a local concentration effect acting at each individual bead. It is the local concentration on the bead that determines the substrate channelling effect. As shown in figure 6.7 the light intensity of the assay increases linearly with bead concentration.

Nevertheless the equation (21) will still determine the effect of enzyme concentrations on the level of light output. The most striking observation on this analysis is that if $k_l > k_a$ the effect of additional luciferase is zero on the light intensity detected. It also means that loss of activity in a preparation will not effect the light output of the system. Hence if luciferase is present in huge excess the concentration of k_r varying with time is likely to be the major source of error.

Since the reductase is not diffusion limited the level of NADH is independent of $[DH]$ the effect upon coupling efficiency in a non diffusion limited system would be zero. As the immobilised system is likely to be diffusion limited for the enzyme some substrate channeling effects will be observed between dehydrogenase and the reductase. The values of ξ for NADH and Lactate and malate coupling show increased efficiency at between both DH and reductase, and reductase and luciferase (table 4.8).

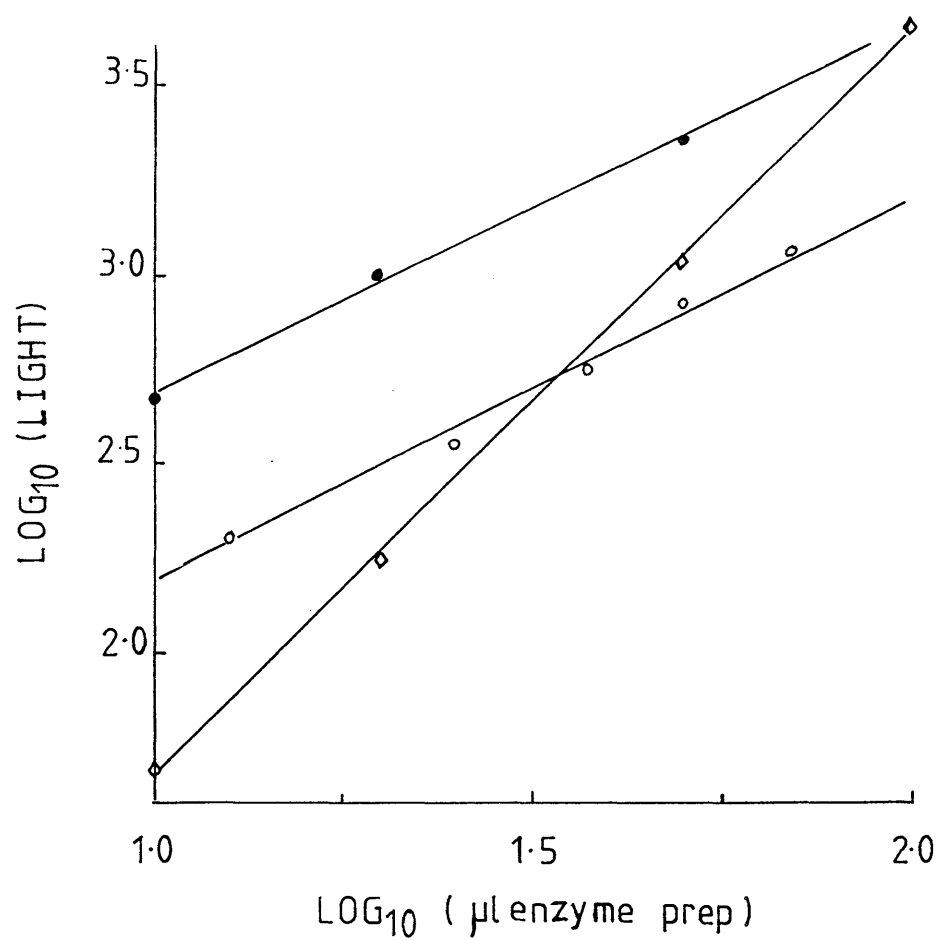


FIGURE 6,7 Effect of increasing quantity of enzyme preparation

- ◇ free solution enzymes
- DE 52 immobilised luciferase and reductase
- Sepharose " " " "

6.4 EXPERIMENTAL TESTING OF KINETIC MODEL

Dependence of NADH assay response time on luciferase and oxygen concentrations

In section 6.2 analysis of the NADH assay kinetics led to a mathematical model of the assay which predicted the time dependence of the light intensity generated. The time constant for the exponential expression developed showed that luciferase and oxygen concentrations defined the response time for the assay. The expression for $[FMNH_2]$ and therefore I developed in that chapter was:

$$[FMNH_2]_t = \frac{k_r [NADH] (1 - e^{-(k_a + k_l)t})}{k_a + k_l}$$

The time constant for this expression is $k_a + k_l$ where:

$$k_a = k_a[O_2]$$

and

$$k_l = \frac{k_{cat} [L]}{K_m}$$

Experiments varying either O_2 or $[L]$ were performed and the effect on the value of $t_{1/2}$ for approach to a plateau light intensity recorded.

Dilutions of a stock solution of luciferase at 1.25 μM concentration were used to obtain light intensity profiles for a standard coupled assay with a fixed concentration of 1.0 μM NADH (two orders of magnitude below saturating $[NADH]$). The experiment worked well producing a straight line plot (figure 6.8) of $\ln 2/t_{1/2}$ against $[L]$. The plot has a correlation coefficient of 0.998 and gives k_a a value of $25 \times 10^{-3} s^{-1}$ and $k_l/[L]$ for luciferase of $1.4 \times 10^6 s^{-1}$. This value is consistent with the known values for k_{cat} ($0.125 s^{-1}$) and K_m ($10^{-6} M$).

Controlling the $[O_2]$ level with glucose oxidase and catalase enzymatically converting a known quantity of glucose to gluconic acid with the consumption of 1/2 mole of O_2 for each mole of D-glucose used proved a far more error prone method for obtaining k_a and k_l values. The results obtained (figure 6.9a) show a curve rather than the expected straight line because of low $t_{1/2}$ at the saturating $[O_2]$. The plotted points themselves have an inaccuracy of $\pm 10\%$, control of the $[O_2]$ particularly at lower levels is difficult without the use of a

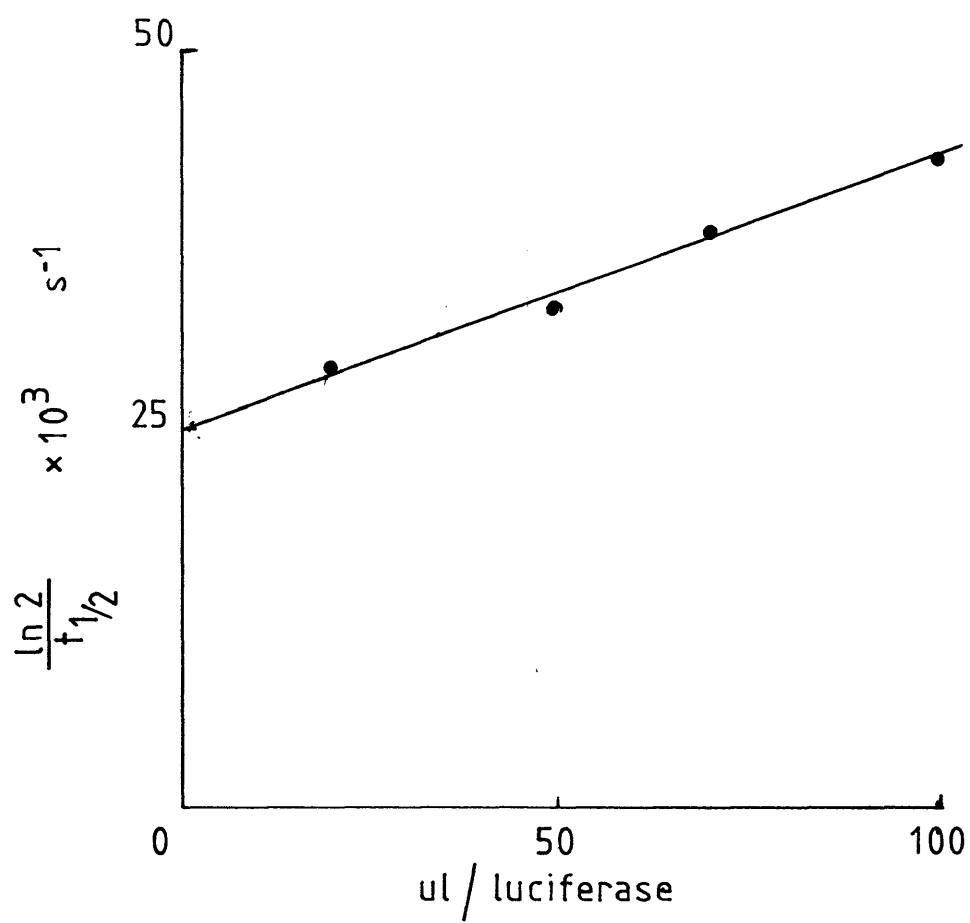


FIGURE 6,8 Relationship between $t_{1/2}$ & [L]

FIGURE 6.9a Effect of $[O_2]$ on $t_{1/2}$

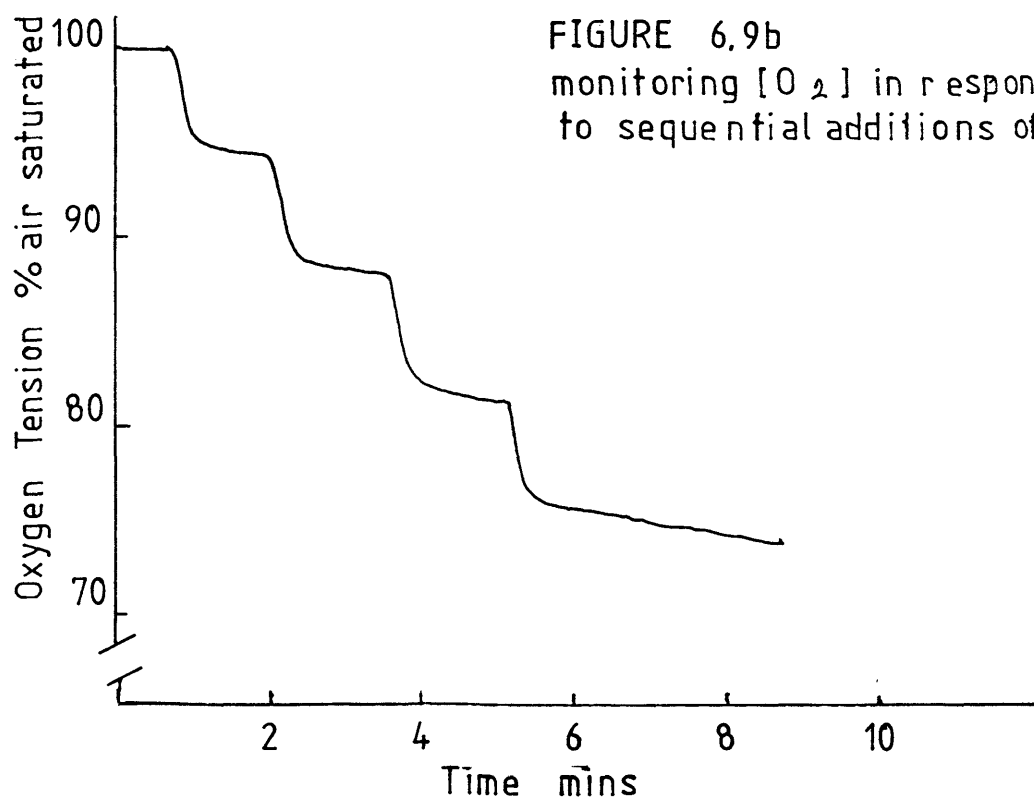
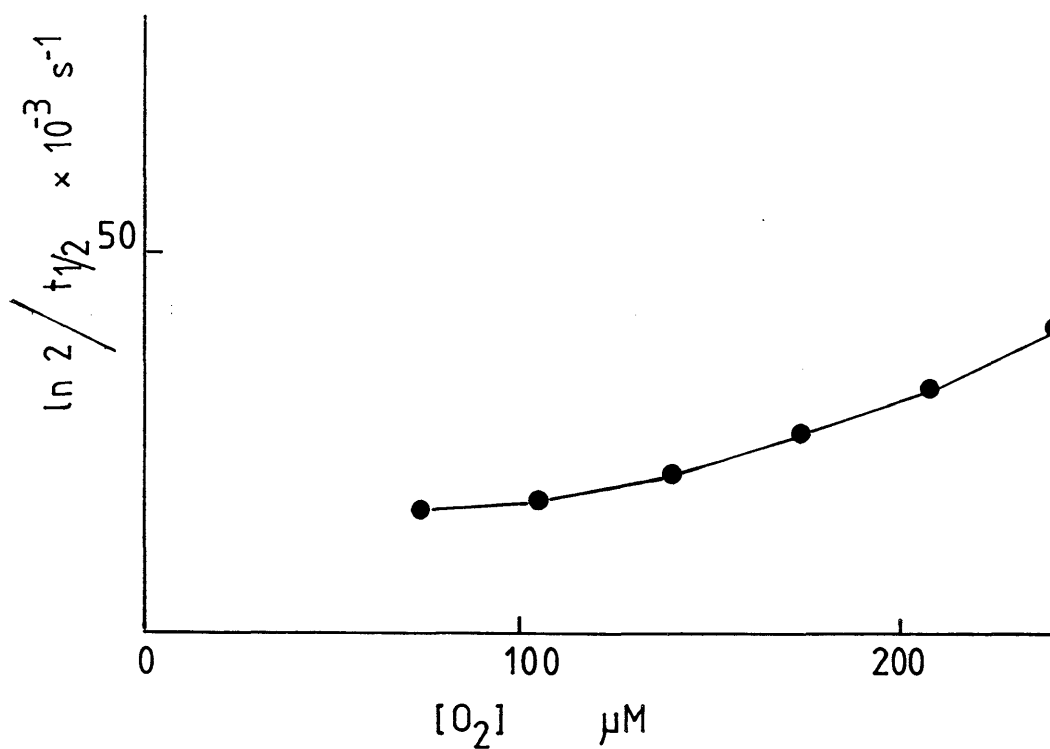


FIGURE 6.9b
monitoring $[O_2]$ in response
to sequential additions of glucose

specially constructed chamber, the O_2 electrode chamber used to calibrate the glucose solution uses a stirred compartment sealed from the air, the assay cuvette is open to the air and unstirred. However within these limitations the very approximate values for k_a and k_l are consistent with those obtained in the previous experiment.

D-glucose solutions were calibrated using an O_2 electrode the resultant rate of O_2 consumption is shown by the traces in figure 6.9b. The rapid removal of 70% of the expected quantity of oxygen is followed by the slower removal of the other 30%. This behaviour reflects the existence of two anomers, α and β D-glucose which in solution exist in the equilibrium. Glucose oxidase catalyses the reaction with the α anomer only accounting for the rapid removal of only 70%.

CHAPTER SEVEN

CONCLUSIONS AND FURTHER DISCUSSION OF THE IMMOBILISATION AND ANALYTICAL USES OF BACTERIAL LUCIFERASE

7.1 Overview

In this concluding chapter I discuss the results and findings of the four preceding sections. In particular the extent to which the work has developed to satisfy the original objectives. As remarked upon in the introduction the chosen bioluminescence system from V.harveyi has been found by previous researchers to possess sensitivity to a wide range of analytes. However all the areas of knowledge about the system's function, character, immobilisation and even purification needed improvement to enable its use as the basis for a commercial analytical system.

7.2. Purification protocols

The choice of protocol must in the end reflect the end requirements, what quantities of enzyme are required and in what purity.

The original techniques of Hastings et al (1978) allowed for the production of small quantities of luciferase, in the region of 100 mg of high purity luciferase and the concomitant crude purification of the FMN:NADH oxidoreductase. The whole protocol would take 1 to 2 weeks. While adequate for research purposes the protocol becomes inadequate when a scale up for immobilisations is required, in addition it was found that for use in a biosensor device the purity of luciferase demanded was not as high as originally used.

The changes described in chapter three have:

- * Scaled up the fermentation of the V.harveyi organism to 400L batches, with every indication that further scale up of this step is possible.

- * High percentages of the luciferase in the cells can be released and purified to a satisfactory purity for immobilisation, 60% + in less than a week. With further purification to 99% luciferase for research use possible in a single FPLC step taking 30 minutes.

It should be borne in mind that the above technique has been developed from the original Hastings protocol using standard purification materials and equipment. Other workers have sought to develop more specific

purification protocols for luciferase, two methods are described in Methods in Enzymology 133 (Baldwin et al 1986, O'Kane et al 1986).

The first method uses an affinity column based on the luciferase inhibitor 2,2-diphenyl propyl. The method (Baldwin et al 1986) describes the use of a 2,2-dipropylamino DOPA affinity gel either in a column or in a batch elution method. While the purification is simplified with the affinity column extracting the luciferase directly from the lysate it has two drawbacks. The method does not co-purify the FMN Oxidoreductase at all, it also requires a large volume of specially manufactured affinity gel - 500 mls is needed for the faster batch elution protocol. Where large scale purification of high purity luciferase is sought the affinity technique has much to offer: however for this project the method developed in chapter 3 uses commonly available materials and provides both the luciferase and reductase enzymes in the required purities.

The second method (O'Kane et al 1986) describes a final purification stage using HPLC chromatography. This method is analogous to the FPLC method developed in chapter 3, only using several columns to replace the low pressure steps remaining in the full purification described in chapter 3. As with the affinity method the reductase is separated earlier and as with FPLC unless preparative columns are available the amounts of protein which can be processed are small.

7.3 Immobilisation techniques

A wide range of immobilisation methods were tried during the course of this project and the results given in chapter 4 show that they met with varying degrees of success. In view of the objective of the project to study the analytical applications of the bioluminescence system, the more exotic and complicated methods were not pursued as a first priority nor were methods where it was quickly apparent that the technique had such severe drawbacks that it could not be reasonably expected to produce a working assay system. Below are some further brief conclusions on the techniques studied and comments on their usefulness and the problems encountered.

Broadly the problems were of three types :-

- * Manufacture of the immobilised product is difficult and does not result in an accurate calibration curve because of poor reproducibility between assays.

- * Loading sufficient enzyme on to the immobilisation surface or carrier to produce an assay which emitted sufficient light to provide a sensitive, reproducible and

measurable assay system.

* Leaching of enzyme was found to be a problem in almost all but the covalent coupling techniques. In some cases it not only caused problems with re-use of an immobilisation preparation but affected the emission profile of the "first use" assay too.

7.3.1 Entrapment techniques

A wide range of entrapment techniques were tried, but success was limited by either or both of the following effects; the leaching of activity from gels and the slow response time observed for coupled assays with gels caused by the carrier/matrix structure impeding the mass transfer of substrates to the enzymes.

The more chemically severe entrapments were largely unsuccessful, in particular those using free radical chemistry - polyacrylamide and HEMA gel techniques - which were shown to destroy the enzymatic activity in solution as well as in the final gel product. The classic protein membrane technique using glutaraldehyde and feeder protein failed for the same reason, inactivation by the immobilisation agent.

The use of cellulose acetate to entrap the enzyme behind a film had limited success. The technique was complicated by the inhibitory effects of solvents used in the formation of the film and the somewhat chaotic nature of the reaction between the film mixture and cuvette walls resulting in uneven distribution of luciferase. Attempts to improve the technique by using solvent compatible liquid ion exchange resin and either lyophilised enzyme or enzyme in solution failed to solve the problem. However after removing loose enzyme from a prepared cuvette re-use of the cuvette in serial assays was possible although slow response time and low levels of emitted light made pursuing this technique unattractive.

As was shown in section 4.1.3 with DE-52, considerable improvements can be made by using simple composite techniques, i.e. the gel entrapment of enzymes already attached to an ion exchange resin. This enabled the problem of leaching associated with gels to be overcome as well as allowing the beads carrying the enzyme to be held in a stable arrangement overcoming the problems of settling associated with freely suspended beads; the settling of these during an assay may be a contributory factor to experimental errors. The problem of slow response time was however not overcome and assay reproducibility between slices of gel also remained a problem. Although further work on improving the gel manufacture and rigidity may have reduced these remaining

problems enabling uniform ultra thin slices of gel to be used.

Since this was really only a problem of how best to present a bead immobilised system in a stable configuration other solutions namely a packed enzyme bed were pursued in preference.

Incorporation of co-factors was tried with the composite gel preparations and although it was found helpful in increasing the level of response the slow build up of emission levels remained a problem and was interpreted as either diffusion limitation of the analyte or decanal, which is not compatible with storage in an aqueous environment. FMN storage was also found to be problematic and a cause of lost activity on some occasions.

7.3.2 Adsorption Techniques

Both ionic and hydrophobic adsorption techniques resulted in binding of active enzyme. Table 7.2 summarises the efficiencies and response times observed for both adsorption to DE-52 and polystyrene surfaces. As was noted above both techniques also gave excellent calibration curves for the assay of NADH.

DE-52 adsorption was both quick and simple to perform, and as such offers a very convenient method of immobilising the bacterial bioluminescence system with a marked increase in the efficiency of substrate channelling. However the tolerance of the flow system to pH and ionic strength in repeated use was not good, compared with covalent coupling, as one would expect from this type of immobilisation. Further most dehydrogenases are cationic making the immobilisation of complete dehydrogenase assay systems much more difficult. As seen in the previous sections it was necessary to immobilise MDH on CM-52 and mix the two preparations for an immobilised malate assay.

In situations where pH and ionic strength are going to be within a narrow range. The method may be compatible with the characteristics of the resin and offers a very useful and cheap solution to immobilisation of the enzyme although not as robust and versatile as the CNBr sepharose product.

Immobilisation on polystyrene surfaces, standard cuvettes and latex beads both appear quite robust and capable of repeated use. As with the DE-52 adsorption the method was successful in producing a good NADH calibration curve. The principle drawbacks to the technique were:

TABLE 7.1

Summary of adsorption immobilisation techniques: activity recovery, coupling efficiency, sensitivity and response times.

Immobilisation	Activity Recovery	Activity Efficiency Beads Σ_1	Soln. Σ_2	Σ_1/Σ_2	Assay Time mins
DE-52	90%	0.16	0.001	160	<0.2
Latex Beads	8%	0.00286	0.00038	7.5	c0.5
Cuvettes	8%	0.00236	0.00049	4.8	c0.5

Activity refers to the luciferase activity recovered and figures are based on the control experiments i.e. The DE-52 will release 90% of adsorbed activity (only 10% is measured when bound), PPL causes upto 92% inactivation of luciferase.

The time response values for Latex beads and cuvettes were difficult to measure since the light levels were so low the real figure is possibly much better than this.

* The low levels of enzyme loading achieved made extensive study of the resultant product difficult with the detection equipment being used. Difficulties arose in assessing the level of enzyme immobilised and inaccuracies resulted from these low activities in some experiments. This led to the conclusion that even with small, 16µm, latex beads insufficient light was produced to progress with the technique. The problem of low enzyme activity was made worse by the fact that poly (phenyllysine) inhibits the bioluminescence reaction by around 90%, a non inhibiting adsorption agent might result in significant improvements in the techniques success.

* As with the DE-52 adsorption an anionic exchange adsorption was used in this technique and even if a heavier enzyme loading were possible there would be no guarantee of co-immobilising the dehydrogenase enzymes with the mix of anionic and hydrophobic forces available.

7.3.3 Covalent Coupling

In the context of the three key areas where problems arise in immobilisation: leaching, diffusion limitation and loading of active enzyme, covalent coupling is the obvious way to get round the first of these and if the attachment is to a surface exposed to the bulk solution and not separated by a membrane of any type it is also a convenient solution to the second problem. Loading of enzyme can however be a problem, firstly the level of enzyme loading will be related to the surface area available, secondly the inactivation of a portion of the enzyme population will further reduce the number of active enzyme molecules that may be loaded onto a surface. Some brief calculations that illustrate this point were presented in section 4.2.2. It was found that this problem was a serious hurdle to direct immobilisation to the cuvette surface as in the experiments using poly (phenyl-lysine), other attempts at immobilising directly to acrylic cuvettes and nylon tubing were abandoned quickly after initial attempts produced insignificant levels of activity.

As a result of this problem the bulk of covalent immobilisation work has concentrated on large surface area carriers which will provide the necessary final activity to study the bioluminescence assay in practical applications considering the sensitivity of the equipment being used.

7.3.3.1 CNBr Sepharose 4B as a carrier

Immobilisation studies confirmed the usefulness of the technique for immobilisation of bioluminescence assay enzymes, and the co-immobilisation of dehydrogenases needed for individual assay applications. As with other methods substrate channelling between reductase and luciferase was observed, an even greater degree of channelling was observed for the complete assay pathway from analyte, ie. malate, through to light emission. Substrate channelling between dehydrogenase and reductase is due to the common effect of increased enzyme concentrations, in the reductase to luciferase step the intermediate is unstable and reduced physical paths between enzymes limits time for the competitive oxidation of FMNH₂ by O₂ to occur.

Intrinsic enzymic properties such as kcat and Km do not appear to have been significantly altered, although the effect of substrate channelling does cause some distortions to the emission profile within an order of magnitude of Km for analyte levels of NADH, as discussed in section 4.3.1.3. The response time and calibration curves for the preparations of immobilised enzymes were good.

One of the applications explored for the assay was the determination of malate in wine. When working with acidic samples such as wine it would be convenient to use an assay with an operational pH close to the sample. A shift in the pH profile for the bioluminescence assay was achieved with DE-52, the incorporation of polylysine into the CNBr sepharose matrix was tried in the hope that by giving the preparation a positive charge the pH profile would shift as for DE-52. While a small shift of half a pH unit did occur, but rather than broadening the pH tolerance as with DE-52 it was noticeably narrowed.

It was concluded that shifting the pH optimum of the assay by choice of carrier characteristics might be useful in some applications. For a general purpose method where samples are likely to be of highly variable character in both pH and ionic strength a simpler route is to use a pre-assay treatment protocol which will standardise the pH and ionic strength to a point within the activity optimum plateau region.

7.3.3.2 Controlled Pore Glass as a carrier

The results using aminopropyl CPG as a carrier were disappointing. The approach was used to find a more efficient method of immobilising the enzyme, ie. one that destroyed less than 90% of the initial enzymatic activity. Published results did not rule out a coupling through carboxyl groups, and the labeling work described in section 4.4 indicates that in mild conditions several

carboxyl groups are preferentially modified with only minor loss of activity.

Indeed the results in section 4.3.2.2 indicate that a relatively high loading of activity can be obtained by simple adsorption to the glass, with little improvement brought about by the use of the carbodiimide as a coupling agent.

It is worth noting that the results described in section 4.4 show that precise conditions under which luciferase reacts with carbodiimide affects the degree to which the enzyme is inactivated. It is a kind of "catch 22" that a porous support is required for the desired enzyme loading to be achieved yet the porous nature of the support removes control over the exposure time of enzyme and coupling reagent by entrapping both in the matrix structure.

A possible strategy to get round this problem would be to hold the beads in a flow cell and then flow a carbodiimide immobilisation mixture over the beads. Since only one Flow cell of each size had been commissioned this would have contaminated the flow cell and so was not tried. However it would have allowed tighter control over exposure to immobilisation agents and more rapid washing of the beads to remove coupling agent.

7.3.4 Chemical modification of Luciferase

It was found that the enzymatic activity of luciferase is sensitive to carbodiimide reagents, the degree and rate at which inactivation of the enzyme took place was found to be dependent on temperature, pH and reagent concentration. The presence of taurine as a labelling agent altered the inactivation profile and allowed some quantification of the extent of modification during inactivation. Although it is significant that biphasic inactivation profiles are also observed in the absence of taurine albeit with some differences in inactivation rates.

Inactivation appears to be in two phases, however many more than two residues per molecule are affected during the biphasic inactivation. The first phase is associated with about 5 moles of taurine becoming attached to each mole of luciferase, the second phase leads to the incorporation of upto 120 moles of taurine per mole of luciferase which indicates that modification of almost all carboxyl groups must be taking place. Although it is worth noting that normally the formation of peptide linkages occurs at a pH of 4.5 due to the reaction of carbodiimide with the protonated carboxyl group i.e. the

carboxylic acid. Obviously the high concentration of taurine used at a pH of 7.0 has nevertheless resulted in near complete modification. The formation of a precipitate when the degree of inactivation approaches 99% is consistent with extensive modification and conformational change to the enzyme with or without the presence of taurine during the inactivation.

SDS-PAGE of modified protein did not show significant change in banding pattern from native protein, although the extent of protein precipitation may have resulted in an unrepresentative portion of enzyme being examined on the gel it does not indicate significant cross linking of subunits. The experiments with substrate protection and reversible protection of thiol residues indicate that although thiol groups are modified in the experiment this modification is not the principle reason for lost activity.

It appears that the inactivation of luciferase is a result of the modification of large numbers of residues, this modification may include intra-molecular and/or inter-molecular cross linking but is associated with the visible precipitation of a large portion of the enzyme material. Precipitation may result from conformational changes, changes in charge distribution on the surface of the enzyme or inter-molecular crosslinking could all cause both precipitation and inactivation. Enzyme activity is known to involve conformational change during the catalytic cycle (AbouKhair *et al* 1985) and hence conformational changes alone could be the cause of inactivation without implication of essential residues.

To further investigate the precise cause of inactivation a substantial investigation into the modifications to the enzyme would have to be undertaken. To start with the modified protein could be treated to give peptide fragments to reveal the modified residues and any intra molecular cross links introduced during inactivation. Such a protein chemistry project might also reveal some additional information about the tertiary structure of luciferase.

7.3.5 Aldehyde (Decanal) and Co-Factor Immobilisation

The non-enzymatic components of the assay also needed to be packaged in some form.

The buffering solution can easily be supplied in a prepared and bottled solution or as a fast dissolving solid.

The two co-factors could also be packaged with the buffering components, however NADH is slowly degraded in

aqueous solution at neutral pH and FMN is very sensitive to exposure to natural or artificial light - the sensitivity to artificial light is much less than natural light which will cause problems if solutions are left exposed to it during preparation of assays - the evidence of the gel entrapment experiments indicated that FMN was the more problematic of the co-factors. For dry preparations the co-factors can easily be stored as part of the preparations. In any final solution the co-factors would have to either be regenerated in situ or constantly replenished, hence either they are supplied at the required concentration in the buffer solution as was the case for most of the studies discussed in this thesis, or the co-factors can be physically immobilised in the same way as the enzymes. Entrapment of the cofactors as mentioned above was tried but it was of limited success and for applications involving re-use leaching would have been a serious problem.

Immobilisation by covalent coupling could offer a solution, however there is little evidence for the successful immobilisation of all components in such a complex system as this. A priori the main problem would be the immobilisation of NADH, which in the assay is used by both the reductase and the dehydrogenase, in a position where it could communicate between the two enzymes. FMN also has to communicate between the reductase and luciferase, these enzymes occur naturally in the same enzymatic system and there is some, not very strong, evidence that they may associate naturally thus increasing the chances of a close immobilisation of all three molecules. A further problem for FMN is that from the kinetics and ratios of the two enzymes needed implies that each reductase molecule is serving many luciferase molecules. Nevertheless the work with CPG and FMN as a substrate protection strategy during carbodi-imide immobilisation indicated that the level of background light emission was raised in the absence of FMN in the buffer solution although there is no way of ascertaining whether this was due to either immobilised FMN or trace levels of FMN adsorbed to the glass and trapped in the structure.

If an attempt were made to immobilise all five components as described above the work would have to involve a considerable amount of protein engineering in the approach. An attempt was made to co-immobilise luciferase and reductase on BSA previously treated with lysine and carbodimide to produce a synthetic enzyme complex; however problems separating an active fraction of specific molecular weight range caused me to abandon what was really an exercise in protein chemistry/engineering in order to concentrate on studying the application and dynamics of the bioluminescence system.

Decanal is the final component in the assay system: unfortunately the substrate has some properties which lead to serious if not irreconcilable problems in incorporating it into an immobilised assay system. As outlined in the introduction decanal is unstable in aqueous solution, undergoing autoxidation to form the fatty acid decanoic acid: it also causes inhibition of the luciferase enzyme activity if present in high concentrations by forming deadend complexes (Kurfast et al 1985). This has the effect in solution assays of causing significant changes to the observed emission profile depending on the order in which the assay components are mixed. For instance in early experiments to define the protocol for the standard assay procedure decanal was added before FMN and the bulk of the buffer solution to the luciferase; this resulted in the level of light being greatly suppressed and rather than forming a plateau quickly the light level slowly rose for the next 10-30 minutes, the time over which the assay was followed.

To overcome these problems the decanal had to be separated from both the aqueous element and enzymatic element during storage. The uses of decanal/solvent mixes was investigated as an extension of the assay's normal protocols where to handle the minute quantities of decanal needed stock mixes were made to store the decanal. The further observation was made that solvents could be used to alter the structure of the polystyrene cuvette and hence deposit material at a defined location on the surface. All solvents used were inhibitors of luciferase, so either the degree of inhibition had to be acceptable or the solvent removed. The technique developed (Manuel and Cass 1985) was found to work well although precisely how the deposited cyclohexane/decanal solution alters the polystyrene surface and delivers the decanal to the assay solution is not clear. Some ideas on this are outlined in section 4.5.3. It is probable that both evaporation, causing removal of solvent, and hydrophobic partitioning are key factors in the method. From the work described earlier the method would lend itself to providing a means of depositing the required decanal in a polystyrene vessel for storage prior to the assay. Then the assay solution/buffer could be passed over the immobilised decanal, such a system could involve either deposition on the cuvette for one shot assays or on the inside of a syringe for the flow injection analysis.

7.4 Flow injection Analysis (f.i.a.) and packed enzyme beds

The different techniques of immobilisation were all

precursors to developing an assay system and studying its applications and behaviour. The immobilised enzymes make the detection part of the system, the next problem is the designing a system to deliver the sample to the sensor for measurement. With biosensors several key considerations are preparation of sample, mechanics of operating the assay, reusability of the system. The chapter (5) on f.i.a. examines one of the solutions to these problems. The f.i.a system proved to be the easiest of the methods to use for assaying samples and in some senses is the best and most sophisticated of the systems discussed in this thesis.

When comparing the f.i.a. system with other solutions to the problem of developing an assay system, washable cuvettes, one shot preparations and other reusable preparations like slices of gels should be considered.

The key advantages of a f.i.a. system were ease of use and the reproducibility between assays. These are both derived from having the same set of immobilised enzyme beads in subsequent assays. As described in chapter five the system compared favourably to that recently described by Vellom and Kricka (1986).

The hydrodynamic flow experiments point to a way of using the system with a substantially lower cost method of driving the buffer flow. In the experiments the flow rate was controlled by a motor driven syringe which could control flow rates to better than 10 μ l/min. However the studies of various flow rates showed a plateau region of assay performance for flow rates of 1.00 $\pm$ 0.20 ml/min, hence for applications where accuracy of only $\pm$ 5-10% is tolerable a much cheaper drive system could be used. The high levels of sensitivity and light emission obtained with the packed beds would also permit a substantially cheaper detector system to be used, i.e. a photodiode rather than a photomultiplier based system.

Sample preparation remained a problem in some cases, particularly the case of some dairy products. However simple sample preparation steps can be developed to clean up samples before analysis. The nature of the enzymes is the problem here and it is difficult to see any way round some basic sample preparation steps since the assay is sensitive to both colouration of samples which would absorb the emitted light and inhibitors of the luciferase enzyme in particular long chain aldehydes, fatty acids and alcohols and proteases which may be present in biological samples. Buffering the sample in a solution of appropriate pH was easily achieved by dilution into the assay buffer.

With the two analytes examined, malate and lactate, methods of using the system were devised which gave

consistent results. The decolourisation of wine samples was straight forward with subsequent assays fitting neatly onto a conventional calibration curve. The assay of dairy products with the f.i.a. for lactate was more complicated and although a method involving three assays gave results consistent with each other across different sample dilutions the results differed from those obtained using a commercial kit - although problems with the kit were also experienced - but the nature of the different degrees of inhibition caused by the different samples was not explained although several suggestions might be made.

- 1) Milk related fats could inhibit the activity of luciferase by competing with decanal for binding sites.

- 2) The fat content of the sample could be causing some partition of decanal between fat droplets and the aqueous solution, despite the choice of decanal concentration, discussed in the introduction, being such that the level of light output is not sensitive to small changes in decanal concentration (Figure 4.6).

The way to gain further insight into this particular problem would be to analyse the dairy samples for their aldehyde, alcohol and fatty acid content and examine the effect of each of these components on the assay system.

7.5. Kinetic Modeling of the bioluminescence assay

The preceding chapter developed a model of the bioluminescence assay, the aim of this was to understand how the assay worked and why it responded in the way that it did to certain conditions. Like all theoretical models of kinetic behavior it makes a number of assumptions, these were itemised during the development of the theory, which rests on the extent of known conditions and behaviour of the assay for their validity. So long as the model holds reasonably true within the limits of the conditions normally experienced it has uses in both explaining phenomena and implications for the design of a biosensor assay based on the bioluminescence assay system. The implications and predictions which arise from the model can be tested against observed behaviour, these tests give weight to the value of the model's implications for designing a biosensor.

Throughout chapter six references were made to the experimental evidence which supported the model. Below some of this evidence is briefly reviewed and the implications of the model to further development of bioluminescence applications is outlined.

- 1) The impact of the autoxidation reaction of FMNH₂ to

FMN in the presence of molecular oxygen can be seen by the way in which the autoxidation reaction rate defined as k_a occurs in the various equations in particular as both part of the denominator in the expression for I_{plat} in equation 21 and as a factor in determining the response time for the bioluminescence assay.

2) The virtually non perturbing nature of the reductase and luciferase assay in monitoring levels of analyte. This arises from the high sensitivity of the assay and ability to detect low levels of emitted light, as a result very low concentrations of the NADH:FMN oxidoreductase enzyme are needed resulting in negligible consumption of analyte.

3) The model explained some of the behavioural anomalies observed during the course of experimental work. Two examples are:

i) The need found, when measuring the half life time to reach the plateau level in assays for malate and lactate, for taking the time interval between reaching 50% and 75% of maximum light emission to get consistent results. This was clear from the lag effect created when the time taken for the dehydrogenase catalysed reaction to reach equilibrium is significant compared to the response time of the bioluminescence enzymes to the level of NADH.

ii) The second example of where theoretical analysis helped explain experimental behaviour was in section 6.3 where the hump in the assay profile observed with immobilised enzyme and high concentrations of NADH was explained with reference to the work of Wigley (1984), showing that the turnover time for a single enzyme is longer than that implicit in the decay of the light emission in the single turnover assay due to a conformational relaxation observed after the reaction has been catalysed. Since this results in the limitation of supply of free luciferase for reaction catalysis it is an exception outside the scope of the model.

4) The dependence of assay response to the concentration of the various assay components showed how potential sources of error could best be minimised.

The effects of varying the concentration of NAD, oxygen and luciferase were specifically looked at in chapter 6 the broad implications for the last two of these were confirmed in the experiments described in section 6.4.

5) For the development of assays for other analytes using different dehydrogenases, the model shows the effect of equilibrium positions for the dehydrogenase enzymes on the assay and allows the relative concentrations of enzymes and co-factors to be optimised

on a theoretical basis reducing the need for empirical formulation of the assay. The next stage would of course now be to take new analyte with a dehydrogenase, preferably dissimilar in kinetic parameters to the two model systems used in this work and see if the results observed were consistent with the model developed alongside these two model systems.

6) Operational and storage stability was found to be improved by the immobilisation of the enzymes on sepharose beads, while there are several potential sources of this improved stability the model does make one important prediction which could lead to improved operational and storage stability. The prediction that light intensity is related to:

$$I_{plat} = Q_b \frac{k_r k_l}{k_a + k_l} (n^+ \text{ ah } k_1/k_{-1})^{1/2}$$

(equation 21 in chapter 6) leads to the prediction that if the concentration of luciferase is sufficiently high the proportional introduction of errors due to lost luciferase activity will be small compared to the lost percentage of active enzyme. This makes an additional reason to seek to improve upon and develop new methods of increasing the achieved loading of active luciferase in immobilisations.

The theoretical model developed thus points to certain lines of investigation which could improve the effectiveness of applications for the bioluminescence systems.

APPENDIX A

BBC Computer Interface to LKB 1250 Luminometer

Introduction

The LKB 1250 luminometer was interfaced to a BBC model B computer enabling data to be easily collected and quickly analysed by the computer.

The two instruments were linked by using a simple step down potentiometer circuit shown in figure A.1

This interface is switchable, giving a choice of two scales. In position A a 15 V signal is seen by the computer as a 1.159 V signal, in position B a 1.5 V signal is seen as 1.125 V by the computer. These signals are detected at the analogue/digital port. The signal, between 0.0 V and 1.8 V, is digitised into a number between 0 and 64564, it is this number which is read into the computer program to represent the light signal from the sample.

The software which constitutes the package consists of 10 programs collectively referred to as BIOPACK and individually by their program names. Figure A.2 shows the relationship between the programs themselves.

The system is accessed by auto-booting the BIOPACK disc, the disc is loaded into the disc drive and the BREAK and SHIFT keys pressed simultaneously. This runs the program:

```
!BOOT
```

```
0001 CHAIN"MENU"
```

Which loads and runs the introduction program MENU giving information on the system and asking the user to choose between different courses of action following the prompt signal:

?

The reply must be one of the letters given in brackets () in the explanatory text followed by pressing the RETURN key

Since most of the programs are written in MODE 0, for high resolution, and contain a 2 x 250 real array for data storage insufficient memory is left for explanatory remarks in the text as REM statements. An explanation of the programs and the procedures is given on the next few pages. The listings of the programs are included at

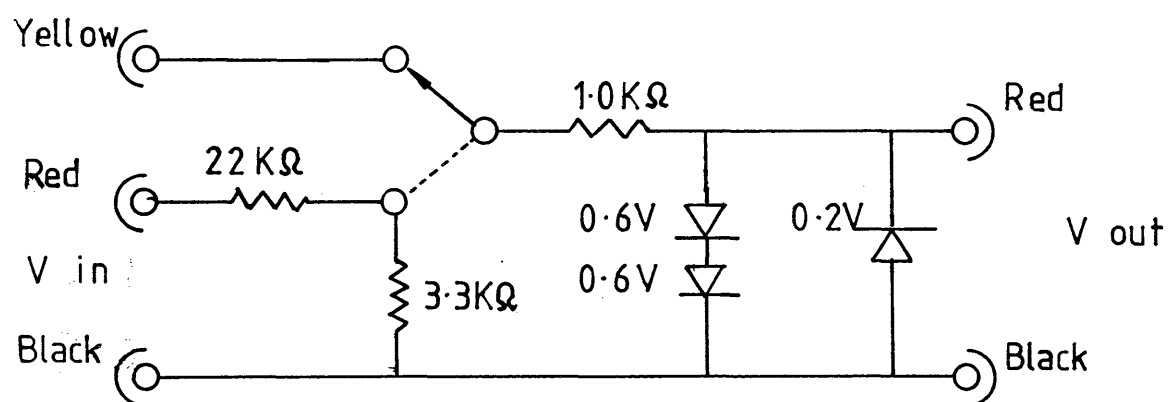


FIGURE A.1

Luminometer to BBC Computer Interface

the end of the appendix.

The figure A.2 is a graphical representation of the inter-relationships between the different programs. Briefly the constituent programs are:

!BOOT This program allows the package to be auto-booted. It introduces the user to the system via "MENU".

MENU Informs the user of the different facilities available in BIOPACK. Allows the user to choose between a variety of programs, for data acquisition or analysis.

LUCIF This program essentially collects data from the analogue/digital port and stores it in a file TEMP before linking to further programs for analysis.

CALDITH One of the analysis programs that is tailored to a specific bioluminescence analysis. It is entered automatically following the selection of choice D, indicating a dithionite assay, in the LUCIF program.

It outputs to the printer the following information:

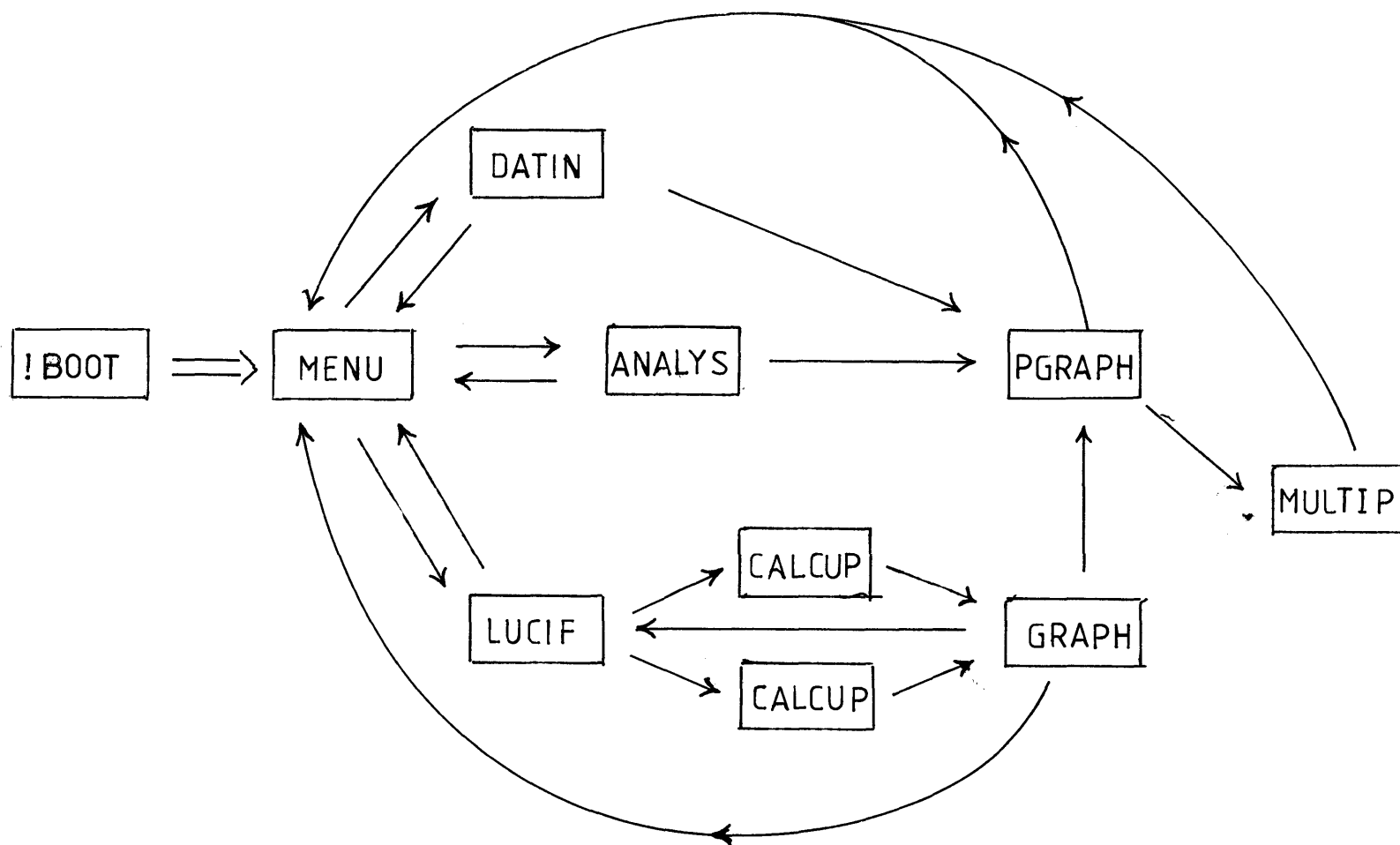
EXPERIMENT : Text as entered by user
PEAK LIGHT : Peak level of light emission
LOG[PEAK] : Log base 10 of the peak
RESPONSE : Time in seconds to reach peak
AREA : Integrated measure of light RLU S
KCAT IS : Turnover time of the enzyme in S
CORRELATION: Correlation of the log plot
used for Kcat calculation

CALCUP Automatically called following LUCIF on the choice of option C, for a coupled assay. The information is more limited but designed to give an idea of the plateauing effect.

EXPERIMENT :Text as entered by user
PEAK LIGHT :Peak of light emission
RESPONSE TIME :Time (seconds) of peak
TIME TO 95% RESPONSE:Time to 95% of peak
light

ANALYS This program offers general analysis utilities enabling the user to: examine data files, edit them, store on disc, call from disc, create a new data set, transform the data into new X and Y

FIGURE A.2 Interrelationship of BIOPACK programs



values as functions $f(x,y)$ and $g(x,y)$, calculate linear regression for a set of data and plot data sets using plotting programs. The program is menu driven and after use the operator can return to the main MENU program.

GRAPH This is the program called by CALDITH and CALCUP to display the recorded data set in graphical form. After displaying the data the operator is given a menu of choices. This allows for further analysis, a hard copy of the plot, or return to LUCIF or the main MENU.

PGRAPH This is the plotting program used by the ANALYSIS program. It allows the user to choose between plotting points, a curve, a regression line or several plots on the same graph through the MULTIPLOT program. The user also selects the limits of and the labels for the X and Y axis and the plot as a whole.

MULTIP As mentioned above this program is accessed through the **PlotGRAPH** program and allows upto five sets of data to be represented on the same plot.

SDUMP A program in machine code which allows the plots on the screen to be printed on the printer as they appear on the screen.

Technical Directory of BIOPACK procedures

The package of programs is built up from a set of procedures some of which are common to several programs others specific for individual programs.

The following is a list of the procedures used in the package with an explanation of what they do, and will enable the user to follow the programs listed at the end of this appendix.

PROCAnaloge/digital Takes a value from the analogue/digital port proportional to the signal from the external/source (luminometer) and stores it in a data array **R**.

PROCALLresults Outputs the results of the **CALCUP** analysis program to the printer.

PROCAREA Defines a routine to calculate the area under a graph by the trapezium rule.

PROCBOX(G) Draws a small box, triangle, cross, diamond, or plus sign at the current/graphics cursor position. The value of **G** defines which symbol is

printed.

PROCBOX(W,Z) Draws a square at the point on a graph defined by the co-ordinates **W,Z**.

PROCCalculate Calculates X,Y values for any function $f(x)$ between given limits of X, the points are at equal intervals of X between these limits.

PROCHOICE Executes the choice made by the user in the main **MENU**.

PROCUTdata While editing data in the analysis program this procedure allows sections of data to be chosen or examination and manipulation.

PROCdisc(K\$) This allows data to be retrieved
PROCDISK(K\$) from a named data file on disc, file **K\$** is called.

PROCEdit Allows the operator to enter the editing mode of the analysis program.

PROCFILE These two procedures execute the
PROCfile users choice in the GRAPH and DATIN programs after displaying a menus.

PROCFUNCTION This recalculates data for storage
as X,Y values with new $X=f(x,y)$ and $Y=g(x,y)$.

PROCGraphAXIS Plots on the screen the X axis and
PROCGraphAxis the Y axis for the graph plotting programs.

PROCGraphPLOT Plots the set of data on the screen to
PROCGraphplot
PROCGraphPlot give a graphical representation of that data.

PROCHold Causes the computer to wait until the value of the external signal reaches a value greater than or equal to the threshold value defined by the user for a particular experiment.

PROCKCAT Within the **CALDITH** program this procedure calculates the time constant for the decay of light as monitored by the analogue digital port. The computer looks at the signal between 10 and 90 percent of the peak light level and calculates the decay constant for this region of the data. It then prints this value and the correlation value for the log plot used to arrive at the decay constant.

PROCLink Links the **LUCIF** data collection program to the appropriate analysis program.

PROCList Lists the current data set while working in the **ANALYSIS** program.

PROCMaximum,minimum This procedure when applied repeatedly to a data set picks out the maximum and minimum values of X and Y.

PROCNumbers Prints the numerical values of X and Y on the screen during **PROCList** listing.

PROCPERMAX This procedure iterates the **PROCAREA** routine to give the area under the graph defined by the data set.

PROCRegression Calculates the best straight line fit to the data and the correlation value of that line.

PROCResults Stores the data from the analogue/digital port in an array called R for Results.

PROCScreen(A,B) Produces the framework for the **PROCScreen(J,K)** listing of data sets by the data **ANALYSIS** program and the **DATAInput** program.

PROCSort Sorts out the data into numerical order after editing.

PROCStore(K\$) Stores the data set currently being worked on in the file K\$ on disc.
PROCSTORE(K\$)

PROCTimer Internal clock allows the LUCIF program to sample the analogue/digital port at regular intervals during the experiment.

```

LIST ===== MENU =====
10MODE0
20COLOUR129:CLS:COLOUR0:PRINTTAB(15);"BIOPACK INTRODUCTION program by D.C.MA
NUEL":COLOUR130:COLOUR1
30VDU28,1,30,78,1:CLS:VDU28,4,29,76,2
40 *KEY 0 RUN!M
50 *KEY1MODE0!M!NLIST!M
60 *KEY 2 "*FX5,1!M:VDU2!M"
70 *KEY 3 VDU3!M
80 *KEY 4 VDU2!M
90 *KEY 5 MODE7!M
100 *KEY 6 MODE4!M
110 *KEY 7 MODE0!M
120 *KEY 8 LOAD
130 *KEY 9 CHAIN
140 CLS:PRINT TAB(26,5);"WELCOME TO BIOPACK",TAB(26,6)"-----"
150 PRINT TAB(9,10);"This is a software package designed to collect data from
any labinstrument and store it for later analysis. It was originally designed
for use with an LKB 1250 luminometer."
160 PRINT TAB(9,13);"The package as a result contains several programs for us
e in the collection and analysis of bioluminescence assay data. However the
package has several programs of general utility"
170 INPUT TAB(9,20);"For futher information please choose:"TAB(9);"<G> G
ENERAL UTILITY PROGRAMS""TAB(9);"<B> BIOLUMINESCENCE""",C#
180 PROCCHOICE
190 GOTO 120
200 CLS:PRINT TAB(30,5);"BIOLUMINESCENCE",TAB(30,6)"-----"
210 PRINT TAB(6,8);"The package is accessed through a program 'LUCIF', for luc
iferase. It allows the collection of data in the form of an analouge signal in t
herange"
220 PRINT "0 to 1.8 volts. Data collection is over a defined time period 'LEN
GTH' in seconds. The number of data points, up to 250, and a threshold value"
230 PRINT "for the collection of data are also set. Two defined sets of these
valuesexist: <D> and <C>."
240 PRINTTAB(6);"Type the appropriate letter for more information "" <L>
To load the 'LUCIF' program"" <G> For more information on the
general utility programs"
250 PRINT"" <D> For the dithionite assay."" <C> For the co
upled assay."
260 INPUT"" <R> For redefining the data collection parameters."" <F
> Links into another program for flow injection assays.""",C#
270 PROCCHOICE
280 CLS:PRINT TAB(20,5);"THE COUPLED ASSAY IN BIOLUMINESCENCE",TAB(20,6)"---
-----"
290 PRINT TAB(6,10);"The coupled assay uses the bioluminescence system of bact

```

```

erial luciferase coupled to an FMN:NADH oxidoreductase to detect either NADH or
another analyte linked to NADH production."
300 PRINT TAB(6,14); "For such coupled assays a response time of several minute
s is common so the experimental option of <C> gives a 500 second time duration
with 200 time points and a threshold of 20 R.L.U."
310 INPUT TAB(10); "Please choose: "" <L> To load 'LUCIF' for data a
quisition"" <S> For more information"" ,C#:PROCCHOICE
320 CLS:PRINT TAB(30,5); "GENERAL UTILITY PROGRAMS",TAB(30,6) "-----
-----"
330 PRINT TAB(6,10); "The analysis packages here are of general use. In additi
on there are two programs 'CALDITH' and 'CALCUP' specifically designed for use wit
h the 'TURNOVER' and 'COUPLED' assays respectively for bioluminescence."
340 INPUT TAB(10); "Please choose: "" <I> For inputting data from keyboard
or disc"" <A> For the package of analysis"" <S> For more informa
tion"" ,C#:PROCCHOICE
350 CLS:PRINT TAB(20,5); "THE DITHIONITE ASSAY IN BIOLUMINESCENCE",TAB(20,6) "----
-----"
360 PRINT TAB(6,10); "The dithionite assay measures luciferase b y means of a s
ingle turnover assay using reduced FMN produced by addition of sodium dithioni
te to an enzyme FMN mixture."
370 PRINT TAB(6,14); "For such assays a response time of about one second is us
ual followed by adeacy of time constant ~7 seconds corresponding to the enzyme's
Kcat so the"
380 PRINT "experimental option of <D> gives a 10 second time duration with 200
time points and a threshold of 100 R.L.U."
390 INPUT TAB(10); "Please choose: "" <L> To load 'LUCIF' for data aq
quisition"" <S> For more information"" ,C#:PROCCHOICE
400 PRINT TAB(6,8); "These suit of programs allow certain analysis to be perfor
med on a set of data already on disc or typed in through the key board. Data c
an also be input from an instrument via 'LUCIF' program and <R> option."
410 PRINT TAB(6,14); "Type the appropriate letter for more information "" <A
> For types of analysis available"" <P> For the graphics plo
ting program"
420 INPUT "" <R> For inputting data from an instrument"" <I>
For inputting data from disc or keyboard"" ,C#
430 PROCCHOICE
440 CLS:PRINT TAB(15,5); "REDEFINING THE PARAMETERS FOR DATA AQUISITION",TAB(20,
6) "-----"
450 PRINT TAB(6,10); "For some experiments it is found desirable to redefine the
parameters for data collection. This can be done by choosing the <R> option w
hen defining the experimental parameters."
460 PRINT TAB(6,14); "This option can also be used to collect data from other in
struments which are interfaced to the BBC."" The maximum number of time p
oints during the data collection period is 250."
470 INPUT TAB(10,20); "Choose one of the following: "" <L> To load 'L

```

```

LIST 470,620
470 INPUT TAB(10,20)"Choose one of the following:""" <L>           To load 'L
UCIF' and start data collection"" <S>           To return to the first menu""
,C#:PROCCHOICE
480 END
490 DEF PROCCHOICE
500 IF C#="A" THEN CHAIN"OTHER"
510 IF C#="B" GOTO 200
520 IF C#="C" GOTO 280
530 IF C#="D" GOTO 350
540 IF C#="F" GOTO
550 IF C#="G" GOTO 320
560 IF C#="I" CHAIN"DATIN"
570 IF C#="L" CHAIN"LUCIF"
580 IF C#="R" GOTO 440
590 IF C#="S" GOTO 140
600 IF C#="P" GOTO
610 GOTO 430
620 ENDPROC

```

```

LIST ===== LUCIF =====
10MODE0
15COLOUR129:CLS:COLOUR0:PRINTTAB(15);"BIOPACK INTERFACING program by D.C.MAN
UEL":COLOUR130:COLOUR1
16VDU28,2,30,78,1:CLS:VDU28,4,29,76,2
20 VDU4
30DIMR(250,2)
40VDU29,150;500;
50VDU24,-150;-80;1129;523;
60VDU28,4,29,39,19
70COLOUR130
80COLOUR1
90GCOL0,128
100CLS
110FORN=1TO20
120MOVE500,250:DRAWNRND(1000),RND(500)
130NEXT
140*FX5,1
150PROCA
160PROCS("TEMP")
170PROCL
180DEFFPROCL
200IFCH#="D"CHAIN"CALDITH"
210IFCH#="C"CHAIN"CALCUP"
215IFCH#="R"CHAIN"OTHER"
218IFCH#="F"CHAIN"CALDITH"
220ENDPROC
230DEFFPROCA
240YMAX=0:YMIN=0:XMIN=0:XAX=0:XIN=0:YAX=0:YIN=0
250CLS:INPUT"Define experimental parameters""<D> For dithionite""<C> For cou
oled""<R> To redefine""<F> For flow cell analysis""<S> To return to main menu
""CH#
260IFCH#="C"THENGOTO330
270IFCH#="D"THENGOTO340
275IFCH#="F"THENGOTO335
280IFCH#="S"THENCHAIN"MENU"
290INPUT"LENGTH in seconds?"X%
300INPUT"NUMBER of points?"A%
310INPUT"THRESHOLD value?"T%
320GOTO350
330X%=500:A%=250:T%=10:GOTO350
335X%=120:A%=240:T%=50:GOTO350

```

```

340X%=10:A%=200:T%=100
350CLS:PRINT"LENGTH          ";X%
360PRINT"NO. OF POINTS      ";A%
370PRINT"THRESHOLD value   ";T%
380VDU24,500;-450;1100;-100;
390ST=X%*100/A%
400VDU29,650;50;
401FORV=0TO9
402MOVE0,V*50:DRAW450,V*50
403MOVEV*50,0:DRAWV*50,450
404NEXT
410YS=100:X5=X%/450
420PROC
430PROCI
440CLS:PRINT" **** DATA COLLECTION FINISHED *****":VDU7
450ENDPROC
460DEFPROC
470LOCAL V%
480REPEAT
490V%=ADVAL(1)
500UNTIL V%>T%
510PRINT" ***** RECORDING NOW *****":VDU7
520ENDPROC
530DEFPROCI
540LOCAL TEM%,C%
550TEM%=TIME:CL%=TIME-TEM%
560C%=CL%
570FORN=1TOA%
580PROC
590PROCR
600PROCM
610NEXT
620ENDPROC
630DEFPROCT
640REPEAT
650CL%=TIME-TEM%
660UNTIL CL%>=C%+ST
670ENDPROC
680DEFPROCR
690C%=CL%
700R(N,0)=C%/100
710R(N,1)=ADVAL(1)

```

```

720X=R(N,0):Y=R(N,1)
730MOVEX/XS,0:DRAWX/XS,Y/YS
740ENDPROC
750DEFPROCS(K#)
760B=OPENOUT K#
770PRINT#B,A%
780FOR N=1 TO A%
790PRINT#B,R(N,0),R(N,1)
800NEXT
810CLOSE#B
820ENDPROC
830DEFPROCM
840IFX>X%THENXMAX%=X:YAX=Y
850IFY>YMAX THEN YMAX=Y:XAX=X
860IF X<XMIN THEN XMIN=X:YIN=Y
870IF Y<YMIN THEN YMIN=Y:XIN=X
880ENDPROC

```

```

LIST ===== CALDITH =====
10 DIM RECDAT(250,2)
20 XMAX%=0:YAX=0:XMIN=0:YMAX=0:XAX=0:YMIN=0:XIN=0
30 PROCDISK("TEMP")
40 PROCPERMAX
50 INPUT"NAME OF EXPERIMENT",F#
60 CLS:VDU2:PRINT"EXPERIMENT :";F#,"PEAK LIGHT :";YMAX,"LOG[PEAK] :";LOGYMA
AX,"RESPONSE :";XAX"AREA :";AREA
70 PROCKCAT
80 PRINT"KCAT IS :";-1/SLOPE:PRINT"CORRELATION:";COREL,"
85VDU3
90 CHAIN"GRAPH"
100 DEF PROCREGRESSION
110 LOCAL A,B,C,D,E
120 A=0:B=0:C=0:D=0:E=0:N=0
130 FOR J=1 TO ACU%
140 X=RECDAT(J,0)
150 Y=RECDAT(J,1)
160 A=A+X
170 E=E+Y
180 C=C+(X*Y)
190 D=D+(X*X)
200 E=E+(Y*Y)
210 N=N+1
220 NEXT
230 SLOPE=(C-(A*B)/N)/(D-A*A/N)
240 CR=(N*C-A*B)^2/((N*D-A*A)*(N*E-B*B))
250 COREL=SQR(CR)
260 ENDPROC
270 DEF PROCPERMAX
280 AREA=0
290 FOR N=0 TO ACU%-1
300 PROCAREA
310 NEXT
320 ENDPROC
330 DEF PROCAREA
340 LOCAL A,B,C,D
350 A=RECDAT(N,0):B=RECDAT(N+1,0)
360 C=RECDAT(N,1):D=RECDAT(N+1,1)
370 AREA=AREA+((B-A)*(C+D)/2)
380 ENDPROC
390 DEF PROCKCAT
400 M=0

```

```

LIST ===== CALCUP =====
10DIM RECDAT(250,2)
20XMAX%=0:YAX=0:YMAX=0:XAX=0:XMIN=0:YIN=0:YMIN=0:XIN=0
30PROC DISK("TEMP")
40PROCALL
50CHAIN"GRAPH"
60DEF PROCALL
70INPUT"EXPERIMENT NAME",F#
80CLS:VDU2:PRINT"EXPERIMENT          :";F#"MAXIMUM LIGHT IS:";YMAX'"RESPONSE TIM
E: ";XAX
90N=1
100REPEAT:N=N+1:RESP=RECDAT(N,0):UNTIL RECDAT(N,1)>0.95*YMAX OR RECDAT(N,1)=0.
95*YMAX
110PRINT"RESPONSE TO 95%:";RESP:VDU3
120ENDPROC
130DEF PROC DISK(K#)
140A=OPENIN K#
150INPUT£A,ACU%
160FOR N=1 TO ACU%
170INPUT£A,RECDAT(N,0),RECDAT(N,1)
180X=RECDAT(N,0):Y=RECDAT(N,1):PROC MAXMIN
190NEXT
200CLOSE£A
210ENDPROC
220DEF PROC MAXMIN
230IF X>XMAX% THEN XMAX%=X:YAX=Y
240IF Y>YMAX THEN YMAX=Y:XAX=X
250IF X<XMIN THEN XMIN=X:YIN=Y
260IF Y<YMIN THEN YMIN=Y:XIN=X
270 ENDPROC

```

```

>LIST ===== ANALYS =====
10 MODE3:VDU14
20COLOUR129:CLS:COLOUR130
30COLOUR129:CLS:COLOUR0:PRINTTAB(15);"ANALYSIS program by D.C.MANUEL":COLOUR
130:COLOUR1
40VDU28,2,23,78,1:CLS:VDU28,4,22,76,2
50J=1:K=1:A%=1:F#="TEMP":X#="X":Y#="Y"
60DIMR(250,2)
70YX=0:XX=0:YN=0:XN=0
80PROC("TEMP")
90IF K=1 THEN K=A%
100VDU28,10,22,65,2:CLS:VDU28,15,22,65,5
110INPUT"New data"TAB(30)"Present data"'"<1> Disc"TAB(30);"<4> Store"'"<2> Ke
yboard"TAB(30);"<5> Edit"'"<3> Catalogue"TAB(30)"<6> List"'"Functions"'"<7> Re
gression"TAB(30)"<11> Find f(X)'"<8> f(X),f(Y)'"<9> Plot"'"<10> Menu"'"',C
120IFC=1THENINPUT"FILENAME",F#:PROC(F#)
130IFC=2THENPROCN
140IFC=4THENINPUT"FILENAME",F#:PROCST(F#)
150IFC=5THENPROCE
160IFC=3THEN *CAT
170IFC=3THEN INPUT"PRESS RETURN TO CONTINUE",C#
180IFC=6THENCLS:P=3:INPUT"Label for X values",X#"Label for Y values",Y#"Prin
t Y/N",C#:IFC#="Y"THENP=2
190IFC=6THENPROCL
200IFC=7 PROCREG
210IFC=8 PROCFUN
220IFC=10THENCHAIN"MENU"
230IFC=9THENPROCST("TEMP")
240IFC=9THENCHAIN"PRGRAPH"
250IFC=11THENPROCC
255IFC=12THENPROCSPECIAL
256IFC=13THENPROCDECANAL
260GOTO90
270END
280DEFPROC(K#)
290A=OPENIN K#
300INPUT#A,A%
310FOR N=1 TO A%
320INPUT#A,R(N,0),R(N,1)
330X=R(N,0):Y=R(N,1):PROC(M
340NEXT
350CLOSE#A
360PROC(M

```

```

370ENDPROC
380DEFPROCST(K#)
390A=OPENOUT K#
400PRINT#A,A%
410FOR N=1 TO A%
420PRINT#A,R(N,0),R(N,1)
430NEXT
440CLOSE#A
450ENDPROC
460DEFPROCREG
470LOCAL A,B,C,D,E
480A=0:B=0:C=0:D=0:E=0:N=0
490FOR M=J TO K
500X=R(M,0):Y=R(M,1)
510A=A+X:B=B+Y:C=C+X*Y:D=D+X*X:E=E+Y*Y:N=N+1
520NEXT
530SLOPE=(C-(A*B)/N)/(D-A*A/N)
540NTCEP=(B-SLOPE*A)/N
550CR=(N*C-A*B)^2/((N*D-A*A)*(N*E-B*B))
560COREL=SQR(CR)
570SX=SQR((N*D-A*A)/(N*(N-1)))
580SY=SQR((N*E-B*B)/(N*(N-1)))
590SE=SQR((N-1)*(SY*SY-(SLOPE*SX)^2)/(N-2))
600SB=SE/(SX*SQR(N-1))
610VDU2:PRINT,F#," POINTS ";J;" TO ";K;"SLOPE"TAB(15),SLOPE;" +/- ";SB;"INTERCEP
T"TAB(15),NTCEP;" +/- ";SE;"CORRELATION"TAB(15),COREL:VDU3
620ENDPROC
630DEFPROCFUN
640INPUT"FUNCTION OF X",X#;"FUNCTION OF Y",Y#
650YX=0:XX=0:YN=0:XN=0
660FOR N=J TO K
670X=R(N,0):Y=R(N,1):XT=EVAL(X#):R(N,0)=XT
680YT=EVAL(Y#):R(N,1)=YT
690NEXT
700ENDPROC
710DEFPROCMM
720IF X>XX THEN XX=X
730IF Y>YX THEN YX=Y
740IF X<XN THEN XN=X
750IF Y<YN THEN YN=Y
760ENDPROC

```

```

770DEFPROCUT
780J=0:K=0
790INPUT"Enter new first and last point no.",J,K
800ENDPROC
810DEFPROCL
820VDU28,15,6,75,2:CLS:VDUP:PRINT"_":PROCS(48,0):PRINT"!";TAB(16)"!";TAB(32)
"!";TAB(48)"!"
830PRINT"! POINT No.      ! ";X#,TAB(32);"! ";Y#,TAB(48);"!":PROCS(49,1):PRINT"
"
840VDU28,15,22,75,6:VDU14
850FOR N=J TO K
860PRINT"!      ";N,TAB(16);"! ";R(N,0),TAB(32);"! ";R(N,1);TAB(48)"!"
870IFB=1 THEN PROCS(49,1):PRINT" ":VDU3:INPUT"PRESS RETURN TO CONTINUE",C#
880NEXT
890ENDPROC
900DEFPROCS(A,B)
910FORM=1 TO A
920IFB=1 AND ((M=1 OR M=17) OR (M=33 OR M=49)) THEN PRINT"!"; ELSE PRINT"_";
930NEXT
940ENDPROC
950DEFPROCN
960CLS:INPUT"Number of data points";A%
970N=0
980REPEAT
990N=N+1:PRINT"DATA POINT";N':INPUT"X=";R(N,0)"Y=";R(N,1)
1000UNTIL N=A%
1010ENDPROC
1020DEFPROCC
1030CLS:A%=250:INPUT"Function X",FX#:"Max X=";X%"Min X=";XN'"Label X";X#"Label Y'
;Y#:XSC=(X%-XN)/250
1040FOR N=1 TO A%
1050R(N,0)=XN+N*XSC:X=R(N,0):R(N,1)=EVAL(FX#)
1060NEXT
1070ENDPROC
1080DEFPROCE
1090CLS:INPUT""<1> To add data""<2> To delete data""<3> Choose section data'
""<4> Leave edit"",0
1100IF0=1 THEN INPUT"DATA""X=";R(A%+1,0),"Y=";R(A%+1,1):A%=A%+1:PROCS0
1110IF0=2 THEN INPUT"No. OF POINT",L:A%=A%-1:FOR N=L TO A%:R(N,0)=R(N+1,0):R(N,1)=
R(N+1,1):NEXT
1120IF0=3 THEN PROCUT
1130P=3:PROCL:IF0=4 THEN GOTO1140 ELSE GOTO1090

```

```

1140ENDPROC
1150DEFFPROCSPECIAL
1160DT=(R(250,0)-R(1,0))/250:LB=0:DLB=0:N=0
1170REPEAT
1175N=N+1
1180DLB=((3-LB)*R(N,1))*DT
1190LB=LB+DLB
1200R(N,1)=LB
1205UNTIL N=25
1206FOR M=N TO A%
1207DLB=((3-LB)*R(M,1)-R(M-25,1)*0.14)*DT
1208LB=LB+DLB
1209R(M,1)=LB
1210NEXT M
1220ENDPROC
1230DEFFPROCDECENAL
1240REM =====
1250REM  CALCULATES FROM A SET OF DATA [S],v THE
1260REM  VALUE OF Kd AND Ki FOR DECENAL
1270REM =====
1280 INPUT"VALUES FOR V0, AND [S0]"V0,S0
1285 SUMVM=0
1290 FOR N=1 TO A%
1300 A=R(N,0)*S0
1310 B=R(N,1)*(S0^2+R(N,0)^2)
1320 VM=V0*(2*R(N,0)*A-B)/(2*V0*A-B)
1330 SUMVM=SUMVM+VM
1333 *FX1,5
1334 VDU2
1335 PRINTA,B,VM
1340 NEXT N
1350 V=SUMVM/N
1360 ALPHA=4*V0/(V-V0)
1370 KD=S0/(ALPHA^0.5)
1380 PRINT"Kd IS";KD;"Ki IS";ALPHA*KD
1385 VDU3
1390 ENDPROC

```

```

LIST ===== GRAPH =====
10MODE0
15COLOUR129:CLS:COLOUR0:PRINTTAB(15);"BIOPACK INTERFACING program by D.C.MAN
UEL":COLOUR130:COLOUR1
16VDU28,2,30,78,1:CLS:VDU28,4,29,76,2
17 DIM RECDAT(250,2)
40 VDU 29, 200; 400;
45 VDU 24,-100;-80; 1100; 500;
50 VDU 28,3,29,39,22
80 GCOL 0,128
90 XMAX%=0:XMIN=0:YMAX=0:YMIN=0:XAX=0:YAX=0:XIN=0:YIN=0
100 PROCDISK("TEMP")
110 PROCGPLOT
120PROCFIL:CLS
125 GOTO 110
130 DEF PROCGPLOT
150YSC=(YMAX-YMIN)/500
160 XSC=(XMAX%-XMIN)/1000
170 YZEO=400-YMIN/YSC
180 XZEO=200-XMIN/XSC
190 VDU 29, XZEO; YZEO;
200 PROCGAXIS
210 MOVE RECDAT(1,0)/XSC,RECDAT(1,1)/YSC
220 FOR N=1 TO ACU%
230 X=RECDAT(N,0)
240 Y=RECDAT(N,1)
250 GCOL 0,1
260 DRAW X/XSC,Y/YSC
270 NEXT
280 ENDPROC
290 DEF PROCGAXIS
300 VDU5
310 MOVE 0,0: DRAW 1000,0
320 MOVE 0,0:DRAW 0,YMAX/YSC
330 MOVE 0,0:DRAW 0,YMIN/YSC
340 MOVE 0,0
350 FOR N=0 TO 5
360 MOVE N*200,0
370 DRAW N*200,-30
380 PRINT;XSC*200*N
390 NEXT N
400 MOVE-140,0:PRINT"0.0"

```

```

410 MOVE-140,YMAX/YSC:PRINT;YMAX
420 MOVE-140,YMIN/YSC:PRINT;YMIN
430 VDU4
440 ENDPROC
450 DEF PROCFILE
460 INPUT"<1>          STORE""<2>          RETREIVE""<3>          RETURN TO START
""<4>          INTRO""<5>          PRINT""<6>          REDRAW AND PRINT",TYPE%
470IF TYPE%=3 THEN CHAIN"LUCIF"
480IF TYPE%=4THEN CHAIN"MENU"
490IF TYPE%=5THEN *SDUMP
495IF TYPE%=6 THEN CHAIN"PRGRAPH"
500IF TYPE%=1 OR TYPE%=2 THEN INPUT "FILENAME",F#
510IF TYPE%=1 THEN PROCSTORE(F#)
520IF TYPE%=2 THEN PROCDISK(F#)
530 GOTO460
540 ENDPROC
550 DEF PROCDISK(K#)
560 A=OPENIN K#
570INPUT£A,ACU%
580 FOR N=1 TO ACU%
590 INPUT£A,RECDAT(N,0),RECDAT(N,1)
600 X=RECDAT(N,0):Y=RECDAT(N,1)
610 PROCMAXMIN
620 NEXT
630 CLOSE£A
640 ENDPROC
650 DEF PROCSTORE(K#)
660 B=OPENOUT K#
670 PRINT£B,ACU%
680 FOR N=1 TO ACU%
690 PRINT£B,RECDAT(N,0),RECDAT(N,1)
700 NEXT
710 CLOSE£B
720 ENDPROC
730 DEF PROCMAXMIN
740 IF X>XMAX% THEN XMAX%=X:YAX=Y
750 IF Y>YMAX THEN YMAX=Y:XAX=X
760 IF X<XMIN THEN XMIN=X:YIN=Y
770 IF Y<YMIN THEN YMIN=Y:XIN=X
780 ENDPROC
790IF TYPE%=4THEN CHAIN"OTHER"

```

```

LIST ===== PRGRAPH =====
10MODE0
20DIMR(250,2)
30COLOUR130
40COLOUR1
50GCOLOR,128
55XX=0: XN=0: YX=0: YN=0
56VDU28,20,20,60,4
60INPUT"Plot"'"<1> As curve"'"<2> As points"'"<3> Regression"'"<4> Load new
data"'"<5> Plot several curves"'"<6> Menu"',C
70IFC<4THENPROC("TEMP")
71IFC=4THENINPUT"FILENAME",F#:PROC(F#)
72IFC=5THENCHAIN"MULTIP"
73IFC=6THENCHAIN"MENU"
80PROC GP
100*SDUMP
105CLS: INPUTTAB(10,15); "Redraw Y/N",C#: IF C#="Y" THEN GOTO 60
120CHAIN"MENU"
130END
140DEFPROC GP
150CLS: PRINT'"XMAX="; XX'"XMIN="; XN, '"YMAX="; YX'"YMIN="; YN, '
160INPUT"Limits for axes:"'"XMAX="; XX, "XMIN="; XN, "YMAX="; YX; "YMIN="; YN, '
170INPUT"Labels for axes:"'"X AXIS "; X#, "Y AXIS "; Y#, "Title"; T#
180VDU24,-150;-80;1129;823;
190YSC=(YX-YN)/750
200XSC=(XX-XN)/1000
210VDU29,200-XN/XSC;200-YN/YSC;
220CLG:PROC G
240MOVER(1,0)/XSC,R(1,1)/YSC
250FOR N=1 TO A%
260X=R(N,0): Y=R(N,1)
280GCOLOR,1
290IFC=1THENDRAWX/XSC,Y/YSC ELSE PROCBOX(X/XSC,Y/YSC)
300NEXT
305IFC=3THENPROC REG
310ENDPROC
320DEFPROC G
330VDU5
340MOVE XN/XSC,YN/YSC: DRAW1000+XN/XSC,YN/YSC
350MOVE XN/XSC,YN/YSC: DRAWXN/XSC,YX/YSC
360@%=&00020205
370FOR N=0T05

```

```

380MOVEXN/XSC+N*200,YN/YSC: DRAWXN/XSC+N*200,-30+YN/YSC: MOVEXN/XSC+N*200-40,-40
+YN/YSC: PRINT; XN+XSC*200*N
390MOVEXN/XSC,YN/YSC+N*150: DRAW-30+XN/XSC,YN/YSC+N*150: MOVE-150+XN/XSC,YN/YSC-
N*150: PRINT; YN+YSC*150*N
400NEXTN
410VDU4
420@%=%90A
430VDU28,0,20,0,5
440PRINTTAB(0,5);Y#;
450VDU28,20,31,60,29
460PRINTX# 'T#
470ENDPROC
480DEFPROCD(K#)
490A=OPENIN K#
500INPUTEA,A%
510FOR N=1 TO A%
520INPUTEA,R(N,0),R(N,1)
530X=R(N,0):Y=R(N,1)
540PROC M
550NEXT
560CLOSEEA
570ENDPROC
600DEFPROCM
610IF X>XX THEN XX=X
620IF Y>YX THEN YX=Y
630IF X<XN THEN XN=X
640IF Y<YN THEN YN=Y
650ENDPROC
660DEFPROC REG
680A=0:B=0:C=0:D=0:E=0:N=0
690FOR M=1 TO A%
700X=R(M,0):Y=R(M,1)
710A=A+X:B=B+Y:C=C+X*Y:D=D+X*X:E=E+Y*Y:N=N+1
711NEXT
712S=(C-(A*B)/N)/(D-A*A/N)
713T=(B-S*A)/N
714MOVEXN/XSC,(S*XN+T)/YSC: DRAWXX/XSC,(S*XX+T)/YSC
715ENDPROC
720DEFPROC BOX(W,Z)
730MOVEW-6,Z+6: DRAWW-6,Z-6: DRAWW+6,Z-6: DRAWW+6,Z+6: DRAWW-6,Z+6
740ENDPROC

```

```

LIST ===== MULTIP =====
100DIMR(250,2)
200IMP$(8)
300MODE0
400COLOUR130
500COLOUR1
600GCOLOR,128
700XX=0: XN=0: YX=0: YN=0
800INPUTTAB(10)"WELCOME TO MULTILOT""How many lines to plot",M
85INPUT"Do you wish to plot as plain curves Y/N",C$
90FORG=1TOM
100INPUT"DATAFILE",P$(G):PROCD(P$(G))
110NEXT
120CLS:PRINT"Max and min values are:"TAB(6);"XMAX=";XX,"XMIN=";XN,"TAB(6);"
MAX=";YX,"YMIN=";YN,
130INPUT"Enter limits for axes on graph:"TAB(6,7);"XMAX=";XX;TAB(22,7);"XMIN="
,XN,TAB(6,8);"YMAX=";YX;TAB(22,8);"YMIN=";YN,
140INPUT"Label axes""TAB(6);"X AXIS ";X$,TAB(6)"Y AXIS ";Y$,TAB(6);"Title";T
150CLS:VDU24,-150,-80;1129;823;
160YSC=(YX-YN)/750
170XSC=(XX-XN)/1000
180VDU29,200-XN/XSC;200-YN/YSC;
190PROC GA
200FORG=1TOM
210PROC D(P$(G))
220PROC G
230NEXT
235INPUT,C$
240 *SDUMP
250CLS
260INPUTTAB(10,15);"Redraw graph Y/N?"TAB(10),C$: IF C$="Y" THEN GOTO 220
270CHAIN"MENU"
280END
290DEF PROC G
300MOVER(1,0)/XSC,R(1,1)/YSC
310FOR N=1TO A%
320X=R(N,0)/XSC: Y=R(N,1)/YSC
330GCOLOR,1
340DRAWX,Y
350PROC BOX(G)
370NEXT
380ENDPROC

```

```

390DEFPROC GA
400VDU5
410MOVE XN/XSC, YN/YSC: DRAW 1000+XN/XSC, YN/YSC
420MOVE XN/XSC, YN/YSC: DRAW XN/XSC, YX/YSC
430@%=&00020205
440FOR N=0 TO 5
450MOVE XN/XSC+N*200, YN/YSC: DRAW XN/XSC+N*200, -30+YN/YSC: MOVE XN/XSC+N*200-40, -4
+YN/YSC: PRINT; XN+XSC*200*N
460MOVE XN/XSC, YN/YSC+N*150: DRAW -30+XN/XSC, YN/YSC+N*150: MOVE -150+XN/XSC, YN/YSC
N*150: PRINT; YN+YSC*150*N
470NEXT N
480VDU4
490@%=&90A
500VDU28,0,20,0,5
510PRINT TAB(0,5) Y#;
520VDU28,20,31,60,29
530PRINT TAB(20,29) X# 'T#;
540ENDPROC
550DEFPROC D(K#)
560A=OPENIN K#
570INPUT EA, A%
580FOR N=1 TO A%
590INPUT EA, R(N,0), R(N,1)
600X=R(N,0): Y=R(N,1)
610PROC M
620NEXT
630CLOSE A
640ENDPROC
650DEFPROC M
660IF X>XX THEN XX=X
670IF Y>YX THEN YX=Y
680IF X<XN THEN XN=X
690IF Y<YN THEN YN=Y
700ENDPROC
710DEFPROC BOX(G)
715IF C#="Y" THEN GOTO 775
720V=720+G*10: GOTO V
730MOVE X-6, Y+6: DRAW X-6, Y-6: DRAW X+6, Y-6: DRAW X+6, Y+6: DRAW X-6, Y+6: MOVE X, Y: GOTO 76
740MOVE X-6, Y-6: DRAW X+6, Y-6: DRAW X, Y+9: DRAW X-6, Y-6: MOVE X, Y: GOTO 780
750MOVE X-6, Y: DRAW X, Y-6: DRAW X+6, Y: DRAW X, Y+6: DRAW X-6, Y: MOVE X, Y: GOTO 780
760MOVE X-6, Y: DRAW X+6, Y: MOVE X, Y-6: DRAW X, Y+6: MOVE X, Y: GOTO 780
770MOVE X-4, Y-4: DRAW X+4, Y+4: MOVE X-4, Y+4: DRAW X+4, Y-4: MOVE X, Y: GOTO 780

```

APPENDIX B

Numerical simulation of bioluminescence assay kinetics

A computer program in fortran IV was written to solve the three non-linear differential equations (23), (24) and (25) developed in chapter 6 to describe the kinetics of NADH and dehydrogenase linked bioluminescence assays. Further IBM PC based software was developed to display the numerical solutions, which amount to computer based simulations of the levels of substrates and products under a given set of conditions. Figure B.1 shows a flow chart describing the overall process from a set of initial conditions through to a graphical display of the data. Filenames and software packages are in bold print.

VTERM, **BASICA** and **WORDSTAR** are all commercially available software for the IBM PC.

FSIM and **NFSIM5.BAS** are two of the programs specifically written for the simulation. Listings of these two programs are given at the end of this appendix. Both **FSIM** and **NFSIM5.BAS** can be simply altered to either simulate slightly different assays or output the data and plot it in several ways.

FSIM

A fortran IV program written to use the **NAG** routine **D02EBF** to provide numerical solutions for the differential equations (23), (24) and (25). The routine **D02EBF** uses a iterative interpolation method to solve the problem. The bulk of the program defines the differential equations and the data output required. A detailed explanation of **D02EBF** can be found in **NAG** library documentation volume 11. The differential equations (23), (24) and (25) are defined in the subroutine **FCN** as the array **F(3)** the three elements being the three differential equations. The array **Y(3)** contains the calculated integrals with time of **F(3)** ie. **Y(1)** represents [NADH]; **Y(2)**, oxidised analyte [A] and **Y(3)**, [FMNH₂]. The initial values of these variables and other data needed to calculate **K1**, **K2**, **K3**, **K4**, **K5** and **K6** representing A, B, B', C, D and E in the equations (23), (24) and (25) are requested from the computer operator in lines 625 - 660.

Subroutine **OUT** describes the output sent to the screen (& **VTERM.LOG**) by the **NAG** routine. The number of data points is defined in the program by **I** while the length of the simulation in terms of time is user defined in line 617.

The program allows for a "family" of simulations to be

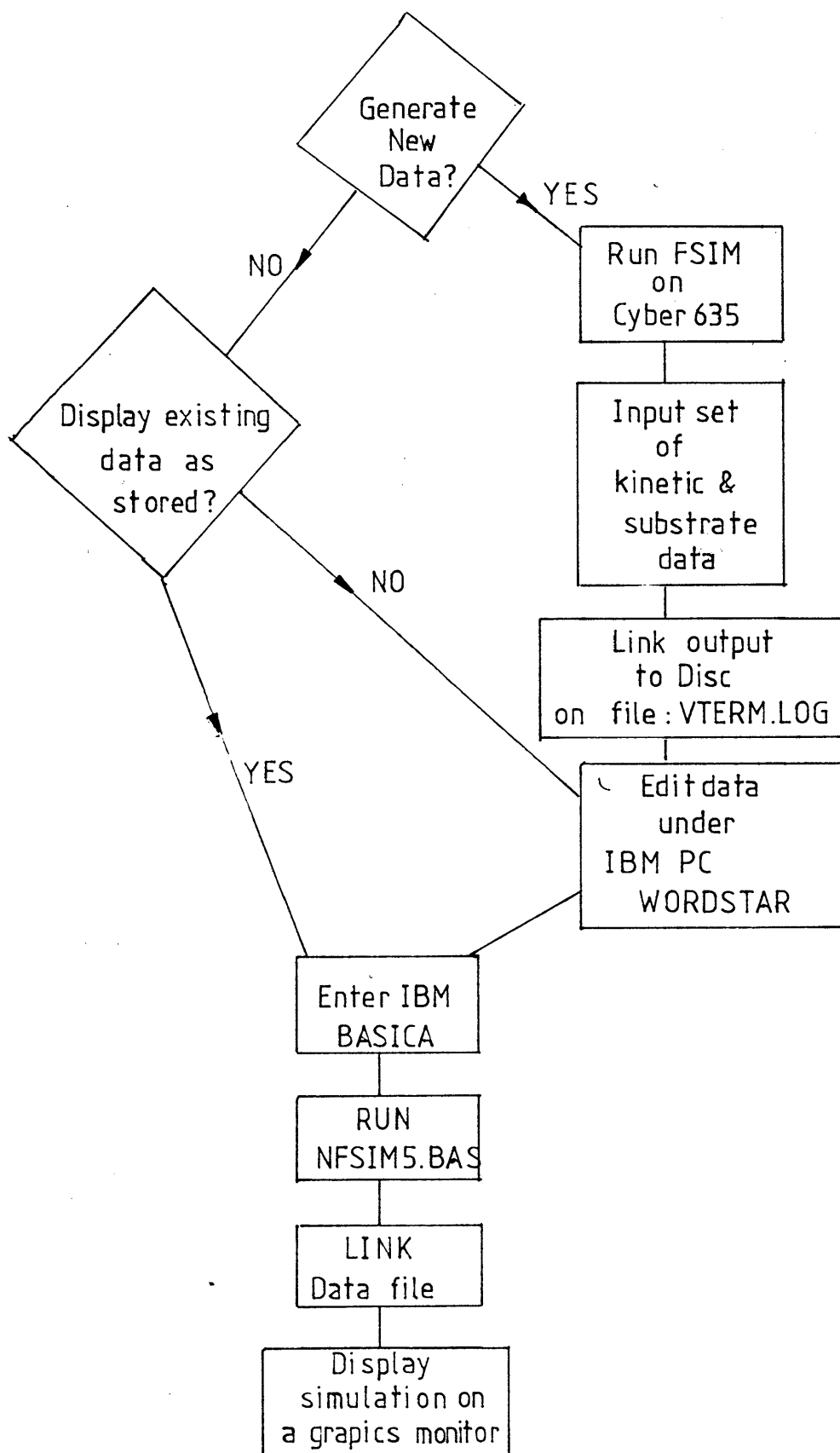


FIGURE B.1

carried out by **FSIM** the lines 618 and 619 define the size and nature of the family.

```
ie.      00618      DO 15 M=1,5

          00619      OR=OR*(10**0.25)
```

00618 instructs the computer to run the body of the program ie. **NAG** routine **D02EBF** 5 times with the same initial data except for the change described in line 00619. In this example variable **OR** representing the kinetic constant **kr** defined in chapter 6 equation (11) is increased by the fourth root of 10 each time the routine is run. These two lines were altered between runs to collect the different sets of data discussed in chapter 6.

Editing data sets

As can be seen from the figure B.1 the data is stored on floppy disk in the IBM PC using the screen dump facility available on **VTERM**. **VTERM.LOG** contains this record of the run of program **FSIM**. To create a data file suitable for **NFSIM5.BAS** this must be edited of unwanted mainframe messages and output. Editing the file is done using the package **WORDSTAR** in non document mode. The resultant data file must follow the format given below:

```

      I
      J
      K1   K2   K3   K4   K5   K6   var
Repeated J Times
      T   [NADH]   [A]   [FMNH2]   Repeated I x
```

I is the number of data points in each simulation, ie. for a simulation from 0 to 200 seconds in 1 second steps there are 201 data points.

J is the number of sets of data, runs of the **NAG** routine **D02EBF** performed in the run of **FSIM**. This number can be edited to display only some of the data sets using **NFSIM5.BAS**.

Graphical display of data

NFSIM5.BAS firstly scans the data sets for the maximum and minimum values of the intermediates and the extent of the time course. Data sets are then plotted on a screen with maximum intermediate concentration equal to 100 % of the Y axis for both **[NADH]** (Green) and **[FMNH₂]** (Red) plots. Beside the graphical display information is

presented on the right hand side of the screen. The values of [NADH] and [FMNH₂] equal to 100 % of the Y axis are given above a list of values for the constants **K1**, **K2** ... **K6** and the variable defined in the program **FSIM** at line 00619 and in **NFSIM5.BAS** by data at point var in the format (figure B.2). These values are tabulated for the current data set as constants 1, 2 7 respectively. The current values for [NADH], [FMNH₂] and time are displayed below these constants as the specific points are being plotted.

Program FSIM

```

list
00100 PROGRAM NSIM (INPUT,OUTPUT,TAPE5=INPUT,TAPE6=OUTPUT)
00110C ...
00200C ...
00210C SCALARS IN COMMON
00220 REAL H,XEND
00230 INTEGER I
00240C ...
00250C LOCAL SCALARS
00400 REAL TOL,X,A,AH,CO,COH,KR1,KR2,DH,OR,K1,K2,K3,K4,K5,K6
00405 INTEGER IFAIL,IR,IW,J,MPED,N,LGTH,M
00410C ...
00420C LOCAL ARRAYS
00430 REAL W(3,199),Y(3)
00440C ...
00450C CALLS NAG ROUTINE D02EBF TO SOLVE THE DIFFERENTIAL EQUATIONS
00460C USING EXTERNAL SUBROUTINES FCN AND OUT
00470 EXTERNAL FCN,OUT,PEDERV
00480 COMMON XEND,H,I
00485 COMMON/CONST/K1,K2,K3,K4,K5,K6
00490C ...
00495C SET UP THE INTIAL PARAMETERS OF THE SIMULATION
00500 PRINT *, 'INPUT INITIAL LEVELS OF SUBSTRATES'
00510 PRINT *, ' (1) CONCENTRATION OF ANALYTE (M)'

00520 PRINT *, ' (2) CONCENTRATION OF PRODUCT ONE (M)'
00530 PRINT *, ' (3) CONCENTRATION OF NAD+ (M)'
00540 PRINT *, ' (4) CONCENTRATION OF NADH (M)'
00550 READ (5,*) AH,A,CO,COH
00560 PRINT *, 'INPUT KINETIC DATA'
00570 PRINT *, ' (1) DETECTING DEHYDROGENASE CONCENTRATION'
00580 PRINT *, ' (2) K1 , FOR AH + NAD+ -> A + NADH (S-1)'
00590 PRINT *, ' (3) K-1, FOR A + NADH -> AH + NAD+ (S-1)'
00600 PRINT *, ' (4) RATE OF NADH CONSUMPTION BY BIOLUMINESCENCE (M S-1)'
00605 PRINT *, ' (5) ESTIMATE OF KA+KL FOR BIOLUMINESCENCE REACTION M S-1'
00610 READ(5,*) DH,KR1,KR2,OR,K6
00612 PRINT *, 'LENGTH OF SIMULATION IN SECONDS'
00617 READ(5,*) LGTH
00618 DO 15 M=1,8
00619 DH=DH*2
00625 K1=KR1*DH*AH*CO
00630 K2=-(KR1*AH*DH + OR)
00640 K3=-KR1*AH*DH
00650 K4=-KR1*DH*CO
00660 K5=(KR1-KR2)*DH
00720 WRITE(6,*) K1,K2,K3,K4,K5,K6
00728 WRITE(6,*) OR
00730 IW=199

00740 MPED=0
00750 N=3
00755 FM=0
00760 IR=1
00770 J=6
00780 TOL=10.**(-J)*AH
00800 X=0
00810 XEND=LGTH
00820 Y(1)=COH
00830 Y(2)=A
00835 Y(3)=FM

```

```

00850      I=199
00860      IFAIL=0
00870      CALL D02EBF(X,XEND,N,Y,TOL,IR,FCN,MPED,PEDERV,OUT,W,IW,IFAIL)
00910 1001 FORMAT (22H CALCULATION WITH TOL=, E8.1)
00920 1002 FORMAT (24H RANGE TOO SHORT FOR TOL)
00930 1003 FORMAT (8H IFAIL=, I1)
00932 15 CONTINUE
00934 STOP
00950 END
00960C ...
00970C SPECIFICATIONS OF THE SUBROUTINES FCN AND OUT

00980C ...
00990 SUBROUTINE FCN(T,Y,F)
01000C ...
01010C SCALAR ARGUMENTS
01020 REAL T
01030C ARRAY ARGUMENTS
01040 REAL F(3),Y(3),K1,K2,K3,K4,K5,K6
01045 COMMON/CONST/K1,K2,K3,K4,K5,K6
01050 COMMON XEND,H,I
01060 F(1)=K1+K2*Y(1)+K4*Y(2)+K5*Y(1)*Y(2)
01070 F(2)=K1+K3*Y(1)+K4*Y(2)+K5*Y(1)*Y(2)
01075 F(3)=(K3-K2)*Y(1)-K6*Y(3)
01080 RETURN
01090 END
01100C ...
01110 SUBROUTINE OUT(X,Y)
01120C ...
01130 REAL X
01140C ARRAY ARGUMENTS
01150 REAL Y(3)
01160C SCALARS IN COMMON
01170 REAL H,XEND
01180 INTEGER I

01190C LOCAL SCALARS
01200 INTEGER J,NOUT
01210 COMMON XEND,H,I
01220 DATA NOUT /6/
01230 WRITE (NOUT,9999)X,(Y(J),J=1,3)
01240 X=XEND-FLOAT(I)*H
01250 I=I-1
01260 RETURN
01270 9999 FORMAT(1H ,F7.2,3E13.5)
01280 END
01290 SUBROUTINE PEDERV
01300 RETURN
01310 END

READY.
IDLE

```

```

LIST
10 '
20 ' This programme displays a graph of concentration against time.
30 ' The program plots the values obtained by running the nsim
40 ' program on the mainframe Cyber computer it plots data sets
50 ' representing (t,c1),(t,c2), where c1 and c2 are the two
60 ' concentrations simulated by the Cyber
70 '
71 ' Define function keys F1 and F2 for pausing and continuing the
72 ' simulation in progress
73 '
74 KEY 15, CHR$(&H40)+CHR$(35)
75 KEY 16, CHR$(&H40)+CHR$(36)
76 ON KEY (15) GOSUB 4000
77 KEY (15) ON
78 ON KEY (16) GOSUB 5000
79 KEY (16) ON
80 CLEAR
90 ON ERROR GOTO 1620
100 COLOR 2,0
110 CLS
120 GOSUB 650
130 COLOR 2,0
140 LO=12
150 '
160 LOCATE LO,1:PRINT "Enter name of data file to be displayed : ";
170 LOCATE LO,43
180 N$=""
190 WHILE D<>13
200 GOSUB 580
210 IF D=59 THEN 400
220 PRINT D$;
230 N$=N$+D$
240 WEND
250 N$=LEFT$(N$,LEN(N$)-1)
260 M$="B:"*N$
270 OPEN M$ FOR INPUT AS #1
280 INPUT #1,LGTH,RPTS
290 GOSUB 940
300 GOSUB 720
310 GOSUB 1090
320 GOSUB 580
330 IF D<>59 THEN 320
340 GOSUB 490
350 END
360 '
370 ' Subroutine to pass output to colour monitor.
380 '
390 DEF SEG=0
400 POKE &H410,(PEEK(&H410) AND &HCF) OR &H10
410 SCREEN 1,0,0,0
420 SCREEN 0
430 SCREEN 1
440 LOCATE ,1,6,7
450 RETURN
460 '
470 ' Subroutine to return output to monochrome monitor.
480 '
490 DEF SEG=0
500 POKE &H410,(PEEK(&H410) OR &H30)
510 SCREEN 0

```

' Find maxima and minima of data.
' Set up the graphics screen.
' Plot data.

```

530 LOCATE ,1,12,13
540 RETURN
550 '
560 ' Subroutine to read the keyboard
570 '
580 D$=INKEY$
590 IF D$="" THEN 580 ELSE D$=RIGHT$(D$,1)
600 D=ASC(D$)
610 RETURN
620 '
630 ' Routine to print program header.
640 '
650 COLOR 0,7
660 LOCATE 1,28
670 PRINT " CONCENTRATION VS. TIME "
680 RETURN
690 '
700 ' Subroutine to set up the graphics screen.
710 '
720 BGSUB 390
730 COLOR 0,0
740 VIEW (1,1)-(315,185),3
750 VIEW (214,15)-(312,32),0,1
760 FOR N=1 TO 10
770 PSET (285+2*N,39-N),1
780 PSET (285+2*N,64-N),2
790 NEXT N
800 VIEW (214,39)-(312,56),0,2
810 VIEW (214,63)-(312,127),0,1
820 VIEW (214,135)-(312,176),0,2
830 VIEW (5,5)-(210,180),0,3
840 WINDOW (TMIN,CMIN)-(TMAX,CMAX)
850 FOR C=1 TO 10
860 LINE (TMAX*C/10,0)-(TMAX*C/10,CMAX/50),3
870 LINE (0,CMAX*C/10)-(TMAX/100,CMAX*C/10),3
880 NEXT C
890 LINE -(0,CMAX*C/10),3
900 RETURN
910 '
920 ' Subroutine to find maxima and minima of concentration and time.
930 '
940 '
950 TMIN=0:TMAX=0
960 CMIN=0:CMAX=0
970 AMIN=0:AMAX=0
980 FOR I=1 TO RPTS
990 INPUT E1, ANC(I),K1(I),K2(I),K3(I),K4(I),K5(I),K6(I)
1000 FOR J=1 TO LGTH
1010 INPUT E1,TIM,COH,DUMMY,A
1020 IF TIM<TMIN THEN TMIN=TIM ELSE IF TIM>TMAX THEN TMAX=TIM
1030 IF COH<CMIN THEN CMIN=COH ELSE IF COH>CMAX THEN CMAX=COH
1040 IF A<AMIN THEN AMIN=A ELSE IF A>AMAX THEN AMAX=A
1050 NEXT J
1060 NEXT I
1070 CLOSE E1
1080 RETURN
1090 '
1100 ' Routine to plot the concentration/time data.
1110 '
1120 OPEN M$ FOR INPUT AS E1
1121 ' Define function keys F1 and F2 for pausing and continuing the
1122 ' simulation in progress
1123 '
1124 KEY 15, CHR$(8H40)+CHR$(35)
1125 KEY 16, CHR$(8H40)+CHR$(36)

```

```

1127 KEY (15) ON
1128 ON KEY (16) GOSUB 5000
1129 KEY (16) ON
1130 INPUT F1,DA,DB
1140 FOR I=1 TO RPTS
1150 INPUT F1,D(1),D(2),D(3),D(4),D(5),D(6),D(7)
1160 LOCATE 3,28
1170 PRINT"100% NADH"
1180 LOCATE 4,28
1190 PRINT USING "F.fffff^";CMAx;
1200 LOCATE 6,28
1210 PRINT"100% FMNH2"
1220 LOCATE 7,28
1230 PRINT USING "F.fffff^";AMAX
1240 FOR G=1 TO 7
1250 LOCATE 9,28
1260 PRINT " Constants"
1270 LOCATE 6+9,28
1280 PRINT USING "F_=";G
1290 LOCATE 6+9,30
1300 PRINT USING "F.fffff^";D(G)
1310 NEXT G
1320 LOCATE 18,28
1330 PRINT"TIME="
1340 LOCATE 19,39
1350 PRINT"S"
1360 LOCATE 19,28
1370 PRINT"[NADH]/M ="
1380 LOCATE 21,28
1390 PRINT"[FMNH2]/M ="
1400 FT=0
1410 PC=0
1420 PA=0
1430 FOR J=1 TO LGTH
1440 INPUT F1,T,COH,DUMMY,A
1450 PSET (T,COH),1
1460 PSET (T,A*CMAx/AMAX),2
1470 LOCATE 18,33
1480 PRINT USING"ffff.f";T
1490 LOCATE 20,29
1500 PRINT USING "F.fffff^";COH
1510 LOCATE 22,29
1520 PRINT USING "F.fffff^";A
1530 FT=T
1540 PC=COH
1550 PA=A*CMAx/AMAX
1560 NEXT J
1570 NEXT I
1580 RETURN
1590
1600 ' Error trapping routine.
1610
1620 END
1630 CLS
1640 GOSUB 650
1650 LOCATE 5,1
1660 PRINT "FILE NOT FOUND OR BAD FILE NAME"
1670 COLOR 2,0
1680 PRINT "Files available : "
1690 PRINT
1700 FILES "b:
1710 LO=27
1720 D=0
1730 RESUME 150
3999
1600 ' Error trapping routine.

```

```
4000 ROUTINE TO PAUSE THE SIMULATION PLOT  
4001 '  
4002 LOCATE 1,32  
4003 PRINT "PAUSED"  
4005 D$=INKEY$  
4006 IF D$="" THEN 4005  
5000 LOCATE 1,33  
5002 PRINT "RUNNING"  
5003 RETURN  
OK
```

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