An Enzyme Amplification Cascade For The Detection Of Alkaline Phosphatase For Use In Diagnostic Procedures.

A Thesis submitted for the Degree of Doctor of Philosophy of the University of London.

by

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Venienti occurrite morbo.

Persius III 63.

Dedicated to the memory of Dr. Michael Robert Hollaway.

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ABSTRACT.

A simple to use, quantitative and extremely sensitive colorimetric assay, designed to be used in a detection system in diagnostic assay systems, is described. The technology of an amplified colorimetric assay, developed by London Biotechnology Limited, is based on a conceptually new principle which is covered by an issued generic patent. The enzyme amplification cascade detects alkaline phosphatase, a widely used enzyme label in immunoassays and gene probes, via dephosphorylation of a novel substrate, FADP, to produce the prosthetic group FAD. This binds stoichiometrically to inactive apo-D-amino acid oxidase, to produce the active holoenzyme which oxidizes D-proline to yield hydrogen peroxide; this in turn is quantitated by a coupled reaction utilizing horseradish peroxidase. The signal-to-noise ratios and sensitivity are enhanced by the kinetics of the amplification cascade.

The criteria for the quality control of the reagents used the the cascade system are much more severe than those used in conventional assay systems. Methodologies for the monitoring of contaminants in the production of these materials have been developed. Thus the detection system itself has been used to trouble-shoot the production processes. The primary substrate, FADP, and apo-D-amino acid oxidase have been prepared in high yield and purified to the level required. FADP has been produced with a contamination level of FAD equivalent to 2 ppm and apo-D-amino acid oxidase has been prepared which contains only 10 ppb alkaline phosphatase activity.

Evaluation of the technology has demonstrated it to be suitable for use on high quality clinical analyzers; and the methodology has been used in conjunction with the releasable linker technology developed by Du Pont. This system has been used to measure TSH across the entire range of clinical interest.

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ABBREVIATIONS.

a	Concentration of cyclic flavin at time t.
a_0	Concentration of cyclic flavin at t_0.
Ab	Antibody.
Abs	Absorbance.
ADP	Adenosine diphosphate.
AMP	Adenosine monophosphate.
AMPPD	Adamantyl-1,2-dioxetane phosphate.
AP	Alkaline phosphatase.
ATP	Adenosine triphosphate.
BCIP	5-bromo-4-chloro-3-indoyl phosphate.
Bis-Tris	Bis(2-hydroxyethyl)imino-tris (hydroxymethyl)methane.
C	Centigrade.
CARD	Catalyzed Reporter Deposition.
CHAPS	3-((3-cholamidopropyl)-diethylammonio)-1-propane sulphonate.
cm	Centimetre.
DCHB	3,5-dichloro-2-hydroxybenzene sulphonic acid.
DNA	Deoxyribosenucleic acid.
dps	Decays per second.
E_0	Total enzyme concentration.
EF	Concentration of active enzyme.
EDTA	Ethylenediamine tetra acetic acid.
EIA	Enzyme Immunoassay.
ELISA	Enzyme linked immunosorbant assay.
EP	Enzyme-product complex.
F_0	Total flavin concentration.
FAD	Flavin adenine dinucleotide.
FADcP	Flavin adenine dinucleotide-2',3'-cyclicphosphate.
FADP	Flavin adenine dinucleotide-3'-phosphate.
FADPase	FAD-phosphatase.
FCFD	Fluorescent capillary fill device.
4-MU	4-methylumbelliferone.
4-MUP	4-methylumbelliferyl phosphate.
FPIA	Fluorescence polarization immunoassay.
g	Gram.
HBsAg	Hepatitis B surface antibody.
HBV_c	Hepatitis B virus core antigen.
HRP	Horseradish peroxidase.
IgG	Immunoglobulin G.
INT-violet	p-iodonitrotetrazolium violet.
IRMA	Immunoradiometric assay.
IU	International units.
K_{cat}	Catalytic constant.
K_m	Michaelis rate constant.
K_p	Dissociation rate constant of EP for an irreversible reaction.
K_D	Dissociation constant.
L	Litre.
L.B.L.	London Biotechnology Limited.
m	Milli.
M	Molar.
MEIA	Micro-particle Enzyme Immunoassay.
MES	2-(N-morpholino)ethane sulphonic acid.

NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form).
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form).
NBT	Nitroblue tetrazolium.
n.d.	Not detectable.
nm	Nanometers.
oPD	o-phenylenediamine.
PEP	Phosphoenol pyruvate.
Pi	Orthophosphate(inorganic).
pI	Isoelectric point.
pNPP	p-nitrophenyl phosphate.
ppb	Parts per billion.
PPi	Pyrophosphate(inorganic).
ppm	Parts per million.
PYR	Pyruvate.
RELIA	Releasable Enzyme Linker Immunoassay.
RIA	Radioimmunoassay.
RVV	Russell's viper venom.
s	Second.
t	Time.
$t_{1/2}$	Half life.
Tris-HCl	Tris(hydroxymethyl)aminomethane.
TSH	Thyroid stimulating hormone.
u	Micro.
V_f	Velocity of an enzyme reaction in the forward direction.

CHAPTER ONE

INTRODUCTION.

1.1. Immunoassays in diagnostics.

The specificity of immunological reactions has long been applied to the detection and measurement of antibodies resulting from infection and the direct detection of bacterial and viral antigens. Immunoassays have become a common diagnostic tool. Initially visual systems were employed based on precipitation or agglutination reactions. Some of these basic systems are still used today eg. for blood group typing. To quantitate the reaction either the antigen or the antibody is labelled. However, the demand for ever increasing sensitivity has led to the development of radioimmunoassays using radioactive labels, such as ^{125}I, instead of chemical or enzyme reporters. The radioimmunoassay as we know it today began with application to insulin antibodies to measure the hormone in plasma (Yalow and Berson, 1960).

Radioimmunoassays (RIA) and immunoradiometric assays (IRMA) both usually employ radioisotope labels of high specific activity and short half-life which limits the shelf-life of the reagent to a few weeks (See Section 1.4). Also, concern over the safety of operatives, the

environmental impact of ionizing radiation, and problems of the disposal of radioactive waste has prompted a movement away from RIA's and the development of non-isotopic methods. Enzyme labels have been used in clinical laboratories (See Section 1.6.2.). However, the limited sensitivity of conventional enzyme immunoassays has frequently been due to the difficulty in detecting the small number of coloured product molecules generated by the enzyme label in a reasonable time period. In theory, using a catalyst as a label would produce a system of infinite sensitivity, limited only by the rate of the reaction, the time period of measurement and interferences. Enzymes with a high turnover of substrate, such as horse radish peroxidase, alkaline phosphatase or β-galactosidase have been attached to an antibody or antigen. A variety of non-isotopic detection systems with enhanced sensitivity have been described, including time delayed fluorescence, enhanced luminescence, amperometric and enzyme amplification (Diamandis, 1988; Whitehead et al., 1983; Stanley et al., 1985).

1.2. Genetic probes.

Mankind is afflicted by more than 3,000 known different inherited disorders. They affect every organ, system and tissue in the human body. Some cause disease even before birth eg. muscular

dystrophy, whereas others are observed only in adulthood eg. Huntingdon's chorea. Some are common eg. cystic fibrosis, and some are rare eg. Tay-Sach's disease. Although some diseases may be associated with a quite common genetic defect, for example 1/20 of the North European population are carriers of cystic fibrosis, genetic diseases in man represent only a small market for diagnostics.

Gene probe assays are based on the fundamental structure of DNA: strings of nucleotides with reverse complementarity form stable double-stranded molecules. Gene probes were originally developed as basic tools in molecular biology. Their uses, however, are not solely restricted to the detection of inherited diseases in pre- or postnatal analysis (Reeders et al., 1986), since they may be used to detect organisms causing transmissible (infectious) disease (Echeverria et al., 1984; Moseley et al., 1980; Gonzalez and Hanna, 1987). Gene probes are of potential value to identify individuals who are predisposed to conditions such as diabetes, coronary heart disease and some forms of cancer. Latent virus infections can also be identified (Malcolm, 1985), and gene probes may have general application in the food industry (Parsons, 1988).

Automated oligonucleotide synthesizers make it possible to generate a suitable hybridization probe

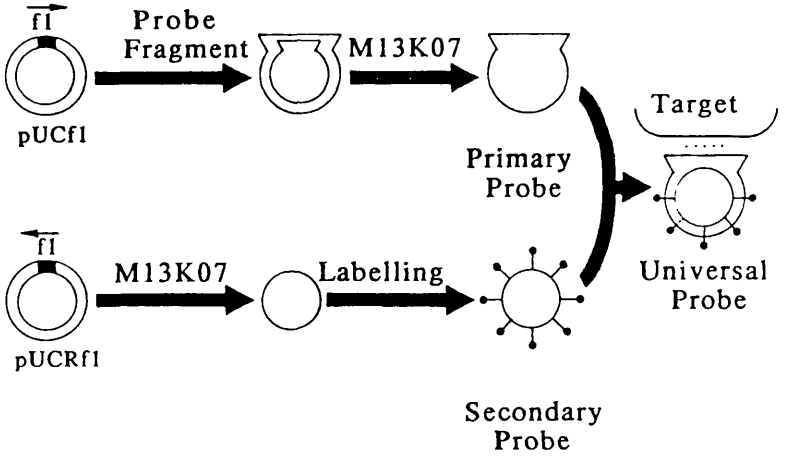
for virtually any nucleic acid of known sequence. To date, gene probes radiolabelled with ^{32}P, and detected by autoradiography, are most commonly used (Lowe, 1986). Commercialization of gene probes as diagnostic tools is dependent upon replacement of radioactive labels with easily detected, long shelf-life, non-radioactive labels (Klausner and Wilson, 1983; Landegren, et al., 1988).

Many non-radioactive gene probe assays have been developed (Renz and Kurz, 1984), most of these involve labelling the probes with biotin (Manning et al., 1975; Langer et al., 1981; Leary et al., 1983). Conventional cytochemical techniques eg. avidin-peroxidase, can be used to detect the biotin after hybridization. Oligonucleotide probes have also been synthesized to incorporate specifically fluorescent, chemiluminescent and enzyme labels (Urdea et al., 1988).

Most enzyme-labelled probes utilize alkaline phosphatase as the preferred label due to its fast turnover and high thermal stability (Jablonski et al., 1986). Previous studies have shown a detection limit of 0.5 femtomol for a non-radioactive biotinylated alkaline phosphatase probe compared to a detection limit of 5.0×10^{-4} femtomol for a ^{32}P labelled probe (Wallace et al., 1985). Improved techniques have rendered sensitivity essentially equal to that of ^{32}P

labelled probes (Takahashi et al., 1989), but procedures are complex and are subject to poor reproducibility. In an attempt to solve this problem Nakagami et al. (1991), synthesized a "Universal Probe System"-based on the principle of the sandwich hybridization assay with two single-stranded DNA probes (See Figure 1.). Each of the primary probes would therefore not be individually labelled with an enzyme, but a unique tailing sequence recognized by a secondary enzyme labelled probe.

Figure 1. Construction of a "Universal Probe."



A target gene fragment is cloned into pUCf1 plasmid. The single-stranded DNA is prepared from the resulting plasmid by helper phage (M13K07) and used as a primary probe. The single-stranded DNA from pUCRf1, which had f1 in orientation opposite to that of pUCf1, was prepared by helper phage and biotinylated.

1.3. Probe sensitivity.

Genetic probes already exist or are being developed for several severe inherited disorders. Prenatal diagnosis by means of gene probes is already carried out on a research basis, but the increasing reliability and range of tests mean that DNA diagnosis is becoming a potential service for all families at high risk.

The opportunity to detect the presence of a genetic disorder *in utero*, by probing DNA isolated from material obtained by chorionic villus sampling or amniocentesis, offers the possibility of early termination (7-10 weeks) avoiding the trauma of termination at a much later date. This is but one application of DNA probe technology. However, it is the basis upon which our target sensitivity is formulated. The amount of DNA isolated by the aforementioned techniques is of the order of 1 microgram (10^{-6} gram). As the effective molecular weight of the human genome is about 2×10^{12}, this amount of DNA corresponds to less than 1 attomol (10^{-18} mol), or fewer than 600,000 copies of any gene that occurs once only in the genome. 1 attomol is therefore the target for the level of detection of a gene probe for the diagnosis of an inherited disease *in utero*.

1.4. Disadvantages of radioisotopes.

1.4.1. Preparation of isotopes.

Production of the radioactive isotopes must be carried out by specialist institutions. Costly apparatus is required and specially trained staff are essential for this type of operation.

1.4.2. Hazard.

Dangerous levels of radioisotopic labels are used in research and clinical laboratories. ^{125}I is readily concentrated in the thyroid and ^{32}P in bone. Special precautions to lessen the potential hazard, such as "hot" rooms, cabinets and personnel screening are therefore essential. Not only must these precautions be incorporated into the handling procedures of such materials but also special facilities are required for waste disposal. All of these factors increase the cost of a radioactive diagnostic process.

1.4.3. Sensitivity and speed of detection.

The detection limit for a radioisotope procedure can be estimated from the half-life ($t_{1/2}$) of its decay. The shorter the half-life, the lower the detection limit. ^{125}I and ^{32}P can be detected in a few minutes at a level of 1-10 attomol using modern counting equipment; however, autoradiography requires hours or days.

Table 1. Detection limits of radioisotopes.

Label Isotope	Particle	$t_{1/2}$	Approximate limit of detection (mol). *
^{14}C	$\beta-$	5568 years	$4x10^{-13}$
^{3}H	$\beta-$	12.26 years	10^{-15}
^{125}I	γ	60 days	10^{-17}
^{32}P	$\beta-$	14.4 days	$3x10^{-18}$
^{131}I	β_i,γ	8.09 days	$2x10^{-18}$

* assuming an ability to detect 10dps over background.
(after A. K. Campbell, 1988).

1.4.4. Consequences of isotope decay.

The most sensitively detectable isotopes are by their very nature the ones that decay most rapidly and they cannot therefore be stored for significant periods of time. Moreover, biological molecules such as proteins are highly susceptible to radiolytic damage. ^{125}I labelled antibodies may self destruct in a few weeks, causing the immunoassay components to possess only a short shelf-life.

1.4.5. The need for a separation step.

In order to count the radioactive label and quantify results, the appropriate material must be separated both before and after the addition of a ^{32}P labelled probe. In autoradiography, unreacted or unbound label must be removed from the microscope section or nitrocellulose filter. For use in immunoassays,

antibody-antigen complexes must be separated from free antibody and antigen. All of these stages introduce some imprecision, as well as complicating automation for clinical application.

1.5. Criteria for acceptability of an alternative system to those employing radioisotopic labels.

1.5.1. Rapid quantification and sensitivity.

The system should be able to easily detect in the femtomol-attomol range, using, ideally, simple and inexpensive apparatus. To compete in the market place with existing RIA's, enzyme immunoassays need relatively short incubation times. A limit of 1 hour is a convenient target.

1.5.2. Visibility of the signal.

For some, but not all applications, it is desirable to observe a visual signal. Other applications may require the formation of a precipitate or luminescent signal. A versatile technology should therefore be capable of coupling to a range of detection systems using a simple amplification methodology.

1.5.3. Attachable to biological molecules.

The label must be capable of attachment to a wide range of molecules of biological and clinical interest. Coupling must not seriously affect either

the label detection or the chemical and biological properties of the molecule to which it is attached.

1.5.4. Stability.

The label, both initially and when attached to the molecule of biological interest should be stable, having a shelf-life of several months.

1.5.5. Non-susceptible to environmental factors.

The signal produced by the label should not be susceptible to interference by chemical factors present in biological samples, or physical factors involved in the assay procedures.

1.5.6. Clinically applicable.

It should be possible to automate both initial assay steps and the detection of the label, whilst retaining precision for the clinical range of the analyte in question (Landegren, 1988).

1.5.7. Safety.

The label should be safe to prepare and to use. It should be non-toxic to humans.

1.5.8. Availability.

The label should be readily available in sufficient quantities and cheap to produce.

1.6. Alternative detection systems.

1.6.1. Chemical labelling of the probes.

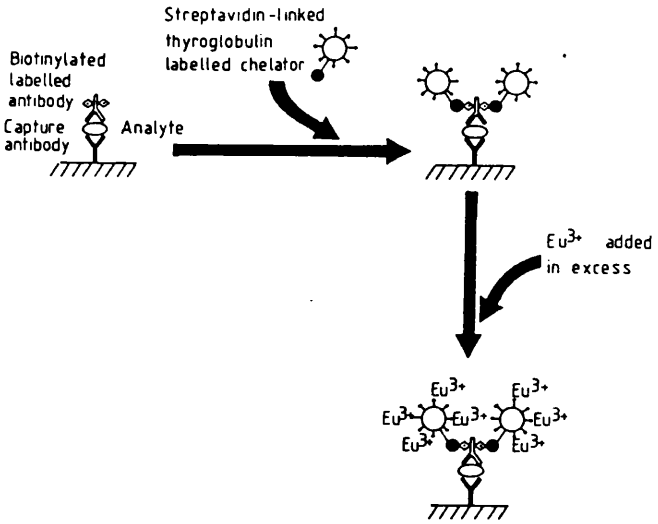
Chemical reporters ,such as fluorescent markers, have been employed in immunoassay procedures (Centifanto and Kaufman, 1971; Davies et al., 1974). However, the sensitivity of these assay systems is poor compared with RIAs. It has only been through the development of reliable automated microfluorometers (Haaijman and Wijnants, 1975) and the simplicity of these assay systems that the fluoroimmunoassay has become a viable process. The most commonly used marker in these assays is fluoroscein which can be used to measure the concentration of analytes in the nanomolar range and above.

Fluoroimmunoassays, like RIAs, have inherent problems. These include separation of fluorescence emission from excitation; scattering of the signal; background fluorescence from cuvettes, optics and sample; non-specific binding of the reagents and fluorescence quenching. Not least of these problems is the characteristic fading of fluorescence which in the past has made quantification difficult (Jongsma, et al., 1971). Development of an assay based on fluorescence has therefore been aimed at overcoming these problems eg. fluorescence fluctuation immunoassays (Elings, et al., 1983) and time resolved fluorescence assays (Diamandis, 1990).

The time resolved fluoresence assay employs a europium or lanthanide chelator as the fluorescent reporter. These complexes have a characteristic long lived fluorescence compared to classical fluorescent probes and narrow emission bands (Diamandis, 1988). Most conventional fluorophores have a lifetime of approximately 100ns or less; the lifetime of lanthanide chelates on the other hand is 100-1000us. The fluoresence of these compounds can therefore be measured following a time delay after excitation (200-600us). The contribution to the signal by background interference from the sample etc. is short lived and therefore eliminated. The major source of error is attributable to non-specific binding of the reporter molecules.

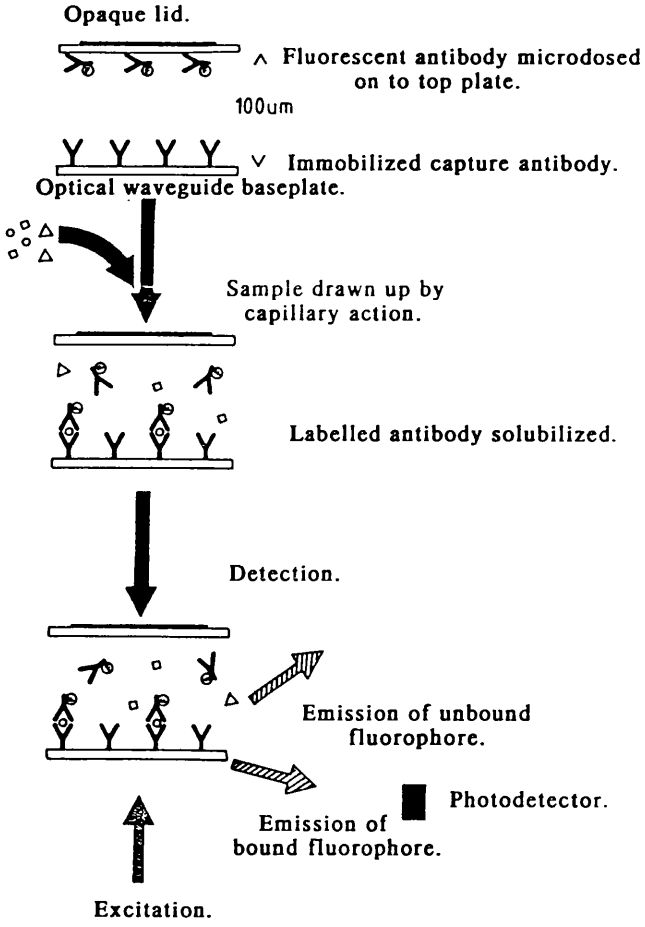
Such europium chelates are the basis of the DELFIA^{R} system. These complexes are of the type $Eu(NTA)_3(TOPO)_2$ where NTA is naphthoyl-trifluoroacetone and TOPO is trioctylphosphine oxide (Diamandis, 1988). The detection limit of this system is approximately 10^{-17}mol analyte. It is however susceptible to europium contamination effects. A second generation system FIAgen^{R} does not suffer from such effects. The chelator in the system is streptavidin-linked and can be used with biotinylated antibodies in a two site immunoassay (See Figure 2.). The smallest concentration of analyte detected by this system is 10^{-12} - 10^{-13}M.

Figure 2. A two site immunoassay employing a fluorescent reporter.



More recently following advances in optical techniques the phenomenon of surface plasmon resonance and the technology of total internal reflection spectroscopy have been employed (Robinson, 1991). The technology of the Fluorescent Capillary Fill Device (FCFD), shown in Figure 3, has been used to create a homogeneous assay system which is aimed at usage outside of the clinical laboratory.

Figure 3. The Fluorescent Capillary Fill Device.



The sample under investigation is drawn by capillarity into the testing device containing the predispensed reagents. The capillary tube is placed into an optical immunosensing system and the amount of bound fluorophore detected (Deacon et al, 1991).

This system tackles some of the associated problems of alternative site immunosensing. The volume of the sample does not require accurate measurement as it is drawn in to the capillary. Assay times are also short (Robinson, 1991), because the reagents only have a small distance over which they must diffuse. The light arising from the bound fluorophore is discriminated from the free fluorophore emission by the evanescent optics of the baseplate waveguide. A washing step is therefore not required to separate material. The system does however require a dedicated analyzer.

1.6.2. Enzyme-linked assay systems.

Enzyme immunoassays (EIA) were introduced in the 1970's (Scharpe *et al.*, 1976). Enzyme linked immunoasborbant assays (ELISA) are based on the same principles as are used in RIA., ie. after an initial incubation of antigens and antibodies, the antigen-antibody complexes are separated from free antigen and antibody and the label detected. No single enzyme-label is universally applicable for use in ELISA. The suitability of an enzyme to a particular assay situation must be assessed. A number of factors should be considered in the choice of an enzyme label (Schuurs and Van Weemen, 1977; Wisdom, 1976):

i) Turnover number of the enzyme.

ii) Purity of the enzyme preparation.

iii) Sensitivity of detection of its product (eg. radioactive, fluorogenic or colorimetric).

iv) Ease and speed of detection of the enzyme reaction.

v) Absence of interferring factors or of enzyme-like activity in the test sample.

vi) Ease of linkage of the enzyme to other molecules while retaining a substantial part of the enzyme activity.

vii) Stability of the enzyme and its conjugates under assay conditions and storage: Alkaline phosphatase is reported to have a shelf-life of over 1 year.

viii) Availabilty and cost of the enzyme and its conjugate.

ix) Suitability of the enzyme for homogeneous EIA (if applcable) ie. capable of inhibition or reactivation when antibody binds to the enzyme-hapten conjugate and that the assay conditions are compatible with hapten-antibody binding.

ELISA may be used in a variety of formats. These can be divided into two basic types, the competitive or the non-competitive assay.

1.6.2.1. Competitive assays.

1.6.2.1.1. ELISA using an antigen-enzyme conjugate.

This technique involves a reaction step in which unlabelled and labelled antigen compete for a limited number of antibody sites (See Figure 4.).

Figure 4. Competitive ELISA (after Engvall, 1980).

1. Attach antibody to solid phase.

2. Incubate with enzyme-labelled antigen in presence or absence of standard antigen or unknown sample.

3. Incubate with enzyme substrate.

The antibody, attached to a solid phase, is incubated with a solution containing a fixed concentration of enzyme-labelled antigen, a known but variable concentration of standard antigen, or an unknown concentration of test antigen. The reaction mixture is incubated until the antigen-antibody reaction attains equilibrium. After washing, to remove free antigen, the enzyme activity of the solid phase is determined by incubation with the appropriate substrate buffer.

1.6.2.1.2. ELISA using an enzyme-labelled antibody.

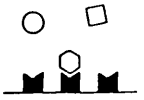
Another type of competitive ELISA employs an enzyme-labelled antibody with the antigen attached to the solid phase. The binding of the enzyme-labelled antibody is competitively inhibited by a known concentration of standard antigen or test antigen. In each case the product concentration is inversely related to the concentration of standard or test antigen in the incubation solution.

1.6.2.2. Non-competitive assays.

These assays take the form of what has become commonly known as the "sandwich assay". Excess immobilized antibody is incubated with the standard or test antigen (See Figure 5.). After washing, the immobilized antibody-antigen complex is incubated with an excess of enzyme-labelled antibody which binds to one or more remaining antigenic sites (Belanger *et al.*, 1973). The concentration of the final product of the enzyme reaction is directly proportional to the concentration of the standard or test antigen.

Figure 5. The "Sandwich Assay" (after Engvall, 1980).

1. Attach antibody to solid phase.



2. Incubate with sample

3. Incubate with enzyme-labelled antibody.

As described above, the labelling of immobilized antigen enables the detection of antibody concentrations in test samples.

1.6.3. The advantages of EIA methods.

i) Specific and sensitive assays can be produced with wide clinical applications. The labelling of antigen, antibody or hapten increases the range of measurements of biological molecules.

ii) The equipment required is relatively cheap and widely available.

iii) Reagents used are relatively cheap and have a long shelf-life eg. Alkaline phosphatase conjugates have a shelf-life greater than 1 year (Halbert and Anken, 1977).

iv) Assays are easy to perform without any special training requirements.

v) Assays may be fast. This can be optimized through the correct choice of label and antiserum with the highest possible affinity. Sensitivity can also be improved by using lower concentrations of antibody and antigen with longer incubation times to attain equilibrium. Multi-site secondary binding in a sandwich assay also enables more of the enzyme label to be isolated for the subsequent signal generation reaction.

vi) A separation stage may not be required. Lysozyme, glucose-6-phosphate dehydrogenase and malate dehydrogenase are well suited to homogeneous EIA. Some competition assays encounter difficulties when incubating enzyme-labelled conjugates with biological samples which may

contain proteases and/or enzyme inhibitors. These will alter the activity of the enzyme used as the label.

vii) A variety of labels are available which may allow multiple, simultaneous assays to be performed.

Table 2. Enzyme labels used in immunoassays (Wisdom, 1976).

Enzyme.	E. C. number.
Malate dehydrogenase	1. 1. 1. 37.
Glucose-6-phosphate dehydrogenase	1. 1. 1. 49.
Peroxidase	1. 1. 1. 7.
Alkaline phosphatase	3. 1. 3. 1.
β-galactosidase	3. 2. 1. 23.
Lysozyme	3. 2. 1. 17.

viii) Automation of ELISA techniques is now well established.

ix) There is no radiation hazard and therefore no expensive handling or monitoring facilities need to be provided.

In general, EIA provide specific and sensitive methods for the identification and quantification of a number of molecules of biological importance (Bidwell et al., 1977; Saunders et al., 1977). The concentration in which drugs can occur in serum and urine are such that detection methods need not be very sensitive, and

therefore only short incubation times are required for these assays. However, for many applications EIA are not as sensitive as the comparable RIA. Table 3 shows the detection limits of enzyme immunoassays employing two widely used enzyme-labels. Some incorporate an element of amplification, whilst others utilize technologies employing specially designed substrates that increase the sensitivity of the enzyme-labelled systems and are comparable to RIA.

Table 3. Comparison of two widely used enzyme-labels employing various detection methodologies (after Kricka, 1991).

	Detection limit amol. Substrate			
Enzyme Label	Colorimetric	Fluorometric	Chemiluminescent	Bioluminescent
Alkaline Phosphatase	50 p-nitrophenyl phosphate 1.0 NADP	0.5 umbelliferyl phosphate 0.1 TM AttoPhos	2.0 AMPPD	0.01 Firefly luciferin-o-phosphate
Horseradish peroxidase	100 TMB 10 o-phenylenediamine	100 4-hydroxyphenyl-acetic acid 10 3-(4-hydroxyphenyl)-propionic acid	25.0 luminol	0.40 Pholas luciferin

Detection via a system of enzyme amplification means that the signal obtained is greatly enhanced over that obtainable by simple use of any one of the enzymes utilized in the system. Such systems generally employ enzyme labels catalyzing the formation of a trigger molecule which activates an "amplifier" resulting in a

detectable change. Amplification results from the combined catalytic effect of the enzyme producing the activator and the catalytic effect of that activator on the secondary system. These secondary systems may be based on another enzyme, a modulator of enzyme activity, or a substrate or cofactor taking part in a cyclic system (Bates, 1987). By reducing background "noise" and improving the signal, it has been possible to produce tests which surpass RIA's in terms of both speed and sensitivity (See Section 1.7.).

Detection via alternative methodologies, based on enzymes are under development. Assays resulting in a fluorescent signal or measurement of luminescence offer the potential for greater sensitivity than colorimetric detectors, and hence much faster assay procedures. At present, immunoassays based on enzymes generating coloured products have proved to be the simplest and most suitable for the clinical market and the "over the counter" market. These coloured products can be measured spectrophotometrically or visually.

1.7. Increasing the sensitivity of enzyme-linked assay systems.

The production of a greater signal in a shorter period of time in enzyme-linked assays has been the focus of much research. The simplest approach is to increase

the relative amount of detectable enzyme label. Oligomeric forms of the enzyme can be linked to the probe molecule (Leary et al, 1983), or labelling may be achieved indirectly through an avidin/biotin complex containing multiple copies of detector enzyme (Kendall et al, 1983; Ternynck and Avrameas, 1990). A variation on this theme, the catalysed reporter deposition (CARD) assay is discussed in Section 1.9.3.

Other developments have included the use of a substrate which is intrinsically detectable with a higher sensitivity, such as luminescence or fluorescence (See Section 1.9.1. and 1.9.4.). Previously, substrates have been designed specifically for this purpose or assays have been coupled to the appropriate detection system (Kato and Suzuki, 1982; Bronstein et al., 1989).

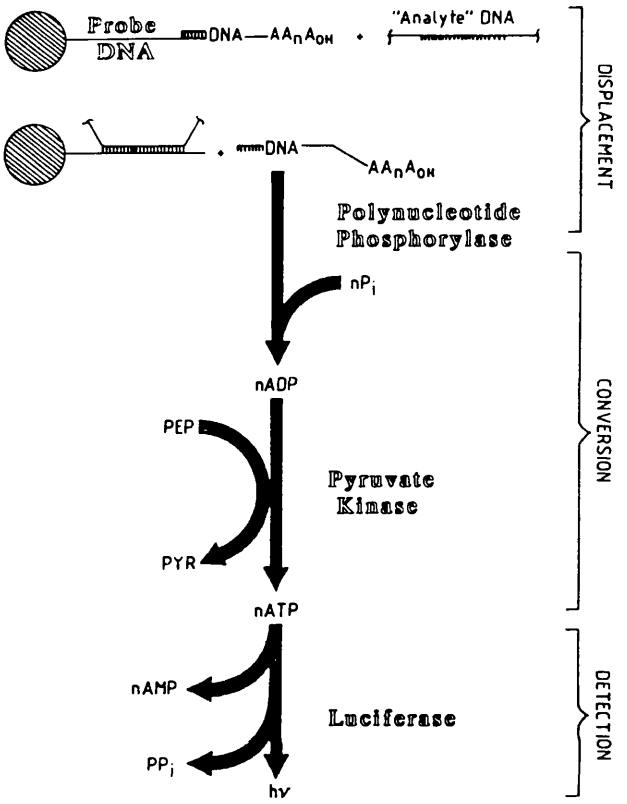
These examples, however do not truly involve enzyme amplification within the definition used here. They produce an amount of the detected substance that is directly and linearly related to the amount of analyte-linked detector enzyme.

True enzyme amplification results from the coupling of the product of the analyte-linked detector to an additional system such as a substrate cycle (Johannsson et al., 1986) or an enzyme cascade (Rabin et al., 1991). The substrate cycle will be discussed

in some detail later (See Section 1.8.1. and 1.9.5.) since it has been commercially developed by IQ (Bio) Ltd. in their alkaline phosphatase detection system. Amplification has also been achieved utilizing the two inactive S-peptide and S-protein fragments of Ribonuclease S (Ehrat et al., 1986). The fragments are immobilized via a "leash" of polycytidylic acid to a Sepharose gel. Hydrolysis of this linkage by Ribonuclease A (E.C. 3.1.27.5.) releases the fragments that recombine to give Ribonuclease S activity. An amplification of 190,000-fold has been achieved over a 20 hour incubation. More recently, β-galactosidase from E. coli has been genetically engineered to produce two inactive fragments that can recombine spontaneously to form the active enzyme in a process called complementation (Khanna, et al., 1989).

A strand displacement assay has also been developed to produce a sensitive detection system (Vary et al., 1986). Typically, hybridization of target DNA sequences by a probe-signal strand complex, displaces the signal strand. This signal strand, containing a 3'-polyadenylated tail is eluted from a solid support and converted to ATP. Luciferase is used in a bioluminescent assay to quantify the ATP produced (Bergmeyer, 1988). The reaction scheme is shown in Figure 6.

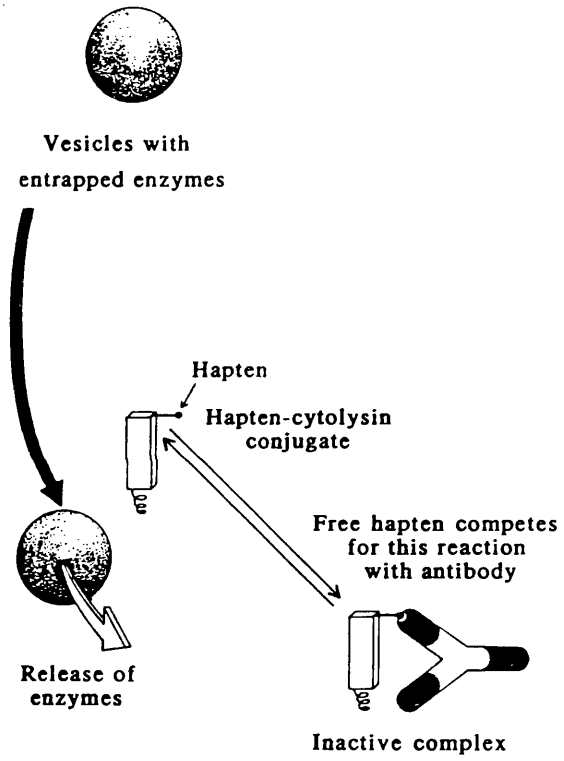
Figure 6. A strand displacement assay.



The release of enzymes from a microcapsule or vesicle can also form the basis of a competitive assay (See Figure 7). Alkaline phosphatase has been used as a marker molecule in such a system. Analyte and analyte-cytolysin conjugate compete for specific antibobies in the assay solution (Litchfield et al, 1984). Once bound by the antibody the cytolysin conjugate is inactive and the vesicles containing the detection enzyme cannot be disrupted. The concentration of free cytolysin conjugate is therefore

proportional to the concentration of analyte. The advantage of such a system is that a whole variety of molecules, such as fluorescent probes, enzymes and enzyme substrates can be used as markers.

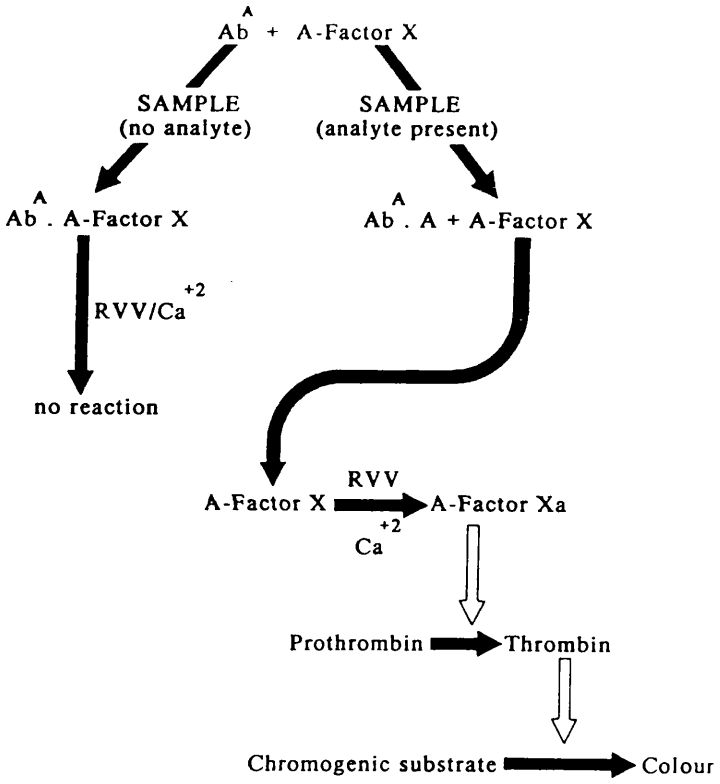
Figure 7. A liposome-based immunoassay employing a novel hapten-cytolysin conjugate.



Enzyme cascades that inhibit an enzyme reaction (Mize et al., 1989), or activate a reaction also result in amplification of the detection system. Classically, the enzyme cascade can be demonstrated by a system that has been developed from part of the natural cascade mechanism of blood clotting (Blake et al, 1984). A protease fraction from Russell's viper venom (RVV) acts upon Factor X, initiating a two-step

cascade that culminates in the generation of thrombin. The activity of the thrombin is measured spectrophotometrically using a chromogenic substrate. The essence of the system is shown in Figure 8.

Figure 8. Zymogen activation immunoassay system.



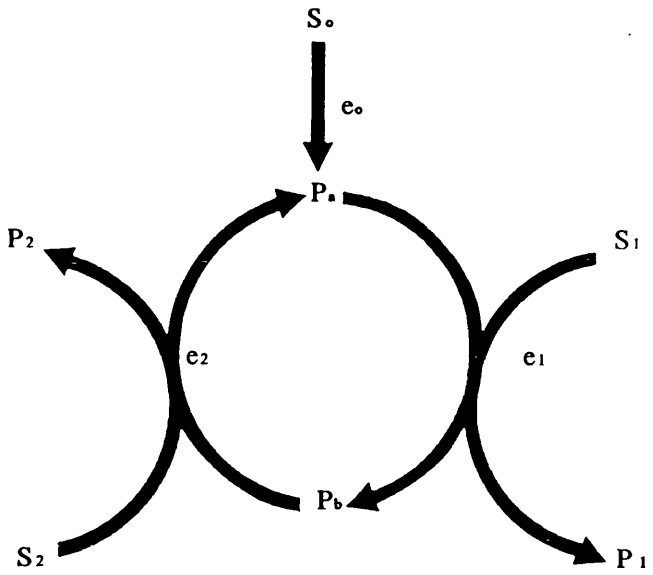
1.8. Enzyme amplification.

None of the aforementioned amplification procedures has been developed into a practical system. The simplest amplification systems that can be applied are based on cycling of a substrate or cofactor which is generated by a primary enzyme label, for example, alkaline phosphatase. Here, two systems will be described. First the substrate cycle reaction, as originally developed by IQ (Bio) Ltd. now part of Novo Biolabs, Cambridge. Secondly, an enzyme cascade system, involving prosthetogenesis (Rabin *et al*, 1990).

1.8.1. Amplification by substrate cycling.

Enzyme cycles that amplify the concentration of substrates were first described some years ago (Lowry *et al*, 1961; Kato, 1975). Application of the principle to produce a simple amplification system has however only been a recent development. The principle of the substrate cycle is illustrated in Figure 9.

Figure 9. An amplification system based on a substrate cycle (Johannsson and Bates, in Kemeny and Challacombe, 1988).



e_0 primary label

S_0 substrate

P product not directly detected that can cycle between two forms, P_a and P_b

e_1 e_2 coupled enzyme cycle to interconvert P_a and P_b

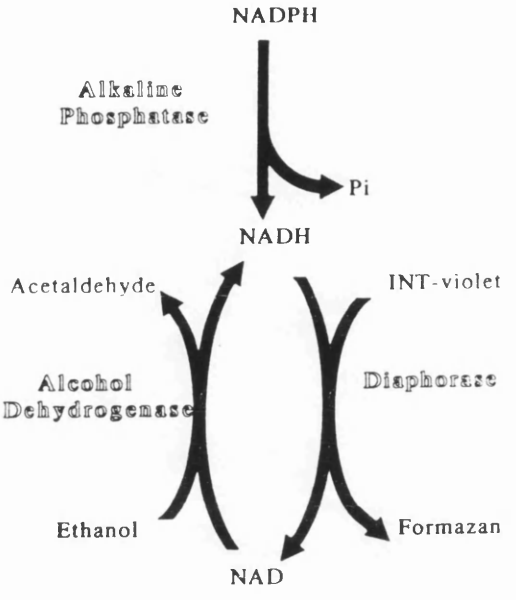
P_1 P_2 products of enzyme cycle that can be simply detected from substrates S_1 and S_2.

Depending on the nature of the enzymes chosen and the compatability of their associated cofactors, substrates and optimum working conditions, it may be possible to allow all three of the enzymes to work simultaneously in a single step. It may be preferable to separate the primary reaction and secondary cycling system into two sequential steps.

Maximum response is achieved when all three substrates S_0, S_1 and S_2 are provided at concentrations above their K_m values. Strict requirements are imposed on the purity of the substrates and specificity of the enzymes. Enzymes e_1 and e_2 must be able to cycle low concentrations of P in the presence of much higher concentrations of S_0. S_0 must therefore not be a substrate for either e_1 or e_2 nor must it compete with P_a or P_b. Also S_0, e_1 and e_2 must not be contaminated with P_a or P_b. The non-enzymic reaction between S_1 and S_2 must not occur at any significant rate.

The simplest method developed for amplifying alkaline phosphatase activity by this procedure is a substrate cycle formatted as a two step or sequential assay (Johannsson and Bates, in Kemeny and Challacombe, 1988). The components of the system are shown in Figure 10.

Figure 10. Enzyme amplification of alkaline phosphatase activity by substrate cycling.



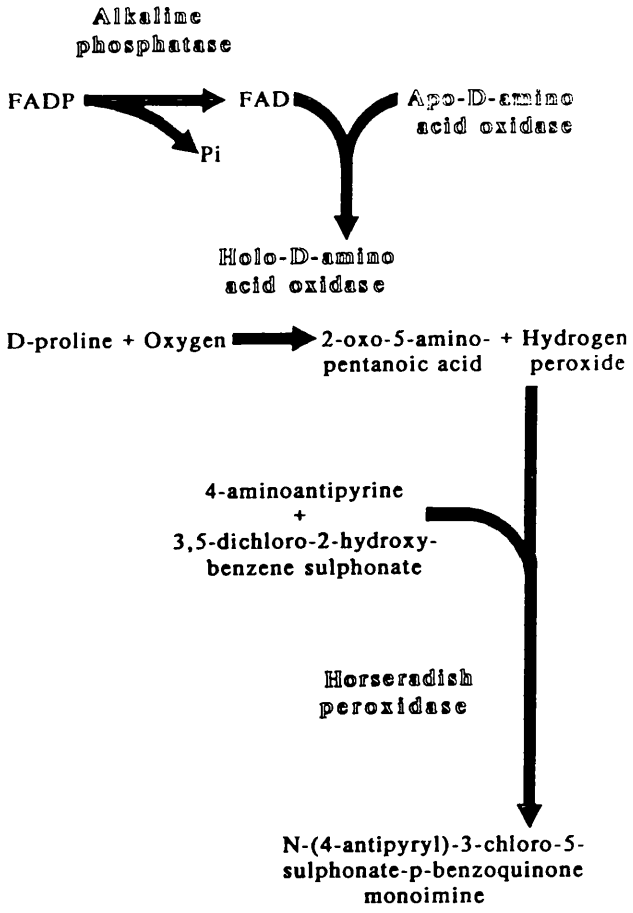
The production of formazan is measured spectrophotometrically at 492nm (See Section 1.9.5.).

1.8.2. Amplification by an enzyme cascade.

Apoenzymes have previously been used in the determination of the concentration of their cofactors in biological samples and in prosthetic group labelled enzyme immunoassays (Ngo and Lenhoff, 1985). Thus apo-D-amino acid oxidase has been used in such a system (Hinkkanen and Decker, 1983) to measure FAD. When coupled to a luminometric detection system, femtomol quantities of FAD were detected. However, an enzyme that could produce FAD from an inactive precursor would be detectable in even lower concentrations. Such

a system is under development by London Biotechnology Ltd. (Figure 11.) and is the subject of this project.

Figure 11. Amplification of alkaline phosphatase activity by an enzyme cascade system.



Enzyme cascade systems, like enzyme substrate cycles have been known for many years. The kinetics of such a system are non-linear, because the concentration of the enzyme that contributes to the visualization reaction, D-amino acid oxidase, is time dependent and increases as more of the active prosthetic group is

released by alkaline phosphatase, the enzyme used to label the probe. The acceleration of response with time is characteristic of an enzyme activation assay. This makes it possible to assay with greater sensitivity at shorter reaction times.

Flavin adenine dinucleotide phosphate (FADP) is a derivatized prosthetic group, flavin adenine dinucleotide (FAD), that does not possess any reconstitutable activity with apo-D-amino acid oxidase (E.C. 1.4.3.3.). Dephosphorylation, by the action of alkaline phosphatase releases the prosthetic group, FAD, which reconstitutes the apo-D-amino acid oxidase to the active holoenzyme. Oxidation of D-proline by this enzyme yields hydrogen peroxide as a product of the reaction. The hydrogen peroxide is measured spectrophotometrically utilizing horseradish peroxidase (E.C. 1.11.1.7.) and a chromogenic substrate. The coloured product is measured at 520nm (Fossati et al., 1980).

The constraints on the purity of each component of the system are particularly strict, as are the criteria for the components operational characteristics.

i) No FAD must be present in any of the components of the system (See Sections 3.6.3 and 3.7.4.).

ii) No active D-amino acid oxidase or other enzyme utilizing the D-amino acid substrate must be present in any of the components.

iii) No "phosphatase activity" must be present in any of the components (See Section 2.6.).

iv) FADP must not be significantly active as a prosthetic group (See Chapter 7).

v) The whole system must be free of hydrogen peroxide and catalase.

vi) The spontaneous reaction between the chromogens used in the visualization system must not occur to any significant degree.

vii) FADP must not compete with FAD for the binding site of apo-D-amino acid oxidase (See Section 5.5.).

viii) D-amino acid oxidase must be able to function at low concentrations of FAD in the presence of high concentrations of FADP (See Section 5.5.).

The compatibility of each component of this cascade system enables the assay to be performed in a single step.

Successive chapters detail the preparation of the macrocomponents of the enzyme cascade assay and the quality control work of each. No independent assay exists that can determine contaminating alkaline phosphatase activity at the concentrations present in these components. Therefore the assay itself is used to trouble-shoot sources of interfering material.

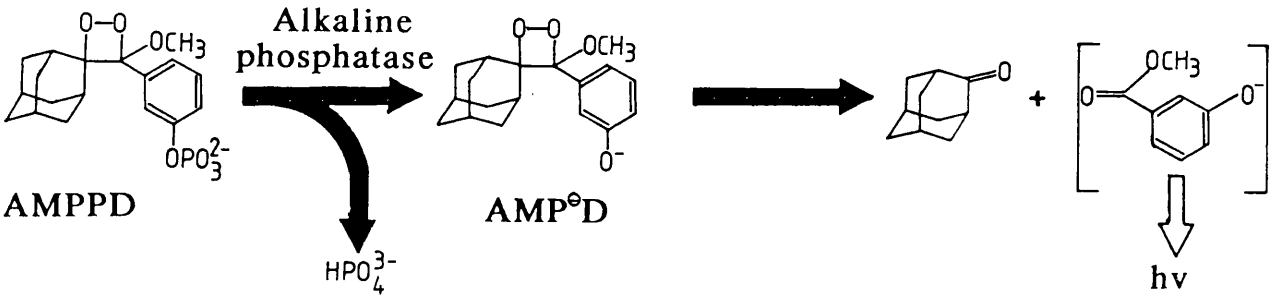
The experiments of quality control and optimization are not presented in chronological order, and therefore a high background signal is apparent in some experiments reported in this thesis. This is because the earlier experiments used materials inferior in quality to those available at the latter stages following development of improved preparative techniques (See Chapter Three) and optimization of the assay conditions (See Chapter Four).

1.9. Non-isotopic probes used in immunoassays and D N A detection.

1.9.1. A Chemiluminescent detection system developed by Tropix Inc. Massachusetts, U.S.A.

A probe has been developed for the detection of hepatitis B virus core antigen plasmid DNA. Sensitivity has been achieved through the development of a new substrate, adamantyl- 1,2-dioxetane phosphate (AMPPD.), for alkaline phosphatase, producing a chemiluminescent product that emits light at 470nm. Following hydrolysis of the substrate by alkaline phosphatase, the enzyme label, the product spontaneously chemiluminesces. The chemistry is illustrated in Figure 12. which shows that hydrolysis results in the formation of a dioxetane anion, which fragments into adamantanone and the excited state of the methylmeta-oxybenzoate anion, the light emitter (Bronstein et al, 1988).

Figure 12. Adamantyl-1,2-dioxetane phosphate, substrate for alkaline phosphatase, and its mechanism of chemiluminescent decomposition (After Bronstein and McGrath, 1989).



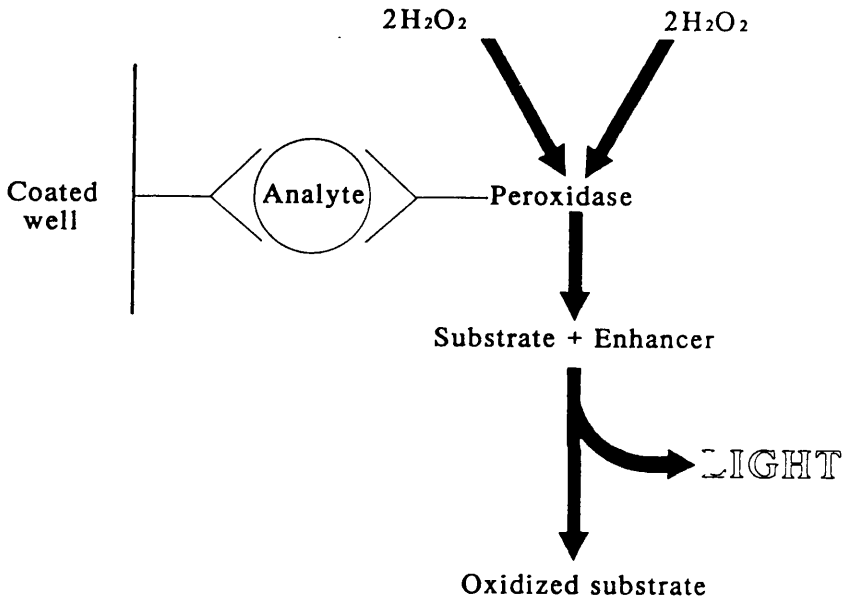
A comparison of the chemiluminescent substrate with a colorimetric substrate (BCIP/NBT) demonstrated an increase of two orders of magnitude in sensitivity in favour of the chemiluminescent assay. After 60 minutes incubation, the chemiluminescent assay can detect 1.18×10^{6} copies (2.0×10^{-18} mol) of HBV_c DNA., whereas the chromogenic substrate could detect 9.63×10^{7} copies (1.6×10^{-16} mol) of HBV_c DNA. (Bronstein et al, 1989).

1.9.2. The Amerlite® System.

Amerlite® is the trade name of a non-radioactive immunoassay system developed by Amersham. The use of enhancers has been optimized to give a continuous

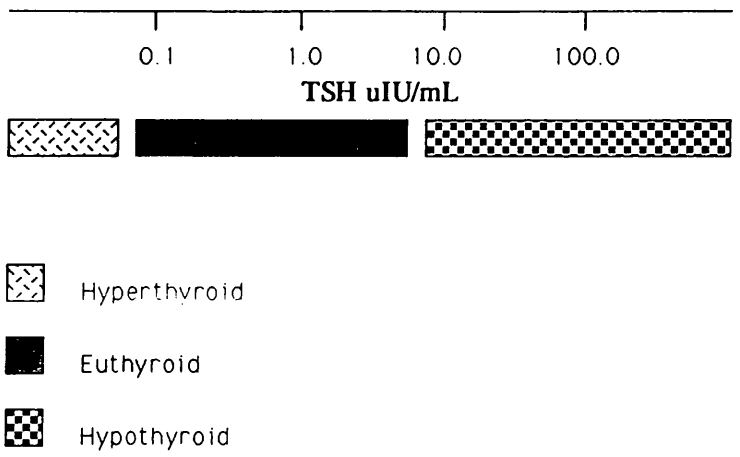
output of light rather than a flash in a luminescent detection system. This enables measurement of the signal without critical timing and re-measurement if desired (Edwards). The principle of enhanced luminescence is shown in Figure 13.

Figure 13. The principle of enhanced luminescence.



The Amerlite system can be used for the assay of both haptens and proteins using either peroxidase labelled antibody or antigen. The Amerlite TSH. assay has a detection limit of 0.04uIU/mL and a wide operating range up to 200uIU/mL so that both high and subnormal values of TSH. can be monitored (See Figure 14.).

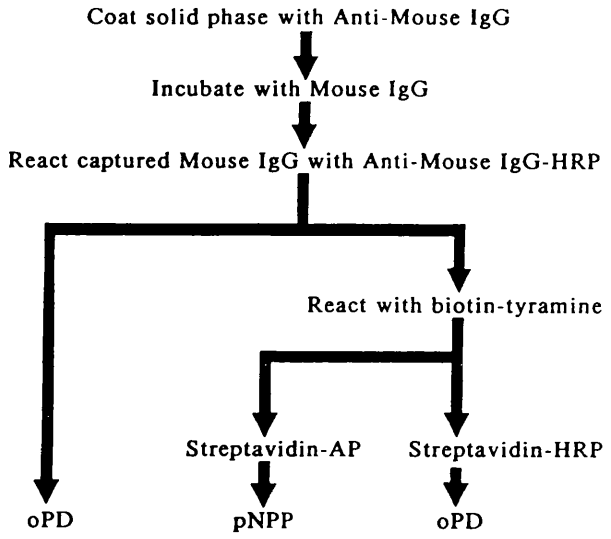
Figure 14. Monitoring of both high and subnormal values of TSH.



1.9.3. Catalyzed Reporter Deposition (CARD) developed by Du Pont.

An analyte-dependent reporter enzyme is used to deposit additional reporter on the surface in a solid phase immunoassay. Horse radish peroxidase is used to catalyse the deposition of biotin labelled phenols. Streptavidin-labelled enzyme is added which binds to the deposited biotin, resulting in amplification of the signal simply by having a higher concentration of the reporter enzyme. The principle of the system is shown in Figure 15.

Figure 15. Flow chart for the HRP mediated deposition of AP and HRP in a mouse IgG assay (Bobrow et al, 1989).



AP = Alkaline phosphatase
HRP = Horseradish peroxidase
pNPP = p-nitrophenylphosphate
oPD = o-phenylenediamine

Using HRP to catalyze the deposition of additional HRP with colorimetric detection has resulted in an amplification of greater than 10-fold for mouse IgG. Detection of a fluorescent signal generated by the deposition of β-galactosidase resulted in a 16-fold improvement of the detection limit compared with a direct HRP labelled detector.

1.9.4. The Abbott IMx automated benchtop immunochemistry analyzer system.

The IMx employs a new technology called Micro-particle Enzyme Immunoassay (MEIA) for the assay of high-molecular mass, low concentration analytes, and a Fluorescence Polarization Immunoassay (FPIA) for the detection of haptens (Flore et al., 1988). This instrument demonstrates the total automation of an immunoassay procedure.

In the MEIA system the enzymic generation of a fluorescent product is quantified by a fluorometer, and a fluorescence polarization optical system is used in a FPIA. The incorporation of these two methodologies in the one instrument widens the range of immunoassay applications.

MEIA technology has been developed through increasing the kinetics of the solid phase by using very small particles and hence a greater surface area for the reaction to occur, followed by an efficient separation of the bound and unbound material. This is achieved by what is effectively a filtration of the reaction solution. MEIA can be used in either a sandwich assay (Brown, 1987), or a competitive ligand format.

The enzyme system employed in both the MEIA assay formats is alkaline phosphatase hydrolysis of 4-methylumbelliferyl phosphate (4-MUP). This substrate

is dephosphorylated to 4-methylumbelliferone (4-MU). Selective excitation of the product enables it to be quantified in the presence of the substrate by comparison with a calibration curve. Concentrations of 4-MU as low as 100nmol L^{-1} can be detected in the presence of 1.2 mmol L^{-1} 4-MUP at a coefficient of variance of under 3%.

1.9.5. The Wellcozyme/IQ (Bio) system.

Hepatitis B surface antigen (HBsAg) is a recognised diagnostic marker for hepatitis B virus. In the Wellcozyme test HBsAg is identified in the samples by simultaneous reaction of two specific monoclonal antibodies which react with different sites on the antigen. The first, coated to the surface of microwells, is a specific mouse monoclonal antibody that captures the antigen whilst the second mouse monoclonal antibody, conjugated to alkaline phosphatase, has a different specificity.* During this incubation period an antibody-antigen conjugated enzyme/antibody complex is formed.

The enzyme label, alkaline phosphatase, is detected by reaction with a substrate to give a coloured product which is produced by a cyclic amplification reaction. This is the process developed by IQ (Bio) Ltd. (Self, 1985).

*Wellcozyme HBsAg product literature enclosed with immunoassay kit.

Different incubation conditions lead to varying sensitivity. The most sensitive being an initial substrate incubation at 37°C followed by detection of the NADH produced by the substrate cycle.

Sensitivity data provided in the users handbook shows a limit of detection of 0.313ng mL^{-1} (E mL^{-1} Paul Ehrlich AD reference) for HBsAg when assayed at a primary incubation of 37°C for 60 minutes followed by second incubation of amplification at 37°C for 20 minutes, & total assay time of 80 minutes.

CHAPTER TWO

ASSAYS FOR THE QUALITY CONTROL OF COMPONENTS USED IN THE AMPLIFICATION ASSAY OF ALKALINE PHOSPHATASE.

2.1. Assay of D-amino acid oxidase (from porcine kidney).

0.1M	Tris-HCl, pH 8.0.
35.0 mM	D-proline.
2.0mM	3,5-dichloro-2-hydroxybenzene-sulphonic acid (DCHB) (Sigma: D 4645).
0.2mM	4-aminoantipyrine (Sigma: A 4382).
0.01mg mL^{-1}	Horseradish peroxidase (Boehringer Mannheim EIA grade).
0.24mM	Flavin adenine dinucleotide.
Total volume:	The components listed above are final concentrations mixed in a total volume of 1.0mL for a spectrophotometric assay.
Temperature:	25°C.
Wavelength:	520nm.
Extinction:	23000M^{-1} for 1cm ligth path.
Activity:	1 unit is the amount of enzyme producing 1umol of hydrogen peroxide in 1 minute.

Omission of the FAD allows the measurement of any residual activity in the apoenzyme preparation.

2.2. Assay of horseradish peroxidase.

At 403nm a 1.0uM solution of horseradish peroxidase has an absorbance of 0.091 Abs Units for a 1.0cm light path (Keesey, 1987).

2.3. Assay of flavin adenine dinucleotide phosphate.

The absorbance of FADP is equal to that of FAD at pH 7.0 (Keesey, 1987).
At 450nm a 1mM solution has an absorbance of 11.3 Abs Units for a 1.0cm light path.

2.4. Assay of alkaline phosphatase.

2.4.1. Unamplified assay of alkaline phosphatase.

0.1M	Tris-HCl, pH 8.0.
0.1mM	$MgSO_4$.
1.0uM	$ZnSO_4$.
0.1mM	4-nitrophenyl phosphate (Aldrich: N2,200-2).
Total volume:	The above components are mixed to give these final concentrations in a volume of 1.0mL for a spectrophotometric assay.
Wavelength:	405nm.
Temperature:	25°C.
Activity:	10pM enzyme gives a change in absorbance per minute of 0.00137 for 1cm light path. This is used as an internal standard to calibrate the amplification assay.

2.4.2. Amplification assay of alkaline phosphatase.

Variable	Tris-HCl, pH variable.
0.1mM	$MgSO_4$
1.0uM	$ZnSO_4$
2.0mM	DCHB.
0.2mM	4-aminoantipyrine.
0.01mg ml^{-1}	Horseradish peroxidase.
Variable	FADP.
Variable	Apo-D-amino acid oxidase (porcine kidney)
Total volume:	The components are mixed to give these final concentrations in a volume of 1.0mL for a spectrophotometric assay or 0.1mL for a microtitre plate assay.
Wavelength:	520nm.
Extinction: of chromophore	23000M^{-1} for 1cm light path.

The variable components are quantified in the experimental details. A cocktail of all of the reagents common to the assay was prepared in each case; in order to minimize assay variability.

2.5. Assay of RNase-T_2.

20.0mM	Bis-Tris, pH 7.0.
0.1mM	Adenosine-2,3-cyclicphosphate-5-phosphate. (Sigma: A 8528).
Total volume:	The above components are mixed to give these final concentrations in a volume of 1.0mL for spectrophotometric assay. The assay is performed in a double beam spectrophotometer against a blank cuvette containing the buffer and substrate.
Wavelength:	270nm.
Extinction:	Differential extinction is $224M^{-1}$ for a 1cm light path
Activity:	1 unit is the amount of enzyme hydrolyzing 1umol of the substrate in 1 minute.

2.6. Assay of "FADPase".

2.6.1. FADPase in apo-D-amino acid oxidase.

0.1M	Tris-HCl, pH 8.0.
0.1mM	$MgSO_4$
1.0uM	$ZnSO_4$
35.0mM	D-proline.
2.0mM	DCHB.
0.2mM	4-aminoantipyrine.
0.02mg mL^{-1}	Horseradish peroxidase.
0.02mM	FADP.
0.1uM	Apo-D-amino acid oxidase.
Total volume:	The above components are mixed to give these concentrations in a total volume of 0.1mL for a spectrophotometric or microtitre plate assay.
Temperature:	37°C.
Wavelength:	520nm.
Activity:	Arbitrary measurement as the difference in the change in absorbance per minute at 5 minutes for the spectrophotometric assay, or at 36 minutes in the microtitre plate assay, between reaction mixtures with and without FADP.

2.6.2. FADPase in horseradish peroxidase or RNase-T_2.

The assay has the same format as above. The sample under investigation is added to the assay mixture. A comparison of the difference in the change in aborbance between reaction mixtures with and without FADP, and with and without the sample allows an estimation of FADPase to be made.

2.7. Assay of FAD in FADP.

0.1M	Tris-HCl, pH 8.0.
35.0mM	D-proline.
2.0mM	DCHB.
0.2mM	4-aminoantipyrine.
0.01mg mL^{-1}	Horseradish peroxidase.
0.14 Units	Apo-D-amino acid oxidase.
Total volume:	The above components are mixed to give these final concentrations in a total volume of 0.1mL for a spectrophotometric or microtitre plate assay.
Temperature:	25°C.
Wavelength:	520nm.

The assay is performed in the absence of FADP, in the presence of 100uM FADP and in the presence of 100uM FADP and 1nM FAD. Comparison of the gradients in the linear region of the curve allows the concentration of FAD to be calculated in the sample of FADP.

CHAPTER THREE.

THE PROPERTIES, PURIFICATION, YIELDS AND QUALITY CONTROL OF THE ASSAY COMPONENTS.

3.1. The structure of D-amino acid oxidase.

D-amino acid oxidase (E.C. 1.4.3.3.), D-amino acid : oxygen oxidoreductase (deaminating), is found abundantly in mammalian kidney, liver and brain (Krebs, 1951). The enzyme catalyses the oxidation of the D-isomer of a number of amino acids to the imino acid which is then hydrolysed, non-enzymically, to the corresponding α-keto acid. Oxygen is the electron acceptor and is converted to hydrogen peroxide (Swenson et al., 1982).

D-amino acid oxidase, isolated from pig kidney, consists of 347 amino acid residues (Ronchi et al., 1982). No disulphide bonds are present. The molecular weight of the apoprotein, calculated from this sequence, is 39,336. It is generally accepted that D-amino acid oxidase from pig kidney undergoes dimerization in aqueous solution (Toji et al., 1985). Previously, only partial understanding of this mechanism has led to inconsistent results in determination of the molecular weight by experimental analysis (Yagi et al., 1967; Miyake et al., 1965). Many variables eg. protein concentration, buffer composition and temperature affect the association-

dissociation phenomena (Yagi et al., 1968).

Cofactor binding is mainly via the adenosyl moiety of the FAD molecule at the amino terminus region of the polypeptide chain. Other regions close to the amino terminus also interact with the ribose and pyrophosphate portions of the cofactor (Hofsteenge et al., 1980; Ronchi et al., 1982).

Chromatographic purification of the enzyme is now well established. Highly purified enzyme is commercially available from Sigma (Poole, Dorset, UK), and Calzyme (San Luis Obispo, California USA).

3.2. Dissociation of FAD from D-amino acid oxidase.

Apoenzyme has been prepared in several ways. The method of Warburg and Christain (1938) involves the liberation of the cofactor/prosthetic group, FAD, at low pH (approximately 2.0) in the presence of high concentrations of ammonium sulphate (50% saturation). This procedure has been incorporated into a purification protocol to isolate the apoprotein (Negelein and Bromel, 1939; Brumby and Massey, 1968). One of the disadvantages of the acidic ammonium sulphate resolution procedure is that the yield of apoprotein is somewhat variable. This is due to denaturation of the protein depending on the pH and temperature at which the separation of precipitated

apoprotein and acid supernatant solution is performed. Typical yields may vary between 60% and 80% (See Section 3.5.1.).

Apoprotein has also been prepared by dialysis against high concentrations of monovalent anions (Walaas and Walaas, 1956). Quantitative dissociation of FAD from the holoenzyme has been obtained at physiological pH values by dialysis against 0.1M pyrophosphate buffer pH 8.5, containing 3.0mM EDTA and 1.0M potassium bromide (Massey and Curti, 1966). The dialyzate was changed three to four times over a two day period, until the yellow colour, due to FAD had disappeared. Potassium bromide was removed by dialysis against 0.1M pyrophosphate buffer pH 8.5 for a further one to two days with several changes of buffer. Preparation of apoprotein by dialysis in the presence of potassium bromide reproducibly yielded close to 100% reconstitutable activity. It is however a lengthy process. The apoprotein prepared by both methods appears to be identical with respect to kinetic and fluorescent studies and FAD titration (Massey and Curti, 1966).

3.3. The structure of horseradish peroxidase.

Peroxidase isoenzymes were first detected by H. Theorell (1942), in horseradish roots. It is the presence of peroxidase isoenzymes that has led to

reported discrepancies in peroxidase literature, since they differ in physiological and kinetic properties. Previous studies (Shannon et al., 1966) have shown that there is no interconversion between the isoenzymes.

Horseradish peroxidase (E.C. 1.11.1.7.) is a haem-containing glycoprotein of molecular weight 40,000. A crude extract from horseradish contains seven major isoenzymes (Shannon et al., 1966) and up to thirteen additional minor isoenzymes (Delincee and Radola, 1970). Each isoenzyme contains a carbohydrate moiety as an integral part of the enzyme (Clarke and Shannon, 1976). However, it seems unlikely that the carbohydrate participates directly in catalytic activity.

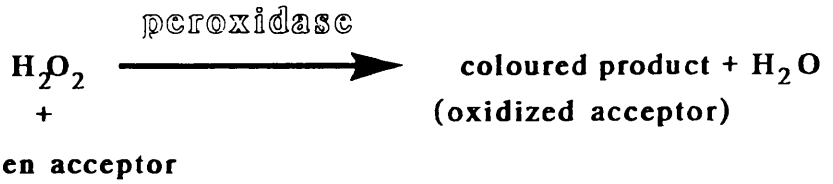
3.4. Clinical diagnostic applications of horseradish peroxidase.

Horseradish peroxidase is a commonly used enzyme label for immunological detection systems (Nakane and Pierce, 1967; Ngo and Lenhoff, 1985). When utilizing hydrogen peroxide as the substrate two molecules of hydrogen peroxide are decomposed to water and oxygen. However, the specificity of peroxidase for the second molecule of hydrogen peroxide is low and many electron donors may be substituted. This has allowed the identification of a number of chromogenic substrates

for the enzyme (Conyers and Kidwell, 1991; Geoghegan et al., 1983; Nakane, 1968).

The concentration of hydrogen peroxide is determined by the general scheme shown below in Figure 16.

Figure 16. The reaction scheme of horseradish peroxidase.



Originally, benzidine was used as an oxygen acceptor in such a chromogenic system. Because of the carcinogenic nature of this compound alternatives have been sought (Barham and Trinder, 1972). The product of this reaction, a quinoneimine dye, has a molar absorptivity four times that of the product of the unsubstituted phenol reaction. The use of various reagents renders the products of the peroxidase reaction soluble (Liem et al., 1979) or insoluble (Kaplow, 1975).

The method of Barham and Trinder, described above, has been used in other coupled reactions (Fossati et al., 1980). The development of colour was shown to be linear and stable over the measured time period. Taking the pH of the L.B.L. system into account, this

method is suitable for coupling to the reaction of D-amino acid oxidase.

3.5. The preparation of apo-D-amino acid oxidase.

3.5.1. Preparation of apo-D-amino acid oxidase by acid precipitation.

D-amino acid oxidase (10mg), supplied as a suspension in ammonium sulphate from Sigma (Cat. No. A 1789), was centrifuged at 18,000g for 2 minutes at +4°C. The supernatant was decanted and the pellet redissolved in in 1.0mL, 0.1M sodium pyrophosphate, pH 8.0. 1.0mL, 3M potassium bromide was added. This was followed by the addition, with mixing over a 20 second period, of 1.0mL saturated ammonium sulphate, pH 1.9. Mixing was continued over a further 40 second period with the rapid addition of another 4.0mL of acidified saturated ammonium sulphate solution. The suspension was centrifuged at 12,000g for 15 minutes, at +4°C. The supernatant was removed as completely as possible by inverting the centrifuge tube to allow the liquid to drain.

The precipitate was dissolved in 5.0mL of 0.1M sodium pyrophosphate, pH 8.0. The enzyme solution was desalted, in two aliquots, on a pre-packed PD-10, G-25 Sephadex column (Pharmacia-LKB Cat No. 17-0851-01), equilibrated with 20mM sodium pyrophosphate, pH 8.0.

The desalted fraction was loaded on to a Cibacron Blue CL6B column (5.0cm x 3.7cm), at +4°C, at a flow rate of 2.2mL min^{-1}. The column was washed with 2.0L of the same pyrophosphate buffer. The apoenzyme was eluted with the same buffer containing 1.0M potassium bromide (Leonii et al., 1985).

Active fractions were pooled. The protein was precipitated at 80% ammonium sulphate saturation (Dawson et al., 1986). The precipitate was separated by centrifugation at 12000g and redissolved in 5.0mL of 20mM bis-tris-propane, containing 0.1M NaCl and 5.0g/L mannitol, pH 8.0. The protein solution was desalted on a PD-10 column as previously described, equilibrated with the bis-tris-propane buffer. The active fraction was frozen at -70°C and lyophilized at 0.05 bar for 24 hours. The lyophilizate was stored at -70°C. The purification and yield for the preparation of apo-D-amino acid oxidase by acid precipitation is summarized in Table 4.

Table 4. Preparation of apo-D-amino acid oxidase by acid precipitation.

	Vol (mL)	Units/ mL(+FAD)	Yld %	Units/ mL(-FAD)	%holo
Start material	5.0	17.8	/	/	100*
Acid treatment	7.0	8.9	70.0	1.0	11.0
Blue Sepharose Eluant	53.5	0.6	36.6	0.0009	0.1
G-25 Eluant	7.0	2.9	22.5	0.002	0.07

The recovery of apo-D-amino acid oxidase during the preparation by acid precipitation was monitored by assaying the activity in the presence of 0.24mM FAD (See Section 2.1.). Omission of FAD from the assay allowed the determination of residual holoenzyme activity.

*100% is a nominal figure since commercial preparations contain free FAD. (also applicable to table 7. and table 8.).

Acid precipitation is a relatively harsh procedure for the preparation of apoenzyme. Table 4 shows that there is an initial reduction in holoenzyme activity to 11%. This is poor when compared to protocols later developed (See tables 7 and 8). The yield from this process is variable due to difficulties involved with adequate mixing and temperature regulation. Production of apoenzyme by acid precipitation is not amenable to scale-up. At most, 10mg of protein can be used in this process.

An inherent instability of the apoenzyme produced in this manner also results in a significant loss of active protein when chromatographed on a Blue Sepharose column (only 52% yield). This is most likely due to damage to the tertiary structure of the protein during the initial stages of apoenzyme preparation. Although this does not appear to effect the reconstitutable activity of the protein (Massey and Curti, 1966) it does alter the chromatographic properties of the apoenzyme.

3.5.2. Effect of pH on the dissociation of FAD from D-amino acid oxidase.

FAD is dissociated from D-amino acid oxidase upon dilution of the holoenzyme (Dixon and Kleppe, 1965a). This phenomenon can be used to calculate the rate of dissociation by assaying the activity of a very dilute sample of holoenzyme and differentiation of the progress curve. The effect of pH on this dissociation was studied by following the progress of the reaction at pH 6-8.

Dilute samples of holoenzyme (less than 10nM) were assayed in a spectrophotometer (See Section 2.1.) using 35mM D-alanine and 0.2M potassium phosphate buffer at pH 6.0, 7.0 and 8.0 at 25°C. Each assay contained 1M potassium bromide. No FAD was included into the assay. The progress of the reaction was followed to completion and the apparent value for the dissociation of FAD calculated from differentiation of the progress curve (See Table 5.).

Table 5. The dissociation of FAD.

pH	E_o nM	EF nM	$\frac{dEF}{dt}$ nM min^{-1}	k_{OFF} min^{-1}	$t_{1/2}$ min
6.0	6.7	0.6	3.5	0.5	1.3
7.0	6.7	0.5	1.5	0.2	3.2
8.0	3.4	0.2	0.6	0.2	4.6

E_o is the total enzyme concentration; EF is equal to the concentration of active enzyme under the assay conditions; k_{OFF} is calculated from the rate of change of EF; $t_{1/2}$ is the half-life of the dissociation of FAD from D-amino acid oxidase and is equal to $0.693/k_{OFF}$.

The dissociation and association of FAD is not an instantaneous process, but requires several minutes (Dixon and Kleppe, 1965a). Neither is it a single step process (Massey and Curti, 1966). FAD binds rapidly to the apoenzyme, followed by a slow conformational change that is required for enzyme activity. The reverse must also be true for the dissociation of FAD. Dixon and Kleppe (1965a) calculated a value of 0.45 min^{-1} for the rate of dissociation at pH 8.5, and reported that the rate of dissociation is decreased in the presence of the enzyme substrate.

The rate of dissociation calculated in Table 5 at pH 6.0 demonstrates that it would be possible to dissociate FAD from the enzyme on a long gel filtration column.

3.5.3. Effect of potassium bromide on the dissociation constant K_D of D-amino acid oxidase for FAD.

An assay mixture was prepared containing:

720uL 1.0M MES, pH 6.0.

180uL 8.0mM 4-aminoantipyrine

180uL 80.0mM DCHB.

720uL 0.1mg mL^{-1} horseradish peroxidase.

apo-D-amino acid oxidase to a final concentration of 0.041 Units in 100uL assay (assessed at pH 8.0; See Section 2.1.).

Sterile water to a final volume of 3.6mL.

50uL of the above assay mixture was added to a microtitre plate well. Each well contained a range of FAD concentrations (0-10uM) in a volume of 40uL and a range of potassium bromide concentrations (0-1.5M). After thorough mixing the assays were left at room temperature for 60 minutes in the dark. 10uL of 0.35M D-methionine was added to each well and mixed thoroughly. The reaction was followed for a further 60 minutes at 520nm using a Titretek II plate reader.

Table 6 shows the equivalent reaction rate obtained at pH 6.0 with D-methionine as the substrate for D-amino acid oxidase when compared to the reaction rate obtained at pH 8.0 with D-alanine as the substrate. These ratios were used to calculate the concentration of active D-amino acid oxidase in each instance.

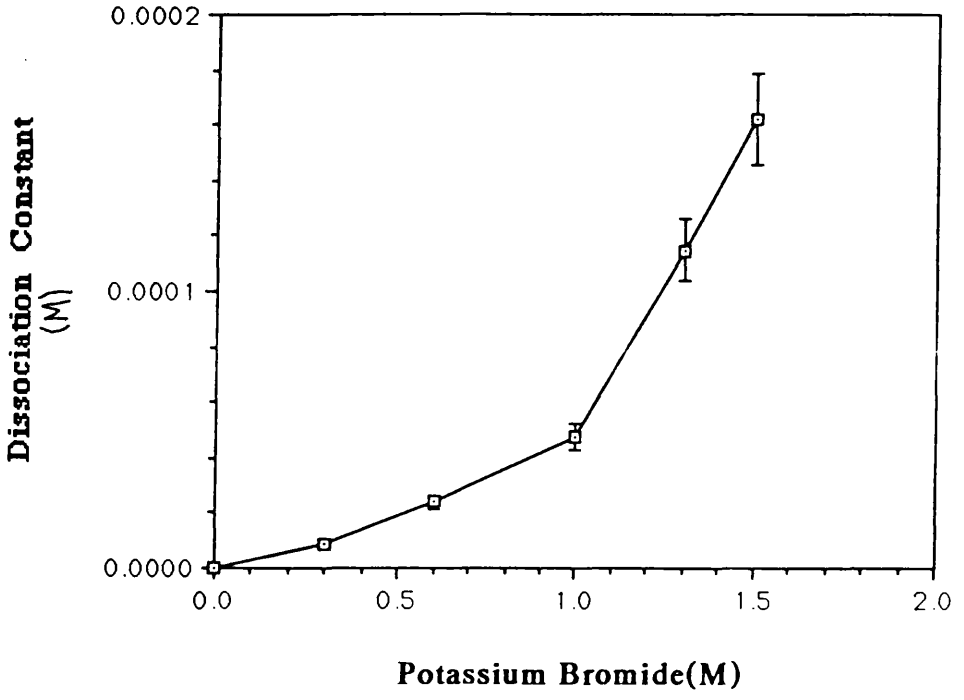
Table 6. Equivalence of 1 Abs unit min^{-1} at pH 8.0 with D-alanine as substrate.

Abs unit min^{-1} at pH 6.0 with D-methionine.	Potassium bromide (M).
0.014	0.3
0.008	0.6
0.007	1.0
0.004	1.3
0.003	1.5

D-amino acid oxidase was assayed in 0.1M tris-HCl, pH 8.0 using 35mM D-alanine as the substrate and in 0.1M MES, pH 6.0 using 35mM D-methionine as the substrate in the presence of potassium bromide at each of the stated concentrations.* The ratio of the rates was used to determine the activity of D-amino acid oxidase. The activity, equated to the assay in 0.1M tris-HCl, pH 8.0 using 35mM D-alanine was used to calculate K_D in each instance. The value of K_D calculated was plotted as a function of the concentration of potassium bromide (See Figure 17).

* D-methionine was used as the substrate at pH 6.0 because of the interdependence of substrate specificity and pH (Dixon and Klappe, 1964c).

Figure 17. Variation of K_D with the concentration of potassium bromide at pH 6.0..



Apo-D-amino acid oxidase (0.041 Units) was assayed in 0.1M MES, pH 6.0, using 35mM D-methionine as the substrate, in the presnce of 0-10uM FAD and 0-1.5M potassium bromide.

The association of apo-D amino acid oxidase and free FAD can be represented by the equation below;

$$E + FAD \rightleftharpoons EFAD \rightleftharpoons holoenzyme$$

where EFAD is an inactive FAD-apoenzyme complex (Massey and Curti, 1966). The equilibrium position of this equation, which would normally lie to the right hand side, is susceptible to many environmental factors. These include protein concentration and composition of the buffer in which the protein is dissolved. The addition of potassium bromide to the buffer shifts the equilibrium position much further to the left hand side of the equation to a point where the protein is precipitated at concentrations of potassium bromide greater than 1.5M and is the upper limit of Figure 17. This is the basis of apoenzyme preparation previously described (Section 3.5.1.).

For the production of apo-D-amino acid oxidase via a gel filtration column, the dissociation of the apoprotein from the FAD must be favoured as much as possible by the buffer composition since the actual protein sample would be relatively concentrated (See Table 7.). A value of 1.0 M potassium bromide was chosen. K_D is greatly increased at these concentrations and it is also low enough not to initiate precipitation of the protein.

3.5.4. Preparation of apo-D-amino acid oxidase by chromatography.

This process is a development of the procedure used by Walaas and Walaas (1956), where the removal of the prosthetic group was achieved by dialysis against a high concentration of potassium bromide. Here the consistent yields obtained by dialysis are achieved by a simple, single chromatographic stage which would be convenient for a productive scale set-up.

D-amino acid oxidase (70mg), purchased as a freeze-dried powder from Calzyme Laboratories Inc. (Cat. No. 052B2000), was dissolved in a minimum volume of 20mM MES, containing 1.0M potassium bromide, pH 6.0. After a 2 minute centrifugation at 18,000g in an Eppendorf microfuge, to remove the small quantity of insoluble components, the enzyme was chromatographed on a column (26mm x 95cm) of Sephadex G50F at a flow rate of 1.0mL min^{-1} at room temperature. The protein was eluted with the same buffer. Fractions of 10mL were collected (See Figure 18.).

The eluate from the G50F column containing apoenzyme was diluted with 20mM sodium pyrophosphate, pH 8.0 to give a solution that was 35mM with respect to potassium bromide. The diluted apoenzyme was loaded on to a Cibacron Blue Sepharose CL6B column (5cm x 10cm) pre-equilibrated with 20mM sodium pyrophosphate buffer, pH 8.0, at 6.7mL min^{-1} at +4°C.

The column was washed with three column volumes of the same buffer before eluting the apoprotein with 20mM sodium pyrophosphate, pH 8.0, containing 1.0M potassium bromide. Active apoenzyme fractions were pooled and desalted on a column (5cm x 20cm) of Sephadex G25C by elution with 10mM bis-tris, pH 7.0, containing 5g L^{-1} mannitol, at 6.7mL min^{-1} at +4°C (See Figure 19.).

Active fractions were pooled and shell frozen in dry ice/methanol and lyophilized at 0.05 bar for 36 hours. The lyophilizate was stored at -70°C. A fraction of the lyophilizate was dissolved in 20mM bis-tris, pH 7.0, containing 5gL^{-1} mannitol and 1.0mM CHAPS and desalted on a G25C column. The purification and yield of apo-D-amino acid oxidase prepared by chromatography is summarized in Table 7.

The protein solution was loaded on to a Mono Q HR10/10 FPLC column via a superloop at a protein concentration of 2.9 Unit mL^{-1}. The apoprotein was eluted with a gradient of 20mM bis-tris, pH 7.0, containing 5g L^{-1} mannitol, 1.0mM CHAPS and 1.0M sodium chloride, at 2.19mM NaCl mL^{-1} after a 41.0mL wash of loading buffer at a flow rate of 4.0mL min^{-1} at room temperature.

Fractions of the eluted protein were assessed for total D-amino acid oxidase activity and phosphatase

activity (See Section 2.1. and 2.6.1.). Fractions containing the highest ratio of D-amino acid oxidase : phosphatase activity were pooled (See Figure 20.).

All glassware used was previously acid washed and all buffers filtered through a 0.45um cellulose filter and sterilized.

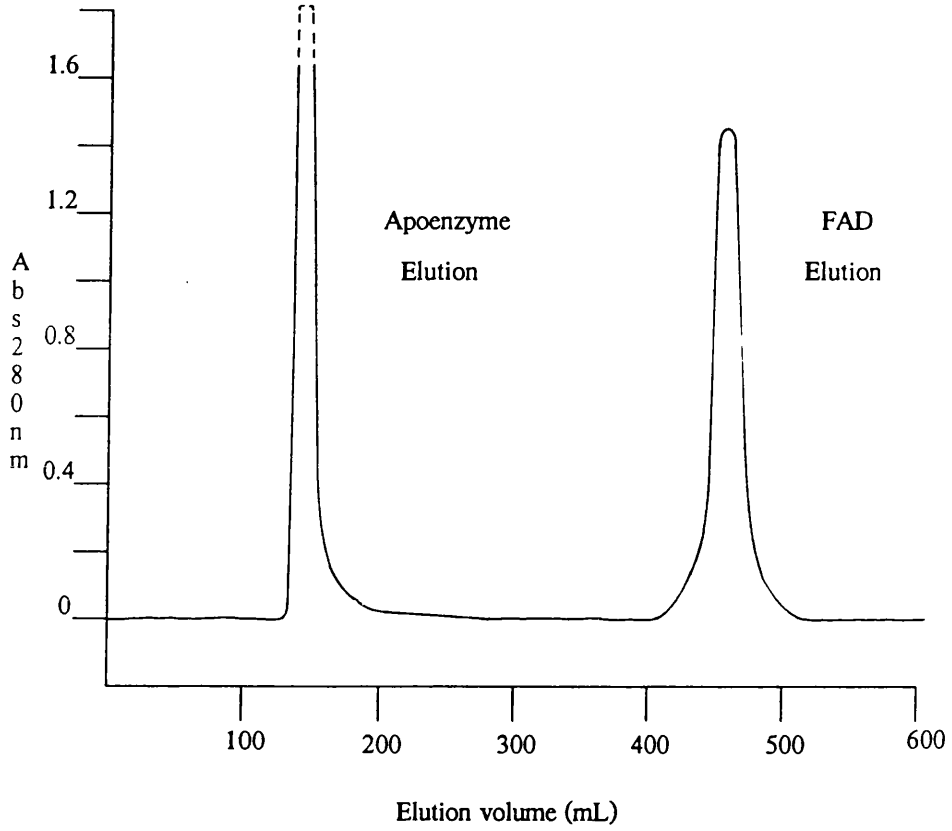
Table 7. Preparation of apo-D-amino acid oxidase by gel filtration.

	Vol (mL)	Units/ mL(+FAD)	Yld %	Units/ mL(-FAD)	%holo
Start Material	3.1	637.0	/	/	100*
G-50 Eluant	30.0	73.9	112	0.1	0.1
Blue Sepharose Eluant	120.0	11.9	72	0.002	0.02
G-25 Eluant	135.0	10.9	74	0.002	0.02

The recovery of apo-D-amino acid oxidase during the assaying the activity in the presence of 0.24mM FAD. by assaying the reconstitutible activity in the presence of 0.24mM FAD. Omission of the FAD allowed the determination of residual holoenzyme activity.

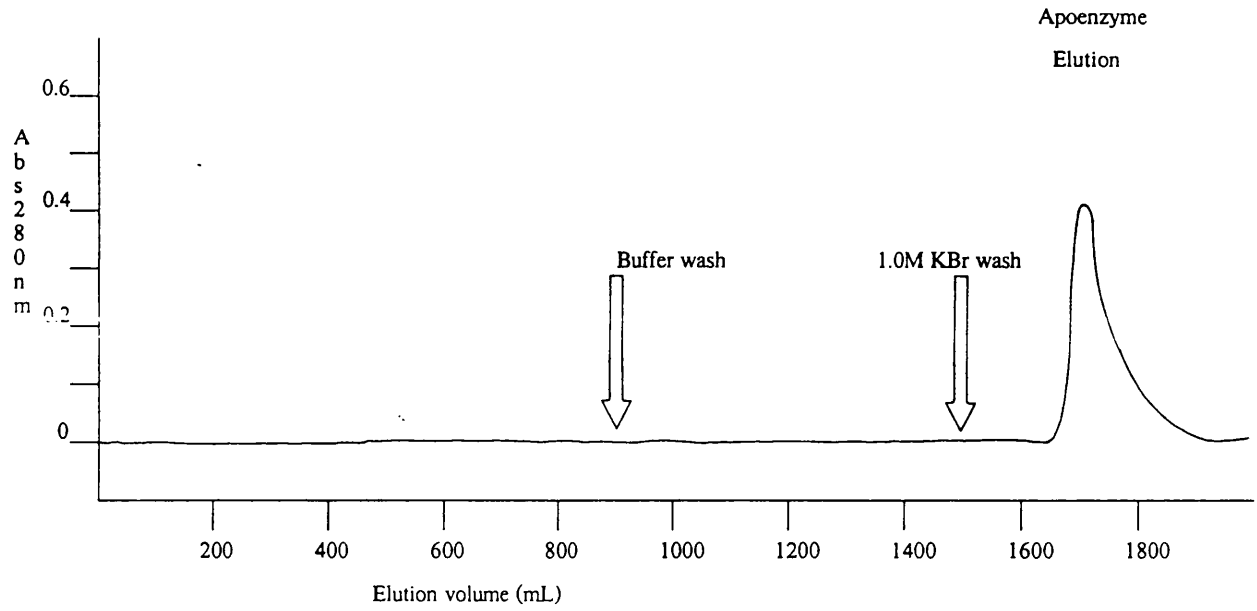
* 100% is a nominal figure (See page 72).

Figure 18. Resolution of apo-D-amino acid oxidase on Sephadex G50F.



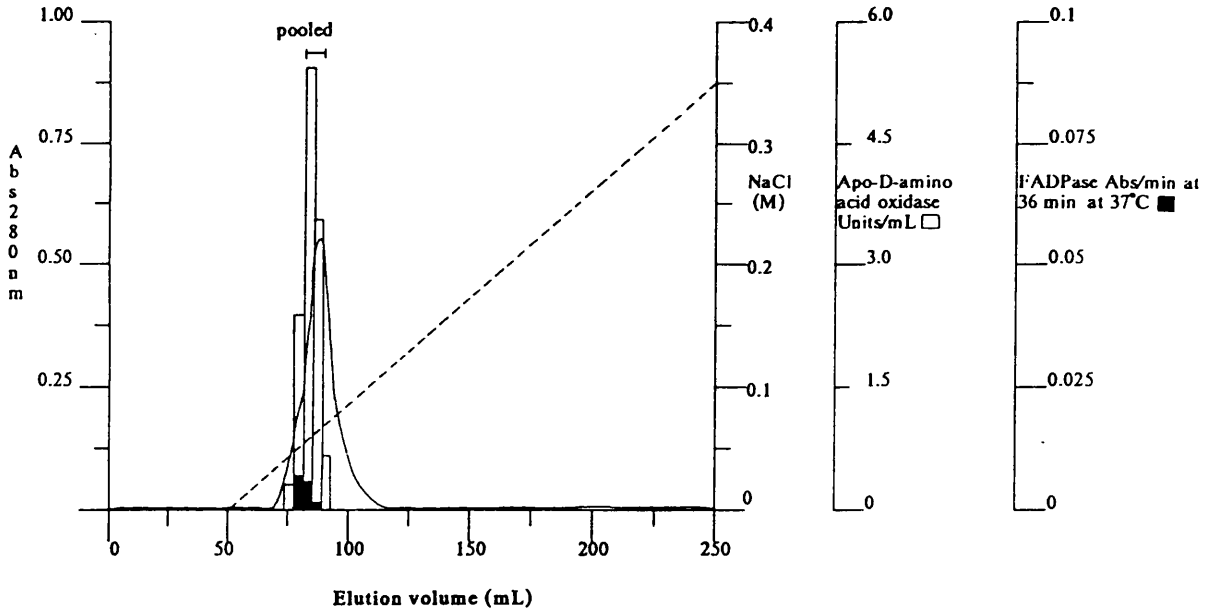
Apoenzyme is eluted in the void volume of the G50F Sephadex column in 20mM MES, pH 6.0, containing 1.0M potassium bromide. Fractions were pooled that had an absorbance reading, at 280nm, 2.0% above that of the background of the elution profile.

Figure 19. Chromatography of apo-D-amino acid oxidase on Blue Sepharose CL6B.



The protein bound to the column was washed with three column volumes of 20mM sodium pyrophosphate buffer, pH 8.0, to remove residual holoenzyme, phosphatases and nucleotidase activity and catalase from the preparation. Apoenzyme, eluted with the same buffer containing 1.0M potassium bromide, was collected and desalted on a column of G25C Sephadex.

Figure 20. Chromatography of apo-D-amino acid oxidase on a Mono Q column.



Apo-D-amino acid oxidase (2.0mg) was chromatographed on a Mono Q HR10/10 column. The column was pre-equilibrated with 20mM bis-tris containing 1.0mM CHAPS, pH 7.0. Protein was eluted from the column with a gradient of 2.19mM NaCl min^{-1} in the same buffer after a 41.0mL loading buffer wash. The reconstititable D-amino acid oxidase activity was determined (See Section 2.1.). FADPase activity was also measured in each fraction (See Section 2.6.1.). Fractions containing the highest ratio of D-amino acid oxidase activity to FADPase activity were pooled.

3.5.5. Preparation of apo-D-amino acid oxidase by diafiltration.

Approximately 500mg of holoenzyme, purchased from Calzyme, was dissolved in 200mL of 20mM MES, pH 6.0, containing 1.0M potassium bromide and 1.0mM CHAPS. Once dissolved, the solution was allowed to stand at room temperature for 30 minutes before centrifuging at 12,000g in a Sorval RC5B centrifuge at +4°C to remove remaining solids.

The supernatant was gently poured into the holding vessel of an Amicon ultrafilter (Model CH2 with a RA2000 reservoir) fitted with a S1 spiral-wound cartridge (cut-off 10,000 molecular weight). After gentle recirculation of the solution through the cartridge, ultrafiltration commenced at a rate of 170mL min^{-1} at a pressure difference of 3psi (9psi inlet; 6psi outlet). The retentate volume was maintained with the addition of more of the same buffer. After 2.0L of ultrafiltrate had been collected, the reservoir buffer was changed to 20mM MES, pH 6.0, containing 1.0M potassium bromide, 1.0mM CHAPS and 5.0mM D-methionine. A further 4.0L of ultrafiltrate was collected. Figure 21 shows the decrease in absorbance as the flavin content is monitored at 450nm in the ultrafiltrate

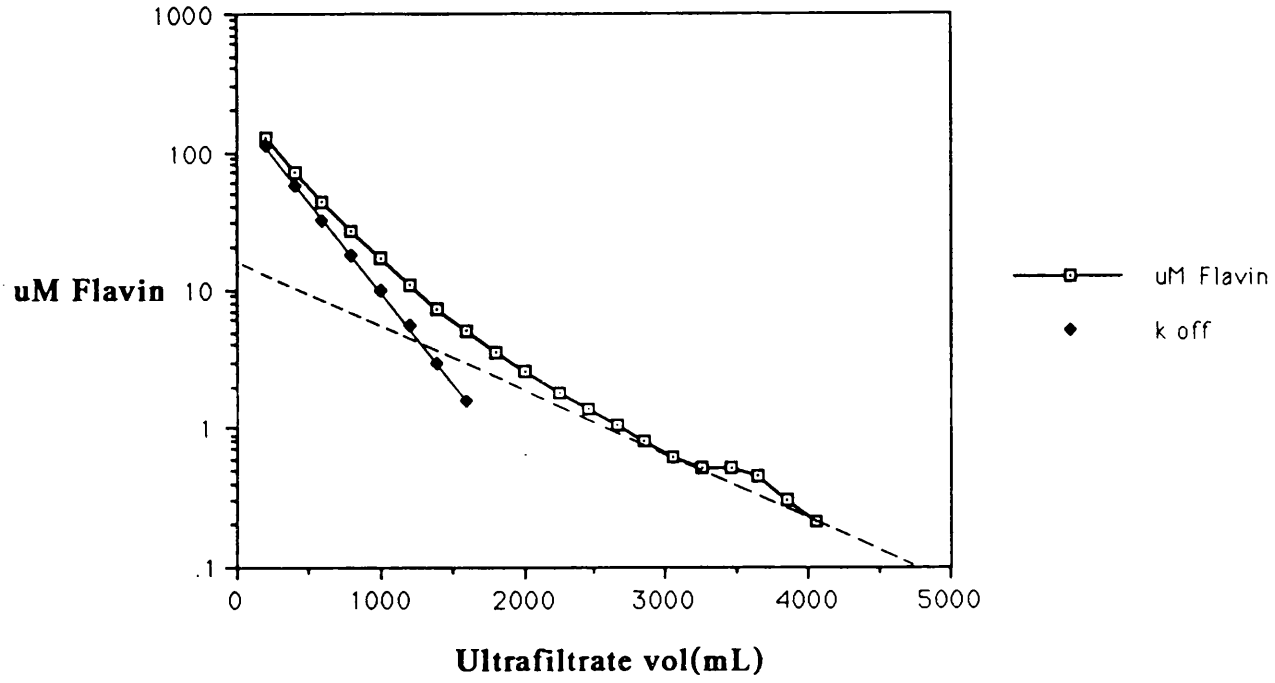
Salt was displaced from the apoenzyme solution by buffer exchange with 1.0L of 20mM tris, pH 8.0. The

desalted solution was removed from the holding vessel. 200mL of the same tris buffer was added to the holding vessel and the ultrafilter was then operated in a recycling mode for a further 15 minutes to wash any residual protein from the cartridge.

The pooled apoenzyme solution and washings were chromatographed on a Cibacron Blue Sepharose CL6B column (5cm x 15cm), pre-equilibrated with 20mM tris buffer, pH 8.0, containing 1.0mM CHAPS, at a rate of 7.1mL min^{-1} at room temperature. The column was washed with three column volumes of the tris-HCl buffer before eluting the apoprotein with the same buffer containing 1.0M potassium bromide. Active fractions were pooled. Potassium bromide was removed by ultrafiltration against 1.0L of 20mM tris-HCl, pH 8.0. After the removal of the concentrated protein solution from the holding vessel, the cartridge was washed with a further 200mL of tris buffer as previously described. The preparation of apo-D-amino acid oxidase by diafiltration is summarized in Table 8.

The pooled apoenzyme solution was frozen in dry ice/methanol and lyophilized at 0.05 bar for 36 hours. The lyophilizate was stored at -70°C. Chromatography of the apoenzyme on a Mono Q column was performed as previously described (See Section 3.5.4.).

Figure 21. The wash-out of flavin by diafiltration.



Ultrafiltration of the D-amino acid oxidase solution was performed at a rate of 170mL min^{-1} at a pressure difference of 3psi through a S1 spiral wound cartridge with a 10,000 molecular weight cut-off. Ultrafiltration was performed with a total volume of 6.0L of buffer.

The reduction of flavin in the holding vessel is dependent on at least two factors. First the rate of dissociation of the FAD, and secondly the rate of wash out from the ultrafilter. The initial rate of dissociation is masked in Figure 21 by the much slower wash-out rate. Subtracting the lower region of the curve, shown by the broken line, from the initial values would reveal the magnitude of the dissociation. The line (—◆—) represents this phenomenon. The gradient of this line is used to calculate k_{OFF}. A value of 0.48 min^{-1} agrees well with the the value of 0.5 derived in Table 5.

Table 8. Preparation of apo-D-amino acid oxidase by diafiltration.

	Vol (mL)	Units/ mL(+FAD)	Yld %	Units/ mL(-FAD)	%holo
Start Material	200	63.8	/	/	100*
U/F Retentate	149	51.0	59.6	0.6	1.2
U/F Washing	168	15.9	20.9	0.2	1.5
Blue Sepharose Eluant	182	45.8	65.3	0.1	0.02
U/F Retentate	142	40.0	44.6	0.006	0.01
U/F Washing	168	11.3	14.9	0.002	0.01
Total Recovery	310	24.5	59.5	0.003	0.01

The recovery of apo-D-amino acid oxidase during the preparation by diafiltration was monitored by assaying the activity in the presence of 0.24mM FAD. Omission of the FAD allowed the determination of residual holoenzyme activity.

* 100% is a nominal figure (see page 72).

Preparation of apo-D-amino acid oxidase by diafiltration represents the most efficient and realistic protocol for a production-scale operation. Preparations of reproducible quality and yield can be manufactured with little alteration of the unit operation to cater for larger quantities of enzyme. There are no increased operational costs due to the quantity of buffers involved in the operation because of the much larger quantity of enzyme that can be produced in this way.

Maintaining a constant concentration of protein in the 2.0L reservoir used here would produce a capacity for 5.0g of protein for the present system (Harbron et al., 1992b) with no increase in processing time. The binding capacity of the Blue Sepharose, approximately 1.0 mg mL^{-1} of gel under the conditions used here (Harbron et al., 1992b), is the only limitation. Under appropriate production conditions the operation of a 5.0L column would not pose any problem.

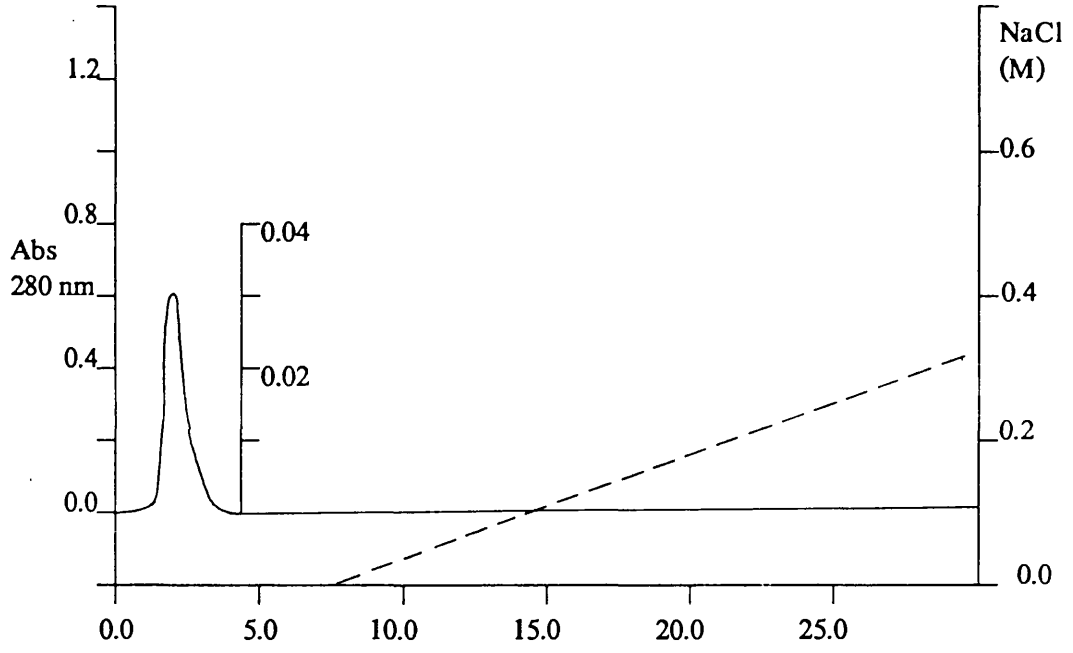
3.6. Preparation of horseradish peroxidase.

EIA-grade horseradish peroxidase was purchased from Boehringer-Mannheim (Cat. No. 814 407). Generally this grade of material was suitable for use without further purification (See Section 3.6.2.). The enzyme must be desalted on a G25C Sephadex column and eluted with 20mM bis-tris, pH 7.0. However, for extended incubation periods of the assay, removal of small traces of phosphatase activity is required.

3.6.1. Purification of horseradish peroxidase on a Mono Q column.

Contaminating phosphatases are removed by chromatography on a Pharmacia-LKB Mono Q HR5/5 column. The column is equilibrated with 20mM bis-tris, pH 7.0. The horseradish peroxidase (20mg mL^{-1}) was first desalted on a Phamacia-LKB NAP-5 column (Cat. No. 17-0853-02), equilibrated with this same buffer. The desalted peroxidase (approximately 5.0mg of enzyme) was loaded on to the Mono Q column at a flow rate of 1.0 ml min^{-1}. The elution buffer was 20mM bis-tris, pH 7.0, containing 1.0M sodium chloride. The gradient used was 17.5mM NaCl min^{-1}, which was started after 8.0 mL of a loading buffer wash (See Figure 22.). The eluate, containing the active peroxidase, was lyophilized as previously described. The lyophilzate was stored at -70°C.

Figure 22. Preparation of horseradish peroxidase on a Mono Q column.



5.0mg of desalted horseradish peroxidase was chromatographed on a Mono Q HR5/5 column pre-equilibrated with 20mM bis-tris, pH 7.0, at a flow rate of 1.0mL min^{-1}. A decrease in the full scale deflection of the monitor after the elution of 5.0 mL from the column failed to reveal any significant contaminating protein.

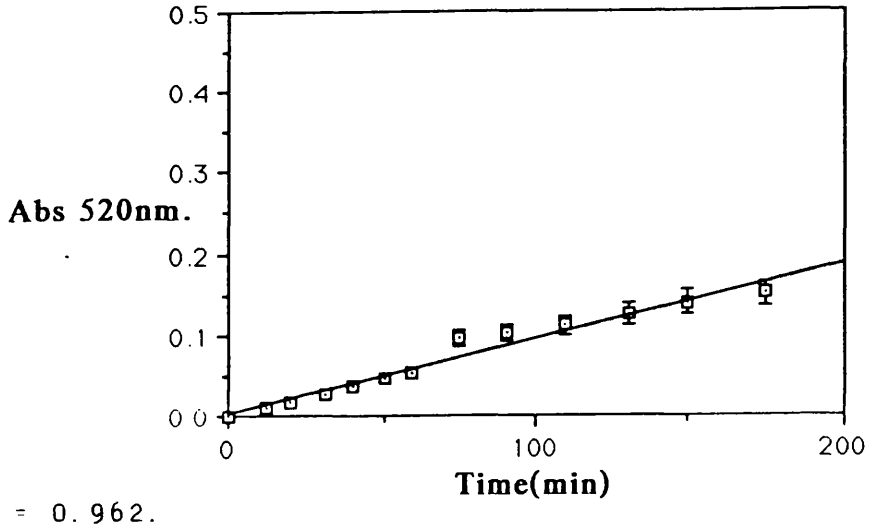
The pI of horse radish peroxidase is in the region of pH 8.0 to 8.9, whereas phosphatases generally have a pI in the acidic region (Keesey, 1987). An efficient separation can therefore be achieved to remove the micro-contamination component that has a phosphatase activity detectable in the amplification assay.

The HR5/5 column is designed for a total binding capacity of 25 mg of protein. Larger quantities of horseradish peroxidase can be loaded onto the column since this protein does not bind under the conditions used. This unit operation is simple to use and rapid.

3.6.2. Determination of "FADPase" in horseradish peroxidase.

A series of amplification assays was prepared at 0.41 Units mL^{-1} apo-D-amino acid oxidase (See Section 2.4.2.), in 0.1M tris, pH 8.0. Horseradish peroxidase concentrations used in each assay were 10ug mL^{-1} and 30ug mL^{-1}. 20uM FADP was added to each assay. A series of blank assays was also prepared which did not contain any FADP, and hence the contribution to the assay from residual holoenzyme activity could be assessed. To evaluate the assay performance, a standard assay incorporating 1.0nM FAD was used. All assays were performed in triplicate (See Figure 23 a-d). The progress curve of each was followed on a Titretek II plate reader.

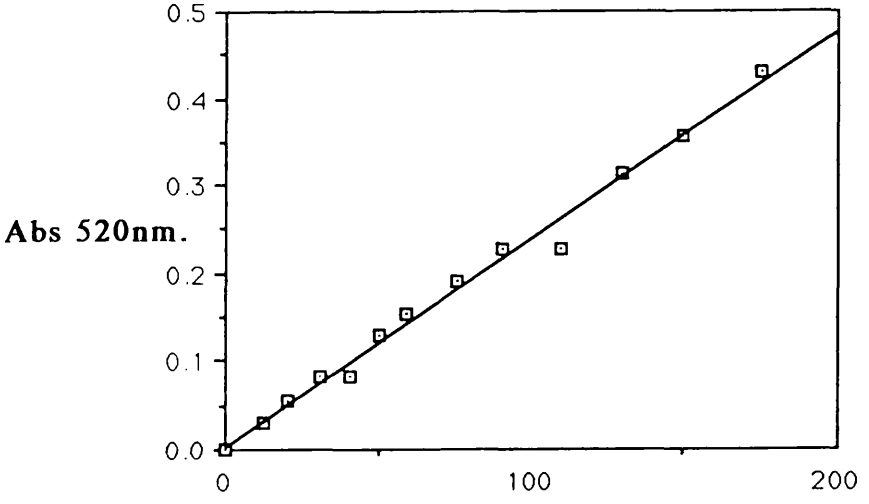
Figure 23a. Residual holoenzyme activity.



R^2 = 0.962.

Residual holoenzyme activity in 0.41 Units/mL^{-1} of apo-D-amino acid oxidase determined in 0.1M tris, pH 8.0 with 10ug mL^{-1} horseradish peroxidase 35mM D-proline, 2.0mM DCHB and 0.2mM 4-aminoantipyrine.

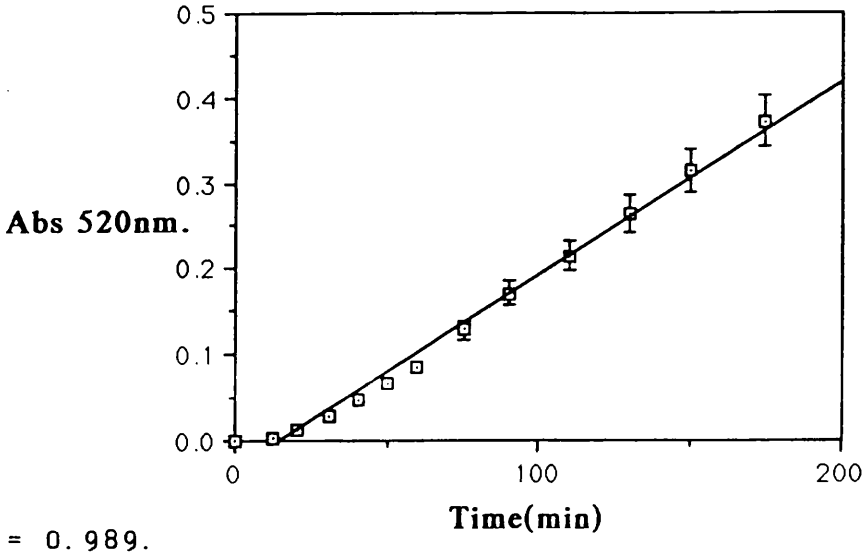
Figure 23b. 1.0nM FAD standard.



R^2 = 0.989.

0.41 Units/mL^{-1} of apo-D-amino acid oxidase was assayed in the presence of 1.0nM FAD.

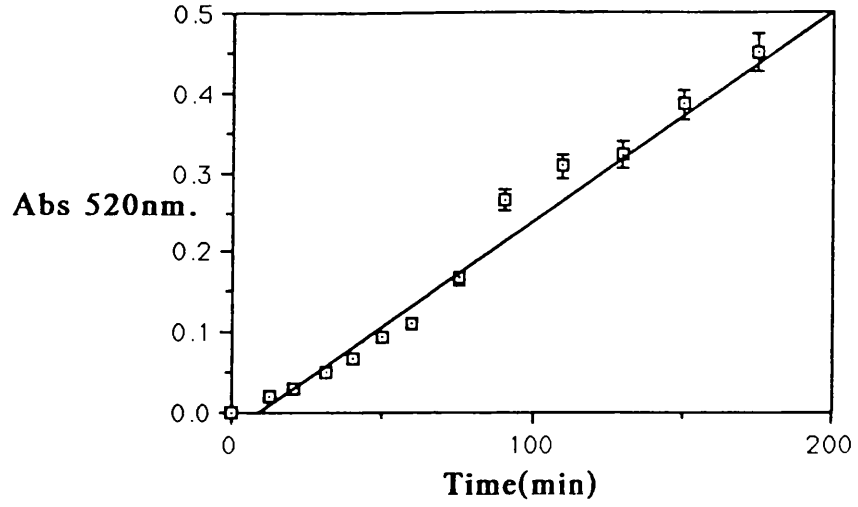
Figure 23c. Assay of D-amino acid oxidase employing 10ug mL^{-1} horseradish peroxidase.



R^2 = 0.989.

0.41 Units/mL^{-1} of apo-D-amino acid oxidase was assayed in the presence of 10ug mL^{-1} horseradish peroxidase and 20uM FADP.

Figure 23d. Assay of D-amino acid oxidase employing 30ug mL^{-1} horseradish peroxidase.



R^2 = 0.981.

0.41 Units/mL^{-1} of apo-D-amino acid oxidase was assayed in the presence of 30ug mL^{-1} horseradish peroxidase and 20uM FADP.

The R^2 term derived by regression analysis of the curves shown in Figure 23c and 23d containing varying concentrations of horseradish peroxidase indicates a good degree of fit to a straight line. An assay that progresses in this fashion is indicative of holoenzyme (FAD) contamination, or other oxidase/peroxidase activity, only. If an "FADPase" activity were present in the assay system, the amplification reaction would be initiated and the progress curve would follow a quadratic function characteristic of the amplification kinetics.

3.6.3. Assessment of horseradish peroxidase for contaminating FAD.

Horseradish peroxidase, prepared by treatment on a Mono Q column was reconstituted in sterile water at a concentration of 10mg mL^{-1} (See Section 2.2.). Various concentrations of the peroxidase were used in an assay of apo-D-amino acid oxidase (See Section 2.1.) at 65.5nM in 0.2M tris, pH 8.0. The concentration of residual holoenzyme was determined, by following the progress of the assay spectrophotometrically at 25°C. Assuming a value for K_D of 4.8×10^{-8}M (See Section 5.6.), the concentration of flavin present in the assay was calculated (See Table 9.).

Table 9. Determination of FAD in horseradish peroxidase.

HRP mg mL^{-1}	$[E_0]$ M	[EF] M	$[F_0]$ M
3.0×10^{-2}	6.55×10^{-8}	1.48×10^{-11}	1.23×10^{-10}
6.0×10^{-2}	6.55×10^{-8}	1.39×10^{-11}	1.16×10^{-10}
9.0×10^{-2}	6.55×10^{-8}	1.04×10^{-11}	8.66×10^{-11}
1.2×10^{-1}	6.55×10^{-8}	1.41×10^{-11}	1.17×10^{-10}

Mean value of $F_0 = 1.0 \times 10^{-10}$M

The residual holoenzyme activity of D-amino acid oxidase was determined in the presence of horseradish peroxidase (0.03 - 0.12mg mL^{-1}) in 0.2 M tris-HCl, pH 8.0. In the laboratory 1 Unit mL^{-1} of D-amino acid oxidase activity is equivalent to a concentration of 0.72uM. Since the original concentration of D-amino acid oxidase (E_0) is known and the value of EF is determined by experimentation the concentration of FAD can be calculated assuming a value of $K_D = 4.8 \times 10^{-7}$M (See Section 5.6.).

$$K_D = \frac{[E]\ [F]}{[EF]}$$

$$K_D = \frac{[E_0 - EF]\ [F_0 - EF]}{[EF]}$$

The value of F_0 calculated is equivalent to a residual holoenzyme activity of 0.17%. This value is larger than the residual holoenzyme content shown in Table 4, but is still typical of the residual activity found in early preparations of the apoenzyme. It can therefore be concluded that there is no FAD present in the horseradish peroxidase preparation.

3.7. Synthesis of the prosthetogen.

3.7.1. Chemical synthesis of FAD-2',3'-cyclic-phosphate.

Cyclic FADP was prepared by Dr. H. J. Egglete. Several approaches have been investigated for the production of cyclic FADP with a view to cost, ease of scale-up and overall yield.

Adaptations of the procedures described by A. M. Michelson (1964a and 1964b), for the synthesis of Coenzyme A have proved to be the most applicable. FMN trioctylamine salt and the bis-trioctylamine salt of adenosine-2,5- and 3,5-bis-phosphate were purified in the laboratory from commercially available reagents (Sigma Cat. No. F 6750 and A 3142 respectively).

Purification of the final product was achieved by anion exchange chromatography followed by reverse phase HPLC. Typically this contained 0.0002% FAD (See Section 3.7.4.).

3.7.2. Enzymic synthesis of FAD-3'-phosphate from FAD-2',3'-cyclicphosphate.

Early production procedures of FADP incorporated a final stage of acid hydrolysis to yield a mixture of the 2' and 3' isomers of the prosthetogen. This procedure however resulted in the production of FAD (0.02%), which was too high a concentration to use in

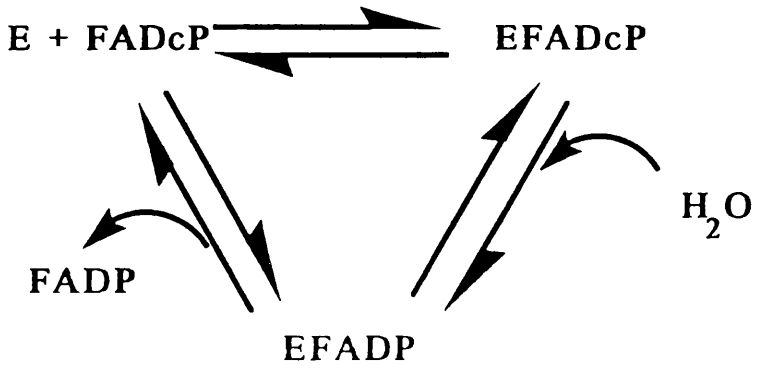
the amplification assay.

Enzymic hydrolysis of the cyclicphosphate was employed as a final step in the production of the prosthetogen. The 2' isomer and the 3' isomer of FADP could therefore be produced independently. All studies described herein were carried out using the FAD-3'-phosphate. The production of the FAD-2'-phosphate is described in Appendix 1.

Purified FAD-2',3'-cyclicphosphate was hydrolyzed to FAD-3'-phosphate by the action of ribonuclease-T_2 (E. C. 3.1.27.1.), (Sigma Cat. No. R 3751.). The catalytic activity of ribonuclease-T_2 was assessed by using adenosine-2,3-cyclicphosphate-5-phosphate as a substrate analogue of FADcP, in 20mM bis-tris, pH 7.0 (See Section 2.5.). The reaction was followed by the spectral change at 270nm. K_p, K_a and V_f were calculated.

The most simple reaction scheme for the production of FADP from FADcP by Ribonuclease-T_2 is shown in Figure 24.

Figure 24. The reaction scheme of Ribonuclease-T_2.



E = enzyme.

FADcP = FAD-2',3'-cyclicphosphate.

FADP = FAD-3'-phosphate.

EFADcP, EFADP = Complexes between E and FADcP, FADP.

The integrated rate equation (Equation 1), derived from this scheme (Cornish-Bowden, 1979), determines the concentration of enzyme required to yield a 99% conversion of the substrate.

Equation 1.

$$V_f = \frac{(1 - K_m/K_p)(a_0 - a) + K_m(1 + a_0/K_p)\mathrm{Ln}(a_0/a)}{t}$$

where V_f = velocity of the forward reaction.

K_s = Michaelis rate constant 2.16×10^{-4}M (S. Harbron, unpublished).

K_p = dissociation constant of EP for an irreversible reaction 1.03×10^{-4}M (S. Harbron, unpublished).

a_0 = concentration of cyclic flavin at t_0.

a = concentration of cyclic flavin at time t.

t = time (minutes).

Providing in the above calculation molar concentrations are used throughout, the number of units of enzyme can be equated to the number of Sigma units for the hydrolysis of polyadenylic acid. This value is obtained fron equation 2.

Equation 2.

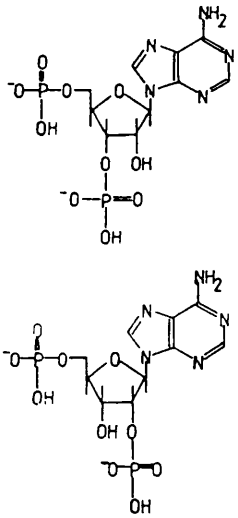
$$\text{Units (Sigma)} = V_f(\text{from 1.}) \times \text{vol.(mL)} \times 7.98 \times 10^3$$

Typically, 8.56mg FAD-2',3'-cyclicphosphate was dissolved in 20mM bis-tris, pH 7.0. 10.3 units of ribonuclease-T_2 was added to give a total volume of 4.0mL. The reaction mixture was incubated at 25°C, in the dark for 60 minutes.

The enzyme was removed by centrifugation at 6,300g through a Centricon 10 ultrafilter (Amicon Cat No. 4205), which was pre-washed with sterile water. Flavin remaining above the membrane was flushed through with an equal volume of sterile water.

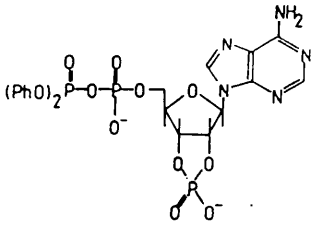
The ultrafiltrate was aliquoted and frozen at -70°C and thawed as required or lyophilized at 0.05 bar for 36 hours in the dark. The lyophilizate was stored at -70°C. The production of FAD-3'-phosphate is outlined in Figure 25. FAD-3'-phosphate has been repeatedly produced in this way. Table 10 demonstrates the reproducibility of the procedure.

Figure 25. The synthesis of FAD-3'-phosphate.
(See Appendix four for a complete description of the synthesis of FAPcP).

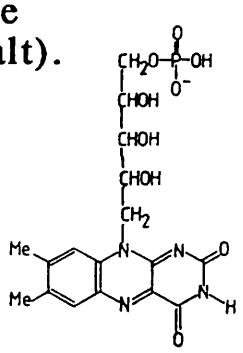


Equimolar mixture of adenosine-2',5', and 3',5'-*bis*-phosphate (*bis*- trioctylamine salt).

Diphenylchlorophosphate/ tributylamine in dioxan.



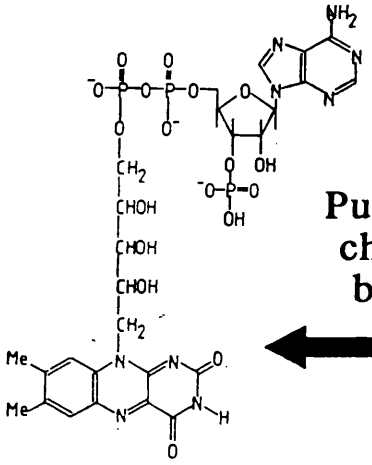
Adenosine-2',3'-cyclic-phosphate-5'-diphenyl-pyrophosphate.



Pyridine/ Formamide.

Ether extraction

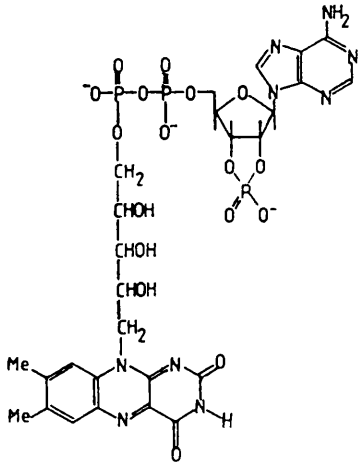
FMN(trioctylamine salt).



Purified by anion exchange chromatography followed by reverse phase HPLC.

Ribonuclease T2 (E.C. 3.1.27.1.).

FAD-3'-phosphate



FAD-2',3'-cyclicphosphate

3.7.3. Production yields for the enzymic conversion of FADcP to FADP.

Table 10. Yield of FAD-3'-phosphate.

Preparation	FADcP mg	FADP mg	Yield %
1	8.56	7.20	84%
2	8.20	7.02	86%
3	12.05	11.25	93%
4	3.88	2.70	70%
5	6.75	5.40	80%

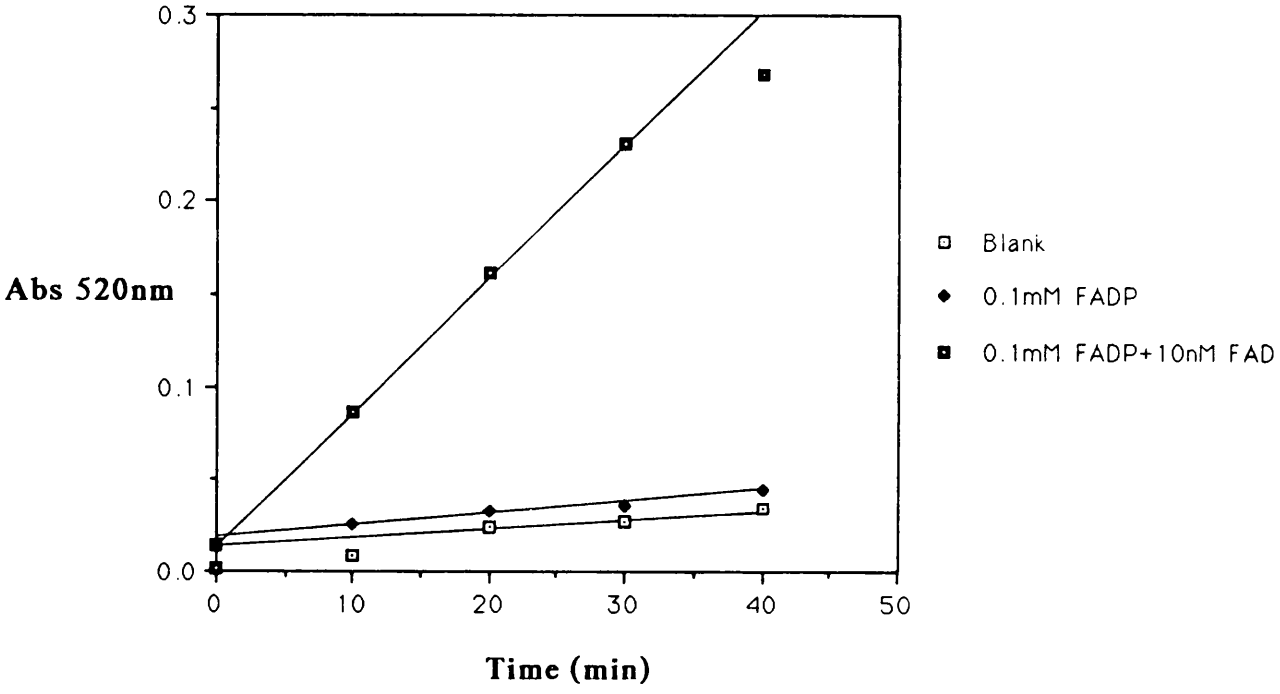
The concentration of flavin was determined by the extinction at 450nm (See Section 2.3.).

Consistently high yields have been obtained from this procedure. The major contribution to the loss of flavin is attributed to the small volume of liquid that is retained above the membrane in the centricon 10 after centrifugation. The average loss throughout these preparations is 1.25mg.

3.7.4. The assay of FAD contaminating FADP.

See Section 2.7.

Figure 26. Determination of FAD contaminating FADP.



The concentration of FAD present in FADP was determined by measuring the residual holoenzyme activity of 0.14 Units mL^{-1} of apo-D-amino acid oxidase in 0.1M tris-HCl, pH 8.0

Gradient of blank = 4.73×10^{-4} min^{-1}

Gradient of 0.1mM FADP = 6.50×10^{-4} min^{-1}

Gradient of 0.1mM FADP + 10nM FAD = 7.34×10^{-3} min^{-1}

% FAD in FADP is therefore:

$$\left[\frac{(6.50\times10^{-4})-(4.73\times10^{-4})}{(7.34\times10^{-3})-(6.50\times10^{-4})}\right] \times 10^{-8} \times 100\% = 2.63\times10^{-4}\%$$

$$1\times10^{-4}$$

3.7.5. Purification of FADP.

Chromatographic methods for the removal of FAD from FADP by FPLC using a Mono Q column have proved unsuccessful. The resolving power of the column was inadequate and the degradation of FADP to FAD under the conditions of chromatography actually increased the levels of contamination. Since FADcP is resistant to hydrolysis by phosphatases and nucleotidases there is a significant advantage in removing FAD contamination at this stage and then converting FADcP to FADP.

Treatment of partially purified FADcP with alkaline phosphatase, which was later removed by ultrafiltration, and rechromatographing on Q-Sepharose removed contaminating dinucleotides that would otherwise have resulted in an effectively lower turnover of FADP by alkaline phosphatase. Typically, this resulted in a preparation of FADcP containing

0.007-0.02% FAD.

An alternative procedure to reverse phase HPLC for the purification of the prosthetogen was assessed. Apoglucose oxidase (See Appendix 2.) was used to reduce the levels of FAD contamination (See Appendix 3.). Commercially available preparations of holoenzyme were found to contain low levels of phosphodiesterase activity that produced FAD from contaminants in FADcP. It was necessary therefore to effect prior removal of the contaminating dinucleotides before employing apoglucose oxidase.

3.7.6. The quality control of a preparation of FADP.

The purity of the final prosthetogen preparation can be determined by chromatography on a Mono Q FPLC column. This procedure has been used for analytical purposes only. Complete resolution of FAD and FADP has not been achieved.

3.7.6.1. The assessment of quality of a preparation of FADP.

To provide an operational measure of the purity of a prosthetogen preparation it was possible to compare it with a standard sample of FADP. When incorporated into an amplification assay of alkaline phosphatase, the signals obtained were compared to those produced by an FAD standard used in place of the FADP. Table 11 shows such a comparison and the ratios determined. The assay of alkaline phosphatase in this instance was carried out in 0.2M tris-HCl pH 8.0, containing 1.0mM $MgSO_4$ and 0.1mM $ZnSO_4$. The concentration of apo-D-amino acid oxidase was 0.41 Units mL^{-1}.

Table 11. Signal ratios of an amplification assay for an FADP preparation compared to an assay of FAD(10nM).

Alkaline Phosphatase (amol)	Time (Minutes)				
	10	20	30	40	50
10.0	/	0.14	1.25	1.32	1.91
30.0	0.06	0.18	2.10	2.82	3.50
100.0	1.30	3.36	8.90	10.75	12.79

Alkaline phosphatase was assayed using 0.41 Units mL^{-1} apo-D-amino acid oxidase, 10ug mL^{-1} HRP, 35mM D-alanine 2.0mM DCHB, 0.2mM 4-aminoantipyrine and 100uM flavin in 0.2M tris-HCl, pH 8.0, containing 1.0mM $MgSO_4$ and 0.1mM $ZnSO_4$. The ratios tabulated above are the signal at each time point compared to the signal generated by a 10nM FAD standard incorporated into an assay that did not contain alkaline phosphatase.

CHAPTER FOUR.

METHODS EMPLOYED IN THE OPTIMIZATION OF THE AMPLIFICATION ASSAY OF ALKALINE PHOSPHATASE.

4.1. Alkaline phosphatase: An introductory note on the problems of optimization.

The term alkaline phosphatase (3.1.3.1.) is applied to a group of enzymes sharing the capacity to hydrolyze phosphate monoesters in an alkaline medium. Hydrolysis occurs by nucleophilic displacement reactions at the phosphorus atom with intermediate formation of a phosphoryl enzyme.

Calf intestine alkaline phosphatase is a dimeric metalloenzyme (Chappelet-Tordo et al., 1974) and a glycoprotein (Engstrom, 1961). The two similar, or identical, subunits of the 69,000 molecular weight monomer associate to form a dimer of 140,000 molecular weight (Chappelet-Tordo et al., 1974). The carbohydrate components are known to contribute to approximately 12% of the total molecular weight of the protein (Fosset et al., 1974). Although the active enzyme is a dimer, at alkaline pH only one of the two active sites can be phosphorylated at any one time. This means that alkaline phosphatase can be classified as a half-site enzyme (Fernley and Walker, 1966; Chappelet-Tordo et al., 1974). The enzyme is

inhibited by its specific product P_i (Martland and Robinson, 1927; Morton, 1955b). This inhibition is strictly competitive (Lazdunski and Ouellet, 1962).

Ethylenediamine tetra acetic acid (EDTA) has been shown to inactivate calf intestinal alkaline phosphatase (Hofstee, 1955), though it was J. C. Mathies (1958) who first demonstrated the presence of zinc in alkaline phosphatase by direct measurement. The activity of many alkaline phosphatases is enhanced by the presence of magnesium in the assay system (Alhers, 1974; Jenner and Kay, 1931).

The exact nature of the interaction with magnesium and its role is not known, though it does protect against denaturation (Garen and Levinthal, 1960). There is evidence that magnesium stabilizes the tertiary structure of alkaline phosphatase in *E. coli* (Anderson *et al.*, 1975). However, the exact nature of the enzyme-magnesium complexes is likely to vary with the source of the enzyme (Posen, 1967; Linden *et al.* 1977). Zinc is essential for catalytic activity and possibly also for maintenance of native enzyme structure (Plocke *et al.*, 1962a and b).

Many of the early studies of alkaline phosphatase employed only partially purified enzyme. This has led to considerable differences in the reported values of kinetic parameters. However, a characteristic feature

of alkaline phosphatase is that the measured value of the pH optimum increases with increasing concentration of substrates (Posen, 1967).

The activation of alkaline phosphatase by amino alcohols was first described by Granger and Fraux in 1947 (in McComb, 1979). Tris-HCl has become a commonly used buffer for the assay of alkaline phosphatase activity. At values higher than pH 9.0 ethanolamine has been used (Hindberg and Laidler, 1972). The optimum activity of intestinal alkaline phosphatase is at pHs between 8.4 to pH 9.4 (Martland and Robinson, 1926; Morton, 1955a) depending on the nature of the substrate and the buffer system employed.

Intestinal alkaline phosphatase follows Michaelis-Menten kinetics between pH 7.5 and pH 10.0 (Chappelet-Tordo et al., 1974). Using p-nitrophenyl phosphate as substrate a large increase in K_m with pH is observed, from about 2.5uM at pH 7.5 to 2.0mM at pH 10.0. There is also a concomitant increase in V_{max} which is dependent on the concentration of the tris-HCl buffer (Hindberg and Laidler, 1973). K_m is known to be independent of tris-HCl concentration (Hindberg and Laidler, 1972). Conditions of high ionic strength have also been shown to increase the activity of alkaline phosphatase at pH 8.0. The variation of activity with ionic strength is abolished above pH 10.

Each assay protocol of alkaline phosphatase must therefore be individually optimized because of this complex inter-dependence between pH optimum and substrate concentration. Enzyme stability, and the pH dependent activation and inhibition by some buffering systems and ionic strength effects that may also be pH related (Morton, 1957; Chappelet-Tordo, 1974) must also be taken into account. When dealing with a coupled enzyme assay system, as used here, the factors that regulate the activity of the other enzymes employed must also be considered in the process of optimization.

4.2. The effect of the concentration of horseradish peroxidase at 25°C and 37°C on the measured activity of D-amino acid oxidase.

For all coupled enzyme reactions the enzyme producing the detectable signal must not be present in limiting concentrations. This would reduce the overall observable rate of the reaction. The effect of the concentration of horseradish peroxidase in the coupled reaction with D-amino acid oxidase was determined by the assay of a known concentration of apo-D-amino acid oxidase reconstituted with a set concentration of FAD to give a fixed reaction rate (See Section 5.5.).

The concentration of horseradish peroxidase was varied (1.0-50ug mL^{-1}) in the assay (See Section 2.1.) of an

FAD standard of 10nM. A cocktail of reagents common to each assay was prepared to give the final concentrations stated, in an assay volume of 100uL:

35.0mM	D-proline.
2.0mM	DCHB.
0.2mM	4-aminoantipyrine

Apo-D-amino acid oxidase, at final concentrations of 0.14 and 0.41 Unit mL^{-1}, was mixed with the standard FAD solution in 0.1M tris-HCl, pH 8.9. After 15 minutes the remaining assay components were added to a final volume of 100uL. The progress of the reaction was followed for a period of 60 minutes on a Dynatech MR 7000 plate reader with a thermostatically regulated plate holder. The rate of each assay was determined in the linear region of the curves.

4.3. Comparison of amino acid substrates at pH 8.0.

D-amino acid oxidase is active across a broad range of pH (Dixon and Kleppe, 1965c). The specificty of the enzyme alters over this pH range. The substrate which is turned over most rapidly at a specified pH is the one that would be incorporated in the amplification assay.

A series of assays was prepared (See Section 2.1.), to assay a standard solution of FAD (10nM). A cocktail of

reagents was prepared to give the final concentrations stated, in an assay volume of 100uL:

0.2M	Tris-HCl, pH 8.0.
2.0mM	DCHB.
0.2mM	4-aminoantipyrine.
0.01mg mL^{-1}	Horseradish peroxidase.
0.24mM	FAD.

Each assay employed a different D-amino acid substrate. The substrates used were, D-alanine, D-methionine and D-proline at a final concentration of 35.0mM. The reaction was initiated by the addition of a fixed volume (5uL) of a solution of apo-D-amino acid oxidase. The reaction was followed on a Titretek II plate reader at 520nm at 22°C and the rate of change of absorbance determined over the linear region of the curve.

4.4. The incorporation of L-amino acids in the assay of alkaline phosphatase activity.

It has been reported that the activity of alkaline phosphatase can be stimulated by the presence of L-amino acids in an assay mixture (Morton, 1955a). Such a phenomenon would be beneficial as incorporation of an L-amino acid into the amplification assay would increase the overall rate of the assay. Incorporation of a D-L mixture of an amino acid in the assay would

also reduce the cost of materials in the system.

The presence of L-amino acid(s) in the assay must not interfere with the activity of D-amino acid oxidase. Certain amino acids have been reported to be inhibitors of this enzyme (Dixon and Kleppe, 1965b). Two experiments were carried out to investigate these phenomena.

A solution of alkaline phosphatase, approximately 10^{-11}M (See Section 2.4.1.), was assayed by following the hydrolysis of p-nitrophenyl phosphate at 405nm. The progress of the assay was also followed with 1.0mM L-histidine or 1.0mM L-proline in the assay buffer.

The possible inhibition of D-amino acid oxidase was studied by determining the total activity of an enzyme preparation (See Section 2.1.) using either 35mM D-proline or 70mM DL-proline as the substrate in 0.2M tris-HCl, pH 8.0, at 25°C. The assay was followed spectrophotometrically at 520nm.

4.5. Reconstitution of apo-D-amino acid oxidase in the presence of high concentrations of the prosthetogen.

The prosthetogen must not inhibit the binding of FAD to apo-D-amino acid oxidase. The rate of association of FAD and apo-D-amino acid oxidase must also be unaffected by the small concentration of FADcP that is likely to be present in any preparation of the FAD-3'-phosphate.

A series of assays for the determination of D-amino acid oxidase activity was prepared (See Section 2.1.) using a cocktail of reagents to give the stated final concentrations, in an assay volume of 100uL:

0.1M	Tris-HCl, pH 8.9.
35.0mM	D-proline.
2.0mM	DCHB.
0.2mM	4-aminoantipyrine.
0.01mg mL^{-1}	Horseradish peroxidase.
0.14 Unit mL^{-1}	Apo-D-amino acid oxidase

The total reconstitutable activity of apo-D-amino acid oxidase was determined by employing a range of concentrations of FAD ($0-10^{-7}$M). The assays were performed in the presence and the absence of 20uM FAD-3'-phosphate. The progress of the reaction was followed under subdued lighting for 120 minutes using

a Dynatech MR 7000 plate reader with a thermostatically regulated plate holder. The experiment was repeated incorporating 10uM FAD-2',3'-cyclicphosphate in the place of FAD-3'-phosphate in 0.1M tris-HCl, pH 8.0.

4.6. Determination of the dissociation constant of D-amino acid oxidase for FAD at pH 8.0.

The dissociation constant of D-amino acid oxidase for FAD was determined by measuring the extent of reconstitution of the apoenzyme with varying concentrations of FAD. Assays were prepared to give the stated concentrations in a final volume of 100uL.

20-200mM	Tris-HCl, pH 8.0.
2.0mM	DCHB.
0.2mM	4-aminoantipyrine.
0.01mg mL^{-1}	Horseradish peroxidase.
0-0.5uM	FAD.
0.14 Unit mL^{-1}	Apo-D-amino acid oxidase.

The reaction was initiated in each well of the microtitre plate by the addition of D-alanine (final concentration 35.0mM). Prior to the addition of the substrate, the apoenzyme/FAD/tris-HCl mixtures were incubated for 15 minutes to attain equilibrium. The reactions were followed on a Titretek II plate reader at 520nm, at 25°C. The concentration of active enzyme

was determined from the progress curves and the value of K_D calculated for each of the apoenzyme/FAD/tris-HCl mixtures.

4.7. The effect of pH on the assay of D-amino acid oxidase.

4.7.1. Reconstitution of holoenzyme activity.

Apo-D-amino acid oxidase (0.14 Units mL^{-1}) was assayed in the presence of 5.0nM FAD (See Section 2.1.). The reaction was studied under varying conditions of pH in buffers comprising of 20mM bis-tris-propane, pH 7.1-9.8, containing 0.1M tris-HCl. The progress of the reaction was followed on a Dynatech MR 7000 plate reader at 25°C for 60 minutes.

4.7.2. Stability of the chromogenic reaction product of horseradish peroxidase.

The stability of the chromogenic reaction product was studied across a pH range suitable for the amplification assay of alkaline phosphatase. The experiment outlined in section 4.7.1. was repeated with 0.14 Units mL^{-1} apo-D-amino acid oxidase and 10nM FAD in the reaction mixture, in a series of five replicates. After 30 minutes incubation, DL-propargylglycine (Sigma Cat No. P 7888) was added to each reaction well of the microtitre plate to a final concentration of 15mM. This compound is an irreverisble inhibitor of D-amino acid oxidase

(Zollner, 1989). The production of hydrogen peroxide is therefore quenched. The chromogenic coupling reaction is terminated and the colour that has already been developed cannot be further oxidized by the action of horseradish peroxidase in the absence of hydrogen peroxide. The reaction progress was followed for a further 90 minutes on a Dynatech MR 7000 plate reader at 520nm at 25°C.

4.8. The effect of pH on the amplification assay of alkaline phosphatase.

The effect of pH on the amplification assay of alkaline phosphatase was assessed by following the progress of a reaction containing 1.0 amol of alkaline phosphatase. A cocktail of reagents was prepared for the assay of alkaline phosphatase containing the final concentrations stated, in an assay volume of 100uL:

0.1mM	$MgSO_4$.
1.0uM	$ZnSO_4$.
2.0mM	DCHB.
0.2mM	4-aminoantipyrine.
0.01mg mL^{-1}	Horseradish peroxidase.
0.14 Unit mL^{-1}	Apo-D-amino acid oxidase.
35.0mM	D-proline
20.0 µM	FADP.

Aliquots of the pre-mix were added to the wells of a microtitre plate containing 1.0 amol of alkaline phosphatase (See Section 2.4.1.) in 20mM bis-tris-

propane buffer, containing 0.1M tris-HCl, at pH 8.0-9.8. A series of blank reagent wells, containing all of the reagents except alkaline phosphatase, was also studied across the pH range. The reaction progress was followed for 120 minutes on a Dynatech MR 7000 plate reader at 520nm at 25°C.

4.9. The determination of the apparent K_m for FADP at 25°C and 37°C.

A series of amplification assay mixtures, differing only in the concentration of FADP, was prepared to determine the kinetic parameters of alkaline phosphatase ($3x10^{-14}$M) (See Section 2.4.1.). Each mixture contained the final concentrations stated, in an assay volume of 100uL:

0.1M	Tris-HCl, pH 8.9.
0.1mM	$MgSO_4$.
1.0uM	$ZnSO_4$.
35.0mM	D-proline.
2.0mM	DCHB.
0.2mM	4-aminoantipyrine.
0.01mg mL^{-1}	Horseradish peroxidase.
0.14 Unit mL^{-1}	Apo-D-amino acid oxidase.

The concentration of FADP was varied from 5.0-50uM. The signals from each reaction well in the microtitre plate were approximately corrected using a blank

containing the same reaction mixture without alkaline phosphatase. The progress of the reaction was monitored at 520nm in a Dynatech MR 7000 plate reader at 25°C. The rate of the reaction was determined at 60 minutes.

The experiment was repeated at 37°C using concentrations of FADP in the range 7.5-50uM. The rate of the reaction was determined at 20 minutes.

4.10. The determination of K_{cat} for alkaline phosphatase and FADP.

Alkaline phosphatase (10^{-14}M) was incubated with 200uM FADP in 0.1M tris-HCl, pH 8.9, containing 0.1mM $MgSO_4$ and 1.0uM $ZnSO_4$, in a total volume of 200uL at 25°C. Aliquots (10uL) were removed at intervals and the concentration of FAD produced was determined (See Section 5.5., Figure 31.) by the extent of the reconstitution of 0.14 Units mL^{-1} apo-D-amino acid oxidase in 0.1M tris-HCl, pH 8.9, containing 10mM sodium phosphate. The signal from each reaction well was approximately corrected for the initially small concentration of FAD present in the preparation of FADP using a blank containing 20uM FADP. The rate of FAD production was used to determine K_{cat} for the reaction of alkaline phosphatase and FADP.

4.11. The effect of the concentration of tris-HCl and FADP on the sensitivity of the amplification assay.

Previously it has been shown that the concentration of tris-HCL effects the dissociation constant and hence reconstitution of D-amino acid oxidase activity (See Section 4.6.). Since tris-HCl stimulates the activity of alkaline phosphatase, a reduction in the concentration of the buffer may have a deleterious effect on the amplification assay. The interaction of the apoenzyme, FAD and the stimulation of alkaline phosphatase activity was studied.

Dilutions of alkaline phosphatase were prepared in 0.1M tris-HCl, pH 8.0, containing 0.1mM $MgSO_4$ and 1.0uM $ZnSO_4$ ($3x10^{-14}$ - 10^{-12}M, final concentration). Amplification assays were prepared (See Section 2.4.2.) varying the concentration of tris-HCl (100-200mM; pH 8.9) and the concentration of FADP (10-20uM). The concentration of apo-D-amino acid oxidase was 0.41 Units mL^{-1}. The progress of the reaction was followed on a Dynatech MR 7000 plate reader at 25°C.

CHAPTER FIVE.

OPTIMIZATION DATA FOR THE AMPLIFICATION ASSAY OF ALKALINE PHOSPHATASE.

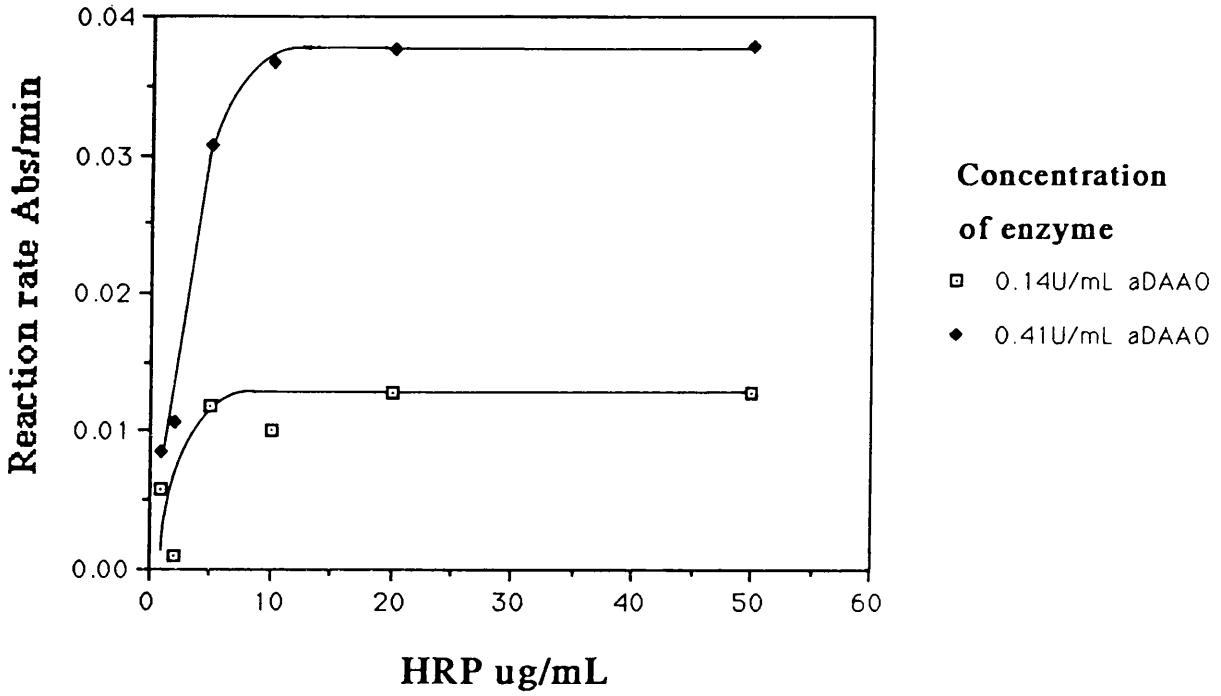
5.1. The effect of varying the concentration of horseradish peroxidase at 25°C and 37°C on the reconstitutable activity of D-amino acid oxidase.

The maximum rate of reaction measured for the activity of D-amino acid oxidase was plotted as a function of the concentration of horseradish peroxidase in each of the assays. Figure 27a shows the effect of varying the concentration of horseradish peroxidase at 25°C and Figure 27b shows the effect of varying the concentration of horseradish peroxidase at 37°C on the reconstitutable activity of D-amino acid oxidase.

Figures 27a/b show no appreciable decrease in the measured reaction rates when the concentration of horseradish peroxidase is maintained at 10ug mL^{-1} and above. Horseradish peroxidase is stable for at least 3 hours in solution (pH 4.0-10.0) at 25°C (Keesey, 1987). The stability of the enzyme is reduced as temperature increases, but this is not significant at 37°C over the period of the assay.

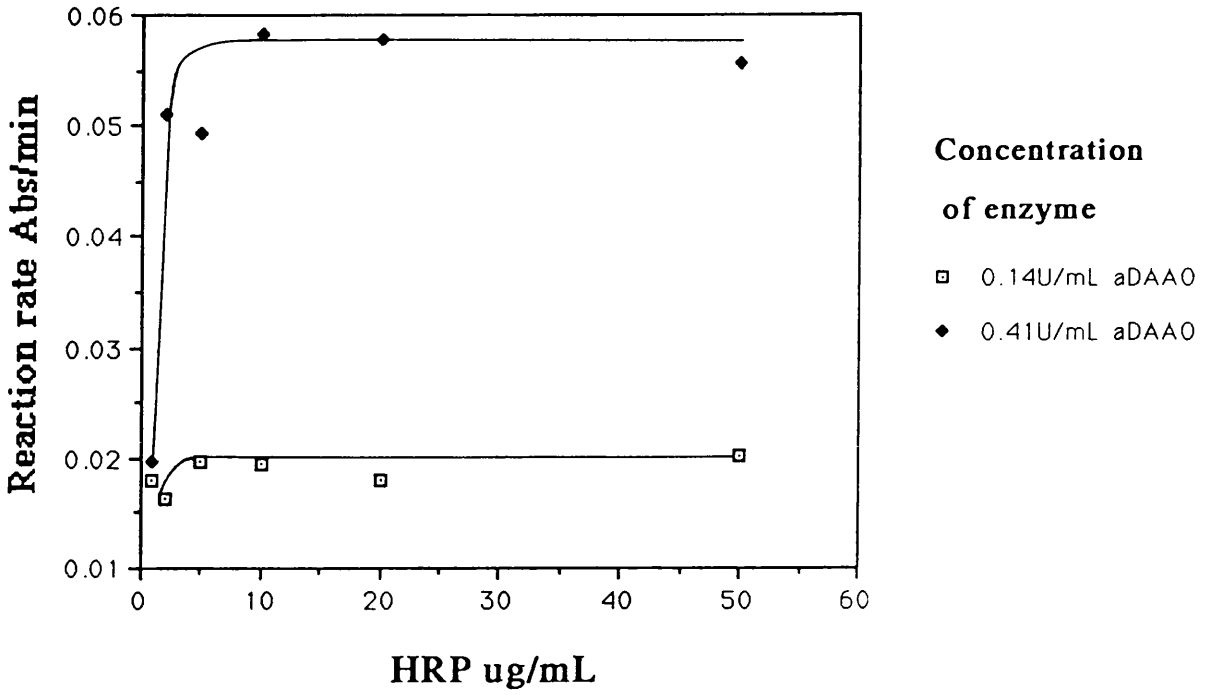
At a concentration of 10ug mL^{-1}, 1.0mg of a preparation of horseradish peroxidase is sufficient for 1000 assays. However, in practical use, a 20% excess of the protein is incorporated into the cocktail of reagents for freeze-drying. Thus, 1.0mg is sufficient for about 830 assays in a microtitre plate format (See Section 6.2.).

Figure 27a. Reaction rates of 0.14/0.41 Units mL^{-1} apo-D-amino acid oxidase reconstituted with 10nM FAD at 25°C.



Apo-D-amino acid oxidase (0.14/0.41 Units mL^{-1}) was reconstituted with 10nM FAD in 0.1M tris-HCl, pH 8.9, in the presence of varying concentrations of HRP (1.0-50ug mL^{-1}) at 25°C. The reaction rates were determined from the linear region of the progress curves.

Figure 27b. Reaction rates of 0.41/0.41 Units mL^{-1} apo-D-amino acid oxidase reconstituted with 10nM FAD at 37°C.



Apo-D-amino acid oxidase (0.14/0.41 Units mL^{-1}) was reconstituted with 10nM FAD in 0.1M tris-HCl, pH 8.9, in the presence of varying concentrations of HRP (1.0-50ug mL^{-1}) at 37°C. The reaction rates were determined from the linear region of the progress curves.

5.2. Comparison of D-amino acid substrates at pH 8.0

The data presented in Table 12 shows that the rate of reaction of D-amino acid oxidase varies with the nature of the amino acid substrate. The best substrate is D-proline and the worst is D-alanine with D-methionine intermediate between the extremes. The ratios of the reaction rates are approximately 3:2.6:1 respectively.

In accordance with Dixon and Kleppe (1965b), D-proline is the substrate which is turned over most rapid by D-amino acid oxidase under the conditions of this study. The increased rate may be due to greater enzyme-substrate affinity or higher turnover of the enzyme-substrate complex, the rate limiting step being the dissociation of the product from the holoenzyme (Massey and Gibson, 1964; Dixon and Kleppe, 1965b). For the purposes of the amplification assay it is the substrate turnover which is important.

A relationship exists between substrate structure and the determined kinetic constants. Dixon and Kleppe (1965b), reported a decrease in K_m, and hence probably an increase in affinity, with increasing chain length (up to four carbon atoms), for a series of straight chain aliphatic amino acids. Higher values of V_{max}, accompanied by lower values of K_m were also observed with sulphur-containing amino acids. A K_m of 2.0mM has been determined for D-proline.

Table 12. Substrate specificity at pH 8.0.

Substrate	Abs min^{-1}	Mean Abs min^{-1}	S.E.
D-alanine	0.76 0.76 0.80 0.80 0.77	0.78	+/- 0.0089
D-methionine	1.80 1.85 1.99 2.31 2.16	2.02	+/- 0.074
D-proline	2.23 2.47 2.53 2.31 2.16	2.34	+/- 0.071

Reconstitutable activity of apo-D-amino acid oxidase was determined with different substrates (35mM D-alanine, D-methionine or D-proline) and 10nM FAD in 0.2M tris-HCl, pH 8.0.

5.3. The effect of L-amino acids on the activity of alkaline phosphatase.

Table 13 shows the reaction rates obtained from a non-amplified assay of alkaline phosphatase employing 0.1mM p-nitrophenyl phosphate in 0.1M tris-HCl, pH 8.0. The data were analyzed for variance between each of the assay conditions (See Table 14.).

The analysis of variance shown in Table 14 illustrates that there is no stimulation of alkaline phosphatase activity by L-amino acids. This is in agreement with Morton (1955a). When freshly prepared enzyme was used no activation of alkaline phosphatase activity was evident. However, activation was shown by Morton on an enzyme solution that was not freshly prepared. This may have been due to contamination of the L-amino acid used with divalent metal ions since activation was also shown upon the addition of magnesium to the solution. The protection afforded by L-amino acids in a long-standing solution of alkaline phosphatase may be due to a decrease in the dissociation of metal ions, zinc and magnesium, from the enzyme or chelation of other potential inhibitors that result in the dissociation of these metals. The dissociation of the divalent metal ions from the native enzyme in solution is however very slow.

Table 13. Rate of reaction of alkaline phosphatase.

Rate of change of absorbance (Abs min^{-1})		
Alkaline phosphatase	Alkaline phosphatase + 1.0 mM L-histidine	Alkaline phosphatase + 1.0 mM L-proline
0.0019	0.0019	0.0019
0.0017	0.0016	0.0020
0.0018	0.0016	0.0025

Alkaline phosphatase (10^{-11}M) was assayed in 0.1M tris-HCl, pH 8.0, containing 0.1mM $MgSO_4$ and 1.0uM $ZnSO_4$. The substrate used was 0.1mM p-nitrophenyl phosphate.

Table 14. Analysis of variance of the assay of alkaline phosphatase.

Source	Degrees of freedom	Sum of squares	Mean square	F ratio
Among assays	2	3.6×10^{-7}	1.8×10^{-7}	4.29
Within assays	6	2.5×10^{-7}	4.2×10^{-8}	
Total	8	6.1×10^{-7}		

Tabulated $F_{0.05}(2,6) = 5.14$

The calculated value of F is smaller than the 5% point of the tabulated F-distribution. The differences between the assays with various amino acids present in the assay mixture is not statistically significant at the 5% level.

5.4. The effect of L-proline on the reaction rate of D-amino acid oxidase.

The data presented in Table 15 shows the effect of L-proline on the activity of D-amino acid oxidase using D-proline as the substrate. The variance of the data was analyzed to assess whether or not there was a significant difference in the measured reaction rates (See Table 16.).

No statistically significant difference was found when L-proline was incorporated into the assay of D-amino acid oxidase using D-proline as the substrate. A mixure of DL-proline can therefore be used in the amplification assay with no deleterious effect on the signal. Commercially, the reduction in the cost of materials is important. If 70mM DL-proline is used in the place of 35mM D-proline in the assay, this translates to a reduction of 35% in the cost of the substrate at present market prices.

Table 15. Reaction rate of D-amino acid oxidase.

Units mL^{-1} of D-amino acid oxidase assayed	
D-proline (35mM)	DL-proline (70mM)
46.38	43.06
44.72	43.06
45.31	44.10
45.80	43.40

The total reconstitutible activity of apo-D-amino acid oxidase was assayed in 0.2M tris-HCl, pH 8.0. The substrates used in each instance were 35mM D-proline and 70mM DL-proline (equal mixture of isomers).

Table 16. Analysis of variance of the assay of D-amino acid oxidase.

Source	Degrees of freedom	Sum of squares	Mean square	F ratio
Among assays	3	9.22	3.07	5.54
Within assays	4	2.22	0.56	
Total	7	11.44		

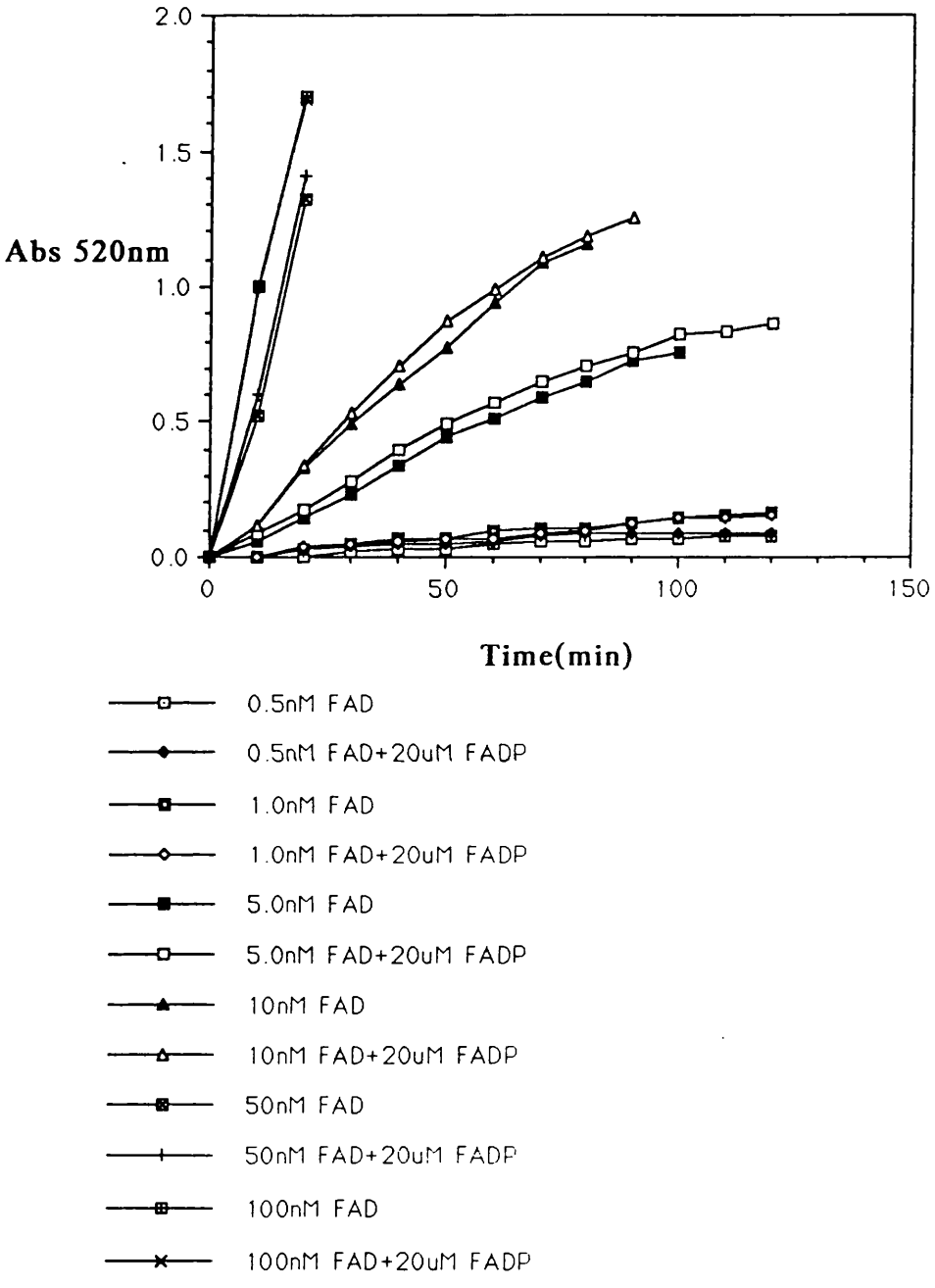
Tabulated value of $F_{0.05}(3,4) = 6.59$

The calculated value of F is smaller than the 5% point of the tabulated F-distribution. The differences between the assays of D-amino acid oxidase with D-proline and DL-proline are not statistically significant at the 5% level.

5.5. The reconstitution of apo-D-amino acid oxidase in the presence of high concentrations of the prosthetogen.

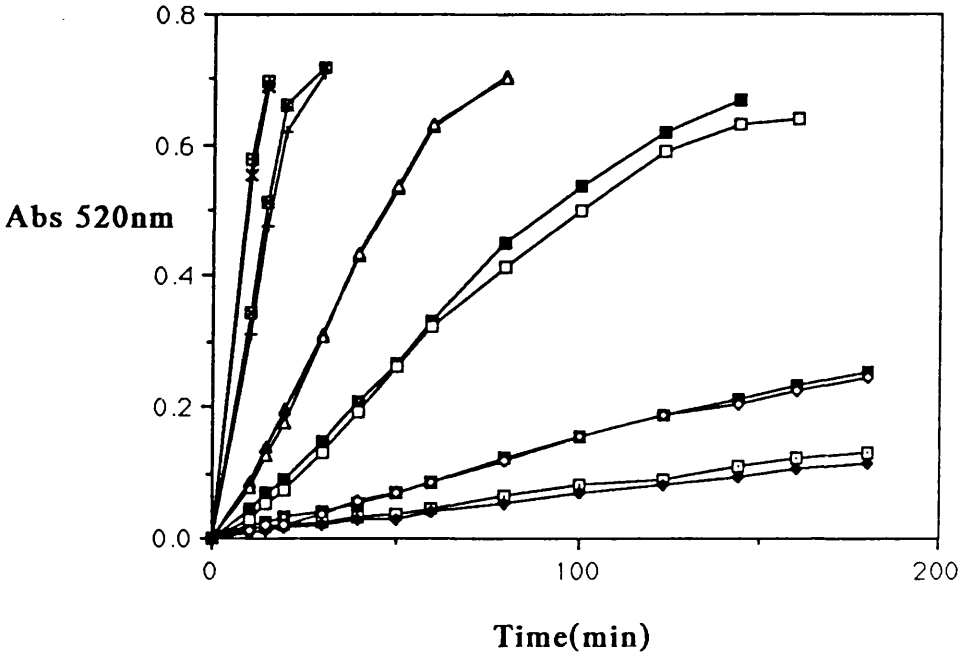
The experiments illustrated in Figures 28a and 28b were developed to ascertain whether FADP or FADcP interfered with the binding of FAD to apo-D-amino acid oxidase. The curves in these figures were derived by subtracting the appropriate background signal in each case. The contribution to the enzymic rate attributable to the small concentration of FAD present in the FADP and FADcP and residual holoenzyme activity has therefore been removed. Both sets of curves can be superimposed on the data obtained from the assay of FAD standards measured in the absence of each derivative of FAD. This demonstrates that the FADP does not compete for the FAD binding site of D-amino acid oxidase under the assay conditions. Also, small concentrations of FADcP that may be present from the incomplete hydrolysis of the cyclic phosphate, do not compete for the FAD binding site of the enzyme.

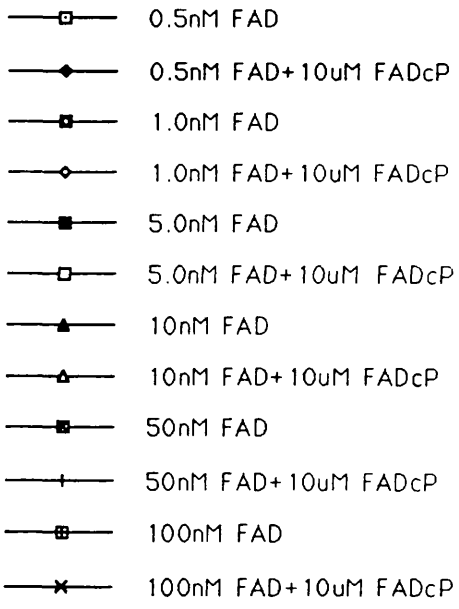
Figure 28a. The reconstitution of holoenzyme activity with FAD in the presence of 20uM FADP.



The reconstitution of D-amino acid oxidase (0.14 Units mL^{-1}) activity was determined with varying concentrations of FAD (0.5-100nM) in the presence of 20uM FADP.

Figure 28b. The reconstitution of holoenzyme activity with FAD in the presence of 10uM FADcP.

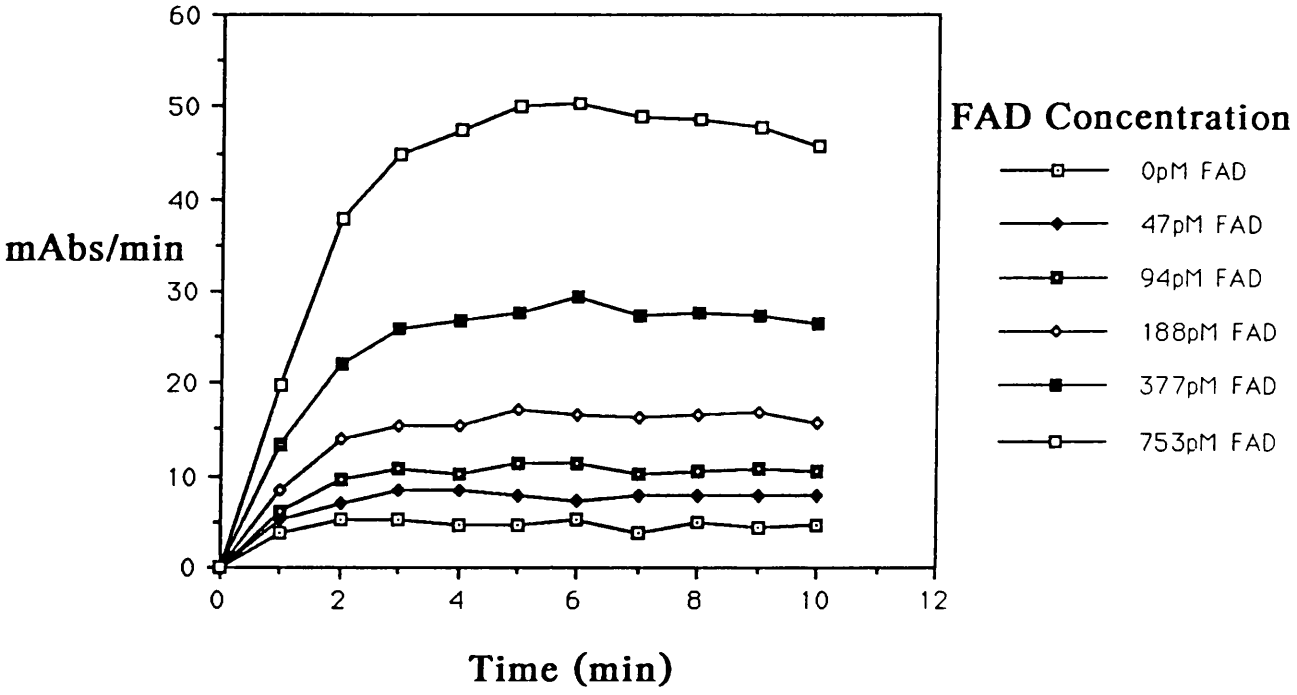




The reconstitution of D-amino acid oxidase (0.14 Units mL^{-1}) activity was determined with varying concentrations of FAD (0.5-100nM) in the presence of 10uM FADcP.

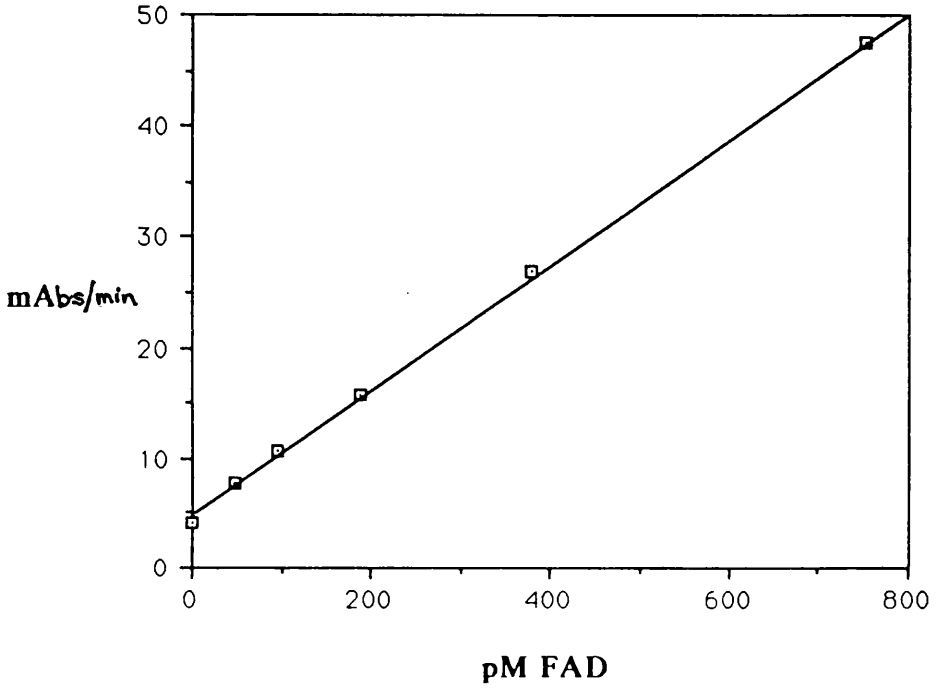
Since the reconstitution of holoenzyme activity was unaffected by the presence of molecules that are very similar to the cofactor it was possible to use the measurement of D-amino acid oxidase activity as an assay for FAD in solution or present as a contaminant in a preparation of FADP (See Section 2.7. and Section 3.7.4.). Figure 29 shows the rates measured in an assay of D-amino acid oxidase reconstituted with varying concentrations of FAD. This experiment was performed by Du Pont during the evaluation of the technology. Within 3 minutes a maximum activity of D-amino acid oxidase is obtained under each of the assay conditions. Figure 30 shows that after this initial period of binding of the cofactor the rate of reaction is directly proportional to the concentration of FAD in solution.

Figure 29. The reconstitution of 0.65 Units mL^{-1} apo-D-amino acid oxidase with varying FAD concentrations at 37°C. (Du Pont).



Apo-D-amino acid oxidase (0.65 Units mL^{-1}) was reconstituted with varying concentrations of FAD (0-753pM) and the activity measured using 35mM D-proline 2.5mM DCHB, 0.25mM 4-aminoantipyrine and 20ug mL^{-1} HRP in 0.1M tris-HCl, pH 8.25 at 37°C. This experiment was performed by Du Pont with materials supplied by London Biotechnology Ltd..

Figure 30. Standard curve of D-amino acid oxidase reconstitutible activity as a function of FAD concentration in the aca® discrete clinical analyzer (Du Pont).

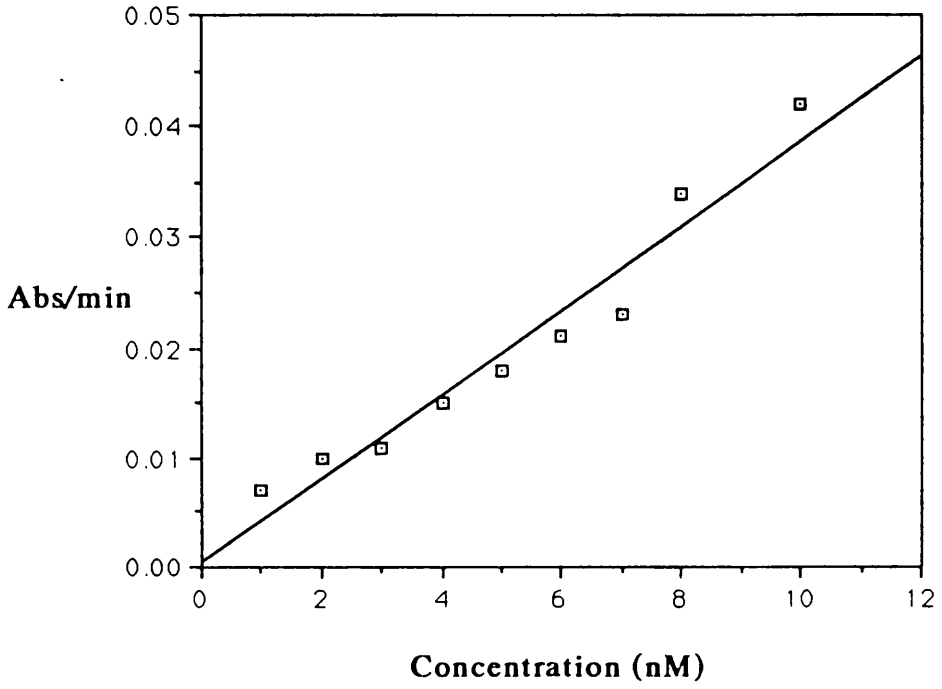


R^2 = 0.999.

Apo-D-amino acid oxidase (0.65 Units mL^{-1}) was reconstituted with varying concentrations of FAD (0-753pM). The activity was measured in 0.1M tris-HCl, pH 8.25, at 37°C. The reaction rate was determined from the plateau region of the curves in Figure 29. This experiment was performed by Du Pont with materials supplied by London Biotechnology Ltd..

The microtitre plate format can also be used for the determination of FAD in solution as shown in Figure 31. The reaction rate was linear over the range of FAD concentrations used. The time required for a maximum activity to be attained in each instance is 3 times longer at 25°C than at 37°C. The kinetics of binding of FAD to the apoenzyme are shown in Figure 32, where the influence of pH on the association of the cofactor has been studied.

Figure 31. Standard curve of D-amino acid oxidase reconstitutible activity as a function of FAD concentration in a microtitre plate assay.



R^2 = 0.946.

Apo-D-amino acid oxidase (0.14 Units mL^{-1}) was assayed in the presence of varying concentrations of FAD (0-10nM). Each assay was performed in a total volume of 0.1mL containing 10ug mL^{-1} HRP, 2.0mM DCHB and 0.2mM 4-aminoantipyrine in 0.1 M tris-HCl, pH 8.9 at 25°C.

5.6. Determination of the dissociation constant of D-amino acid oxidase for FAD and the effect of buffer concentration.

As shown in Table 17 the apparent value of K_D in 100mM tris-HCl is half that in 200mM tris-HCl. It follows therfore that twice as much holoenzyme will be produced at the lower buffer concentration. This would increase the reaction rate by a factor of 2, since the effective concentration of active D-amino acid oxidase would be double. However a reduction of the nucleophile concentration has a deleterious effect on the activity of alkaline phosphatase. Chappelet-Tordo *et al.* (1974), demonstrated that, when p-nitrophenyl phosphate was used as the substrate for alkaline phosphatase, under such conditions, there was a resulting loss of 14% of enzyme activity. Assuming that there is a similar reduction in the rate of FADP hydrolysis, the resulting increase in the rate of colour formation in the detection of the amplification assay would be 1.7 fold (See Section 5.12.) at least as compared to the higher buffer concentration.

Table 18. shows previous analytical determinations of K_D. Experiments have been conducted at a variety of temperatures in pyrophosphate buffer at different concentrations.

Table 17. Variation of K_D with the concentration of tris-HCl buffer.

E_o uM	F_o uM	EF (uM) 20-100mM tris-HCl	EF (uM) 200mM tris-HCl	K_D (uM) 20-100mM tris-HCl	K_D (uM) 200mM tris-HCl
0.10	0.050	0.014	0.008	0.22	0.48
	0.063	0.017	0.010	0.22	0.47
	0.083	0.019	0.011	0.27	/
	0.13	0.029	0.014	0.24	/
	0.50	0.052	0.048	/	0.49

K_D at 20-100mM tris-HCl = 2.4×10^{-7} +/- 1.7×10^{-8} M

K_D at 200mM tris-HCl = 4.8×10^{-7} +/- 5.8×10^{-9} M

E_o = Total concentration of enzyme.

F_o = Total concentration of FAD.

EF = Concentration of holoenzyme.

The concentration of active D-amino acid oxidase was determined by reconstitution of the active enzyme in the presence of varying concentrations of FAD (50-500nM). The influence of buffer composition was assessed in each instance by varying the concentration of tris-HCl, pH 8.0 (20mM-200mM). K_D was calculated from the known concentrations of total reconstitutable apo-D-amino acid oxidase and FAD and the extent of reconstitution measured under the various conditions in each assay. The results of assays performed in 100mM tris-HCl and below have been averaged since no difference was discernable between these assays.

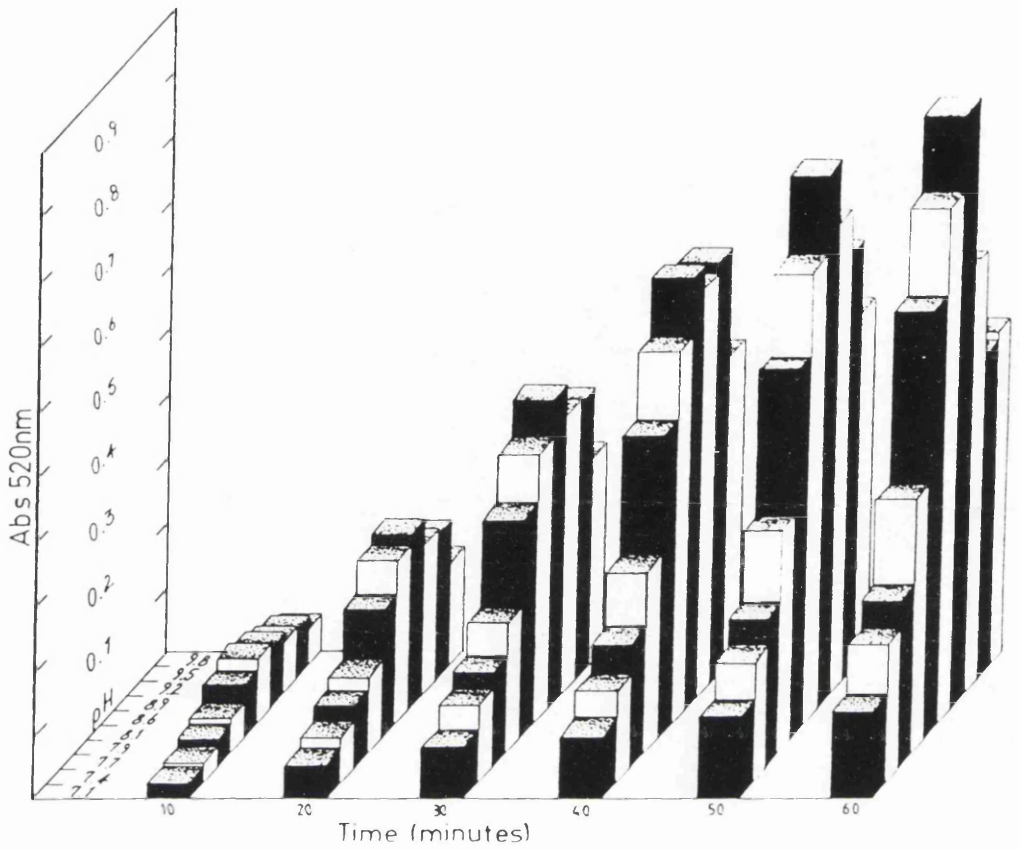
Table 18. The variation of the dissociation constant of D-amino acid oxidase for FAD with pH and temperature (after Brumby *et al.*, 1968).

pH	Temperature °C	K_D (M)
9.0	38	0.6×10^{-7}
8.5	25	$2.5 - 2.8 \times 10^{-7}$
8.5	0	2.2×10^{-7}
8.3	38	$1.2 - 4.7 \times 10^{-7}$
7.5	38	6.0×10^{-7}
8.0	25 (20-100mM tris)	$2.23 - 2.57 \times 10^{-7}$
8.0	25 (200mM tris)	$4.74 - 4.86 \times 10^{-7}$
6.0	25 (See Figure 17.1.	6.0×10^{-6}

5.7. The effect of pH on the assay of D-amino acid oxidase.

The effect of pH on the reconstitution of apo-D-amino acid oxidase activity as measured by the chromogenic reaction of horseradish peroxidase is shown in Figure 32. The binding of FAD to the apoenzyme is insensitive to the change in pH across the range studied. The turnover of D-proline by D-amino acid oxidase and production of hydrogen peroxide and the subsequent production of colour is maximal at pH 8.9. The decrease in the signal as the pH of the reaction is raised above 8.9 is not due to a loss of stability of the final product (See Section 5.8), but is due to a reduction in enzyme activity, either of D-amino acid oxidase (Dixon and Kleppe, 1964c), or horseradish peroxidase or both. The reduction in the maximum absorbance as the pH of the reaction is increased above pH 8.9 shows that the chromogenic substrates are more labile under these assay conditions.

Figure 32. The reconstitution of holoenzyme activity with 5.0nM FAD as a function of pH.



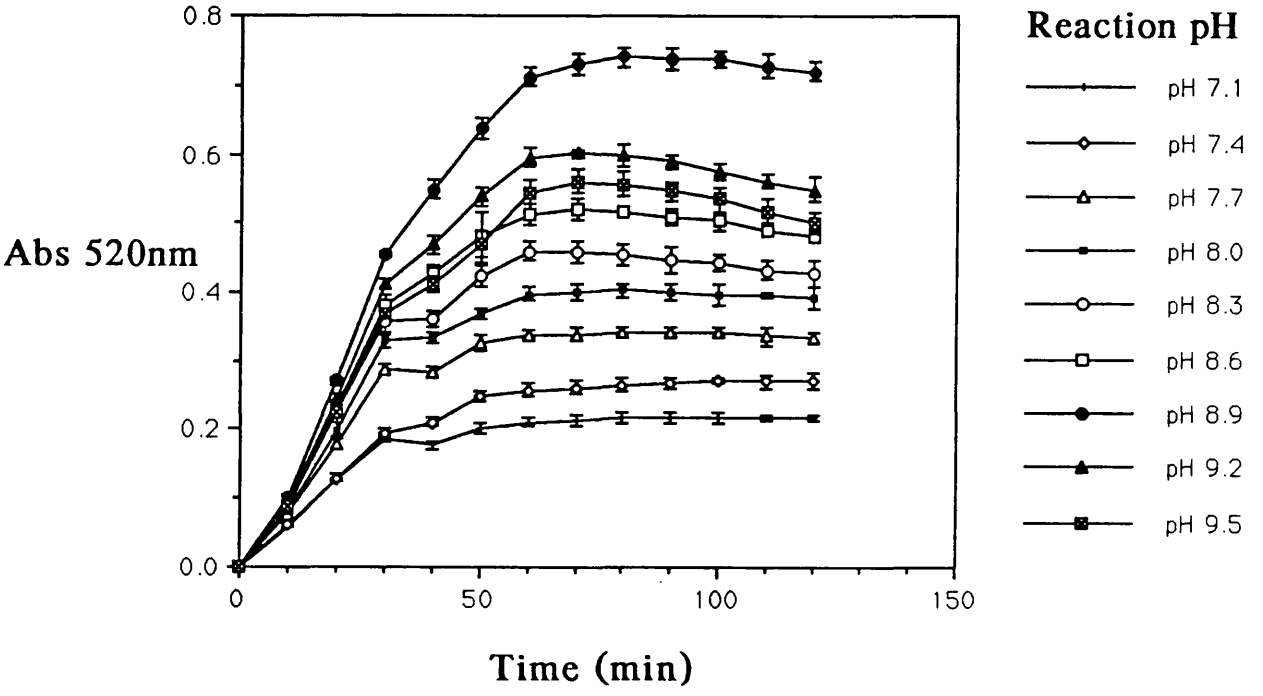
The change in absorbance as a function of time and pH due to the reconstitution of apo-D-amino acid oxidase (0.14 Units mL^{-1}) was measured in the presence of 5.0nM FAD in a microtitre plate (total volume 0.1mL). The assays were performed over a pH range of 7.1-9.8 in 20mM bis-tris-propane, containing 0.1M tris-HCl at 25°C.

5.8. The effect of pH on the stability of the chromogenic reaction product of horseradish peroxidase.

The experiments illustrated in Figure 33 were designed to give information on the stability of the coloured product as a function of time and pH. The principle employed was to add an irreversible inhibitor (DL-propargylglycine at a desired time and monitor the decay of the signal. Initially it was thought that the addition of a small quantity of catalase would serve to halt the reaction because of its high turnover of hydrogen peroxide (approximately 1,000,000 mol substrate mol enzyme^{-1} min^{-1}; Keesey, 1987.). However this enzyme could not utilize all of the hydrogen peroxide in the assay and further colour developed in the reaction mixture.

The addition of the irreversible inhibitor halted the reaction of D-amino acid oxidase within 20 minutes. Any change thereafter in the colour of the reaction solution could be attributed to the instability of the dye in the buffered reaction mixture. The rate of degeneration of the colour was monitored for a further 60 minutes. The rate of colour degeneration in each instance is shown in Table 19.

Figure 33. Stability of the chromogenic reaction of horseradish peroxidase across a pH range viable for the amplification assay (n=5).



The extent of reconstitution of D-amino acid oxidase activity (0.14 Units mL^{-1}) by 10nM FAD was followed in a series of assays performed in 20mM bis-tris-propane, pH 7.1-9.5, containing 0.1M tris-HCl, in a microtitre plate. DL-propargylglycine (15mM) was added to the reaction mixtures after 30 minutes. The decay of the chromogen was monitored at 520nm.

The coloured reaction product is very stable at neutral pH. Increasing the pH above 8.0 causes the coloured product to decay. The rate of degradation is, however, very small when compared to the decrease in intensity that has been observed in some assays. The major contribution to the decay is therefore a bleaching effect due to oxidation of the coloured product by horseradish peroxidase when the 4-aminoantipyrine becomes limiting in solution (See Section 7.3.). The separate components of the chromogenic reaction are stable in solutions buffered between pH 7.1-8.9. Under these conditions the assay mixture can be stored and reused without a loss of signal.

Table 19. Rate of colour degeneration in buffers of varying pH.

Buffer pH	Rate of colour degeneration Abs min^{-1}
7.1	n.d.
7.4	n.d.
7.7	2.3×10^{-4}
8.0	2.3×10^{-4}
8.3	1.1×10^{-3}
8.6	1.0×10^{-3}
8.9	9.6×10^{-4}
9.2	1.4×10^{-3}
9.5	1.7×10^{-3}

The degeneration of the chromogen was monitored over a 60 minute period after the irreversible inhibition of D-amino acid oxidase activity had been achieved by the addition of DL-propargylglycine.

5.9. The amplification assay and pH variation.

The decay of the final colour of the amplification assay with time has been found to be insignificant over the period of the assay, providing that none of the reagents are at limiting concentrations. Therefore, the effect of pH on the complete amplification system was assessed. Figures 34a-f show the amplification assay of 0 amol and 1.0 amol of alkaline phosphatase in a microtitre plate format employing 0.14 Units mL^{-1} apo-D-amino acid oxidase and 0.1M tris-HCl at various pH values (8.0-9.8) at 25°C.

The ratios of the signal produced by 1.0 amol of alkaline phosphatase to the background signal are shown in Table 20.

Figure 34a. The assay of 1.0 amol of alkaline phosphatase at pH 8.0.

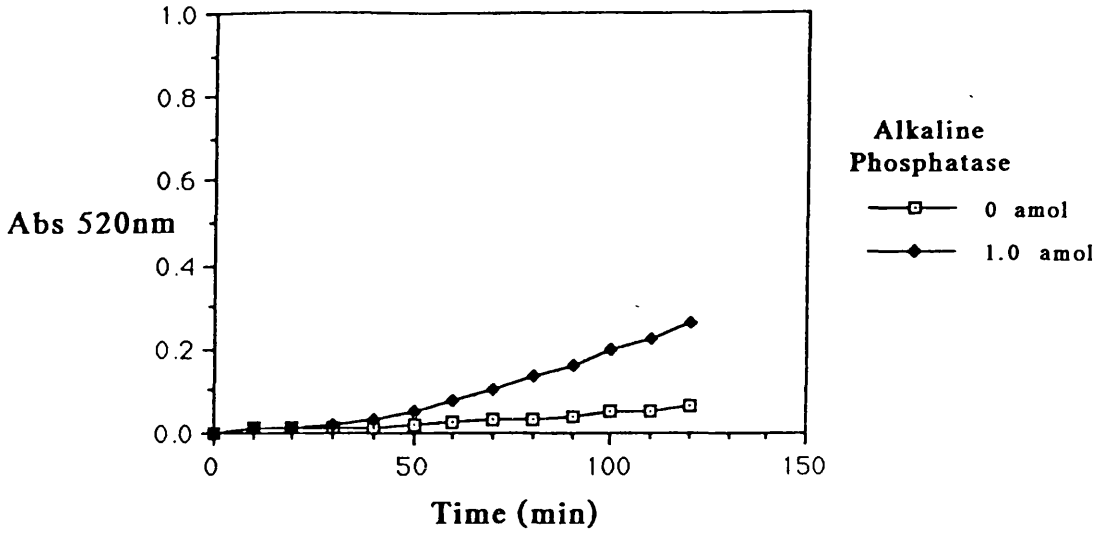


Figure 34b. The assay of 1.0 amol of alkaline phosphatase at pH 8.6.

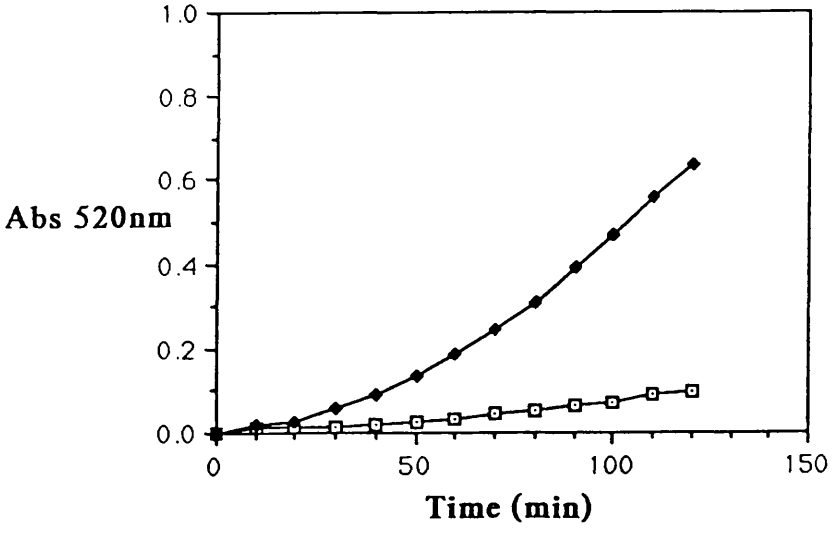


Figure 34c. The assay of 1.0 amol of alkaline phosphatase at pH 8.9.

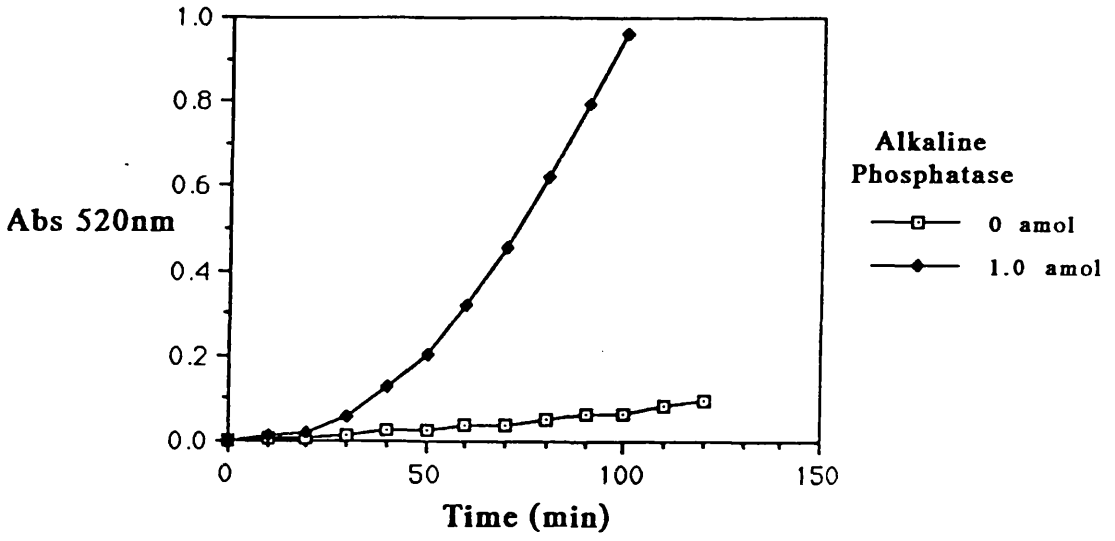


Figure 34d. The assay of 1.0 amol of alkaline phosphatase at pH 9.2.

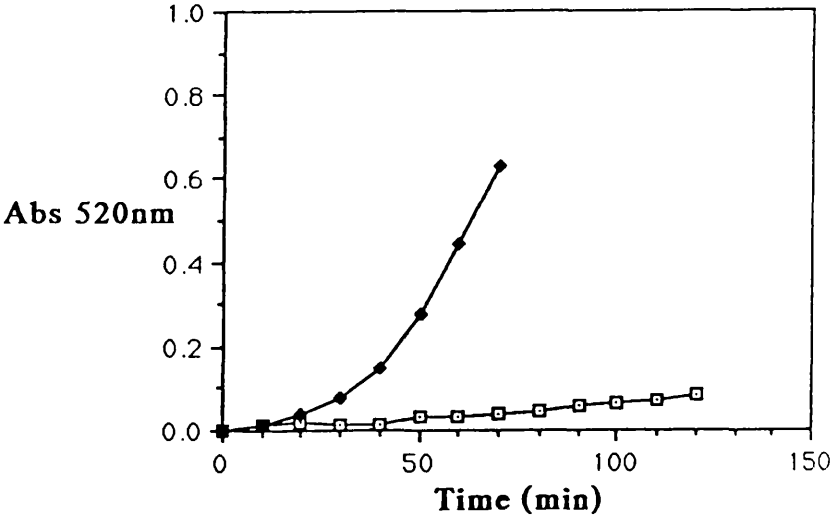


Figure 34e. The assay of 1.0 amol of alkaline phosphatase at pH 9.5.

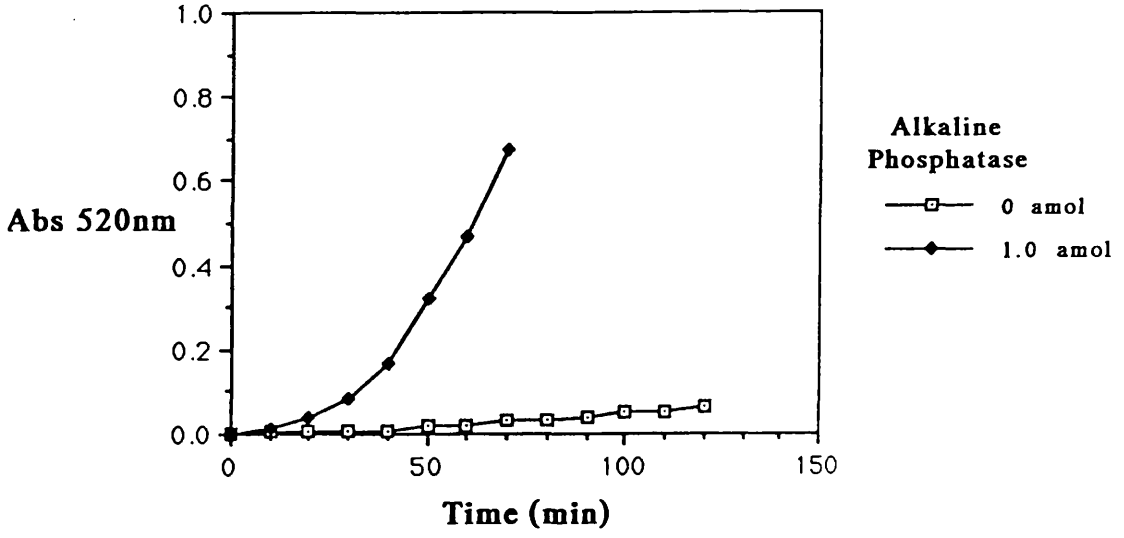
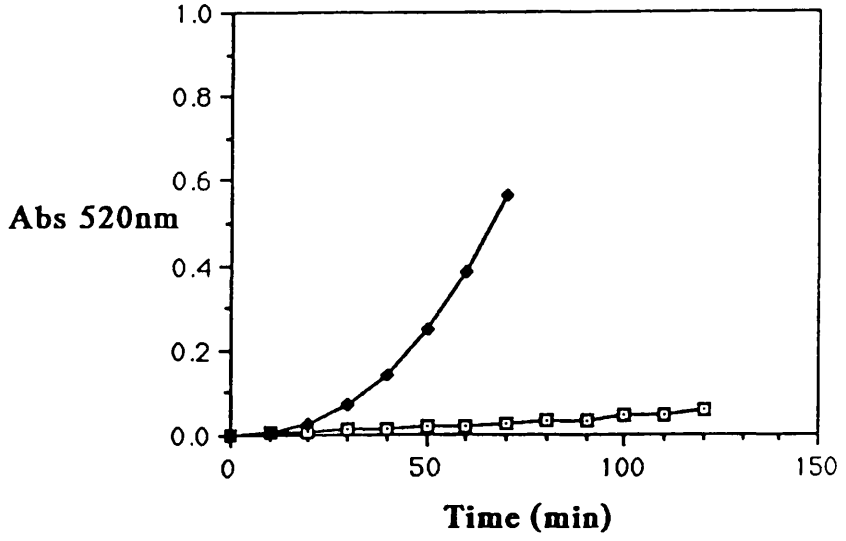


Figure 34f. The assay of 1.0 amol of akaline phosphatase at pH 9.8.



The ratios in Table 20 show that the amplification assay of alkaline phosphatase activity is fastest at pH 9.5. The decrease of the ratio at the later time points in the higher pH range is due to the utilization of the substrate of the chromogenic reaction and the concentration of the components ultimately limiting the the rate of the reaction. The instability of the final product of the reaction under the conditions of elevated pH also leads to a decrease in the overall signal, though this is small (See Section 5.8.). For an extended assay incubation period, a reaction at pH 8.9 is most favourable. This is because of the stability of the reagents during the assay and the magnitude of the signal produced on hydrolysis of the FADP by alkaline phosphatase. However, under different circumstances a test result may be required in a short time. In this instance the assay may be carried out in a buffer medium at a higher pH. Alternatively, a more sophisticated measurement determined on a rate basis produces signal:noise ratios that are even larger (See Section 7.6.).

Table 20. Signal: noise ratios for the detection of 1.0 amol of alkaline phosphatase and their dependence on pH. (Derived from Figures 34a-f.).

Time (min)	pH					
	8.0	8.6	8.9	9.2	9.5	9.8
20	/	1.5	1.5	1.5	5.0	3.5
30	/	3.0	3.7	4.1	12.8	5.6
40	1.0	3.2	6.6	8.8	19.5	9.8
50	1.5	4.4	6.8	8.5	16.7	13.9
60	2.0	4.9	10.0	14.2	26.6	21.2
70	2.3	4.4	10.7	14.3	18.6	19.9
80	2.8	5.3	10.8	/	/	/
90	3.2	5.2	13.3	/	/	/
100	2.7	5.7	/	/	/	/
110	2.6	5.3	/	/	/	/
120	3.2	5.4	/	/	/	/

Signal: noise ratios were determined at each time point by the equation given below:

$$Ratio_t = \frac{Signal_t - Background_t}{Background_t}$$

5.10. The determination of K_s for alkaline phosphatase and FADP at 25°C and 37°C.

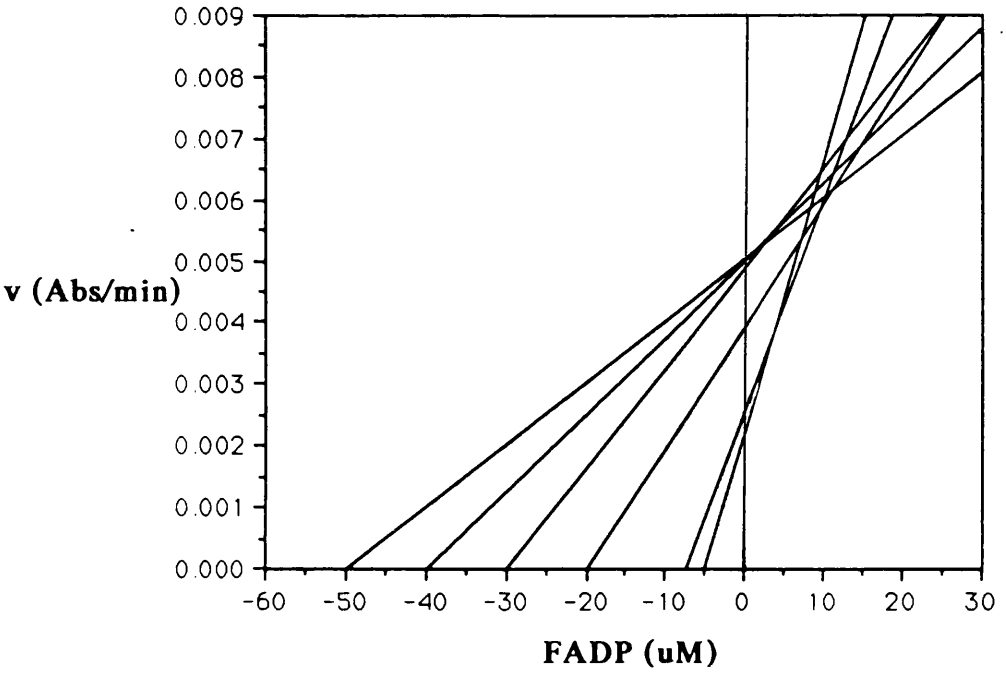
Classically the determination of the K_s of an enzyme reaction has involved the measurement of a series of initial velocities at different substrate concentrations. A rearrangement of the Michaelis-Menton equation then permits the results to be plotted as a straight line. The double reciprocal plot of Lineweaver-Burk has been most commonly used. This plot however gives a very misleading impression of the experimental error. In principle, this can be overcome by the use of suitable weights on each of the data points, but this often leads to a line of "best fit" which actually appears to fit very poorly. Statistically, the least-squares regression analysis is the most convenient approach. However, this assumes a normal distribution of the error of the data; that the error is independent of any other variable in the experiment; that the correct weights of each of the experimental points are known and that systematic error can be ignored ie. the distribution of each error has a mean value of zero.

The direct linear plot attempts to introduce a determination that is independent of the distribution of the data. The median value for the data is chosen directly from the plot because it is insensitive to extreme values. When these extreme values occur they appear as almost parallel lines in the direct linear

plot.

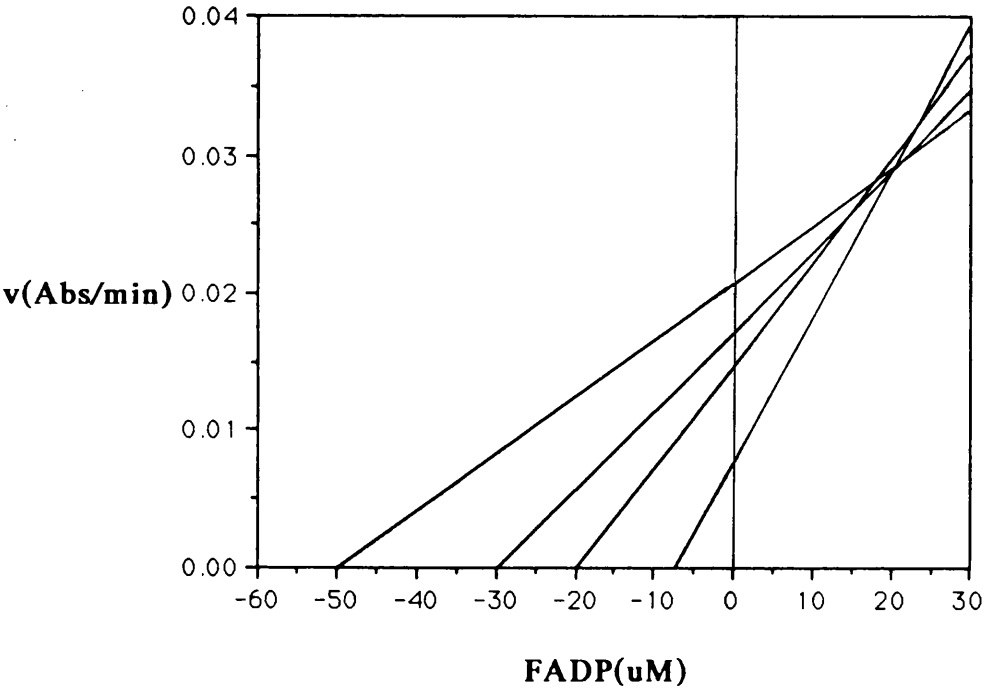
The K_m of alkaline phosphatase under the conditions of the assay is 9.6uM at 25°C and 20uM at 37°C. These values are determined by the median value of the direct linear plot in Figure 35 and 36. This plot treats V and K_m as variables and s and v as constants. Since s and v have been determined experimentally, any honest analysis of the results will leave them unchanged. Only the point of intersection will represent the value of K_m that fits all of the data (Cornish-Bowden, 1979).

Figure 35. The K_m of alkaline phosphatase under assay conditions at 25°C.



Alkaline phosphatase activity ($3x10^{-14}$M) was determined in a microtitre plate amplification assay employing 0.14 Units mL^{-1} apo-D-amino acid oxidase, 10ug mL^{-1} HRP and 5.0-50uM FADP in 0.1M tris-HCl, pH 8.5, at 25°C. The rate of the reaction was measured at 60 minutes. Each line represents one observation of the rate of the reaction and is drawn with intercepts -s on the abscissa and v on the ordinate. The value of K_m was obtained from the median intersect and is 9.6uM.

Figure 36. The K_m of alkaline phosphatase under assay conditions at 37°C.

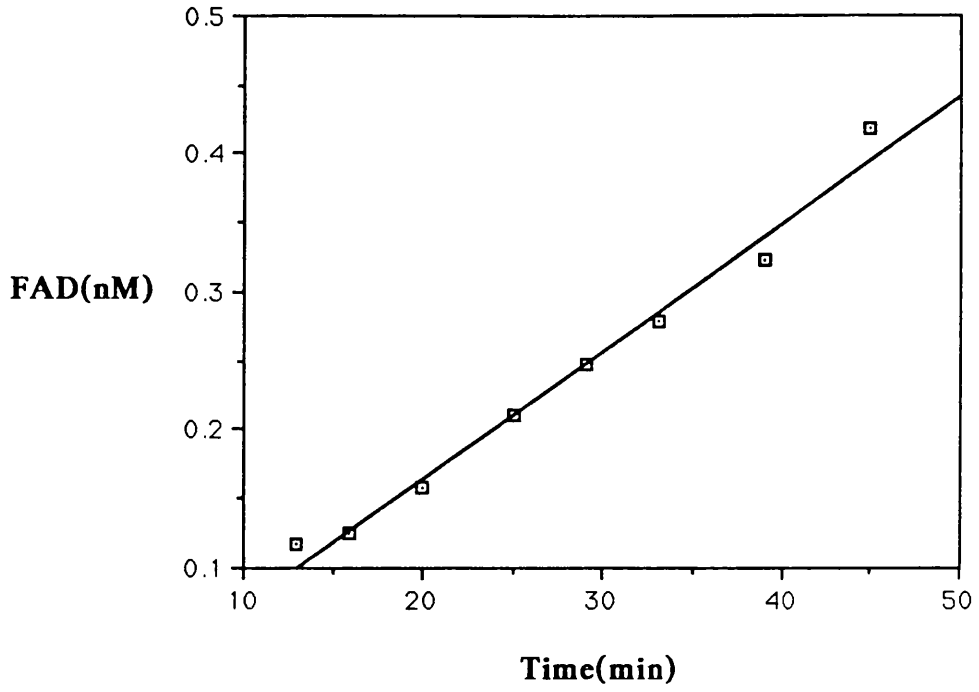


Alkaline phosphatase activity ($3x10^{-14}$M) was determined in a microtitre plate amplification assay employing 0.14 Units mL^{-1} apo-D-amino acid oxidase, 10ug mL^{-1} HRP and 7.5-50uM FADP in 0.1M tris-HCl, pH 8.5 at 37°C. The reaction rate was measured at 20 minutes. Each line represents one observation of the rate of the reaction and is drawn with intercepts of -s on the abscissa and v on the ordinate. The value of K_m was obtained from the median value of the intersect and is 20.0uM.

5.11. The determination of K_{cat} of alkaline phosphatase for FADP.

The amount of FAD produced from FADP in an incubation with alkaline phosphatase in 0.1M tris-HCl, pH 8.9 was determined by assay and use of the standard curve for FAD (See Figure 31). The increase in the concentration of FAD during the incubation is shown in Figure 37. The gradient of this line was used to calculate the value of K_{cat}.

Figure 37. The production of FAD from FADP by alkaline phosphatase.



R^2 = 0.985.

Alkaline phosphatase (10^{-14}M) was incubated with 200uM FADP in 0.1M tris-HCl, pH 8.9, at 25°C. Aliquots (10uL) were removed at intervals and the concentration of FAD determined presence of 10mM sodium phosphate by measuring the extent of reconstitution of apo-D-amino acid oxidase (0.14 Units mL^{-1}).

Gradient (Figure 37) = 9.24×10^{-3} nM min^{-1}

Since a 10uL aliquot was assayed in a total volume of 100uL in a microtitre plate assay the actual rate of production of FAD was 9.24×10^{-2} nM min^{-1}. K_{cat} can be calculated from this gradient:

Equation 2.

$$v = \frac{E_0 K_{cat} S_0}{K_m + S_0}$$

Rearranging,

Equation 3.

$$K_{cat} = \frac{v(K_m + S_0)}{E_0 S_0}$$

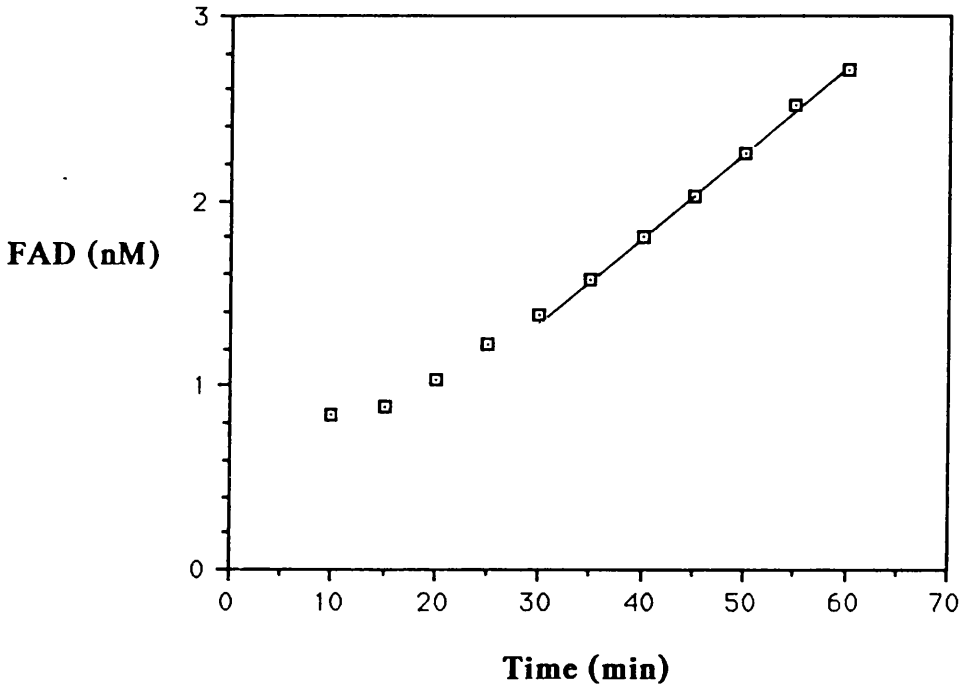
$$K_{cat} = \frac{9.24 \times 10^{-11}(9.6 \times 10^{-6} + 2.0 \times 10^{-4})}{1.0 \times 10^{-14} \times 2.0 \times 10^{-4}} \ min^{-1}$$

$$= 9683.5 \ min^{-1}$$

$$= 161 \ s^{-1}$$

K_{cat} can also be measured directly under assay conditions by comparison of the progress curve of an amplification reaction of alkaline phosphatase with that of a reaction incorporating a standard concentration of FAD. The production of FAD from the prosthetogen by 1.0 amol of alkaline phosphatase is shown in Figure 38 which is derived by the division of the progress of the amplification reaction by the progress curve of the standard FAD reaction. The gradient of this line is used to calculate K_{cat}.

Figure 38. The production of FAD by 1.0 amol of alkaline phosphatase in a single pot assay.



The activity of 1.0 amol of alkaline phosphatase was measured in 0.1M tris-HCl, pH 8.9 by the reconstitution of 0.14 units mL^{-1} apo-D-amino acid oxidase. The concentration of FAD produced at each time point was calculated from the progress curve of a reaction of 1.0nM FAD under identical conditions. The rate of production of FAD is equal to the gradient of the line and is $4.8 \times 10^{-11} M\ min^{-1}$.

From Equation 3.

$$K_{cat} = \frac{v(K_m + S_0)}{E_0 S_0}$$

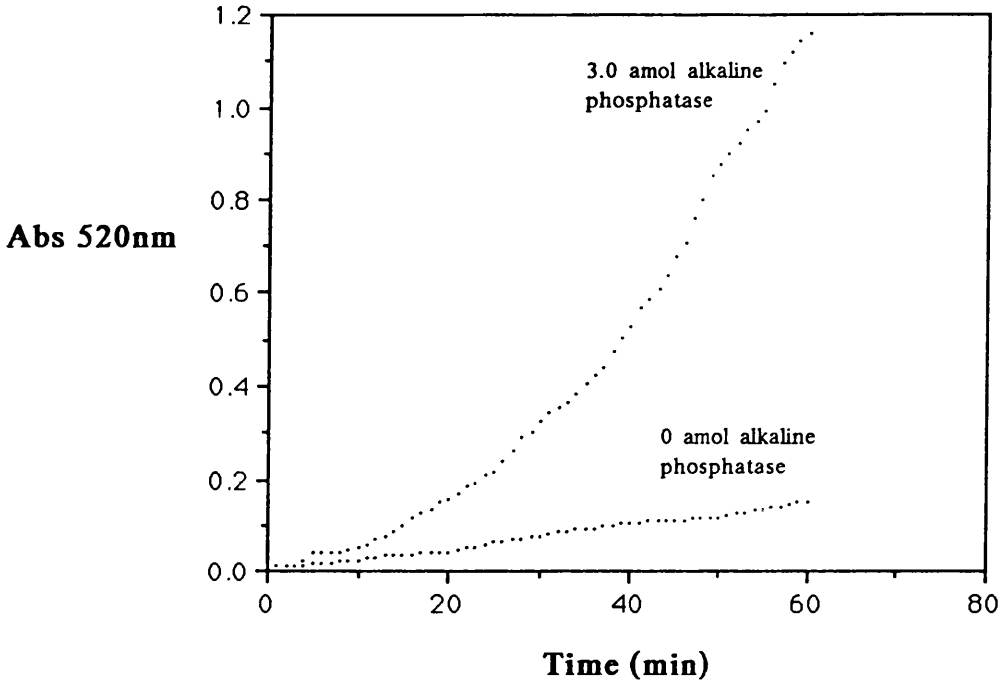
$$K_{cat} = \frac{4.8 \times 10^{-11} (9.6 \times 10^{-6} + 2.0 \times 10^{-5})}{1.0 \times 10^{-14} \times 2.0 \times 10^{-5}} \text{ min}^{-1}$$

$$= 7104 \text{ min}^{-1}$$

$$= 118 \text{ s}^{-1}$$

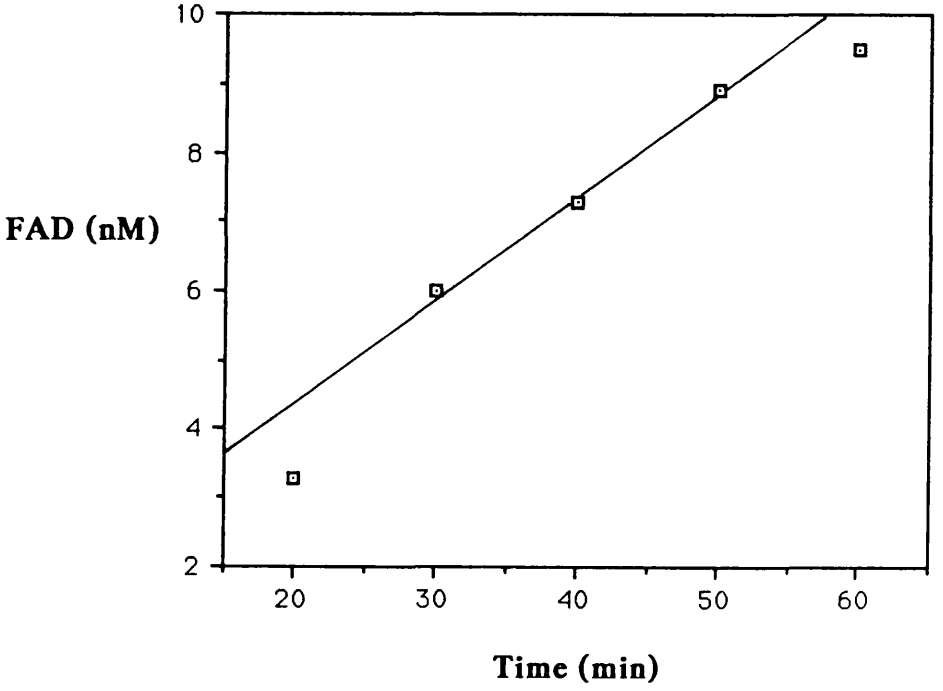
Alternatively, K_{cat} can be calculated directly from the progress curve of an amplification reaction of alkaline phosphatase. Figure 39 shows the progress curve of reactions containing 0 and 3.0 amol of alkaline phosphatase in 0.1M tris-HCl, pH 8.9. The gradient of the progress curve was calculated at specified time intervals over the period of the assay in order to determine the concentration of active D-amino acid oxidase. The concentration of FAD in solution was calculated assuming that the dissociation constant of the enzyme D-amino acid oxidase for FAD was 2.4×10^{-7}M under assay conditions (See Section 5.6.). The concentration of flavin produced as a function of time, by 3.0 amols of alkaline phosphatase, is shown in Figure 40.

Figure 39. The amplification assay of 0 and 3.0 amol of alkaline phosphatase.



The activity of alkaline phosphatase was measured with 0.14 units mL^{-1} apo-D-amino acid oxidase, 35mM D-proline, 10ug mL^{-1} HRP, 0.2mM 4-aminoantipyrine, 2.0mM DCHB, and 20uM FADP, in 0.1M tris-HCl, pH 8.9, containing 0.1mM $MgSO_4$ and 10uM $ZnSO_4$.

Figure 40. The formation of FAD and active D-amino acid oxidase in the assay of 3.0 amol of alkaline phosphatase.



The concentration of FAD produced by 3.0 amol of alkaline phosphatase from FADP was calculated from the activity of D-amino acid oxidase at each time point from the gradient of the progress curve (See Figure 39.). The concentration of FAD in solution and not bound by the apoenzyme was calculated assuming a value of K_D equal to 2.4×10^{-7} M.

As previously described the gradient of the line in Figure 40. is used to calculate the value of K_{cat} under the conditions of the assay.

From Equation 3.

$$K_{cat} = \frac{0.155 \times 10^{-9} (9.6 \times 10^{-6} + 2.0 \times 10^{-5})}{3 \times 10^{-14} \times 2 \times 10^{-5}} \text{ min}^{-1}$$

$$= 7646 \text{ min}^{-1}.$$

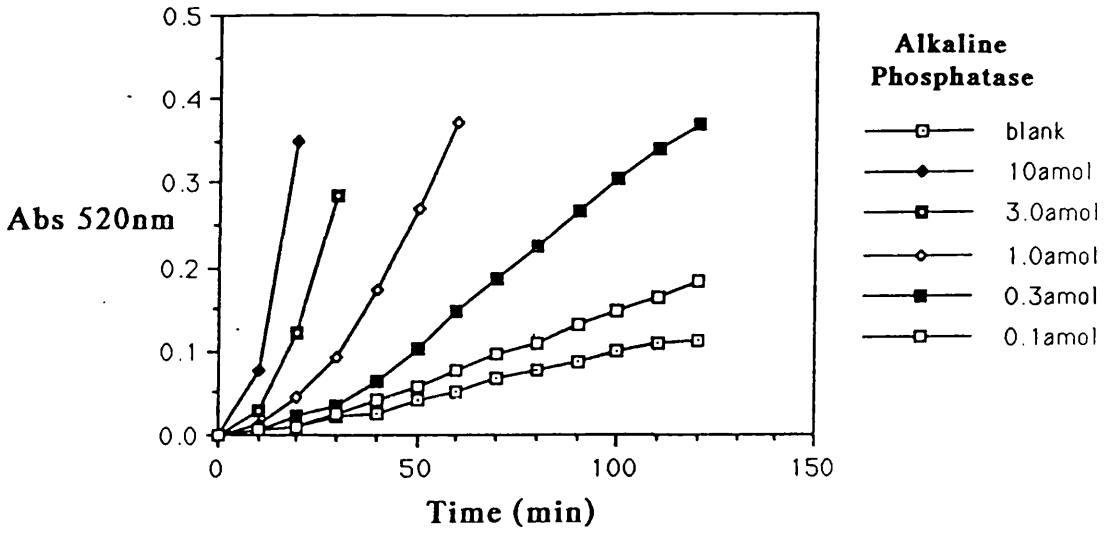
$$= 127 \text{ s}^{-1}.$$

The value of K_{cat} calculated in the single pot assays are lower than those calculated in the two pot system. This phenomenon could be due to the interaction of the apoenzyme and the alkaline phosphatase resulting in a decreased activity of the analyte.

5.12. The effect of the concentration of tris-HCl and FADP on the performance of the amplification assay.

The amplification assay of alkaline phosphatase has been shown to be sensitive to the concentration and pH of the buffer system as well as the concentration of the substrate. The activity of a series of dilutions of alkaline phosphatase was measured under conditions of varying buffer concentration and varying substrate concentration to illustrate the interaction of these two components in the assay system and their effect on the sensitivity of the assay. Figures 41-44 show the progress curves obtained from the series of experiments described. The signal: noise ratios have been determined in each instance (Tables 21-24) to illustrate the variation in sensitivity under each of the conditions of the assay. The assay is most sensitive under the conditions employing 0.1M tris-HCl and 20uM FADP.

Figure 41. The amplification assay of alkaline phosphatase employing 0.2M tris-HCl, pH 8.9 and 20uM FADP.



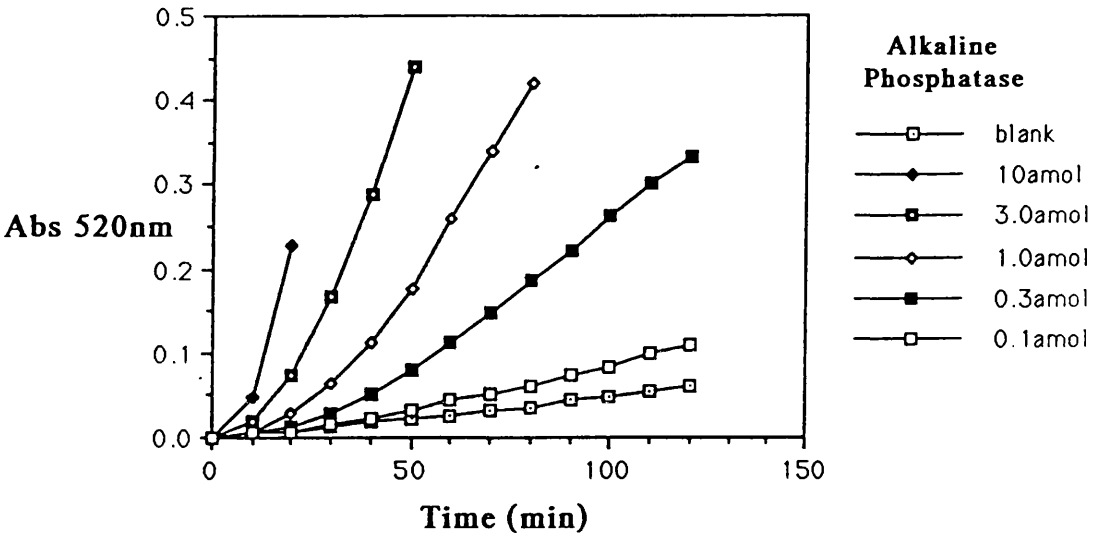
The activity of alkaline phosphatase was determined in an microtitre plate amplification assay using 0.2M tris-HCl, pH 8.9, at 25°C, 0.41 Units mL^{-1} apo-D-amino acid oxidase, 10ug mL^{-1} HRP and 20uM FADP.

Table 21. Signal: noise ratios of the assay employing 0.2M tris-HCl, pH 8.9 and 20uM FADP.

Time(min)	amol alkaline phosphatase.				
	10.0	3.0	1.0	0.3	0.1
10	14.6	5.0	1.4	n.d.	n.d.
20	30.6	10.6	3.2	n.d.	n.d.
30	/	17.4	3.4	n.d.	n.d.
40	/	/	5.9	1.5	n.d.
50	/	/	5.3	1.5	n.d.
60	/	/	6.4	2.0	n.d.

$$Ratio_t = \frac{Signal_t - Background_t}{Background_t}$$

Figure 42. The amplification assay of alkaline phosphatase employing 0.2M tris-HCl, pH 8.9 and 10uM FADP.



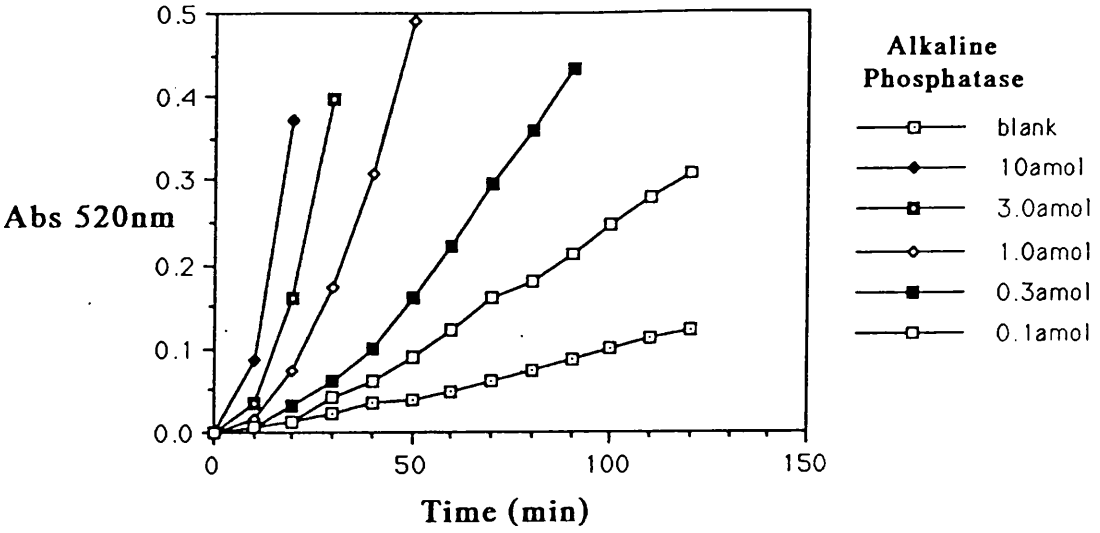
Alkaline phosphatase activity was determined in a microtitre plate amplification assay using 0.2M tris-HCl, pH 8.9, at 25°C, 0.41 Units mL^{-1} apo-D-amino acid oxidase, 10ug mL^{-1} HRP and 10uM FADP.

Table 22. Signal: noise ratios of the assay employing 0.2M tris-HCl, pH 8.9 and 10uM FADP.

Time(min)	amol alkaline phosphatase				
	10.0	3.0	1.0	0.3	0.1
10	7.0	2.2	n.d.	n.d.	n.d.
20	31.6	9.4	3.1	n.d.	n.d.
30	/	12.8	4.3	1.4	n.d.
40	/	15.1	5.2	1.8	n.d.
50	/	20.2	7.3	2.8	n.d.
60	/	/	8.6	3.1	n.d.

$$Ratio_t = \frac{Signal_t - Background_t}{Background_t}$$

Figure 43. The amplification assay of alkaline phosphatase employing 0.1M tris-HCl, pH 8.9 and 20uM FADP.



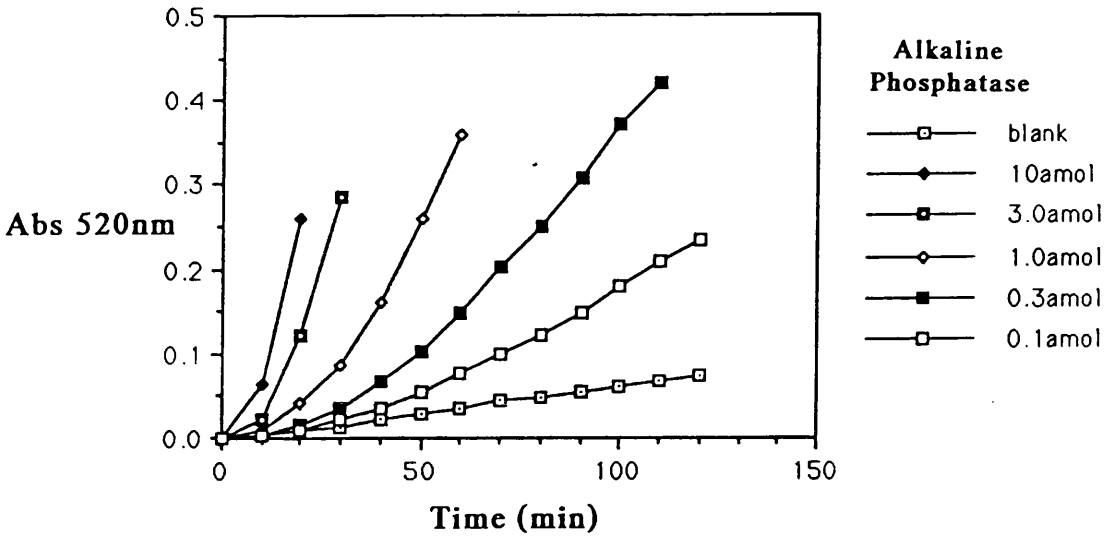
Alkaline phosphatase activity was determined in a microtitre plate amplification assay using 0.1M tris-HCl, pH 8.9, at 25°C, 0.41 Units mL^{-1} apo-D-amino acid oxidase, 10ug mL^{-1} HRP and 20uM FADP.

Table 23. Signal: noise ratios of the assay employing 0.1M tris-HCl, pH 8.9 and 20uM FADP.

Time(min)	amol alkaline phosphatase				
	10.0	3.0	1.0	0.3	0.1
10	11.1	4.1	1.3	n.d.	n.d.
20	25.6	10.4	5.2	1.3	n.d.
30	/	16.2	6.5	1.7	n.d.
40	/	/	8.8	1.8	n.d.
50	/	/	11.5	3.1	1.3
60	/	/	/	3.5	1.5

$$Ratio_t = \frac{Signal_t - Background_t}{Background_t}$$

Figure 44. The amplification assay of alkaline phosphatase employing 0.1M tris-HCl, pH 8.9 and 10uM FADP.



Alkaline phosphatase activity was determined in a microtitre plate amplification assay using 0.1M tris-HCl, pH 8.9, at 25°C, 0.41 Units mL^{-1} apo-D-amino acid oxidase, 10ug mL^{-1} HRP and 10uM FADP.

Table 24. Signal: noise ratios of the assay employing 0.1M tris-HCl, pH 8.9 and 10uM FADP.

Time(min)	amol alkaline phosphatase				
	10.0	3.0	1.0	0.3	0.1
10	15.0	5.0	1.8	n.d.	n.d.
20	22.5	10.2	2.7	n.d.	n.d.
30	/	19.3	5.1	1.6	n.d.
40	/	/	5.7	1.8	n.d.
50	/	/	7.9	2.6	n.d.
60	/	/	9.2	3.2	1.2

$$Ratio_t = \frac{Signal_t - Background_t}{Background_t}$$

A comparison of the signal:noise ratios of the series of assays employing 20uM FADP demonstrates the influence of the concentration of tris-HCl on the assay system. When the concentration of tris-HCl is reduced from 0.2M to 0.1M the signal ratios are doubled. This is illustrated in Table 25. This is marginally better than was assumed from the determination of K_D in Section 5.6.

Table 25. Signal ratios of amplification assays employing 20uM FADP and 0.1M/0.2M tris-HCl, pH 8.9.

Time(min)	amol alkaline phosphatase Signal ratio	
	1.0	0.3
20	1.69	/
30	2.08	1.71
40	1.86	1.51
50	1.99	1.95
60	/	1.77
70	/	1.97
80	/	1.92
90	/	1.93

$$Ratio_t = \frac{Ratio(0.1M\ tris)_t}{Ratio(0.2M\ tris)_t}$$

CHAPTER SIX.

FORMATTING OF THE AMPLIFICATION ASSAY OF ALKALINE PHOSPHATASE FOR COMMERCIAL USE.

6.1. Formatting of the amplification assay of alkaline phosphatase. The one-pot versus the two-pot assay.

For use in a microtitre plate assay, the reagents can be formatted in a number of ways. The simplest procedure for practical usage is to add the total amount of reagents necessary for the measurement of alkaline phosphatase activity as a single premixed cocktail. To this end, the system has been optimized to give the greatest sensitivity in the shortest possible time. However, when the two stages of the assay, hydrolysis of the primary substrate by alkaline phosphatase, and the detection cascade, are separated, the conditions of the assay can be optimized for each stage to improve the assay performance.

For the single-pot assay technology a cocktail of reagents was prepared to give the final concentrations stated, in an assay volume of 100uL:

0.1M	Tris-HCl, pH 8.9.
0.1mM	$MgSO_4$.
1.0uM	$ZnSO_4$.
35mM	D-proline.
0.4mM	4-aminoantipyrine.
2.0mM	DCHB.
0.01mg mL^{-1}	Horseradish peroxidase.
0.14 Units mL^{-1}	Apo-D-amino acid oxidase.
20uM	FADP.

For the two-pot assay a cocktail of reagents was prepared to give the final concentrations stated in the primary incubation, in an assay volume of 50uL:

0.2M	Tris-HCl, pH 8.9.
0.1mM	$MgSO_4$.
1.0uM	$ZnSO_4$.
20uM	FADP

A second cocktail was prepared containing the reagents of the detection cascade. The initial incubation of alkaline phosphatase and FADP was allowed to proceed for 30 minutes. The second cocktail of reagents was then added, with thorough mixing, to a final volume of 100uL, to give the secondary incubation with the final concentrations of components as stated:

35mM	D-proline.
0.2mM	4-aminoantipyrine.
2.0mM	DCHB.
0.01mg mL^{-1}	Horseradish peroxidase.
0.14 Units mL^{-1}	Apo-D-amino acid oxidase.
10mM	Sodium phosphate.

The progress of the reactions for the one-pot and the two-pot assay were monitored on a Dynatech MR 7000 microtitre plate reader at 25°C fitted with a thermostatically controlled plate holder. By formatting the two-pot assay in this manner the activity of alkaline phosphatase is increased in the initial incubation due to the increased tris-HCl concentration. The incorporation of 10mM sodium phosphate in the second incubation inhibits the further hydrolysis of FADP by alkaline phosphatase. It also alters the kinetic characteristics of the assay to give a linear progress curve after a short initial period corresponding to the reconstitution of D-amino acid oxidase activity. The background signal of the assay is also reduced.

6.2. Preparation of a single premix; a stability study.

For use in a microtitre plate format, the assay system can be supplied as a kit comprising of a single bottle of freeze-dried components. This can be reconstituted

with a pre-measured quantity of sterile buffer. The possibility of errors when using the kit and the risk of contamination of any of the components is therefore reduced.

To assemble a cocktail of the components for freeze-drying, each must first be prepared in sterile distilled water. A 20% excess of each of the assay components is added to the vial to compensate for any loses of material that may occur during the freeze-drying process. The cocktails were prepared in amber glass vials, to minimize the exposure of the prosthetogen to light, and frozen at -70°C. The cocktails were lyophilized at 0.05 bar for 36 hours in the dark.

The recipe below shows the concentration of the reagents for a reaction mixture to perform 100 assays, each of 100uL, at 25°C.

Apo-D-amino acid oxidase.	1.68 Units (See Section 2.5.).
D-proline.	1.2mL of 0.35M solution (40.2mg mL^{-1}.).
4-aminoantipyrine.	0.3mL of 80mM solution (1.6mg mL^{-1}.).
DCHB.	0.3mL of 8mM solution (21.2mg mL^{-1}.).
Horseradish peroxidase.	120ug (E_{403} = 91mM^{-1} cm^{-1}; MW = 44,000.).
FADP.	250ug (E_{450} = 11.3mM^{-1} cm^{-1}; MW = 1000.).

This premix may be reconstituted with 10mL of 0.1M tris-HCl, pH 8.0, containing 0.1mM $MgSO_4$ and 1.0uM $ZnSO_4$.

Prior to reconstitution with sterile buffer the freeze-dried mixtures were stored under different conditions to assess the stability of the preparations. One pot was stored at -70°C for 3 days and the other was left at room temperature for the same period of time. Each preparation was reconstituted with the specified quantity of sterile buffer. The performance of the assay mixtures was assessed with dilutions of alkaline phosphatase by monitoring the progress of the reactions at 520nm on a Titretek II plate reader at room temperature.

6.3. Variation of the concentration of apo-D-amino acid oxidase and the sensitivity of the amplification assay for the single premix technology.

The nature of the amplification system results in an ever increasing reaction rate as more active D-amino acid oxidase is produced with time in the incubation of the assay components with the analyte, alkaline phosphatase. The performance of the assay was studied employing partially purified apo-D-amino acid oxidase (prepared by diafiltration and chromatography on Blue Sepharose only) and highly purified apo-D-amino acid

oxidase (prepared by further purification on an anion exchange Mono Q column). The concentration of the components of the assays were as stated below:

0.1M	Tris-HCl, pH 8.9.
35.0mM	D-proline.
0.4mM	4-aminoantipyrine.
2.0mM	DCHB.
0.01mg mL^{-1}	Horseradish peroxidase.
20uM	FADP.
0.1mM	$MgSO_4$.
1.0uM	$ZnSO_4$.

The partially purified apo-D-amino acid oxidase was employed at a final concentration of 0.14 Units mL^{-1}. The highly purified apo-D-amino acid oxidase was employed at a final concentration of 0.41 Units mL^{-1}. The progress of each reaction was monitored at 520nm using a Dynatech MR 7000 microtitre plate reader at 25°C.

6.4. Reproducibility of the amplification assay.

An assay that is used in clinical diagnostic procedures must be reproducible to a high degree in order that the results can be validated and that comparisons can be made between samples over a period of time with confidence in the analysis. A measure of reproducibility must therefore be carried out on both

an intra-assay and an inter-assay basis.

The activity of a series of solutions of alkaline phosphatase was measured in a microtitre plate assay Each assay contained the stated concentrations of reagents, in a final volume of 100uL:

0.1M	Tris-HCl, pH 8.9.
35.0mM	D-proline.
0.4mM	4-aminoantipyrine.
2.0mM	DCHB.
0.14 Units mL^{-1}	Apo-D-amino acid oxidase.
0.01mg mL^{-1}	Horseradish peroxidase.
20uM	FADP.
0.1mM	$MgSO_4$.
1.0uM	$ZnSO_4$

For each solution of alkaline phosphatase (10^{-12} - 3 x 10^{-15}M, final concentration), 5 replicates were assayed. The progress of each reaction was followed for 120 minutes on a Dynatech MR 7000 plate reader at 520nm at 25°C.

The experiment was repeated to determine the inter-assay reproducibility employing individually prepared dilution of alkaline phosphatase for each assay and different preparations of apo-D-amino acid oxidase, HRP and FADP in different combinations. These assays were performed over a period of 6 months.

6.5. A comparison of enzyme amplification by substrate cycling and the enzyme cascade.

The technology of the enzyme cascade system was compared to the performance of the system based on a substrate cycle described in Section 1.8. This system is commercially available under the trademark AMPAK and was purchased from Novo BioLabs.

The AMPAK system may be used in a variety of ways varying the temperature and time of each of the substrate utilization and amplification incubation stages in a microtitre plate assay.

The activity of a number of solutions of alkaline phosphatase was measured (10^{-16} - 10^{-12}M final concentration) employing the AMPAK assay kit. Firstly, a series of assays were performed using a primary incubation time with the substrate, NADP, of 40 minutes at 23°C and a secondary amplification time of 10 minutes at 23°C. This assay was used as a direct comparison of the assay performance against that of the enzyme cascade system performed at the same temperature. Secondly, a series of assays was performed employing a primary substrate incubation time of 20 minutes at 37°C and a secondary amplification time of 10 minutes at 23°C. These conditions are optimal for the AMPAK assay system. The progress of the reaction was monitored at 492nm on

a Titretek II microtitre plate reader. In each instance, a comparison was made against an enzyme cascade assay employing the following concentrations of reagents, in an assay volume of 100uL:

0.1M	Tris-HCl, pH 8.0.
35mM	D-proline.
0.2mM	4-aminoantipyrine.
2.0mM	DCHB.
0.14 Units mL^{-1}	Apo-D-amino acid oxidase.
0.01mg mL^{-1}	Horseradish peroxidase.
20uM	FADP.
0.1mM	$MgSO_4$.
1.0uM	$ZnSO_4$.

6.6. Measurement of TSH using the FADP cascade technology formatted onto the aca® discrete clinical analyzer.

The FADP-cascade has been adapted for use on the aca® discrete clinical analyzer. The following experiment was carried out at Du Pont with materials supplied by London Biotechnology Ltd. (Obzansky et al., 1991).

TSH standards (500uL) were incubated with 50uL of capture antibody-CrO_2 particles (125ug of CrO_2) and 500uL of dethiobiotin-conjugated second antibody (0.5ug) at 37°C for 30 minutes with periodic mixing. This resulted in the formation of a sandwich structure consisting of capture antibody immobilized to CrO_2 particles, the TSH analyte and second antibody covalently conjugated with dethiobiotin. The immobilized immune complex was washed 3 times with 1.0mL aliquots of 0.25M tris-HCl, pH 7.85 at 25°C, containing 50mM sodium borate.

The washed immune complex was incubated with 500uL of a 1.0mg L^{-1} streptavidin-alkaline phosphatase conjugate solution at 37°C for 15 minutes. The newly formed complex was separated magnetically from the bulk of the solution and excess conjugate was removed by washing 4 times with 1.0mL aliquots of the tris-HCl wash buffer. The particles were incubated with 1.0mL of release reagent (68mg biotin L^{-1} of alkaline

phosphatase free BSA - 50mg L^{-1} in 0.1M tris-HCl, pH 8.5 at 25°C) for 10 minutes.

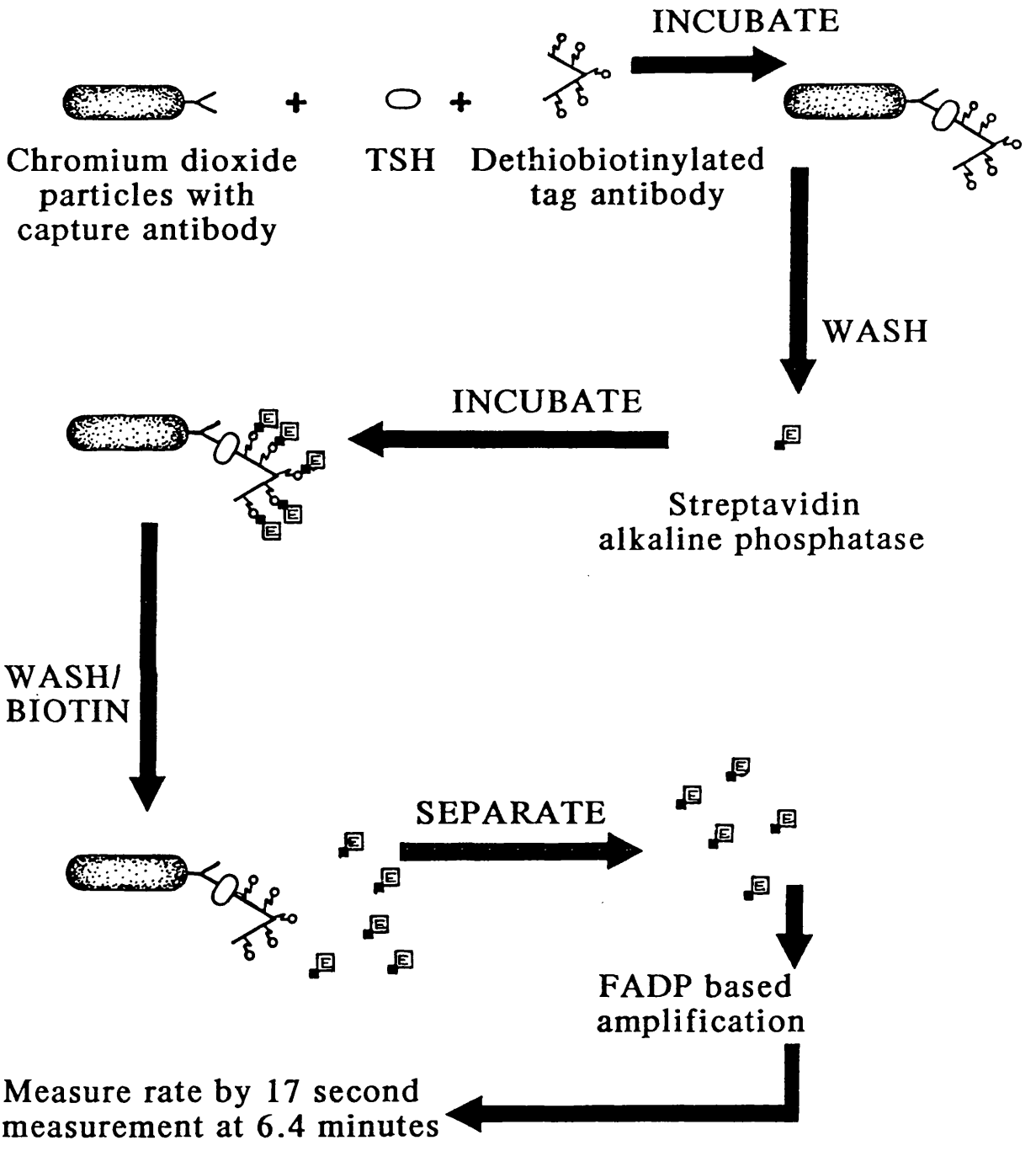
The released CrO_2 particles were separated magnetically from the bulk of the solution. An aliquot of the supernatent was used in the aca^R discrete clinical analyzer to measure the activity of the label alkaline phosphatase using the FADP-based enzyme amplification assay. The scheme of the RELIA technology is shown in Figure 45.

The enzyme cascade formatted for use on the aca^R analyzer employed the following concentration of reagents:

0.1M	Tris-HCl, pH 8.5 at 25°C.
0.1mM	$MgSO_4$.
1.0uM	$ZnSO_4$.
35mM	D-proline.
0.25mM	4-aminoantipyrine.
2.5mM	DCHB.
0.057 Units mL^{-1}	Apo-D-amino acid oxidase.
0.02mg mL^{-1}	Horseradish peroxidase.
20uM	FADP.

All reactions were performed at 37°C in a volume of 5.0mL (Obzansky et al., 1991).

Figure 46. The use of RELIA and the FADP cascade for the measurment of TSH.



t_0	Alkaline phosphatase and buffer
$t_{1.3}$	Apo-D-amino acid oxidase, proline and FADP
$t_{5.6}$	Horse radish peroxidase and chromogens
$t_{6.4}$	17 second kinetic measurement

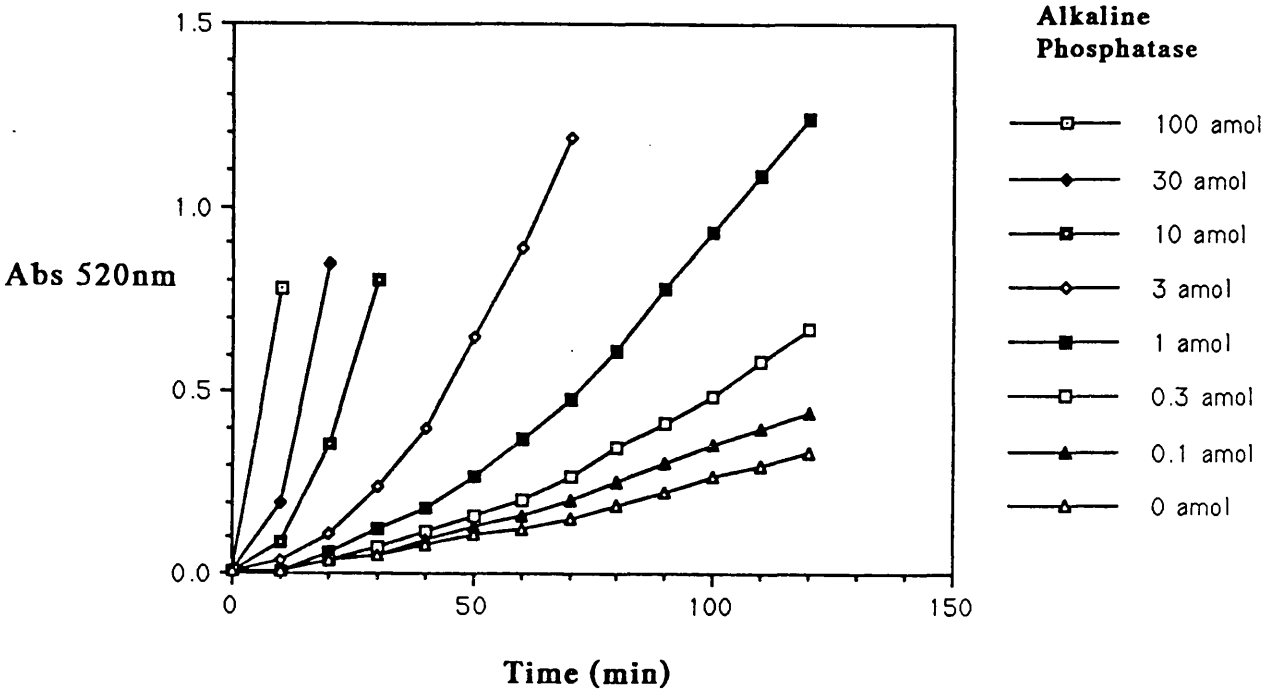
CHAPTER SEVEN.

REAGENT FORMATTING, SENSITIVITY AND USE OF THE AMPLIFICATION ASSAY OF ALKALINE PHOSPHATASE.

7.1. Formatting of the assay reagents into a one-pot and a two-pot system.

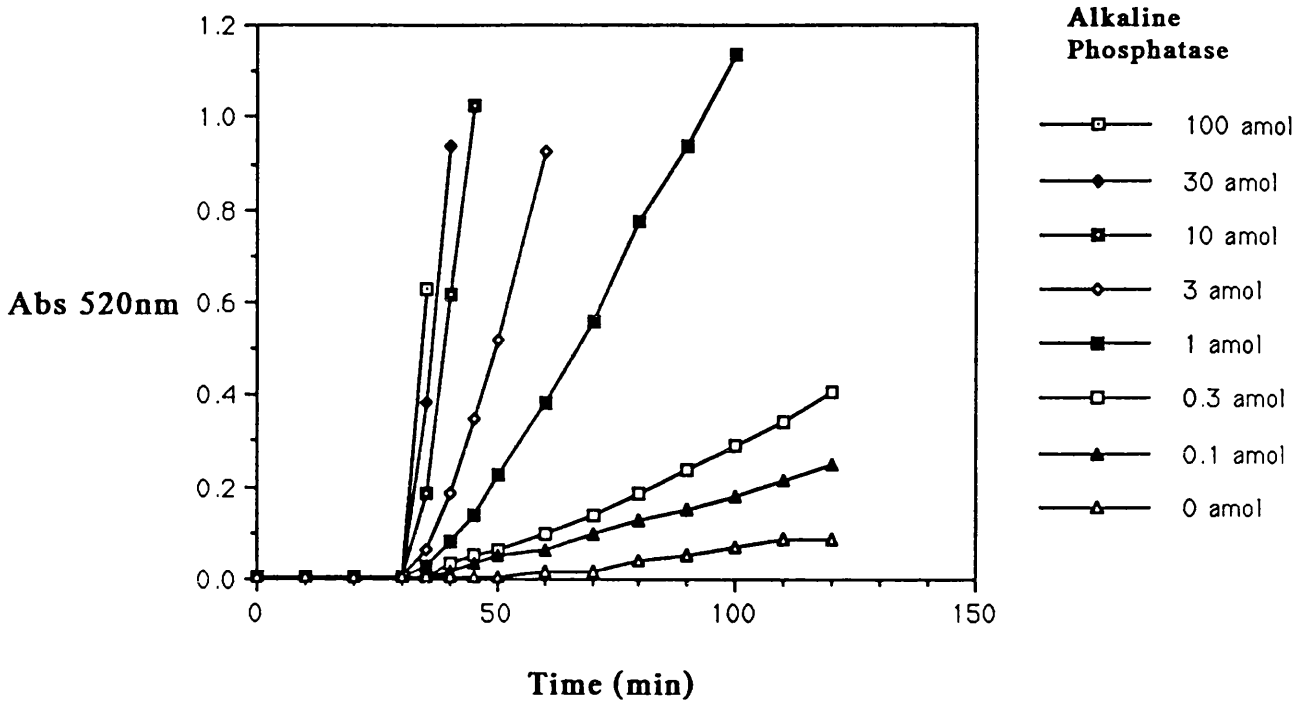
Figures 46 and 47 show the assay of alkaline phosphatase employing the amplification assay formatted as a one-pot and a two-pot assay respectively. The progress of the reaction of the one-pot assay follows reaction kinetics that vary quadratically with respect to time, characteristic of the amplification assay. The two-pot assay employed a 30 minute incubation of 20uM FADP and alkaline phosphatase, followed by the addition of the other components of the detection system in a 10mM sodium phosphate solution (final concentration). The reaction kinetics in this system are linear with respect to time after the initial binding of the FAD produced during the first incubation period.

Figure 46. The measurement of alkaline phosphatase activity in a one-pot amplification assay.



Alkaline phosphatase activity was measured in a one-pot amplification assay employing 35mM D-proline, 0.4mM 4-aminoantipyrine, 2.0mM DCHB, 0.14 Units mL^{-1} apo-D-amino acid oxidase, 10ug mL^{-1} HRP and 20uM FADP in 0.1M tris-HCl, pH 8.9, containing 0.1mM $MgSO_4$ and 1.0uM $ZnSO_4$.

Figure 47. The measurement of alkaline phosphatase activity in a two-pot amplification assay.



The activity of alkaline phosphatase was measured in a two-pot amplification assay. The initial substrate incubation (50uL) was carried out in 0.2M tris-HCl, pH 8.9, containing 0.1mM $MgSO_4$, 1.0uM $ZnSO_4$ and 20uM FADP. After 30 minutes the remaining detection system components were added to final concentrations of 35mM D-proline 0.4mM 4-aminoantipyrine, 2.0mM DCHB, 0.14 Units mL^{-1} apo-D-amino acid oxidase, 10ug mL^{-1} HRP and 10mM sodium phosphate in a total volume of 100uL.

K_{cat} can be determined (See Section 5.11.) for both the one-pot and the two-pot assay.

The one-pot assay:

The rate of production of FAD by 1 amol of alkaline phosphatase is equivalent to 0.049nM min^{-1} at 30 minutes.

Assuming simple Michaelis kinetics:

$$K_{cat} = \frac{v(K_m + S_0)}{E_0 S_0}$$

$$K_{cat} = \frac{0.49 \times 10^{-10} (9.6 \times 10^{-6} + 2.0 \times 10^{-5})}{1.0 \times 10^{-14} \times 2.0 \times 10^{-5}} \ min^{-1}$$

$$= 7252 \ min^{-1}$$

$$= 121 \ s^{-1}$$

This result compares well with the value of K_{cat} (118 s^{-1} and 127 s^{-1}) determined in Section 5.11 for the one-pot assay system. Similarly, the value of k_{cat} can be calculated for the two-pot assay system since the linear kinetics are proportional to the concentration of FAD produced from FADP in 30 minutes.

The two-pot assay:

When 20uM FADP is incubated with 3 amol of alkaline phosphatase the rate of production of FAD is equivalent to 0.27nM min^{-1}.

Assuming simple Michaelis kinetics:

$$K_{cat} = \frac{0.27 \times 10^{-9} (9.6 \times 10^{-6} + 2.0 \times 10^{-5})}{3 \times 10^{-14} \times 2.0 \times 10^{-5}} \ min^{-1}$$

$$= 13320 \ min^{-1}$$

$$= 222 \ s^{-1}$$

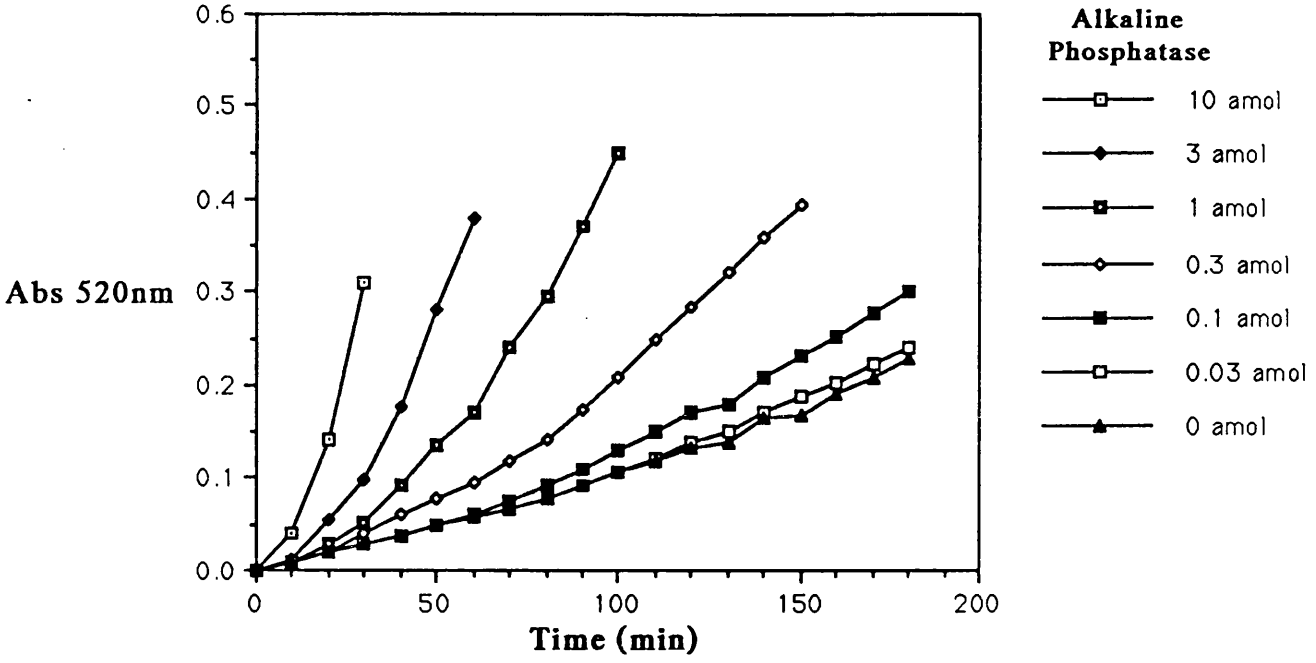
The value of K_{cat} determined above is higher than that determined in the two-pot system in Section 5.11. This is due to the increased concentration of tris-HCl (0.2M) used in the initial incubation period. This is borne out by the fact that the gradients of the progress curves in the two-pot system are greater than the gradients of the progress curves at 30 minutes in the one-pot system.

The one-pot assay system is a convenient simple format to use for microtitre plate assays. The single addition of a cocktail of reagents in the assay protocol renders it extremely "user friendly" and the technology is able to measure the activity of alkaline phosphatsae across a range encompassing three orders of magnitude in less than 90 minutes (See Section 7.4.). However, separation of the components into a substrate hydrolysis and signal generating phase increases the sensitivity of the assay (a higher signal-to-noise ratio in a shorter period of time). Separation of the components in this way enables each of the enzyme reactions to by performed under conditions which are more favorable than those in the one-pot system. The increased K_{cat} of alkaline phosphatase when D-amino acid oxidase is not present in the medium (See Section 5.11) leads to an increased perfomance when the components are added to generate the signal.

7.2. The stability of the freeze-dried premix of the assay components.

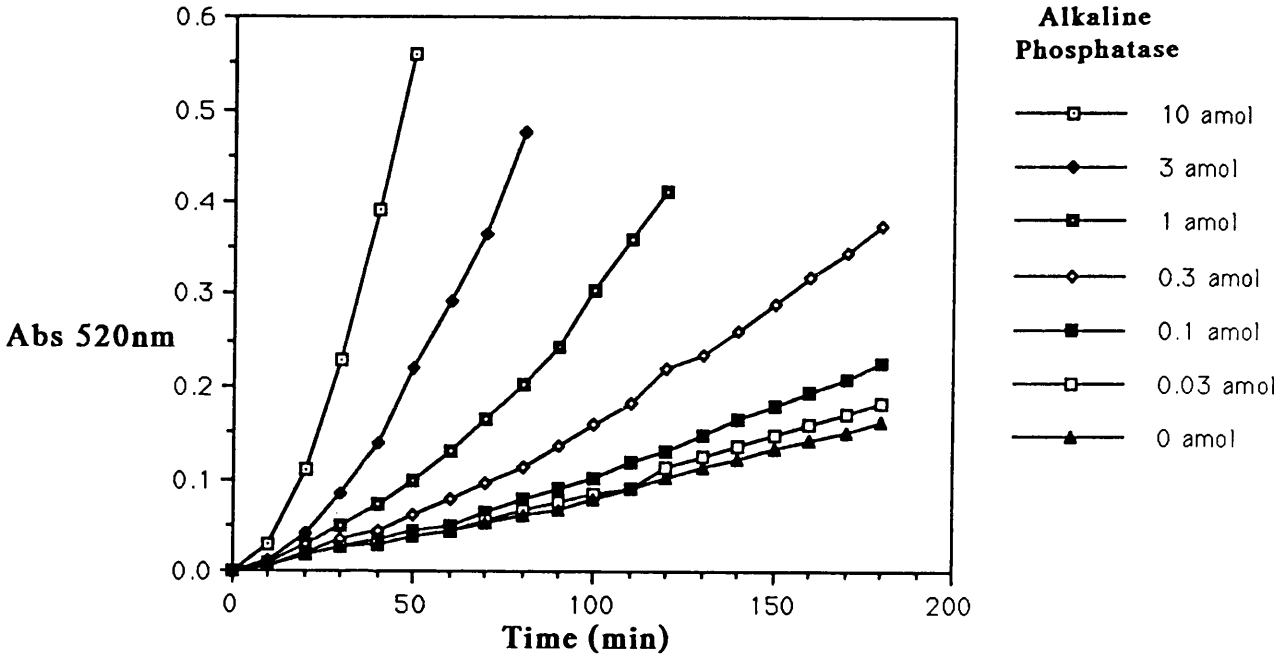
The assay formatted as a single freeze-dried premix reduces the risk of contamination of the reagents during their use and also provides an easy, ready-to-use assay with minimal variability between assays. Figure 48. shows the performance of such a premix, stored at -70°C for 3 days after reconstitution of the assay components with sterile buffer (0.1M tris-HCl, pH 8.0, containing 0.1mM $MgSO_4$ and 1.0uM $ZnSO_4$). To simulate conditions of transit, a similar freeze-dried premix was left at room temerature for the same period of time. The performance of this assay preparation after reconstitution with sterile buffer is shown in Figure 49.

Figure 48. The assay of alkaline phosphatase employing a freeze-dried premix stored at -70°C.



The activity of alkaline phosphatase was measured in a mictrotitre plate assay employing a single freeze-dried premix which had been stored at -70°C for 72 hours.

Figure 49. The assay of alkaline phosphatase employing a freeze-dried premix stored at room temperature.



The activity of alkaline phosphatase was measured in a microtitre plate assay employing a single freeze-dried premix which had been stored at room temperature for 72 hours.

The single premix is highly stable and no deterioration in the performance of the assay can be detected. Such assay formats have been transported by international courier with similar results. Indeed, reagents packaged in this way have been stored at -70°C for periods exceeding 6 months with no loss of performance.

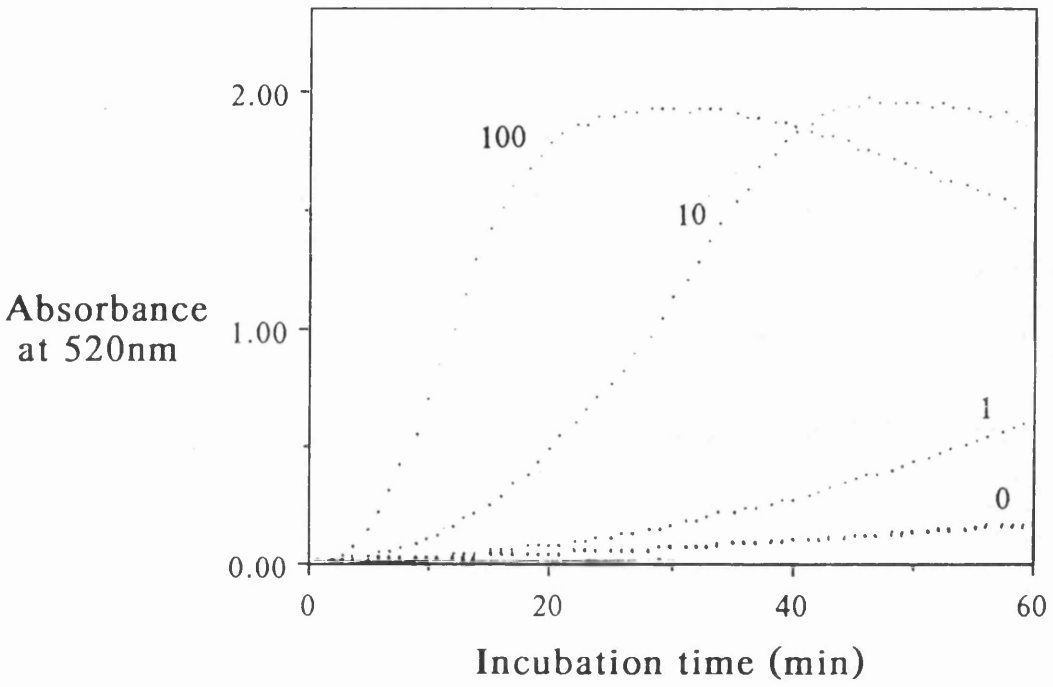
7.3. Increased assay sensitivity using highly purified apo-D-amino acid oxidase.

A comparison of the assay performance is illustrated by Figures 50 and 51 employing 0.14 Units mL^{-1} apo-D-amino acid oxidase and 0.41 Units mL^{-1}, highly purified apo-D-amino acid oxidase respectively. Using partially purified apo-D-amino acid oxidase (prepared by diafiltration and chromatography on Blue Sepharose only), alkaline phosphatase could be assayed in the 1 - 100 amol range after a 20 minute incubation at 25°C (Figure 50.). The sensitivity of this assay could not be improved by longer incubation periods or by employing a higher concentration of apo-D-amino acid oxidase because impurities in the apoenzyme preparation (nucleotidase and phosphatase activity) led to an unacceptable rise in the background signal. Longer incubation times also resulted in some bleaching of the colour produced at the highest levels of alkaline phosphatase measured. This is evident from the tail-off shown in Figure 50. This latter effect

could be eliminated by the use of a greater concentration of 4-aminoantipyrine in the assay and is due, mainly, to further oxidation of the coloured product by horseradish peroxidase as the concentration of 4-aminoantipyrine becomes limiting in solution. Figure 52 shows that the rate at which the coloured product of the reaction of horseradish peroxidase decays is proportional to the rate of production of hydrogen peroxide and hence the concentration of active D-amino acid oxidase.

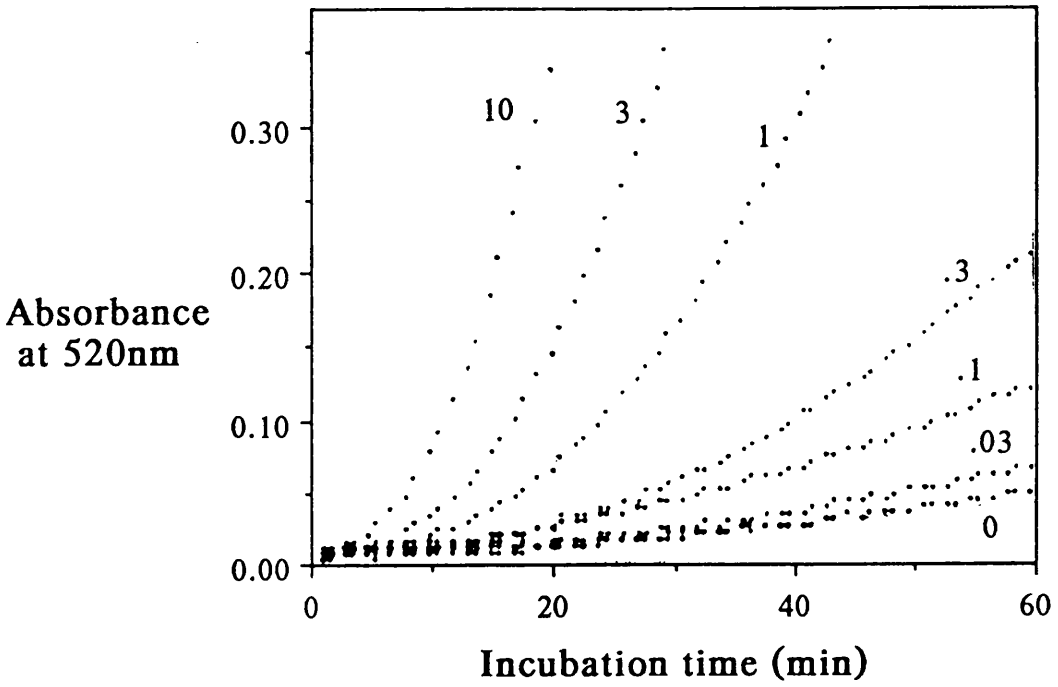
Further purification of the apoenzyme was achieved by chromatography on a Mono Q column (See Section 3.12.3.). This removed at least 80% of the remaining nucleotidase and phosphatase contaminants. When used in the amplification assay at a concentration of 0.41 Units mL^{-1} the activity of alkaline phosphatase could be measured in the range 0.03 -0.3 amol after 60 minutes incubation at 25°C (Figure 51.).

Figure 50. The measurement of the activity of alkaline phosphatase employing 0.14 Units mL^{-1} apo-D-amino acid oxidase.



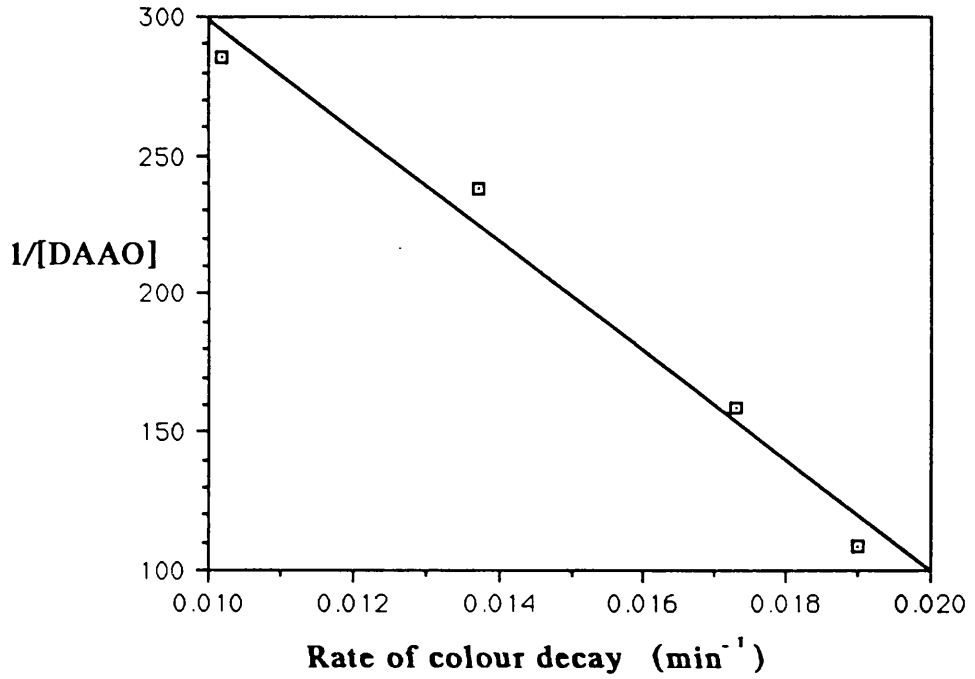
Alkaline phosphatase activity was measured in a microtitre plate assay employing 0.1M tris-HCl, pH 8.9, 35mM D-proline , 0.4mM 4-aminoantipyrine, 2.0mM DCHB, 10ug mL^{-1} HRP, 20uM FADP and 0.14 Units mL^{-1} apo-D-amino acid oxidase prepared by diafiltration and chromatography on Blue Sepharose only.

Figure 51. The measurement of the activity of alkaline phosphatase employing 0.41 Units mL^{-1} apo-D-amino acid oxidase.



The activity of alkaline phosphatase was measured in a microtitre plate assay employing 0.1M tris-HCl, pH 8.9, 35mM D-proline, 0.2mM 4-aminoantipyrine, 2.0mM DCHB, 10ug mL^{-1} HRP, 20uM FADP and 0.41 Units mL^{-1} apo-D-amino acid oxidase prepared by diafiltration, Chromatography on Blue Sepharose and chromatography by ion exchange (Mono Q).

Figure 52. The decay of the coloured product of the reaction of horseradish peroxidase with respect to the concentration of active D-amino acid oxidase.



R^2 = 0.980

The activity of alkaline phosphatase was measured in an amplified assay employing 0.14 Units mL^{-1} apo-D-amino acid oxidase in 0.1M tris-HCl, pH 8.9. The progress of the reaction was monitored on a Dynatech MR7000 microtitre plate reader at 25°C. The concentration of active D-amino acid oxidase was calculated at an absorbance equivalent to 1.0 for each of the concentrations of alkaline phosphatase measured and plotted against the rate of decay of the coloured reaction product.

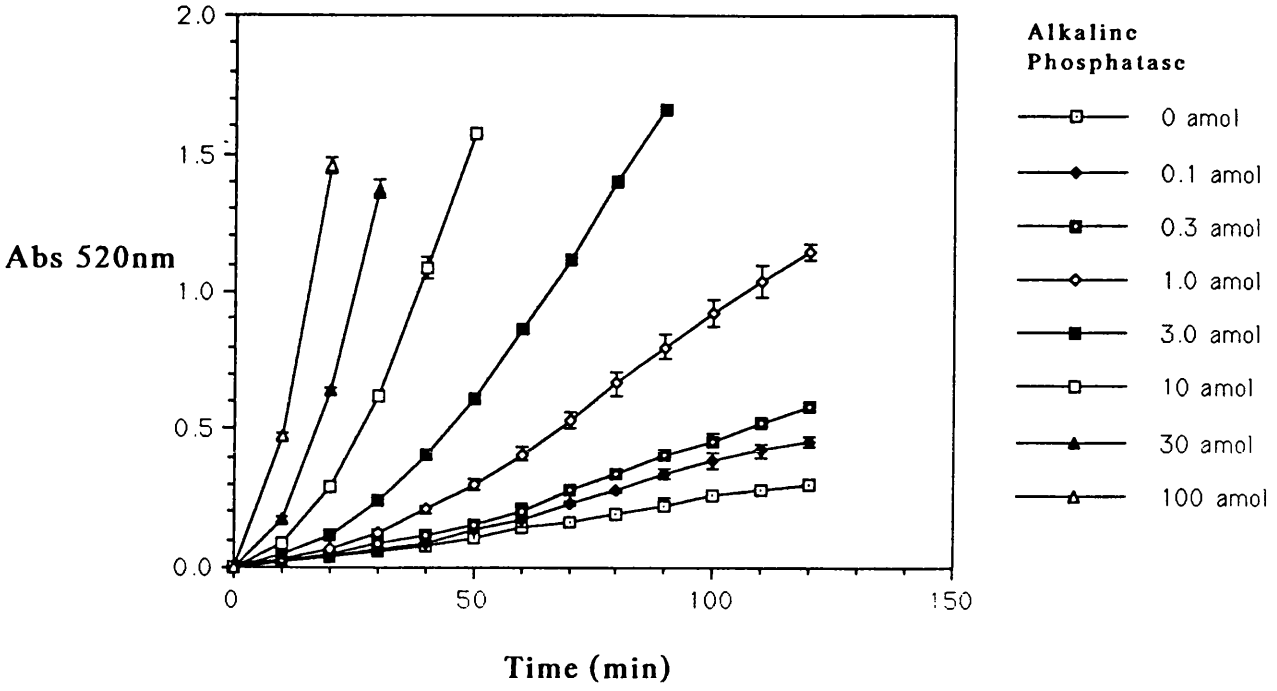
7.4. Reproducibility and the detection limit of the microtitre plate one-pot assay.

7.4.1. Intra-assay precision.

The reproducibility of the assay of alkaline phosphatase is shown in Figure 53. The standard deviation of each time point on the reaction progress curve for a one-pot assay was calculated from a series of five replicate assays. The intra-assay coefficient of variance is less than 8% and lies within acceptable assay limits. Reports on other amplification techniques show an increased coefficient of variance above non-amplified assays (Bobrow et al., 1989).

The definition of the detection limit of an assay is variable in the literature (Kaspar and Khanna, 1991; Thompson et al., 1991; Johannsson et al., 1986; Jackson and Ekins, 1986 and Harbron et al., 1991). The worst scenario is used here and is equivalent to 3 times the standard deviation above a mean reading for an assay containing no alkaline phophatase. Table 26 shows the detection limit of the data presented in Figure 53. The assay is capable of quantitation of amounts of alkaline phosphatase ranging from 0.1 - 100 amol in less than 90 minutes.

Figure 53. Intra-assay precision for the amplification assay of alkaline phosphatase (n = 5).



The activity of alkaline phosphatase was measured in an amplification assay employing a freeze-dried pre-mix containing 0.14 Units mL^{-1} apo-D-amino acid oxidase, 10ug mL^{-1} HRP, 35mM D-proline, 4.0mM 4-aminoantipyrine, 2.0mM DCHB and 20uM FADP per assay (100uL) reconstituted in 0.1M tris-HCl, pH 8.9, containing 0.1mM $MgSO_4$ and 1.0uM $ZnSO_4$. The progress of the reaction was monitored on a Dynatech MR 7000 microtitre plate reader at 25°C.

Table 26. Detection limits of the assay of alkaline phosphatase employing the FADP-cascade.

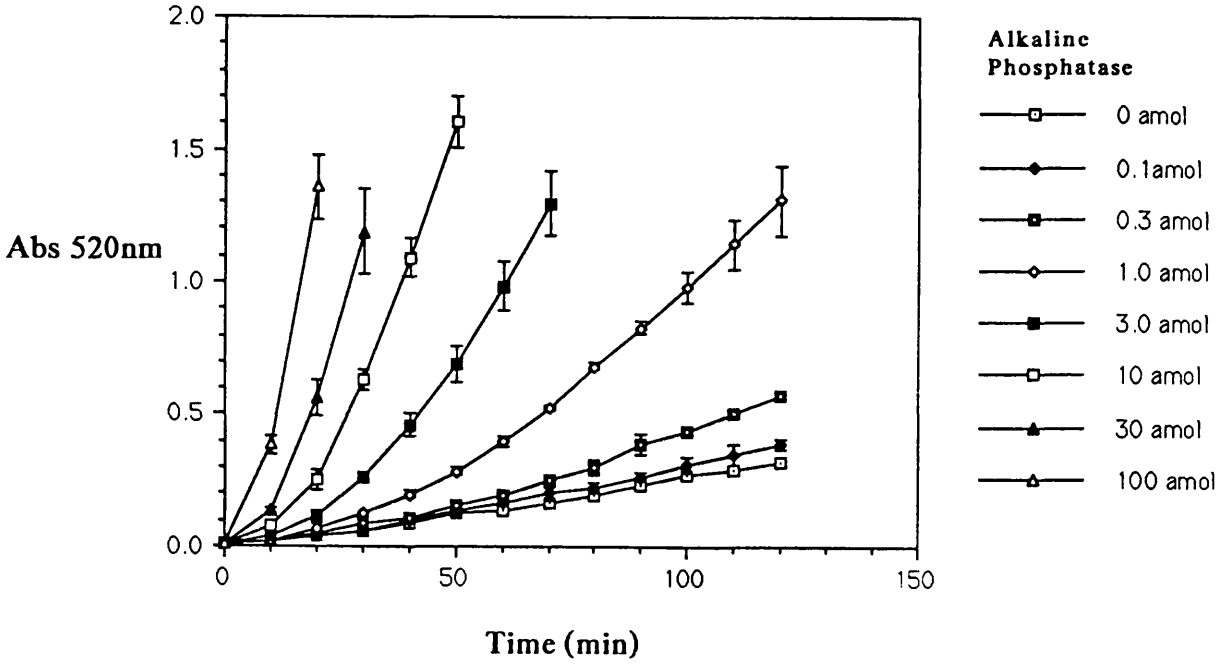
Time (min)	0 amol Signal+/-SD	Cut-off 0 amol+3SD	Detection limit Signal+/-SD
10	0.020+/- 0.008	0.044	10 amol 0.091+/-0.005
20	0.038+/-0.009	0.065	3 amol 0.113+/-0.010
30	0.057+/-0.013	0.096	1 amol 0.129+/-0.010
70	0.168+/-0.019	0.225	0.3 amol 0.0280+/-0.009
80	0.198+/-0.019	0.243	0.1 amol 0.278+/-0.008

The cut-off value at each time point is equal to the signal produced by 0 amol of alkaline phosphatase plus three standard deviations of the mean signal. Each concentration of analyte is detectable if the signal minus the standard deviation of the signal is above the cut-off value.

7.4.2. Inter-assay precision.

The reproducibility of the measurement of the activity of alkaline phosphatase over a period of time is also an important criteria in the suitability of an assay to be used in a clinical laboratory. Figure 54 shows the variation in the assay over a 6 month period using different preparations of FADP, apo-D-amino acid oxidase and horseradish peroxidase. The inter-assay coefficient of variance is less than 11% and lies within acceptable assay limits.

Figure 54. Inter-assay precision for the amplification assay of alkaline phosphatase (n = 5).

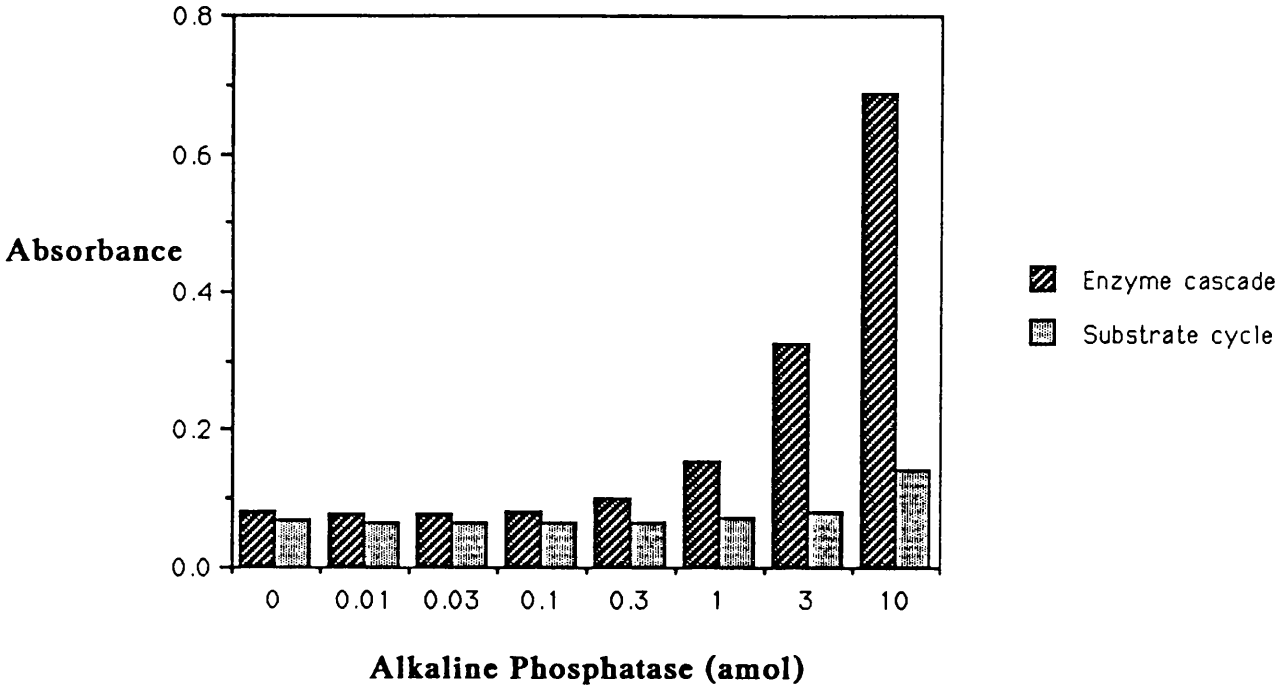


The activity of alkaline phosphatase was measured over a 6 months period. Each assay contained 0.14 Units mL^{-1} apo-D-amino acid oxidase, 10ug mL^{-1} HRP, 35mM D-proline, 4.0mM 4-aminoantipyrine, 2.0mM DCHB and 20uM FADP in 0.1M tris-HCl pH 8.9, containing 0.1mM $MgSO_4$ and 1.0uM $ZnSO_4$ in a total volume of 100uL. The progress of the assays was monitored on a Dynatech MR 7000 microtitre plate reader at 25 °C.

7.5. Performance comparison of enzyme amplification by substrate cycling and the enzyme cascade.

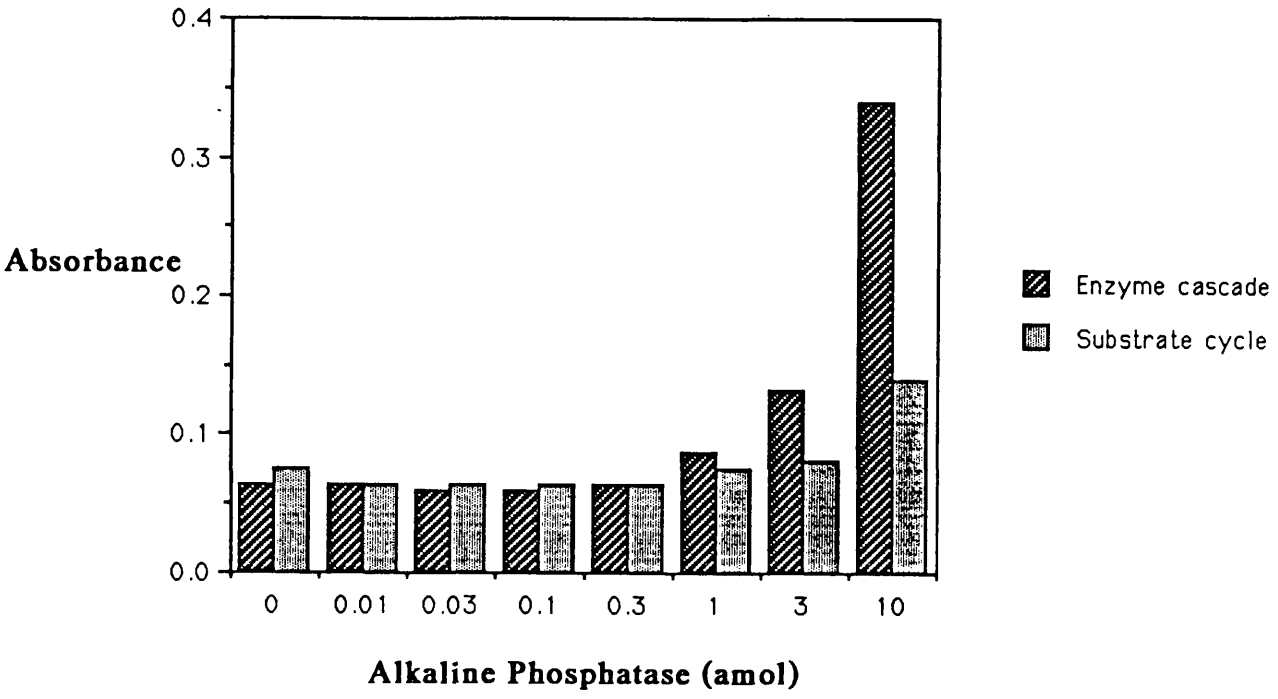
When both the assay employing the enzyme cascade and the substrate cycle are performad at 25°C the signal-to-noise ratios of the enzyme cascade are larger than those of the substrate cycle. Figure 55 shows the difference in the signals produced at a total assay time of 50 minutes for each of the amplification technologies. The detection limit of the enzyme cascade is at least one order of magnitude lower than that of the substrate cycle assay. However, the substrate cycle assay is performed in two separate stages, each at a different temperature optimum to increase the overall assay performance. Figure 56 shows the signals produced by each of the assay methodologies when the substrate cycle is used under optimum conditions. At a total assay time of 30 minutes the assay of alkaline phosphatase activity by the enzyme cascade is still superior to that of the substrate cycle. Although the detection limits of each of the assay systems is now on a more equal footing the signal-to-noise ratios of the enzyme cascade are much more favorable and will increase as a function of time whereas the kinetics of the substrate cycle are linear.

Figure 55. A comparison of the assay performance of the enzyme cascade and the substrate cycle at 23°C.



The substrate cycle assay was performed using an initial substrate incubation time of 40 minutes followed by a 10 minute incubation with the reagents for the amplification of the signal. The enzyme cascade assay was performed in a single-pot over a total incubation time of 50 minutes.

Figure 56. A comparison of the assay performance of the enzyme cascade and the substrate cycle at optimum temperatures.

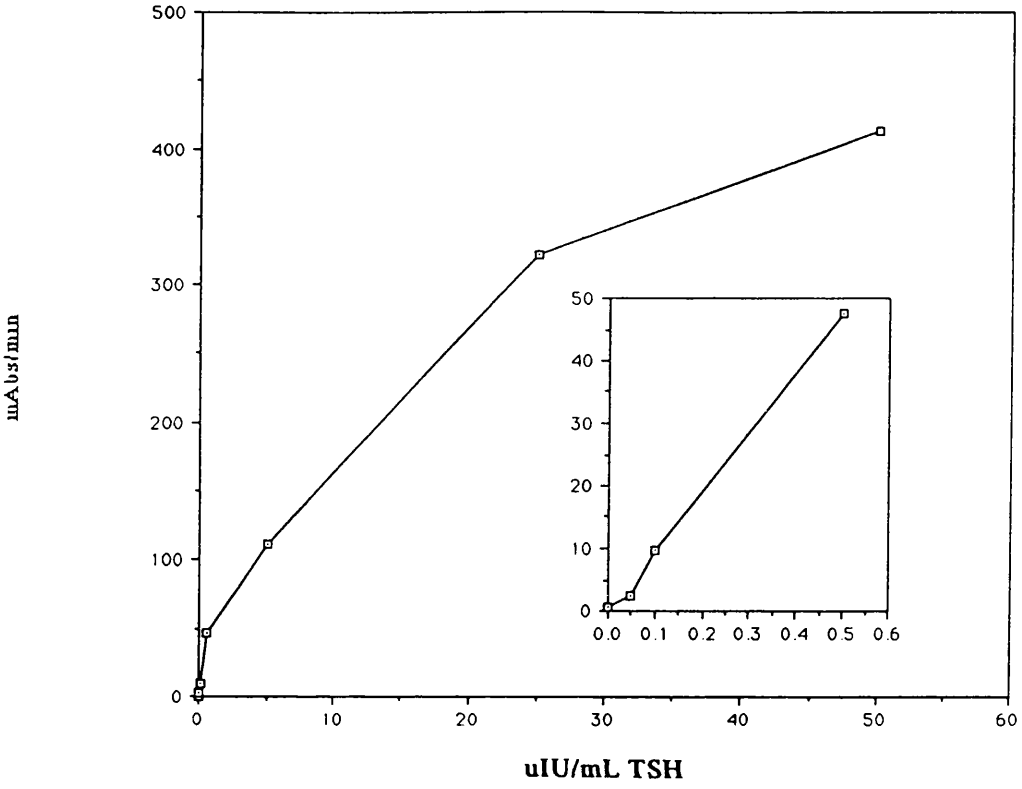


The substrate cycle assay was performed as a two-pot system, initially employing a substrate utilization incubation at 37°C for 20 minutes followed by an amplification of the signal at 23°C for 10 minutes. The enzyme cascade was performed as a single-pot assay over a 30 minute period at 23°C.

7.6. The measurement of TSH on the aca® dicrete clinical analyzer.

The RELIA technology is a generic way to release a signal generator (the alkaline phosphatase-avidin-biotin complex) from a specific immunocomplex containing the analyte (TSH). In essence the determination of TSH uses a dual amplification system. The effective concentration of the enzyme-label is increased through this interaction, and the signal is amplified via the cascade mechanism. This has enabled the measurement of TSH to be carried out across the entire clinical range of interest in a short assay time. Figure 57 shows the signal produced by the combination of the RELIA and the FADP cascade technologies.

Figure 57. TSH standard curve in the releasable linker immunoassay.



The measurement of TSH was performed on the aca® discrete clinical analyzer employing a combination of the RELIA and the FADP cascade technologies. The concentration of TSH was determined on a kinetic measurement over a total assay period of 6.4 minutes.

CHAPTER EIGHT.

DISCUSSION.

8.1. Preparation of apo-D-amino acid oxidase.

The preparation of apo-D-amino acid oxidase by diafiltration is an extremely fast and efficient process. The apoenzyme produced must be free of residual holoenzyme, catalase, phosphatase and nucleotidase activities. Chromatography on Blue Sepharose CL6B has been used to remove the residual holoenzyme and catalase activity. It is the removal of catalase during this process that can give an apparent increase in yield of the D-amino acid oxidase activity. Blue Sepharose has also been used to purify alkaline phosphatase from calf intestine under similar conditions (Lindner *et al.*, 1989). Alkaline phosphatase was specifically eluted free of phosphodiesterase activity with inorganic phosphate. When the apo-D-amino acid oxidase is bound to the Blue Sepharose the incorporation of a two column volume 10mM sodium phosphate wash in 20mM tris-HCl, pH 8.0, containing 1mM CHAPS has reduced the background level of contamination of the apoenzyme without loss of yield.

L-histidyldiazobenzylphosphonic acid agarose has been used as an affinity column medium (Landt *et al.*,

1978), to purify alkaline phosphatase. Passage of the apoenzyme through such a column did not reduce the level of phosphatase-like contamination, inferring that the contaminating activity is due to a nucleotidase enzyme. A range of immobilized dye ligands (Sigma Cat No: RDL-9) have also been studied to resolve this activity but without success.

The quality of the apoenzyme produced by diafiltration and chromatography on Blue Sepharose was sufficient for use in the aca^{R} discrete clinical analyzer by Du Pont. In an automated kinetic assay, 1.3 fmol L^{-1} alkaline phosphatase was detected in less than 10 minutes at 37°C (Obzansky et al., 1991), equivalent to 0.13 amol of analyte in a microtitre plate assay. Enough material was produced in a single batch process for 15,000 assays, each of 5.0mL. When used in a microtitre plate-based assay, at 25°C, 0.1 - 100 amol of analyte could be detected in 90 minutes (See Table 26). However, as Figure 51 shows, for extended incubation times and higher sensitivity, further purification of the apoenzyme on a Mono Q column is required. The incomplete resolution of phosphatase-like activity and D-amino acid oxidase activity (See Figure 20) requires detailed analysis of the column fractions to isolate the bulk of the contaminating material.

The components of the FADP cascade, although requiring purification to a degree not normally employed in enzyme assays, have been produced in the laboratory under conditions of "good house-keeping" without specially designed clean-rooms. Equipment has been dedicated in some circumstances, whilst other apparatus has been autoclaved or chemically cleaned. All components have been produced on a large scale without a reduction in the purity.

8.2. Preparation of horseradish peroxidase.

Highly purified horseradish peroxidase is commercially available. The large difference in pI's of horseradish peroxidase and alkaline phosphatases in general, enable complete resolution of the two or more enzymes. The minute quantity of contaminating phosphatase activity in the commercial preparations of horseradish peroxidase is removed in a simple, single chromatographic step. This involves inexpensive buffer preparations and the final purified protein can be readily freeze-dried without loss of activity.

8.3. Preparation of FADP.

FADP can be prepared to the required level of purity (containing less than 0.0003% FAD, See Section 3.7.4.) by employing a combination of chemical and enzymic techniques New methodologies have been developed to

reduce the level of FAD in the preparations of FADP. Apo-glucose oxidase can be used as a reagent in a selective binding process and is subsequently removed by ultrafiltration (See Appendix 3.). Immobilization of ribonuclease-T_2 and/or PDE, used in the production of FADP from FADcP would enable more efficient use of these components. This would also result in isolation of the enzyme free of any contaminating phosphatase activity that results in an increase in the concentration of FAD in the preparation during the process of opening the cyclic phosphate moiety of the prosthetogen.

8.4. Formatting of the amplification assay of alkaline phosphatase.

The assay performs extremely well in a microtitre plate format and lends itself to use on high quality, automated clinical analyzers (See Section 7.4 and 7.6). It can be formatted as separate components which can be optimized to suit a designated protocol or as a single premix with a long shelf-life. Overall, the assay is robust, user friendly and highly reproducible.

The operational effectiveness of the system derives from the fact that the prosthetogen substrate, FADP, neither reconstitutes the apoenzyme nor interferes with the binding of FAD to the apoenzyme (See Section

5.5.). The concentration of the primary substrate, and the apoenzyme detector, apo-D-amino acid oxidase, remain substantially unaltered throughout the time course of the assay. Thus, after 10 minutes in an assay containing 0.14 Units mL^{-1} (Figure 50.), 100 amol of alkaline phosphatase hydrolyses 0.065uM (0.33%) of the FADP and the concentration of apo-D-amino acid oxidase in the assay is reduced by about 20%. After 60 minutes in the higher sensitivity assay, containing 0.41 Units mL^{-1} apo-D-amino acid oxidase (Figure 51.), 0.03 amol of alkaline phosphatase hydrolyzes 0.12nM (0.0006%) of the FADP and the apo-D-amino acid oxidase is reduced by 0.000082 Units mL^{-1} (0.02%). Hence, despite the fact that the measured signal varies quadratically with time, at any given time point the measured absorbance is proportional to the concentration of alkaline phosphatase present in the assay (Harbron et al., 1992a). The ability to quantitate analytes with comparable precision over a 3333-fold concentration range (0.3fM - 1.0pM) is a uniquely advantageous aspect of the amplification system.

8.5. Enzyme-labels and detection systems.

Alkaline phosphatase and horseradish peroxidase are the two most widely employed enzyme labels (Gosling, 1990). A sensitive detection system for solid phase enzyme detection has been developed employing alkaline

phosphatase as a label with the substrates 5-bromo-4-chloro-3-indoylphosphate (BCIP) and nitro blue tetrazolium (NBT) (Leary et al., 1983). Alkaline phosphatase has therefore been utilized in a larger number of sensitive detection systems than other enzyme-labels. A direct comparison of the sensitivity of different enzyme systems is not strictly valid because different numbers of enzyme molecules may be conjugated in each enzyme labelled system.

The use of mammalian alkaline phosphatase in binding assays does however have disadvantages. Some enzyme preparations are of inadequate purity for coupling to assay system. Higher background readings are also believed to occur due to the covalently linked carbohydrates that contribute to non-specific binding in the assay procedures. Some conjugates also have a low thermal stability. Thus alkaline phosphatase isolated from E. coli has been considered as a potential label-enzyme. This enzyme can be easily purified and has a higher thermal stability as well as a broad specificity. However, the turnover of the wild type enzyme is much lower than that of calf intestinal alkaline phosphatase when p-nitrophenyl phosphate is used as the substrate. Site directed mutagenesis techniques have been used to improve the turnover of this enzyme (Mandecki, 1991).

The liberation of hydrogen peroxide during an assay and coupling of different dye substrates by horseradish peroxidase has been employed in a number of assays incorporating different methodologies across a wide range of pH. Systems employing a luminometric and a precipitating substrate are also under development for use with the FADP cascade. Different colorimetric systems for the detection of hydrogen peroxide have been used in other detection systems and are pH dependent (Porstmann et al., 1981). A reaction system for the oxidative coupling of 3-methyl-2-benzothiazolinone hydrozone hydrochloride (MBTH) and 3-(dimethylamino)benzoic acid (DMAB) which produces an indamine dye with a molar extinction coefficient of 47,600cm^{-1} has been developed (Ngo and Lenhoff, 1980). This system has been employed in coupled enzyme reactions resulting in a sensitive detection system (Obzansky and Richardson, 1983). The rate of colour development however is not constant and there is a significant degree of spontaneous reaction between the reagents resulting in a high background signal (Ngo and Lenhoff, 1985).

8.6. Alternative detection methodologies and diagnostic procedures.

Alkaline phosphatase can be covalently linked to an antibody or a gene probe that recognizes, directly or otherwise, the analyte being measured. Alternatively

it can be attached to a component of an avidin-biotin detection system, with the other component analyte-linked where this system is employed (Ternynck and Avameas, 1990). Hybridization conditions employing enzyme-labelled oligonucleotide probes can be optimized to distinguish between a single base change in a nuceic acid sequence (Wallace et al., 1985). Such factors include variation of salt concentration, temperature and the concentration of DNA used.

To date, nucleic acid tests have not been commercially feasible because they require isolation of DNA or RNA from a biologically complex sample, involving complex analytical procedures and use of a specific detection probe labelled with a short-lived radioactive isotope. The most widely used label in DNA hybridization assays is ^{32}P. The detection techniques employed, Southern blotting and dot blots (Thomas, 1980), are labour intensive, time consuming and not amenable to automation. It is therefore unlikely that these techniques will find application outside the research laboratory. Immunoprecipitable DNA probes, which are hybridized by established procedures and immunochemically detected have been developed as an alternative to radioisotopic labels (Tchen et al., 1984).

Pre-natal diagnostic tests have been limited to those that have used the most sensitive detection methods

because the amount of fetal material available is often very small. With the development of the Polymerase Chain Reaction (PCR), (Bugawan et al., 1988), it is feasible that less sensitive and more readily automated methodologies will be employed. PCR has already been used to diagnose a number of genetic diseases, including phenylketonuria, β-thalassemia, muscular dystrophy and recently cystic fibrosis (Arnhelm and Levenson, 1990).

PCR is not 100% specific. Non-specific products of PCR may also appear, caused by the mismatched annealling of primers or primer dimerization. The more cycles of PCR that are carried out, the more likely it is that such non-specific primer events will occur. A sensitive detection system used after PCR will therefore still be required to minimize the number of cycles should PCR ever find application outside of the research laboratory. The advent of PCR has made it possible to detect DNA sequences that were beyond the range of other systems already automated. However, the use of a single detection system ie. a highly sensitive system would still be preferable to a multi-step process employing an integration of methodologies (Nicholls et al., 1989).

APPENDIX 1.

1.1. Production of FAD-2'-phosphate from FAD-2'3'-cyclicphosphate.

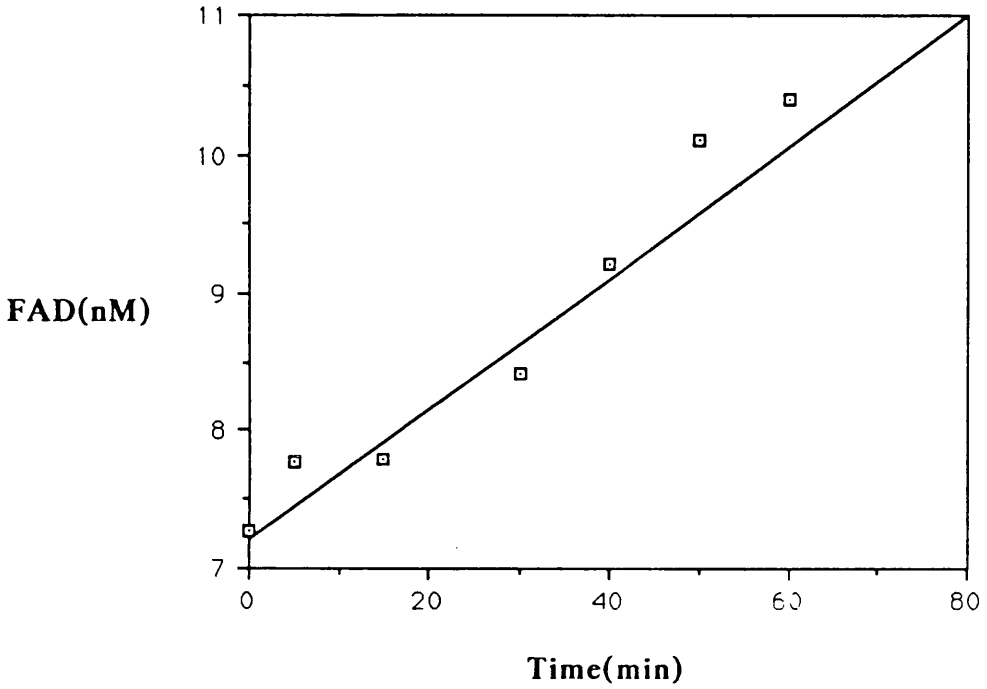
3'-phosphodiesterase, 2':3'-cyclic nucleotide (Sigma Cat No. P 6274) was used to produce the 2' isomer of the prosthetogen. The procedure used was as described in Section 2.14.2., except that the hydrolysis of the cyclicphosphate was carried out in 20mM MES, pH 6.0 and using a value of $K_m = 44.2 \times 10^{-6}$ M and $K_p = 1.2 \times 10^{-3}$ M to calculate the concentration of enzyme required to produce the 2'-isomer of FADP in 10 minutes.

During the production of FAD-2'-phosphate the concentration of FAD in the FADP preparation increased. Phosphatase activity could be detected in the preparation of PDE used (See Appendix 1, Section 1.2.).

1.2. 3'-Phosphodiesterase, 2':3'-cyclic nucleotide and the production of FAD from FADcP.

FADcP (1.0mM) containing an initial contamination level of FAD of 0.007% was incubated with PDE in 20mM MES, pH 6.0 to give 99% conversion of the substrate in 10 minutes at room temperature (See Appendix 1, Section 1.1.). An aliquot (10uL) of the incubation mixture was removed periodically over an incubation period of 1 hour. Figure 58 shows the FAD content determined in each of the aliquots.

Figure 58. The production of FAD during the hydrolysis of FADcP by PDE.



$R^2 = 0.961$

FADcP (1.0mM) was incubated with PDE in 20mM MES to yield 99% conversion of the substrate in 10 minutes. Aliquots were removed at intervals and the concentration of FAD determined by measuring the extent of reconstitution of 0.14 Units mL^{-1} of apo-D-amino acid oxidase in 0.1M tris-HCl, pH 8.0.

APPENDIX 2.

2.1. Preparation of apo-glucose oxidase.

The procedure described below is adapted from that used by Morris and Buckler (1983).

2.1.1. Preparation procedure for apo-glucose oxidase.

33g of Bio-Gel P-10 (Bio Rad Cat No: 150-1040) was gradually added to 600mL of 25mM sodium phosphate buffer, pH 1.7, containing 30% glycerol. The gel was allowed to hydrate at room temperature for four hours. The gel was poured into a K50 column and equilibrated with 600mL of the same buffer.

Glucose oxidase (320mg Sigma: Type X-S Cat No. G 7141) was dissolved in 3.5mL Of 25mM sodium phosphate buffer, pH 6.0, containing 30% glycerol. The enzyme solution was cooled to 0°C and the pH adjusted to pH 1.7 by the dropwise addition of ice-cold 25mM sodium phosphate pH 1.1, containing 30% glycerol. The mixture was incubated at 0°C for 30 minutes and chromatographed on the Bio-Gel column. The protein was eluted from the column with the phosphate buffer, pH 1.7 at a flow rate of 2.25ml min^{-1}.

Protein that eluted from the column at an absorbance of greater than 0.05 Abs units was collected into 2.0mL of 0.4M sodium phosphate, pH 8.0, containing

10mg of Dextran 70 (Pharmacia Cat No. 17-0280-01) and 300mg of activated charcoal (Sigma Cat No. C 4386). This mixture was incubated at 0°C for 60 minutes before the pH was adjusted to pH 7.0 with 0.1M sodium hydroxide. The whole was filtered through a Whatman 54 filter paper attached to a Buchner funnel.

The protein was desalted on a Sephadex G25C column (5.0 x 25cm). Protein was eluted from the column with 20mM bis-tris-propane, pH 7.0, containing 5g L^{-1} mannitol at a flow rate of 5.6mL min^{-1}. Active fractions were frozen and lyophilized in 50mg aliquots at 0.05 bar for 36 hours. The lyophilizate was stored at -70°C.

2.1.2. Yield of apo-glucose oxidase preparation.

Table 27. Preparation of apo-glucose oxidase.

	Vol (mL)	Units/ mL(+FAD)	Yld %	Units/ mL(-FAD)	%holo	Protein mg ml^{-1}
Start material	3.5	1740	/	/	/	/
G-25	130	20	74	0.012	0.06	1.9

Apo-glucose oxidase was prepared by a process of acid dissociation of the cofactor, FAD, which was separated on a Bio-Gel P-10 coloumn. The final solution was desalted on a Sephadex G25C column.

2.2. The assay of glucose oxidase.

0.1M	Potassium phosphate, pH 7.0.
0.1M	D-Glucose.
2.0mM	DCHB.
0.2mM	4-aminoantipyrine.
0.1mg mL^{-1}	Horseradish peroxidase.
0.24mM	Flavin adenine dinucleotide.

Total volume: The components listed above are final concentrations mixed in a total volume of 1.0mL for a spectrophotometric assay.

Temperature: 25°C.

Wavelength: 520nm.

Extinction: 23000M^{-1} for 1cm light path.

Activity: 1 unit is the amount of enzyme producing 1umol of hydrogen peroxide in 1 minute.

Omission of the FAD allows the measurement of any residual activity in the apoenzyme preparation.

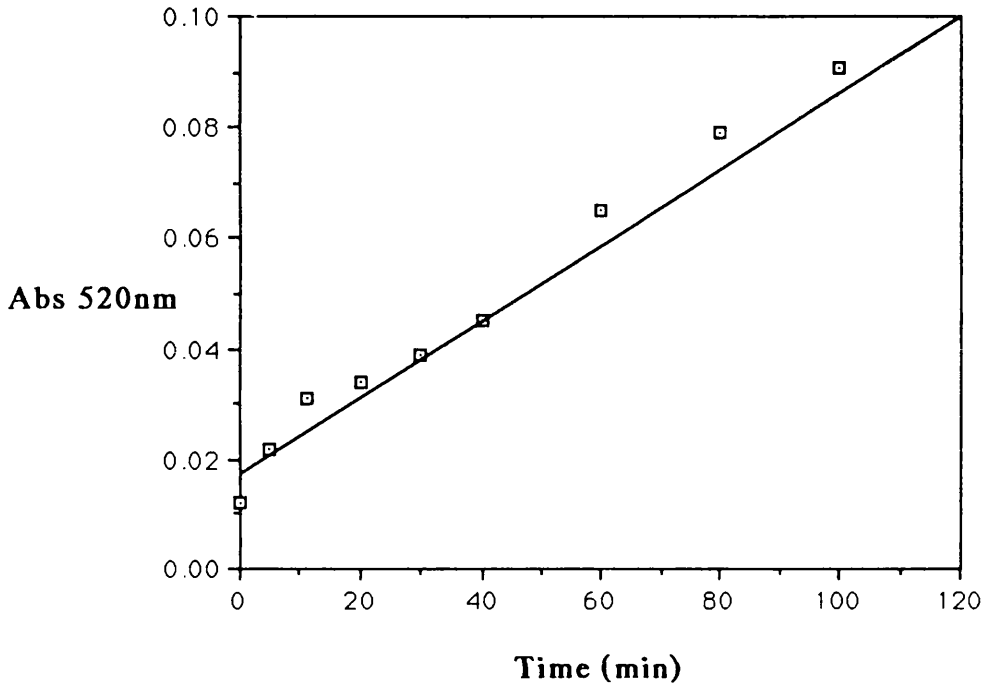
APPENDIX 3.

3.1. The removal of FAD from FADcP employing apo-glucose oxidase.

Apo-glucose oxidase (31.5 Units mL^{-1} and 63.0 Units mL^{-1}) was incubated with 500uM FADcP in 20mM bis-tris, pH 6.0, at room temerature in the dark. After an incubation period of 1 hour the mixture was ultrafiltered on a Centricon 10 microconcentrator to remove the glucose oxidase. The ultrafiltrate was assayed for FAD content by determining the extent of reconstitution of activity of apo-D-amino acid (See Section 2.1.).

3.2. The measurement of FAD in FAD-2',3'-cyclicphosphate after treatment with apo-glucose oxidase

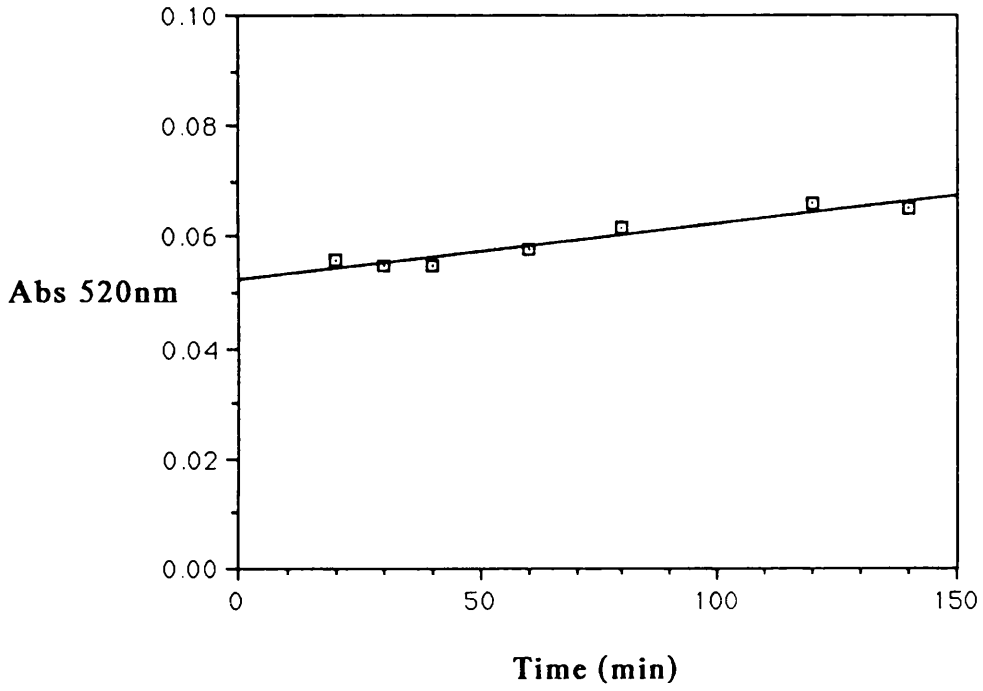
Figure 59a. Untreated FADcP.



R^2 = 0.986.

The concentration of FAD contaminating a preparation of FADcP was determined by measuring the reconstituted activity of apo-D-amino oxidase and comparison with an FAD standard assay.

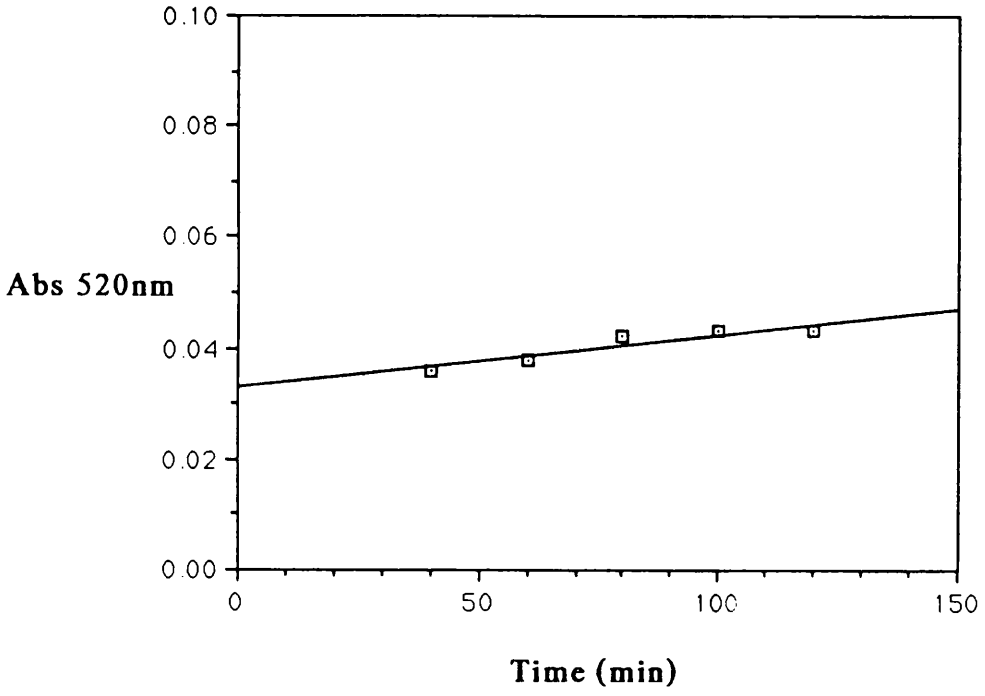
Figure 59b. 31.5 Units mL^{-1} apo-glucose oxidase incubation.



R^2 = 0.921

Apo-glucose oxidase (31.5 Units mL^{-1}) was incubated with 500uM FADcP in 20mM bis-tris, pH 6.0. Residual FAD was determined by measuring the extent of reconstitution of 0.14 Units mL^{-1} apo-D-amino acid oxidase in 0.1M tris-HCl, pH 8.0.

Figure 59c. 63.0 Units mL^{-1} apo-glucose oxidase incubation.



R^2 = 0.876.

Apo-glucose oxidase (63.0 Units mL^{-1}) was incubated with 500uM FADcP in 20mM bis-tris, pH 6.0. Residual FAD was determined by measuring the extent of reconstitution of 0.14 Units mL^{-1} apo-D-amino acid oxidase in 0.1M tris-HCl, pH 8.0.

Table 28a. Analysis of variance of untreated FADcP.

Source	Degrees of freedom	Sum of squares	Mean squares	F ratio
Regression	1	3.22×10^{-2}	3.22×10^{-2}	489.5
Residual	7	4.60×10^{-4}	6.60×10^{-5}	
Total	8	3.26×10^{-2}		

Tabulated $F_{0.05}(1,8) = 5.32$.

The calculated value of F is greater than the tabulated value of F. The slope is therefore significantly different to zero at the 5% level.

Table 28b. Analysis of variance of incubation with 31.5 Units mL^{-1} apo-glucose oxidase.

Source	Degrees of freedom	Sum of squares	Mean squares	F ratio
Regression	1	1.29×10^{-3}	1.29×10^{-3}	58.6
Residual	5	1.10×10^{-4}	2.20×10^{-5}	
Total	6	1.40×10^{-3}		

Tabulated $F_{0.05}(1,6) = 5.99$.

The calculated value of F is greater than the tabulated value of F. The slope is therefore significantly different to zero at the 5% level.

Table 28c. Analysis of variance of incubation with 63.0 Units mL^{-1} apo-glucose oxidase.

Source	Degrees of freedom	Sum of squares	Mean squares	F ratio
Regression	1	3.61×10^{-5}	3.61×10^{-5}	21.23
Residual	3	5.10×10^{-6}	1.7×10^{-6}	
Total	4	4.12×10^{-5}		

Tabulated $F_{0.05}(1,4) = 7.71$.

The calculated value of F is greater than the tabulated value of F. The slope is therefore significantly different to zero at the 5% level.

Table 29. Limits of the slopes.

Sample	Gradient of slope	Upper limit	Lower limit
Untreated FADcP	7.50×10^{-3}	5.60×10^{-4}	2.70×10^{-4}
31.5 Units mL^{-1} incubation	9.80×10^{-5}	2.10×10^{-4}	-9.50×10^{-6}
63.0 Units mL^{-1} incubation	9.50×10^{-5}	1.60×10^{-3}	2.80×10^{-5}

95% confidence limits were calculated for each of the gradients using the tabulated T-value (Clarke, 1980).

The limits of the slopes for the incubations employing apo-glucose oxidase reflect the degree of inaccuracy of the linear regression analysis corresponding to the value of R^2. Although the slopes are significantly diffent to zero there is no significant difference between these two slopes, and no requirement to utilize a large excess of apo-glucose oxidase to "mop up" the FAD which is in solution.

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