ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

A thesis entitled

METHODOLOGICAL AND BIOLOGICAL STUDIES OF THE AMINO ACID OXIDASES

presented by

DAVID JAMES HOLME F.I.M.L.S.

in part fulfilment of the requirements
for the Degree of

MASTER OF PHILOSOPHY

of the

COUNCIL FOR NATIONAL ACADEMIC AWARDS.

Department of Biological Sciences
Sheffield City Polytechnic
Pond Street,
Sheffield. S1. 1WB.

August 1978.

ACKNOWLEDGEMENTS

Professor D.M. Goldberg provided the inspiration, direction and guidance for the work described in this thesis and under his aegis a paper was published in 1975. I wish to express my very sincere thanks for his never failing enthusiasm and encouragement throughout the course of this project.

When the practical work was virtually completed Professor Goldberg moved to Toronto, Canada, as Professor and Chairman of the Department of Clinical Biochemistry, University of Toronto, and Dr. J.B. Turner assumed the responsibility of the Director of Studies and supervised the preparation of this thesis. I should like to thank Dr. Turner for his invaluable help and encouragement during this period. I am also indebted to Dr.D. Atkins of the Department of Chemical Pathology, Sheffield University for his consideration and advice.

I should also like to express my thanks to Dr. B.H. Fletcher Head of the Department of Biological Sciences for his encouragement and for the use of departmental facilities and the flexibility in timetables that he permitted.

The willing help of Mr. G. Anderson, of the Department of Pathology, Hallamshire Hospital in organising a supply of tissue samples is also gratefully acknowledged.

David J. Holme.

ABBREVIATIONS.

Throughout this thesis the nomenclature and style of presentation suggested by the Editorial Board of the Biochemical Journal has been used. Abbreviations acceptable to the above Board have been used and are listed below.

ADP	Adenosine 5'-pyrophosphate
FAD	Flavin-adenine dinucleotide
FMN	Flavin mononucleotide
NAD^+	Nicotinamide-adenine dinucleotide (oxidized)
NADH	Nicotinamide-adenine dinucleotide (reduced)
Tris	tris (hydroxymethyl) amino methane
Tricine	N-tris (hydroxymethyl)methylglycine

The abbreviation GDH has been used to indicate the enzyme glutamate dehydrogenase in some tables of methods, where the lack of space prevented the use of the full name.

CONTENTS

SUMMARY

Methods are described in which the liberation of ammonia from amino acid substrates by the D- and L-amino acid oxidases is coupled to the NADH dependent reductive amination of 2-oxoglutarate, the reaction being catalysed by exogenous glutamate dehydrogenase (L-glutamate: NAD(P) oxidoreductase (deaminating), EC 1.4.1.3.) The inhibition of D-amino acid oxidase (D-amino acid: oxygen oxidoreductase (deaminating), EC 1.4.3.3.) by the ADP needed to activate and stabilise glutamate dehydrogenase was relieved by FAD. The Michaelis constant (Km) for the enzyme was 3.3 mmol l^{-1} with D-alanine as the substrate which, when used in the assay at a concentration of 17 mmol l^{-1}, permitted 84% of the maximum velocity. Neither FAD nor FMN were required in the L-amino acid oxidase (L-amino acid: oxygen oxidoreductase (deaminating) EC 1.4.3.2.) assay which utilized L-leucine as substrate (Km 0.6 mmol l^{-1}) at a concentration of 3.3 mmol l^{-1}. This concentration of substrate was low enough to avoid any significant effect of substrate inhibition and yet permitted 85% of the maximum velocity.

The oxidation of NADH was monitored both as a fall in absorbance at 340 nm and by the increase in fluorescence due to NAD^+ in alkaline solution (excitation maximum 365 nm, emission maximum 455 nm). The former provided the basis for a kinetic spectrophotometric assay which was sensitive and precise and lent itself to valid kinetic studies of the enzymes. The fluorescence due to NAD^+ in a solution of 6 mol l^{-1} NaOH was stabilised by the presence of 10 mmol l^{-1} imidazole and formed the basis of a sensitive, fixed time assay.

A study of human tissues demonstrated the presence of significant concentrations of D-amino acid oxidase in kidney and liver with lower concentrations in samples of brain tissue. L-amino acid oxidase could only be detected in kidney and liver and no other tissues investigated showed any amino acid oxidase activity.

The specificities of human, hog and snake venom amino acid oxidases were shown to be significantly different.

STATEMENT OF THE OBJECTIVES

The physiological rôle of the amino acid oxidases in mammals has been a matter of debate since the subject was first raised by Krebs, (1933) and one of the major difficulties in the study of these enzymes has been the lack of a suitable method of assay. This is borne out by the large number of different assay methods which have been published over the last forty five years, many of which were either technically laborious or restricted to the use of a limited number of potential substrates.

Among the long term objectives in the present development of an assay method for the amino acid oxidases was the possibility that it might provide a means of assessing or monitoring diseased tissue and also that variations in serum levels of the enzyme might be used to monitor tissue destruction. The reputed presence of these enzymes in kidney tissue, for instance, might offer a method of assessing the condition of damaged or transplanted kidneys. There was also the possibility that in the wide specificity apparently shown by the amino acid oxidases there might be some variation between enzymes from different tissues and thus provide an enzyme or substrate for that enzyme which would permit the study of a specific tissue. Certainly the availability and use of a flexible and sensitive assay method for the study of amino acid oxidases in tissue and body fluids would help in the elucidation of a physiological rôle for the amino acid oxidases in man.

In searching for a suitable method of assay, an important criterion, second only to specificity, is the degree of sensitivity shown by the method. A high degree of sensitivity is necessary not only to detect small quantities of enzyme in a given sample but also to provide a tool to enable the examination of samples which by their very nature contain only small amounts of the enzyme. In the preparation of tissue homogenates, dilution is always a difficulty and some workers have overcome this to some extent by the use of large samples of tissue. However the availability of a method with the characteristics defined above would permit the assay of homogenates prepared from the small samples which would be available in any

critical study of the distribution of these enzymes in different tissues or sub-cellular fractions.

The selected method, in addition to being a valid method of quantitation, must be suitable for the study of the kinetic properties of the enzymes concerned and, to this end, must permit the use of any potential substrate. Methods which involve the measurement of initial rates of reaction are the most acceptable for the study of the kinetic properties of an enzyme and this information is obtained most satisfactorily by the use of continuous monitoring techniques, a feature which lends itself to the subsequent automation of the assay method.

Summary of objectives

1. Assay Method.
   a) specific;
   b) sensitive;
   c) kinetic;
   d) continuous monitoring.
2. Enzyme Studies.
   a) distribution in tissues;
   b) kinetic characteristics;
   c) variations due to disease or damage.

INTRODUCTION

A review of the literature available on the amino acid oxidases fell naturally into two main areas because of the dual objectives of the present work. The methodological requirements necessitated an appraisal of the methods currently available for the assay of both D- and L-amino acid oxidase. Information was also required on the current understanding of the biological role of the enzymes if the relevance of such data to the effective functioning of tissues or organs was to be investigated.

Review of the amino acid oxidases

The fact that the deamination of amino acids with its concomitant release of ammonia was an oxidative process involving the alpha carbon was elucidated by Knoop & Oesterlin (1925). The equation for the overall reaction was proposed to be

$$RCH(NH_2)COOH + \tfrac{1}{2}O_2 \rightarrow RCOCOOH + NH_3 \quad \text{..... Equation 1}$$

Undoubtedly the foremost worker in this area was Krebs (1935) who demonstrated that fresh slices of mammalian liver and kidney, particularly the latter, were able to deaminate amino acids and that the concurrent uptake of oxygen could be monitored and used as an indicator of the reaction. He proposed that the reaction proceeded via the formation of an imino acid intermediate and that in tissue slices the oxoacids which were formed were very quickly removed in an, as then, unknown manner.

$$R\underset{NH_2}{\underset{|}{C}}HCOOH \rightarrow R\underset{NH}{\underset{||}{C}}COOH \rightarrow RCOCOOH + NH_3$$

This loss of oxoacids was subsequently shown to be due to either enzymic transamination or non-enzymic oxidation by the hydrogen peroxide which was also formed during the reaction.

Krebs also reported that there were two enzyme systems involved in the process which varied mainly in their stereospecificity and he named these enzymes "d-amino acid deaminase" and "l-amino acid deaminase" indicating their stereospecificity. He also demonstrated that the two enzyme systems showed different kinetic and physical characteristics, (Table 1. below).

TABLE 1

Summary of differences between enzymes responsible for the oxidation of amino acids. Krebs, H.A. (1935)

Treatment	d-amino acid deaminase	l-amino acid deaminase
Drying the tissue	not destroyed	destroyed
Extraction in water from fresh tissue	extractable	not extractable
Effect of cyanide	not inhibited	inhibited
Effect of octyl alcohol	not inhibited	inhibited
Effect of pyruvate	not inhibited	inhibited
Effect of inorganic salts	inhibited	not inhibited
Optimum pH	8.8	7.4

Bernheim & Bernheim (1932) studied the oxidation of proline by rat liver and monitored the reaction by the reduction of methylene blue and the uptake of oxygen. In a further paper published in 1934 they noted that the oxidation of proline did not result in ammonia formation. They also compared the rate of oxidation of proline and

alanine by various tissue homogenates and concluded that the oxidation of each of these amino acids was catalysed by a different enzyme. However they did not distinguish between the two stereospecific forms of the enzymes nor were they aware of the effect of hydrogen peroxide and the varying catalase content of the tissue on the overall reaction.

Krebs (1935) discussed the deamination of amino acids and he demonstrated quantitatively the existence of the two stereospecific enzymes by the inhibition and extraction studies indicated earlier (Table 1.p 8). He also reported that the two enzymes varied in their activity towards different amino acids of the same configuration.

Whilst investigating the ratio of ammonia formed to oxygen utilized, Krebs noticed that although the expected stoichiometric ratio of 2:1 (Equation 1.p 7) was true in most tissue preparations there were several samples where the ratio was nearly 1:1, notably in trout and frog kidney. He concluded at this stage that the apparent increase in oxygen utilization was due to the presence of a suitable but unknown substrate or the presence of an unknown catalyst. This was in fact the first observation related to the formation of hydrogen peroxide in the reaction.

In the same paper Krebs also investigated extensively the specificity of "d-amino acid deaminase" but did not study the "l-amino acid deaminase" as fully, (Table 2.p 10). He noted that the "d-amino acid deaminase" was present in the kidney and liver of all the vertebrates that he investigated (eleven) and that tissue from the kidney contained approximately four times as much as that from the liver. However he could not demonstrate the enzyme in any other tissues. "l-amino acid deaminase" was found in the renal cortex but only small amounts were present in the liver and intestinal wall.

The following year, Keilin & Hartree (1936) confirmed the findings of Krebs although they debated some of his conclusions, particularly the suggestion that the enzymes functioned differently in tissue slices from the way in which they functioned in tissue extracts. These workers also introduced the term "amino acid oxidase" instead of the term deaminase used by Krebs. A major conclusion from their work was that hydrogen peroxide was formed in the reaction.

TABLE 2

Specificity of the amino acid oxidases - Krebs, H.A.

Date of paper	1935		1951	
Stereospecific nature of enzyme	D	L	D	L
pH of assay	8.3	7.4	8.8	7.2

Amino acid	Enzyme activity expressed as a percentage			
Alanine	100	38	100	
Arginine	33			
Aspartic acid	12	26	3	
Cystine	47		3	15
Glutamic acid	4	144	0	
Glycine	0			
Histidine	10		9	9
Iso-leucine	129		31	71
Leucine	37	100	22	100
Lysine			2	
Methionine	193		125	81
Nor-leucine	86			
Nor-valine	35			
Phenylalanine	121		41	45
Proline			231	77
Serine	60		66	
Tryptophan	12		58	40
Tyrosine	37		296	20
Valine	82	47	55	28

The data above was collected from two papers published by Krebs, in 1935 and 1951 and although the results were reported as oxygen uptake, they have been recalculated as percentages of the oxygen uptake in the presence of the two reference substrates namely D-alanine and L-leucine.

Working initially with uricase they demonstrated that the oxygen to ammonia ratio of 2:1 could be reduced to 1:1 by coupling the reaction to the oxidation of p-phenylenediamine by means of the enzyme peroxidase. The omission of peroxidase resulted in a slow or partial oxidation of the p-phenylenediamine and subsequent oxygen to ammonia ratios between 2:1 and 1:1. A ratio of 1:1 was produced by the use of ethyl alcohol alone which is more readily oxidised than p-phenylenediamine. Keilin and Hartree found it more difficult to demonstrate the formation of hydrogen peroxide in the amino acid oxidase systems due to the inhibitory nature of p-phenylenediamine towards those enzymes.

The subject was further developed in a paper by Bernheim et al. (1936) in which they described the conversion of haemoglobin to methaemoglobin as a test for the presence of hydrogen peroxide. The main finding in this paper was the detection of the oxoacid formed during the reaction. Krebs (1935) had utilized the formation of the dinitrophenylhydrazones to demonstrate the production of oxoacids by D-amino acid oxidase in tissue poisoned by arsenic. Bernheim et al., however, attempted to quantitate the oxoacids so formed by both a gravimetric method involving the phenylhydrazones and a titrimetric method involving the back titration of excess sodium bisulphite with potassium permanganate. They could not however consistently demonstrate the complete formation of the stoichiometric amounts of the oxoacid for all the ten amino acids studied, their results varying from 55% to 100%. They did claim to demonstrate that L-proline was oxidised by their preparation of D-amino acid oxidase.

The conjugated protein nature of D-amino acid oxidase was demonstrated by Warburg & Christian (1938) who identified FAD as the prosthetic group of the enzyme, an observation confirmed by Negelein & Bromel (1939). Most of this earlier work concentrated on the D-amino acid oxidase, due no doubt to its ready availability compared with the L-amino acid oxidase. The discovery by Zeller & Maritz (1944) that snake venom contained L-amino acid oxidase not only provided a more accessible source of this enzyme but also complicated subsequent studies in that the enzymes of snake venom

were later demonstrated to be different from the L-amino acid oxidase of mammalian tissue. Singer & Kearney (1950) demonstrated that snake venom L-amino acid oxidase was a flavoprotein with FAD as the prosthetic group whilst Blanchard et al. (1945) identified FMN as the prosthetic group of the L-amino acid oxidase from rat kidney.

Blanchard et al. (1944) studied the L-amino acid oxidase of rat tissue and clarified the overall nature of the reaction with regard to the formation of hydrogen peroxide. They demonstrated that the hydrogen peroxide was utilized in either the regeneration of oxygen in the presence of catalase or the oxidation of other compounds, particularly the oxoacids. Those oxoacids which were formed from methionine, tryptophan and tyrosine were more susceptible to such an oxidative reaction than other oxoacids. In a similar manner, ethyl alcohol was oxidised by the hydrogen peroxide. These reactions can be summarised thus:

$$RCHNH_2COOH + O_2 \rightarrow RCOCOOH + H_2O_2 + NH_3 \quad \text{..... Equation 2}$$

$$H_2O_2 \rightarrow H_2O + \tfrac{1}{2}O_2$$

$$RCHNH_2COOH + \tfrac{1}{2}O_2 \rightarrow RCOCOOH + NH_3$$

In the same paper, these workers demonstrated an optimum pH of 10.0 for L-amino acid oxidase compared to the present value of pH 7.5. Their studies on the substrate specificity of the enzyme excluded glycine, the L-isomers of threonine and serine, the dicarboxylic amino acids and the dibasic amino acids, lysine, ornithine and arginine.

Krebs (1951) reviewed the nature and characteristics of the amino acid oxidases in an extremely comprehensive paper. He classified the various oxidases in the following way.

General D-amino acid oxidases

1. Mammalian D-amino acid oxidases
2. D-amino acid oxidases of moulds
3. Bacterial D-amino acid oxidases

General L-amino acid oxidases

1. Mammalian L-amino acid oxidases
2. Ophio L-amino acid oxidases
3. L-amino acid oxidases of moulds
4. Bacterial L-amino acid oxidases

Specific amino acid oxidases

1. Specific D-amino acid oxidases
   - aspartic acid
2. Specific L-amino acid oxidases
   - glutamic acid
   - cysteine
   - proline and hydroxyproline
   - phenylalanine and tyrosine
   - histidine
3. Specific ω-amino acid oxidases
   - glycine
   - ornithine
   - lysine

He again reviewed the specificity of the enzymes, the relevent information being incorporated into Table 2.p 10. He noted that the D-amino acid oxidases were present in the liver and kidney of all vertebrates investigated and that the highest concentrations of the enzymes were found in carnivorous animals and the lowest in the herbivorous animals. The only other mammalian source of the enzyme was possibly brain tissue although the levels demonstrated were very low. Krebs discounted both of his previous suggestions, namely that mammalian D-amino acid oxidase was a fragment of the L-amino acid oxidase and that the resolution of racemic mixtures of amino acids was a possible role of the enzyme but he could offer no satisfactory alternative suggestion for its physiological role.

The study of the amino acid oxidases then moved into another stage in which the elucidation of the reaction mechanism and the

development of a sensitive method of assay became major objectives, Meister et al. (1960). These authors discussed at some length the possible physiological roles of the enzymes and suggested that L-amino acid oxidase might provide a route for the formation of oxoacids. In effect this would be an alternative to the transamination reaction and it was envisaged that the L-amino acid oxidase would act in a similar manner to that of glutamate dehydrogenase

Transamination

a) alanine amino transferase

2-oxoglutarate + amino acid → L-glutamate + oxoacid

b) glutamate dehydrogenase

L-glutamate + NAD → 2-oxoglutarate + NH_3 + NADH

c) L-amino acid oxidase

L-amino acid + oxygen → oxoacid + NH_3

They also suggested that the possible metabolism of amino acids such as lysine, the transamination of which had not been demonstrated, could be facilitated by an L-amino acid oxidase system.

The same authors discussed several possible roles for the D-amino acid oxidase of mammalian tissues but considered the most likely to be the elimination or utilisation of D-amino acids. These amino acids may have been ingested or formed in the intestinal tract by bacterial action and subsequently absorbed by the animal. The possibility of a natural substrate for D-amino acid oxidase not yet discovered was also suggested. They could not demonstrate any activity of the enzyme preparation with dicarboxylic amino acids, nor with dibasic amino acids (except lysine which showed only slight activity). They did find some activity with glycine and claimed that maximal activity was demonstrated with proline.

It is evident that whilst there was broad agreement on the

conditions necessary for maximal enzyme activity there were some discrepancies on the question of enzyme specificity. This subject is discussed later in this thesis in relation to the data presented in the study of the amino acid oxidases.

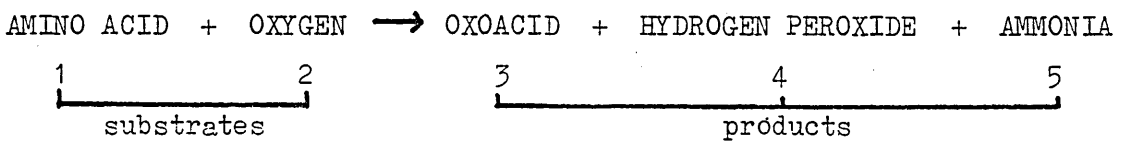
Review of amino acid oxidase methodology

The changing approach to the assay of the amino acid oxidases which reflects among other things, the advances made in analytical techniques over the years particularly during the period 1960 to 1970 is apparent from the following summary.

Date	Technique
1934	Warburg manometry
1944	Kjeldahl distillation
1960	U.V. spectrophotometry
1960	Colorimetry
1964	Oxygen polarography
1964	Enzyme inhibition
1966	Fluorimetry
1967	Coupled fluorimetry
1971	Vibrating electrode polarography

In attempting to review the large number of methods that have been described for the assay of the amino acid oxidases, some difficulty was experienced in deciding upon a suitable classification system. The methods could be grouped according to the technique used to monitor the reaction as indicated above but, in addition, some consideration had to be given to the kinetic basis of the method employed, at least in the simplest terms of being either a kinetic or fixed time assay. The flexibility of the method in terms of the range of amino acids that could be used as substrates and also the sensitivity of the method were aspects that also had to be considered.

An examination of the reaction catalysed by the amino acid oxidases indicated several possible parameters that could be monitored in quantitative assay:



Hence for the purpose of this thesis, the methods have been classified on the basis of the component of the chemical reaction which is quantitatively monitored.

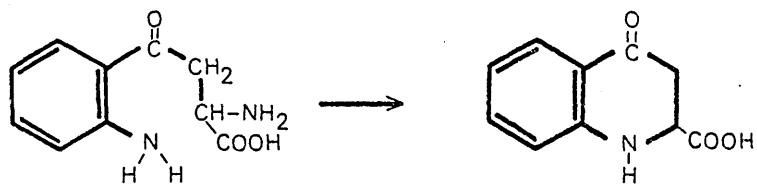
1. Variable substrate - amino acid
2. Obligatory substrate - oxygen
3. Variable product - oxoacid
4. Obligatory product - hydrogen peroxide
5. Obligatory product - ammonia

1. Variable substrate - amino acid or derivative.

Amino acids in general do not show any physicochemical characteristic that would lend itself to a specific monitoring technique. Many of the methods which have been described have utilized an amino acid analogue which showed a particular spectral characteristic which was suitable for use in a quantitative method. Such methods obviously did not lend themselves to a critical study of the catalytic nature of the enzymes but were suitable as a convenient method of detection and quantitation. They also had the disadvantage of being suitable for only one of the enzymes and hence two different assay methods would have been required for the present study.

Weissbach et al. (1960) described a method for the assay of L-amino acid oxidase which utilized the conversion of L-kynurenine (I)

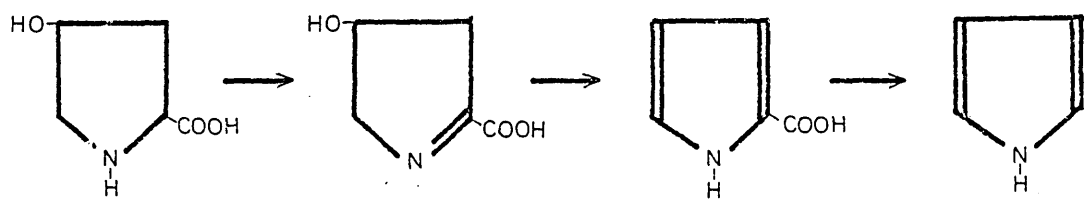
(absorbance maximum 360 mm) to kynurenic acid (II) (absorbance maximum 331 mm).



I II

They proposed that the enzyme reaction could be followed by monitoring the increase in absorbance at 331 nm and they quoted the Km for L-amino acid oxidase as 2.0×10^{-4} mol l^{-1} but, due to substrate inhibition, they used a substrate concentration of 5.0×10^{-4} mol l^{-1}. An additional problem lay in the high absorbance at 331 nm due to the presence of substrate which in the relatively high concentration used resulted in a high blank reading.

Corrigan et al. (1963) described a method for the determination of D-amino acid oxidase utilising D-allohydroxyproline (III) which was oxidised to Δ' pyrroline-4-hydroxy-2-carboxylic acid (IV) by the enzyme. This was subsequently converted to pyrrole (VI) via the intermediate pyrrole-2-carboxylic acid (V) under acid conditions, and the pyrrole so formed condensed with p-dimethylamino-benzaldehyde (PAB) giving a red coloured complex which had an absorbance maximum at 550 nm.



III IV V VI

The method as described was fairly lengthy involving thirty minutes incubation for the catalysed reaction, heating at 70°C in acid for reactions IV to VI followed by ten minutes for the reaction with PAB to reach a maximum. Although most of their work was with the D-amino acid oxidase of insect tissue they did demonstrate the presence of enzyme in the liver and kidney of guinea pig.

The conversion of D-phenylglycine to benzoylformic acid (absorbance maximum 253 nm) was proposed as an assay method for D-amino acid oxidase by Fonda & Anderson (1967). Using a pyrophosphate buffer pH 8.5 containing D-phenylglycine the increase in absorbance at 253 nm was monitored after initiating the reaction by the addition of the enzyme. After a lag period of increasing reaction velocity a constant maximum velocity was attained after about six minutes. The main difficulty, apart from the use of a substrate analogue, was the cost of an instrument that functioned effectively at 253 nm.

2. Obligatory substrate - oxygen.

It is not absolutely correct to refer to oxygen as an obligatory substrate since it has been demonstrated that some other hydrogen acceptors can be used. Dixon & Kleppe (1965b) demonstrated that whilst the amino acid oxidases are very specific for oxygen, methylene blue and dichlorophenolindophenol show slight activity as alternative acceptors and in fact methylene blue reduction had been used in an assay method by Bernheim & Bernheim (1932). L-amino acid oxidase can utilize, in addition to these compounds, ferricyanide as an electron acceptor, Marcus & Feeley (1962).

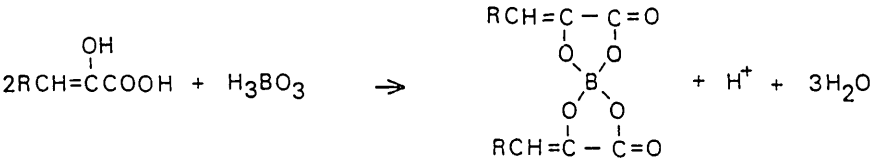
The majority of the original work on the amino acid oxidases utilized the basic gasometric characteristics of the reaction by employing Warburg manometric techniques. The work of Krebs with this method was particularly impressive. A difficulty in the correlation of results from different workers, in addition to the variations in pH and temperature which were used, was the varying extent to which catalase was present in the reaction system; this resulted in

considerable variations in oxygen uptake. Warburg manometry obviously presented technical problems and although the use of the Gilson differential respirometer utilising volumetric measurements considerably simplified the technique and even introduced the possibility of a chart recording system, it could not improve the inherent low sensitivity of gasometric measurements.

A paper by Clark (1956) describing an oxygen electrode led to several workers adapting the electrode for use in monitoring amino acid oxidase reactions. Dixon & Kleppe (1965a) described a closed system using a membrane electrode and reagents saturated with air. Catalase was excluded from the system and they demonstrated that hydrogen peroxide had no effect on the oxoacid formed under the conditions of the assay. They also demonstrated that the effect of pH on the decomposition of hydrogen peroxide was not significant below pH 11. Luppa & Aurich (1971) described a vibrating platinum electrode system in an open vessel which they claimed was more sensitive and showed a quicker response than the original electrode.

3. Variable product - oxoacid.

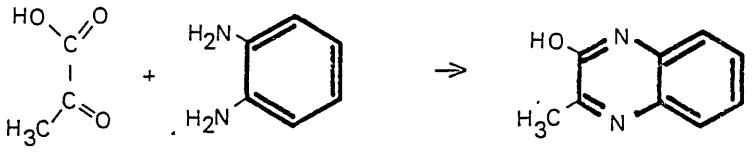
Bernheim et al (1936) had tried to measure the amount of oxoacid formed in the reaction by gravimetric and titrimetric methods without a great deal of success. However Knox & Pitt (1957) whilst studying a complex (VIII) which was formed between borate and oxoacids and which showed a high absorbance in the ultraviolet, demonstrated that the reaction involved the enol tautomer of the oxoacid (VII) and that the normal keto-enol equilibrium could be displaced to the enol by the presence of either an enzyme which they called a tautomerase or arsenate ions.



VII VIII

This information was utilized by Wellner & Lichtenberg (1971) who used phenylalanine as the substrate and, after a fixed time of incubation, complexed the phenylpyruvate which was formed with borate in the presence of arsenate ions. Catalase was incorporated in the original mixture to protect the oxoacid from the hydrogen peroxide which was also formed during the reaction. The concentration of the complex was subsequently monitored at 300 nm but unfortunately the authors quoted the enzyme activity in arbitrary units rather than in international units.

Verity et al. (1967) utilized a condensation reaction between pyruvic acid and o-phenylenediamine in acid solution to produce 2-hydroxy-3-methylquinoxaline(IX) which fluoresces strongly (excitation maximum 375 nm, emission maximum 480 nm) when heated at 60°C in the presence of concentrated sulphuric acid.



IX

Soda (1967) published a similar method which involved the complexing of the oxoacid formed after 45 minutes incubation, with 3-methyl-2-benzothiazolone hydrazone hydrochloride by heating at 50°C and pH 5.0. The actual wavelength used to quantitate the complex varied depending upon the actual oxoacid involved but was usually between 316 nm and 325 nm.

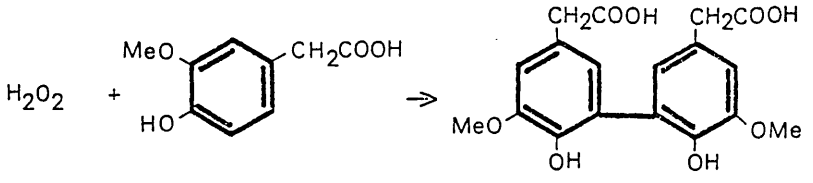
The methods involving the assay of the oxoacids were obviously very complex and also show a major disadvantage in being fixed time assays and were either restricted to a specific substrate or showed variable absorption characteristics depending upon the actual substrate used.

4. Obligatory product - hydrogen peroxide.

Despite the fact that initially hydrogen peroxide was the most obscure of the products, its chemical reactivity led to the development of several very novel assay methods. Margoliash & Novogrodsky (1958) found that catalase was irreversibly inhibited by hydrogen peroxide in the presence of 3-amino-1,2,4-triazole and this fact was utilized by Scannone et al (1964) in a method which related the degree of inhibition of catalase to the amount of hydrogen peroxide and hence to the activity of the amino acid oxidase. A buffered reaction mixture containing catalase, 3-amino-1,2,4-triazole, amino acid oxidase and an amino acid substrate was incubated for sixty minutes and the activity of the remaining catalase was assessed by its ability to hydrolyse perborate, the residual perborate being back titrated with potassium permanganate.

The method was claimed to be very sensitive but there were obviously many factors that had to be carefully controlled. The authors also expressed concern over the relatively slow rates of the inhibition reaction and the discrepancies that might occur due to the rapid formation of hydrogen peroxide.

Guilbault & Heiserman (1968) described a coupled fluorimetric assay which utilized the oxidation of homovanillic acid (X) by hydrogen peroxide and peroxidase to the highly fluorescent 2,2'-dihydroxy-3,3'-dimethoxybiphenyl-5,5'-diacetic acid (XI) (excitation maximum 315 nm, emission maximum 425 nm).



X XI

Although the method was claimed to be very sensitive, no explanation was given for the considerable difference in sensitivity for D-amino acid oxidase (1.2 $i.u.l^{-1}$) and the L-amino acid oxidase (0.03 $i.u.l^{-1}$).

A similar fluorimetric method was described by Lichtenberg & Wellner (1968) in which the oxidation of the fluorescent compound scopoletin by hydrogen peroxide resulted in the formation of a non-fluorescent product. The method involved raising the pH of the reaction mixture to 10 in order to measure the fluorescence (excitation maximum 395 nm, emission maximum 470 nm) and so resulted in a fixed time assay rather than a kinetic assay.

All of these methods involving hydrogen peroxide have the advantage of utilizing all amino acids as potential substrates and were claimed to be very sensitive, but the fact that most were fixed time assays was a disadvantage and there was some doubt as to the precision obtainable by these methods.

5. Obligatory product - ammonia.

The fundamental physical characteristics of ammonia, namely its stability and solubility, and the fact that it is a product common to the oxidation of all amino acids made it very suitable as the basis of many assay methods, although imino acids such as proline do not react in this manner. Blanchard et al (1944) used a micro-Kjeldahl technique to monitor the rate of ammonia formation but this was obviously a laborious method and relatively insensitive. Nagatsu & Yagi (1966) described a colorimetric method utilizing the formation of indophenol (absorbance maximum 610 nm) a reaction described initially by Lubochinsky & Zalta (1954). The enzyme and substrate reaction mixture was incubated for sixty minutes and although the method was relatively sensitive the presence of some components of the reaction mixture resulted in inhibition of the colour development.

The value of the formation of ammonia became much more significant in the search for a suitable assay method for the amino acid oxidases when the potential of the glutamate dehydrogenase system

was realized. In the method developed in this thesis, the ammonia formed during the oxidation of the amino acid is coupled by the enzyme glutamate dehydrogenase to the oxidation of the coenzyme NADH to NAD^+. The spectral properties of these coenzymes are such that the fall in concentration of NADH can be monitored by the fall in absorbance at 340 nm. with no interference from the NAD^+ which is simultaneously formed. The method described is a kinetic method which permits the use of any potential substrate of the amino acid oxidases provided that one of the products is ammonia, and utilizes a recording spectrophotometer with a thermostatically controlled cell holder. Absorbance changes at 340 nm are monitored and the rate of change of NADH concentration calculated from the rate of change of absorbance using the molar absorbance coefficient of NADH.

Amino acid oxidase reaction:

$$RCHNH_2COOH + O_2 \rightarrow RCOCOOH + H_2O_2 + NH_3$$

Glutamate dehydrogenase reaction:

$$NH_3 + HOOCCH_2CH_2COCOOH + NADH \rightarrow HOOCCH_2CH_2CHNH_2COOH + NAD^+$$

The fluorimetric method also described in this thesis is a modification of the kinetic method in which the extent of oxidation of NADH is measured fluorimetrically after the initial reaction has proceeded for one hour. Excess NADH is destroyed by the addition of acid and the remaining NAD^+ is converted to a fluorescent derivative (excitation maximum 365 nm, emission maximum 455 nm) by heating in an alkaline solution, the fluorescent derivative being stabilized by the presence of imidazole.

Some of the methods reviewed are listed in Table 3.1 24 which also indicates the major features of each method. The kinetic method described in this thesis is an extremely reliable, precise and technically simple method which shows a sensitivity which is better than most. The fluorimetric method which was designed as a back up method shows an increase in sensitivity over the kinetic method.

TABLE 3

Comparison of some methods available for the assay of the amino acid oxidases.

Author	Specificity	Technique	Sensitivity (lowest measurable activity in i.u. l^{-1})	Time of assay (min)	Substrate range
Blanchard (1944)	D-, L-	Manometric	90	V	General
Corrigan (1963)	D-	Colorimetric	0.17	60	Restricted
Scannone (1964)	D-, L-	Titrimetric	0.03	60	General
Luppa (1971)	L-	Polarographic	0.6	K	General
Verity (1967)	D-	Fluorimetric	1.3	15	Restricted
Lichtenberg (1968)	D-, L-	Fluorimetric	0.3	15	General
Guilbault (1968)	D-, L-	Fluorimetric	0.05	K	General
Weissbach (1960)	L-	Spectrophotometric	0.5	K	Restricted
Dunn (1963)	D-	Spectrophotometric	0.17	30	Restricted
Fonda (1967)	D-	Spectrophotometric	138	K	Restricted
Soda (1968)	D-	Spectrophotometric	5	10	General
Wellner (1971)	D-, L-	Spectrophotometric	30	15	Restricted
Present	D-, L-	Spectrophotometric	1.0	K	General
Present	D-, L-	Fluorimetric	0.05	60	General

By " General " it is implied that the method can be used with all amino acids as substrates and by " Restricted " it is implied that the method can only be used with one or two amino acids or amino acid analogues.

A variable time of assay is indicated by V and kinetic assays are indicated by K otherwise the time of assay is indicated.

CHAPTER 1

DEVELOPMENT OF THE ASSAY METHODS

Introduction.

The spectral properties of the NAD(P) coenzymes were first suggested by Warburg & Christian (1938) as a suitable means of monitoring enzyme reactions which involved these coenzymes. From this suggestion Negelein & Haas (1935) described a method for the determination of glucose-6-phosphate based on the increase in absorbance in the near ultraviolet due to the formation of NADH during the reaction. Subsequently many coupled assays have been described in which the reaction under investigation is linked to the oxidation or reduction of one of these coenzymes in an indicator reaction.

The enzyme glutamate dehydrogenase was used by Kirsten et al (1963) for the determination of ammonia by monitoring the fall in absorbance at 340 nm due to the oxidation of NADH. Previous experience in applying this technique to other assays provided the basis for the development of the present assay method for the amino acid oxidases, Ellis & Goldberg (1970).

In attempting to establish a coupled assay method for the amino acid oxidases it was necessary to elucidate the optimal conditions for each of the three enzymes involved and to assess to what extent the defined conditions were compatible for each of the coupled reactions. It was also necessary to establish what activity of glutamate dehydrogenase was required in order that this indicator reaction would not be rate limiting under any possible assay conditions.

Subsequent to the development of the kinetic assay method it was felt desirable to develop a more sensitive method for the assay of enzyme activities which were near the sensitivity limit of the kinetic assay, in order to demonstrate the presence of very low enzyme activities in samples which might otherwise be reported as

negative. The fluorescent properties of the pyrimidine coenzymes had previously been used in quantitative determinations by Theorell et al (1954) and they had also been extensively investigated by Lowry & Passoneau (1972) who had clarified the effects of solvents, pH and light on the stability of the fluorescence. It was on the basis of this information that the present fluorimetric method was developed.

Experimental details.

1. Glutamate dehydrogenase.

1.1. Stability of the enzyme.

The enzyme glutamate dehydrogenase was the key to the coupled assay and it was essential for the indicator reaction to be as efficient as possible in order for valid kinetic studies to be undertaken on the amino acid oxidases. Di Prisco & Strecker (1966), had reported that glutamate dehydrogenase showed instability at pH 8.0 in aqueous solution and that this evidenced itself as a loss of catalytic activity. As a result of their investigations they concluded that the ionic species present in solution and the pH of the solution altered the protein structure of glutamate dehydrogenase in such a way as to have a marked effect on the catalytic activity of the enzyme. In studying solutions of the enzyme at pH 7.0, Ellis & Goldberg (1970), noted that there was a slow precipitation of the protein, which they monitored at 340 nm. Their investigations revealed that the rate of precipitation increased at higher pH

values and also in the presence of NADH and 2-oxoglutarate. They also noticed that NADH itself was unstable in acid solutions and that even at pH 7.5 the rate of auto-oxidation was small but significant.

In devising a method of assay for the amino acid oxidases, it was decided to study initially, the stability of glutamate dehydrogenase in the presence of substrates which would have to be present in the final assay and to assess the instability of the enzyme in terms of the extent of precipitation, correlating this with any simultaneous loss of activity. It was appreciated that turbidimetric methods were more sensitive when radiation from the blue region of the visible spectum was used due to the fact that shorter wavelengths show a greater degree of scattering than do the longer wavelengths. However because a wavelength of 340 nm would show any variation due to the instability of NADH, it was decided to monitor the extent of precipitation at 400 nm, where neither NAD^+ nor NADH showed any significant absorbance.

Using barbitone buffer at pH 8.0 and incorporating NADH and 2-oxoglutarate, the turbidity of the solution was monitored at 400 nm after the addition of glutamate dehydrogenase. An aliquot of 3.0 cm^3 was removed at 0, 15 and 30 minutes and the catalytic activity assessed by the rate of fall in absorbance at 340 nm, after initiating the reaction by the addition of 0.1 cm^3 of a solution containing 10 mmol l^{-1} of ammonia. The results showed that there was a parallel between the extent of precipitation and the loss of catalytic activity and under the conditions of the test approximately 80% of the enzymic activity was lost after 30 minutes, correlating with an increase in absorbance of 0.34 at 400 nm. The data reported was a mean of two observations (Table 4, p 29).

Following the conclusions of Di Prisco & Strecker (1966) regarding the effect of ionic species on the stability of glutamate dehydrogenase, it was decided to study the effectiveness of various buffers in maintaining the enzyme in a stable form. Four buffers were selected on the basis of previous work by Ellis & Goldberg (1972a) but bearing in mind that pH conditions would probably be different in the final assay from those used by these authors. The buffers

Table 4

The effect of precipitation on the activity of glutamate dehydrogenase

TIME	ABSORBANCE 400 nm	REACTION RATE $\Delta E_{340\ nm}\ min^{-1}$	ACTIVITY %
0	0	0.43	100
15	0.13	0.26	60
30	0.34	0.09	21

PROTOCOL

Reagent	Concentration	Volume (cm^3)
Barbitone buffer pH8.0	0.1 mol l^{-1}	25.0
2-oxoglutarate	0.2 mol l^{-1}	2.0
NADH	2.0 mmol l^{-1}	2.0
GDH		0.2
Monitor absorbance at 400 nm. Remove aliquots of 3.0 cm^3 at the required times and initiate the enzymic reaction by the addition of		
Ammonia	10 mmol l^{-1}	0.1
Monitor absorbance at 340 nm.		

selected were barbitone (5,5'-diethyl barbituric acid, pKa 7.98), tris(hydroxymethyl)aminomethane, pKa 8.3), Tricine (N-tris(hydroxymethyl)methylglycine, pKa 8.15), and glycylglycine (pKa 8.4).

The stability of the enzyme was studied in these buffers over the pH range 7.0 - 9.0 using the extent of precipitation in a 30 minute period as an assessment of instability, and again incorporating both NADH and 2-oxoglutarate in the system. It was demonstrated that the enzyme showed the greatest instability at around pH 8.0 but that this instability was least in the glycylglycine and Tricine buffers (Figure 1, p 31).

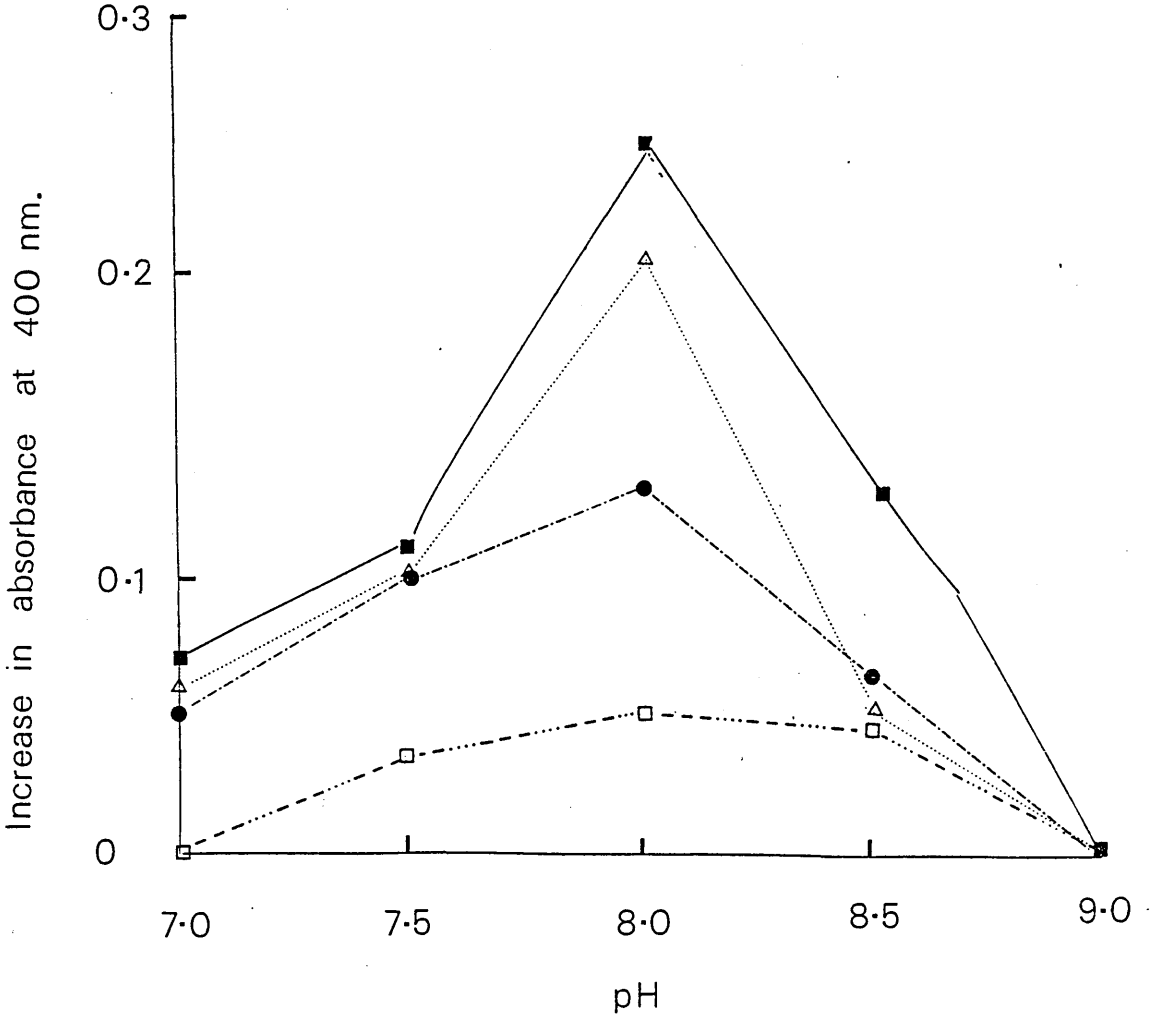
Di Prisco & Strecker (1966) had also noted that the enzyme is stabilized by the addition of some inorganic ionic compounds and they found that potassium phosphate (10 mmol l^{-1}) and ammonium sulphate (5.0 mmol l^{-1}) showed an appreciable effect. However the most effective stabilizing agent was the nucleotide ADP (0.5 mmol l^{-1}). Ellis & Goldberg (1972b) similarly confirmed the stabilizing effect of ADP and stated that the concentration of the stabilizing agent required depended upon the concentration of glutamate dehydrogenase involved. They demonstrated that concentrations of 0.3 mmol l^{-1} ADP and 1.0 mol l^{-1} NaCl were required to stabilize 0.01 cm^3 of the Böehringer preparation of glutamate dehydrogenase.

It was realised that the nucleotide FAD might be required to optimise the conditions for the amino acid oxidases and it was decided to investigate the possible effects of the flavin nucleotides on glutamate dehydrogenase and at the same time attempt to confirm the findings of Ellis and Goldberg. The use of ammonium salts in stabilizing the enzyme, as suggested by Di Prisco and Strecker, was obviously inappropriate due to the fact that ammonia was the linking substrate between the two coupled reactions.

The results of the investigation confirmed that the incorporation of 0.3 mmol l^{-1} ADP and 1.0 mol l^{-1} NaCl into all the four buffers studied showed stabilization of the glutamate dehydrogenase by the turbidometric method. No such stabilizing effects were shown by the flavin nucleotides at these concentrations.

1.2. Optimum pH of the enzyme.

Instability of glutamate dehydrogenase



The turbidity due to the precipitation of the enzyme was measured as the increase in absorbance at 400 nm in each of the four buffers using the method indicated below.

■——■ barbitone. △·····△ tris
●-·-·● Tricine □-··-□ glycylglycine

Protocol

Reagent	Concentration	Volume (cm^3)
Buffer	0.1 mol l^{-1}	2.6
2-oxoglutarate	0.2 mol l^{-1}	0.2
NADH	2.0 mmol l^{-1}	0.2
GDH		0.02

Measure absorbance at 400 nm after 30 minutes.

Although the optimum pH for glutamate dehydrogenase has been generally reported to be pH 8.0, Barman (1969), some workers have reported values of pH 8.5-8.6, Strecker, (1955), and pH 7.4, Ellis & Goldberg, (1972b). The fact that there are several sources of this enzyme e.g. corn, bovine liver, frog liver, and human serum, together with the fact that different buffers were used by these workers, may have caused the apparent discrepancies. In the light of these statements it was decided to clarify the optimum pH in the two most suitable buffers.

A series of solutions containing 0.1 mol l^{-1} of the selected buffers, glycylglycine and Tricine, were prepared and the pH values adjusted by the addition of 1.0 mol l^{-1} NaOH to give a range of values from pH 7.0 to pH 9.0. The activity of 0.01 cm^3 of glutamate dehydrogenase was measured in each buffer by monitoring the oxidation of NADH after initiating the reaction by the addition of ammonia as indicated earlier (p 28.). The results confirmed an optimum pH of 8.0 and demonstrated greater activity in the glycylglycine buffer than in the Tricine buffer. (Figure 2, p33).

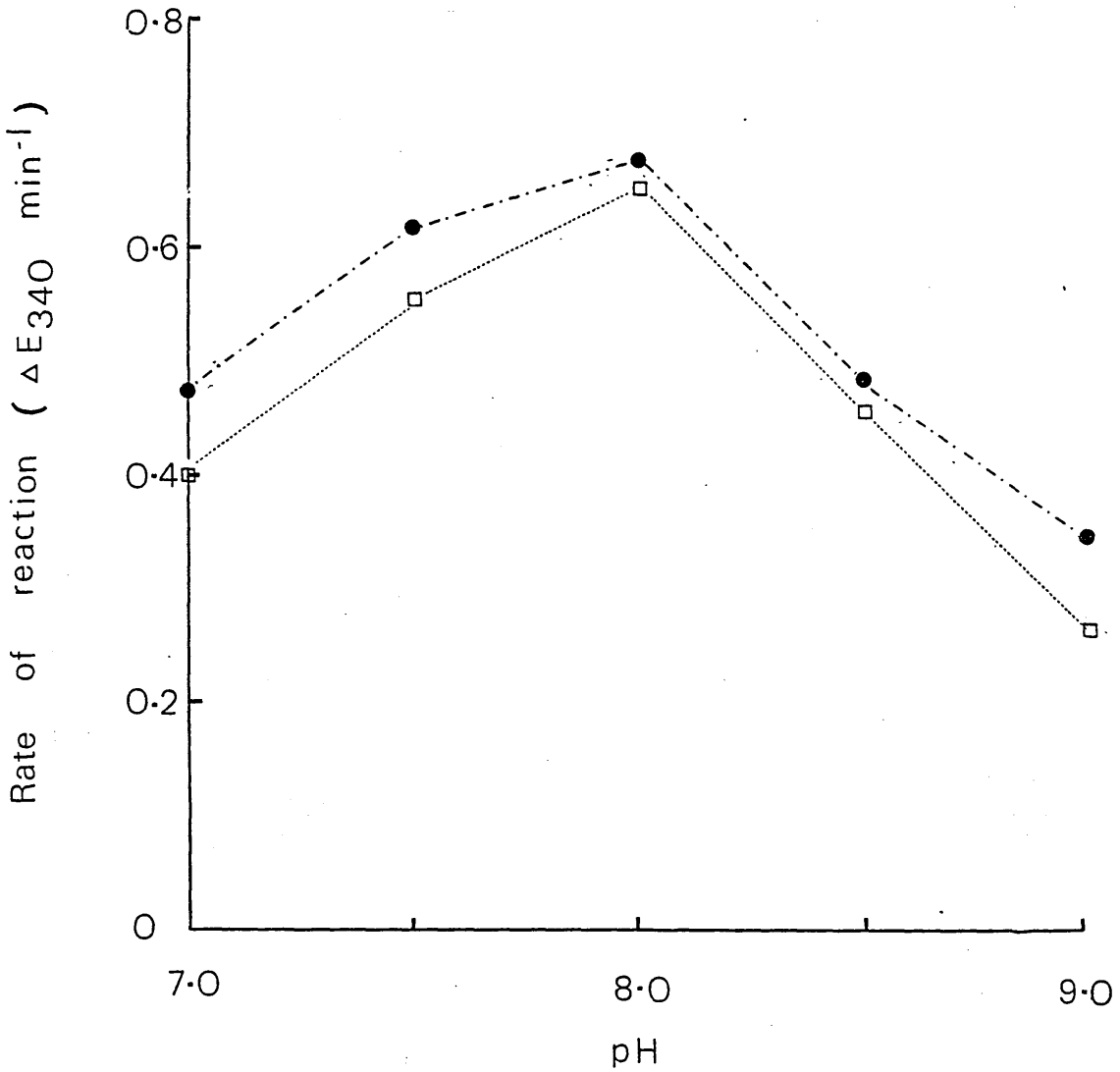
1.3. Activation of the enzyme.

There was considerable evidence that ADP, as well as having a stabilising effect of glutamate dehydrogenase, also had an activating effect, Ellis & Goldberg (1970). These authors also reported an activating effect of adenosine and AMP, although the effectiveness of these compounds was considerably less than that of ADP. The same authors (1972b) demonstrated that L-leucine also had an activating effect although in the present study the use of this compound was obviously inappropriate. Evidence that the activating effect of nucleotides on some enzymes could be competitively inhibited by other nucleotides, (Fonda & Anderson, 1968), suggested that the effect of ADP should also be assessed in the presence of the flavin nucleotides.

The experimental results (Table 5. p 34.) confirmed the activating effect of ADP and demonstrated that, in the absence of ADP, the nucleotide FMN was inhibitory to glutamate dehydrogenase especially in glycylglycine and Tricine. However in the presence

Figure 2

Optimum pH of glutamate dehydrogenase



The activity of glutamate dehydrogenase was measured as the rate of fall in absorbance at 340 nm in two buffer solutions using the method indicated below.

□·····□ Tricine ●-·-·-● glycylglycine

PROTOCOL

Reagent	Concentration	Volume (cm^3)
Buffer	0.1 mol l^{-1}	2.5
2-oxoglutarate	0.2 mol l^{-1}	0.2
NADH	2.0 mmol l^{-1}	0.2
GDH		0.01
Monitor absorbance at 340 nm and initiate the reaction by the addition of		
Ammonia	10 mmol l^{-1}	0.2

Table 5

Effect of various co-enzymes on the activity of glutamate dehydrogenase

Buffer pH 8.0	Rate of reaction ($\Delta E_{340\ nm}\ min^{-1}$)					
	Coenzyme					
	None	ADP	FMN	FAD	ADP+FAD	ADP+FMN
Barbitone	0.10	0.44	0.14	0.20	0.37	0.38
Tris	0.10	0.42	0.06	0.06	0.35	0.40
Tricine	0.34	0.68	0.20	0.38	0.43	0.66
Glycylglycine	0.26	0.66	0.08	0.24	0.43	0.66

PROTOCOL

Reagent	Concentration	Volume (cm^3)
Buffer	0.1 mol l^{-1}	2.4
2-oxoglutarate	0.2 mol l^{-1}	0.2
NADH	2.0 mmol l^{-1}	0.2
GDH		0.01
Coenzyme	10.0 mmol l^{-1}	0.1
Monitor the absorbance at 340 nm and initiate the reaction by the addition of		
Ammonia	10.0 mmol l^{-1}	0.1

of 0.3 mmol l^{-1} ADP it showed no inhibitory effect on the activity of the enzyme. The other flavin nucleotide studied, FAD, showed no inhibitory effect but it did reduce the activating influence of ADP to some extent. It was shown that the activating effect of ADP was progressive as the concentration of ADP was raised giving a maximum effect at a concentration of about 0.5 mmol l^{-1} (Table 6, p 36).

1.4. Effect of NADH concentration.

As indicated earlier, Ellis & Goldberg, (1970) had noticed that the presence of NADH increased the instability of glutamate dehydrogenase, whilst Yielding et al. (1964) stated that NADH showed substrate inhibition at concentrations above 0.4 mmol l^{-1}. In considering this report it was calculated that the stated concentration of NADH would give an absorbance at 340 nm of approximately 2.4 and it was felt that at this high value, the Beer-Lambert relationship might not hold true.

A series of dilutions of NADH were prepared containing from 0.05 mmol l^{-1} to 0.75 mmol l^{-1} and the absorbance of each solution at 340 nm was measured. A plot of the data (Figure 3. p 37.) revealed that the linear relationship between concentration and absorbance at 340 nm was only experimentally valid up to an absorbance value of 1.5 or a concentration of NADH of approximately 0.24 mmol l^{-1}.

A study of the effect of NADH concentration on the rate of change of absorbance at 340 nm resulting from the presence of a fixed amount of glutamate dehydrogenase demonstrated an apparent inhibition above a concentration of NADH of 0.13 mmol l^{-1} (Figure 4, p38). In order to compensate for the invalidity of the Beer-Lambert relationship at high concentrations of NADH, the change in NADH concentration during the first minute of the reaction was calculated and expressed in mmol l^{-1} min^{-1} and used as a measure of reaction rate. This was done by converting the zero time and one minute absorbance values into terms of NADH concentration using the NADH calibration curve and calculating the difference between the two values.

Effect of ADP on the activity of glutamate dehydrogenase

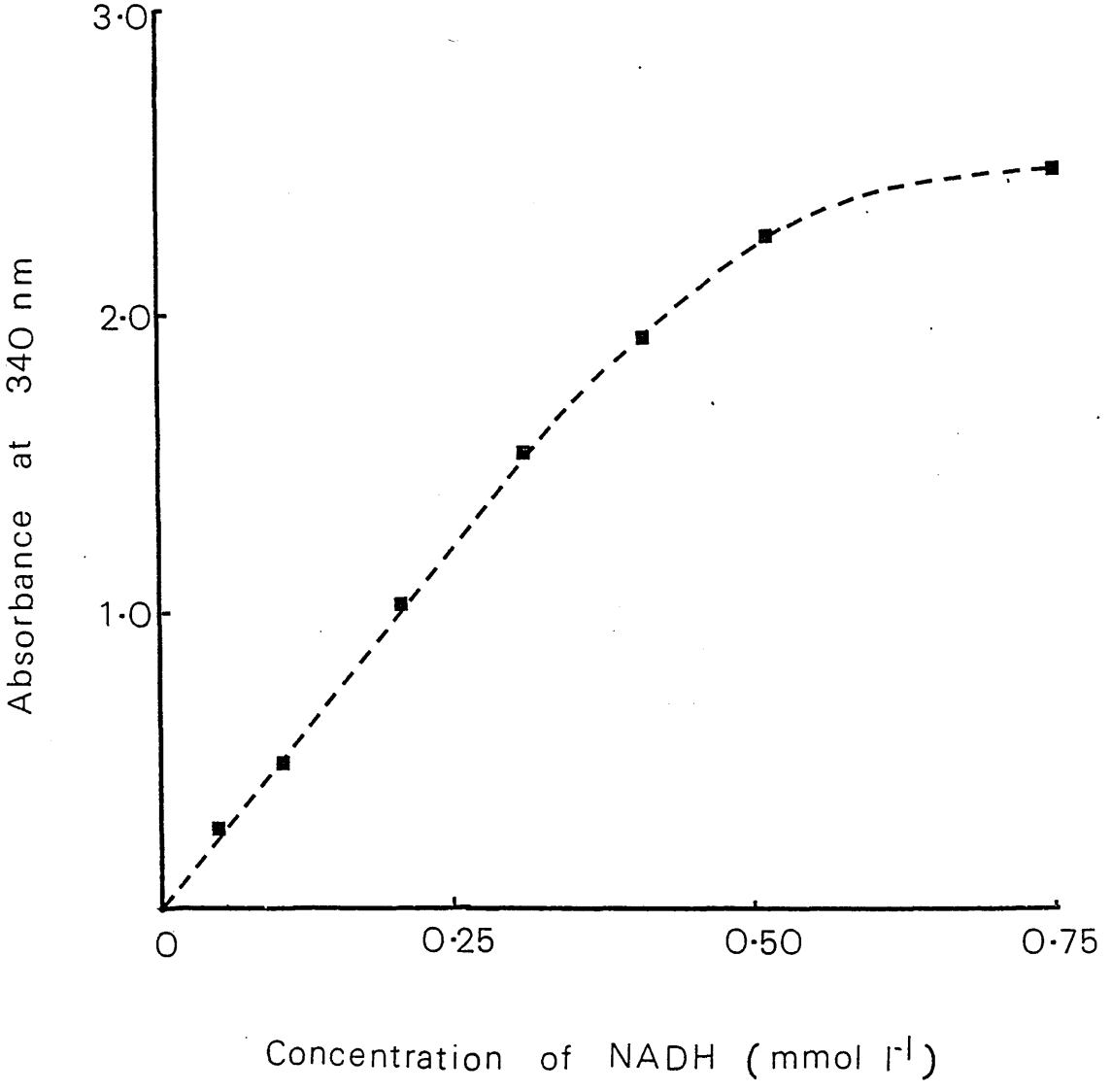
Final Concentration ADP (mmol l^{-1})	Rate of reaction ($\Delta E_{340\ nm}\ min^{-1}$)
0	0.04
0.001	0.03
0.005	0.03
0.01	0.04
0.05	0.07
0.10	0.09
0.25	0.10
0.50	0.11
0.75	0.12
1.00	0.12
5.00	0.12

PROTOCOL

Reagent	Concentration	Volume (cm^3)
Glycylglycine buffer	0.1 mol l^{-1}	2.4
2-oxoglutarate	0.2 mol l^{-1}	0.2
NADH	2.0 mmol l^{-1}	0.2
GDH		0.01
Coenzyme		0.1
Monitor absorbance at 340 nm and initiate the reaction by the addition of		
Ammonia	10.0 mmol l^{-1}	0.1

Figure 3

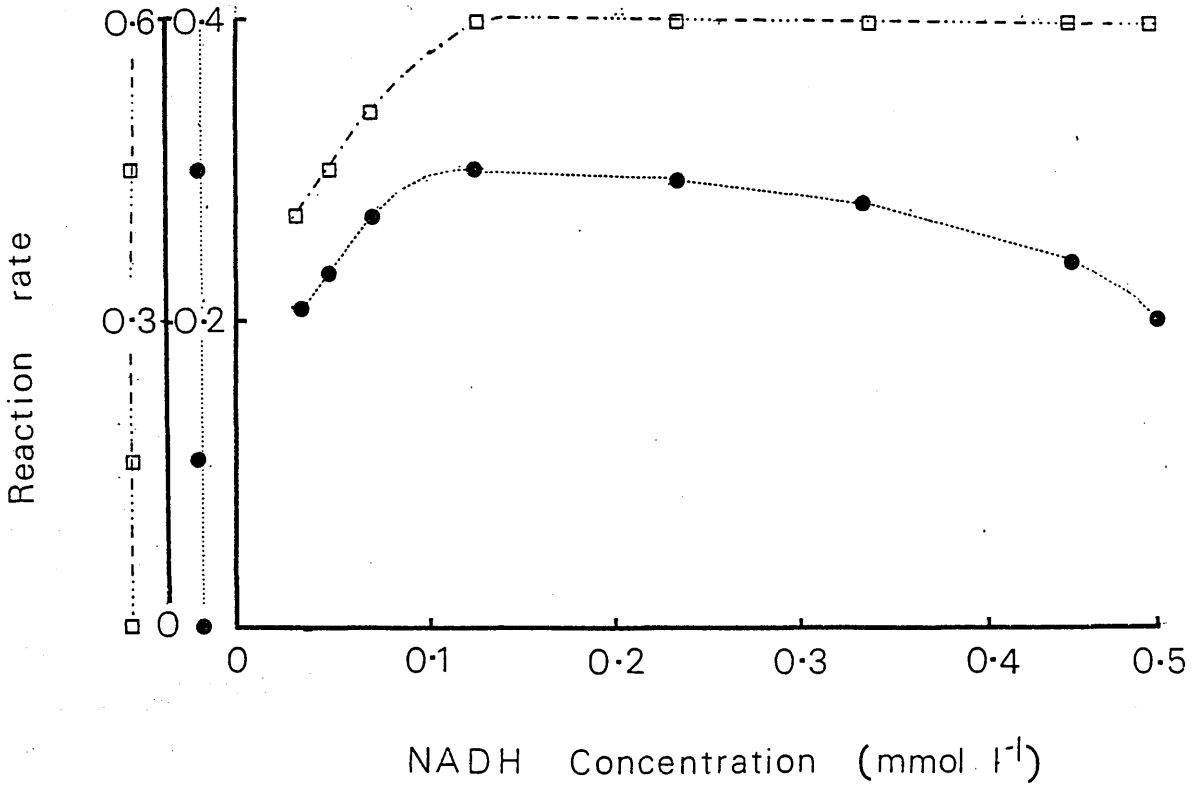
Beer-Lambert relationship for NADH



Concentration of NADH (mmol l^{-1})	Absorbance 340 nm
0.05	0.26
0.10	0.48
0.20	1.02
0.30	1.52
0.40	1.93
0.50	2.25
0.75	2.47

Figure 4

Apparent inhibition of glutamate dehydrogenase by NADH



An apparent inhibitory effect of high concentrations of NADH was demonstrated when the observed rate of reaction was used. ($\Delta E_{340\ nm}\ min^{-1}$ ●·····●). This effect was not demonstrated when the rate of reaction was calculated. (ΔNADH mmol min^{-1} □-·-·-□)

PROTOCOL

Reagent	Concentration	Volume (cm^3)
Glycylglycine pH 8.3	0.1 mol l^{-1}	2.4
2-oxoglutarate	0.2 mol l^{-1}	0.2
ADP	15.0 mmol l^{-1}	0.1
NADH		0.2
GDH		0.01
Monitor absorbance at 340 nm and initiate the reaction by the addition of		
Ammonia	10.0 mmol l^{-1}	0.1

These data were used instead of the rate of change in absorbance and the resulting graph (Figure 4. p 38.) showed no inhibition effect but the reaction showed maximum velocity at concentrations of NADH above 0.13 mmol l^{-1}.

The results of the investigations described above are summarised below.

GLUTAMATE DEHYDROGENASE.

A summary of optimal assay conditions and inhibitors.

Characteristic	Reagent	Concentration
Optimum pH	8.0	
Optimum buffer	glycylglycine	0.1 mol l^{-1}
Stabilizer	ADP	0.3 mmol l^{-1}
	Sodium chloride	1.0 mol l^{-1}
Activator	ADP	0.5 mmol l^{-1}
Inhibitor	FMN	0.3 mmol l^{-1}

2. D-amino acid oxidase.

2.1. Optimum pH of the enzyme.

The main commercial source of the enzyme D-amino acid oxidase is from hog kidney and this has been fairly extensively studied using a variety of methods which are described in the Introduction to this thesis.

Burton (1955), in reviewing the properties of the enzyme, described the manometric method of assay and quoted the optimum pH as 8.8, whilst Brumby et al. (1967) after quoting the optimum pH as 8.5 made the comment that the actual value varied with the ionic composition of the buffer and with the amino acid used as the substrate. Both of these authors used D-alanine as the assay substrate and Dixon & Kleppe (1965b) stated that the enzyme showed

greatest activity with D-alanine as the substrate and that D-methionine was also oxidised very rapidly.

It was decided therefore to use D-alanine in the initial studies of D-amino acid oxidase and in order to clarify the effect of pH on hog kidney D-amino acid oxidase, the activity of the enzyme was studied in glycylglycine and Tricine buffers using the gasometric method (Method 1. p117). The pH values of a series of solutions of the buffers each containing 0.1 mol l^{-1} were adjusted, by the addition of 0.1 mol l^{-1} sodium hydroxide, to gives values over the range pH 7.0 to 10.0 and the rate of oxygen uptake by a fixed amount of the enzyme in the presence of 3.3 g l^{-1} DL-alanine was monitored over a thirty minute period using a Gilson Differential Respirometer. The enzyme showed an optimum pH in both buffers pH 8.3, with the activity in the glycylglycine buffer being slightly greater than in the Tricine buffer. (Figure 5 p 41). Using the same method, the activity of the enzyme at pH 8.3 was studied in all the four buffers referred to on page 30. The activity in glycylglycine was again demonstrated to be marginally greater than in Tricine but the enzyme activity in both buffers was substantially greater than that in either barbitone or Tris buffers (Table 7. p 42).

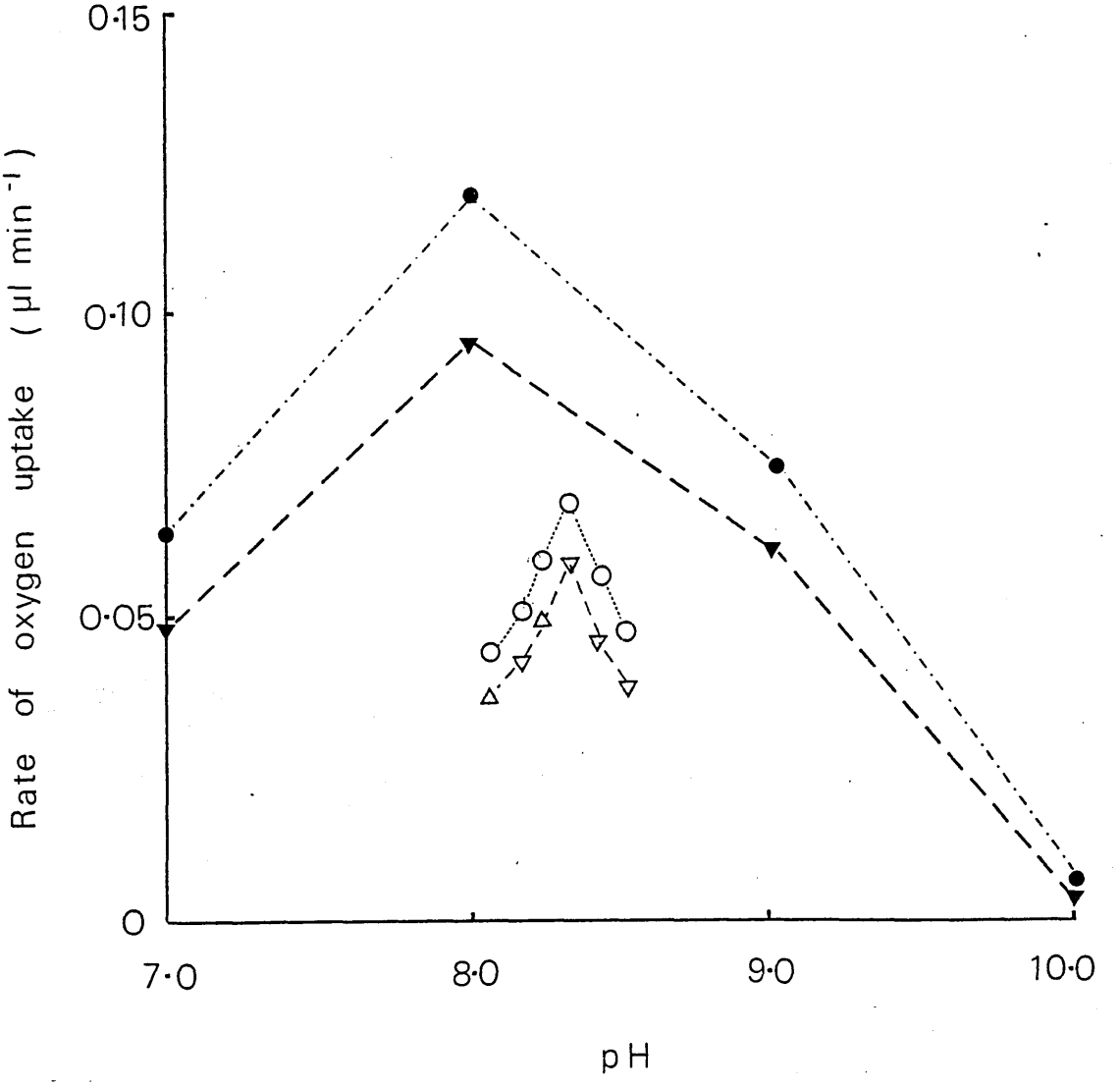
2.2. Effect of coenzymes.

As indicated earlier, the function of FAD as a prosthetic group had been elucidated as early as 1938 and Dixon & Kleppe (1965a) demonstrated that there was spontaneous dissociation of the D-amino acid oxidase holoenzyme into the apoenzyme and FAD, the rate of the dissociation being increased as the degree of dilution increased. Fonda & Anderson (1968) studied the binding of FAD by the apoenzyme of D-amino acid oxidase and noted that various adenosine nucleotides, notably ADP and AMP competitively inhibited the enzyme with respect to FAD. They reported that inhibition by FMN only occured as a result of photoactivation of the flavin derivative and was prevented by the presence of FAD.

The dissociation of the FAD and the apoenzyme was confirmed by dialyzing a solution of the enzyme in 0.1 mol l^{-1} phosphate buffer, (pH 7.0) against the buffer for 24 hours after which the activity of

Figure 5

Optimum pH of D-amino acid oxidase



The activity of D-amino acid oxidase in two buffer solutions was measured as the rate of oxygen uptake in µl min^{-1} using the method indicated below.

▼— - — ▼ Tricine

●-·-·-· ● glycylglycine
○·····○

PROTOCOL

Compartment	Reagent	Concentration	Volume (cm^3)
Main vessel	Buffer	0.1 mol l^{-1}	2.3
	Catalase prepn.		0.3
	Enzyme sample		0.2
Side arm	DL-alanine	50 g l^{-1}	0.2
Well	Sodium hydroxide	100 g l^{-1}	0.2

Monitor oxygen uptake for 30 minutes.

Table 7

Activity of D-amino acid oxidase in various buffers

Buffer pH 8.3	Rate of oxygen uptake ($\mu l\ min^{-1}$)
Barbitone	0.11
Tris	0.11
Tricine	0.15
Glycylglycine	0.16

PROTOCOL

Compartment	Reagent	Concentration	Volume (cm^3)
Main vessel	Buffer pH 8.3	0.1 mol l^{-1}	2.3
	Catalase prepn.		0.3
	Enzyme sample		0.2
Side arm	DL-alanine	50 g l^{-1}	0.2
Well	Sodium hydroxide	100 g l^{-1}	0.2
Monitor oxygen uptake for 30 minutes.			

the preparation was only approximately 40% of the undialyzed sample. Addition of 0.3 mmol l^{-1} FAD increased the activity of this dialysis residue to more than twice that of the original preparation. (Table 8. p 44). This dialysed preparation was subsequently used to assess the extent of activation by FAD and the effect of ADP and FMN on the activity of the enzyme.

A series of experiments were set up as for glutamate dehydrogenase in which various nucleotides were incorporated in the assay in concentrations of 0.3 mmol l^{-1}. (Table 8. p 44). The results confirmed the activation of the enzyme by FAD but failed to demonstrate any significant variation in the reaction rate in the presence of FMN. The presence of 0.3 mmol l^{-1} ADP caused a significant degree of inhibition which was completely reversed by the presence of a similar concentration of FAD.

In order to assess the optimal concentration of FAD for the defined assay method, a series of dilutions of the coenzyme were prepared and introduced into the gasometric enzyme assay. These experiments were also repeated using the same concentrations of FAD but incorporating 0.5 mmol l^{-1} ADP into the assay as well. (Table 9. p 45). The enzyme showed maximal activity in a concentration of FAD of about 3.0×10^{-3} mmol l^{-1} and although the presence of ADP reduced the activating influence of FAD at the lower concentrations, no reduction in activity was demonstrated at concentrations of FAD above 6.0×10^{-3} mmol l^{-1}. A final assay concentration of 1.0×10^{-2} mmol l^{-1} FAD was selected, this concentration effectively eliminated the inhibitory effects of 0.5 mmol l^{-1} ADP on D-amino acid oxidase and yet was still very much less than that concentration of FAD required to cause any possible inhibition of glutamate dehydrogenase.

The results of the investigations described above are summarised on page 46.

Table 8

Effect of various coenzymes on the activity of D-amino acid oxidase

	Coenzyme						un-dialysed sample
	None	FAD	FMN	ADP	FAD/ADP	FMN/ADP	
Rate of oxygen uptake $\mu l\ min^{-1}$	0.11	0.60	0.18	0.02	0.61	0.10	0.29

PROTOCOL

Compartment	Reagent	Concentration	Volume (cm^3)
Main vessel	Glycylglycine pH 8.3	0.1 mol l^{-1}	2.1
	Catalase prep.		0.3
	Enzyme sample		0.2
	Coenzyme	10 mmol l^{-1}	0.2
Side arm	DL-alanine	50 g l^{-1}	0.2
Well	Sodium hydroxide	100 g l^{-1}	0.2
Monitor oxygen uptake for 30 minutes			

Table 9

Effect of FAD and ADP on the activity of D-amino acid oxidase.

	Concentration of FAD (mmol l^{-1})						
		$x10^{-4}$		$x10^{-3}$		$x10^{-2}$	
	0	3.0	6.0	3.0	6.0	3.0	6.0
Rate of oxygen uptake (µl min^{-1})	0.10	0.16	0.36	0.50	0.48	0.52	0.52
Rate of oxygen uptake in the presence of 0.5 mmol l^{-1} ADP (µl min^{-1})	0.03	0.08	0.30	0.49	0.49	0.52	0.51

PROTOCOL

Compartment	Reagent	Concentration	Volume (cm^3)
Main vessel	Glycylglycine pH 8.3	0.1 mol l^{-1}	2.1
	Catalase prep.		0.3
	Enzyme sample		0.2
	Coenzyme		0.2
Side arm	DL-alanine	50 g l^{-1}	0.2
Well	Sodium hydroxide	100 g l^{-1}	0.2
Monitor oxygen uptake for 30 minutes.			

D-AMINO ACID OXIDASE.

A summary of optimal assay conditions and inhibitors.

Characteristic	Reagent	Concentrations
Optimum pH	8.3	
Optimum buffer	glycylglycine	0.1 mol l^{-1}
Activator	FAD	0.01mmol l^{-1}
Inhibitor	ADP	0.3 mmol l^{-1}

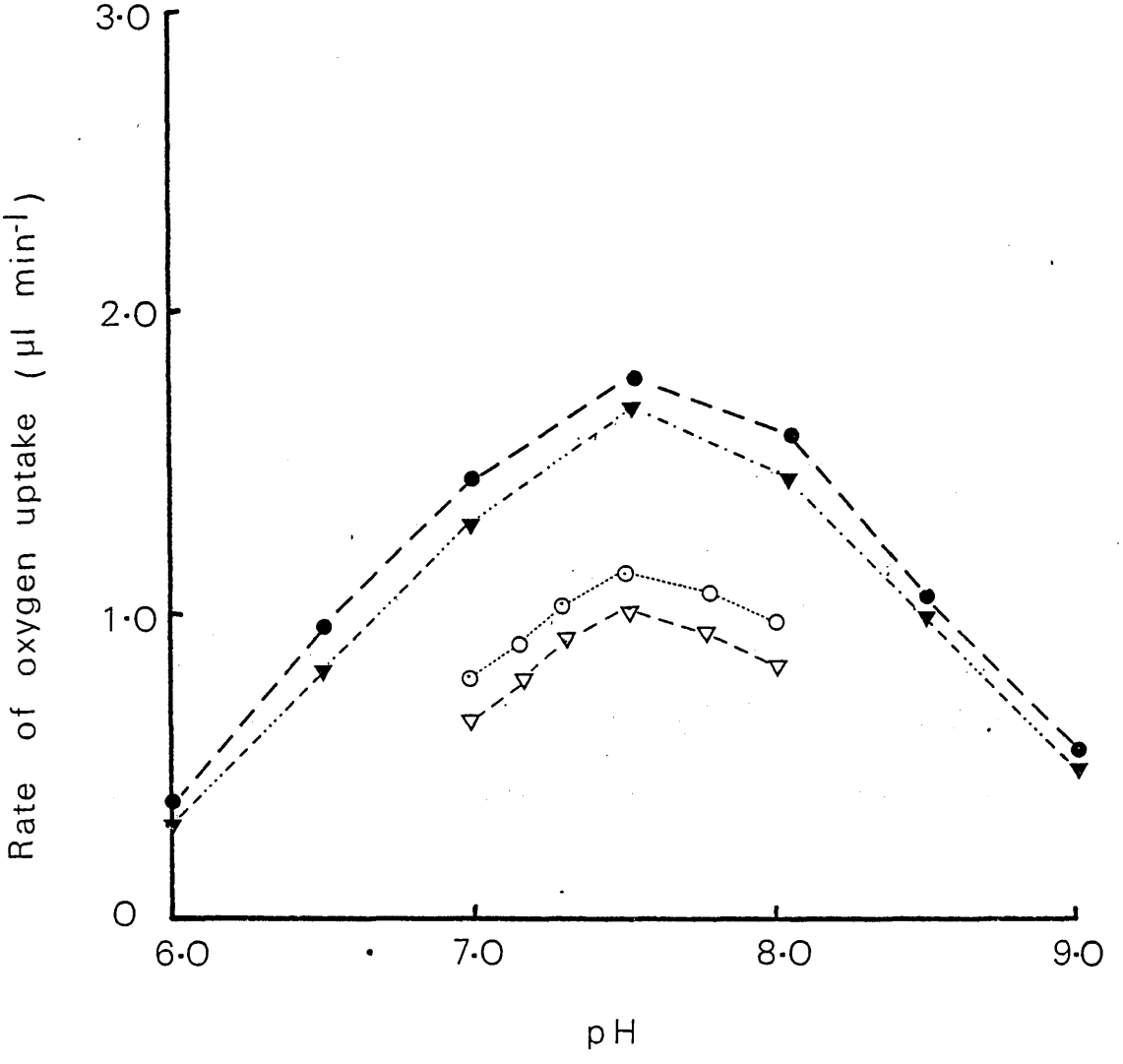
3. L-amino acid oxidase.

3.1. Optimum pH of the enzyme.

It has already been mentioned in the context of apparently conflicting reviews (p 12.) that there are two main sources of L-amino acid oxidase, namely snake venom and rat liver or kidney, and that even the enzymes isolated from the venom of different snakes show some catalytic differences. Values quoted for the optimum pH varied from pH 7.0 to pH 10.0 although again this was further complicated by the use of different buffer systems. L-leucine has been consistently used as the substrate, Ratner (1955a), and although a degree of substrate inhibition has been reported, it was decided initially to use L-leucine as the substrate for the present study of L-amino acid oxidase.

Using the volumetric measurement of oxygen uptake (Method 1 p117), the effect of pH on the activity of commercially available L-amino acid oxidase from snake venom was studied in glycylglycine and Tricine buffers, initially over the pH range 6.0 - 9.0 and subsequently over the range 7.0 - 8.0 (Figure 6. p 47). The enzyme preparation showed maximal activity at pH 7.5 and as with the D-amino acid oxidase the activity in glycylglycine buffer was marginally superior to that in Tricine. Comparison of the effectiveness of all four buffers at pH 7.5 demonstrated again the marginal superiority of glycylglycine although the enzyme activity in all four buffers was very similar (Table 10. p 48).

Optimum pH of L-amino acid oxidase



The activity of L-amino acid oxidase in two buffer solutions was measured as the rate of oxygen uptake in µl min^{-1} using the method indicated below.

Tricine

glycylglycine

PROTOCOL

Compartment	Reagent	Concentration	Volume (cm^3)
Main vessel	Buffer	0.1 mol l^{-1}	2.3
	Catalase prep.		0.3
	Enzyme sample		0.2
Side arm	L-leucine	20 g l^{-1}	0.2
Well	Sodium hydroxide	100 g l^{-1}	0.2
Monitor oxygen uptake for 30 minutes.			

Table 10

Activity of L- amino acid oxidase in various buffers

Buffer pH 7.5	Rate of oxygen uptake ($\mu l\ min^{-1}$)
Barbitone	0.23
Tris	0.24
Tricine	0.24
Glycylglycine	0.26

PROTOCOL

Compartment	Reagent	Concentration	Volume (cm^3)
Main vessel	Buffer pH 7.5.	0.1 mol l^{-1}	2.3
	Catalase prepn.		0.3
	Enzyme sample		0.2
Side arm	L-leucine	20 g l^{-1}	0.2
Well	Sodium hydroxide	100 g l^{-1}	0.2
Monitor oxygen uptake for 30 minutes.			

3.2. Effect of coenzymes.

No author has suggested that any coenzyme showed an inhibitory effect on the L-amino acid oxidases but it has been reported by Ratner (1955a), that the prosthetic group for the snake venom enzyme was FAD whilst that for the rat tissues enzyme was FMN. Wellner (1971), had reported that the FAD prosthetic group for snake venom L-amino acid oxidase was very tightly bound to the apoenzyme and suggested that in any assay method there was no need for additional coenzyme to be included.

For the present study it was necessary to assess the requirements of the enzyme for additional coenzymes and also to confirm the reported lack of inhibition by other nucleotides. To this end a sample of the enzyme was dissolved in 0.1 mol l^{-1} phosphate buffer pH 7.0 and dialysed against the buffer for 48 hours. Although the activity of the preparation fell during dialysis by approximately 40%, possibly due to denaturation of the enzyme, no increase in activity could be subsequently demonstrated by the addition of either FAD or FMN, nor did the presence of ADP reduce the activity of the enzyme preparation.

The results of the investigations described above are summarised below.

L-AMINO ACID OXIDASE

A summary of optimal assay conditions and inhibitors.

Characteristic	Reagent	Concentration
Optimum pH	7.5	
Optimum buffer	glycylglycine	0.1 mol l^{-1}
Activator	none	
Inhibitor	L-leucine	3.0 mmol l^{-1}

4. Spectrophotometric assay.

4.1. Concentration of 2-oxoglutarate.

It was necessary, having elucidated the optimal conditions for each of the enzymes, to decide on the conditions for the coupled assay. The necessary substrates for each of the two assay methods were evident from an examination of the basic equations for the reactions (Equation 1 & 2, p 7 & 12). Both assay methods required 2-oxoglutarate and NADH as substrates for the indicator reaction; the oxygen required for the test reaction was available as dissolved oxygen in the solutions.

The reported Km value for 2-oxoglutarate was 0.7 mmol l^{-1} Barman (1969), and final concentrations of 13.3 mmol l^{-1} had been used previously, Ellis & Goldberg (1970). The concentration of NADH used would depend upon the maximum absorbance which was capable of being accurately measured (p 35).

4.2. Effect of coenzymes.

It was evident from the studies on the activation and inhibition of the three enzymes that there would be some inhibition effect in the coupled assay, namely ADP activating glutamate hydrogenase but inhibiting D-amino acid oxidase. However the evidence was that at the concentration selected, namely 0.5 mmol l^{-1}, and in the presence of FAD the inhibitory effect would be negligible (Table 9. p 45).

4.3. Assay pH.

There was some difficulty over the selection of the pH at which the assays would be performed. In order to gain maximum sensitivity, it was desirable to select the optimum pH of the appropriate amino acid oxidase as the working pH but, as neither of these values were the most appropriate for the glutamate dehydrogenase, the effect would be to suppress the activity of that enzyme. From the studies on the effect of pH on glutamate dehydrogenase, described earlier

(Figure 2. p 33.), it was calculated that at pH 7.5, glutamate dehydrogenase showed approximately 91% of maximal activity, whilst at pH 8.3 it showed approximately 85% of maximal activity.

4.4. Amount of glutamate dehydrogenase.

In order that the indicator reaction should not be rate limiting, it was decided that the amount of glutamate dehydrogenase that would permit a 100 fold increase in reaction rate over that actually expected, should be used. On the basis of previous experience with kinetic assays utilizing the oxidation of NADH, it was felt that a rate of reaction comparable with a fall in absorbance of 0.1 per minute could be regarded as an absolute maximum, Ellis & Goldberg (1970). This reaction rate would equate with the formation of 0.5×10^{-1} micromole of ammonia in one minute in the 3.0 cm^3 of the assay and on this basis the coupled assay would require a minimum of 5 units of glutamate dehydrogenase per 3.0 cm^3 of reaction mixture. The volume of glutamate dehydrogenase subsequently used in the assay was 0.05 cm^3 which, according to the specifications of the preparation contained 60 units of activity, was more than enough even allowing for the effect of pH on the activity of the enzyme.

4.5. Amino acid substrates.

In standardizing the assay methods, it was essential to know the concentration of the amino acid substrate necessary to give zero order kinetics and in order to assess this value, it was necessary to determine the Michaelis constant (Km) for each enzyme using the substrate indicated. A series of dilutions of D-alanine and L-leucine containing 5 - 200 mmol l^{-1} were prepared from stock solutions of each amino acid which, when incorporated into the assay, gave final concentrations from 0.16 mmol l^{-1} to 6.66 mmol l^{-1}. Using Method 2 (p 119), the rate of reaction produced by a fixed amount of the appropriate enzyme in the presence of these concentrations of substrate, was measured in terms of absorbance change at 340 nm (Figure 7 p 52).

Using the statistical method indicated (Method 3. p121), the Km value for D-amino acid oxidase was calculated to be 3.3 mmol l^{-1}.

Figure 7

Effect of substrate concentration on the activity of the amino acid oxidases.

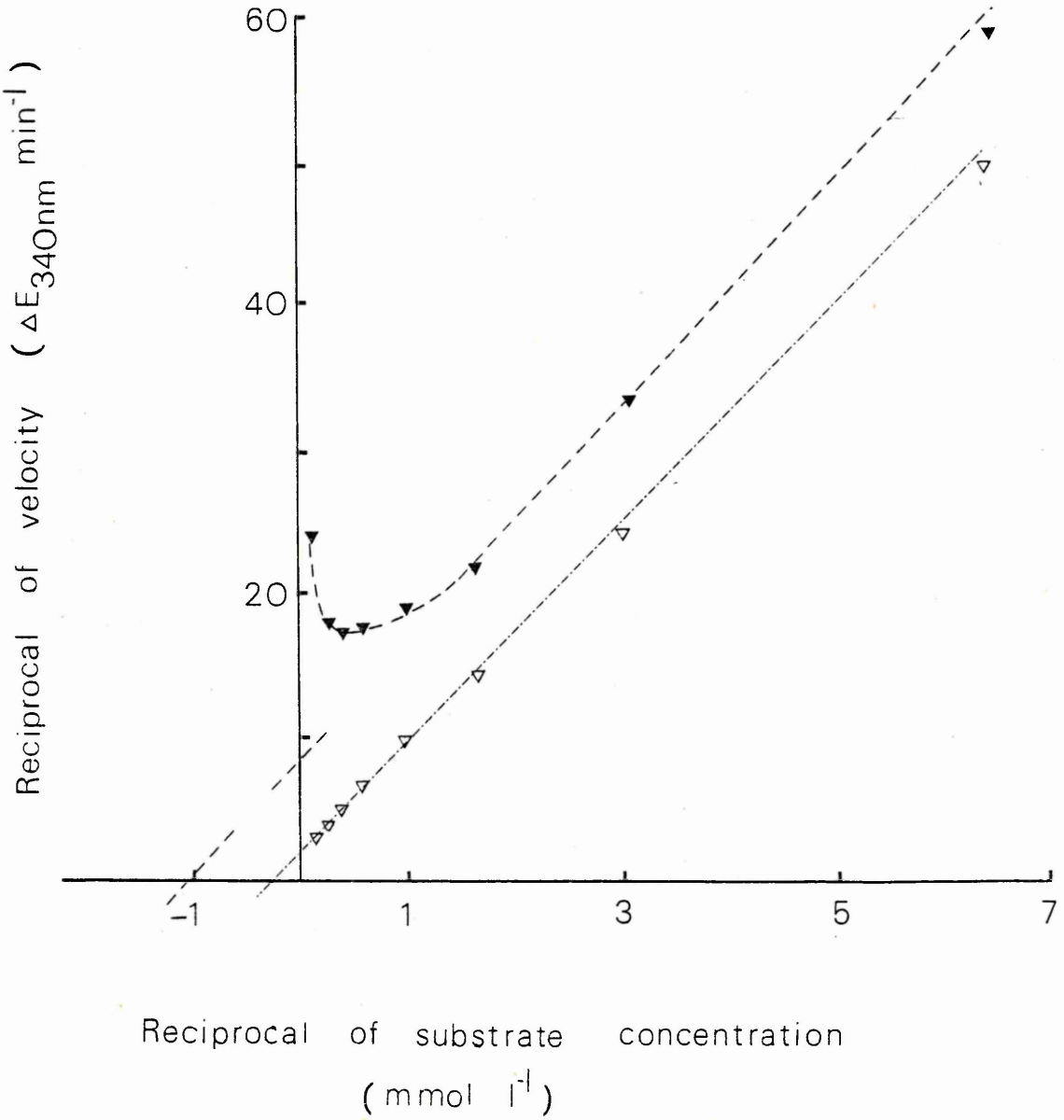


Table 11

Effect of substrate concentration on the activity of the amino acid oxidases

Final Concentration of substrate (mmol l^{-1})	Rate of reaction ($\Delta E_{340\ nm}$)	
	D-amino acid oxidase (D-alanine)	L-amino acid oxidase (L-leucine)
0.16	0.004	0.017
0.33	0.008	0.030
0.66	0.014	0.045
1.00	0.020	0.052
1.66	0.030	0.056
2.50	0.039	0.057
3.33	0.046	0.056
6.66	0.058	0.041

Method	Michaelis Constant (mmol l^{-1})	
Graphical	3·3	0·7
Statistical	3·3±0·12	0·6±0·16

(Table 11. p 53). Burton (1955), and Ratner (1955b), quoted values between 2 - 9 mmol l^{-1}.

For the L-amino acid oxidase, however, the results indicated a significant degree of substrate inhibition at concentrations of L-leucine above 3.0 mmol l^{-1}, the inhibition becoming progressivly greater as the concentration increased. (Figure 7. p 52). These results were not completely unexpected as Ratner (1955a), had stated that concentrations of L-leucine above 10 mmol l^{-1} were inhibitory. She also noted that ammonium ions inhibited at the same concentration.

The non-inhibited portion of the Lineweaver - Burke plot was used to calculate the Michaelis constant and it gave a value of 0.6 mmol l^{-1} (Table 11. p 53). Ratner (1955a), had quoted a value of about 1.0 mmol l^{-1} whilst Nakano & Danowski (1971) gave a value of 1.3 mmol l^{-1}.

A concentration of D-alanine of 0.5 mol l^{-1} was chosen for the working substrate for the D-amino acid oxidase assay which, when used as described in (Method 2. p119), gave a final assay concentration of 17 mmol l^{-1}. This concentration was approximately five times greater than the Km value for the substrate and permitted 84% of the theoretical maximum velocity. In selecting a suitable concentration of L-leucine for the L-amino acid oxidase assay, not only was the inhibitory nature of L-leucine to be considered but also the fact that it showed only a limited solubility of approximately 0.2 mol l^{-1}, Weast (1967). As inhibition only became evident at concentrations in excess of 3.0 mmol l^{-1} it was decided to use a working solution containing 0.1 mol l^{-1} which, as well as being within the solubility range of L-leucine, also provided as assay concentration of 3.3 mmol l^{-1} and theoretically permitted 85% maximum velocity.

4.6. Assay procedure.

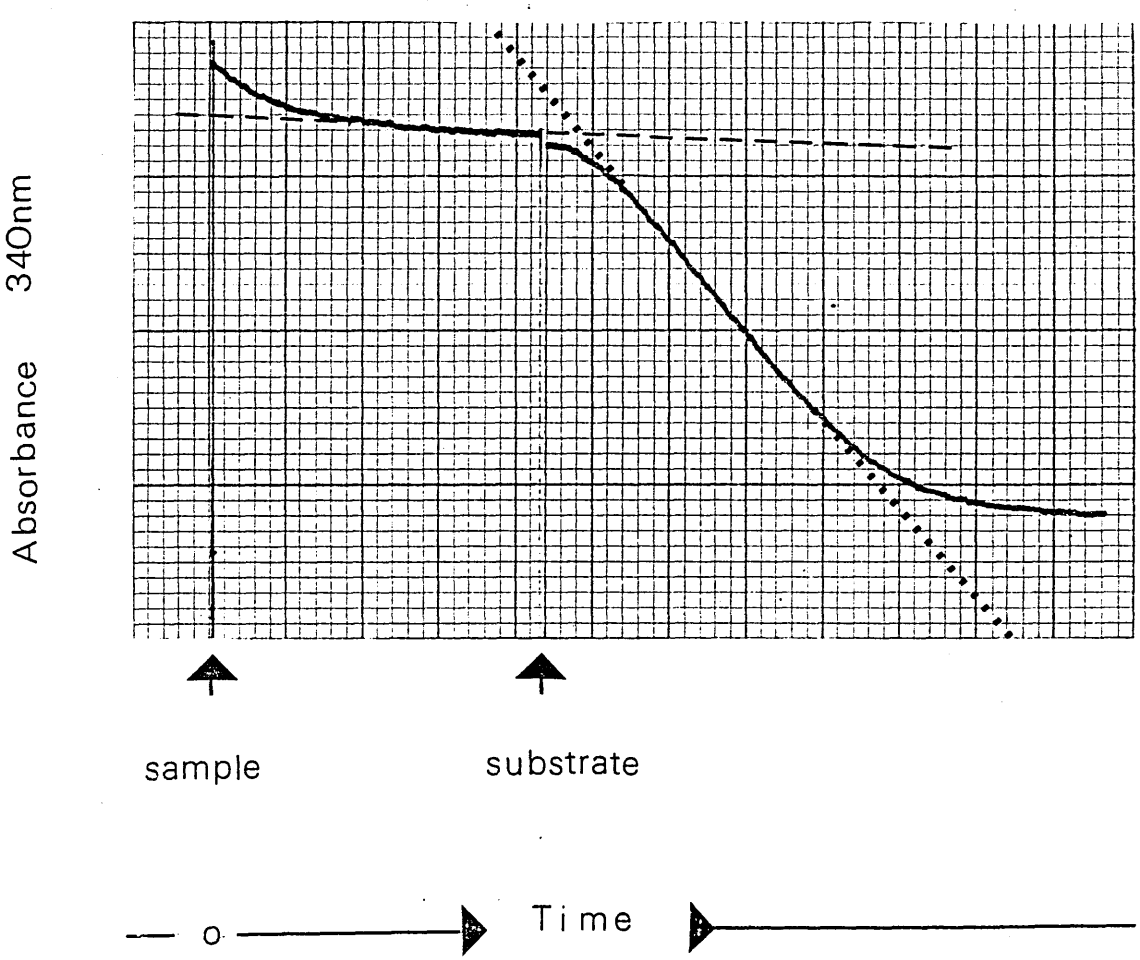
A typical trace of an assay of either D-amino acid oxidase or L-amino acid oxidase by the method described is shown in Figure 8 (p 55). On addition of the sample to the reaction mixture from which the substrate had been omitted, there was a rapid fall in absorbance at 340 nm due to the oxidation of NADH by ammonia present in the sample. The absorbance either stabilized or continued to fall at a constant

Figure 8

Typical trace of a spectrophotometric assay

_ _ _ Blank reaction

..... Test reaction



Typical trace of a spectrophotometric assay using a Unicam SP1800 spectrophotometer. The arrows indicate the time of addition of sample and substrate.

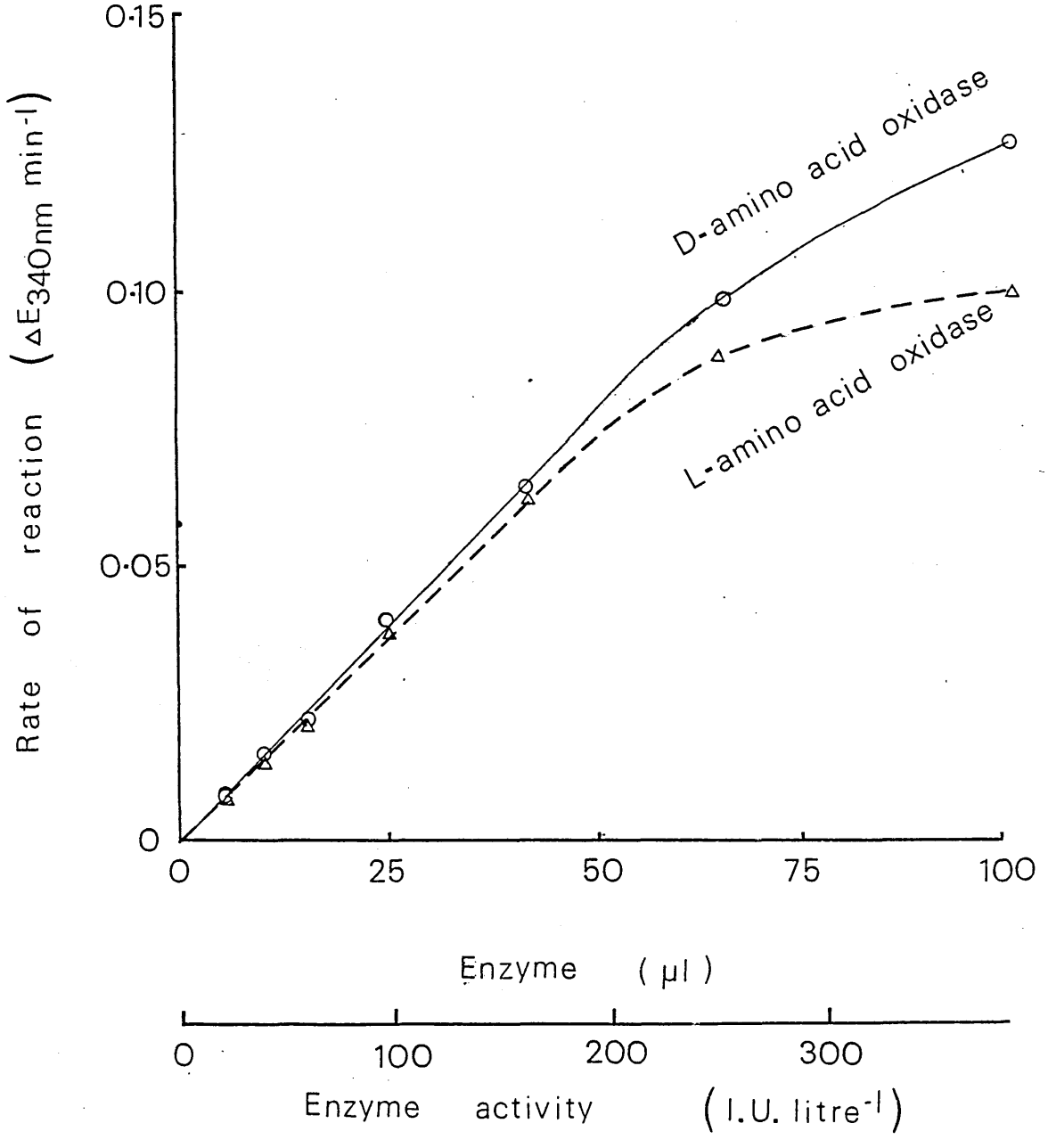
rate depending upon the extent of endogenous ammonia formation by the sample. This represented the blank reaction which was measured and subsequently subtracted from the test reaction rate. The appropriate substrate was then added to initiate the reaction and after a lag period, during which the reaction rate increased to a maximum, the rate of reaction held constant until the concentration of NADH fell to a level at which it became rate limiting. The reaction rate during this linear part of the reaction was measured at 340 nm in terms of absorbance change per minute and the enzyme activity of the sample was calculated in International Units per litre of sample. (Method 4. p122).

This calculation assumed that the only rate limiting factor in the assay was the activity of the test enzyme but it was appreciated that there were other factors which could become rate limiting under certain conditions. Although steps had been taken to maintain high concentrations of substrates and indicator enzyme in order to eliminate or minimise these effects, nevertheless the concentration of NADH particularly had to be held relatively low because of its high absorption at 340 nm. It was felt necessary therefore to test the validity of the equation over a wide range of enzyme activities and in order to do this, a series of dilutions of samples with high concentrations of each amino acid oxidase were assayed in the normal way and the measured rate of reaction plotted against the percentage concentration of the enzyme in the sample (Figure 9. p57). The results indicated that the linear relationship between the rate of reaction and enzyme concentration held true for both assays only up to an enzyme activity of about 150 i.u. l^{-1} (an absorbance change at 340 nm of 0.06 units per minute). Enzyme activities beyond this level resulted in an increasing deviation from the linear relationship, particularly for the L-amino acid oxidase assay. Using this method it was possible to detect activities as low as 2.4 units l^{-1} without difficulty; this being equivalent to an absorbance change of 0.001 units per minute, and with care it was possible to detect activities as low as 1.0 i.u. l^{-1}.

The precision of each method was assessed by performing ten replicate assays on samples with mean activities of approximately

Figure 9

Analytical range of the coupled assay



Relationship of enzyme concentration for D-amino acid oxidase (o——o) and L-amino acid oxidase (▽— — —▽) with the rate of reaction and showing linearity up to an enzyme activity of approximately 150 i.u. l^{-1}.

20,100, and 250 i.u. l^{-1}. The results indicated that the overall precision was very good particularly for activities of about 100 i.u. l^{-1} giving coefficients of variation between 1.2% and 0.2% (Table 12. p 59). Precision was less at the two extremes of the activity range but was still very satisfactory.

4.7. Substrate specificity of the enzymes.

It was necessary to investigate the specificity of the two amino acid oxidases in order to confirm the selection of D-alanine and L-leucine as the assay substrates, and also to compare the results obtained using the present assay method with previously published data. Solutions of chromatographically pure D- and L-stereoisomers of some amino acids were prepared at the same concentrations as those used in the assays involving D-alanine and L-leucine, namely 0.5 mol l^{-1} and 0.1 mol l^{-1} respectively. However when the solubility of an amino acid would not permit these concentrations, a solution of lower concentration was prepared and an appropriate volume, calculated to give the final concentration of amino acid required, was used in the assay.

Both amino acid oxidases were tested with all the chosen amino acids of both configurations and the activity of the enzyme shown with each amino acid was expressed as a percentage of that given with the normal substrate for the enzyme (Table 13. p 60). A four cell autochanger system was used in the spectrophotometer and the first cell always contained the normal substrate for the enzyme. Although ultimately neither amino acid oxidase showed any reaction with amino acids of the opposite configuration to that specified for the enzyme, there was initially one apparent exception in that both enzymes showed an appreciable activity with both the D- and L- isomers of aspartic acid. This was subsequently proved to be an artifact and was traced to contamination of each sample of aspartic acid with ammonia. This contamination could be demonstrated most easily by the addition of the substrate to the assay reagent in the absence of the amino acid oxidase.

Table 12

Precision of assay methods

	Activity (I.U. l^{-1})		
	Mean	SD	CV
Spectrophotometric method			
	28	1.0	3.6
	93	0.2	0.2
	234	0.8	0.3
	343	4.1	1.2
Fluorimetric method			
	1.1	0.1	16.3
	12.8	1.3	10.4

These results are based on ten replicate assays at each activity under the conditions of the standard assay. The data is expressed as International Units per litre.

Table 13

Specificity of the amino acid oxidases

Amino acid	L-amino acid oxidase snake venom	D-amino acid oxidase		
		hog kidney	Human Liver	Kidney
Alanine	0	100	100	100
Arginine	1	1	0	0
Aspartic acid	0	30	30	34
Cystine	40	0	0	0
Glutamic acid	0	0	0	0
Glycine	0	0	0	0
Histidine	10	0	24	24
Iso-leucine	30	80	46	49
Leucine	100	60	22	19
Lysine	0	0	0	0
Methionine	110	100	40	41
Phenylalanine	60	70	38	39
Serine	0	1	27	28
Threonine	0	0	0	0
Tryptophan	80	0	0	0
Tyrosine	65	80	73	76
Valine	0	50	34	33

Data for specific D- and L- isomers of the amino acids above are the mean of three observations and are given as a percentage of the activity of the enzyme with either D-alanine or L-leucine and in the same final concentration.

5. Fluorimetric method.

5.1. Fluorescent properties of NAD^+.

It was decided to use the fluorescent properties of the nicotinamide coenzymes as the basis of a back-up assay method for two main reasons. A method had been described by Lowry & Passonneau, (1972), for the quantitation of NAD^+ based on its fluorescent properties in alkaline solution and it was felt that this method could be modified to provide an assay method for the amino acid oxidases. Another reason was that a fluorimetric assay method, although unsuitable for kinetic studies of amino acid oxidase activity, reputedly offered greater sensitivity than absorptiometric methods.

A study of the fluorescent properties of NAD^+ in alkaline solution using a recording spectrofluorimeter confirmed an excitation maximum at 365 nm and an emission maximum at 455 nm (Figure 10. p 62).

The pH conditions necessary for the destruction of NADH and the development of maximum fluorescence from NAD^+ were reputedly critical. Lowry & Passonneau stated that 99% of NADH was destroyed in just over 1 minute at pH2 at 23°C, and that for maximum fluorescence from NAD^+, a pH of 10.5 had to be achieved. They suggested a final concentration of 6 mol l^{-1} sodium hydroxide as being the most suitable means of achieving the latter.

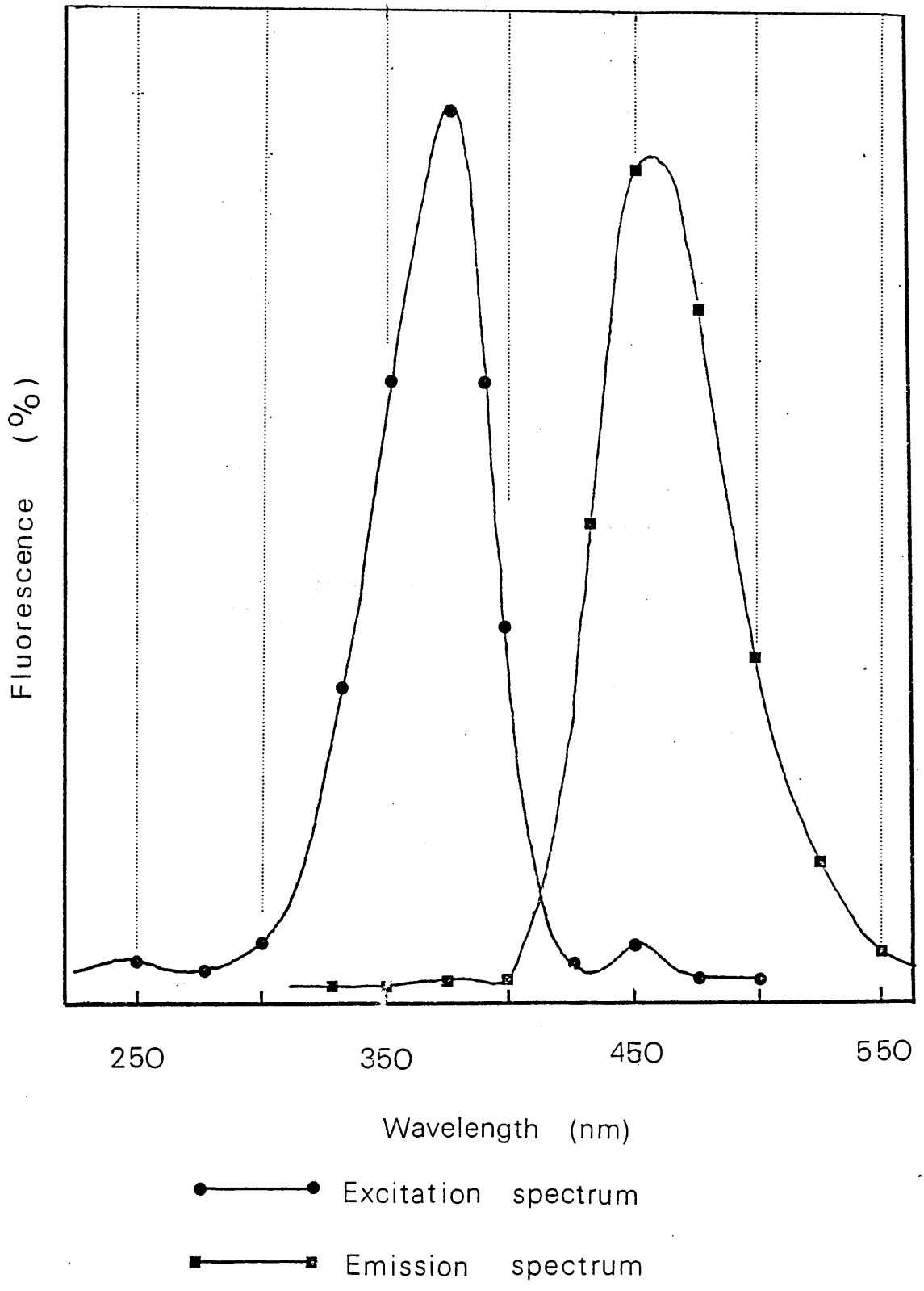
In the development of a method for maximum sensitivity, it was obviously desirable to minimise any dilution factors and therefore any reagents used to modify pH conditions had to be as concentrated as possible. Using the same assay buffer system, namely 0.1 mol l^{-1} glycylglycine pH 8.3, it was discovered that the addition of a tenth of its volume of 2.0 mol l^{-1} HCl reduced the pH to 1.4 and subsequently the addition of two volumes of 10 mol l^{-1} sodium hydroxide, as well as giving a final concentration of approximately 6.5 mol l^{-1}, resulted in a solution of pH 11.5.

5.2. Stability of NAD^+ fluorescence.

One of the difficulties with the fluorescence of NAD^+ in alkaline solution was that the intensity of fluorescence diminished on

Figure 10

Fluorescent properties of NAD^+



exposure to natural light and specifically to ultraviolet radiation, a fact noted by Lowry and Passoneau in their original work. However, Lowry & Carter (1974), stated that this fluorescence was stabilized by the presence of 10 mmol l^{-1} imidazole in the sodium hydroxide solution.

This statement was investigated by preparing three solutions, each in duplicate, of NAD^+ of varying concentration and producing the fluorescent derivative in (a) sodium hydroxide solution only, and (b) in sodium hydroxide solution containing 10 mmol l^{-1} imidazole. After heating at 56°C for 15 minutes an aliquot of each sample was exposed to bright sunlight whilst the remainder was kept in the dark. The intensity of fluorescence of each aliquot was measured initially, and after 30 minutes, against a reference solution which was also kept in the dark. The intensity of fluorescence of those samples in sodium hydroxide alone fell on average by 36% over the 30 minute period whilst no other sample showed any significant variation (Table 14. p 64).

5.3. Effect of NAD^+ concentration.

The fluorimetric method utilized the same reaction mixture as the kinetic method and therefore the maximum concentration of NAD^+ generated would be 0.15 mmol l^{-1}, due to the oxidation of all the NADH incorporated in the assay reagent. The relationship between the intensity of fluorescence and the concentration of NAD^+ up to this maximum concentration was studied by preparing a range of dilutions of a stock solution of 0.15 mmol l^{-1} NAD^+. The fluorescence was developed by heating 1.0 cm^3 of these solutions with 2.0 cm^3 10 mol l^{-1} sodium hydroxide containing 10 mmol l^{-1} imidazole at 56°C for 15 minutes and the intensity of fluorescence was measured as a percentage of that produced by the highest concentration of NAD^+. The results indicated that over this concentration range there was a linear relationship between concentration of NAD^+ and the percentage fluorescence (Figure 11. p 65). At the same time a duplicate set of solutions was set up to which 0.1 cm^3 0.15 mmol l^{-1} NADH had been added. Prior to the addition of the sodium hydroxide, 0.1 cm^3 2.0 mol l^{-1} HCl was added and the

Table 14

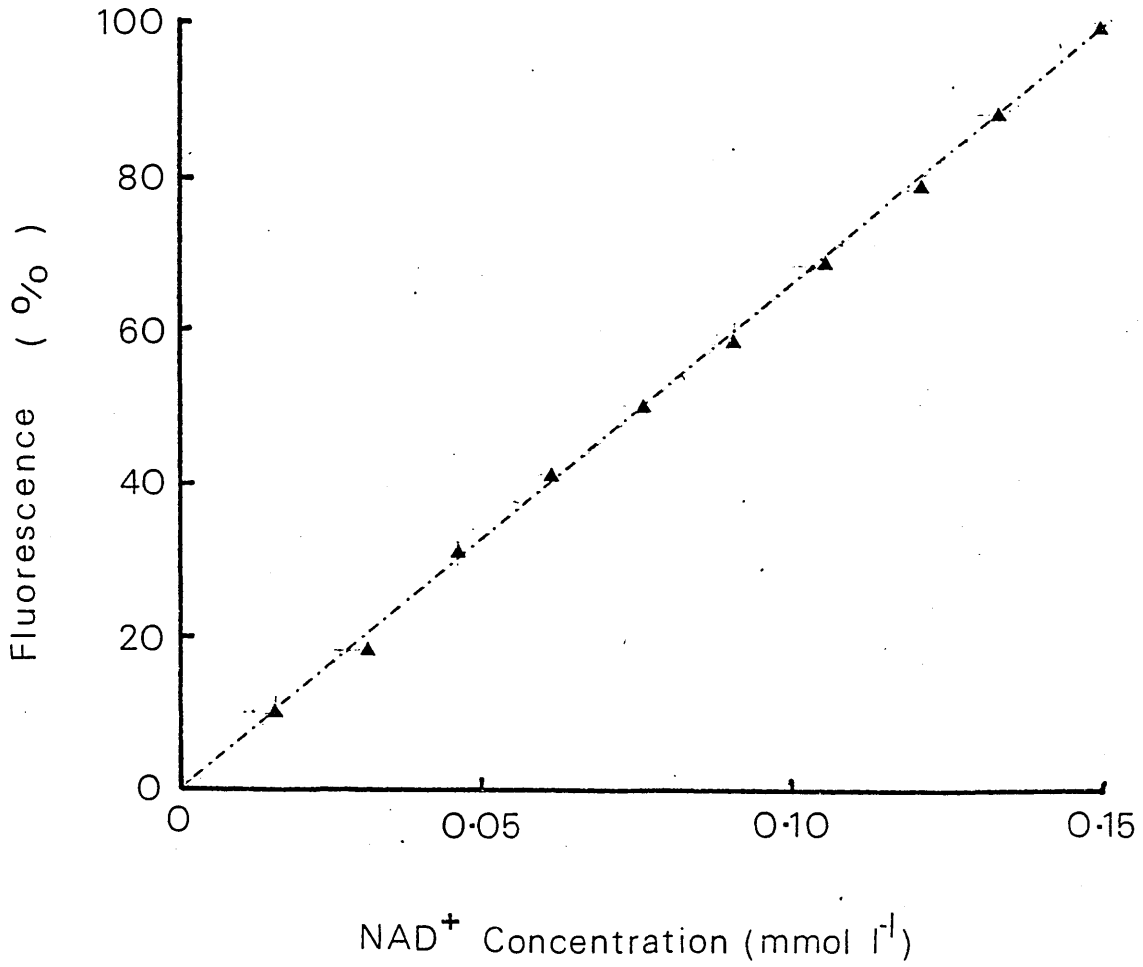
Photosensitivity of NAD^+ fluorescence

NAD^+ Concentration (mmol l^{-1})	Intensity of fluorescence (%)			
	Without imidazole		With imidazole	
	Sunlight	Dark	Sunlight	Dark
0.015	64	100	99	99
0.010	42	65	66	66
0.005	19	31	32	33

Each sample was prepared in duplicate by heating 1.0 cm^3 of the NAD^+ solution at 56°C for 15 minutes with either 2.0 cm^3 NaOH solution (10 mol l^{-1}) or 2.0 cm^3 NaOH solution containing 10 mmol l^{-1} imidazole. The resulting fluorescence was measured after allowing the mixtures to stand for 30 minutes in either the dark or bright sunlight.

Figure 11

Fluorescence of NAD^+ solutions



PROTOCOL

Reagent	Concentration	Volume (cm^3)
NAD^+		1.0
NaOH with imidazole	10 mol l^{-1} 10 mmol l^{-1}	2.0
Heat at 56°C for 15 minutes and measure the fluorescence at 455 nm (excitation 365 nm)		

mixture allowed to stand for 15 minutes. The results showed no difference from the unmodified solutions and indicated that the NADH was effectively destroyed under the specified conditions.

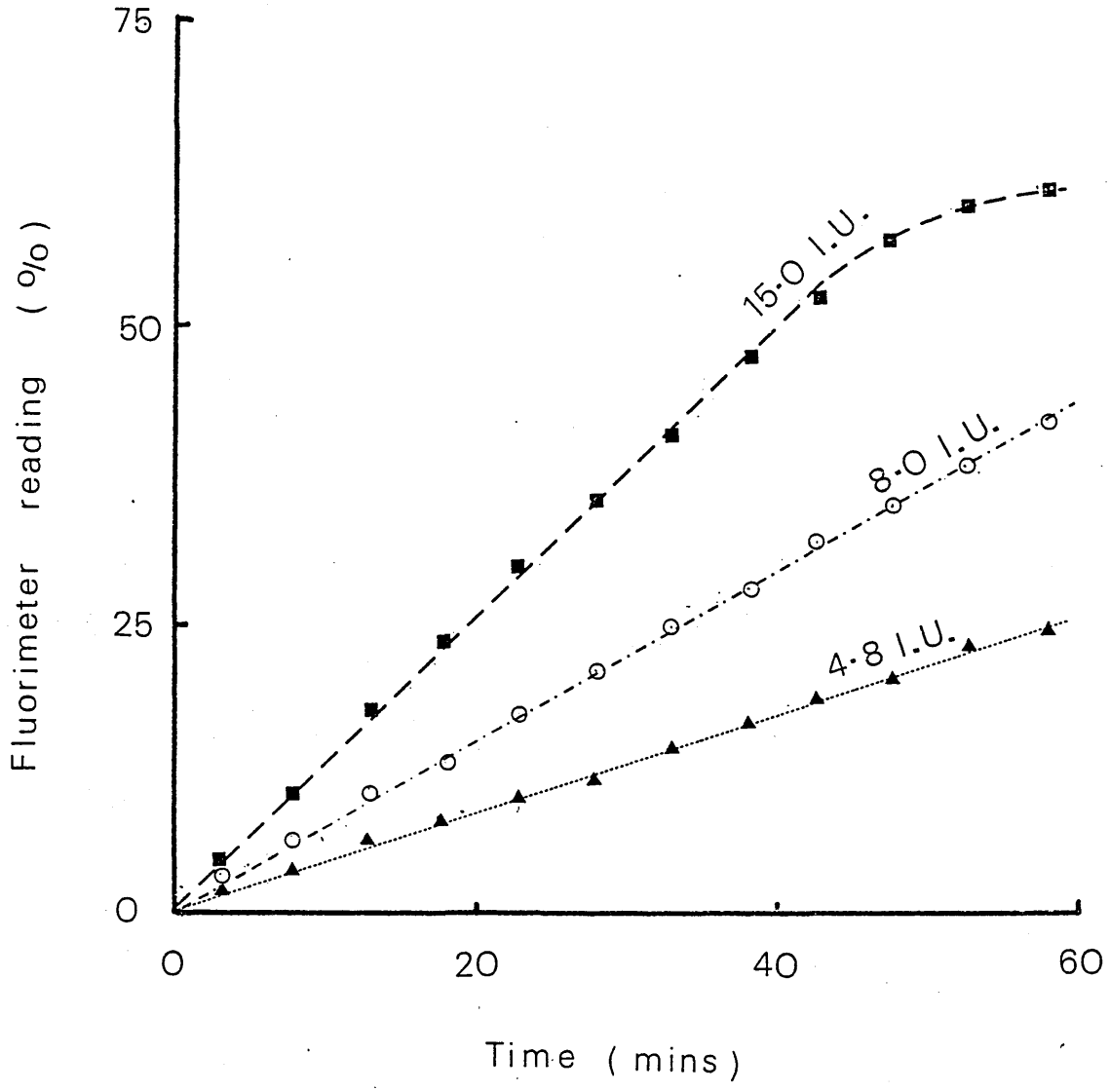
5.4. Rate of NAD^+ formation.

It was evident from an examination of the plot of the kinetic method of assay (Figure 8. p 55.), that the oxidation of NADH showed a linear relationship with respect to time, from about the third minute onwards and continued for a variable period of time depending upon the availability of NADH, and the concentration of the enzyme in the sample. In order to assess the effective range of the fixed time assay it was necessary to define more clearly the period of time for which the linear relationship held true. To this end three samples of D-amino acid oxidase were prepared, the activity of each was measured by the kinetic method and calculated to be 15.0, 8.0 and 4.8 i.u. l^{-1}. An assay was set up for each enzyme preparation as indicated in (Method 2 p119) but using volumes 10 times greater than specified, and duplicate 1.0 cm^3 aliquots were taken after 3 minutes and subsequently at 5 minute intervals up to 63 minutes. The fluorescence produced by the NAD^+ in each of these aliquots was measured using the described technique, (Method 5.p123). A plot of the intensity of fluorescence against reaction time (Figure 12. p67), indicated that for enzyme activities of 8.0 i.u. l^{-1} and less, the rate of formation of NAD^+ was linear for in excess of 60 minutes, whilst for enzyme activities of 15 i.u. l^{-1} the reaction deviated from linearity after about 40 minutes.

The initial decision to take samples at 3 and 63 minutes was based upon the observation that the reaction rate was not linear until about the third minute. Whilst this was certainly true for high concentrations of enzyme it was felt that at low activities this might be less of a problem. To clarify this situation the rate of formation of NAD^+ over the first ten minutes of the reaction was monitored in the same way, using two enzyme preparations with activities of 15 i.u. l^{-1} and 1.8 i.u. l^{-1}. Although the concentration of NAD^+ produced was very low, it was possible to demonstrate that for low activities of enzyme there was no noticeable deviation from

Figure 12

Rate of formation of NAD^+



The rate of formation of NAD^+ in the coupled assay was measured using the fluorimetric method and three different activities of D-amino acid oxidase. 1.0 cm^3 aliquots were heated at 56°C with 2.0 cm^3 volumes of 10 mol l^{-1} NaOH which contained 10 mmol l^{-1} imidazole. The fluorescence of each sample was measured at 455 nm (excitation 365 nm) after heating for 15 minutes.

linearity during the first few minutes, whilst for the higher activity the deviation was only slight (Figure 13. p 69). It was on this evidence that the sampling times were set at 0 and 60 minutes as indicated in Method 5 (p123).

5.5. Assay procedure.

In order to assess the effective range of the method, a series of dilutions of preparations of both D-amino acid oxidase and L-amino acid oxidase of approximately 18 i.u. l^{-1} were prepared and assayed by both the kinetic and fluorimetric methods (Methods 2 & 5, p119&123). The results indicated that the effective range for the fluorimetric method was up to an activity of approximately 10 i.u. l^{-1} and the results by both methods for enzyme activities up to this value were very comparable (Figure 14. p 70).

Studies on the precision of the method over the range of enzyme activity indicated, showed that ten replicate samples with a mean activity of 12.8 i.u. l^{-1} gave a standard deviation of 1.34 i.u. l^{-1} and a coefficient of variation of 10.4%, whilst samples with a mean activity of 1.1 i.u. l^{-1} gave a standard deviation of 0.18 i.u. l^{-1} and a coefficient of variation of 16.3%. (Table 12. p59). The lowest activity that could be detected was 0.05 i.u. l^{-1} which can be compared with the kinetic method which could detect to 1.0 i.u. l^{-1}.

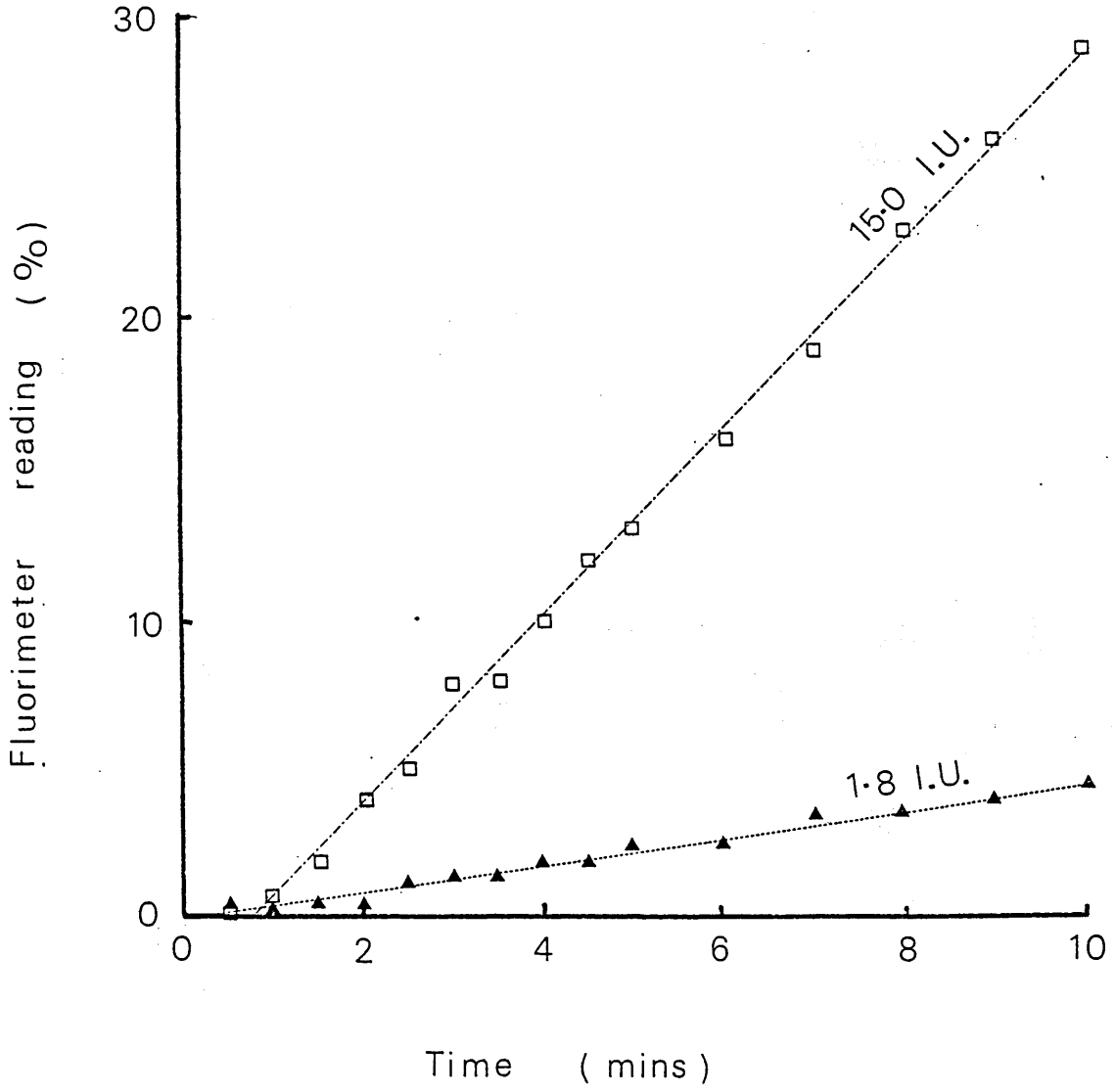
6. Discussion.

The sensitivity of an assay method is only of significance when compared to that of other assay methods and is basically determined by the physicochemical characteristics of the compound being measured. The inherent sensitivity of a method can, however, be reduced if care is not exercised in the choice of assay conditions and instrumentation.

The degree of sensitivity required in an analytical method is related to the analytical use of the method, the prior knowledge of the expected enzyme activities in the test samples, and what order of variation constitutes a significant change. In the present study very

Figure 13

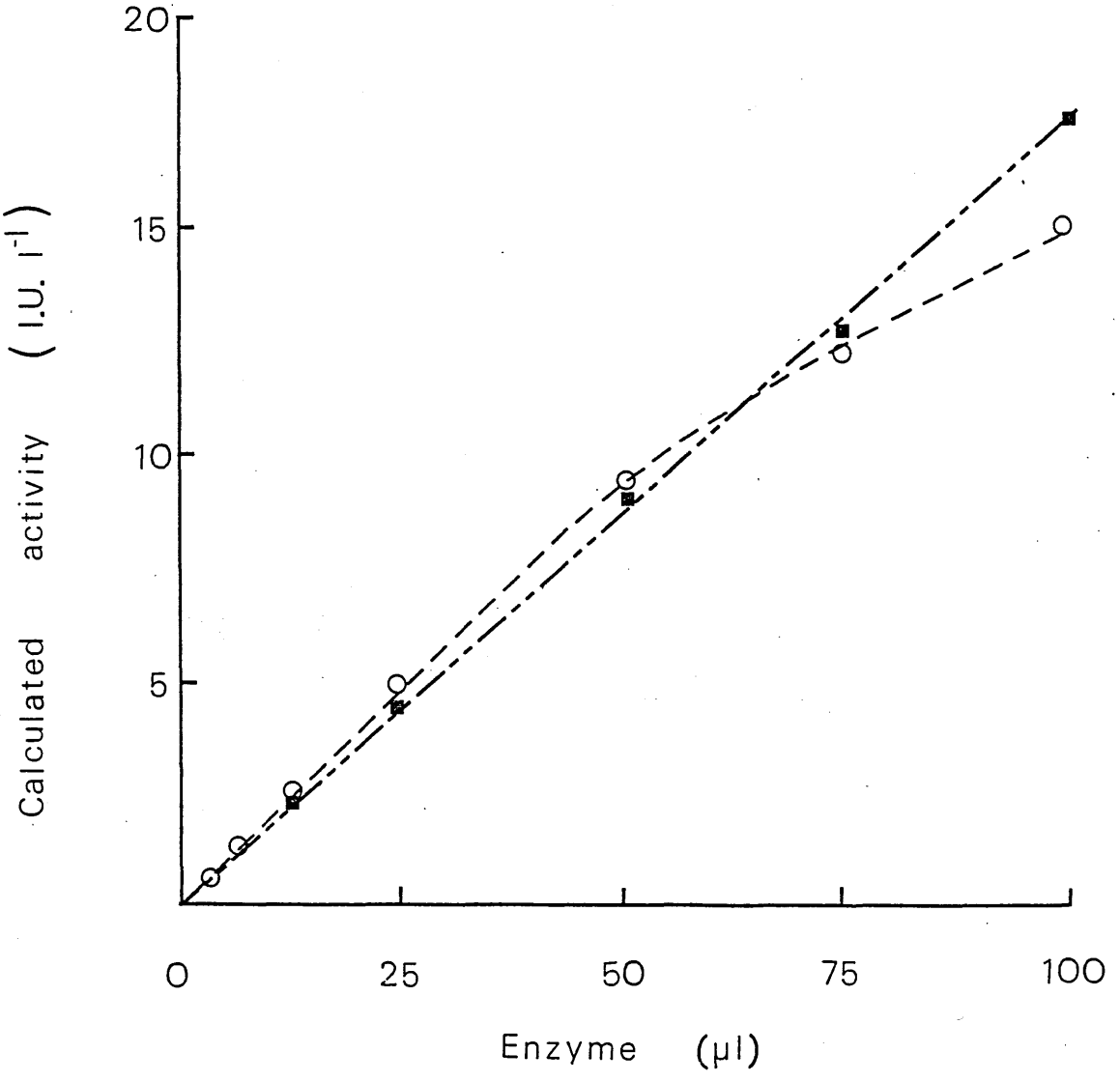
Rate of NAD^+ formation



The rate of NAD^+ formation during the first ten minutes of the coupled assay was measured using the fluorimetric method and two different activities of D-amino acid oxidase.

Figure 14

Analytical range of the fluorimetric assay



Relationship of enzyme concentration with the rate of reaction using the fluorimetric method (Θ — — Θ) and the spectrophotometric method (■—---—■) with a maximum enzyme activity of 18 i.u. l^{-1}.

little information was available on these latter points and so the use of a very sensitive method in attempting to define these limits was essential.

Apart from the clarification of optimum pH, activators and inhibitors, the choice of a particular amino acid substrate would seem to be of significance in amino acid oxidase assays. A comparison of the activities of the enzymes with different amino acids (Table 13. p 60.), confirmed the validity of the initial selection of D-alanine and L-leucine as being the most suitable substrates for the D-amino acid oxidase and the L-amino acid oxidase assay methods respectively. The use of L-methionine instead of L-leucine would have given an increase of approximately 10% in the rate of reaction with the L-amino acid oxidase. Apart from the selection of a totally unsuitable substrate the use of any other amino acid than L-leucine would have only resulted in a reduction in the reaction rate of approximately 50%. The sensitivity could vary therefore by a factor of 2 depending upon the selection of the amino acid substrate.

In considering the effects of instrumentation on the sensitivity of a method, some thought had to be given to the choice of spectrophotometer. The use of an instrument with a valid scale expansion facility might give up to a five fold improvement in sensitivity. Some instruments, although having expansion facilities, also show increased noise levels or encourage the quite erroneous use of absorbance values above 2.0. Care must obviously be exercised in the selection of a valid instrumental system of analysis.

The inherent sensitivity of the present spectrophotometric method is due to the molar absorption of NADH. It is of the order of 10^{-4} mol l^{-1} and can be modified depending upon the analytical conditions by a factor of about 10 and although this figure is not large it is nevertheless significant.

Theoretically, the fluorescent properties of NAD^+ and NADH are about 1×10^3 times greater than their absorptive properties, and this implies that fluorimetric methods should show a comparable increase in sensitivity. However the data given for the sensitivities of the present fluorimetric and spectrophotometric methods only show a 20 fold increase in sensitivity, and with the subsequent use of tissue homogenates as samples even this increase was not maintained.

The inherent advantage of fluorimetric methods lies in the basic fact that in such methods an increase in signal over a zero background is measured, whilst in spectrophotometric methods, the decrease in a large standing current is measured. This asset is largely lost when a blank which also shows fluorescent properties, is used in the assay method. The use of partially purified enzymes minimized this effect of the blank but tissue homogenates containing, not only the added nucleotides but also endogenous material, resulted in a relatively high blank fluoresence.

An examination of the sensitivities of the methods reviewed earlier (Table 3. p 24.) indicated that the sensitivity of the present spectrophotometric method, capable of detecting as little as 1.0 i.u. of enzyme per litre sample, compared favourably with all the other reported kinetic methods, except the fluorimetric method of Guilbault & Hieserman (1968). There must be some doubt as to the validity of this latter extremely sensitive method because of the significant difference in sensitivity between the D-amino acid oxidase assay (1.2 i.u. l^{-1}) and the L-amino acid oxidase assay (0.03 i.u. l^{-1}). Although the indicator system was exactly the same for both assays the L-amino acid oxidase assay unaccountably showed a 50 fold increase in sensitivity over the D-amino acid oxidase assay. In fact, the sensitivity claimed by Guilbault & Heiserman for this latter fluorimetric method is the same as the sensitivity of the present spectrophotometric method.

The present spectrophotometric method is a simple method to perform and shows a considerable degree of precision, particularly for enzyme activities in excess of 20 i.u. l^{-1}. Due to the absorption characteristics of both NAD^+ and NADH the need for standardization does not arise and the method could be very easily adapted for use with automated enzyme rate analysers, thus enabling large numbers of assays to be done relatively easily. This latter feature is of particular importance in view of the dramatic increase in both quantity and sophistication of automated analysis systems in use in routine biochemistry laboratories. The method could also be modified to measure the fall in concentration of NADH by monitoring the fall in fluorescence at the assay pH. This might give some increase in sensitivity without dispensing with the kinetic nature

of the assay but the inherent blank problem indicated above would remain.

The sensitivity of the fluorimetric assay could probably be improved by utilizing a lower concentration of standard and possibly by increasing the degree of dilution, which should affect the blank reading to a greater extent than the test. An increase in reaction time would also increase the sensitivity but this might result in a method which is too tedious. There is certainly room for improvement in the fluorimetric method and it is possible that a greater degree of sensitivity could be achieved by careful design of the method but the problem of a blank signal would always act to reduce the sensitivity.

The potential danger of contamination of the solutions with ammonia was highlighted in the initial studies on enzyme specificity. The apparent reaction of the enzymes with both stereoisomers of aspartic acid demonstrated that, without adequate controls, the presence of ammonia in the last reagent to be added to the reaction mixture, usually the substrate, could result in an undetected incorrect result. Graphically the reaction was different in that the usual lag period did not occur and the initial velocity was the maximum velocity as is the case in most non-coupled reactions. Thus it is essential when using any new substrate solutions to check for the presence of ammonia. The simplest way to do this, is to add the substrate to the reaction mixture in the absence of any amino acid oxidase and any subsequent fall in absorbance is most likely to be due to the presence of ammonia.

CHAPTER 2

TISSUE HOMOGENATES.

Introduction

The second of the two major objectives in the present work, was the study of the distribution and nature of the amino acid oxidases in human tissues. Due to the obvious difficulties of obtaining more than a minimal supply of human tissue, it was decided to use rat tissue initially, in order to clarify some of the basic problems in the preparation of tissue homogenates.

There are a variety of ways of studying enzyme activity in cells and tissues but not all are appropriate for all situations. The use of tissue slices is suitable for such techniques as Warburg manometry but is obviously unsuitable for spectrophotometric techniques, whilst the homogenisation of tissue has drawbacks due to the labile nature of enzymes.

It was decided in the present study to utilize tissue homogenates in which the cells were mechanically disrupted and to clarify the soluble fraction which resulted, by centrifugation. It was felt necessary to adopt this technique for two main reasons. Within the cells, the activity of an enzyme is modified by other factors which influence the transport of either substrate or products across the cell membrane, and so in order to produce maximum enzyme activity in any preparation it would be necessary to remove this barrier. In doing this however, the conditions necessary for cell disruption may result in the denaturation of some protein. The second reason for the use of tissue homogenates was that the centrifugation of the homogenate would result in a clear supernatant suitable for spectrophotometric studies. Whilst this was not necessary for the gasometric studies it was felt desirable to use a standard method throughout the investigation. A difficulty with such a method lay in the fact that membrane bound enzymes would separate with the cellular debris on centrifugation, and hence not be available in the supernatant for subsequent assay. It was desirable therefore to utilize some agent which would enable the release of

protein from cell membranes.

Due mainly to the availability of suitable instrumentation, it was decided to use a combination of two main disruptive techniques. After preliminary disintegration of the tissue into a fine brei in 1.0 mol l^{-1} KCl solution, it was subjected to two treatments, each of 30 second duration, with a blade homogeniser at high speed (14 000 rpm) and subsequently to two 30 second periods of ultrasonic disruption. The homogenate was then centrifuged at 50 000 rpm (150 000 x g) for 30 minutes in a refrigerated centrifuge and the supernatant passed through a small Sephadex G25 column, primarily to remove amino acids and ammonia.

Although it was desirable to release as much protein as possible during the homogenisation process, it was not essential that all the protein should be made available in the supernatant provided that a standard technique was adhered to and the effectiveness of disruption assessed in some way. This was done by measuring the protein content of the supernatant and expressing the enzyme activity of the preparation in terms of units of activity per gram of soluble protein.

1. Preparation of homogenates.

1.1. Protein releasing agents.

The fact that a significant proportion of cellular protein is bound to membranes, meant that some technique to release as much protein as possible had to be employed. The efficiency of two potential releasing agents, deoxycholate and Triton X100 was investigated by incorporating various concentrations of the two agents in aliquots of a suspension of rat kidney tissue, and subjecting each aliquot, including a control, to the defined homogenisation procedure (Method 6.p125). The D-amino acid oxidase and L-amino acid oxidase content of each supernatant sample was measured using the kinetic assay method and the protein content was measured using the biuret method. (Method 7a.p126).

Triton X100 in concentrations ranging from 0.05% to 0.5% (v/v), resulted in the release of approximately twice the amount of protein

than in the control and consistently more than that achieved with deoxycholate. (Table 15. p 78). The enzyme activity of the homogenates however, was generally less in Triton X100 than in the deoxycholate homogenates. The difference was most noticeable in the values for the specific activity, the values for Triton X100 being lower not only than the deoxycholate homogenates but also lower than the control homogenate. Further studies on the effectiveness of deoxycholate revealed that maximum release of both D- and L-amino acid oxidases in terms of specific activity of the homogenates occured in a concentration of 1 mmol l^{-1} of the detergent.(Table 15. p 78).

1.2. Storage conditions for tissue and homogenates.

It was appreciated that it would be necessary on occasions to store both tissue and homogenates prior to examination and therefore it was essential to determine the most satisfactory storage conditions. A sample of rat kidney homogenate was prepared in 1.0 mol l^{-1} KCl and divided into three aliquots. To one aliquot, bovine serum albumin was added to give a final concentration of 10 g l^{-1}, whilst an equal volume of glycerol was added to the second aliquot. The third aliquot was not modified in any way. A sample of each aliquot was stored at each of three temperatures, room temperature, 4°C, and -20°C, and the activity of D- and L- amino acid oxidases assayed at intervals over the following twelve weeks and reported as a percentage of the activity on day 1, (Table 16. p 79).

The results showed conclusively that storage in 50% (v/v) glycerol at -20°C was the most effective in maintaining enzymic activity. Only 10% of the original activity was lost in the first 14 days and in the case of D-amino acid oxidase approximately 75% of the initial activity remained after twelve weeks. L-amino acid oxidase similarly showed greatest stability under these conditions but appeared to be less stable than the D-amino acid oxidase. However due to the low initial L-amino acid oxidase activity, measurements of reduced activity were more difficult than for the D-amino acid oxidase.

A study of the effect of storage conditions on fresh tissue was difficult due to the limited amount of tissue available at one

Study of protein releasing agents in tissue homogenisation.

KIDNEY TISSUE - SAMPLE A

		D-amino acid oxidase		L-amino acid oxidase	
	Protein ($g\ l^{-1}$)	Activity ($IU\ l^{-1}$)	Specific activity per g protein ($IU\ g^{-1}$)	Activity ($IU\ l^{-1}$)	Specific activity per g protein ($IU\ g^{-1}$)
No detergent	4.0	48	12.0	6.0	1.5
Deoxycholate					
10 mmol l^{-1}	4.8	66	13.8	9.5	2.0
5	5.7	85	15.0	14.0	2.5
1	6.0	97	16.2	18.6	3.1
Triton X 100					
0.5% (v/v)	8.0	41	5.1	6.0	0.7
0.25%	8.2	64	7.8	9.0	1.1
0.05%	7.8	72	9.2	11.0	1.4

KIDNEY TISSUE - SAMPLE B

No detergent	3.5	70	20	6.0	1.7
Deoxycholate $mmol\ l^{-1}$					
5	4.5	110	24	8.0	1.8
1	4.8	150	31	10.2	2.0
0.5	4.2	95	22	6.8	1.6
0.1	4.0	80	20	6.0	1.5

Varying concentrations of protein releasing agents were incorporated into aliquots of a suspension of rat kidney and after homogenisation by the methods specified, the protein content and the enzyme activity of the samples were determined in duplicate assays.

Table 16

Effect of storage conditions on tissue homogenates

Day	Enzyme activity of the stored sample expressed as a percentage of the original activity					
	D-amino acid oxidase			L-amino acid oxidase		
	KCl 1.0 mol l^{-1}	Bovine Serum Albumin 10 g l^{-1}	Glycerol 50% (v/v)	KCl 1.0 mol l^{-1}	Bovine Serum Albumin	Glycerol 50% (v/v)
ROOM TEMPERATURE						
1	100	100	100	100	100	100
2	26	25	93	17	22	65
3	0	0	90	0	0	35
4			88			0
5			85			
7			75			
14			35			
28			0			
4° CENTIGRADE						
1	100	100	100	100	100	100
2	75	79	98	55	95	90
3	45	61	97	18	95	85
4	20	56	98	0	80	90
5	12	44	96		60	75
7	8	28	96		42	50
14	0	0	90		0	20
28			82			0
56			62			
84			35			
-20° CENTIGRADE						
1	100	100	100	100	100	100
7	95	95	98	75	95	98
14	72	80	90	70	65	90
28	39	57	86	0	35	90
56	12	34	80		0	60
84	0	20	76			50

Tissue homogenates were stored under the conditions indicated and the enzyme content of each sample was determined in duplicate on the days indicated.

time and also due to normal variations in enzyme content of tissues from different rats. The kidneys from four rats were chopped up, and the tissue mixed, and then divided into four portions, three of which were immediately frozen at -20°C and the fourth immediately homogenised and assayed the same day. Subsequently the three remaining samples of tissue were homogenised and assayed at fortnightly intervals.

The results, although showing variation in enzyme activity between the four homogenates, did give comparable results for the specific activities, although again the results for the L-amino acid oxidase were less satisfactory than for the D-amino acid oxidase. (Table 17. p 81). It was concluded that it would be acceptable to store tissue at -20°C for short periods if it was not convenient to prepare the homogenates immediately the tissue was available.

1.3. Protein determination.

It was noticed, when determining the protein content of samples by the biuret method, that very high blank readings were obtained when glycerol was involved. A study of the absorption characteristics of the copper-glycerol complex revealed an absorption maximum at 520 nm. This was very close to the absorption maximum of the copper-protein complex at 540 nm and both maxima showed considerable overlap. (Figure 15. p 82). An investigation into the effect of the concentration of glycerol on the absorbance of biuret reagent measured at 540 nm, showed that maximum colour was developed with glycerol solutions exceeding 10% (v/v) (Table 18. p83). Due to the fact that it was difficult to prepare a 50% glycerol solution with a high degree of precision, it was felt necessary to investigate the effect of such variation in glycerol concentration, on the precision of the protein determination. This was done by incorporating 30% and 50% glycerol in a prepared series of dilutions of 20 g l^{-1} albumin and measuring the absorbance at 540 nm after the addition of the appropriate volume of biuret reagent.

The results indicated that the effects on the accuracy of protein determinations was minimal, but due to the slope of the standard graph being significantly reduced, the sensitivity of the method would be

Table 17

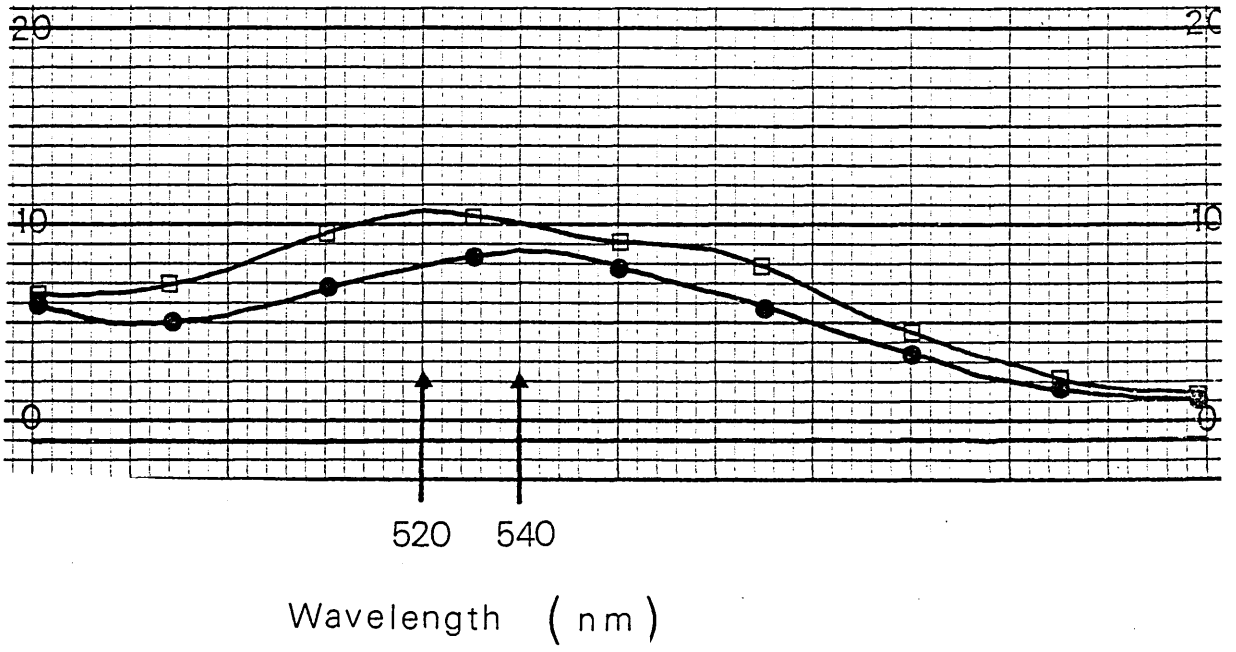
Effect of storage at -20 °C on fresh tissue

		D-amino acid oxidase		L-amino acid oxidase	
Day	Protein (g l^{-1})	Activity (IU l^{-1})	Specific Activity (IU g^{-1})	Activity (IU l^{-1})	Specific Activity (IU g^{-1})
1	5.0	112	22	10.5	2.1
14	4.0	74	18	7.0	1.7
28	5.1	102	20	8.0	1.5
42	3.6	60	16	4.5	1.2

Data indicates the enzyme content of homogenates prepared from the same sample of tissue after storing at -20°C for varying periods of time. All assays were done in duplicate.

Figure 15

Absorption spectrum of the glycerol-biuret complex



Absorption spectrum of the protein-biuret complex (●——●) shows an absorption maximum at 540 nm whilst the glycerol-biuret complex (□——□) shows an absorption maximum at 520 nm. The two spectra show considerable overlap.

Table 18

Reaction between glycerol and Biuret reagent

Glycerol Concentration (% v/v)	Absorbance 540 nm
0	0
10	0.090
20	0.110
30	0.120
40	0.125
50	0.125

PROTOCOL

Reagent	Volume (cm^3)
Glycerol	0.5
Biuret reagent	4.5

Mix and allow to stand at room temperature for 30 minutes. Measure absorbance at 540 nm.

impaired. (Table 19, p 85).

Investigations revealed that glycerol had no effect on the tyrosine method of protein determination, Lowry et al (1951) which, in any case, was more sensitive than the biuret method. For these reasons it was decided to use the tyrosine method for the determination of protein in tissue homogenates (Method 7b. p126).

2. Assay of tissue homogenates.

2.1. Blank reaction at pH 7.5

During the preliminary work on rat tissue homogenates, the assay of L- amino acid oxidase activity was always complicated by the presence of a very high blank reaction. The blank reaction was minimal in the assay of commercially prepared enzymes and for the D-amino acid oxidase assay at pH 8.3 tissue homogenates gave blank reaction rates in the region of ΔE_{340} 0.0050 min^{-1}, whereas for the assay of L-amino acid oxidase activity at pH 7.5 the blank reaction rates were as high as ΔE_{340} 0.0200 min^{-1}. This meant that there was no significant increase in the rate of reaction on the addition of the substrate, in the assay of samples with low enzyme activities. This was obviously very unsatisfactory and it was felt necessary to identify the reason and to determine suitable conditions for the assay of the enzyme.

In order to be able to consider alternative assay methods it was necessary initially to identify the components of the present assay system which resulted in this blank reaction. To this end a series of tests were prepared in glycylglycine buffer at pH 7.5 in which one component of the assay system was omitted from each test except the control. The fall in absorbance at 340 nm due to the blank reaction was monitored for three hours.

The results showed that the blank reaction in the control which showed a fall in absorbance of 0.53 $hour^{-1}$, was not produced if any component of the assay system was omitted (Table 20. p 86). In order to check the effect of omitting ADP from the assay, the glutamate dehydrogenase was stabilized by the incorporation of 1.0 mol l^{-1} NaCl

Table 19

Effect of glycerol on protein determinations using the Biuret reaction

Protein Content (g l^{-1})	Absorbance 540 nm	
	30% Glycerol	50% Glycerol
0.2	0.035	0.035
0.4	0.080	0.080
0.6	0.120	0.120
0.8	0.160	0.160
1.0	0.195	0.195
1.2	0.230	0.230
1.4	0.280	0.280
1.6	0.310	0.310
1.8	0.340	0.340
2.0	0.380	0.380

The absorbance of samples containing the indicated glycerol concentration was measured against a reagent blank which incorporated an aqueous solution of glycerol (50% v/v).

Table 20

Blank reaction - effect of assay components

Component omitted from the assay mixture	Rate of reaction ΔE_{340nm} hour^{-1}
None	0.53
Homogenate sample	0.01
Glutamate dehydrogenase	0.06
2-oxoglutarate	0.08
ADP	0.05
Glutamate dehydrogenase) 2-oxoglutarate) ADP)	0.08

The blank reaction was monitored as indicated in Method 2 when using an assay pH of 7.5 and tissue homogenate as the source of enzyme.
In the absence of ADP the stability of the glutamate dehydrogenase was maintained by the incorporation of 1.0 mol l^{-1} NaCl solution.

which had been demonstrated to be effective in previous studies (p 30). The addition of extra NADH to the control when all the initial coenzyme had been oxidized, resulted in a reaction rate which was greater than the initial rate and this suggested that there was a possible build up situation which was not dependent upon the presence of NADH.

The only component not checked by this series of experiments was the glycylglycine buffer, and subsequent investigation suggested that this compound was primarily responsible, in the presence of the homogenate, for this blank reaction. A series of tests were made in which the glycylglycine buffer was replaced with 0.1 mol l^{-1} phosphate buffer pH 7.5 and in which glycylglycine was incorporated in concentrations varying from 0 - 0.024 mol l^{-1}. The fall in absorbance was monitored after the addition of homogenate. Compared with a rate of 0.019 min^{-1} in the usual glycylglycine buffer, the rate in the phosphate buffer was only 0.0050 min^{-1}. The addition of glycylglycine to the phosphate buffer caused the reaction rate to increase from a value of 0.0050 min^{-1} to a value of 0.018 min^{-1} (at a glycylglycine concentration of 0.024 mol l^{-1}). This was comparable with the maximum rate of 0.019 min^{-1} achieved in the glycylglycine buffer. (Table 21. p 88).

The fact that the rate of the blank reaction at pH 8.3 was acceptable, suggested that pH might be an important factor, and a study of the pH profile of the blank reaction in glycylglycine buffer over the pH range 7.0 - 8.5 indicated a maximum rate at about pH 7.5 (Figure 16. p 89). Gasometric studies of the reaction using several samples of homogenates evidencing the high blank reaction failed to demonstrate any uptake of oxygen during the reaction, and thus excluded any process involving the oxidation of residual L-amino acids. The use of dialysed homogenates showed no significant diminution of the blank reaction whereas boiling the homogenate did effectively eliminate the reaction. These results suggested, that whilst the reaction was not due to the amino acid oxidases, it was nevertheless enzymic in nature.

Table 21

Blank reaction - rôle of glycylglycine

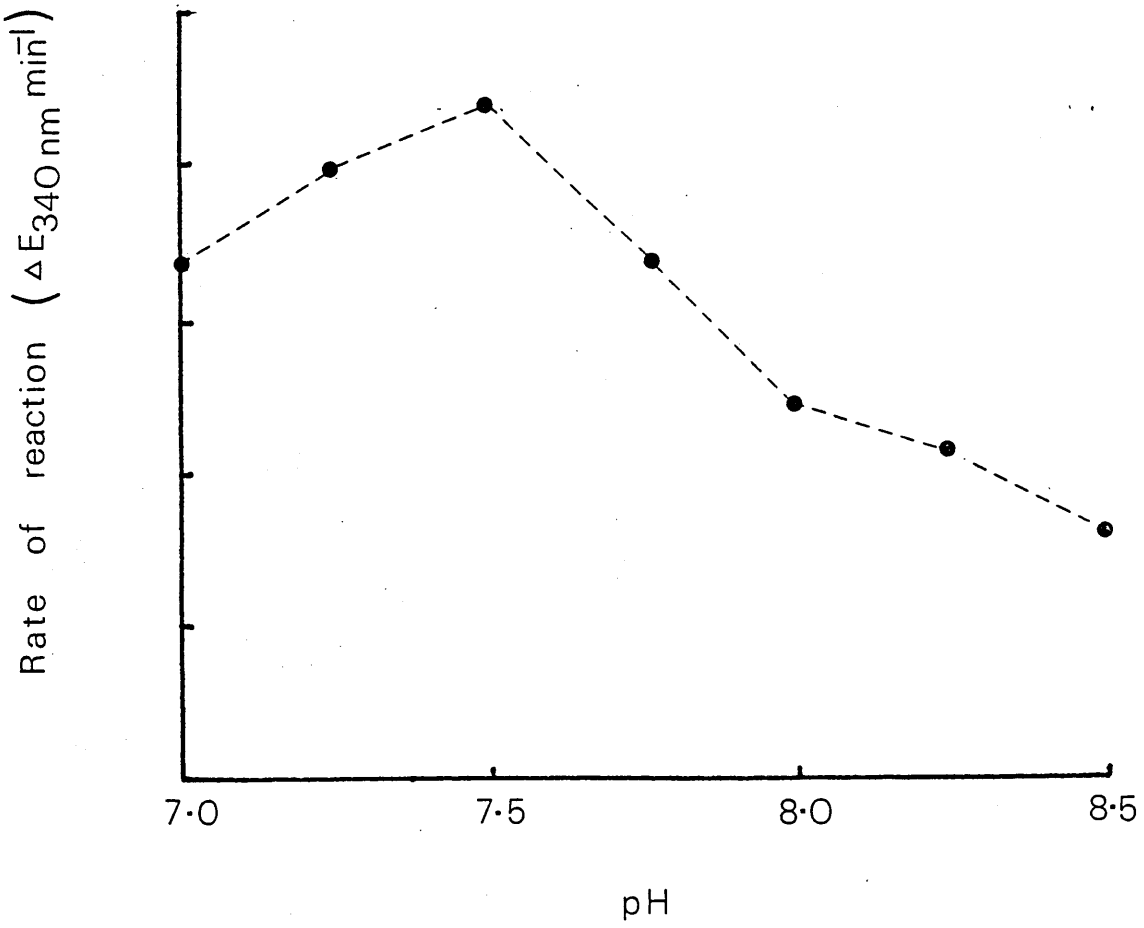
Final Concentration of glycylglycine (mol l^{-1})	Rate of Reaction ΔE_{340nm} min^{-1}
0	0.005
0.006	0.008
0.012	0.012
0.018	0.015
0.024	0.018

PROTOCOL

Reagent	Concentration	Volume (cm^3)
Phosphate buffer pH 7.5	0.1 mol l^{-1}	2.15
Glycylglycine		0.10
2-oxoglutarate	0.2 mol l^{-1}	0.20
NADH	2.25 mmol l^{-1}	0.20
ADP	15.0 mmol l^{-1}	0.10
GDH		0.05
Sample		0.20
Monitor absorbance at 340 nm.		

Figure 16

Blank reaction - effect of pH



The rate of the reaction ($\Delta E_{340\ nm}\ min^{-1}$) before the addition of the substrate (the blank reaction) was measured in buffer solutions of varying pH values.

PROTOCOL

Reagent	Concentration	Volume (cm^3)
Glycylglycine buffer	0.1 mol l^{-1}	2.05
2-oxoglutarate	0.2 mol l^{-1}	0.2
NADH	2.25 mmol l^{-1}	0.2
ADP	15.0 mmol l^{-1}	0.1
GDH		0.05
Homogenate sample		0.2

Monitor absorbance at 340 nm.

2.2. Utilisation of NADH.

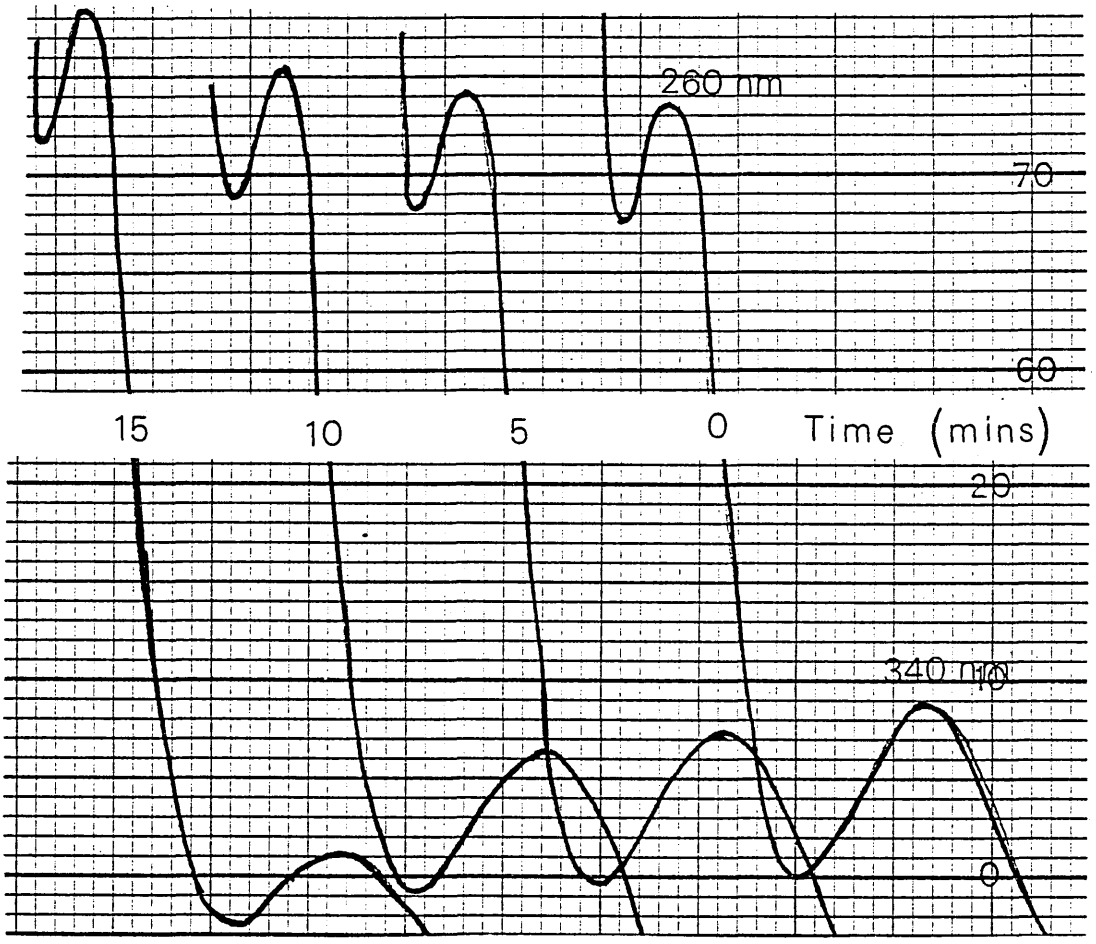
The main observable feature of the blank reaction was the fall in absorbance at 340 nm and it was assumed that this was due to the oxidation of NADH to NAD^+. It was felt necessary to clarify the fate of the NADH, there being the possibility that the NADH was being removed by some reaction other than the oxidation to NAD^+. This problem was approached in two ways, namely, the spectral changes occuring during the oxidation of NADH and also the fluorimetric quantitation of NAD^+ formation.

Normally it would be expected that the oxidation of NADH (absorption maximum 340 nm) to NAD^+ (absorption maximum 260 nm) would be accompanied by a fall in absorbance at 340 nm and an increase in absorbance at 260 nm. If, however, the observable fall in absorbance at 340 nm was related to the destruction of NADH rather than its oxidation, then some alternative spectral changes might occur. Studies of absorption spectra were complicated by the presence of ADP (absorption maximum 260 nm)and to overcome this difficulty the experiments were performed in the presence of 1.0 mol l^{-1} NaCl to stabilize the glutamate dehydrogenase. In control experiments utilizing commercial L-amino acid oxidase,it was possible to demonstrate that the fall in absorbance at 340 nm was paralleled by an increase in absorbance at 260 nm (Figure 17. p 91). However, in experiments using tissue homogenates, due to the high absorbance produced by the nature of the sample,it was not possible to demonstrate that the absorbance at 260 nm rose significantly.

The formation of NAD^+ during the reaction was however conclusively demonstrated by the use of the fluorimetric method. The NAD^+ content of aliquots taken from the reaction mixture were compared with the fall in absorbance at 340 nm of the reaction mixture. The rate of fall in absorbance at 340 nm expressed as a percentage of the total fall, showed a mean value of 3.0%,which compared favourably with the mean value for the percentage rate of increase in fluorescence of 2.8%. (Table 22. p 92).

Figure 17

Blank reaction - utilisation of NADH



Successive absorption spectra of the reaction mixture indicated below were recorded at the times indicated and show the absorption peaks at 260 nm and 340 nm.

PROTOCOL

Reagent	Concentration	Volume (cm^3)
Glycylglycine buffer	0.1 mol l^{-1}	2.0
2-oxoglutarate	0.2 mol l^{-1}	0.2
NADH	2.25 mmol l^{-1}	0.2
NaCl	15.0 mol l^{-1}	0.2
GDH		0.05
Homogenate sample		0.2

Scan absorbance over the wavelength range 240 - 360 nm at specified time intervals.

Table 22

Blank reaction - utilisation of NADH

Time of sample	Absorbance at 340 nm	Fluorescence %
0	0.88	8
5	0.72	30
15	0.48	45
25	0.22	63
35	0	88
Rate of reaction	0.026 min^{-1}	2.3 min^{-1}
Rate as a percentage of total change	3.0	2.8

The absorbance at 340 nm of a reaction mixture prepared as indicated below, was monitored for 35 minutes and the NAD^+ content of samples removed from the reaction mixture, determined using the fluorimetric method (Method 5).

PROTOCOL

Reagent	Concentration	Volume (cm^3)
Glycylglycine buffer	0.1 mol l^{-1}	2.05
2-oxoglutarate	0.2 mol l^{-1}	0.2
NADH	2.25 mmol l^{-1}	0.2
ADP	15.0 mmol l^{-1}	0.1
GDH		0.05
Homogenate sample		0.2

Monitor absorbance at 340 nm and remove samples for fluorimetric assay (Method 5).

2.3. Generation of ammonia.

The fact that both glutamate dehydrogenase and oxoglutarate were required for the blank reaction suggested that ammonia was being formed and subsequently utilized by the indicator enzyme system. It was decided therefore to monitor the ammonia levels in a reaction mixture which contained all the necessary components but lacked the indicator enzyme glutamate dehydrogenase. The hypochlorite - nitroprusside method was used for the determination of ammonia (Method 8. p128) Lubochinsky & Zalta (1954), but it was very soon realized that glycylglycine also reacted in a somewhat similar manner to ammonia and, at the high concentration used in the buffer, completely masked any colour development due to the presence of ammonia. Protein also acted in a similar manner and at a concentration of 5.0 g l^{-1} masked any colour development which may have been due to low concentrations of ammonia.

Bearing in mind the restrictions discussed above, a series of experiments were undertaken in which minimal amounts of homogenate and glycylglycine were used in a reaction mixture based on 0.1 mol l^{-1} phosphate buffer pH 7.5, the amount of ammonia formed being monitored over a 1 hour period. Using the information gained in the previous studies on the effect of glycylglycine concentrations on the rate of the blank reaction, a final concentration of 0.005 mol l^{-1} was selected. Reaction mixtures were prepared which contained all the necessary components and to the test, both homogenate and glycylglycine were added, whilst the controls contained either homogenate or glycylglycine.

Only the test mixture showed a continuous fall in absorbance at 340 nm which, due to the small quantity of sample used, amounted to a rate of 0.008 min^{-1}. Samples were taken from all three mixtures at 0, 30 and 60 minutes for ammonia determinations and the intensity of the resulting colour was measured in terms of absorbance at 560 nm. Both controls showed elevated but relatively constant colour development over the one hour period, whilst the test mixture showed a gradual increase in absorbance due to generation of ammonia (Table 23. p 94). Although the actual amount of ammonia generated could not be accurately calculated, due to the interference effect of

Table 23

Blank reaction - ammonia generation

Reagent	Volume (cm^3)		
	Test	Control	Blank
Phosphate buffer pH 7.5	6.0	6.4	6.0
Glycylglycine 0.1 mol l^{-1}	0.4	-	0.4
Homogenate	0.1	0.1	-
2-oxoglutarate	0.4	0.4	0.4
ADP	0.2	0.2	0.2
NADH	0.4	0.4	0.4
Fall in absorbance ΔE_{340nm} min^{-1}	0.008	nil.	nil
Ammonia content E_{560nm}			
zero time	0.17	0.04	0.18
30 mins	0.20	0.05	0.18
60 mins	0.26	0.05	0.20

Ammonia was measured using Method 8 and reported as absorbance at 560 nm

protein and glycylglycine, the total change in absorbance suggested the presence of approximately 0.08 µmole ammonia.

2.4. Choice of assay pH.

From the earlier study on the effect of pH on the activity of L-amino acid oxidase (Figure 6. p 47) it was noted that the pH profile showed a fairly broad peak and it was decided to investigate the possibility of using some pH other than 7.5 for the assay of L-amino acid oxidase. A comparative experiment was set up (Table 24. p 96.) in which the activity of the L-amino acid oxidase in a tissue homogenate was assayed in different buffers at pH 7.5, and also in glycylglycine at pH 8.3. The results indicated that glycylglycine at pH 8.3 was superior to any of the other buffers used, namely Tricine, phosphate or borate at pH 7.5. The activity of tissue L-amino acid oxidase at pH 8.3 in the presence of various alternative amino acid substrates was also investigated (Table 25. p 97) The amino acids, methionine, tyrosine, phenylalanine and tryptophan were selected on the basis of earlier studies on the specificity of snake venom L-amino acid oxidase, but L-leucine still proved to be the most suitable.

It was decided therefore, that the assay of L-amino acid oxidase in tissue homogenates should be done in glycylglycine buffer at pH 8.3 instead of the reputed optimum pH of 7.5.

3. Human tissues.

3.1. Assay conditions.

Homogenates were prepared from samples of human tissues using the method detailed on page 125 and were used to re-examine some of the basic problems investigated during the development of the assay methods.

The use of glycylglycine buffer at pH 7.5 still resulted in a very high blank reaction rate, compared to that obtained at pH 8.3. An examination of the blank reaction given by three samples of

Table 24

The activity of rat kidney L-amino acid oxidase in various buffers

Buffer	Reaction Rate $\Delta E_{340\ nm}\ min^{-1}$		
	Blank	Test	Difference
Glycylglycine pH 7.5	0.022	0.029	0.007
Tricine pH 7.5	0.018	0.024	0.006
Phosphate pH 7.5	0.005	0.007	0.002
Borate pH 7.5	0.001	0.002	0.001
Glycylglycine pH 8.3	0.005	0.017	0.012

The method used was as described for the spectrophotometric assay of L-amino acid oxidase (Method 2) and the results are the mean of duplicate assays.

Table 25

Activity of rat kidney L-amino acid oxidase with various substrates

Amino acid Substrate	Reaction Rate $\Delta E_{340\ nm}\ min^{-1}$		
	Blank	Test	Difference
L-leucine	0.008	0.019	0.011
L-methionine	0.009	0.019	0.010
L-tryptophan	0.009	0.017	0.008
L-phenylalanine	0.008	0.015	0.007
L-tyrosine	0.007	0.014	0.007

The L-amino acid oxidase content of rat kidney homogenates were assayed using the specified method (Method 2) using a buffer pH of 8.3

homogenates from liver and kidney showed that in glycylglycine buffer at pH 8.3 the blank reaction was on average only 20-25% of the rate produced in glycylglycine buffer at pH 7.5 (Table 26. 99). It was also confirmed that human D-amino acid oxidase required the presence of FAD in order for maximum activity of the enzyme to be demonstrated. In the same three homogenates, the ommission of FAD from the assay reagent resulted in a loss of nearly 80% of the activity whilst the incorporation of FMN did not improve on this figure (Table 27. p 100).

3.2. Kinetic characteristics of human amino acid oxidases.

The kinetic characteristics of the D-amino acid oxidase from human tissues were studied for comparison with the commercial preparations of the enzyme from hog kidney, used in the initial stages of the project. The low activity of L-amino acid oxidase in the homogenates examined, however, made it impossible to obtain valid data for a similar comparison with snake venom L-amino acid oxidase.

The effect of substrate concentrations on the D-amino acid oxidase present in homogenates of liver and kidney was determined using a series of solutions of D-alanine ranging from 1.3 mmol l^{-1} to 6.6 mmol l^{-1}. (Table 28. p 101). The values of the Michaelis constants for the two enzyme preparations were calculated from these data using the statistical method indicated (Method 3. p121). The Km value for the enzyme from liver was 7.2 mmol l^{-1} whilst that for the enzyme from kidney was 5.9 mmol l^{-1}. Graphical plots of the data (Figure 18. p 102.) gave values which were even closer to one another, being 6.7 mmol l^{-1} and 6.0 mmol l^{-1} respectively.

Specificity studies on human D-amino acid oxidase were undertaken, using the same approach as described for similar studies with the commercial enzyme preparations (p58). Additional problems lay in the volume of samples available and the relatively low activity of enzyme in the homogenates even when using the normal substrate. Where adequate tissue was available, homogenates were prepared in larger volumes of approximately 6 cm^3 but even so it was necessary to use different homogenates and cross check the activity of each homogenate against the same amino acids.

Table 26

Blank reaction - human tissue homogenates

pH	Rate of reaction (ΔE_{340nm} min^{-1})	
	Liver	Kidney
7.5	0.021 (100%)	0.028 (100%)
8.3	0.004 (19%)	0.005 (18%)

The rate of reaction given by the homogenates of three samples of human liver and kidney in the absence of substrate, was measured in buffer solutions at the two pH values indicated. The data presented are the mean of three observations.

PROTOCOL

Reagent	Concentration	Volume (cm^3)
Glycylglycine buffer	0.1 mol l^{-1}	2.05
2-oxoglutarate	0.2 mol l^{-1}	0.2
NADH	2.25 mmol l^{-1}	0.2
ADP	15.0 mmol l^{-1}	0.1
FAD	0.3 mmol l^{-1}	0.1
GDH		0.05
Homogenate		0.2
Monitor the absorbance at 340 nm		

Table 27

Human D-amino acid oxidase - coenzyme requirements

Coenzyme added	Rate of reaction (ΔE_{340nm} min^{-1})	
	Kidney	Liver
FAD	0.0170 (100%)	0.0150 (100%)
FMN	0.0040 (23%)	0.0030 (20%)
none	0.0035 (21%)	0.0020 (13%)

The activity of the D-amino acid oxidase from three samples of human liver and kidney was measured in the presence of FAD and FMN. The data presented is the mean of the three observations.

PROTOCOL

Reagent	Concentration	Volume (cm^3)
Glycylglycine buffer pH 8.3	0.1 mol l^{-1}	2.05
2-oxoglutarate	0.2 mol l^{-1}	0.2
NADH	2.25 mmol l^{-1}	0.2
ADP	15.0 mmol l^{-1}	0.1
Coenzyme	0.3 mmol l^{-1}	0.1
GDH		0.05
Homogenate		0.2
Monitor absorbance at 340 nm and add:		
D-alanine	0.5 mol l^{-1}	0.1

Table 28.

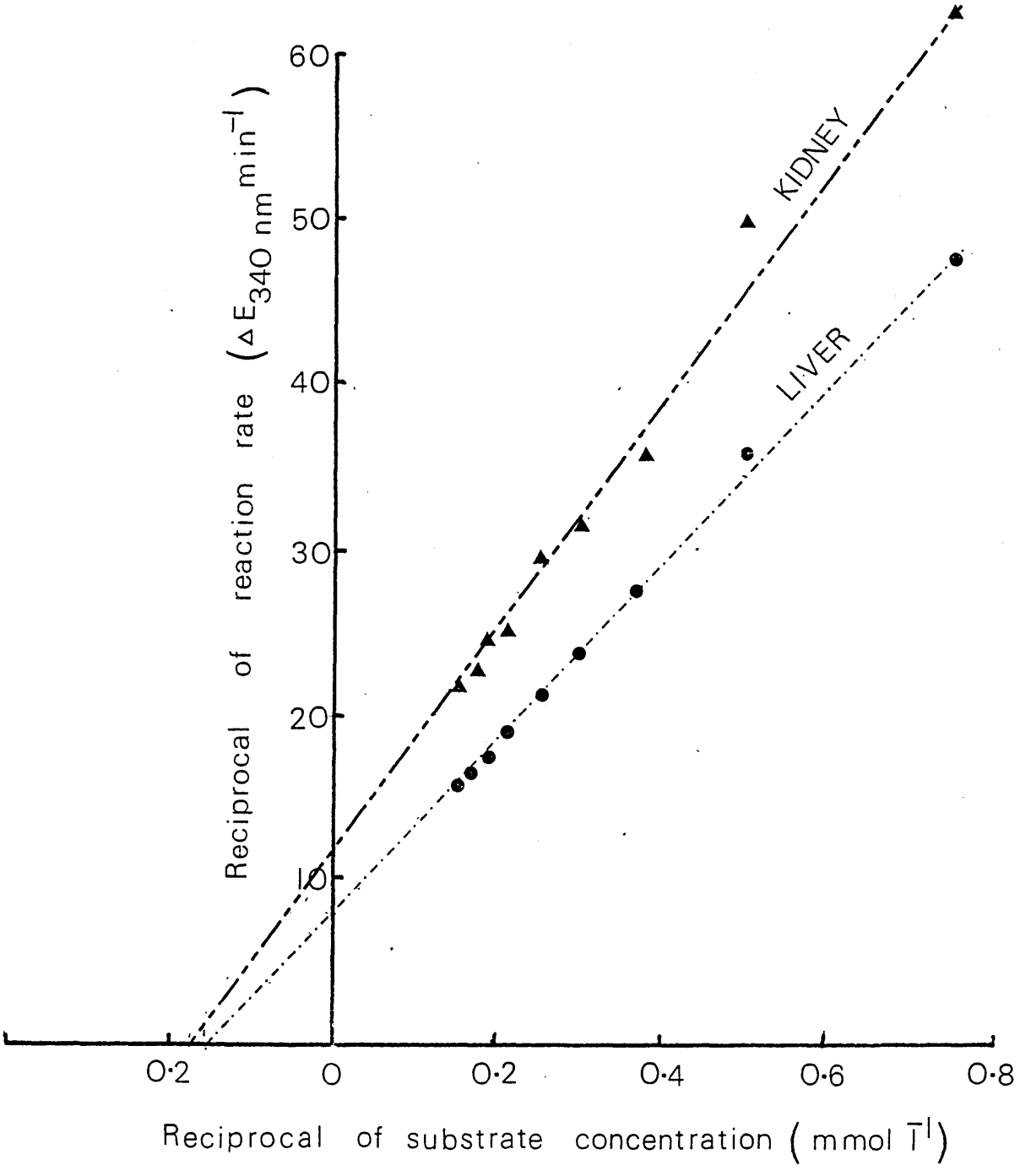
Human D-amino acid oxidase - determination of Michaelis constant

Final Concentration of substrate (mmol l^{-1})	Rate of reaction (ΔE_{340nm} min^{-1})	
	Liver	Kidney
1.33	0.0210	0.0160
2.00	0.0280	0.0200
2.66	0.0365	0.0280
3.33	0.0420	0.0320
4.00	0.0470	0.0335
4.66	0.0530	0.0400
5.33	0.0586	0.0405
6.00	0.0610	0.0440
6.66	0.0625	0.0455

Method	Michaelis Constant (mmol l^{-1})	
Graphical	6.7	6.0
Statistical	7.2 ±0.71	5.9 ±0.56

Figure 18

Human D-amino acid oxidase - effect of substrate concentration



The problem of ammonia contamination of amino acids was again encountered (p 58.) and was detected mainly because of the different shape of the reaction trace in respect of the lag reaction. Subsequently all amino acid solutions were checked for the presence of ammonia, using the same reaction mixture but lacking the amino acid oxidase. Those amino acids which contained only small amounts of ammonia when so tested, were allowed to react until a stable absorbance was achieved and if there was still an adequate amount of NADH, as indicated by the absorbance value, the assay reaction was initiated by the addition of the test enzyme. Aspartic acid, due to the relatively large amount of ammonia present, was treated in bulk with glutamate dehydrogenase, oxoglutarate and NADH before checking again in the manner indicated.

The specificities of D-amino acid oxidase from both liver and kidney were examined in this way and proved to be very similar and the results are listed in Table 13. (p 60) together with the data from the commercial enzyme preparation.

3.3. Amino acid oxidase content of human tissues.

One of the initial objectives of this research was to investigate the distribution of the amino acid oxidases in human tissues, with a view to clarifying any potential relationship between enzyme distribution and tissue dysfunction. However organisational difficulties in the later stages of the work made it difficult to obtain the wide range of suitable specimens initially envisaged. As a result, the experimentation had to be restricted to an examination of the distribution of the amino acid oxidases in a limited number of tissues.

The tissues examined were brain, heart, kidney, liver, lung, and spleen and were random specimens obtained from post mortems at a large general hospital, with no prior knowledge of the precise disease processes involved. The tissues were taken within 12 hours of death and immediately frozen at $-20^{\circ}C$. All the tissues selected were superficially normal in appearance and were from donors in the 55 - 65 years of age range, death being as a result of either vascular disease or secondary malignancies. In all ten sets of

tissues were examined.

Homogenates were prepared in the manner described (Method 6. p125) and assayed immediately for the presence of D- and L-amino acid oxidases using both the kinetic and fluorimetric assay methods. Subsequently the protein content of the homogenates was determined using the tyrosine method (p.126)and the enzyme content of each tissue expressed as the number of International Units of activity per gram of soluble protein (Table 29. p 105).

The results showed that of the six tissues examined , in each case, only three consistently showed any amino acid oxidase activity. Kidney generally had the highest content of D-amino acid oxidase with liver containing similar, although usually lower activities. The samples of brain examined showed an activity of D-amino acid oxidase which was approximately one tenth that of kidney. Only kidney and liver contained any L-amino acid oxidase, the activity of which was relatively low.

Five samples of freshly separated serum from normal adults failed to demonstrate any D- or L-amino acid oxidase activity.

4. Discussion.

A major problem encountered during the assay of tissue homogenates was the rapid utilization of NADH in the presence of the homogenate at pH 7.5. The subsequent decision to measure the activity of L-amino acid oxidase at pH of 7.5 only circumvented the problem without identifying the cause. The selection of pH 8.3 as the assay pH for the enzyme reduced the observed rate of reaction by approximately 25% (Figure 6. p 47) which although undesirable, did not have a serious effect on fundamental sensitivity of the method. Certainly compared with the use of pH 7.5 the selection of pH 8.3 significantly improved the sensitivity (Table 24. p 96) and also considerably simplified the assay procedure in that only one buffer was required for both assays.

The presence of glycylglycine was obviously essential for this enzymic generation of ammonia although its exact role was not clear. The most likely function was that of substrate,although it also could

Table 29

Amino acid oxidase content of various human tissues

Tissue	Enzyme	Enzyme content (I.U. g^{-1} protein)									
Brain	D-AAO	0.4	0	0.3	0.1	0	0.4	0.8	0.2	0.3	0.2
	L-AAO	0	0	0	0	0	0	0	0	0	0
Heart	D-AAO	0	0	0	0	0	0	0	0	0	0
	L-AAO	0	0	0	0	0	0	0	0	0	0
Kidney	D-AAO	6.6	1.3	6.5	4.2	1.3	5.3	3.1	2.1	5.1	4.0
	L-AAO	0.2	0	0.4	0.2	0.2	0.4	0.3	0.2	0.4	0.5
Liver	D-AAO	5.2	2.7	4.7	0.5	4.5	2.6	2.4	1.8	3.6	2.9
	L-AAO	0.1	0.1	0.3	0.2	0.4	0.6	0.6	0	0.5	0.6
Lung	D-AAO	0	0	0	0	0	0	0	0	0	0
	L-AAO	0	0	0	0	0	0	0	0	0	0
Spleen	D-AAO	0	0	0	0	0	0	0	0	0	0
	L-AAO	0	0	0	0	0	0	0	0	0	0
Serum	D-AAO	0	0	0	0	0					
	L-AAO	0	0	0	0	0					

The above data were obtained by the spectrophotometric method and each figure is the mean of two assays. No enzymic activity in any sample reported negative could be detected by the use of the fluorimetric method.

have acted as an activator of some other system. It was obviously not a substrate for the L-amino acid oxidase as evidenced by its non-reaction with the snake venom enzyme.

The identification of the precise nature of this reaction would be desirable and, if known, might suggest an alternative solution to the problem. Further investigation could also be undertaken to find a suitable buffer system other than glycylglycine which would not be susceptible to this reaction, possibly a compound other than an amine.

The examination of the amino acid oxidase content of human tissue revealed that only two tissues contained the enzymes in any appreciable amounts, namely kidney and liver. The predominant enzyme was the D-amino acid oxidase and although the presence of L-amino acid oxidase was demonstrated, its activity in the preparations was not sufficient to enable critical kinetic studies to be satisfactorily undertaken. In order to attempt such studies using the present methods, some preliminary purification and concentration of the enzyme would be necessary.

Studies of the D-amino acid oxidase from liver and kidney revealed almost identical specificities (Table 13. p 60.) and the Km values calculated for each of the enzyme preparations were very similar suggesting that the two enzymes were identical in nature. This conclusion could be clarified if studies of the physical characteristics of these enzymes were undertaken.

A study of the specificities of human and hog D-amino acid oxidases revealed both considerable differences and similarities. The enzymes from both sources failed to exhibit any activity with cystine, glutamic acid, glycine, lysine, threonine, and tryptophan. The enzyme from hog kidney only showed very slight activity with arginine but both enzymes showed similar levels of activity with aspartic acid, tyrosine and possibly valine.

The two enzymes differed more significantly in their reactivity towards leucine, iso-leucine, and phenylalanine, with which the hog kidney D-amino acid oxidase demonstrated an activity of approximately twice that of the human enzyme. A considerable variation also occured in the activity of the enzymes towards histidine and serine, neither of which showed any significant reaction with the hog D-amino

acid oxidase but did show an appreciable rate of oxidation with the human enzyme. The two enzymes also showed a difference in the values calculated for the Michaelis constants. The Km value of human D-amino acid oxidase showed a mean value of 6.5 mmol l^{-1} whilst the hog kidney D-amino acid oxidase gave a value of 3.3 mmol l^{-1}. It seems fairly certain that there are considerable differences between these two enzymes and as suggested earlier, a study of their physical characteristics would help to confirm this belief.

The survey of the literature which was reported in the Introduction, indicated that there was general agreement on the matter of the distribution of the enzymes in tissues. Kidney and liver were stated to be the richest sources of both D- and L-amino acid oxidase in mammals. Krebs (1935), suggested that there was approximately four times as much D-amino acid oxidase in kidney than there was in liver. However the results presented in this thesis show the relative amounts in both tissues to be much closer but with kidney showing consistently higher levels of activity.

Krebs (1951), suggested that D-amino acid oxidase was also present in brain tissue, a point also made by Dunn & Perkoff (1963), who detected the enzyme in brain stem as well as in liver and kidney. They did not however, detect the enzyme in any other tissue nor in samples of serum.

These results agree with the present findings in which the presence of D-amino acid oxidase in brain tissue was consistently demonstrated in a concentration approximately one tenth of that in kidney tissue.

L-amino acid oxidase was only demonstrated in liver and kidney, the levels in both tissues being approximately the same, albeit very low. L-amino acid oxidase however could not be detected in brain tissue nor in serum samples. Mecher & Masszi (1969), demonstrated the presence of an L-amino acid oxidase in the serum from patients suffering from pemphigus and herpes zoster which, they suggested, was of viral origin. They noted that the specificity of this L-amino acid oxidase differed considerably from other amino acid oxidases. This statement is also true with respect to the present studies on enzyme specificity, and the major differences between the two are

indicated in Table 30. (p 109).

Studies on the specificities of the amino acid oxidases by different workers have, as indicated in the Introduction, often varied considerably. An examination of Table 2. (p 10) shows the variation that Krebs found in studies conducted in 1935 and 1951. A valid comparison of the data from various sources was difficult due to the different ways in which the data were reported but nevertheless, several apparent areas of disagreement became evident. These areas are summarized in Table 31. (p 110).

The variations with L-amino acid oxidase would seem to be due almost entirely to the different sources of enzymes used in the studies, whilst the studies with D-amino acid oxidase show variations which are probably more significant. Meister & Wellner (1963), reported some activity of mammalian D-amino acid oxidase with glycine and Dixon & Kleppe (1965b), indicated an activity of approximately 10% of that with D-alanine. These results conflict with the present work which failed to demonstrate any activity of either hog or human D-amino acid oxidase with that amino acid. It has been suggested that the enzyme exhibits an optimum pH of about 10 for glycine and that this accounts for such discrepancies, Neims & Hellerman (1970). However the latter authors measured the enzyme activity at pH 8.5 whilst Meister & Wellner (1963) did not specify the assay pH. Berger et al. (1975), utilized a D-amino acid oxidase preparation for the quantitative determination of glycine at pH 9.7 by means of an enzymic cycling system, after allowing the D-amino acid oxidase to react with the glycine for up to 24 hours. It would appear, therefore that the activity of D-amino acid oxidase with glycine as a substrate at about pH 10, although measurable, is very low.

The considerable debate on the subject of specificity of these enzymes only serves to demonstrate the complexity of the enzymes concerned, and the considerable effect of external factors on the activity of an enzyme with any potential substrate. However the main value in the specificity studies in the present work, apart from the selection of the assay substrate, was to demonstrate identity between the two D-amino acid oxidases of human tissue and the difference between these enzymes and that from hog kidney.

Table 30

Specificity of L-amino acid oxidase in pemphigus serum.

Amino Acid	Snake venom L-AAO	Pemphigus L-AAO
leucine	100	100
alanine	0	500
aspartic acid	0	650
cystine	40	150
methionine	110	100
tryptophan	80	0

Data on the L-amino acid oxidase content of pemphigus serum were calculated from oxygen uptake measurements by Mecher, T., Masszi, J. (1969)

Activity of enzyme preparation is reported as a percentage of that resulting in the presence of L-leucine.

Table 31

Summary of variations in reported specificities of the amino acid oxidases.

L-amino acid oxidase.

Source Author	Snake venom Present work	Snake venom Lichtenberg(1968)	Rat tissue Krebs (1935)
Amino acid	Activity as a percentage of that with L-leucine		
Alanine	0	3	38
Aspartic acid	0	0.1	26
Glutamic acid	0	0.5	144
Valine	0	8	47

D-amino acid oxidase

Source Author	Human Present work	Hog Present work	Hog Dixon (1965)	Rat Krebs (1935)	Sheep Krebs (1951)
Amino acid	Activity as a percentage of that with D-alanine				
Arginine	0	1	0	33	-
Aspartic acid	32	30	0	12	3
Cystine	0	0	0	47	3
Glycine	0	0	10	0	-
Histidine	24	0	25	10	9
Lysine	0	0	0	-	2
Methionine	40	100	70	193	125
Serine	27	1	60	60	66
Tryptophan	73	80	40	12	58

CHAPTER 3

CONCLUSIONS

A major objective of this present work was the development of a valid assay method for the amino acid oxidases. High on the list of priorities was the ability to be able to study the catalytic properties of these enzymes in a kinetically acceptable manner. The methods reviewed in the introductory part of this thesis failed to provide the combined flexibility and sensitivity required. Prior to the development of the present method, no glutamate dehydrogenase coupled assay for the amino acid oxidases had been described and the majority of the methods did not lend themselves to continuous monitoring techniques with the resulting potential for subsequent automation.

The ultraviolet spectrophotometric method described in this thesis meets the requirements for an assay outlined in the Statement of Objectives (p 5). It is extremely reliable and simple to perform and, provided that reliable instrumentation is available, offers a valid assay method, useful over a wide range of enzyme activities. Being a kinetic method, it is ideal for the study of the kinetic characteristics of the enzymes and, due mainly to the spectral properties of NADH, offers a high degree of sensitivity not available in other assay methods. It is not an expensive assay to perform, a fact which makes it very suitable for an automated assay where cost becomes a significant factor. The enzyme glutamate dehydrogenase is the most expensive component of the assay but, due to the high specific activity of the commercial preparation available, it would be feasible to use up to a tenth of the volume specified in this present method. The current total cost is approximately 12 pence per assay (1978), of which 7.5 pence is due to the cost of the glutamate dehydrogenase.

The fluorimetric method described in this thesis is a very sensitive method for the assay of the amino acid oxidases and, although being a fixed time assay, still has many of the advantages of the kinetic method. As indicated in Chapter 1 (p 73), it should be possible to improve even further the high degree of sensitivity shown

by this method, a feature which becomes more significant with the development of high quality recording spectrofluorimeters of reasonable cost.

It is no reflection on the value of the fluorimetric method that, in the study of tissue homogenates as described in Chapter 2, no information on the amino acid oxidase content of tissue homogenates was gained by the use of the fluorimetric method that was not gained by the use of the spectrophotometric method. No enzyme activity was demonstrated by the fluorimetric method in samples which gave negative results using the kinetic method.

The data presented on the amino acid oxidase content of human tissues, show a high degree of consistency and the variation that was shown may have been due, at least in part, to the organisational difficulties experienced in obtaining the specimens. Liver and kidney are the major sources of the amino acid oxidases in human tissues and although the role of these enzymes is not clear, the suggestions made earlier (p14.) do not seem unreasonable. However, the presence of D-amino acid oxidase in brain tissue is not satisfactorily explained by any of these suggestions and further work on the catalysis of substrates other than amino acids might be informative.

It would be very desirable to study the distribution of the enzymes in tissues and serum from patients suffering from known diseases and to assess whether there is a relationship between the distribution and levels of the amino acid oxidases and a specific disease. Similarly a critical study of the distribution of the enzymes in specific tissues and sub-cellular fractions from appropriate organs, could be very enlightening. Certainly, the methods which have been described would be very suitable for such studies.

On the question of the specificity of the amino acid oxidases, a comprehensive study of the effect of pH on the oxidation of each amino acid would be desirable. For such a survey, the gasometric method would have to be used, but despite the limitations of such a method, the use of the Gilson Differential Respirometer, in experienced hands, offers a reliable method of assay which would be enhanced by the use of the continuous recording facility.

MATERIALS

Enzymes

Glutamate dehydrogenase (E.C.1.4.1.3.)

Source - bovine liver

Activity 1200 U. cm^{-3}

(127 710 Boehringer Mannheim, London).

D-amino acid oxidase (E.C.1.4.3.3.)

Source - hog kidney

Activity 0.02 U. mg^{-1}

(A9128, Sigma Chemical Co, London).

L-amino acid oxidase (E.C.1.4.3.2.)

Source - Bothrops Atrox Venon

Activity 0.65 U. mg^{-1}

(A4257, Sigma Chemical Co. London).

Chemicals. (Sigma Chemical Co. London).

D-alanine (A7377)

L-leucine (L8C00)

D-amino acids (DAA-16)

L-amino acids (LAA-21)

5,5'-diethylbarbituric acid (B0375)

Tris (tris(hydroxymethyl)aminomethane) T1378

Tricine (N-tris(hydroxymethyl)methylglycine) T0377

Glycylglycine (G1002)

NAD^+ (N7004)

NADH (N8129)

ADP (A0127)

FAD (F6625)

FMN (F6750)

2-oxoglutarate, sodium salt (K1875).

Imidazole (I0250)

Sodium deoxycholate (D6750)

Triton X100

Folin and Ciocalteu phenol reagent J/4100
(Fisons Scientific Apparatus, Loughborough)

Equipment

Unicam SP1800 U.V. spectrophotometer
(Pye Unicam Ltd, Cambridge.)

Gilson Differential Respirometer, Model G14.
(Gilson Medical Electronics, Villiers-le-Bel, France).

Aminco-Bowman Spectrofluorimeter.
(American Instrument Co. Inc. Silver Springs, Maryland, U.S.A.)

M.S.E. Superspeed 65, Preparative Ultracentrifuge.
(Measuring and Scientific Instruments, London).

M.S.E. Ultrasonic Disintegrator, 100 watt.
(Measuring and Scientific Instruments, London).

Polytron Tissue Disintegrator.
(Kinematica GMBH, Luzern, Switzerland).

Sephadex G25 Columns PD.10.
(Pharmacia Fine Chemicals, Uppsala, Sweden).

METHOD 1

Gasometric assay of D- and L-amino acid oxidases using a Gilson Differential Respirometer.

Reagents

1. Glycylglycine buffer

0.1 mol l^{-1} adjusted to pH 8.3 for the D-amino acid oxidase assay or pH7.5 for the L-amino acid oxidase assay.

2. Coenzyme

FAD. 0.3 mmol l^{-1}.

3. Sodium hydroxide solution 100 g l^{-1}

4. Substrates

D-alanine 0.5 mol l^{-1}

L-leucine 0.1 mol l^{-1}

5. Catalase preparation

1% (v/v) dilution of fresh red blood cells in water.

Protocol

Compartment	Reagent	Volume (cm^3)	
		D-AAO Assay	L-AAO Assay
Main Vessel	Buffer solution	1.2	1.3
	D-alanine	0.1	-
	L-leucine	-	0.1
	FAD	0.1	-
Side Arm	Sample	0.1	0.1
Well	Sodium hydroxide	0.1	0.1

Method.

1. The flasks were set up according to the protocol above and after sealing the joints, the flasks were equilibrated at 37°C for 15 minutes with the system open to the air.

2. The system was closed to the air and the volume readings monitored for a further 15 minutes.

3. The reaction was initiated by carefully mixing the contents of the main vessel and the side arm and the change in volume of the gas monitored for 30 minutes and recorded as µl per minute.

METHOD 2

Spectrophotometric assay of D- and L-amino acid oxidases in tissue homogenates

Reagents

1. Assay reagent

This reagent was often prepared in bulk and stored on ice until required, up to a maximum of three hours.

Reagent	Concentration	Volume (cm^3)
Glycylglycine buffer pH 8.3	0.1 mol l^{-1}	2.05
2-oxoglutarate	0.20 mol l^{-1}	0.20
NADH	2.25 mmol l^{-1}	0.20
ADP	15.0 mmol l^{-1}	0.10
FAD	0.3 mmol l^{-1}	0.10
Glutamate dehydrogenase	1200 I.U.cm^{-3}	0.05

2. Substrates

D-alanine	0.5 mol l^{-1}
L-leucine	0.1 mol l^{-1}

Method

1. 2.7 cm^3 of the assay reagent was pipetted into a clean, dry glass cuvette and the mixture allowed to attain a temperature of 37°C in a thermostatted cell holder.

2. The absorbance of the solution at 340 nm was continuously recorded and 0.2 cm^3 of the sample was added using an automatic pipette and mixed carefully.

3. When the absorbance showed either no further fall or a slow but steady fall (the blank reaction) the reaction was initiated by the addition of 0.1 cm^3 of the appropriate substrate.

4. The reaction was monitored for at least five minutes or until the linear section of the reaction trace could be clearly defined.

5. The rates of the blank reaction and the total reaction were determined graphically from the reaction trace and reported as the change in absorbance at 340 nm in one minute.

METHOD 3

Statistical methods

A. Standard deviation and mean.

$$SD = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}}$$

$$\text{Mean } (\bar{x}) = \frac{\sum x}{n}$$

B. Coefficient of variation. (CV)

$$CV = \frac{s}{\bar{x}} .100\ \%$$

C. Correlation coefficient. (r)

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

D. Linear regression.

$$y = a + bx$$

$$b = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sum (x - \bar{x})^2}$$

$$a = \bar{y} - b\bar{x}$$

$$\frac{1}{v} = \frac{1}{V_{max.}} + \frac{Km}{V_{max.}} \cdot \frac{1}{s}$$

METHOD 4

Calculation of enzyme activity from data gained using the spectrophotometric method.

Data needed.

1. Rate of blank reaction in terms of absorbance change at 340 nm per minute.
2. Rate of test reaction in terms of absorbance change at 340 nm per minute.

Calculation.

The enzyme activity in International Units per litre of sample was calculated from the equation:

$$\frac{(\Delta E_{340}\text{TEST} - \Delta E_{340}\text{BLANK})}{6.22 \times 10^3} \times \frac{10^6}{1} \times \frac{3.0}{10^3} \times \frac{10^3}{0.2}$$

A B C D

The various steps in the calculation were:

A. The use of the molar absorption coefficient of NADH to calculate the concentration change in mol l^{-1}.

B. The conversion of moles to micromoles.

C. Calculation of amount of NADH oxidised in the 3.0 cm^3 of the reaction mixture.

D. Correction for the volume of sample used.

The equation simplified to give a working equation of:

$$(\Delta E_{340}\ \text{TEST} - \Delta E_{340}\ \text{BLANK}) \times 2.41 \times 10^3 \text{ i.u. } l^{-1}$$

METHOD 5

Fluorimetric assay of D- and L-amino acid oxidases in tissue homogenates.

Reagents

1. Assay reagent

This was a bulk preparation of the assay reagent as for the spectrophotometric assay.

2. Sodium hydroxide solution

10 mol l^{-1} sodium hydroxide solution containing 10 mmol l^{-1} imidazole.

3. Standard solution

NAD^+ 1.5 mmol l^{-1} in water

4. Substrates

D-alanine 0.5 mol.l^{-1}

L-leucine 0.1 mol l^{-1}

Method

1. 2.7 cm^3 of the assay reagent was pipetted into each of two test tubes labelled TEST and BLANK and 0.2 cm^3 of sample was added to each tube.

2. After incubating at 37°C for about five minutes the reaction was initiated in the TEST by the addition of 0.1 cm^3 of the appropriate substrate.

3. Both tubes were incubated for a further 60 minutes.

4. To the BLANK, 0.1 cm^3 of the same substrate was added and immediately 1.0 cm^3 of solution from each tube was transferred to appropriately labelled tubes each containing 0.1 cm^3 2 mol l^{-1} HCl.

5. The contents were mixed and allowed to stand at room temperature for 15 minutes.

6. To both tubes 2.0 cm^3 of the sodium hydroxide/imidazole reagent was added and the tubes heated at 56°C for 15 minutes in

the dark.

7. The intensity of fluorescence (excitation 365 nm, emission 455 nm) was measured against that of a standard of NAD^+ which had been treated in exactly the same manner as the sample.

METHOD 6

Preparation of tissue homogenates.

1. Tissue samples were either stored on ice and the homogenates prepared within several hours or frozen immediately and stored at $-20^{\circ}C$ until required.

2. The tissue samples were freed from fat, washed in ice-cold 1.0 mol l^{-1} KCl solution and chopped up in three volumes (w/v) of ice-cold 1.0 mol l^{-1} KCl which contained 1.0 mmol l^{-1} sodium deoxycholate.

3. The sample was converted to a fine brei by two 30 second treatments with the Polytron tissue disintegrator used at low speed. In between each treatment the tissue preparation was cooled in ice.

4. The tissue was homogenised with two 30 second treatments with the Polytron used at top speed (14 000rpm) and two 30 second treatments by sonication using the MSE Ultrasonic Disintegrator, tuned to an amplitude of 8 microns. The sample was again cooled between each treatment.

5. The homogenate was centrifuged in a chilled rotor at 50 000 rpm (150 000 x g)

6. The supernatant solution was desalted by passing a 2.5 cm^3 aliquot through a pre-packed Sephadex G 25 column PD-10, previously cooled in the refrigerator.

7. The eluate was diluted with an equal volume of glycerol and stored at $-20^{\circ}C$ until required.

METHOD 7

Determination of the protein content of tissue homogenates

A. Biuret method

Reagents

1. Biuret reagent

1.5g copper sulphate and 6.0g sodium potassium tartrate were dissolved in approximately 500 cm^3 water. 300 cm^3 of solution containing 100g l^{-1} sodium hydroxide was added and the mixture made up to 1 litre.

2. Standard protein solution containing 10g l^{-1} albumin.

Method

1. To 0.5 cm^3 of sample or standard solution, 4.5 cm^3 of the Biuret reagent was added and the mixture allowed to stand at room temperature for 30 minutes.

2. The absorbance of the resulting solution was measured at 540 nm against a reagent blank.

B. Tyrosine Method (Lowry et al 1951)

Reagents

1. Alkaline copper reagent

1.0 cm^3 of both 20g l^{-1} copper sulphate solution and 20g l^{-1} sodium potassium tartarte solution were added to 98 cm^3 of a 20g l^{-1} solution of sodium carbonate in 0.1 mol l^{-1} sodium hydroxide.

2. Standard protein solution

1.0g l^{-1} albumin in water.

Method

1. To 6.0 cm^3 of the alkaline copper reagent, 0.5 cm^3 of the sample or standard protein solution was added and the mixture allowed to stand at room temperature for 10 minutes.

2. To each tube 0.5 cm^3 of Folin and Ciocalteu's reagent was added and the mixture allowed to stand for a further 30 minutes.

3. The absorbance of the resulting solution was measured at 600 nm against a reagent blank.

METHOD 8

Colorimetic determination of ammonia

Reagents

1. Phenol reagent

10g crystalline phenol and 50mg sodium nitroprusside were dissolved in water and made up to 1 litre. The solution was stored in a dark bottle at 4°C for a maximum of 1 month.

2. Alkaline hypochlorite reagent

To 8.0 cm^3 of a solution of hypochlorite (approximately 50g l^{-1}) 0.125 mol l^{-1} sodium hydroxide solution was added to make a total volume of 1 litre. The solution was stored in a dark bottle at 4°C for a maximum of 1 month.

3. Standard ammonia solution

A stock solution of ammonium sulphate containing 1.0 mmol l^{-1} was diluted to give final concentrations of 0.05 and 0.1 mmol l^{-1}.

Method

1. To 1.0 cm^3 of sample or standard solution, 4.0 cm^3 of phenol reagent and 5.0 cm^3 of alkaline hypochlorite reagent were added and mixed.

2. The mixture was heated at 37°C for 20 minutes.

3. The absorbance of the resulting solution was measured at 560 nm against a reagent blank.

References.

Barman, T.E. (1969)
ed. Enzyme Handbook. p. 170 - 171.
Springer - Verlag, Berlin, New York.

Berger, S.J., Carter, J.A., Lowry, O.H. (1975)
Analyt. Biochem. 65. 232 - 240.

Bernheim, F., Bernheim, M.L.C. (1932)
J. Biol. Chem. 96. 325 - 339.

Bernheim, F., Bernheim, M.L.C. (1934)
J.Biol. Chem. 106. 79 - 86.

Bernheim, F., Bernheim, M.L.C., Gillaspie, A.G. (1936)
J. Biol. Chem. 114. 657 - 663.

Blanchard, M., Green, D.E., Nocito, V., Ratner, S. (1944)
J. Biol. Chem. 155. (2). 421 - 440.

Blanchard, M., Green, D.E., Nocito, V., Ratner, S. (1945)
J. Biol. Chem. 161. (2). 583 - 597.

Brumby, P.E., Massey, V., Wellner, D. (1967)
in Biochemical Preparations, vol 12, p 29 - 41.
ed. Lands, W.E.H.
John Wiley and Sons. London.

Burton, K. (1955)
in Methods in Enzymology. vol. 2. p. 199 - 203.
eds. Colowick, S.P., Kaplan, N.O.
Academic Press. New York, London.

Clark, L.C. (1956)
Trans. Amer. Soc. Artif. internal Organs. 2. 41 - 65.

Corrigan, J.J., Wellner, D., Meister, A. (1963)
Biochim. Biophys. Acta. 73. 50 - 56.

DiPrisco, G., Strecker, H.J. (1966)
Biochim. Biophys. Acta. 122. 413 - 422.

Dixon, M., Kleppe, K. (1965a)
Biochim. Biophys. Acta. 96. 357 - 367.

Dixon, M., Kleppe, K. (1965b)
Biochim. Biophys. Acta. 96. 368 - 382.

Dunn, J.T., Perkoff, G.T. (1963)
Biochim. Biophys. Acta. 73. 327 - 331.

Ellis, G., Goldberg, D.M. (1970)
J. Lab. Clin. Med. 76. (3) 507 - 517.

Ellis, G., Goldberg, D.M. (1972a)
Clin. Chim. Acta. 39. 472 - 474.

Ellis, G., Goldberg, D.M. (1972b)
Clin. Chem. 18. 523 - 527.

Fonda, M.L., Anderson, B.M. (1967)
J. Biol. Chem. 242. (17) 3957 - 3962.

Fonda, M.L., Anderson, B.M. (1968)
J. Biol. Chem. 243. (21) 5635 - 5643.

Guilbault, G.G., Heiserman, J.E., (1963)
Analyt. Biochem. 26. 1 - 11.

Keilin, D., Hartree, E.F. (1936)
Proc. Roy. Soc. 119. 114 - 140.

Kirsten, E., Gerez, C., Kirsten, R. (1963)
Biochem. Z. 337. 312 - 319.

Knoop, K., Oesterlin, H. (1925)
Z. Physiol. Chem. 148. 294 - 315.

Knox, W.E., Pitt, B.M. (1957)
Chem. 225. 675 - 688.

Krebs, H.A. (1933)
Z. Physiol. Chem. 217. 351 - 372.

Krebs. H.A. (1935)
Biochem. J. 29. (7) 1620 - 1644.

Krebs, H.A. (1951)
in The Enzymes, vol 2. part 1. p. 499 - 535.
ed. Boyer, P.D.
Academic Press, New York, London.

Lichtenberg, L.A., Wellner, D. (1968)
Analyt. Biochem. 26. 313 - 319.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951)
J. Biol. Chem. 193. 265 - 275.

Lowry, O.H., Passonneau, J.V. (1972)
in A Flexible System of Enzymatic Analysis p. 3 - 20.
Academic Press, New York. London.

Lowry, O.H., Carter, J.G. (1974)
Analyt. Biochem. 59. 639 -642.

Lubochinsky, B., Zalta, J.P. (1954)
J. Bull. Soc. Chim. Biol. 36. 1363 - 1366.

Luppa, D., Aurich, H. (1971)
Enzyme. 12. 688 - 693.

Marcus, A., Feeley, J. (1962)
Biochim. Biophys. Acta. 59. 398 - 407.

Margoliash, E., Novogrodsky, A. (1958)
Biochem. J. 68. 468 - 475.

Mecher, T., Masszi, J. (1969)
Clin. Chim. Acta. 24. 111 - 120.

Meister, A., Wellner, D., Scott, S.J. (1960)
J. Natl. Cancer. Inst. 24. (1) 31 - 49.

Meister, A., Wellner, D. (1963)
in The Enzymes. vol 7. p. 609 - 648.
ed. Boyer, P.D.
Academic Press. New York. London.

Nagatsu, T., Yagi, K. (1966)
J. Biochem. 60. (2) 219 - 221.

Nakano, M., Danowski, T.S. (1971)
in Methods in Enzymology. vol 17 (B) p. 601 - 605
eds. Tabor, H., Tabor, C.W.
Academic Press. New York, London.

Negelein, E., Haas, E. (1935)
Biochem. Z. 282. 206 - 220.

Negelein, E., Bromel, H. (1939)
Biochem. Z. 300. 225 - 239.

Neims, A.H., Hellerman, L. (1970)
in Ann. Rev. Biochem. vol 39. p. 867 - 888.
ed. Snell. E.E.
Annual Reviews, Palo Alto, U.S.A.

Ratner, S. (1955a)
in Methods in Enzymology vol 2. p 204 - 211.
eds. Colowick, S.P., Kaplan, N.O.
Academic Press, New York, London.

Ratner, S. (1955b)
in Biochemists Handbook (1968) p. 361 - 362.
ed. Long, C.
E. & F.N. Spon Ltd, London.

Scannone, H., Wellner, D., Novogrodski, A. (1964)
Biochem. 3 (11) 1742 - 1745.

Singer, T.P., Kearney, E.B. (1950)
Arch. Biochem. 27 348 - 363.

Soda, K. (1968)
Analyt. Biochem. 25. 228 - 235.

Strecker, H.J. (1955)
in Methods in Enzymology, vol 2. p. 220 - 225
eds. Colowick, S.P., Kaplan, N.O.
Academic Press, New York, London.

Theorell, H., Nygaard, A.P., Bonnischen, F.K. (1954)
Acta. Chem. Scand. 8. 1490 - 1491.

Verity, A.M., Gallagher, R., Brown, W.J. (1967)
Biochem. J. 103. 375 - 381.

Warburg, O., Christian, W. (1938)
Biochem. Z. 98. 150 - 168.

Weast, R.C. (1967) ed.
in Handbook of Chemistry and Physics.
48th edition p. C705.
Chemical Rubber Co. Cleveland. U.S.A.

Weissbach, H., Robertson, A.V., Witkop, B., Udenfriend, S. (1960)
Analyt. Biochem. 1. 286 - 290.

Wellner, D., Lichtenberg, L.A. (1971)
in Methods in Enzymology, vol 17. (B) p. 593 - 596.
eds Tabor, H., Tabor, C.W.
Academic Press, New York, London.

Wellner, D. (1971)
in Methods in Enzymology, vol 17 (B) p. 597 - 600.
eds. Tabor, H., Tabor, C.W.
Academic Press, New York. London.

Yielding, K.L., Tomkins, G.M., Trundle, D.S. (1964)
Biochim. Biophys. Acta. 85. 342 - 345.

Zeller, E.A., Maritz, A. (1944)
Helv. Chim. Acta. 27. 1888 - 1903.

Reading List.

Bergmeyer, H.U. (1974) ed.
Methods of Enzymatic Analysis, vol. 1.
Academic Press, New York, London.

Colowick, S.P., Kaplan, N.O. (1955) eds.
Methods in Enzymology vol. 1. p. 1 - 146.
Academic Press, New York, London.

Guilbault, G.G. (1976)
Handbook of Enzymatic Methods of Analysis.
Marcel Dekker, Inc. New York, Basel.

Moss, D.W. (1977)
Automatic Enzyme Analyzers.
Advances in Clinical Chemistry, vol. 19. p. 1 - 56.
Academic Press, New York, London.

Schwimmer, S., Pardee, A.B. (1953)
in Advances in Enzymology, vol 14. p. 375 - 409.
Interscience Publishers, Inc. New York, London.

Wilkinson, J.H. (1976)
The Principles and Practice of Diagnostic Enzymology.
Edward Arnold, London.