STABILISED ENZYME BASED DIAGNOSTIC SYSTEMS.

BY

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The detection and quantitation of substances using analytical techniques is an important area in many fields. Accuracy, dependability, reproducibility, specificity and stability of the techniques used are all important. The first three parameters are largely operator dependent, however, specificity and stability of the components of the tests also play a part.

The specificity of analysis may be determined chemically or biologically by using enzymes, immunological reagents or receptors of some sort. However, biological molecules are often unstable in purified, isolated forms and must be stabilised in some way to retain activity.

The work reported here attempts to increase the knowledge of enzyme stabilisation, using the enzyme alcohol oxidase as a test enzyme.

This enzyme was used in :

(i) A manual assay for ethanol determination.

(ii) An automated assay using both soluble and immobilised enzyme in segmented flow and flow injection analysis.

(iii) A dry phase stabilised enzyme based test for ethanol in saliva.

During the course of the work a method for stabilising the enzyme was discovered. This has been applied to a number of other enzyme systems, successfully stabilising them in most cases. This work forms the basis of a patent application for enzyme stabilisation.

A novel detection system allowing semi-quantitative results to be obtained, using stabilised dry phase technology has also been discovered. A second patent application has been filed and the system has been applied to analytes measured by oxidase enzymes.

The final area of investigation was to develop an enzymic assay for diacetyl. This substance is a contaminant in beer and as such requires accurate detection at low levels. The purification and characterisation of diacetyl reductase from a new source, (chicken liver) enabled various assay formats to be investigated. These included dye linked assays and enzyme amplified recycling assays to determine diacetyl in aqueous samples.

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The accomplishment and success of this work is due to the involvement of one person above all others.

To Him this work is dedicated.

To

Jesus Christ the Righteous One,

Lord Of Lords, King of Kings.

The Image and Glory of Almighty God.

"Worthy art thou, our Lord and God,

to receive glory and honour and power,

for thou dids't create all things

and by thy will they existed and were created".

REVELATION 4:11.

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ABBREVIATIONS.

4-AAP	4-Aminoantipyrine
ABTS	2,2'-Azino-di(3-ethylbenzothiazoline 6-sulphonic acid)
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
AMPS	Ammonium persulphate
ATP	Adenosine triphosphate
BDH	British Drug Houses
BSA	Bovine serum albumin
CM	Carboxymethyl-
DEAE	Diethyl aminoethyl-
Dmc	Double mixing coil
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbance assay
EMIT	Enzyme multiplied immunoassay technique
EtOH	Ethanol
FC	Flow cell
FIA	Flow injection analysis
FIG/fig	Figure
FMN	Flavin mononucleotide
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
IEF	Isoelectric focusing
INT	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride
K	Potassium
MBTH	3-Methyl 2-benzothiazolinone hydrazone
MOPS	3-(N-Morpholino) propane sulphonic acid
Mr	Relative molecular weight
MTT	3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide
Na	Sodium
NAD	Nicotinamide adenine dinucleotide, oxidised form
NADH	Nicotinamide adenine dinucleotide, reduced form
NAD(H)	Nicotinamide adenine dinucleotide, both or either forms
NADP	Nicotinamide adenine dinucleotide phosphate, oxidised form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NADP(H)	Nicotinamide adenine dinucleotide phosphate, both or either forms
NAD(P)H	Nicotinamide adenine dinucleotide and/or phosphate, reduced form
O_2	Oxygen
PAGE/P.A.G.E.	Polyacrylamide gel electrophoresis
PBE-94	Polybuffer exchanger, pH range 9-4
PMS	Phenazine methosulphate
PMSF	Phenyl methyl sulphonyl fluoride
SDS	Sodium dodecyl sulphate
SFA	Segmented flow analysis
Smc	Single mixing coil
SO_4	Sulphate
TMB	3,3',5,5'-Tetramethylbenzidine
TPCK	N-Tosyl L-phenylalanine chloromethyl ketone
Tris	Tris(hydroxymethyl)aminomethane
U.V.	Ultra violet

Å	Angstrom
°C	Degrees centigrade
cm	Centimetre
g	Grammes or gravities (in centrifugation)
hr/hrs	Hour/hours
l	Litres
M	Molar
m	Metres
mA	Milliamps
mg	Milligrammes
min/mins	Minute/minutes
ml	Millilitres
mm	Millimetres
mM	Millimolar
mol	Moles
n	Nano- or total number of
nm	Nanometres
p	Pico-
r	Correlation coefficient
U	Units
µ	Microns
µg	Microgrammes
µl	Microlitres
v/v	Volume to volume
w/v	Weight to volume

CHAPTER 1.

INTRODUCTION.

1.A. Enzymes, what are they?

Enzymes are biological catalysts which are present in all living tissues. The concept of catalysis was proposed by Jakob Berzelius in 1836. A catalyst may be defined as a substance which promotes or accelerates a chemical reaction without any overall change itself.

Some of the earliest evidences of catalytic action of living tissues were discovered by Planch in 1810 where plant roots were found to promote oxidation of a solution of guaiacum, Thenard in 1818 found hydrogen peroxide could be decomposed by animal tissue and Dubrunfaut in 1830 found he could obtain sugars from starch by an aqueous extract of barley malt.

Such biological activity was described as a "ferment" derived from the obvious chemical changes during a fermentative process such as alcohol production by yeast. However the same term was used to indicate the catalyst causing some biochemical change in vitro. Even today this nomenclature lingers on e.g. Zwischenferment, SIGMA Catalogue 1990 p514.

The name, enzyme derived from the greek meaning "in yeast" was proposed by Fredrich Wilhelm Kuhne in 1878 as the name for such biological substances that showed catalytic activity, (Hoffmann-Ostenhof 1978). This name was also used to describe similar substances from other organisms, beside yeast itself. The name gradually gained acceptance and helped to end misunderstandings and disputes over descriptive terminology of the catalysts involved. The development of enzymology, as the study of enzymes is called, continued with discoveries of enzymes from many sources such as yeast (invertase), pancreas (trypsin), germinating grain (amylase) and also

the finding of enzyme activity in blood, (amylase, lipase).

Enzymes were found to be pH dependent in 1912 by Sorenson, also Bertraud in 1897 found certain small molecules were needed for catalytic activity the so called co-enzymes, the identity of which being discovered in 1935 by Otto Warburg.

That enzymes were proteins was proved positively by the crystallisation of urease by J.B Sumner in 1926, this being followed by trypsin and pepsin shortly after.

From that time to the present many enzymes have been discovered, isolated and studied. The chemical reactions of living tissue have been found to be almost all enzyme dependent. It has been said that the nucleic acids form the molecular basis for life, then it could also be said that enzymes provide the workforce for the chemistry of life, (all references from Bergmeyer and Gawehu 1983).

1.B.1. How enzymes work.

The most definitive character of enzymes is their ability to specifically recognise and interact with their own particular substrate or class of substrates. The recognition of this fact inspired Emil Fischer propose the "lock and key" theory of enzyme action in 1894. He postulated that enzymes acted rather like a template for the specific binding and subsequent reaction to take place. The basic concept of the theory helped explain the action of enzymes, and in 1959 Koshland added the concept of an "induced fit" of the substrate to the enzyme, in which he proposed conformational changes in the enzyme upon reaction with its substrate. This has been observed by X-ray crystallography for certain enzymes. A more detailed account is given in Dixon and Webb (1979).

The action of the enzyme upon its substrate effectively lowers

the energy required to cause a chemical reaction to occur. This is the basic feature of a true catalyst, and a good illustrative account of this effect is given in Lehninger (1982).

1.B.2 Nomenclature

J. Duclaux in 1883 proposed the naming of enzymes by adding the suffix "- ase" to the name root of the substrate of the enzyme. For example, enzymes that hydrolyse lipids become lipases, whilst the enzyme that metabolises urea becomes urease. Alcohol, (ethanol) is oxidised to acetaldehyde by dehydrogenation of the molecule. The enzyme that catalyses the reaction being alcohol dehydrogenase.

The vast majority of enzymes are named in this way, with a few exceptions such as trypsin, pepsin and papain. As more new enzymes were discovered a definitive nomenclature system was proposed by the Enzyme Commission set up in 1955. The final report of this body was published in 1961 and proposed the classification of nomenclature that we know today, yet the general naming of enzymes remains true to the proposal of Duclaux, (Enzyme Nomenclature 1974).

Enzymes are primarily classified according to their main type of reaction mechanism.

1.	Oxidoreductases.	Oxidation/reduction reactions.
2.	Transferases.	Group transfer between molecules.
3.	Hydrolases.	Hydrolysis of molecules.
4.	Lyases.	Group addition to double bonds or removal, (not hydrolytic), leaving double bonds.
5.	Isomerases.	Interconversion of isomers.
6.	Ligases.	Synthesis, joining two molecules together utilising nucleoside triphosphates and breaking the pyrophosphate bond.

Within these classes each enzyme is systematically defined by two further parameters.

(a) The nature of the chemical group acted on or type of molecule accepted by the enzyme.

(b) The acceptor group or molecule for the reaction, which may be the cofactor for the enzyme.

The Enzyme Commission nomenclature takes into account the nature and type of the overall chemical reaction catalysed.

e.g.) Alcohol dehydrogenase is given the designation E.C.1.1.1.1. in this system. E.C. is Enzyme Commission.

The first 1 is the class of oxidoreductases.

The second 1 is the subclass of alcohols or hemiacetals.

The third 1 is the sub-subclass of the acceptor molecule, the cofactor NADH.

The fourth 1 is the number of the enzyme in the sub- subclass.

The systematic name of the enzyme is alcohol : NAD^+ oxido-reductase.

Similarly for alcohol oxidase ; E.C.1.1.3.13.

1 is class of oxidoreductases.

1 is the subclass of alcohols and hemiacetals.

3 is the sub-subclass of molecular oxygen as acceptor

13 is the number of the enzyme in the sub-subclass.

The systematic name being, alcohol : oxygen oxido-reductase.

This systematic nomenclature classification is designed to provide insight into the overall reaction catalysed by any enzyme and thus eliminate errors in terminology. In practice many enzymes are referred to by trivial names or abbreviations, usually this is more convenient and causes few problems after the enzyme and its reaction

is well defined.

1.C.1. General Application of Enzymes.

The very nature of enzyme catalysed reactions makes them of great interest, and they are used in many different fields of study. The specificity of the reaction, the ability of enzymes to catalyse reactions under mild or moderate conditions of temperature and pH and the production of well defined products, with minimal side reactions, all contribute to the usefulness of enzymes. The measurement of reactions by various methods, may be used as a means of quantifying the substrate, product or the enzyme itself, thus enabling analytical estimation of the parameter in question.

Historically, enzymes have been used indirectly for centuries. e.g. yeast to produce alcohol in the fermentative process, and CO_2 during baking and the enzymes in germinating seeds during the process of malting. Isolated and purified enzymes however, are relative newcomers and yet are proving invaluable in many applications, some of which are described below.

1.C.2. Industrial Uses of Enzymes.

This area includes the food industry, the beverage industry, the chemical and pharmaceutical industries and clothing industries. Enzymes are utilised within many areas, a few examples will illustrate the diversity of applications.

(a) Amylases used in the production of malt extracts.

(b) Glucose oxidase as an antioxidant in fruit or for colour control in wine.

(c) Catalase for hydrogen peroxide removal in food.

(d) Proteases for detergent additives.

(e) Epoxysuccinate hydrolase for tartaric acid production.

(f) Tyrosinase and tryptophanase to produce L-tyrosine, L-DOPA and L-5-hydroxy tryptophan.

(g) Diisopropylphosphofluoridase to degrade redundant stocks of nerve gas.

These are but a few of the many industrial uses of enzymes. For a more thorough review see Cheetham (1985).

1.C.3. Medical use of Enzymes.

The main medical use of enzymes is in the diagnostic or analytical areas which will be discussed in greater detail in the next section. Other uses of enzymes in medicine have been reported. L-Asparaginase posseses anti-tumour activity,(Mauer and Simone 1976), trypsin and collagenase have been used to remove dead tissue from wound sites, (Sizer 1972), streptokinase is used for the dissolution of blood clots and keratinase for the removal of skin callouses or the excess keratin formed in skin diseases such as psoriasis.

1.D.1. The Concept and Development of Enzymes as Analytical Reagents.

Enzymes have been used as analytical reagents as far back as 1862 when Dragendorff used malt extract to estimate starch concentration in grain flour. Other crude enzyme preparations were used by Schonbein in 1868 for the detection of hydrogen peroxide to 1 part in 10^7 parts and O' Sullivan in 1875, to detect starch by means of purified diastase preparation. Also, the activity of enzymes present in body fluids, particularly in blood was suggested by Schmidt in 1850. Actual enzyme activities were first measured by Wohlgemuth in 1910 using amylase to measure pancreatic function, (Bergmeyer and Gawahu 1983).

Improved instrumentation and purified enzyme preparations have both contributed to the development of repoducible and accurate

enzyme based analytical techniques. The development of enzymes as analytical tools may be seen clearly in the published literature. An example to illustrate this is the publication entitled " Methods in Enzymatic Analysis" edited by Hans Ulrich Bergmeyer. The first edition appeared in 1963 in one volume. The second edition in 1974 in four volumes and the third current edition started in 1983 consists of twelve volumes, indicating the phenomenal increase of use of enzymes as analytical reagents since 1963.

1.D.2. Direct Enzyme Analysis.

The measurement of a concentration of substrate using enzymes, or the measurement of an enzyme concentration in which the catalytic reaction is directly followed by some means, are examples of direct enzyme analysis. The sensitivity of such methods are usually dependent on the limit of direct detection of the reactants involved and equally the type of detection system used determines the concentration that may be detected. Fortunately, the majority of biologically important substances assayed using enzymes fall into the detection range of such methods and so in practice this type of assay is the most widely used.

End-point or equilibrium assays involve the addition of relatively large amounts of enzyme to a limiting amount of substrate and the reaction is allowed to proceed to give full, or very nearly full, conversion of the substrate to product.

Kinetic assays involve the addition of substrate to a small amount of enzyme, any co-factors needed are added in excess and the reaction rate is measured. The former has the advantage of being less susceptible to interferences and requires no special equipment however, large amounts of expensive enzyme and co-factor may be used

and the incubation time may be prolonged. The latter is susceptible to any interference in the kinetics of the reaction, so pH and temperature must be strictly controlled. This may require specialised and dedicated equipment, however the enzyme consumption and time for assay are both greatly reduced. A detailed comparison may be found in Gould and Rocks (1985).

Examples of direct enzyme reactions are:

$$\text{Glucose} + \text{NAD}^+ \rightleftharpoons \text{Gluconolactone} + \text{NADH} + \text{H}^+$$

This reaction is catalysed by the enzyme glucose dehydrogenase, (Vormbrock 1984) and it is seen that the reaction is in equilibrium. In such a case where equilibrium conditions exist, care has to be exercised in choosing the reaction conditions that are necessary to force the reaction to completion or, alternatively, to remove one of the products from the reaction, thereby pulling the reaction to completion: e.g. semicarbazide is used to react with the acetaldehyde produced in the determination of ethanol by alcohol dehydrogenase, (Lundquist 1957).

$$\text{Ethanol} + \text{NAD}^+ \longrightarrow \text{Acetaldehyde} + \text{NADH} + \text{H}^+$$

$$\text{Acetaldehyde} + \text{semicarbazide} \longrightarrow \text{Semicarbazone}$$

In both of the cases above,detection of the NADH produced gives a measure of the original substrate concentration,(see section 1.E.2).

Similarly glucose may be estimated by consumption of oxygen, (Updike and Hicks 1967) or production of hydrogen peroxide,(Clark 1970) in the following reaction.

$$\text{Glucose} + \text{O}_2 \longrightarrow \text{Gluconolactone} + \text{H}_2\text{O}_2$$

catalysed by glucose oxidase. The reverse reaction does not occur in this case.

Coupled enzyme reactions for direct determinations are used where the detection of the products for the primary reaction is difficult. Thus, for reactions involving phosphorylations with ATP, a linked enzyme system may be used, (Vallins and Baumberg 1985).

(1) Streptomycin + ATP ⟶ Streptomycin 6-phosphate + ADP

(2) ADP + Phosphoenolpyruvate ⟶ ATP + Pyruvate

(3) Pyruvate + NADH + H^+ ⟶ Lactate + NAD^+

The enzymes are: (1) streptomycin 6-kinase, (2) pyruvate kinase, (3) lactate dehydrogenase and the reaction is followed by the removal of NADH from the system.

Detection of hydrogen peroxide formed from the glucose oxidase reaction may be linked to a further enzyme reaction catalysed by peroxidase, (Trinder 1969).

Glucose + O_2 ⟶ Gluconolactone + H_2O_2

H_2O_2 + acceptor ⟶ 2 H_2O + Oxidised acceptor

Usually the oxidised acceptor is some sort of intensely coloured dye, (this chapter,section 1.E.3).

More complex coupled reactions are also possible.

(1) Triglyceride + H_2O ⟶ Glycerol + Fatty acids

(2) Glycerol + ATP ⟶ L-α- Glycerophosphate + ADP

(3) L -α- glycerophosphate + O_2 → Di OH acetone P + H_2O_2

(4) H_2O_2 + acceptor ⟶ 2 H_2O + oxidised acceptor

The enzymes are: (1) lipase, (2) glycerol kinase,(3) L-D-α- glycerophosphate oxidase, (4) peroxidase.

This series of reactions forms the basis of a commercial triglyceride assay, (Spayd et al 1978).

1.D.3. Indirect Enzyme Analysis.

The measurement of a compound which does not take part directly in the enzyme reaction occurring or where the enzyme is used as a means of detecting a reaction or amplifying a reaction, may be termed indirect enzyme analysis. Examples of such assays include: the determination of activators of enzyme reactions, such as magnesium in the following reaction sequence.

(1) Glucose + ATP $\longrightarrow$ Glucose 6-Phosphate + ADP

(2) Glucose 6-P + $NADP^+$ $\longrightarrow$ Gluconate-6-P + NADPH + H^+

Enzymes: 1) Hexokinase (Mg^{2+} dependent).

2) Glucose 6-phosphate dehydrogenase.

The rate of reaction is dependent on the concentration of magnesium ions in the solution, (Hoffmann 1984).

Similarly, the reaction of acetyl cholinesterase on the yellow compound indophenyl acetate, produces an intense blue dye. The presence of organophosphorus or carbamate pesticides inhibits the reaction, the concentration being directly related to the decrease of the rate of the reaction, (Huber 1984).

Immunological reactions, which involve the interaction of antibodies with antigens, have been used for many years to quantify substances which are not direct substrates for enzymes. Antibodies which specifically recognise a particular compound, (the analyte to be measured), may be chemically attached to enzymes such as alkaline phosphatase, peroxidase and β-galactosidase. The attached enzymes may then be used as a means of quantifying the immunological reaction by the amount of associated activity present and so, indirectly, the original amount of analyte present. This type of indirect enzyme analysis is termed enzyme linked immunosorbance assay or E.L.I.S.A.

for solid phase tests and enzyme multiplied immunoassay technique or E.M.I.T. for soluble systems, (Oellerich 1984).

The sensitivity of direct reaction may be enhanced by the use of enzymes to cycle a substrate around a reaction sequence and so chemically amplify a signal. Various reactions can be employed to cycle such compounds as NAD, NADP, glutathione and ATP, (Lowry 1980, Blaedel and Boguslaski 1978). Also, enzyme cycling may be used in conjunction with E.L.I.S.A. techniques to amplify the signal and thus increase sensitivity, (Stanley et al 1985).

1.E.1 <u>The Detection and Measurement of Enzyme Reactions.</u>

To detect and quantify the extent of an enzymatic reaction is a prerequisite of analysis. The appearance of product or the disappearance of substrate may be measured by a variety of techniques including spectrophotometry,fluorometry,luminometry, reflectometry, and electrochemistry.

The absorption, emission or reflection of light from an enzyme system are the basis of measurement of the first four techniques respectively. Electrochemistry involves a direct or coupled measurement of enzyme activity through some sort of electrical sensing system and forms the basis of so called biosensors, (Haar et al 1984, Urbank 1984, Wulff 1984, Kricka and Thorpe 1986, Werner and Rittersdorf 1984, Vadgama 1986, Turner et al 1987). Probably the most commonly used detection system is spectrophotometry, the concentration of reactant in a sample being calculated according to the Lambert Beer law.

$$A = E \times C \times L.$$

A = absorbance, E = molar absorption coefficient, C =

concentration and L = pathlength of the optical cell used.

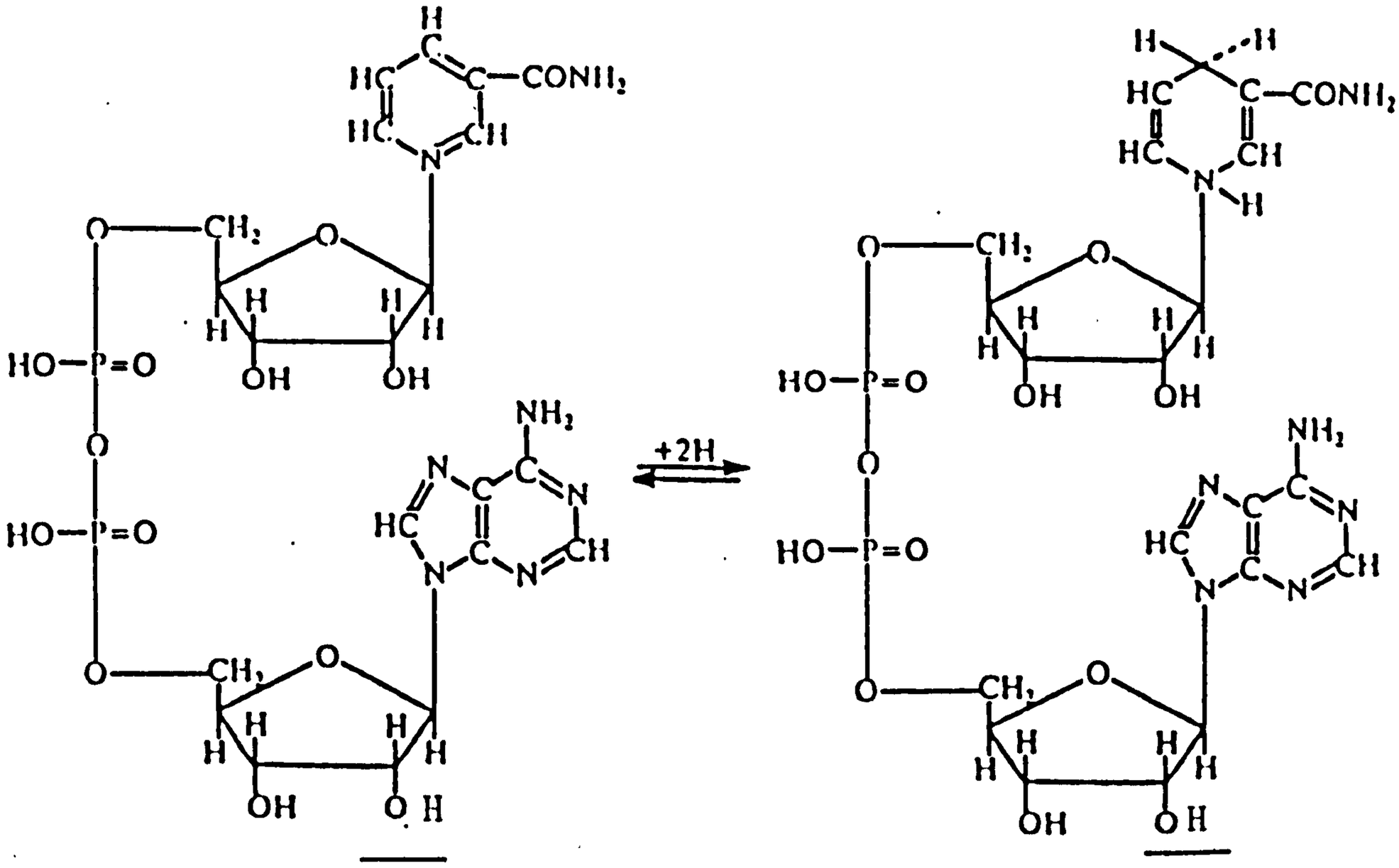
1.E.2 The Detection of Pyridine Cofactors.

Nicotinamide adenine dinucleotide (NAD) and its phosphate (NADP) are co factors for a large number of oxidoreductases. NAD(P) occurs both in the oxidised and in the reduced (NAD(P)H) forms, this interconversion being the fundamental property of the molecule, fig 1.

NAD(P)H has a distinctive absorption spectrum, absorbing in the ultraviolet region at a wavelength of 339nm, with an absorption coefficient (E) of 6.31 x 10^3 $mol.l^{-1}.cm^{-1}$. NAD(P), however, does not absorb at this wavelength, giving a very useful means of detection of many enzyme reactions producing or consuming NAD(P)H, (Beutler 1984, Smith 1984, Schalhorn and Willman 1984, Vassault 1984, Gould and Rocks 1985). Analogues of NAD(H), e.g. 3-iodopyridine adenine dinucleotide, (Abdallah and Biellman 1980), and acetyl pyridine adenine dinucleotide or APAD(H), (Witt 1974), behave similarly with dehydrogenase enzymes.

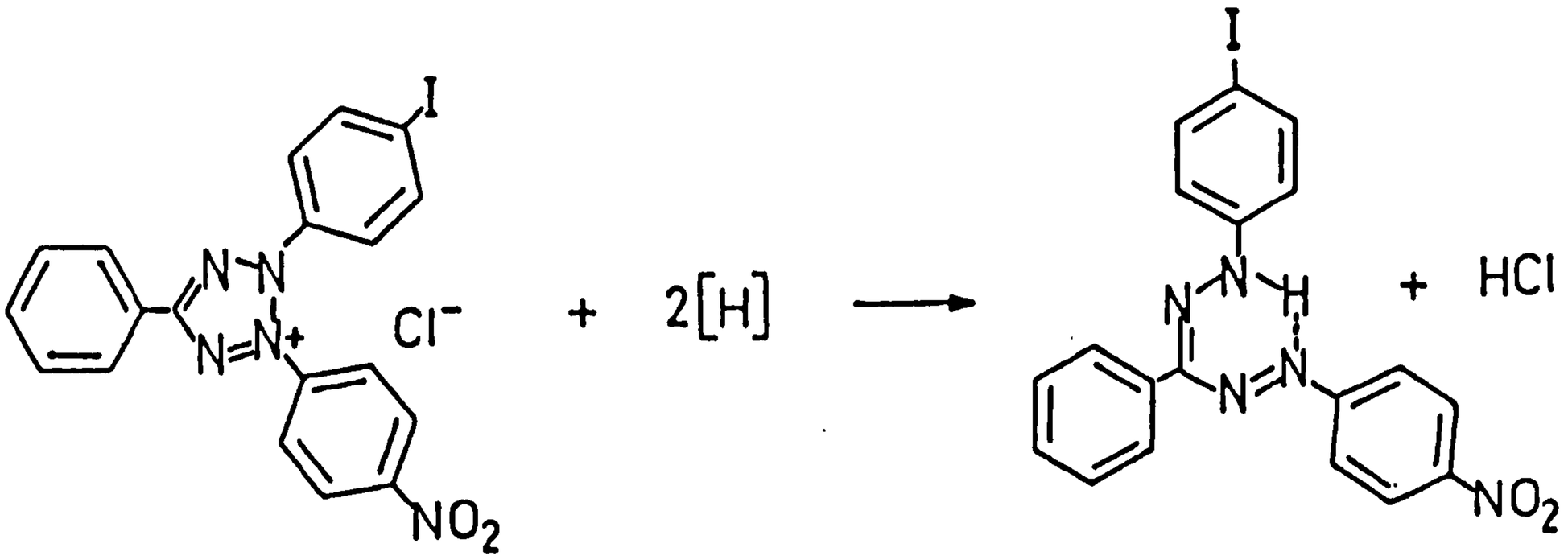
The ultraviolet determination of oxidoreductase reactions is very important, especially in clinical assays, but it does necessitate instrumentation able to accurately measure absorbance in the U.V. region of the spectrum. Combination of NAD(P)H with dye forming reactions, enables visualisation of the enzyme reaction and measurement in the visible region of the spectrum. Normally this is carried out by some sort of hydrogen carrier such as the enzyme diaphorase, (Coburn and Carroll 1973) or phenazonium compounds such as phenazine methosulphate, (Babson and Babson 1973, Worsfold 1977) or phenazine ethosulphate, (Ghosh 1979), since NAD(P)H is unable to transfer hydrogen ions directly to the commonly used dyes.

FIG.1 NAD / NADH REACTION



Nicotinamide Adenine Dinucleotide(NAD) is converted to its reduced form (NADH) by addition of hydrogen to the nicotinamide moiety. Phosphorylation of the underlined hydroxyl group gives NADP / NADPH.

FIG.2 TETRAZOLIUM SALT / FORMAZAN REACTION.



INT. Formazan. (500nm)

2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) is reduced to its red formazan by addition of hydrogen. This is usually carried out using an auxiliary reaction with hydrogen transfer reagents such as phenazine methosulphate or meldola blue being used.

Early work used dyes such as methylene blue and 2,6-dichlorophenol indophenol, (Smith and Olson 1975), however these have been largely superseded by the use of tetrazolium salts, which are substituted 1,2,3,4-tetrazole compounds. These are reduced to intensely coloured formazans by the coupled reaction with NAD(P)H, (Nineham 1955, Altman 1976). The example in fig.2 is a monotetrazolium salt, 2-(4-iodophenyl), 3-(4-nitrophenyl) 5-phenyltetrazolium chloride or INT. The formazan is an intense red with an absorption maximum at 500 nm, (Whitaker 1969, Buttery et al 1977, Michal et al 1983). Ditetrazolium salts tend to give blue or black diformazans on reduction.

Direct estimation of NAD(P)H may be carried out using the dye meldola blue, (8-dimethylamino 2,3-benzophenoxazine) in stabilised solution, (Orsonneau et al 1982). Other indicator reactions include coupling to mono-oxygenase enzymes such as salicylate hydroxylase, (Michal et al 1983), the reduction of ferric to ferrous ions and subsequent determination with ferrozine, (Fu 1972) or 2,2-dipyridyl, (Whitaker 1969) and reduction of 4- nitrosodimethylaniline, (λ max 440nM), in a coupled cycling reaction catalysed by liver alcohol dehydrogenase, (Skursky et al 1979).

NAD(P)H also fluoresces when excited by incident light at 340nm, emitting light at 460nm, (Lowry and Passoneau 1972). Many analytical assays are based on this method of which the assay of D-galactose and its 1-phosphate are examples, (Fujimura 1984) and the assay of 3-hydroxy bile acids is an example of a coupled fluorescence assay, (Stava et al 1984).

Bacterial luciferase isolated mainly from marine bacteria Vibrio fisheri and Vibrio harveyi (now classified as Photobacterium) reacts

with NAD(P)H in a coupled system containing NAD(P)H : FMN oxidoreductase, FMN and n-decyl aldehyde. The reaction produces light which may be measured at 460 nm. This type of luminescent system has been used to measure 7 - hydroxy bile acids, (Roda et al 1984) and rubella IgG, (Jablonski 1985).

One other remarkable property of NAD(P) and its reduced form NAD(P)H is their reversal in stability to acid and base. NAD(P) is stable in acidic solutions for hours, yet heating to 60°C for 10 minutes in basic solution at pH 12.0 destroys it. Conversely NAD(P)H is stable in basic solution yet incubation for 5 minutes at 25 °C in an acidic solution at pH 2 destroys it, (Lowry 1980).

This fact enables the NAD(P) or NAD(P)H, generated in a primary reaction, to be selectively amplified by using an enzyme cycling reaction, increasing the sensitivity of the primary reaction considerably. Ethanol has been estimated in this way, (Kovar 1984), direct NAD concentrations in single cells, (Kato et al 1973) and enhancement of the sensitivity of immunoassays has been suggested by Stanley et al (1985).

Electrochemical detection of NAD(P)H has also been used to quantify enzymatic analyses. Direct detection of NAD(P)H is possible using modified electrodes, where some sort of electrochemical mediator is coupled to the electrode surface: e.g. meldola blue, (Emneus et al 1986), 3 - β - napthoyl nile blue, (Schelter Graf et al 1984) or the bis-benzophenoxazinyl derivative of terephthal ic acid, (Appelqvist 1987). This technique has been used particularly in flow systems to determine L-lactate, (Gorton and Hedlund 1988), xylose and xylulose, (Dominguez et al 1988) and many substrates of other NAD(P)H dependent dehydrogenases, (Schelter Graf et al 1984). In addition to

direct electrochemical detection, NAD(P)H can be oxidised by molecular oxygen in the presence of the same phenazonium compounds used in the colorimetric detection process to quantitatively produce hydrogen peroxide, (Huck et al 1984). This has been used to estimate blood lactate levels by detecting either 0_2 consumption or H_20_2 production with a Clark oxygen electrode, (Vadgama et al 1986).

1.E.3. The Detection of Hydrogen Peroxide.

Hydrogen peroxide is a product of many enzyme reactions, generally those catalysed by oxidases which utilise molecular oxygen. Hydrogen peroxide may be detected by direct ultraviolet absorbance, or by colorimetric, luminometric, fluorimetric or electrochemical techniques. U.V. absorbance is little used except to estimate catalase activity, (Aebi 1984) as the molar absorption coefficient is low, (39.4 mol l^{-1} cm^{-1}) and the chemical reactivity of H_20_2 is very high, accounting for its instability in solution. This reactivity of H_20_2 lends itself to its detection by the formation of highly visible dyes in a variety of spectrophotometric systems.

(a) Redox Dyes.

Redox dyes were among the first type of compounds used to detect H_20_2, benzidine, (4-diaminodiphenyl) and related chromagens o-tolidine and o-dianisidine being used in the colorimetric detection of glucose using the coupled reaction of glucose oxidase and peroxidase, (Bergmeyer and Bernt 1973). Similarly galactose was estimated using galactose oxidase, (Frings and Pardue 1964). These compounds however constitute a health hazard due to their potential carcinogenicity and their use has largely been discontinued. 3,3'5,5'-Tetramethylbenzidine has been claimed to be non-carcinogenic and is widely used to detect H_20_2 giving an intensely blue coloured

intermediate followed by the fully oxidised brown red products, (Josephy et al 1982, Holland 1974, Liem et al 1979), fig.3.

2, 2' Azino - di (3-ethylbenzothiazoline sulphonic acid) or ABTS is a sensitive, stable chromagen producing a stable intense green radical cation on oxidation, (Childs and Bardsley 1975), fig.4. It has been used for various assays including ethanol, (Majkic-Singh and Berkes 1977), uric acid, (Majkic-Singh et al 1981), glucose, (Bergmeyer and Bernt 1973), cholesterol (Majkic and Berkes 1977) and in an ELISA for myelin basic protein, (Groom 1980).

Another redox dye used for a rapid glucose assay was sodium diphenylamine sulphonate. This gives a reddish violet dye on oxidation, (Morin and Prox 1973).

(b) Leuco Dyes.

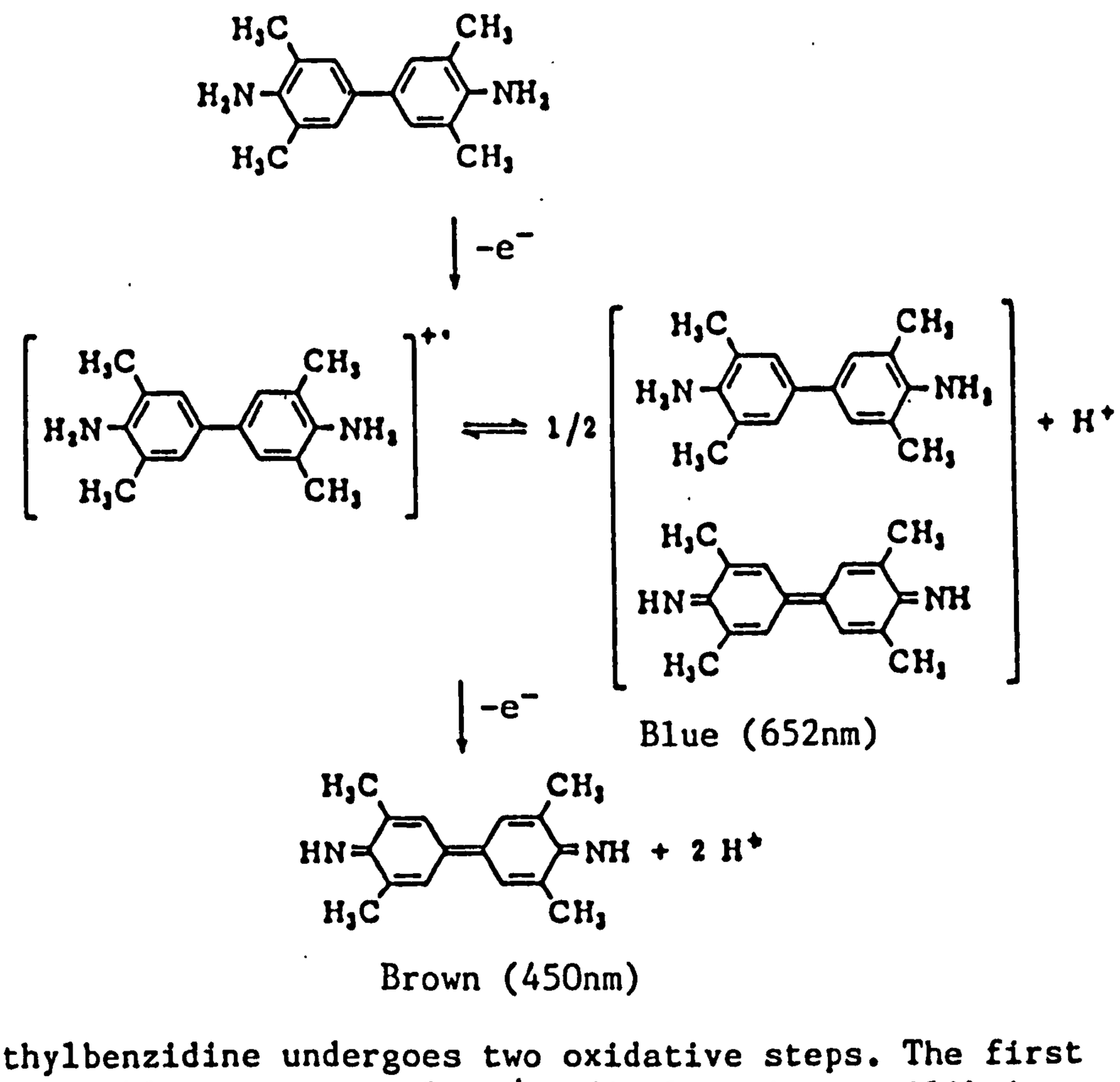
Leuco dyes, which are effectively the stable reduced derivatives of such compounds as methylene blue, (Milke et al 1982) or crystal violet, (Mottola et al 1970), have been used as chromagens in H_2O_2 detection. The oxidising system regenerates the dye from the colourless leuco derivative. The hydroxydiaryl imidazole derivatives used in the Boehringer Reflotron system for uric acid, (Merdes 1986) glutamic pyruvic and glutamic oxaloacetic transaminases belong to this class, (Deneke 1986).

(c) Condensation Reactions.

Oxidative condensation of two reactive molecules constitutes one of the largest group of peroxidase development systems. The chemistry involved is analogous to the production of coloured images in photography, (Bailey and Williams 1971) and the dying of hair with peroxide oxidised dyestuffs, (Corbett 1971).

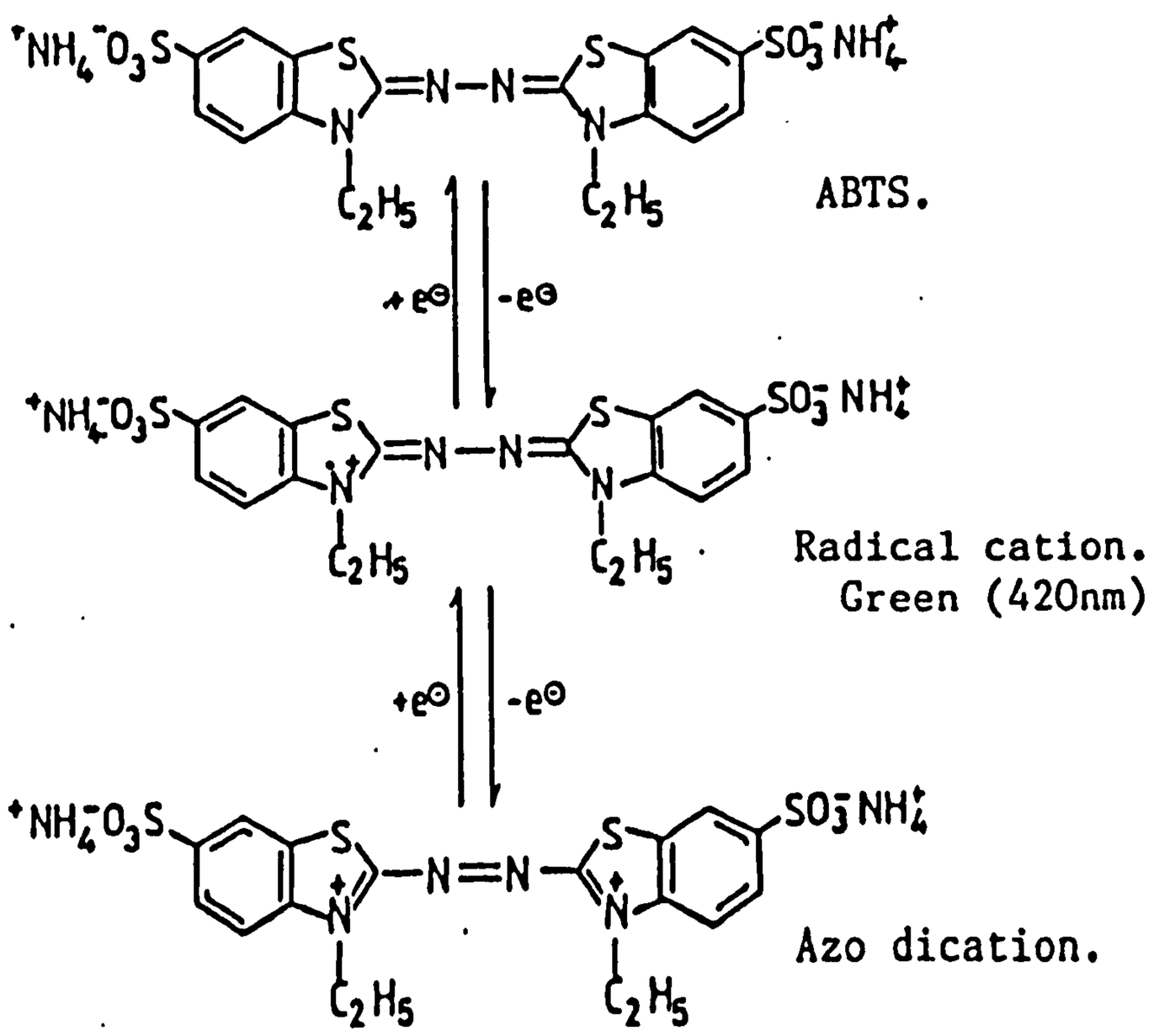
Heterocyclic compounds such as 4-aminoantipyrine, (4-AAP) and

FIG.3 TETRAMETHYLBENZIDINE REACTION.



Tetramethylbenzidine undergoes two oxidative steps. The first results in a blue-green complex / radical cation equilibrium. The second gives a brown diimine.

FIG.4 ABTS REACTION.



ABTS undergoes reversible oxidation to produce a stable green

3-methyl 2- benzothiazolinone hydrazone, (MBTH) will couple with a variety of aromatic compounds. Usually the bond is formed opposite hydroxy, amino or substituted amino groups in the presence of peroxidase and H_2O_2, producing intensely coloured dyes. These coupled systems tend to be rather more stable than individual chromagens especially in formats where the two halves of the dye producing reaction are mixed immediately before assay.

4-Aminoantipyrine was first used in an enzyme reaction by Trinder in 1969 to measure glucose in its oxidative reaction with phenol, which gives a red quinoneimine dye of molar absorbance 6.9 x 10^3 mol. l^{-1}. cm^{-1} at 505nm, (Trinder 1969), fig.5.

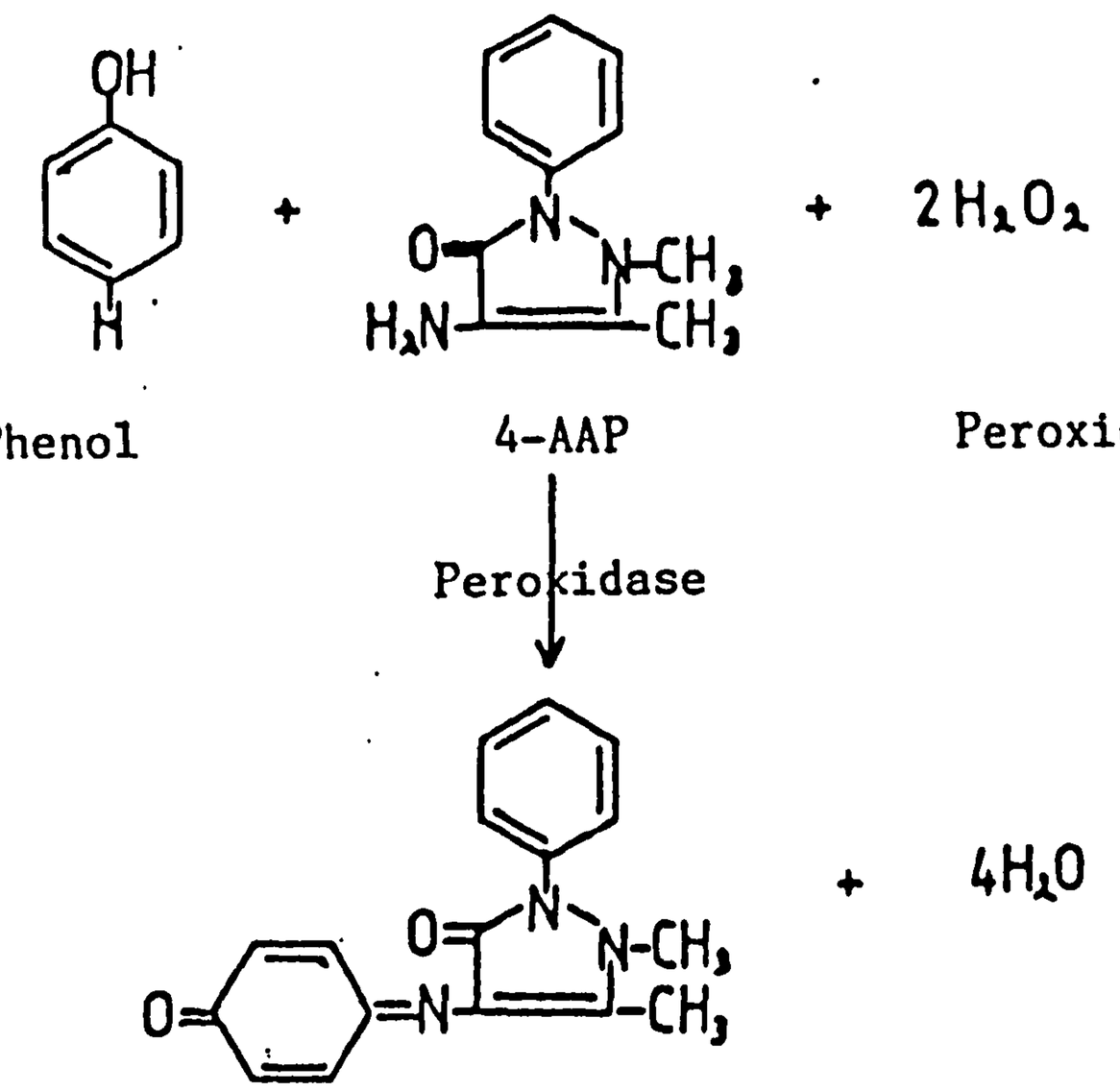
Previous to this, 4-aminoantipyrine had been used to detect phenols in oxidative conditions with inorganic oxidants, (Emerson 1943) and further work compared 4-aminoantipyrine to other developers with a range of coupling compounds, (Emerson et al 1944). The reaction with amines was reported earlier, (Eiesenstaedt 1939). Many other couples have since been used in oxidase catalysed reactions, table 1.

The development of hydrazones and specifically MBTH as colour reagents stemmed from the work of Hunig and co-workers, (Hunig et al 1958, Hunig 1969). MBTH was subsequently used to detect aldehydes, (Sawicki 1961) and aromatic amines, (Sawicki 1961) using ferric chloride as oxidant. The reaction between MBTH and N,N-dimethyl aniline was used to measure uric acid using uricase and peroxidase in an automated system, (Gochman and Schmitz 1971). Subsequently the same reagents were adapted and evaluated in the analysis of glucose, (Gochman and Schmitz 1972, Carey et al 1974). Other coupling agents have been used such as 3-dimethylamino benzoic acid, (Ngo and Lenhoff

Table 1. Chromagens used in Hydrogen Peroxide Detection.

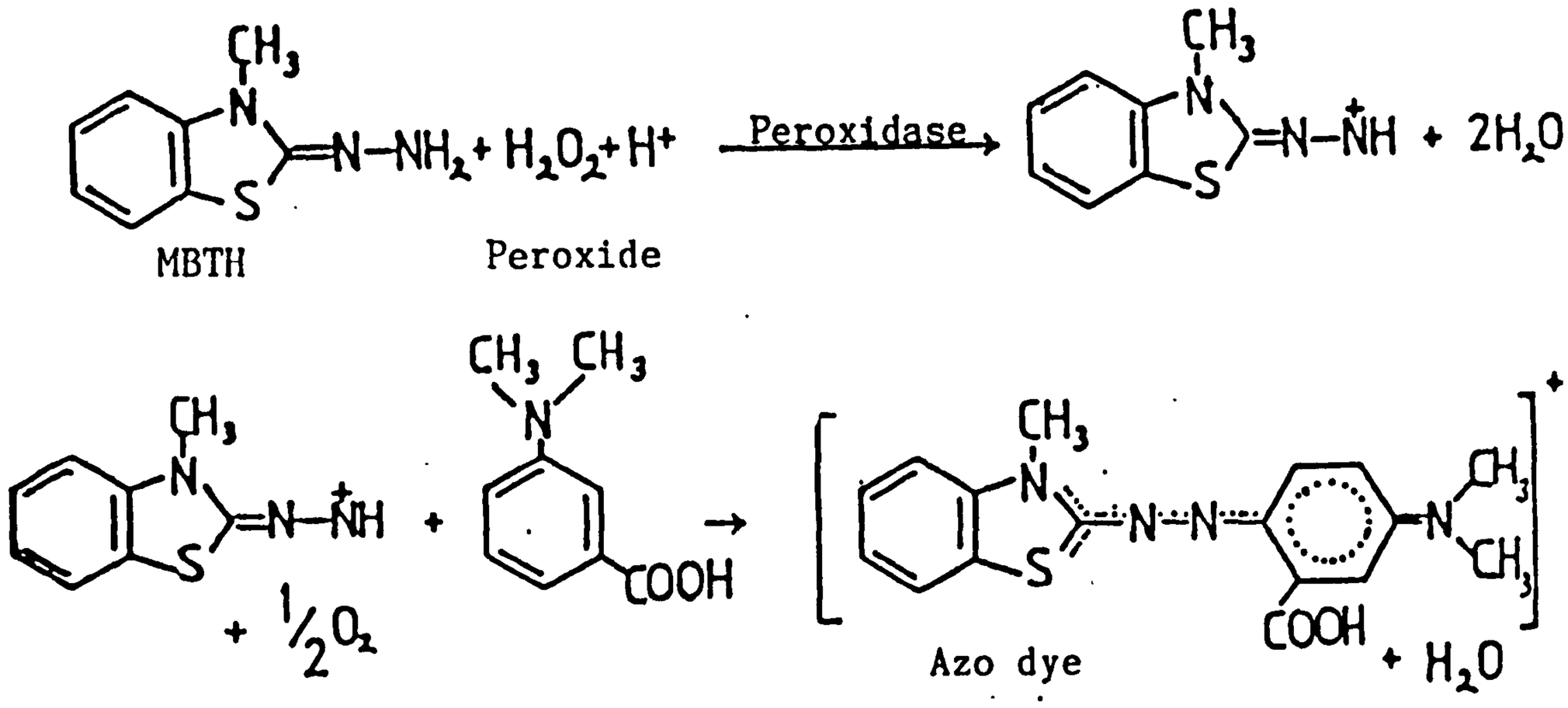
Coupler.	Reference.
Chlorophenol sulphonic acid	Barham and Trinder 1972
Chlorophenol sulphonic acid	Fossati et al. 1980
Chlorophenol sulphonic acid	Grillo et al. 1981
Chlorophenol sulphonic acid	Artiss et al. 1981
N,Ethyl N,sulphopropyl aromatic amines	Tamaoku et al.1981
2,4,6 Tribromophenol	Kabasakalian et al.1973
N,N, Diethylaniline	Kabasakalian et al.1974
N,Ethyl N,hydroxysulphopropyl aromatic amines	Tamaoku et al. 1982
4-Hydroxybenzoic Acid	Meiatelini et al. 1978
1,7-Dihydroxy naphthalene	Curme et al. 1978
2,4,6-Tribromo 3-hydroxy benzoic acid	Trinder and Webster.1984
Miscellaneous Dyes.	
O-Phenylenediamine	Wolder et al. 1976
4-Methoxy 1-naphthol	Guilbault and Kramer 1964
3-Amino 9-ethyl carbazole	Graham et al. 1965
3-Amino 9-ethyl carbazole	Kaplow 1975
4-Chloro 1-naphthol	Nakane 1968
5-Amino salicylic acid	Ellens and Gielkens 1980
3,3-Diamino benzidine	Nakane and Pierce 1967

FIG.5 4-AAP / PHENOL CONDENSATION REACTION.



Condensation reactions such as the one above produce intensely coloured quinoneimine dyes. The reaction depicted is the well known "Trinder" reaction,which uses 4-Aminoantipyrine and Phenol.

FIG.6 MBTH / 3-DIMETHYLAMINO BENZOIC ACID CONDENSATION REACTION.



Condensation reactions of this type using MBTH and substituted amino compounds are very sensitive. The dye produced is of the azo type and is very stable.

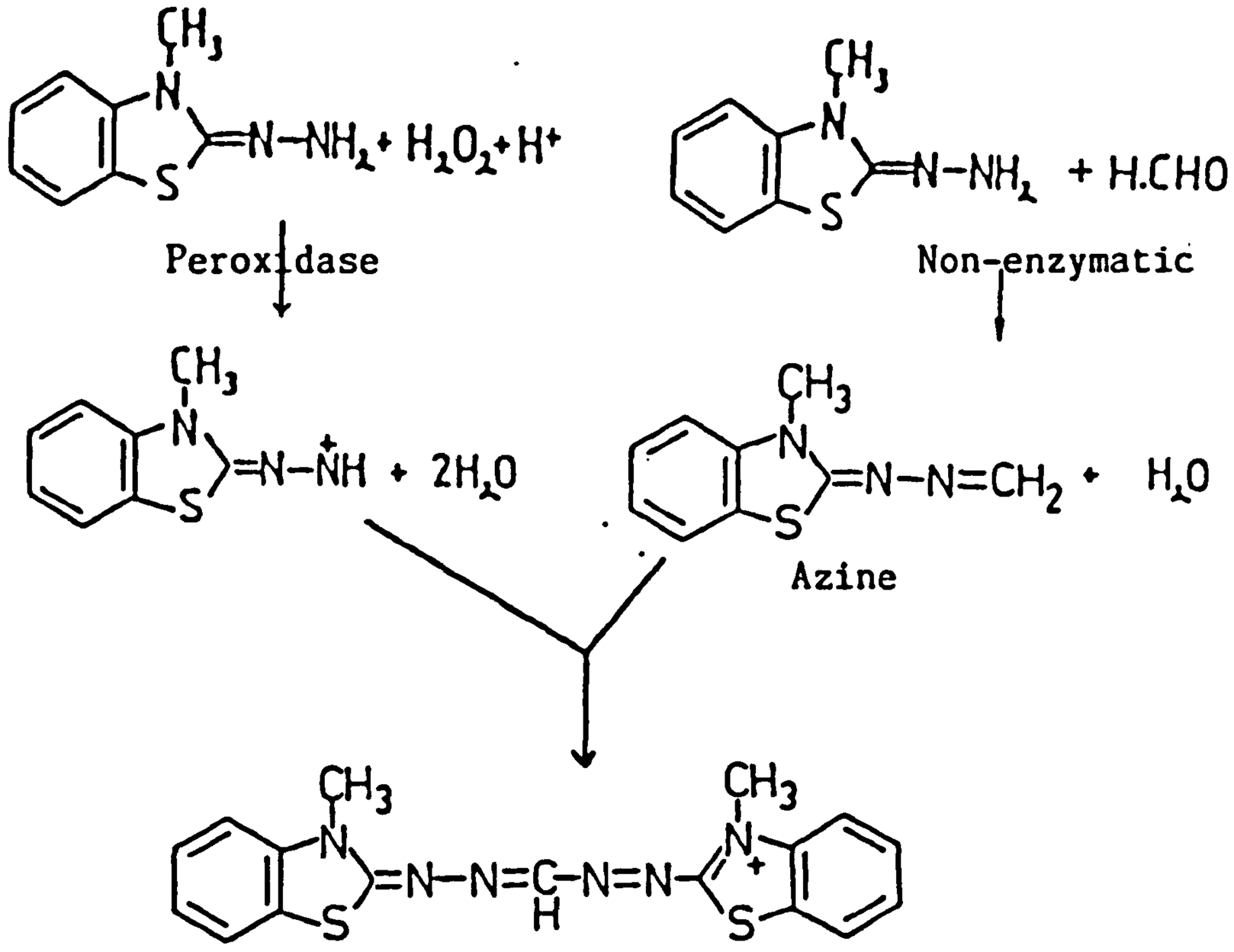
1980) and 2-hydroxy 3,5 dichloro benzene sulphonic acid,(Artiss et al 1983). The former dye has a calculated molar extinction coefficient of 4.7×10^4 $mol.l^{-1}.cm^{-1}$, the reaction is shown in fig 6.

The reaction of MBTH with aldehydes has been exploited to produce a self-coupling reaction which produces a tetra-azapentamethine compound, fig 7. The reaction has been used to measure H_2O_2 , glucose and choline, (Capaldi and Taylor 1982).

The two developing reagents 4-aminoantipyrine and MBTH are the most widely used compounds in analytical condensation reactions, producing relatively stable colours, the dyes produced by MBTH being the most stable. However oxidative condensation reactions involving other molecules are possible. The generation of a quinoneimine reactive intermediate, in an enzyme cycling assay for ethanol and its subsequent oxidative coupling to salicyaldehyde, (Kovar et al 1983) indicated that photographic chemicals of the 1,4-phenylene diamine type may be used for peroxidative reactions, (see chapter 6). Also substituted N-phenyl p-phenylene diamines have been used in histochemical detection of cytochrome oxidase, (Burstone 1960) with the suggestion that peroxidase could be detected in a similar fashion using H_2O_2 as substrate. Various other compounds have been used for colour production in the peroxidase system particularly in immunoassays, table 1.

An alternative colour development for H_2O_2 has been used to assay sulphite using tetrazolium salts, sulphite oxidase and NADH peroxidase. This overcomes the interference of sulphite on the normal peroxidase catalysed colour reactions, (Beutler 1985). Catalase too has been used to measure H_2O_2 in the so called "Hantzsch" reaction, fig 8. Methanol is utilised to produce formaldehyde which then

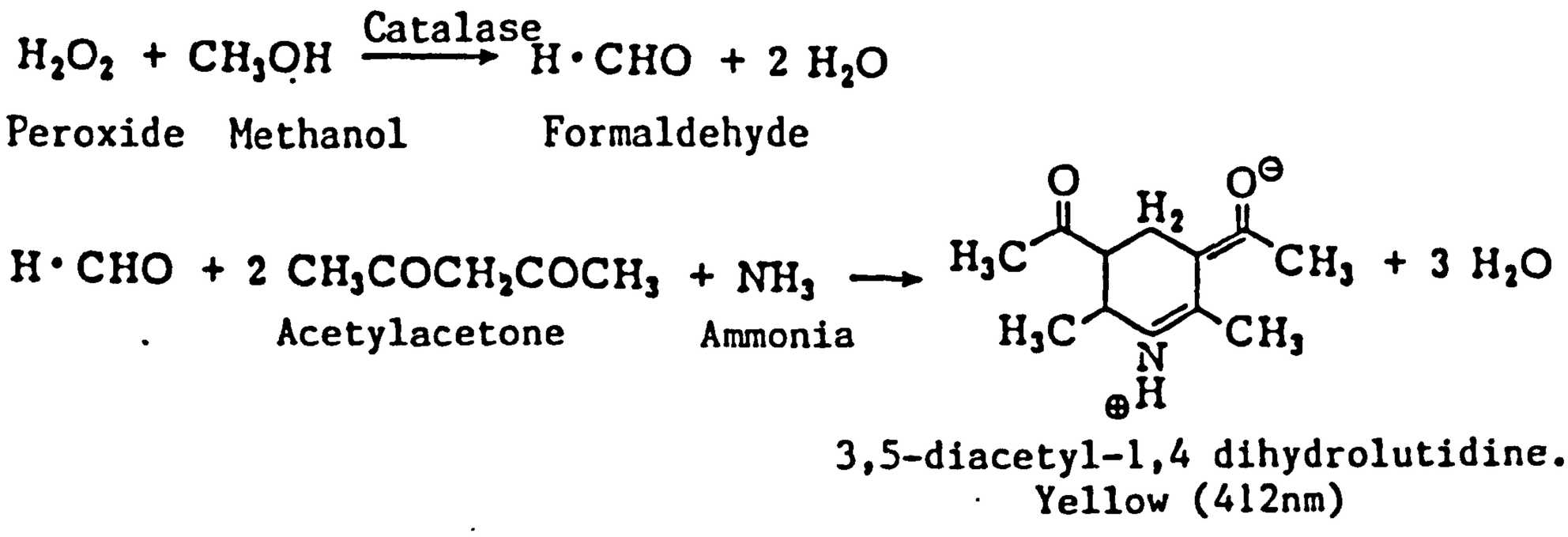
FIG.7 MBTH SELF-COUPLING REACTION.



Tetraazopentamethine dye.

MBTH when reacted with an aldehyde (here formaldehyde) produces a reactive azine. In the presence of peroxide and peroxidase this couples to give an intense blue tetraazopentamethine dye.

FIG.8 HANTZSCH REACTION.



The reaction sequence above is one of the few to use catalase. The lower reaction occurs non-enzymatically to produce the yellow 3,5-diacetyl-1,4 dihydrolutidine.

couples with ammonia and acetylacetone to give yellow 3,5-diacetyl 1,4-dihydrolutidine, (Nash 1953). This reaction has been used to assay uric acid in serum, (Kageyama 1971).

(d) Luminometry.

Luminometric detection of H_2O_2 is primarily chemical in nature, the reaction between H_2O_2 and a luminescent substrate being catalysed by peroxidase. Such substrates are luminol, (5-amino 2,3-dihydro 1,4-phthalazinedione) and isoluminol (6-amino 2,3-dihydro 1,4-phthalazinedione). These have been used to detect H_2O_2 in immunoassay procedures where peroxidase is the enzyme label, (Roda et al 1984, Thorp et al 1985). Bis (2,4,6-trichlorophenyl) oxalate reacts with H_2O_2 to generate peroxyoxalate which in conjunction with 8-anilinonaphthylene 1-sulphonic acid emits light. This system has been used in an enzyme immunoassay for phenytoin, (Takayasu et al 1985).

(e) Fluorimetry.

Fluorimetric analysis of H_2O_2 depends on oxidative coupling of non-fluorescent precursors to give fluorescent products in the presence of peroxidase. Homovanillic acid has been used to measure oxidative enzyme activity, (Guilbault et al 1967) but p-hydroxy phenyl acetic acid was found to be superior, (Guilbault et al 1968, Guilbault et al 1969). This method being the basis of a fluorimetric immunoassay for insulin in serum, (Hinsberg et al 1981).

(f) Electrochemistry.

The electrochemical detection of H_2O_2 has been used in conjunction with many oxidase enzymes to produce biosensors for many analytes. Usually a platinum electrode is used and cellulose acetate is employed to reduce interference on the electrode surface, a

silver/silver chloride electrode being the reference. This type of electrode forms the basis of the Yellow Springs Model 27 glucose analyser where a sandwich of glucose oxidase is held in close physical contact with the detecting electrode, (Clarke 1970, YSI Manual 1988). By varying the enzyme used, sensors have been made for lactate, (Mascini et al 1988, YSI Manual 1988) pyruvate, (Mascini et al 1988), ethanol, (YSI Manual 1988), galactose, (Taylor et al 1977) and sucrose, (YSI Manual 1988). Glucose analysis may be carried out using this type of system on undiluted samples by using silane treated membranes, (Mullen et al 1986). Also electrochemical peroxide detection has been employed in flow systems for ethanol, (Gibson and Woodward 1988).

1.E.4 Miscellaneous Detection Reactions for Enzymes.

Both NAD(P)H and H_2O_2 are by far the most commonly detected species in enzyme reactions and any series of reactions which can be coupled to produce them is usually the method preferred. The diversity and ease of detection of these species makes them an obvious choice for quantitative enzyme analysis. Many other enzymes may be detected by using other reactive species produced, or by using synthetic substrate molecules designed to make quantification simple.

Phosphorylation reactions involving ATP may be estimated directly by the firefly luciferin / luciferase assay. The luminometric assay is one of the most sensitive direct enzyme assays and will measure down to 0.3 p mole of ATP, (Carrea et al 1986).

Enzymes such as creatinine iminohydrolase produce ammonia from creatinine. Ammonia has been assayed colorimetrically to give a commercial dry test for creatinine, (Sundberg et al 1983) and to measure urea electochemically, (Mascini and Guilbault 1977).

Thioesters may be used to measure esterases such as acetylcholinesterase, the sulphydryl containing products being reacted with 5,5'-(dithiobis) 2-nitrobenzoic acid and measured at 412 nm, (Ellman et al 1961). Also synthetic esters of 2-naphthol and other compounds are used, which liberate coloured products on reaction with esterases, (Huber 1984, Loyda et al 1973.)

4-Nitroaniline and 4-nitrophenyl conjugates of many sugars and peptides are used as chromogenic substrates for their respective class of enzymes. Hydrolytic cleavage of the ester bond liberates the nitro aromatic which is measured around the 400 nm region. Many such assays for proteases such as trypsin, (Barns and Elmslie 1975) and chymotrypsin, (Del Mar et al 1979) and for disaccharidases such as galactosidase, (Buonocore et al 1980) and glucosidase, (Weber and Fink 1980) have been described. (For a review on protease substrates see Friberger 1982). Substituted Indoxyl reagents have been used to demonstrate galactosidase, (Lojda et al 1973). Galactosidase is used as an enzyme label in immunoassays, (Oellerich 1984) and a fluorogenic immunoassay for gentamycin has been described using galactosyl umbelliferone-sisomycin as substrate, (Burd et al 1977). Amylase has been detected using a dye conjugated starch which forms the basis for a quantitative amylase assay, (Spayd et al 1978).

Alkaline phosphatase is another important enzyme used in immunoassays. Direct determination may be done by several substrates such as 4-nitrophenyl phosphate, (Bessey and Love 1952), 5-bromo 4-chloro 3-indoxyl phosphate, (Kohno et al 1983) and phenolphthalein monophosphate, (Blake et al 1982). For reviews on enzymes in immunoassays see Schuurs and Van Weeman (1977) and O'Sullivan et al (1979).

Acid phosphatase has been estimated using thymolphthalein monophosphate, (Bowers et al 1981). Recent luminometric assays using luciferin o-phosphate as a substrate for alkaline phosphatase labelled antibodies have been described, (Miska and Geiger 1987, Geiger and Miska 1987).

A direct assay for paracetamol has been described using indophenol dye production at 620nm to detect the reaction, (Price et al 1986).

Enzymes amplification reactions other than NAD(P)H have been described for reduced / oxidised glutathione and ATP/ADP, (Lowry 1980).

The examples given here indicate the scope of analytical reactions available for enzyme analysis. They are by no means exhaustive and for a more comprehensive overview, reference to the literature is necessary, (Bergmeyer 1984-1986).

1.F The Advantages and Disadvantages of Enzymes as Analytical Reagents.

The main advantages of enzymes as analytical reagents are the specific nature of the reaction catalysed and the mild conditions of pH and temperature needed for reaction to take place. The specificity of enzymes often enables a single analyte to be quantified in the presence of many other compounds. It is this property which is at the root of all enzymatic analysis. Also the specificity may be such that the enzyme will only accept one stereoisomer or conformation of a particular compound. Glucose oxidase will only accept β - D- glucose as substrate not the α - D- form, (Bergmayer and Bernt 1973).

Similarly D-aminoacid oxidase is specific for D-amino acids and

is used in their analysis, (Hinkannen and Decker 1985), whereas L-amino acid oxidase is specific for L-aminoacids. Electrochemical sensors for both L and D-aminoacids have been reported, (Guilbault 1980).

Enzymes catalyse reactions in living organisms and therefore most of them work within defined limits of pH and temperature. Rapid analytical procedures are usually possible in mild conditions, minimising risks from harsh reagents.

The main disadvantage of many enzymes is their relative lack of stability. Dry enzyme preparations often show better stability than those in solution. Many analytical enzymes used are stored at -20°C in a dry state or alternatively supplied as a precipitate in a high salt medium to ensure that enzyme activity is retained. Solutions of enzymes, particulary if dilute, often exhibit loss of activity especially at elevated temperatures. For this reason the external enviroment of enzymes is usually controlled by using buffer compounds to maintain a constant pH and keeping the reagents cold to minimise thermal inactivation. Additives may be present to protect the complex protein molecule from such influences as metals, salts, oxidisers, reducing agents, free radicals and other enzymes, such as non-specific proteases which would attack the protein chain. These protective effects are very important for the stability and therefore the catalytic activity of enzymes. This process is usually termed stabilisation.

Another disadvantage is the cost of enzymes. The purity required for analytical enzymes necessitates thorough procedures to remove unwanted material, particulary associated enzyme activities. The price of the preparation tends to reflect on the relative expense of

isolation and purification of the enzyme. Costs however may be offset somewhat by immobilizing the enzyme and its subsequent "re-use", (see section 1.G.3).

1.G.1 Stabilisation of Enzymes.

The stability and consequent retention of catalytic activity of an enzyme is of paramount importance in enzyme analysis. Stability is a reflection of the amino acid content, sequence and subsequent molecular conformation,(tertiary structure) of the enzyme. It is also influenced by external factors such as ionic interactions, temperature, pH, solvation in various solvents, (usually aqueous), detergents, sugars, cofactors, substrate, other proteins, lipids, covalent attachments and hydrophobic interactions. The theory and processes relating to stability and stabilisation are complex and beyond the scope of discussion here, however many reviews and papers have been published which give insight into these areas, (Jencks 1969, Wiseman 1978, Schmid 1979, Torchilin and Martinek 1979, Klibanov 1979, Barker 1982, Klibanov 1983, Monsan and Combes 1984, Tombs 1985, Martinek and Torchilin 1988, Sadana and Henley 1988).

Ideally, the enzyme or enzymes that are used for analysis need to be stable, which in an analytical context refers directly to the retention of catalytic activity upon storage, in use and in the presence of possible destabilising agents. The measure of biological stability is usually carried out using the technique of accelerated degradation, in which samples are incubated at elevated temperatures and activity is compared to samples that have been stored under optimum conditions to retain activity. Relating the rate of inactivation to temperature statistically, a prediction of stability for chosen storage conditions can be obtained, (Kirkwood 1977).

The various methods which have been used to enhance the stability of enzymes are discussed below. Also many enzymes which have been isolated from thermophilic microorganisms exhibit natural stability under extreme conditions. The study of such enzymes are giving insight into mechanisms of stabilisation of proteins.

1.G.2 Naturally Stable Enzymes.

There are a number of microorganisms which thrive in extreme environments such as hot thermal springs. Enzymes isolated from such organisms usually exhibit enhanced thermal and chemical stability and usually catalyse reactions at high temperatures, (Buonocore et al 1980, Lamed and Zeikus 1982, Oshima et al 1982, Malik 1989, Saha and Zeikus 1990).

Structural studies of such thermostable proteins are giving insight into the nature of the stability of enzymes, which appears to be related to the hydrophobicity of the internal part of the molecule, the ion-paring capability, the interaction of metals and the degree of glycosylation. Proteins may be engineered to produce changes in amino acid sequence of the primary structure, using protein engineering techniques. This can produce enhancement in stability due to conformational changes within the molecule, (Yutani et al 1977, Ahern et al 1987). For a fuller discussion see Ward and Moo-Young (1988) and Ljungdahl (1979). Of the many thermostable enzymes isolated and studied, the external factors contributing to stability appear to be the same as for their more labile counterparts, (Ward and Moo-Young 1988).

Other sources of thermostable proteins include plants, (Winer et al 1984) and yeast, (Johnson and Brougham 1981). Thermostable enzymes are of great use industrially where elevated reaction rates, usually

at elevated temperatures and the stability of enzymes reflect in the economy and the feasibility of a process, (Ward and Moo-Young 1988).

A thermostable alcohol dehydrogenase has been used in the construction of an enzyme probe, (Lamed et al 1981), also a thermostable NADH oxidase from Thermus species as been used to measure NADH and ethanol concentrations amperometrically, (McNeil et al 1989).

1.G.3 Immobilisation of Enzymes.

Enzymes, being proteins, are constructed of amino acids which contain reactive groups such as; amino (lysine and n-terminus), thiol (cysteine), carboxyl (aspartate, glutamate and c-terminus), aromatic hydroxyl (tyrosine) and aliphatic hydroxyl (serine and threonine). By causing a chemical, ionic or chelation reaction with such groups, (or a combination of groups) and an insoluble support, which may include glass, nylon, synthetic polymers, natural polymers, ceramics, graphite, metals, or even biological materials such as seeds, (Melo et al 1986), an enzyme may be immobilised.

Recently a novel fluorocarbon immobilisation method was proposed, using perfluoroalkylating reagents to derivatise the enzyme. The perfluoroalkylated enzyme was then adsorbed onto fluorocarbon supports, (Kobos et al 1989). Physical methods such as entrapment or encapsulation within some gelatinous or membranous material may also effectively immobilise an enzyme, (see section 1.G.6).

Immobilised enzymes are useful in the sense that they may be "re-used" which may amount to a substantial economic saving, especially if the free enzyme is expensive. Reviews of enzyme immobilisation have been published and for a thorough discussion see Kennedy and Cabral (1987). Melrose, (1971) lists 54 enzymes and

their respective immobilisation methods and activities. Thirty of the immobilised enzymes exhibited a higher stability on immobilisation, whereas for the remainder, no apparent differences were detected and even in some cases a destabilisation was noted.

In analysis, immobilised enzymes are commonly used especially in flow systems. They have been used to measure a large number of substances, a range of which are shown in table 2.

Nylon tube immobilised reactors for analytical procedures are reviewed in Sunderam (1982) and immobilised enzymes for biosensors in Guilbault (1980).

Immunological techniques take advantage of the effect of physical adsorption of proteins onto hydrophobic surfaces, effectively immobilising the antibody or antigen employed. The amount of protein adsorbed is small (µg), however it is sufficient for most immunoassays. Co-valent attachment of antigens to supports have been carried out, (Gyenes and Sehon 1960, Saito and Nagai 1983).

Immobilised enzymes are usually claimed to be more stable than the soluble counterparts that they are prepared from, two examples being hexokinase and glucose 6-phosphate dehydrogenase, (Morris et al 1975). Although this may be the case, immobilisation onto some sort of support does not guarantee an increase in stability, (Klibanov 1979). A combination of immobilisation and formation of a microenvironment around the immobilised enzyme often enhances stability, (Kricka and Carter 1977, Onyezili and Onitiri 1981).

A thermostable alcohol dehydrogenase has been immobilised onto CN-Br Sepharose 4B. It was found to be 2.5 times more stable than normal yeast alcohol dehydrogenase, (Johnson and Brougham 1981). The

Table 2. Some Analytes Measured using Immobilised Enzymes

Analyte Measured.	Reference.
Glucose	Campbell et al. 1975
Glucose	Hornby et al. 1977
Hydrogen peroxide	Miller et al. 1976
Alcohol	Kuan et al. 1978
Alcohol	Gibson and Woodward. 1988
Alcohol	Gulberg and Christian. 1981
Urea / citrulline	Sunderam et al. 1978
Uric acid	Sunderam et al. 1978
Oxalate	Bais et al. 1980
Cholesterol	Tabata et al. 1981
Cholesterol	Mascini et al. 1983
Starch	Emneus et al. 1986
ATP	Carrea et al. 1986
Fructose	Matsumoto et al. 1986
NAD(P)H / FMN	Nabi and Worsfold. 1986
Lactate	Gorton and Hedlund. 1988
Xylose / xylulose	Dominguez et al. 1988

reagents used for immobilisation are often important factors in the stabilisation of individual enzymes. Luciferase immobilised using cyanogen bromide exhibited 1000 fold increase in stability compared to the free enzyme, but if glutaraldehyde or carbodiimide were used for immobilisation, inactivation occurred, (Ugarova 1982).

1.G.4 Chemical or Carbohydrate Modification of Enzymes.

The modification of reactive groups in proteins without insolubilisation of the molecule has been used to enhance stability of enzymes. Schmid (1979) reviewed a whole range of procedures to produce soluble stable enzymes including chemical modification and grafting or co-valently linking to polysaccharides or synthetic polymers, (pp 68-90). Alkylation, acylation and amino acid modifications are described. Other reactive substituents such as methyl acetimidate have been used. Lactate dehydrogenase exhibits enhanced stability after such modification, (Tuengler and Pfleiderer 1977).

Coupling of sugars or polysaccharides to enzymes mimics glycosylation in native proteins, which is known to enhance their stability, (Pazur et al 1970, Pazur et al 1972). 1-aminoglucose has been used to modify chymotrypsinogen, (Wriston 1973), lactose has been coupled to albumen, (Grey 1974). Dextran has been covalently linked to catalase, this markedly enhanced stability, (Marshall and Rabinowitz 1976) and CM cellulose and DEAE dextran were linked to amylase, giving elevated stability at neutral pH, (Wykes et al 1971). For an overview see Marshall (1978).

A recent method of enzyme modification forms a molecular cage around the protein molecule. The resulting "encaged" enzymes exhibit enhanced thermal stabilities, (Tor et al 1989).

1.G.5 Crosslinking of Enzymes.

Reacting enzymes with bifunctional reagents, (review Wold 1968) such as glutaraldehyde (pentanedial), dicarboxylic acids, diisocyanates, bisimidates and diamines, has been thought to promote crosslinking reactions within the enzyme molecule (intermolecular), or across the subunits if the enzyme is oligomeric (intramolecular). The stability of such crosslinked enzymes is often enhanced considerably, this is thought to be due to the rigidity or clamp-like effect of extra molecular bonding. However, Torchilin and Martinek, (1979) argue that some crosslinking reactions may be only one point modifications and not true crosslinks. Experiments have been carried out using bifunctional reagents with increasing chain lengths, such as dithiols, (Torchilin and Martinek 1979) and dicarboxylic acids, (Torchilin et al 1983).

The chain length of the bifunctional molecule was found to be important for the formation of crosslinks, thus α-chymotrypsin showed maximum stabilisation with 1,5 pentamethylene dithiol, i.e. a relative chain length of 5 methylene units. In contrast, glyceraldehyde 3-phosphate dehydrogenase showed maximum stabilisation using succinic acid, i.e. a chain length of 2 methylene units. Control reactions using monofunctional derivatives confirmed the effect observed was due to crosslinking reactions.

Glucose oxidase has been crosslinked using glutaraldehyde, (Solomon et al 1977) and this method is often used for the preparation of biosensors, usually coupled with some sort of membrane entrapment, (Taylor et al 1977, Petersson 1988). Crosslinking reactions have been reviewed by Martinek and Torchilin (1988).

1.G.6 Physical Entrapment of Enzymes.

Enzymes may be physically entrapped within the matrices of gels or other polymers or sandwiches of membrane systems or microencapsulated in micelles. One advantage of this is that the enzyme itself is not chemically modified but only restrained. Stability may be improved dramatically in such cases, as conformation of the molecule is retained by the rigidity of the gel matrix around it, (Pennington et al 1968, Martinek et al 1977, Serralheiro et al 1990). Modification of the protein to produce reactive groups that polymerise on gel formation is also a possibility. See reviews by Klibanov (1979) pp 5-7, Kennedy and Cabral (1987) pp 384-392 and Melrose (1971).

Glucose oxidase has been sandwiched between dialysis membranes for the automated estimation of glucose, (Campbell et al 1977), no loss of activity was reported after 10 days at 37°C with intermittent use. Entrapment may also be used to form enzyme electrodes, (Guilbault 1980). L-lysine was assayed using a gelatin entrapped enzyme which was then modified by glutaraldehyde treatment, (Romette et al 1983), and oxygen analysis for kinetic measurements in non-aqueous solvents was carried out using micellar entrapped enzymes, (Escobar et al 1990).

1.G.7 Stabilisation with Additives.

The addition of many different chemical compounds to solutions of proteins has been found to enhance stability in many cases. Indeed this is probably one of the major areas of enzyme stabilisation due to the simplicity of the method. The mechanisms by which additives stabilise enzymes are, however, far from simple and are probably due to several concomitant interactions, see Jencks

(1969), Wiseman (1978) pp 283-291, Torchilin and Martinek (1979), Schmid (1979) pp 57-68 and Klibanov (1983) pp17-18.

Neutral salts such as ammonium sulphate and sodium chloride tend to stabilise enzymes, probably by their effect on the hydrophobicity of the polypeptide chain, (e.g. glucose dehydrogenase, Hornby et al 1977). If the concentration of salt becomes high enough precipitation may ensue, (salting out). This effect has applications in the purification of proteins and in stabilisations. Some enzymes are sold as a precipitate in salt solutions, necessitating removal of the high salt concentration before the enzyme is used for analysis. Low concentrations of certain salts may also stabilise enzymes, particularly metalloenzymes. Calcium, for instance, is a stabiliser for α-amylase, (Schmid 1979 p64).

Substrates also may have a pronounced effect on stability. Invertase is stabilised by its substrate sucrose when present above a concentration of 1.5M, however the enzyme activity is greatly reduced, (Monsan and Combes 1984). Co-factors such as NAD, NADH and analogues also stabilise enzymes such as glucose 6-phosphate dehydrogenase, (Cancedda et al 1973) and diacetyl reductase, (Bryn et al 1970).

Reducing agents such as thiol compounds help to stabilise enzymes which contain free sulphydryl residues necessary for catalytic activity including D-fructose dehydrogenase, (Ameyana et al 1981), diacetyl reductase, (Bryn et al 1970), alcohol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase, (Kricka and Carter 1977).

Polyhydric alcohols are well known stabilisers of proteins. The lower chain length molecules such as ethylene glycol and glycerol, are not as effective in stabilising enzymes as molecules of longer

chain length alcohols, of which the most effective are those of intermediate chain length, i.e. 4 - 6 hydroxyl units, (Monsan and Combes 1984). High concentrations of glycerol are useful in stabilising alcohol dehydrogenase, (Brougham and Johnson 1981) and have also been used as a cryoprotectant, (Schmid 1979, p61). Various studies on the stabilisation effect of polyhydric alcohols have been carried out, (Back et al 1979, Fujita et al 1982, Combes and Monsan 1984, Monsan and Combes 1984). The results in each case indicated a greater stabilisation effect as the number of hydroxyl groups increased.

Glucose oxidase has been stabilised in solution using various polyhydric alcohols, (Ye et al 1988) and glucosidase by sorbitol, inositol and mannitol, (Bernier and Stutzenberger 1988).

Drying enzymes in the presence of polyhydric alcohols to produce dry stabilised preparations has also been used and is of particular importance to diagnostic enzymes in various analytical formats, (Phillips 1985).

Sugars have been used to stabilise proteins in much the same way as polyhydric alcohols. Their effect is much the same as polyhydric alcohols, (Back et al 1979, Arakawa and Timasheff 1982). Drying enzymes in the presence of sugars, usually results in enhanced retention of enzyme activity, (review Crowe et al 1987). Disaccharides, particularly trehalose, which is found in organisms where natural dehydration takes place, e.g. brine shrimps, have a particularly good stabilising effect. Trehalose has been claimed as a stabiliser for proteins, (Roser 1986).

Polymers which may be natural or synthetic in origin have also been used to enhance protein stability. Proteins themselves have been

used to enhance stability, e.g. bovine serum albumin or BSA, (Wolf et al 1972). Natural polymers such as soluble celluloses (carboxymethyl, ethyl-hydroxypropyl), alginate, polygalacturonic acid, dextrans and starches have been used to stabilise enzymes, (Wiseman 1978, Schmid 1979, Barker 1982). Polyethylene glycols of various molecular weights have been used to stabilise invertase, (Monsan and Combes 1984) and glucose oxidase, (Ye et al 1988). Polyvinyl alcohol was used to stabilise apoglucose oxidase in conjunction with BSA, (Tabb and Tyhach 1982).

A comprehensive review of the technical applications of protein stabilisation, and the type of stabilisers, used may be found in Schmid (1979) pp 90-103 and 116-118. Many such stabilisers and their applications may be found in the patent literature.

1.H <u>The Aims of the Work and Diagnostic Development.</u>

The assay of substances using enzymes is a wide and expansive field of work, with new developments occurring frequently. The development of analytical techniques with new enzymes is fairly frequent in the area of research, but not so common in the commercial and industrial fields. The production of very simple, stable, "user friendly", analytical tests, is the real object of the work described in this report. This type of test involves a substantial amount of technology to control parameters within the test format, so making the accuracy of the analytical technique more a function of the test, not of the person who uses it. Such user independent assays would have obvious advantages in the rapid detection and quantitation of analytes in real situations, which are not under laboratory conditions.

Oxidoreductase enzymes were chosen for initial study, as it is

on this group that the majority of diagnostic tests are based. The detection techniques are many and well documented, (sections 1.E.2 and 1.E.3). Stabilisation of such systems are however, relatively few, the majority of the literature being concerned with proteases and other industrially important enzymes. The stability of diagnostic tests is of particular importance to the shelf life of a product and perhaps this is one of the major limitations to the production and acceptance of many other enzyme based analytical systems. With this in mind, the enzyme alcohol oxidase from various sources has been chosen as a test enzyme.

This enzyme is very stable in certain enviroments, such as an ammonium sulphate precipitate, however in a purified, ready to use format it has a tendency to autoxidise with subsequent inactivation. This is probably due to reaction with bound formaldehyde which is released on ageing or drying of the enzyme, (Hopkins and Mueller 1987). The enzyme has been used to measure ethanol, (Majkic-Singh and Berkes 1980, Gulberg and Christian 1981, Phillips 1984, Barzana et al 1989) and methanol from hydrolysis of pectins, (Herzenberg and Rogerson 1985, Klavons and Bennett 1986). Enzyme electrodes have also been produced using alcohol oxidase, (Verduyn et al 1983, Belghith et al 1987).

In our laboratory a prototype, mediator linked, alcohol probe has been developed, (Parker, Higgins and Woodward , unpublished results) using N,methyl phenazinium-tetracyano quinodimethane, (NMP-TCNQ) to link the peroxidase reaction directly to a glassy carbon electrode.

The development of a simple enzymic procedure to measure ethanol over a wide range of concentration forms the basis of the work, (chapter 3). A subsequent stabilisation procedure discovered for the

enzyme isolated from the yeast Hansenula polymorpha,was then extended to include alcohol oxidases from other sources and a range of other enzymes, (chapter 5). The applications of this part of the work have been submitted for patent registrations in the area of stabilisation of enzymes, (Gibson and Woodward 1989, Gibson and Woodward 1990) and the use of stabilised enzymes in a differential control, dry diagnostic test for ethanol and other analytes, (Gibson and Woodward 1989). The production of dry reagent analytical tests is discussed in chapter 6.

Chapter 4 reports the production of immobilised enzyme derivatives, which led to the development of flow analysis for ethanol and its application in on-line estimation of analytes during fermentation, (Gibson and Woodward 1986, Gibson and Woodward 1988).

One potential application of the alcohol assay systems produced, was to measure alcohol concentration in the drinks industry. In communication with breweries during this research, the analysis of an important flavour compound, diacetyl, (2,3 butanedione), was discussed. This compound is difficult to analyse as it is usually present at very low levels (0.05 - 0.5mg.l^{-1}). Gas liquid chromatography is the method most often used for analysis, (Scherrer 1972). Also chemical methods have been used after some sort of extraction procedure, (Westerfield 1945, Owades and Jakovac 1963).

The development of an enzymic method to quantify diacetyl, using the enzyme diacetyl reductase has been carried out. The isolation, purification and characterisation of the enzyme from chicken livers was undertaken, since the enzymes from microbial sources and beef liver were found to be unsuitable for the assay, (chapter 7). This work has been submitted and accepted for publication, (Gibson et al

1990).

Further research on commercial products stemming from the results obtained in this work is continuing. The stabilisation system used for alcohol oxidase has been already adapted for use in the Yellow Springs Instruments Ethanol analyser and is currently under investigation in antibody-enzyme conjugate dry phase stabilisation within a dry analytical format.

CHAPTER 2.

MATERIALS and METHODS.

2.A.1 Bacterial Culture. Klebsiella Aerogenes. FG9.

This bacterium formerly known as Aerobacter aerogenes was cultured in the following medium.

Tryptone	10g
Glucose	10g
Sodium citrate $2H_2O$	20g
Yeast extract	5g
K_2HPO_4	1g
$MgSO_4$ $7H_2O$	1g

The glucose was normally sterilised separately at 110°C for 15 minutes and added aseptically to the medium which was sterilised at 121°C for 40 minutes in 10 litre batches. The organism was either grown in shake flasks or in an aerated fermenter at 30°C, at an initial pH of 7.0. During growth the pH was maintained around pH 7.0 by addition of 10% NaOH solution. The pH was monitored by a sterilisable pH probe immersed in the media. The organisms were harvested after 48 hours using an Alfa-Laval continuous clarifier. The cells were washed by centrifugation in 50 mM phosphate buffer pH 7.0 and resuspended in the same buffer.

2.A.2. Streptococcus Lactis Subspecies Diacetylactis 18-16.

The bacteria were cultured in the following medium which was adapted from that of Harvey and Collins (1961).

Yeast extract	15g
Tryptone	10g
KH_2PO_4	0.5g
$MgSO_4$ $7H_2O$	0.2g
Glucose	20g

Trisodium citrate $2H_2O$	5g
Sodium acetate $3H_2O$	2g

The medium was sterilised at 121°C for 15 minutes. The incubation was carried out for 18 hours at 30°C in static culture with no aeration. The cells were harvested by centrifugation, (MSE Coolspin) in 1 litre aliquots at 7250g for 60 minutes at 4°C. They were then washed twice with 100mM tris/HC1 buffer, pH 7.3 containing 1.0mM Cysteine, (Sorvall RC-5B centrifuge).

The total yield was 85.8 g for 16 litres medium.

2.B.1.Enzyme Purification. Alcohol Oxidase from Hansenula Polymorpha.

Frozen yeast cells supplied by Dr J.P.Van Djiken, University of Delft, Delft, Holland, (750g) were added to 3 times their weight of buffer, (25mM potassium phosphate, pH 7.9 containing phenyl methyl sulphonyl fluoride, (PMSF) 25 mM and N-tosyl L-phenylalanine chloromethyl ketone, (TPCK) 4 mM as protease inhibitors and thawed at 4°C overnight. To the thin cream coloured suspension, 1M potassium hydroxide was added to give a pH of 8.5 and then the suspension was homogenised by passage through a Dyno Mill, bead mill, using 0.4 mm glass beads. The chamber was kept at 4-8°C using recirculating ice water and the flow rate was kept between 125-175 $ml.min^{-1}$. The thicker effluent was kept at pH 7.5 - 8.5 using 1M potassium hydroxide and centrifuged at 17,000g at 4°C in a Sorvall RC-5B refrigerated centrifuge for 50 minutes. The supernatant, (cell free extract) was decanted and pooled. Volume, protein and enzyme activities were recorded at this stage.

All buffers used from this point forward contained PMSF 25mM and TPCK 4mM and all steps were carried out at 4°C. The cell free extract was loaded onto Fast Flow DEAE sepharose in a Pharmacia

Bio Process column, pre-equilibrated with 25mM potassium phosphate buffer, pH 7.9. The column was washed with 5 litres of the same buffer and then with buffer containing:

(a) 0.4% w/v sodium chloride (10 litres)

(b) 0.6% w/v sodium chloride (10 litres)

(c) 0.8% w/v sodium chloride (15 litres)

This removed the main contaminant which was catalase.

The alcohol oxidase was eluted using buffer containing 4.0% w/v sodium chloride. The fractions, (500ml) containing the highest activity were pooled, (volume, protein, enzyme activities taken) and the enzyme precipitated using ammonium sulphate to 65% saturation, (chart Appendix I).

During precipitation the pH was kept above 7.5 using ammonium hydroxide solution. The suspension was held at 4°C for at least 30 minutes with stirring and then centrifuged at 4°C at 15,000g for 30 minutes.The pellets were pooled and stored in a sterile glass jar at 4°C.

2.B.2. Diacetyl Reductase from Klebsiella Aerogenes. FG9.

The cells grown as described in 2.A.1 were disrupted in a Braun homogeniser using 0.1 mm glass beads in 60ml of 100mM phosphate buffer, pH 7.0. The extract was filtered through two layers of muslin and centrifuged at 17,500g for 15 minutes. Solid ammonium sulphate was added to the supernatant to 32 % saturation and the precipitate removed by centrifugation at 10,000g for 15 minutes and discarded. Solid ammonium sulphate was then added to the supernatant to 85 % saturation and the precipitate collected in the same manner as before. The pellets were redissolved in a minimum volume of 50mM phosphate buffer, pH 7.0 and dialysed three times against the same

buffer at 4°C.

The dialysate was loaded onto a Pharmacia DEAE Fast Flow sepharose column, (45mm x 210mm) pre-equilibrated with the same buffer and eluted at 0.7 $ml.min^{-1}$ with a linear gradient of sodium chloride, (up to 0.5M), in the same buffer at 4°C. The highest activity fractions were pooled and precipitated using solid ammonium sulphate at 85 % saturation. The pellets were collected by centrifugation at 31,000g for 20 minutes, redissolved in 50mM phosphate buffer, pH 7.0 containing 2-mercaptoethanol 10mM and glycerol 20% v/v and stored at -20°C.

2.B.3. Diacetyl Reductase from S.Lactis sub.Diacetylactis 18-16.

Tris/HCl buffer, 100mM containing 1.0mM cysteine was used throughout and the temperature was kept at 4°C. The washed cells were suspended in 4 times their volume of buffer and homogenised in a Braun homogeniser using 0.1mm glass beads. The extract was filtered through two layers of muslin and centrifuged at 17,500g for 15 minutes, (Sorvall RC-5B). Solid ammonium sulphate was added to the supernatant to 32% saturation and the mixture was centrifuged at 10,000g for 15 minutes. To the supernatant, solid ammonium sulphate was added to 65% saturation and the mixture centrifuged for 30 minutes at 17,000g. The pellets were dissolved in buffer and dialysed against 100mM tris/HCl buffer, pH 7.3 with 2 changes of buffer. The dialysis time was kept as short as possible, (about 4-6 hours) and cysteine was added to a final concentration of 1.0mM to the to the dialysate immediately after recovery.

The solution was then loaded onto a column, (45mm x 210mm) of DEAE Fast Flow sephrose, (Pharmacia) pre-equilibrated with the same buffer and eluted at 0.70 $ml.min^{-1}$ with a linear gradient of sodium

chloride from 0.2M to 0.8M in the same buffer. The highest activity fractions were pooled and precipitated with solid ammonium sulphate at 56% saturation and centrifuged for 20 minutes at 31,000g. The pellet was redissolved in buffer and loaded onto a column, (25mm x 510mm) of Pharmacia sephacryl S-200. Active enzyme was eluted at a flow rate of 0.5ml.min^{-1} with tris/HCl/cysteine buffer. The highest activity fractions were pooled and precipitated with ammonium sulphate and centrifuged as before.

2.B.4. Diacetyl Reductase from Beef Liver.

The purification procedure according to Burgos and Martin (1972) was carried out with the modification of using sepharose ion exchanger instead of cellulose. Beef liver, (2.1 kg) obtained from a freshly killed bullock was packed in ice and transported to the laboratory. This was then decapsulated, finely chopped and homogenised for 1 minute in acetone, (4.21) at -15°C in a large waring blender. The suspension was added to a further 16 litres of cold acetone and stirred for 30 minutes. The solids were filtered under vacuum and rehomogenised in a further 4 litres of cold acetone, refiltered and pressed in a vice to remove excess acetone. The flat, dry cakes were stored at -20°C where they were stable for 3 months.

The crude protein fraction was prepared by briefly homogenising 1 volume of acetone cake with 4 volumes of ice cold distilled water and then stirring for 30 minutes at 4°C. The suspension was centrifuged at 17,000g for 10 minutes and the precipitate discarded. Acetone, (0.95 volumes) at -15 °C was added slowly, with stirring to the supernatant. The suspension was centrifuged as before and the precipitate discarded. A further 0.35 volumes of acetone at -15°C was added with stirring and the mixture stirred for 30 minutes at

4°C. The fine suspension was centrifuged at 17,000g for 15 minutes and the precipitate was redissolved in a minimum of ice cold distilled water and freeze dried.

The crude powder fraction was disolved and loaded onto a column, (28mmx250mm) of Pharmacia Fast Flow DEAE sepharose, pre-equilibrated with 25mM tris/HCl buffer pH 7.5 containing sucrose, (250mM). The protein was eluted with a linear gradient of sodium chloride from 0 to 500mM in the same buffer at a flow rate of 0.4ml.min^{-1}. Activity peaks were pooled, dialysed against 3 changes of the same buffer and freeze dried.

The freeze dried powder was redissolved in a minimum volume of ice cold distilled water and loaded onto a column, (16mm x 800mm) of Pharmacia sephacryl S-200, pre-equilibrated with the same sucrose/tris/HCl buffer used for ion exchange. The protein was eluted using the same buffer at a flow rate of 4.0ml.hr^{-1}, the highest activity peaks being pooled and freeze dried.

2.B.5. <u>Diacetyl Reductase from Chicken Liver.</u>

The purification procedure was that of Bernardo et al (1984) with certain modifications. Fresh chicken livers were rapidly frozen in liquid nitrogen and stored at -70°C until needed. 300g of liver was thawed and homogenised in a waring blender with 1200 ml of ice cold distilled water. The extract was centrifuged at 20,000g for 50 minutes at 4°C, (sorvall RC-5B) 1.1 volumes of acetone at -15°C was slowly added to the supernatant, the precipitate was removed by centrifugation at 13,000g for 10 minutes at 4°C. A further 1.4 volumes of acetone at -15°C was added and the suspension allowed to stand for 10 min. The suspension was centrifuged, (13,000g, 10 minutes 4°C) and the precipitate dissolved in a minimum of ice cold

distilled water and freeze dried.

A solution of the crude acetone precipitate, (325mg in 3ml of buffer), was loaded onto a column, (26mm x 440mm) of Pharmacia sephadex G-100, pre-equilibrated with 5mM sodium/potassium phosphate buffer, pH 8.2. The protein was eluted using using the same buffer at a flow rate of 10.5ml. hr^{-1} at 4°C.

The highest activity fractions were pooled and loaded onto a column, (10mm. x 120mm) of Whatman DE-52 DEAE cellulose ion exchanger, pre-equilibrated with the same buffer as above. The protein was eluted using a concave gradient formed from 120ml of the 5mM phosphate buffer, pH 8.2 and 60ml of 500mM sodium/potasium phosphate buffer, pH 7.0 at a flow rate of 0.175 $ml.min^{-1}$ at 4°C.

The highest activity fractions were pooled and dialysed against 3 changes of 5mM ammonium bicarbonate solution at 4°C over 6-8 hours, then frozen rapidly and freeze dried. The solids were then dissolved 25mM imidazole/HCl buffer, pH 7.4 and applied to a column, (10mm x 100mm) of Pharmacia polybuffer exchanger 94, (PBE 94), pre-equilibrated with the same buffer. The protein was eluted using Pharmacia polybuffer 74 at pH 5.0. The highest activity fractions were pooled and the protein precipitated with ammonium sulphate at 100% saturation for 30min at 4°C. The suspension was centrifuged at 36,000g for 30 minutes at 4°C and the precipitate dissolved in a minimum of 5mM phosphate buffer, pH 8.2. This was dialysed against 2 changes of 5mM ammonium bicarbonate solution at 4°C over 4-5 hours and freeze dried. The dry solids were stored at -70°C over silica gel.

2.C.1. P.A.G.E. Solutions and Buffers.

Acrylamide-Bisacrylamide.

Acrylamide (BDH Electran Grade)	30g.
N,N, Methylenebisacrylamide. (BDH Electran)	0.8g.
Distilled water	to 100ml.

The solids were weighed out carefully, dissolved with stirring and stored in a cool place out of direct sunlight.

Stacking buffer. (0.5M Tris/HCl).

Sigma 7-9 tris (6.0g) was dissolved in 75ml distilled water and the pH was adjusted to 6.8 with concentrated HCl. The volume was made up to 100ml with distilled water.

Resolving buffer. (3.0 M Tris/HCl).

Sigma 7-9 tris (36.3g) was dissolved in 50-60ml distilled water and the pH was adjusted to 8.8 with concentrated HCl. Because of the long equilibrium times of such concentrated tris solutions on some pH electrodes, the pH was tested and adjusted after several hours. The volume was then made up to 100ml with distilled water.

Detergents.

10% w/v solutions of the following detergents were prepared by dissolving 10g of detergent in distilled water to a final volume of 100ml.

Sodium dodecyl sulphate (SDS), triton X-100, sarkosyl NL30 (all from BDH), sodium dodecyl sulphonate (Sigma) and 3-(N,N.-dimethylmyristylammonio) propane sulphate or DMAPS (Fluka).

AMPS.

Ammonium persulphate, (BDH) 100mg was dissolved in 1.0ml of distilled water and used immediately.

Non-dissociating resolving buffers.

(1) Tris/phosphate stacking buffer. Sigma 7-9 tris (4.95g) was dissolved in 50ml distilled water and the pH was adjusted to 5.5 using orthophosphoric acid (BDH). The volume was made up to 100ml with distilled water.

(2) Tris/HCl resolving buffer (2M). Sigma 7.9 tris (24.2g) was dissolved in 50-60ml distilled water and adjusted to pH 7.5 with concentrated HCl in the same manner as the resolving gel for dissociating systems. The volume was made up to 100ml with distilled water.

Reservoir buffers.

(1) Tris/glycine 10x concentrate. (pH 8.3)

Sigma 7-9 tris.	30g
Glycine (Sigma).	144g
Distilled water	up to 1.0 l.

The stock was diluted in distilled water 1 in 10 before use. Detergent was added at this stage at 10ml of 10% w/v per litre of diluted buffer.

(2) Tris/barbitone. (pH 7.0)

Sigma 7-9 tris	10g
Diethyl barbituric acid (BDH)	5.52g
Detergent 10% w/v	10g
Distilled water	up to 1.0 l.

The buffer was freshly prepared and used undiluted.

Sample buffers.

(1) Dissociating sample buffer.

Tris/HCl stacking buffer,(pH 6.8).	0.75ml
Glycerol ("Analar" BDH)	2.0ml
SDS	0.8g
Distilled water	up to 9.0ml

Water soluble bromophenol blue, (BDH) was added to the buffer as a marker dye, (about 0.5mg). Just before use 0.1ml of 2-mercaptoethanol, (Sigma) was added to 0.9ml of buffer.

(2) Non-Dissociating Sample buffer.

Tris/phosphate stacking buffer.	0.75ml
Glycerol	2.0ml
Non-ionic or zwitterionic detergent.	0.8g
Distilled water	up to 10.0ml

Water soluble bromophenol blue was added as before but no 2-mercaptoethanol was used.

2.C.2. P.A.G.E. Dissociating Gels.

Dissociating gels were prepared and developed using the method of Hames and Rickwood (1981). Three types of slab gel apparatus were used throughout the work. (a) A commercial system marketed by Bio-Rad Ltd and (b)/(c) two homemade systems which produce gels of 160mm x 145mm and 80mm x 70mm respectively, (mini gel system).

The gel composition for the mini gel system is shown below, multiply by 4 to obtain values for systems a and b.

Table 3. Electrophoresis Gel Composition.

Solution.	volumes in ml. Stacking gel.	Resolving Gel. 5%	10%	15%	20%
Acrylamide-Bisacrylamide.	1.0	1.34	2.66	4.00	5.34
Stacking buffer.	0.5	-	-	-	-
Resolving buffer.	-	2.0	2.0	2.0	2.0
Detergent 10% w/v.	0.08	0.08	0.08	0.08	0.08
AMPS 10% w/v.	0.04	.028	.028	.028	.028
Distilled water.	4.88	4.56	3.22	1.90	0.56
TEMED.*	.006	.003	.003	.003	.003

* N,N,N,N, Tetramethyl ethylenediamine (Sigma)

The gel components were mixed together, (SDS was used as detergent in dissociating gels) and then the TEMED added. The solution was then pipetted into the slab gel cassette, formed using acetone polished glass plates clamped together with waterproof tape or the plastic clamps of the Bio-Rad system and a 1.0mm or 1.5mm thick plastic spacers. The surface of the gel solution was quickly overlaid with a 1 in 4 dilution of resolving buffer to produce a smooth top surface and allowed to polymerise. The gels so formed could be kept at 4°C covered in clingfilm to prevent dehydration for up to 1 week before use.

Gradient gels were formed using a miniature gradient former manufactured in perspex. Half volume of gel solutions were added to the two chambers, the higher acrylamide concentrations to the front which was stirred. TEMED was added to both chambers, mixed well and the solutions were pumped into the gel cassette using a Gilson

Minipuls MK 2 pump set at 1-2 ml.min^{-1}. The gradient gel so formed was overlaid manually as before, allowed to polymerise and run in the same way as the linear gels. The stacking gel was mixed and poured on the top surface of the resolving gel. Rinsing of the surface with stacking gel solution facilitated a good contact at the interface. The combs used to form the wells for protein loading varied in size depending on numbers and volume of samples being run. The combs were inserted into the stacking gel before polymerisation. After polymerisation the comb was removed and the gel inserted into the running apparatus, making sure the lower surface of the gel slab was exposed to the running buffer. Both the top and bottom tanks were filled with running buffer and the apparatus was checked for leaks.

Samples were prepared by boiling in an equal volume of dissociating sample buffer for 10 minutes, cooling and centrifuging in an MSE microcentrifuge at 13.500 rpm for 2-3 minutes. The samples were then loaded into the wells using a Hamilton syringe. The gel was then connected to the power supply and run at 15-20mA through the stacking gel and 25-40mA through the resolving gel,according to size.

2.C.3. P.A.G.E. Non-Dissociating Gels.

This method was essentially the same as for dissociating gels, (table 3) however the resolving buffer was tris/HC1 pH7.5 and the running buffer tris/barbitone pH 7.0. The detergents used were either non-ionic, (triton X-100, sarkosyl NL30) or zwitterionic, (DMAPS) and the samples were added to non-dissociating sample buffer and not boiled. Gels were run in the cold, (4°C) to retain enzyme activity during separation.

2.C.4. I.E.F. Diacetyl Reductase.

Isoelectric focusing of purified preparations of chicken liver

diacetyl reductase were carried out using pre-cast thin layer polyacrylamide gels, (LKB) pH 3.5 - 9.5 in an LKB Multiphor system according to the instruction manual supplied, (Winter et al 1977).

2.C.5. Gel Activity Staining for Diactyl Reductase.

Gel Overlay Technique.

Low melting point agarose, (Sigma) was dissolved to 1% w/v by heating in 100mM phosphate buffer pH 6.1 or pH 7.0. This was cooled to 45°C in a water bath and poured over a clean glass plate and allowed to set. A solution of NADH, (10mg) and diacetyl, (0.3ml, 5% w/v), in 4ml, pH 6.1 phosphate buffer was layered on the surface of the gel, incubated for 30 minutes and blotted dry. After electrophoresis, the non-dissociating gel was briefly washed with distilled water, blotted dry and laid on the surface of the agarose gel. The sandwich was incubated at room temperature for 30 minutes, then the polyacrylamide gel was removed. A solution of 3 - (4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide, (MTT,Sigma) 0.40mM and meldola blue 0.01mM (Sigma) in 100mM phosphate buffer, pH 8.2 was layered on the surface of the agarose gel. Enzyme activity appeared as clear zones on a dark purple background. The background intensity was controlled by varying the time of incubation in MTT and meldola blue.

Direct Staining.

The activity staining technique was a modification of that of Provecho et al (1984). After electrophoresis, the non-dissociating gel was washed briefly in distilled water and then incubated in phosphate buffer 500mM, pH 6.1 or pH 7.0 containing diacetyl, (20mM) and NADH, (1.0mM) for 10-15 minutes at room temperature. The gel was then blotted dry and incubated for a further 15 minutes in a dry

state on moist filter paper to exhaust cofactor in the gel. MTT, (0.4mM) and meldola blue, (0.01mM) in phosphate buffer, (100mM, pH 8.2) were added to develop the colour. Enzyme activity showed up as colourless zones on a dark purple background, (see chapter 7).

Diacetyl Reductase Activity Staining in SDS Gels.

This method was adapted from Lacks and Springhorn (1980). SDS gels were prepared in the normal way with the inclusion of bovine serum albumen, (BSA, 10mg/ml, Fraction V Sigma) in the gel matrix. Samples were boiled for 2-3 minutes only before applying to gel. After electrophoresis the gel was washed 3 times for 30 minutes in fresh changes of sodium phosphate buffer, (pH 7.0, 50mM). The gel was then blotted dry and stained for activity by the direct method.

2.C.6. Gel Protein Staining.

Protein bands were visualised in polyacrylamide gels using 0.1% coomassie blue R, (Sigma) in methanol / acetic acid / distilled water, (ratio 5:2:5 parts respectively). Staining was carried out for a minimum of 4 hours at room temperature or 1.5 hours at 60°C. Gel destaining was carried out using the same solvent mixture minus the dye. Several changes were usually required to give an acceptable background. The spent destain was regenerated by passage through an activated carbon column and re-used.

2.D.1. Buffers used in Analytical Procedures.

Phosphate.

Recipes for phosphate buffers are given in table 4. The volume is kept standard at 1.0 litre. Sodium phosphates, (BDH "Analar") and potassium phosphates, (Aldrich) were used.

Tris/HC1.

Sigma 7-9 tris, (tris (hydroxy methyl) aminoethane) was used to

Table 4. Phosphate Buffers. $g.l^{-1}$

pH.	Conc	$NaH_2PO_4 2H_2O$	$Na_2HPO_4 12H_2O$	KH_2PO_4	K_2HPO_4
6.0	100mM	13.68	4.43	-	-
6.5	100mM	10.69	11.35	-	-
7.0	100mM	6.08	21.85	-	-
7.5	100mM	2.50	30.23	-	-
8.0	100mM	0.83	34.09	-	-
7.0	100mM	-	-	6.04	9.78
6.1	100mM	-	5.24	11.63	-
7.5	25mM	-	7.07	0.73	-
8.2	100mM	-	34.09	0.73	-
7.9	25mM	0.36	-	-	3.96
7.9	250mM	3.55	-	-	39.60
7.0	500mM	-	-	30.20	48.90
7.1	500mM	-	109.80	26.54	-
7.9	100mM	0.83	33.90	-	-

produce tris buffers. pH adjustment was made with BDH "Analar" concentrated HC1.

Table 5. Tris Buffers.

pH.	Concentration	Sigma 7-9 tris $g.l^{-1}$
7.5	25mM	3.03
8.8	250mM	30.28
8.8	100mM	12.11

MOPS

3-(N-Morpholino) propane sulphonic acid, (MOPS) was prepared by two methods. The free acid was dissolved to the required concentration and the pH was adjusted using 4M NaOH or the sodium salt of MOPS was dissolved together with the free acid to provide the required pH. Both methods gave acceptable buffers.

Table 6. MOPS Buffers.

pH.	concentration.	$g.l^{-1}$ MOPS.	MOPS.NaSalt.
7.0	100mM	11.13	10.83
7.8	100mM	4.93	17.63
7.9	100mM	4.21	18.41
8.0	100mM	3.55	19.15
7.0	100mM	20.93	4 M NaOH
7.9	100mM	20.93	to
7.0	1.0M	209.3	required
7.9	1.0M	209.3	pH.

Sucrose buffers.

Sucrose was added to 25mm phosphate or tris/HCl pH 7.5 to give a

concentration of 250mM, (85.58g l^{-1}).

Borate buffers.

Two methods were used to prepare borate buffers. Borate buffer pH 8.5, 200mM was prepared by mixing free acid and the sodium salt as follows:-

Boric acid (FSA Chemicals) 12.4g, di-sodium tetraborate (BDH) 6.67g dissolved in 1.0l distilled water.

Other borate buffers were prepared by adding NaOH or HC1 to sodium tetraborate.

Table 7. Borate Buffers.

pH	concentration	sodium tetraborate g	0.1MNaOH ml	0.1MHC1. ml
9.02	100mM	8.925	-	46.0
9.90	100mM	8.925	183.0	-
10.65	100mM	8.925	238.0	-

Citrate/Phosphate buffers.

Recipes for citrate/phosphate buffers are given in table 8.

Table 8. Citrate/Phosphate Buffers. $g.l^{-1}$

pH.	concentration	citric acid (BDH)	Na_2HPO_4 $12H_2O$.
4.0	50mM	5.90	13.82
5.0	50mM	4.67	18.40

Glycine buffer.

Glycine buffer 200mM, pH 8.9 was prepared by dissolving 15.01g glycine (Sigma) in 800ml distilled water adjusting the pH to 8.9 with 0.2M NaOH and making up to 1.0l.

Carbonate buffer.

Carbonate buffer 100mM, pH 10.6 was prepared by dissolving 4.505g anhydrous sodium carbonate (BDH) and 0.63g sodium bicarbonate in distilled water and making up to 1.0l.

pH Profile Buffers. Diacetyl Reductase.

Small volumes, (50ml) of 0.1M citrate/phosphate or 0.1M phosphate buffers were prepared by mixing various proportions of Na_2HPO_4 $12H_2O$ solution, ($35.85g.l^{-1}$) with 0.1M citric acid solution, ($19.21g\ l^{-1}$) or 0.1M NaH_2PO_4 $2H_2O$ solution, ($15.60g.l^{-1}$) to give a range of pH's from 5.0 to 8.25.

2.D.2. Activity Measurement of Oxidase Enzymes.

A Clark oxygen electrode, (Rank Bros) was routinely used to assay oxidase enzymes. For alcohol oxidase, 100mM phosphate buffer, pH 7.0 was gassed with air for a minimum of 20 minutes before use and simultaneously incubated at 37°C. The buffer stock was gassed continuously thereafter, 2.96 ml volumes being withdrawn for assay. The electrode cell was water jacketed at 37°C and the buffer was stirred continuously. The oxygen concentration was monitored using a B.B.C. Servogor 120 flat bed recorder with chart speed of $30mm.min^{-1}$. 30 µl of 10% vol/vol methanol solution (BDH) was injected into the chamber and a steady baseline recorded, then 10 µl of a diluted enzyme solution, (typically 100x dilution), was added and the initial rate recorded.

For purified enzyme solutions or dried preparations a pre-incubation step of 20 minutes at 37°C or 10 minutes at 45°C was used to ensure the enzyme had full activity. For enzyme activity estimation during purification where catalase was present, 10 µl of 10.000 $U.ml^{-1}$. bovine liver catalase, (Sigma) was added and the

recorded activity was doubled. (Stoichiometrically catalase produces half a molecule of oxygen per molecule of hydrogen peroxide decomposed, effectively halving the rate of oxygen consumption). Oxidase activity was calculated as follows:-

$$\text{Activity Units ml}^{-1} = \frac{\text{Rate}}{100} \times O_2 \text{ conc}^n \times \text{dilution correction} \times \text{volume correction.}$$

where 100 = Full scale deflection of recorder

Rate = Recorder deflection per minute (slope)

O_2 conc^n = The total oxygen concentration of the buffer in µ moles.

1 unit is defined as the amount of oxygen in µ moles consumed per minute at 37°C at pH 7.0 in phosphate buffer with methanol as substrate. (O_2 concentration at 37°C equivalant to 0.21 µmol.l^{-1}). Changes in buffer composition, temperature, pH and substrate used permit a range of oxidases to be estimated using this method.

2.D.3. Activity Measurement of Catalase.

Catalase activity was estimated spectophotometrically by the method of Aebi (1983). 100mM phosphate buffer pH 7.0, was used containing 160 µl of 30% wt/vol, (100 volume) hydrogen peroxide solution, (BDH) in 100ml. This was incubated at 25°C and 1.0 ml aliquots were pipetted into quartz micro-cuvettes. A Pye Unicam PU 8600 spectrophotometer fitted with a thermostatted cuvette holder and connected via a digital interface to a Phillips PM 8251 chart recorder was used to follow the reaction. The intial absorbance was approximately 0.5 at a wavelength of 240 nm. Diluted enzyme was added, (10 µl) and the initial reaction rate plotted. The catalase concentration was calculated using an extinction coefficient of 39.4 mol.l^{-1}.cm^{-1}. Catalase U.ml^{-1}. $= \frac{\text{Rate}}{39.4} \times \text{Dilution correction} \times \text{Volume correction.}$

2.D.4. Activity Measurement of Diacetyl Reductase.

Liver diacetyl reductase activity was measured at pH 6.1 and the bacterial enzyme at pH 7.0 in 100mM phosphate buffers containing NADH at 0.25mM. The buffer was pre-incubated at 25°C and a 0.98ml volume was pipetted into a quartz micro-cuvette. 10 ul of diluted enzyme was added and a baseline rate was recorded, (same equipment as for catalase assay). Then 10 µl of 5% wt/vol diacetyl in water was added, (5.8mM final conc.) and the initial rate recorded. The activity was calculated from:-

$$\text{Diacetyl Reductase } U.ml^{-1} = \frac{\text{Rate}}{6.20} \times \text{Dilution correction} \times \text{volume correction}$$

Where 6.20 is the millimolar absorption coefficient of NADH and 1 unit is defined as the amount that oxidises 1 µ mole of NADH per minute at pH 6.1 and 25°C with diacetyl as substrate.

2.D.5. Colorimetric Measurement of Oxidase Enzymes.

Colorimetric measurement was carried out using the same equipment as for catalase. The reaction buffers are shown below.

Phosphate buffer pH 7.0 (or other) 100mM,

containing:-

Peroxidase, (horseradish, Sigma or Biozyme)	$2U.ml^{-1}$
4-Aminoantipyrine (Sigma or Aldrich)	0.4mM
Phenol sulphonic acid Na salt (BDH)	25.0mM
or alternatively, 3,5-dichloro 2-hydroxybenzene sulphonic acid Na salt, (Sigma)	10.0mM

The buffer was incubated at 25°C and 3.95ml volumes were pipetted into disposable macro cuvettes. 100 µl of enzyme substrate ($2g.l^{-1}$) was added and a baseline established. The reaction was started with 50 µl of diluted enzyme and the initial rate was measured at 500nm or

520nm for the dichloro derivative.

2.D.6. Quantitative Protein Measurement

The protein concentrations of enzyme solutions were determined using the dye binding assay of Bradford (1976). This is marketed by Bio-Rad Ltd as a stock solution which is diluted 5 fold with distilled water and filtered. This working solution was then placed in a auto-dispenser with a brown glass reservoir, set to deliver 0.9ml aliquots. 100 µl volumes of diluted enzyme solutions were pipetted into disposable micro-cuvettes and 0.9ml of Bradford reagent was added and the cuvette inverted to mix thoroughly. After 5 minutes the absorbance was measured at 595nm and protein concentration was read off a standard curve prepared using BSA standards, (10-80 $\mu g.ml^{-1}$), graph 1.

2.E.1. Substrate Specificity Measurement. Alcohol Oxidase.

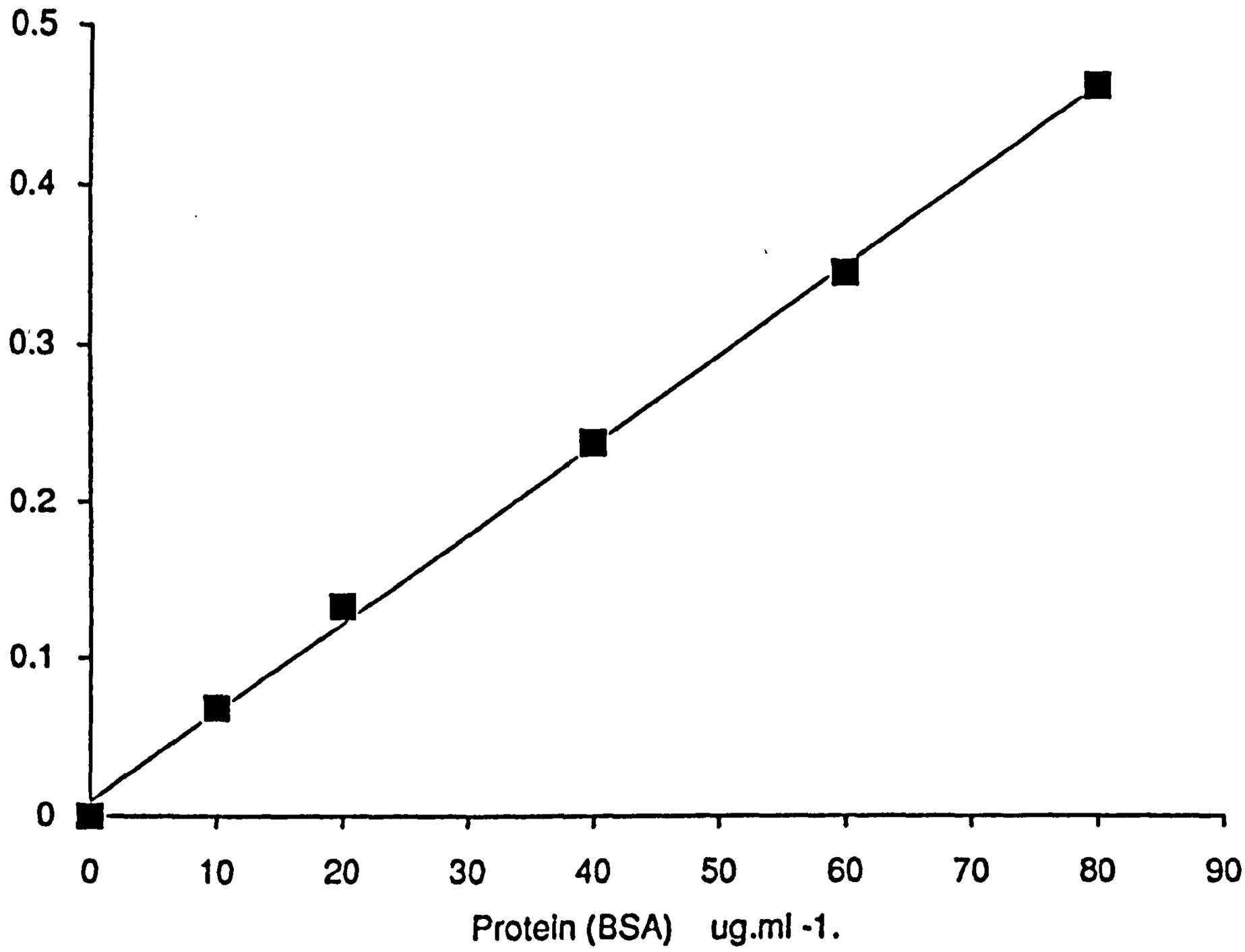
Both the colorimetric assay procedure using phenolsulphonic acid as colour reagent and the Clark electrode oxidase assay were used. In the colorimetric procedure substrate concentration was kept constant at 100 $mg.l^{-1}$.

Assays using formaldehyde as substrate, however, were complicated by the fact that solutions of this compound are stabilised by methanol, which is itself a substrate for the enzyme. A saturated solution of paraformaldehyde, (BDH) in distilled water was therefore prepared, (cloudy solution) which releases free formaldehyde spontaneously. This was used, (30 µl) in the Clark electrode assay to measure the specificity of the enzyme for formaldehyde.

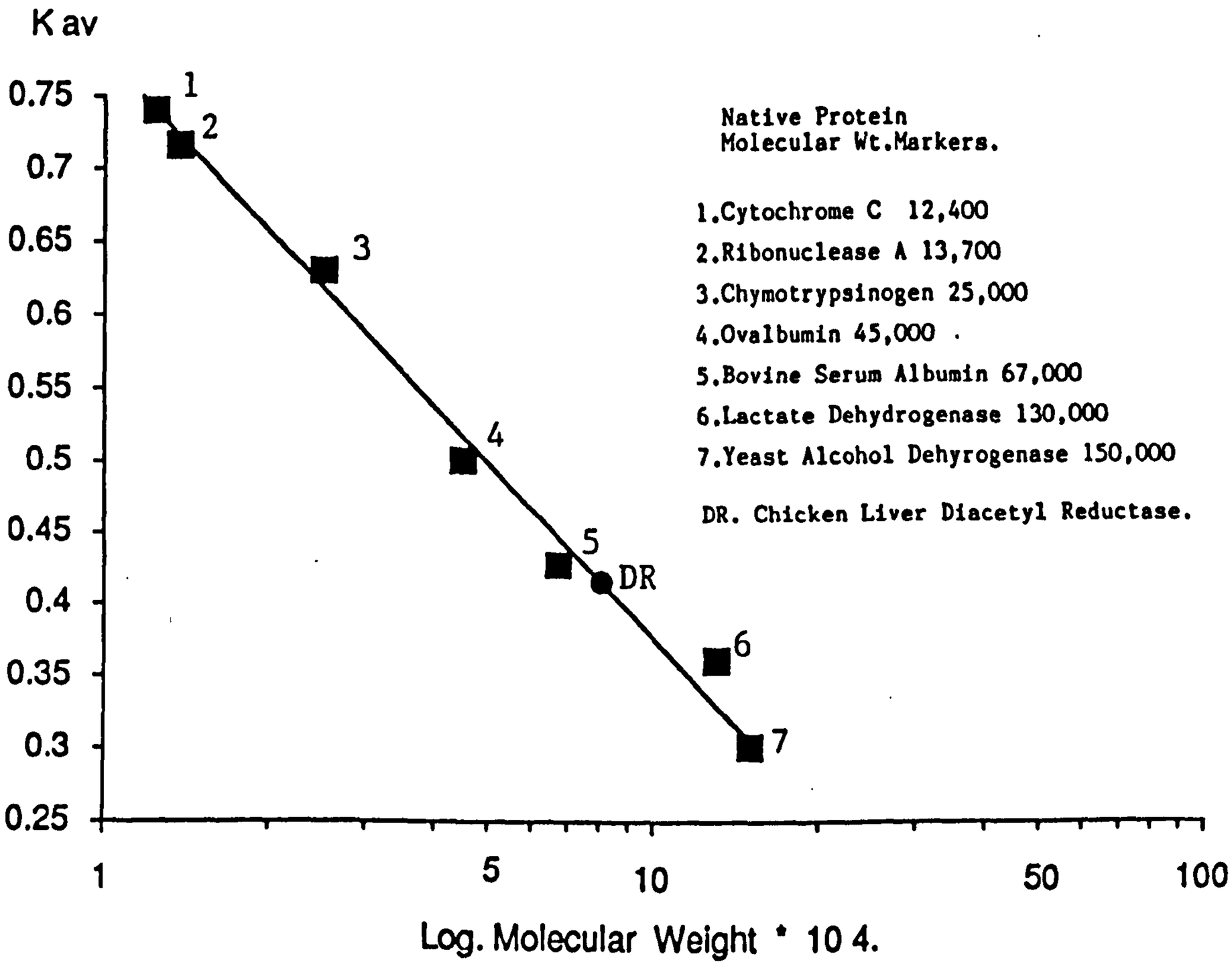
2.E.2. Substrate Specificity Measurement. Diacetyl Reductase.

The standard assay for the enzyme was used to determine substrate specificity keeping NADH, (or NADPH) concentration at 0.25mM and the

Graph 1. PROTEIN STANDARD CURVE.



Graph 2. NATIVE PROTEIN. Molecular Weight Standard Curve.



various substrates tested at 10mM final concentration.

2.E.3. Kinetic Affinity Studies. Diacetyl Reductase.

The affinity of diacetyl reductase to its substrate was measured using the standard assay proceedure with one substrate being varied in concentration. The relative reaction rates were monitored continually using a Beckman DU-50 Spectrophotometer fitted with a Beckman Kinetics Soft-Pac Module and a dot matrix printer. Up to 6 individual reactions could be measured at the same time as the machine was fitted with a 6 place thermostatted cuvette carriage.

NADH and NADPH concentrations were varied between 0.01mM and 0.30mM with diacetyl held constant at 10mM. For the other substrates, NADH was held constant at 0.20mM whilst they were varied between 0.25mM and 55.5mM. Values of V max and Km were estimated from Lineweaver-Burke plots of the results obtained.

2.E.4. Molecular Weight Estimation. Diacetyl Reductase.

Native Protein.

The molecular weight of native diacetyl reductase was measured by Sephadex G-200 gel filtration by the method of Andrews (1981). The column, (16mm x 462mm) had a void volume of 29.1ml measured by blue dextran in 100mM phosphate buffer pH 8.2 at a flow rate of 50μl.min^{-1} at room temperature.

Proteins of known molecular weights were passed down the column as a mixture in the same buffer to produce a standard curve, graph 2. This procedure was repeated several times. Protein peaks were measured by absorbance at 280nm and by activity profiles where appropriate. Diacetyl reductase samples were run in triplicate, and both protein and activity peaks were measured.

Subunits.

Diacetyl reductase subunit molecular weights were measured using SDS gradient slab polyacrylamide gel electrophoresis by the method of Weber and Osborn (1969). SDS gradient gels were poured according to section 2.C.2. using system B and low molecular weight standards, (Pharmacia) were run in duplicate alongside samples to produce a standard curve, graph 3. The procedure was repeated to give an average value for the subunit size.

2.E.5. pH Profile. Diacetyl Reductase from Chicken Liver.

Buffers of pH range 5.0 to 8.25 were prepared, (section 2.D.1) and pre-incubated at 25°C. Standard diacetyl reductase assay conditions were used, (section 2.D.4) with a rate of reaction recorded in duplicate for each buffer used.

2.E.6. Temperature Profile. Diacetyl Reductase from Chicken Liver.

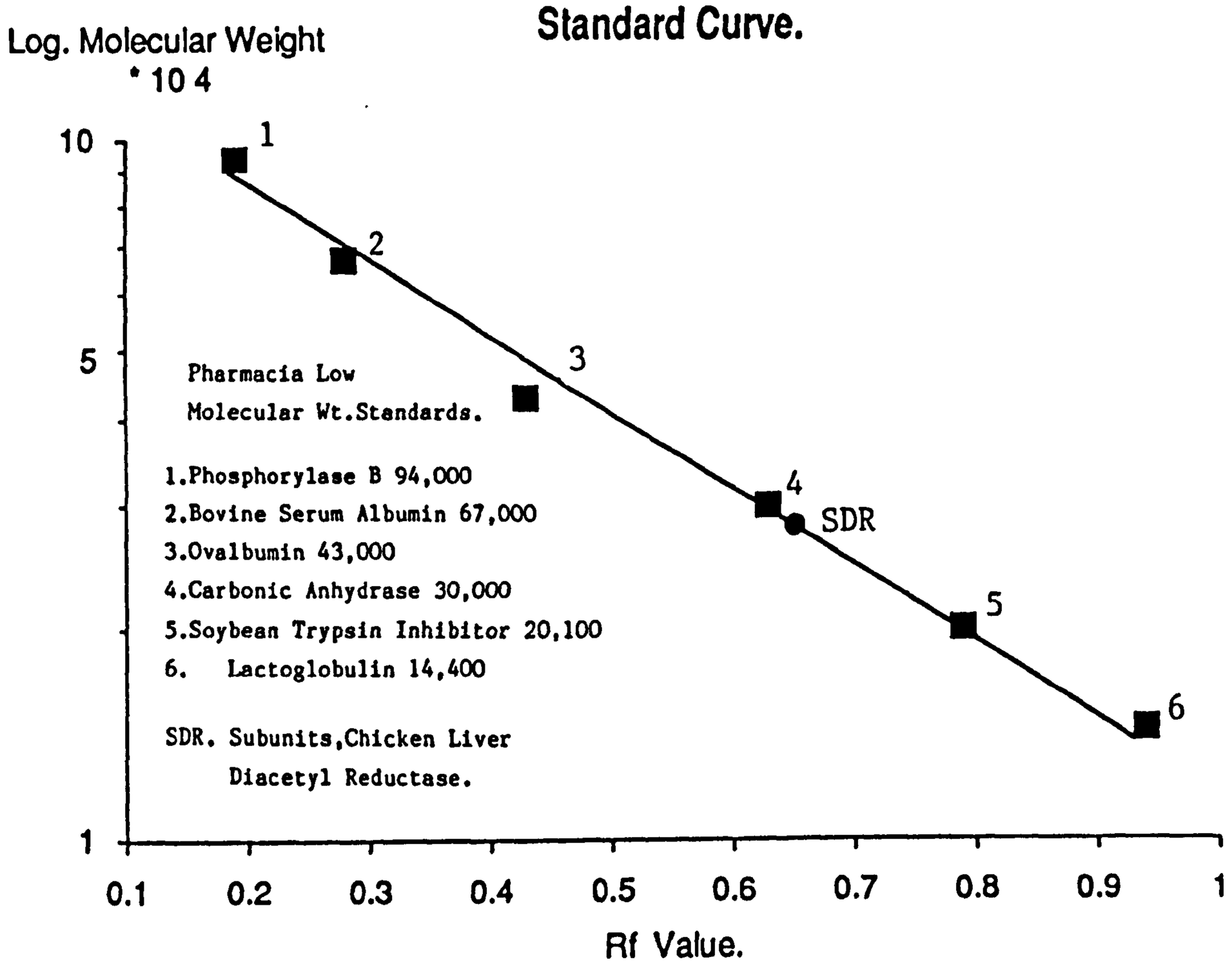
100mM phosphate buffer pH 6.1, was pre-incubated at a set temperature in a water bath. 1.0ml aliquots of buffer were added to 10 - 15 µl of 2 $U.ml^{-1}$ diacetyl reductase and pre-incubated a further 5 min. 10 µl of diacetyl, (580mM) and 20 µl of 12.5mM NADH were added and the reaction rate followed in a thermostatted cell in the spectrophotometer, (section 2.D.3). This procedure was repeated several times at the same temperature to give an average rate of reaction. The complete process was carried out at a range of set temperatures to give a profile of activity in relation to temperature.

2.F.1. Direct Chemical Measurement of Diacetyl.

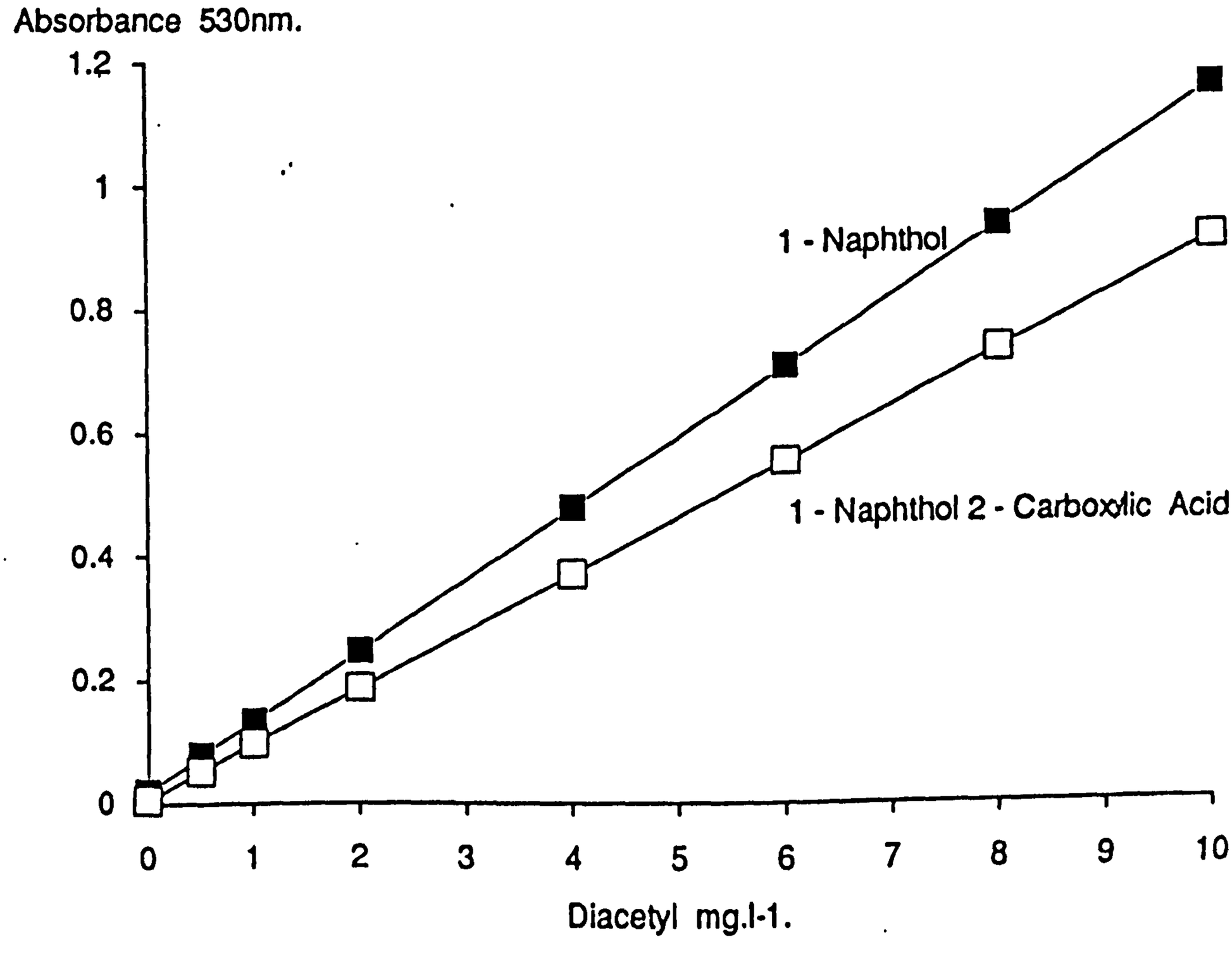
1-Naphthol/Creatine Method.

The method of Westerfield (1945) was adapted to measure diacetyl in aqueous samples. The reagents were as follows:-

Graph 3. PROTEIN SUBUNITS. Molecular Weight Standard Curve.



Graph 4. DIACETYL. 1 - Naphthol / Creatine Method.



(a) Creatine (Sigma) 5.0g

Distilled water up to 100ml

(b) 1-Naphthol (BDH Analar) 1.0g

Sodium hydroxide (BDH Analar) 2.0g

Distilled water up to 20ml

1.0ml of aqueous sample or standard was added to 0.5 ml of (a),followed by 0.5ml of (b). The assays were mixed well and incubated for 10 minutes at room temperature. The absorbance was measured at 530nm against a reagent blank. An alternative reagent was tested by replacing 1-naphthol with 1-naphthol 2-carboxylic acid (Sigma) at the same concentration and with the same method, graph 4.

Girard T Method.

This method was an adaptation of that described by Mitchel and Birnboim (1977) for the estimation of α - dicarbonyl compounds by Girard T reagent, (trimethylamino acetohydrazide chloride). The reaction was carried out at pH 2.9 in formate buffer.

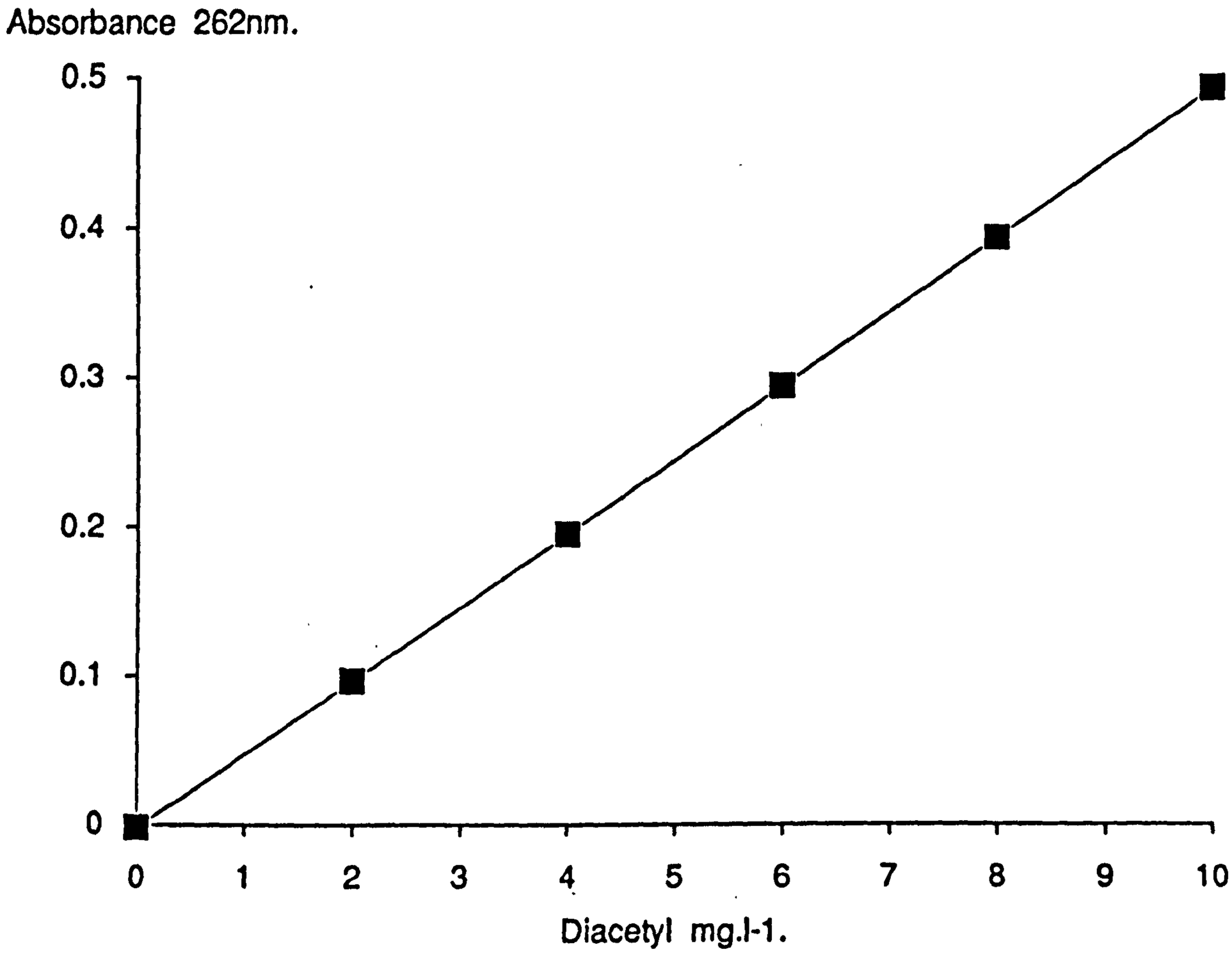
(a) Sodium formate (Sigma) 0.5 M. 3.40g was dissolved in 50 ml distilled water, made to pH 2.9 with 0.5 M formic acid, (BDH,23g.l^{-1}) and made up to total volume of 100ml with distilled water.

(b) Girard T reagent (Sigma) 0.1 M. 1.68g was dissolved in 100ml distilled water.

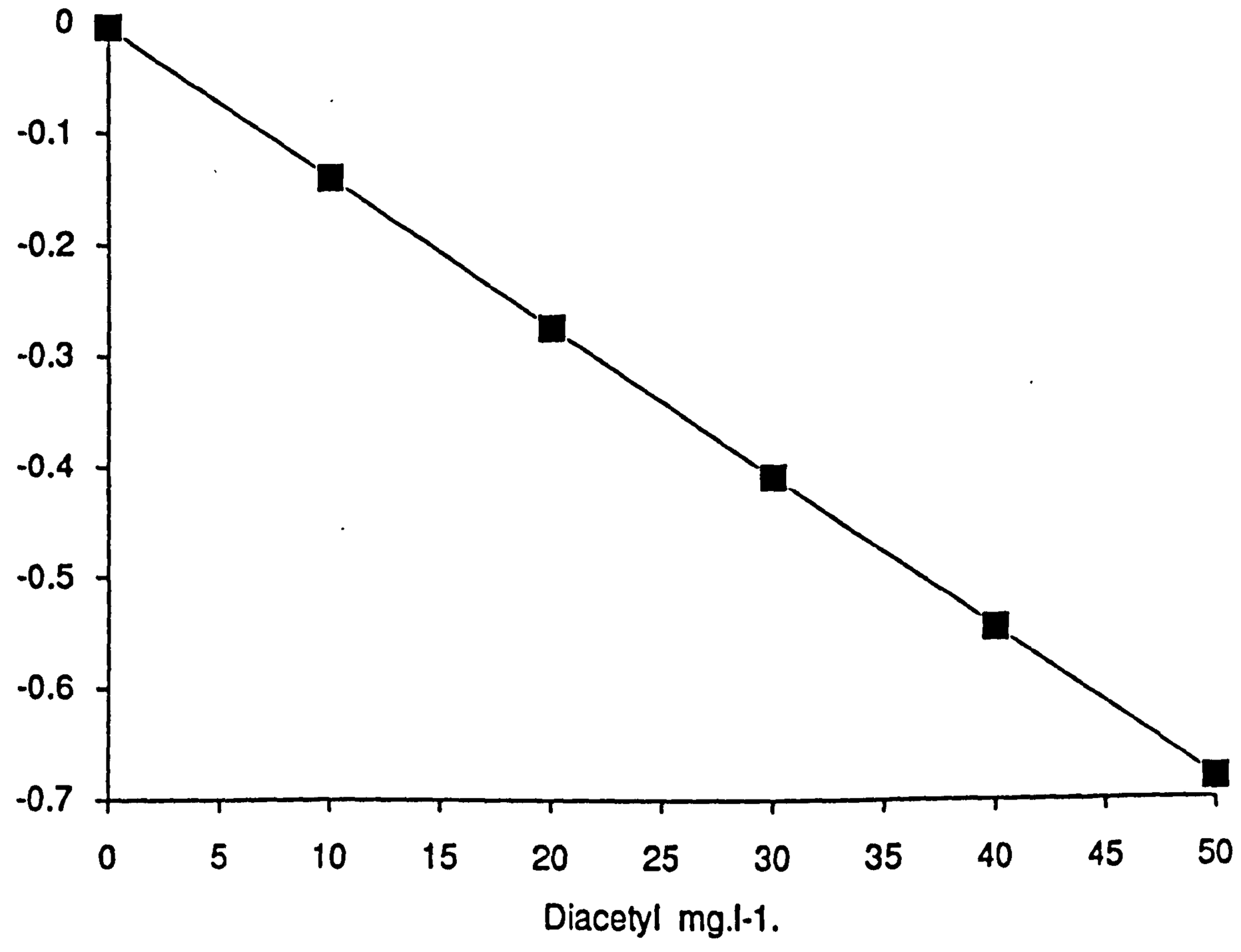
(c) Sodium formate 0.1 M. A 1 in 5 dilution of (a) was made in distilled water.

Method. 600 µl of aqueous sample or standard was added to 200 µl of (a) and 200 µl of (b). The reactants were mixed and incubated for 10 minutes at 30°C. 1.0 ml of (c) was added and the absorbance recorded at 262nm against a reagent blank, graph 5.

Graph 5. DIACETYL. Girard T Method.



Graph 6. DIACETYL. Enzymic Analysis. UV Detection.



2.F.2. Enzymic measurement of Diacetyl.

Diacetyl standards in distilled water were prepared as follows. 5.0g diacetyl was weighed into a 100ml volumetric flask and dissolved in distilled water. This stock solution was stable for 3 months at 4°C.

Dilutions were made into distilled water using volumetric flasks and bulb pipettes. Concentrations of 2-50 $mg.l^{-1}$ were made up fresh each week and stored at 4°C. Lower concentrations, up to 2.0 $mg.l^{-1}$ were made up fresh on the day and discarded at the end of the experiment.

(a) Chicken liver diacetyl reductase preparations were made up at 10 $U.ml^{-1}$ in 5.0 mM phosphate buffer pH 8.2, and stored at -70°C in 100 µl aliquots.

(b) NADH 1.53 $mg.ml^{-1}$ (2mM) was made up fresh in 1.0M phosphate buffer pH 6.1.

(c) NADH 9.55 $mg.ml^{-1}$ (12.5mM) was made up fresh in 1.0M phosphate buffer pH 6.1.

(d) NADH 1.91 $mg.ml^{-1}$ (2.5mM) was made up fresh in 250mM phosphate buffer pH 7.1.

Diacetyl standards or samples were measured by three methods.

(1) 200 µl standard or sample was added to 14 µl of (a), 100 µl of (b) and 400 µl distilled water. The assays were incubated at 37°C for 20 minutes in capped tubes and the residual NADH determined by the U.V. method, (section 2.F.3(i)) against a reagent blank, (omit NADH).

(2) 100 µl standard or sample was added to 10 µl of (c) and 10 µl of (a). Incubation as in (1). The residual NADH was measured using formazan, (section 2.F.3.iii) or meldola blue, (section

2.F.3.iv).

(3) 100 µl of standard or sample was added to 50 µl of (d) and 5 µl of (a). Incubation as in (1). Residual NADH was measured using meldola blue, (section 2.F.3.iv.).

Recycling techniques are different in the respect that NAD production is measured rather than residual NADH. See section 2.F.4.

2.F.3. Measurement of NAD(P)H in Enzyme Methods.

(i) U.V. Method.

Direct detection of NAD(P)H was carried out at 340nm using quartz cuvettes in a Pye Unicam or Beckman DU-50 spectrophotometer. Absorbance was linearly related to residual concentration over the range of diacetyl concentration used, graph 6.

(ii) Iron II formation and detection with 2,2'-Dipyridyl and Ferrozine.

NAD(P)H will reduce Iron III to Iron II in the presence of electron coupling agents such as phenazine methosulphate, (PMS). The final concentration of Iron II in the solution may then be detected using complexing reagents such as 2,2'-dipyridyl, (Whitaker 1969) or ferrozine, (Stooky 1970, Ceriotti et al 1980).

(a) Ferric ammonium sulphate $24H_20$. (Sigma) (i) 482.1 mg l^{-1} ; (ii) 1.21g l^{-1} in 1.0% v/v acetic acid in deionised water.

(b) PMS. (Sigma) 153.1 mg l^{-1} in deionised water, stored at 4°C.

(c) 2,2'-Dipyridyl (Sigma) 1.0 $g.l^{-1}$ in 3% v/v acetic acid in deionsed water.

(d) Ferrozine (Sigma) 1.5 $g.l^{-1}$ in 1% acetic acid in deionised water.

(e) Sodium fluoride (BDH) 1.5$g.l^{-1}$ in 3% v/v hydrogen peroxide

(BDH) in deionised water.

(f) Hydrochloric Acid (2M) 205.5 ml concentrated HCl to 1 litre deionised water.

Dipyridyl reagent was made up by adding equal volumes of a(i), (b) and (c) just before use. Ferrozine reagent was made up by adding equal volumes of a(ii), (b) and (d) just before use.

NADH, (sample volume 0.5ml) was measured by adding 2.0ml of dipyridyl or ferrozine reagent to dilutions in phosphate buffer pH6.1 or 7.0, reacting for 1 minute and then adding either sodium fluoride reagent, 0.5ml for the dipyridyl assays or HCl, 0.5ml to the ferrozine assays. Absorbance was measured at 520nm for the dipyridyl reagent or 560nm for the ferrozine reagent against reagent blanks. Residual NADH in enzyme assays was measured by the same procedure, graphs 7a and 7b.

(iii) Detection with Formazans.

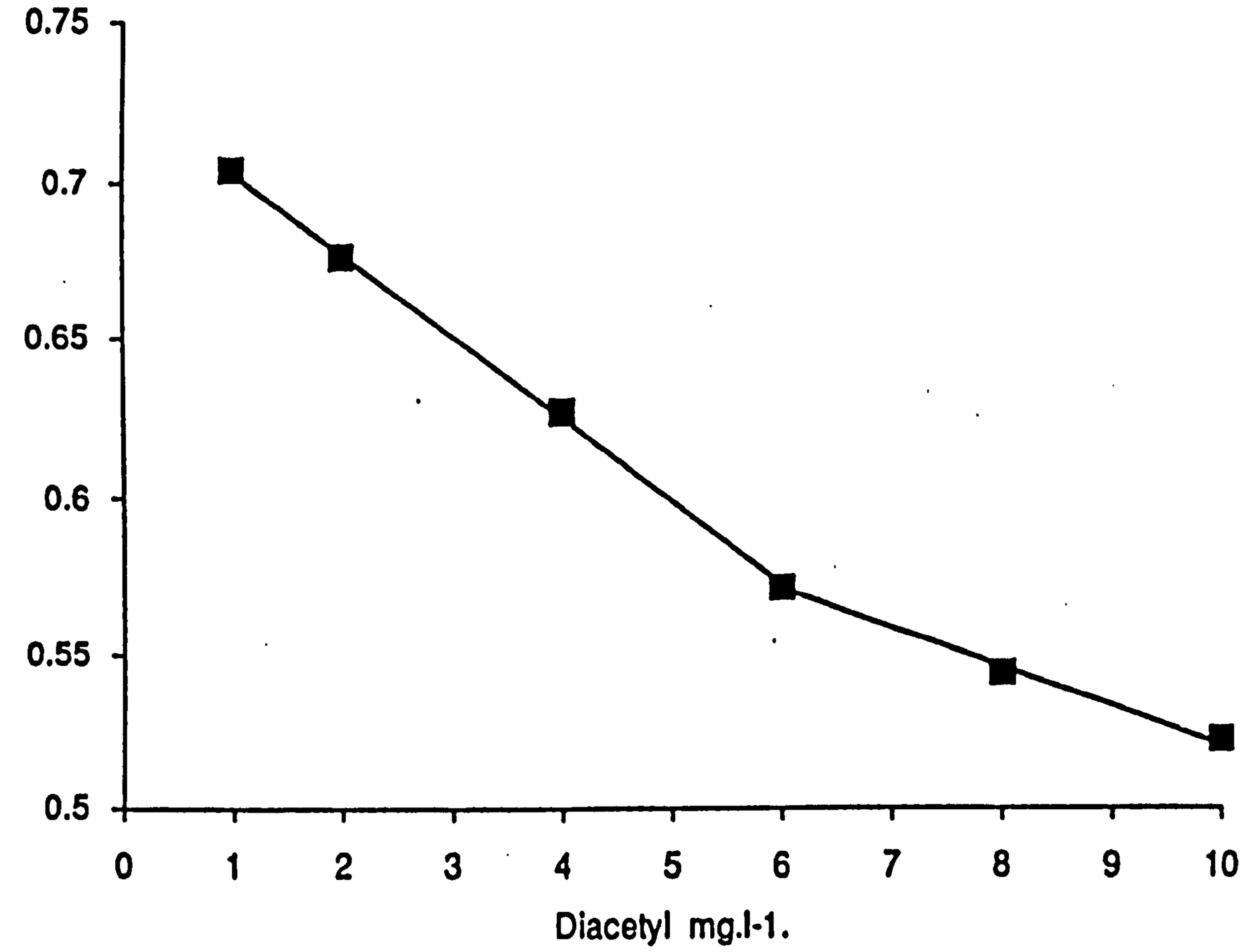
The method of Whitaker (1969) was used.

(a) Tetrazolium salt, (INT, nitroblue or MTT), (Sigma) 800mg l^{-1}, PMS, (Sigma) 200mg.l^{-1} in 1% v/v tween 20, (Sigma) in distilled water, stored at 4°C in a foil covered bottle.

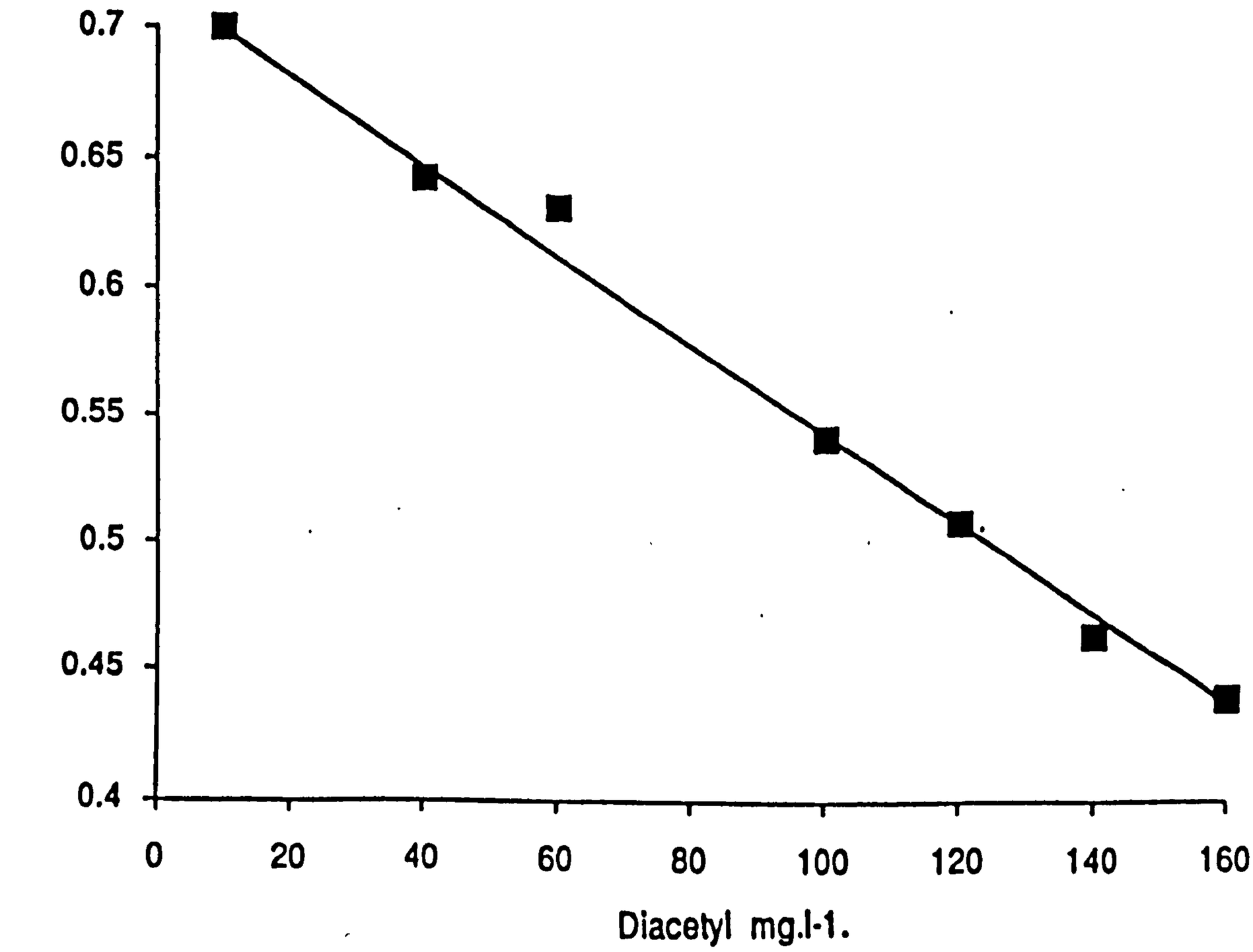
(b) Potassium hydrogen phthalate, (BDH) 10.21g.l^{-1} , 204ml 0.1M HCl in 1% v/v tween 20 in distilled water, (pH 3.0).

NADH, (sample volume 0.5ml) was estimated by adding 0.5ml of (a), reacting 1 minute then adding 1.5ml of (b). The absorbance was measured at 500 nm (INT) 540 nm (nitroblue) or 580 nm (MTT), against reagent blanks, an example is given in graph 8. Residual NADH in enzyme assays was measured by the same procedure.

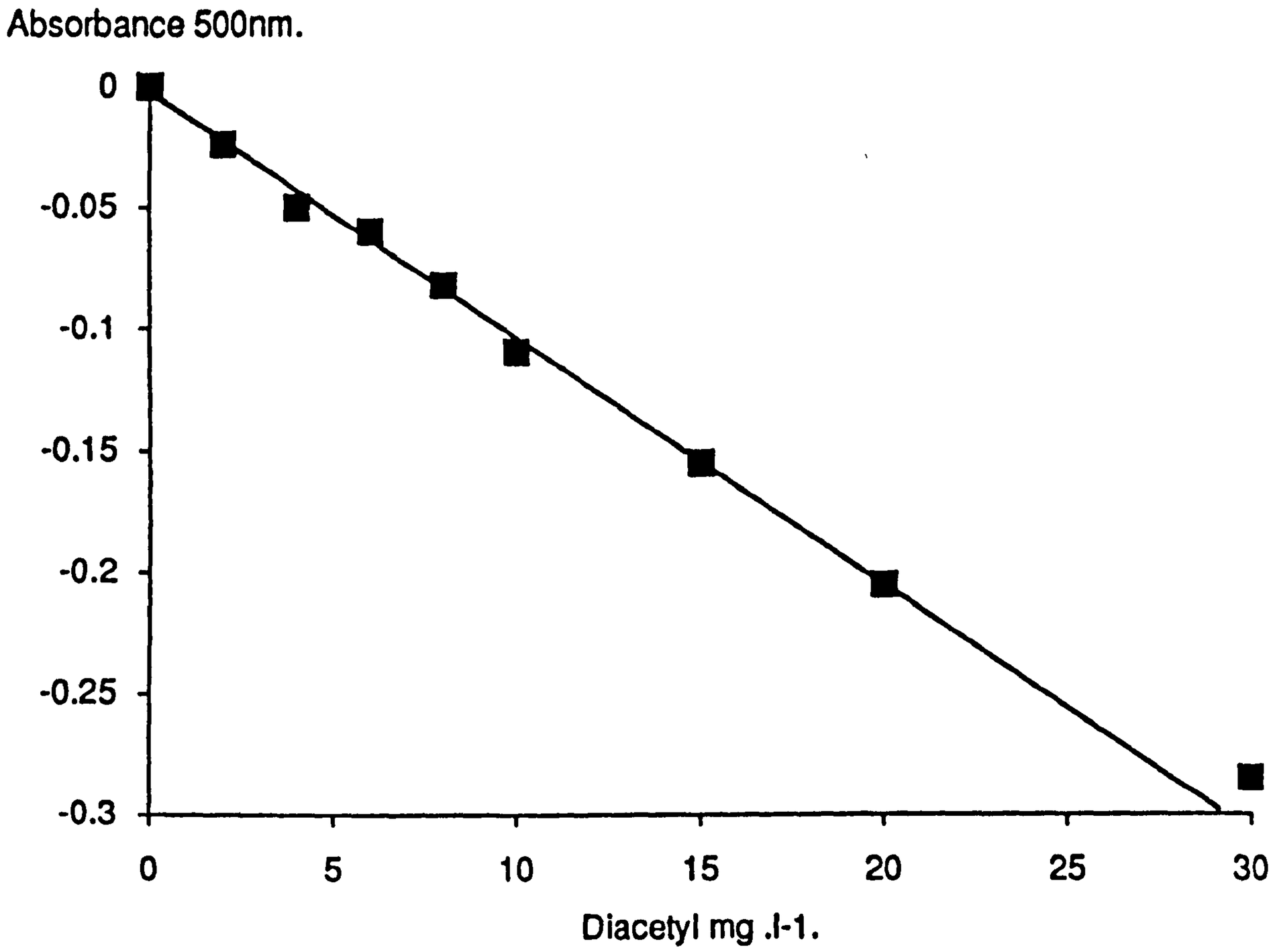
Graph 7a. DIACETYL. Ferrozine Method.



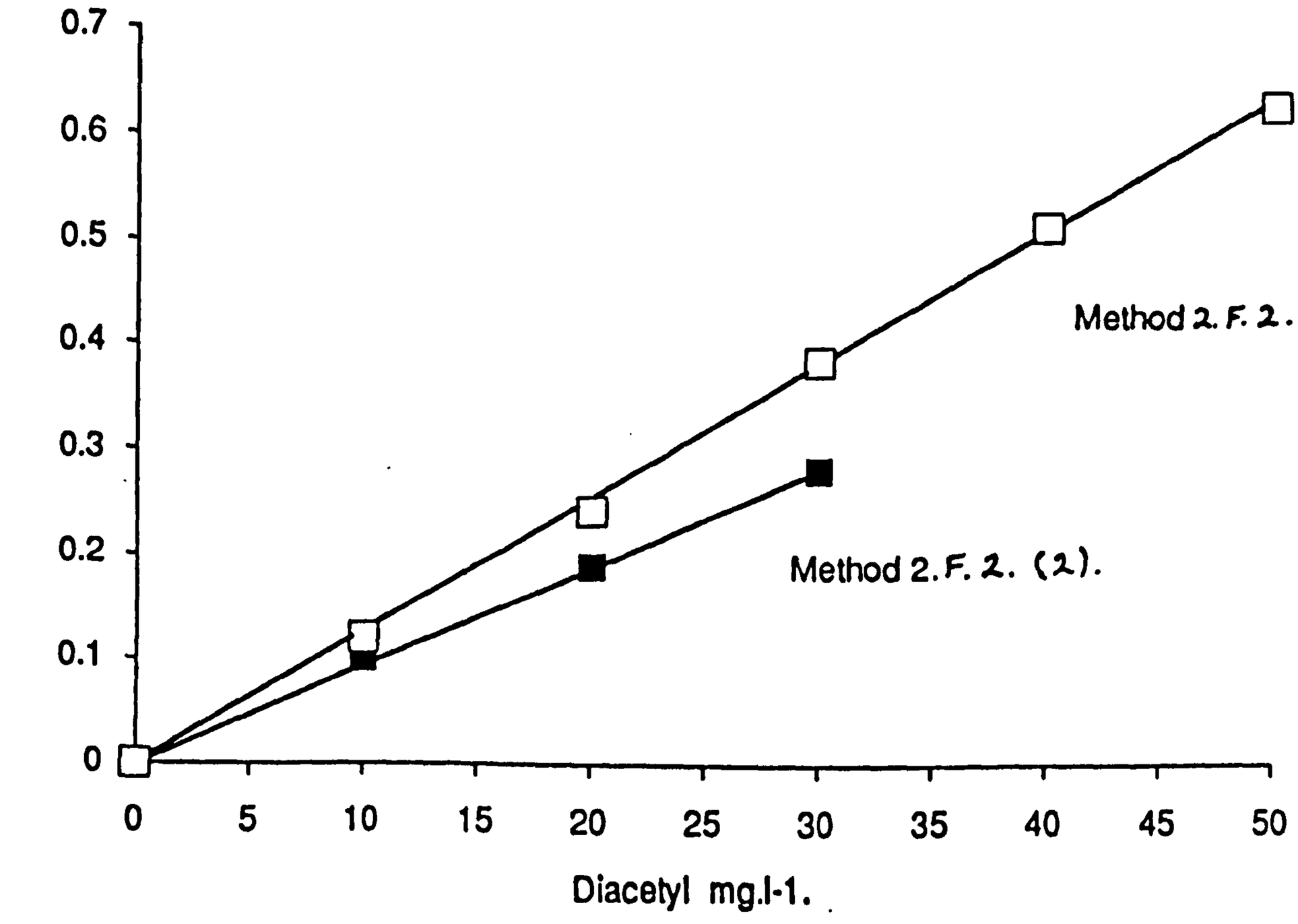
Graph 7b. DIACETYL. 2 , 2' Dipyridyl Method.



Graph 8. DIACETYL. Enzyme Analysis. Formazan Detection. (INT).



Graph 9. DIACETYL. Enzyme Analysis. Meldola Blue Detection.



(iv) Meldola Blue Decolourisation.

This method was adapted from that of Orssoneau et al (1982).

(a) Meldola blue 3.11g l^{-1} (10mM) in distilled water.

(b) Citric acid (BDH) 42.0 $g.l^{-1}$ (200mM) in 3.75% w/v triton X-100 (BDH) in distilled water.

NADH was measured by adding 1.5 ml of (a) to 98.5 ml of (b), and mixing thoroughly. To 0.5ml volumes of standard or sample 1.0ml of the mixed reagent was added. The mixed solution was allowed to stand for 2 minutes and the absorbance was measured at 565nm against a water blank, graph 9.

2.F.4. Enzyme Recycling of NADH.

The single enzyme methods of Rasmusson et al (1972) and Kovar et al (1984) were used, with the modification of using guaiacol sulphonic acid as coupling agent as opposed to salicaldehyde.

(a) 4-Nitroso N,N-dimethylaniline (Sigma) 44 mg was dissolved in 1.0ml 95% ethanol and added to 1.86ml cyclohexanol (Sigma). This stock solution was stored in the dark, stability 2 weeks.

(b) Guaiacol sulphonic acid, K salt. (Sigma) 363.4 $mg.l^{-1}$ in 250 mM tris/HCl buffer , pH 8.8.

(c) Liver alcohol dehydrogenase (equine) 10 $units.ml^{-1}$ in 50% v/v glycerol in 250mM tris/HCl buffer, pH 8.8. Store at -20°C.

(d) NADH for use as standards and substrate was made up according to the method of Payne et al (1982). 3.82 mg NADH in 1.0ml carbonate buffer, pH 10.6 were sub-aliquotted, (200 µl) and stored at -70°C. The reagent was stable for 3 weeks.

(e) For use, an aliquot was heated for 20 minutes on a boiling water bath and then diluted 1 in 10 immediately prior to use in 0.5 M phosphate buffer, pH 7.1.

(f) Concentrated HCl (205.5 ml) was diluted to 1.0 l with distilled water.

Diacetyl samples or standards were measured by the following method:- Samples or standards, 50 µl were added to 50 µl of (e) and 2 µl diacetyl reductase, (section 2.F.2, reagent a). Incubation was carried out at 37°C in capped tubes for 20 minutes. 20 µl of (f) was added and the reagents were completely mixed, (this step is very important) and incubated at 37°C for a further 5 minutes. Working recycle buffer prepared from 236 ul of (a), 100 ml of (b) and 200 µl of (c) was pre-incubated at 37°C for 5 minutes. 1.0 ml of this was added to the reactants and incubated for 10 minutes at 37°C. The tubes were then cooled on ice and the absorbance measured at 680nm against a reagent blank within 2 minutes, graph 10. Alternatively the reaction could be stopped by adding NaOH, (100 µl, 2.5M) and reading the absorbance within 5 minutes.

2.F.5. Measurement of Hydrogen Peroxide.

(i) Redox dyes.

ABTS was used to detect hydrogen peroxide produced by oxidase reactions, particularly at low substrate concentrations. Also sodium diphenylamine sulphonate was tested as a single reagent for glucose estimation after Morin and Prox (1973).

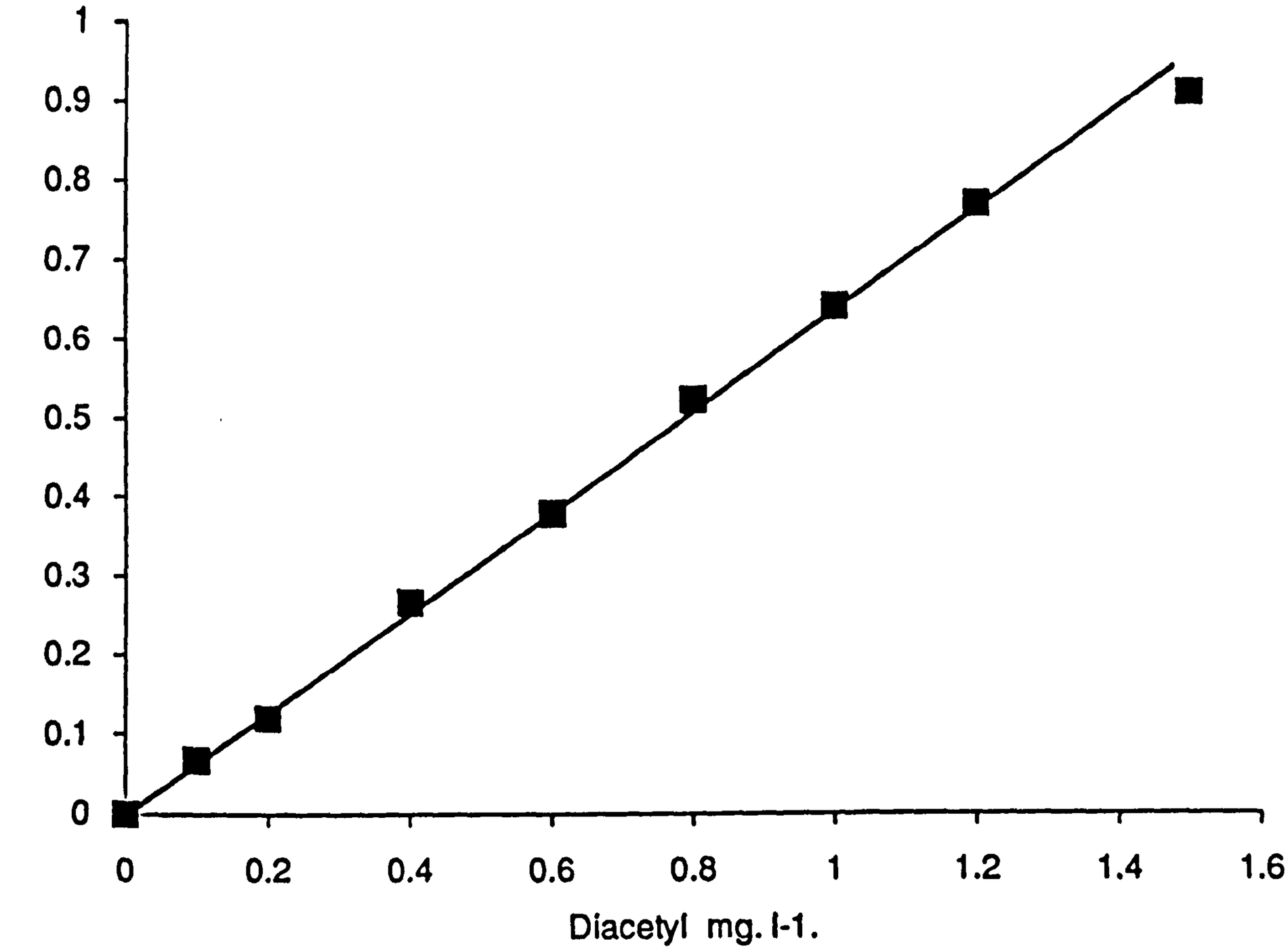
(a) ABTS ammonium salt (Sigma) 1mg.ml^{-1} dissolved in phosphate buffer, (various pH's).

(b) Peroxidase 4000 units.ml^{-1} in 100mM phosphate buffer pH 7.0.

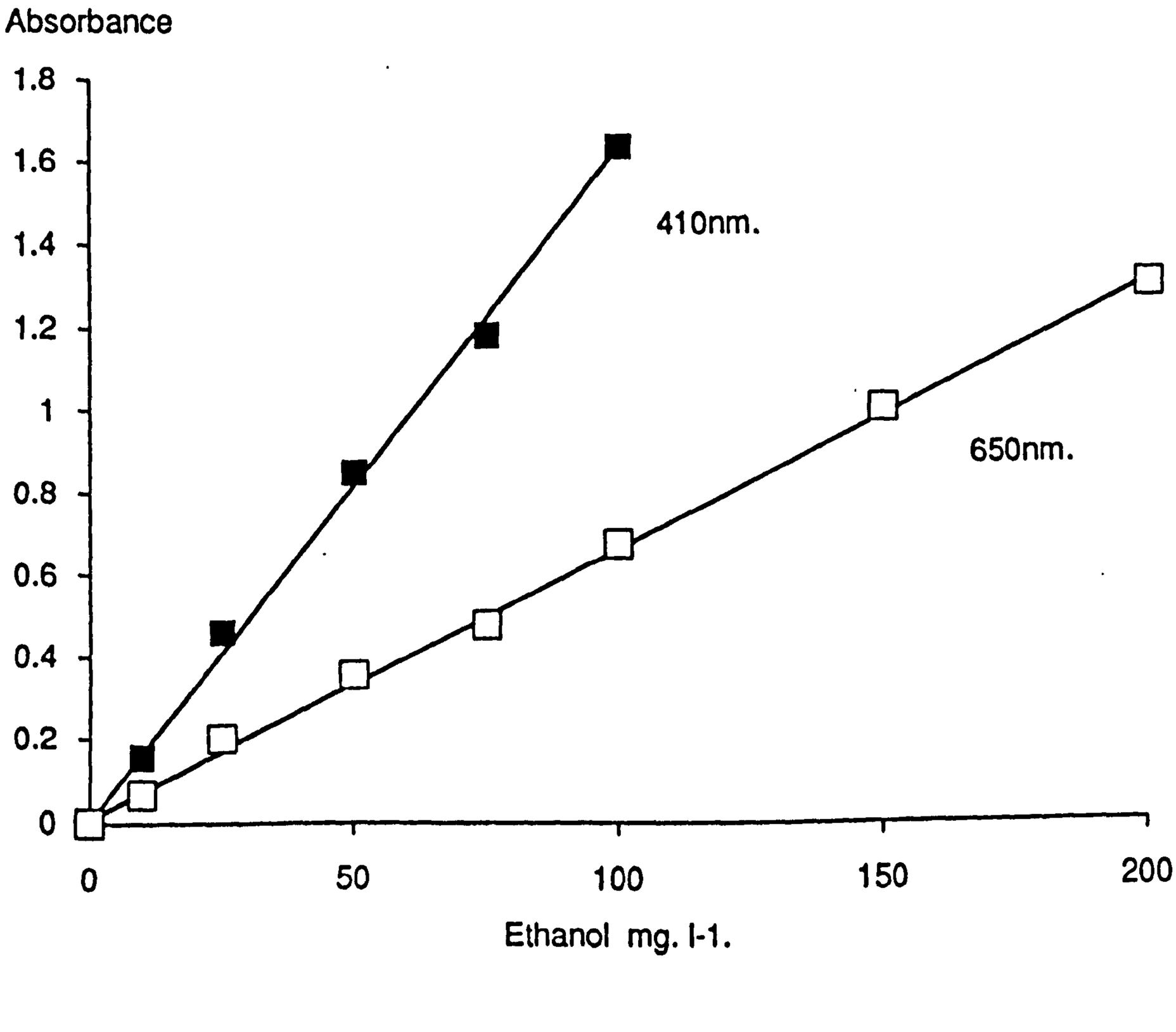
(c) Alcohol oxidase. Concentration dependent on preparation.

100ml of (a) was mixed with 60 µl of (b) and sufficient (c) to give between 2 and 5 units alcohol oxidase.ml^{-1}. 1.0ml aliquots were incubated for 30 minutes at room temperature with 50 µl of sample.

Graph 10. DIACETYL. Enzyme Recycling Assay.



Graph 11. ETHANOL. ABTS Detection.



Absorbance was measured at 410 or 650nm against a reagent blank, graph 11.

(d) Sodium diphenylamine sulphonate 0.9mg $.ml^{-1}$ in 100mM citrate buffer, pH 5.5 containing 48U$.ml^{-1}$ peroxidase and 11.4U$.ml^{-1}$ glucose oxidase.

50 µl standard or sample was added to 2.5ml reagent (d) and incubated for 2 minutes at room temperature. Absorbance was measured at 470 nm against a reagent blank, graph 12.

(ii) Condensation Reactions.

Many Reactions involving condensation of reagents to form dyes have been tested in oxidative systems. (Appendices II and III). In practice for "wet" enzyme methods only 4-aminoantipyrine was used with various different colour coupling reagents. MBTH and substituted phenylene diamines were used in dry systems, (see chapter 6).

(a) 4-Aminoantipyrine 40mM in distilled water. Stored at 4°C in dark bottle, stable 3 months.

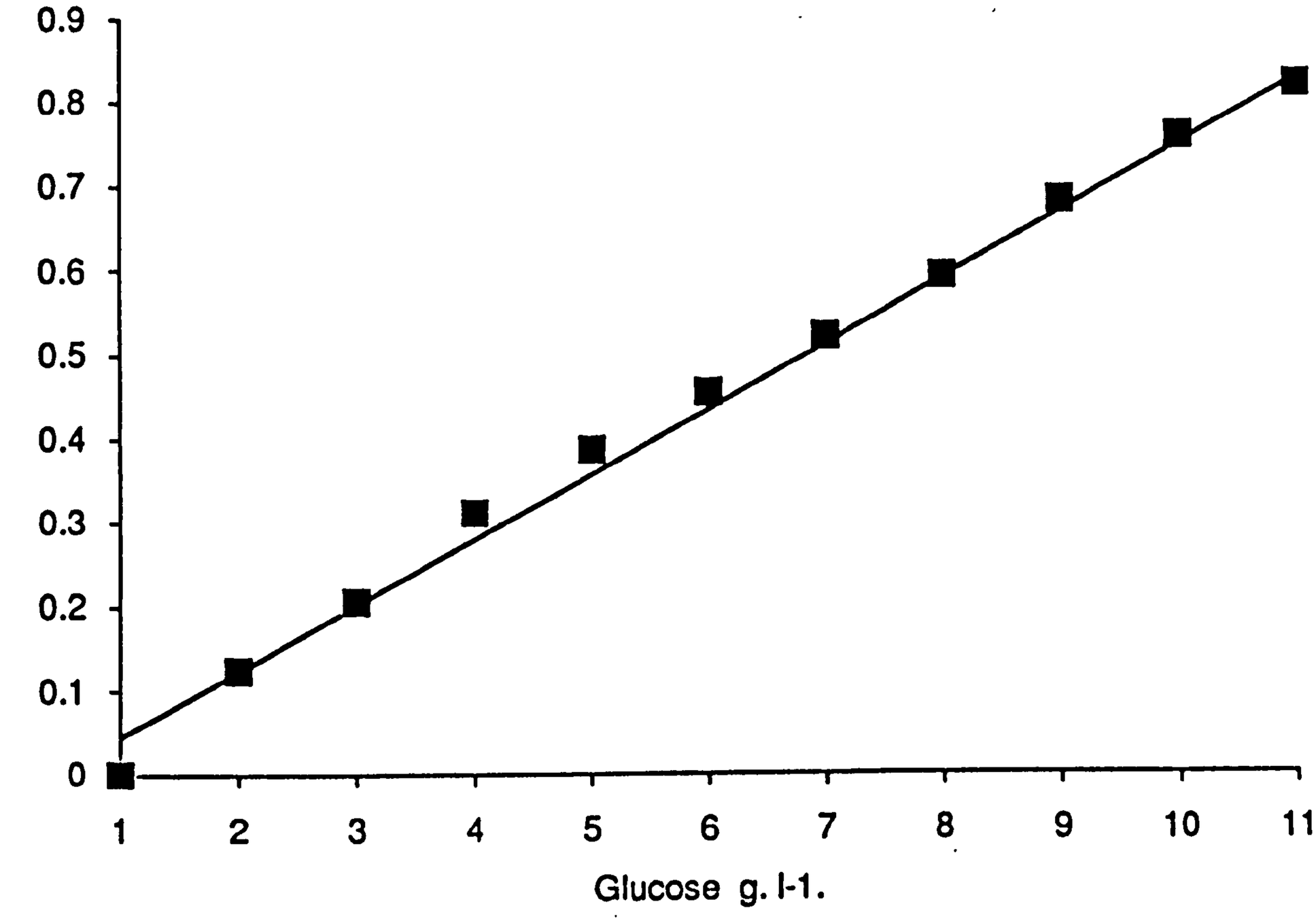
(b) Peroxidase 4000U$.ml^{-1}$ in 100mM phosphate buffer pH 7.0.

(c) Colour coupler (vary) 25mM in buffer (vary).

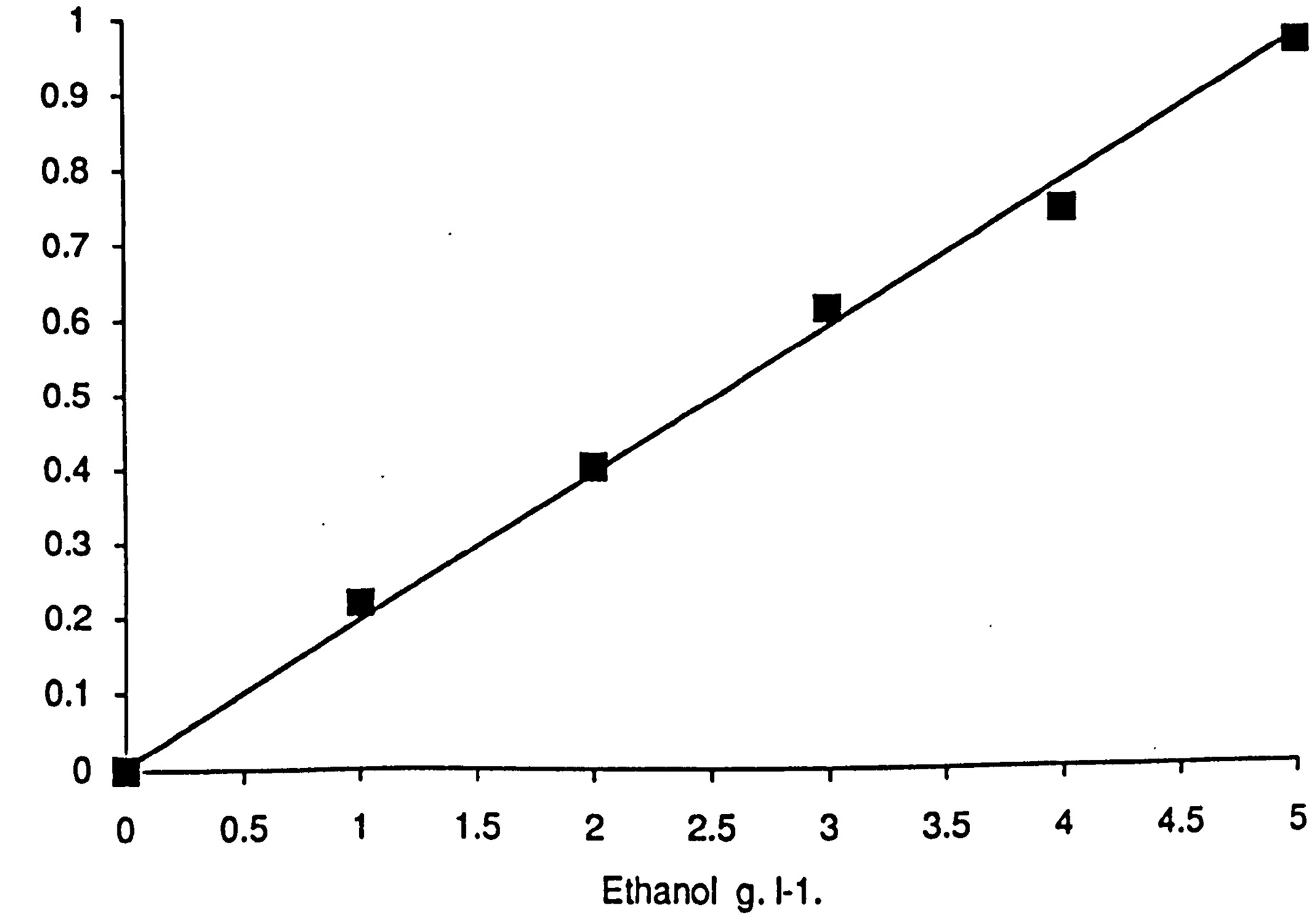
(d) Oxidase enzyme. (Typically 500U$.ml^{-1}$).

e.g. Alcohol reagent. 1.0ml of (a), 80 µl of (b) and 50-200 µl alcohol oxidase (d) were mixed with 99ml of (c), (phenolsulphonic acid sodium salt in 100mM phosphate buffer, pH 7.0). 100 µl of sample or standard was added to 4ml reagent. Absorbance was then measured after 5 minutes, (concentration range 1.0 - 5.0g$.l^{-1}$ ethanol) at room temperature or after 30 minutes at 30°C, (concentration range 0 - 1.0g$.l^{-1}$ ethanol) at 500nm against a reagent blank, graphs 13 and 14.

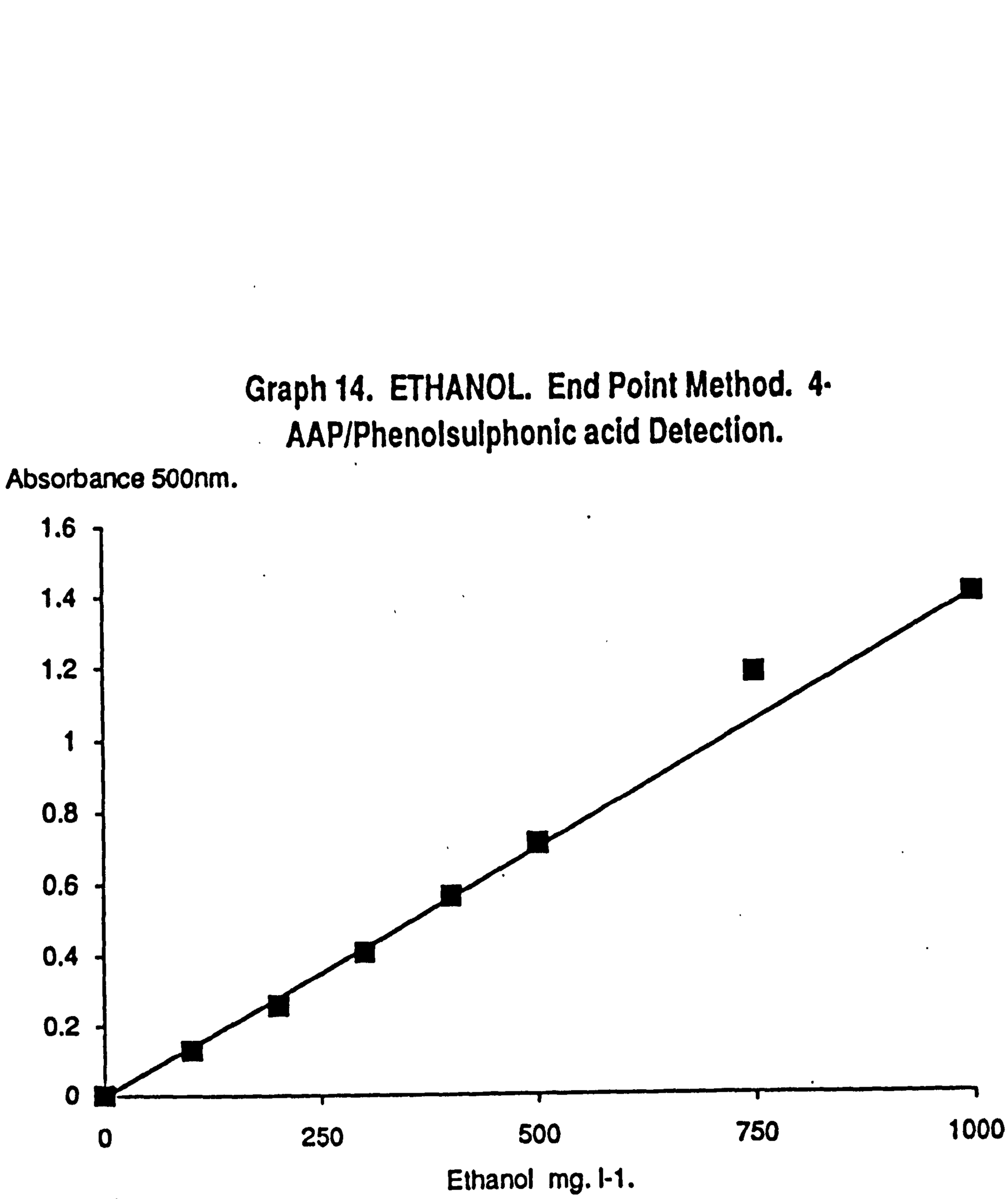
Graph 12. GLUCOSE. Diphenylamine Sulphonate Detection.



Graph 13. ETHANOL. Rapid Kinetic Method. 4-AAP/Phenolsulphonic Acid Detection.



Graph 14. ETHANOL. End Point Method. 4-AAP/Phenolsulphonic acid Detection.



(iii) Electrochemical Detection.

Peroxide probes , (Yellow Springs Instruments Inc.) were used to measure hydrogen peroxide concentration directly. Proprietary buffer salts were used with alcohol and glucose being detected in a flow system using immobilised enzyme coils, (sections 2.I.2 and 2.I.4).

YSI 2357 Buffer.

Sodium dihydrogen orthphosphate	0.76g.
Disodium hydrogen orthophosphate	3.47g.
Sodium chloride	1.37g.
EDTA dipotassuim salt. $2H_2O$	0.28g.
Sodium benzoate	0.46g.
Gentamycin sulphate	0.004g

The buffer salts were supplied dehydrated in sachet form and were dissolved in 450ml of distilled water before use.

2.G.1. Immobilisation Procedures. Nylon Supports.

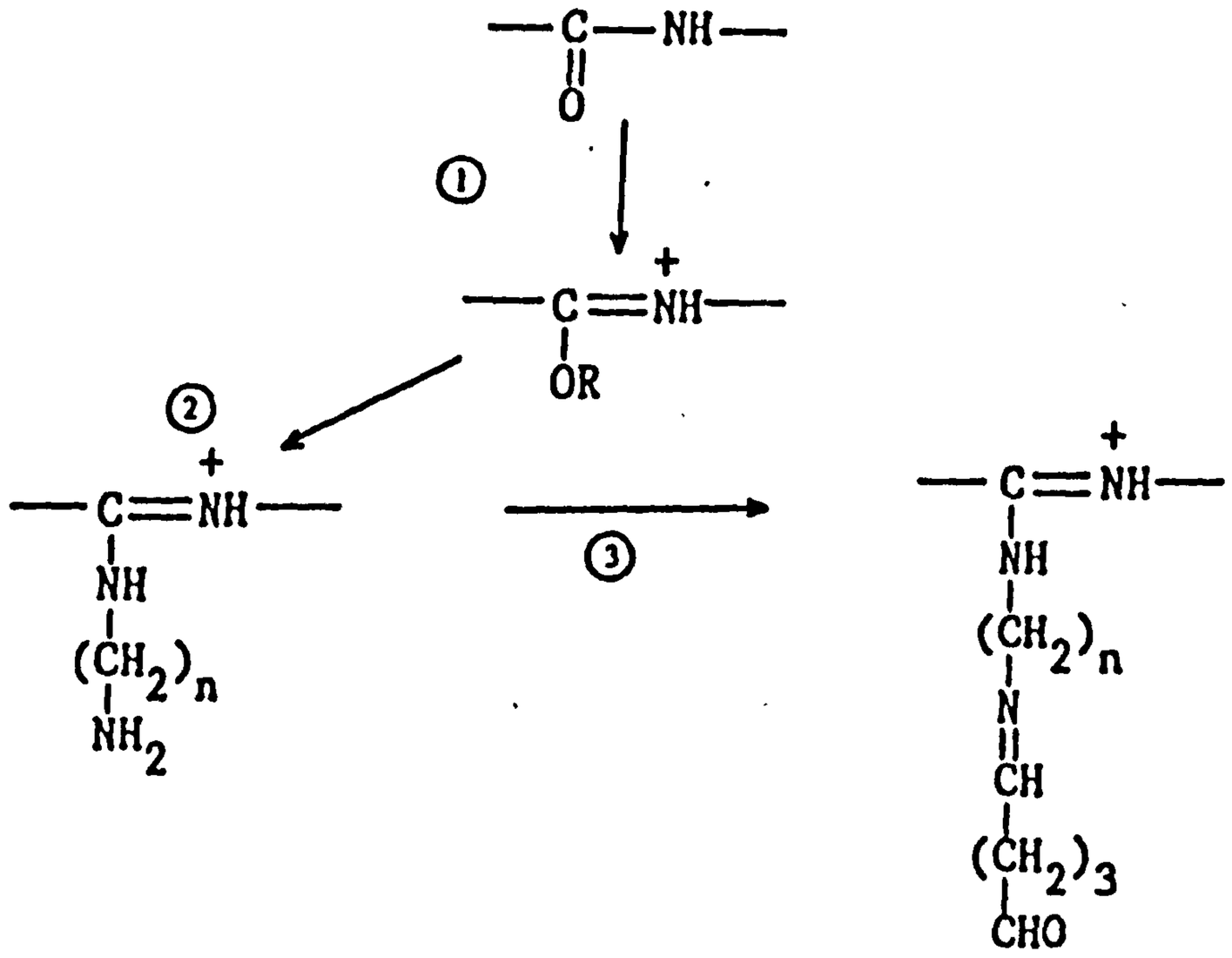
Nylon 6 tubing, (Portex) with internal diameters of 1.0mm and 2.0mm was used. Coils were produced by wrapping the tubing around a glass rod or tube as a former and holding in place by tightly wrapping autoclave tape along the whole length of tubing. Heating to 100°C in a boiling water bath for 10 minutes, with subsequent cooling under running cold water for 5 minutes, gave helical coils that retained their shape at temperatures up to 55°C. These could be supported using a central core of cardboard or expanded polystyrene before incorporation into a Technicon AutoAnalyzer system.

Derivatisation and Immobilisation of Enzymes.

This was carried out by the method of Morris et al (1975). The reaction sequence is shown in fig.9.

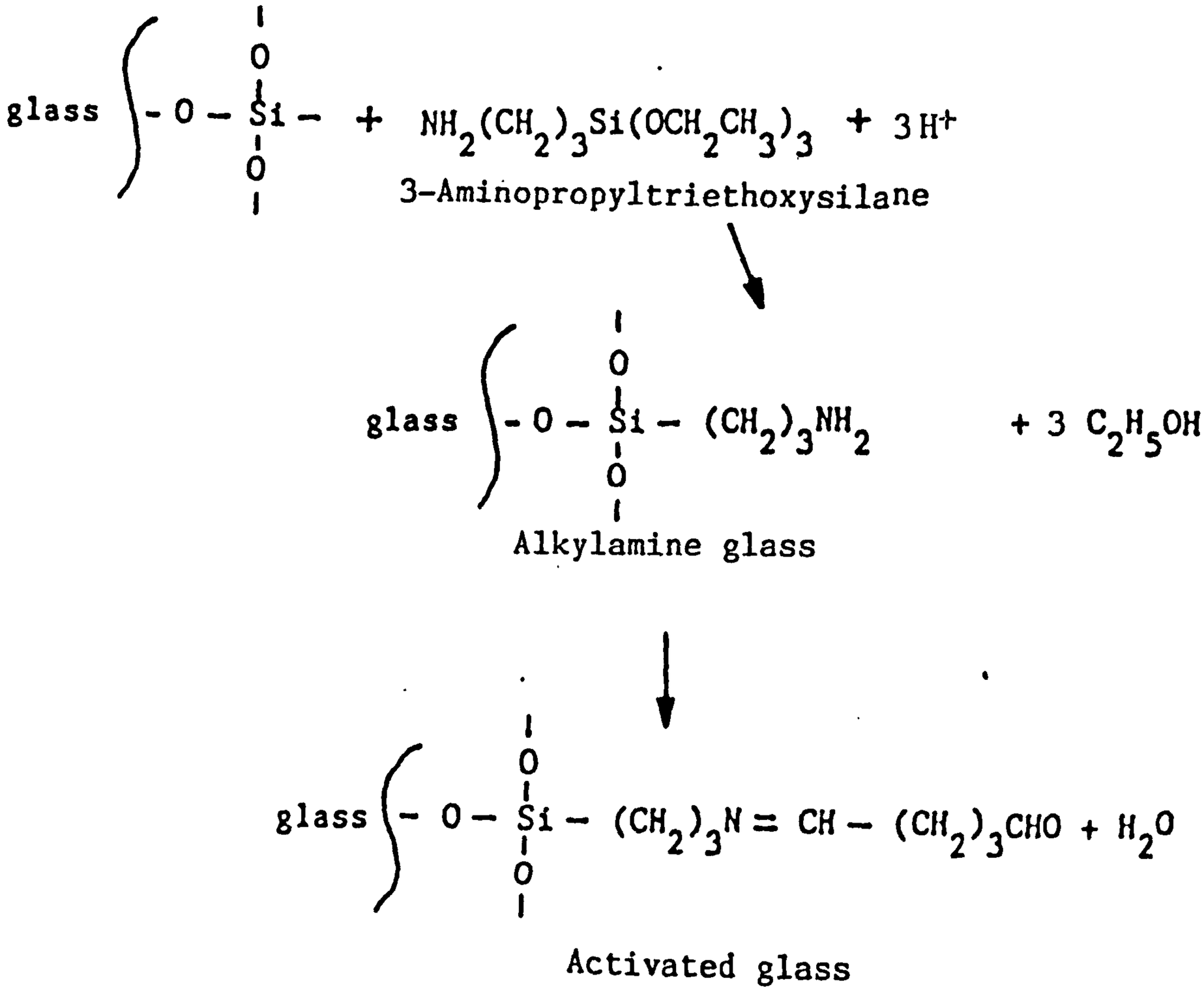
Triethyloxonium tetrafluoroborate (Fluka) 12.5% w/v in

FIG.9 NYLON ACTIVATION. REACTION SEQUENCE.



Reagents. 1.Triethyloxonium Tetrafluoroborate.
2.1,n Diamino-Alkane. Other spacers include,
3.Glutaraldehyde. Adipic acid dihydrazide.
Polyamine dextran.
Pectinamine.

FIG.10 CONTROLLED PORE GLASS ACTIVATION. REACTION SEQUENCE.



3-Aminopropyltri e thoxysilane reacts with silane groups on the glass surface to give alkylamine glass,which is then activated using glutaraldehyde.

dichloromethane (Fluka) was pumped into dry, formed nylon tubing and incubated at 25°C for 15 minutes. The tubing was washed with dichloromethane, (10-15ml) and filled with one of a variety of activating compounds, table 9.

Table 9. Activating Compounds for Nylon Tube Reactors.

Diaminoethane (Sigma) Undiluted.
Adipic acid dihydrazide (Fluka) 3% w/v in formamide (Sigma).
Polyamine dextran, (section 2.M.4.) 8% w/v in 200mM borate buffer, pH 8.5.
Pectinamine, (section 2.M.6.) 2% w/v in distilled water.

After incubation for 2 hours at room temperature the tubes were washed exhaustively with distilled water and perfused at 1.0ml.min^{-1} with 5% v/v glutaraldehyde solution, (Agar Aids) in 200mM borate buffer, pH 8.5 for 15 minutes. The tubes were then washed with borate buffer containing 150mM sodium chloride at 3.0ml.min^{-1} for 15 minutes. The activated tubes were then filled with enzyme in 100mM phosphate buffer, pH 7.0, table 10.

Table 10. Enzymes used in Nylon Tube Reactors.

Enzyme	Protein mg.ml^{-1}	Activity U.ml^{-1}
Alcohol oxidase (various preparations)	2-7	18.0
Glucose oxidase (Sigma Type X)	0.27	30.0
Cholesterol oxidase (Sigma) *	0.88	20.0
Cholesterol esterase (BDH) *	2.5	10.0

* The cholesterol oxidase and cholesterol esterase were co-immobilised onto the nylon tube.

After incubation overnight at 4°C the tubes were washed with 100mM

phosphate buffer, pH 7.0 containing 1.0M sodium chloride at a flow of 3ml.min^{-1} for 10-15 minutes to remove any adsorbed enzyme. After a final wash with 100mM phosphate buffer, pH 7.0, (3ml.min^{-1}, 15 minutes) the tubes were ready for use. The enzyme coils were stored at 4°C filled with phosphate buffer when not in use.

2.G.2. Glass Supports.

The method of Weetall (1977) was used to immobilise enzymes to controlled pore glass, (Sigma). The enzyme - glass was then loaded into micro-bore columns of 2 or 3mm internal diameter of various lengths (20-80mm) for incorporation into a flow injection system.

Derivatisation and Immobilisation of Enzyme.

Various grades of control pore glass from 500 Å to 1273 Å were washed with 5% v/v nitric acid (BDH) at 90°C in a water bath to clean the surface. The cleaned glass was washed exhaustively on a sintered glass funnel with distilled water and then dried in an oven at 115°C.

One gramme of glass was then added to 1% v/v 3-amino propyl triethoxysilane, (Sigma) in distilled water adjusted to pH 3-4 with 6M HC1. The aqueous silanisation was carried out for 2 hours at 75°C in a water bath, then the glass was washed exhaustively with distilled water and dried at 115°C. The alkylamine glass so formed was stable and could be stored dry indefinitely.

To 200mg of alkylamine glass, 5ml of 5% w/v glutaraldehyde in 100mM borate buffer, pH 8.5 was added and the suspension was incubated for 1 hour at room temperature with occasional shaking. During this procedure a colour change from white to pink or purple-brown occurred. The glass was then washed exhaustively with distilled water and then rinsed in buffer. The enzyme to be

immobilised was then added in a suitable buffer, e.g.100mM phosphate, pH 7.0 at a concentration of 20-40 $mg.ml^{-1}$, and incubated overnight at 4°C. After incubation the glass was washed well with buffer and then stored in fresh buffer at 4°C. The reaction sequence is shown in fig.10.

2.H.1. Dialysis Probe.Standardisation / Characterisation.

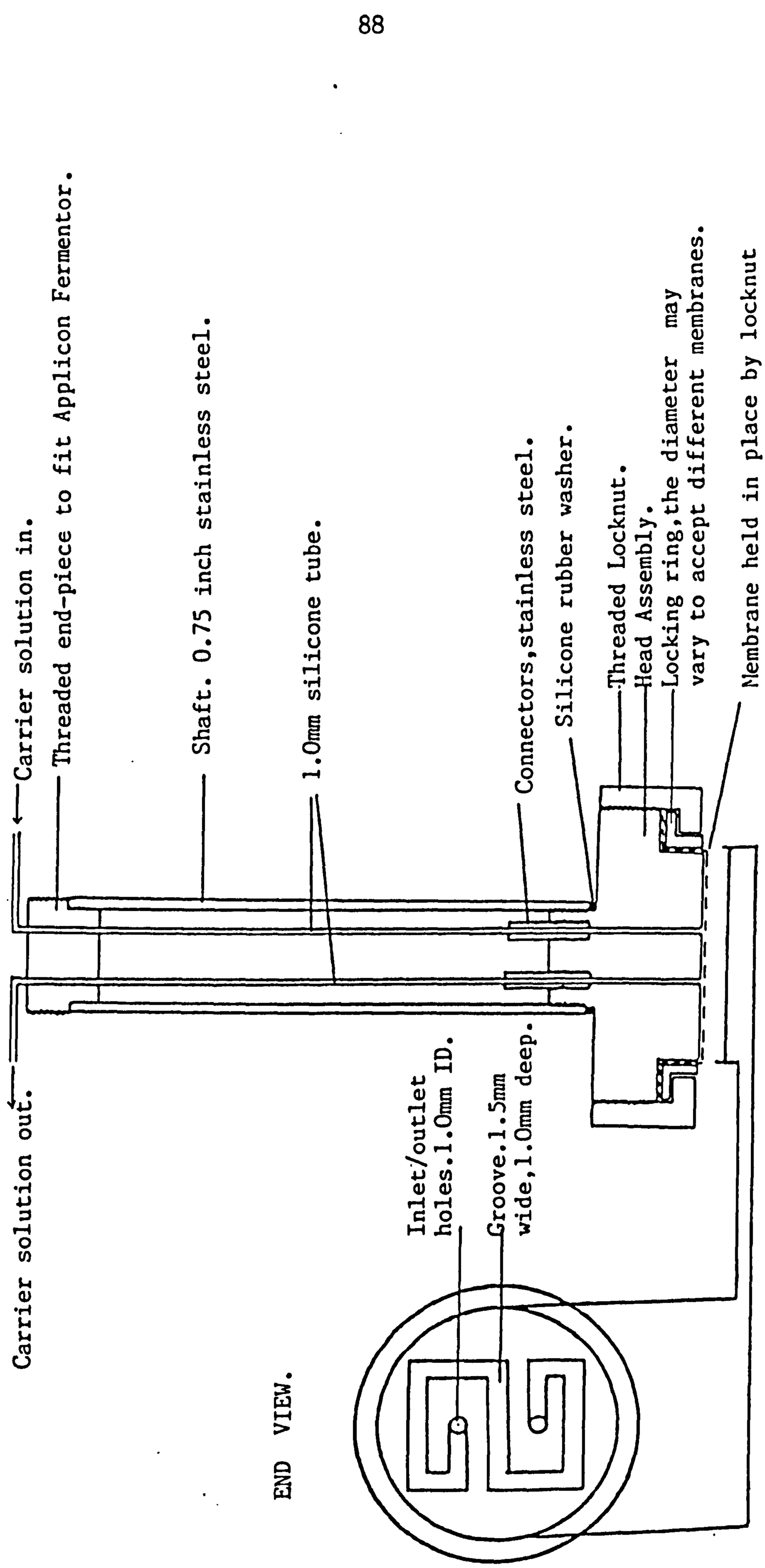
The dialysis probe was designed in the Biotechnology Unit and manufactured in the Biophysics workshop. It was made as an on- line sampling device for fermentation analysis. The probe is depicted in fig 11. In use, isosmotic carrier solution is pulled through the probe, producing a slight negative pressure which keeps the dialysis membrane, (Medicell) taut. Low molecular weight analytes cross the membrane by dialysis and may be analysed. A range of concentrations may be measured directly by this method, the sensitivity may be controlled by varying the flow rate of the carrier solution.

Standardisation of the probe was carried out with mixed standard solutions in a thermostatted vessel with stirring. Isosmotic sucrose solution was drawn at $0.26ml.min^{-1}$ through the probe. The probe head was immersed in the standard solution and left to equilibrate for 5-10 minutes. The effluent solution from the probe was then collected and analysed in a flow system,(section 2.I.). The process was repeated for several standards and the results plotted to give standard curves for each analyte, graphs 15 and 16. Using the same apparatus temperature dependence, response time, pH dependence and the response of various analytes were studied and the results are reported in chapter 4.

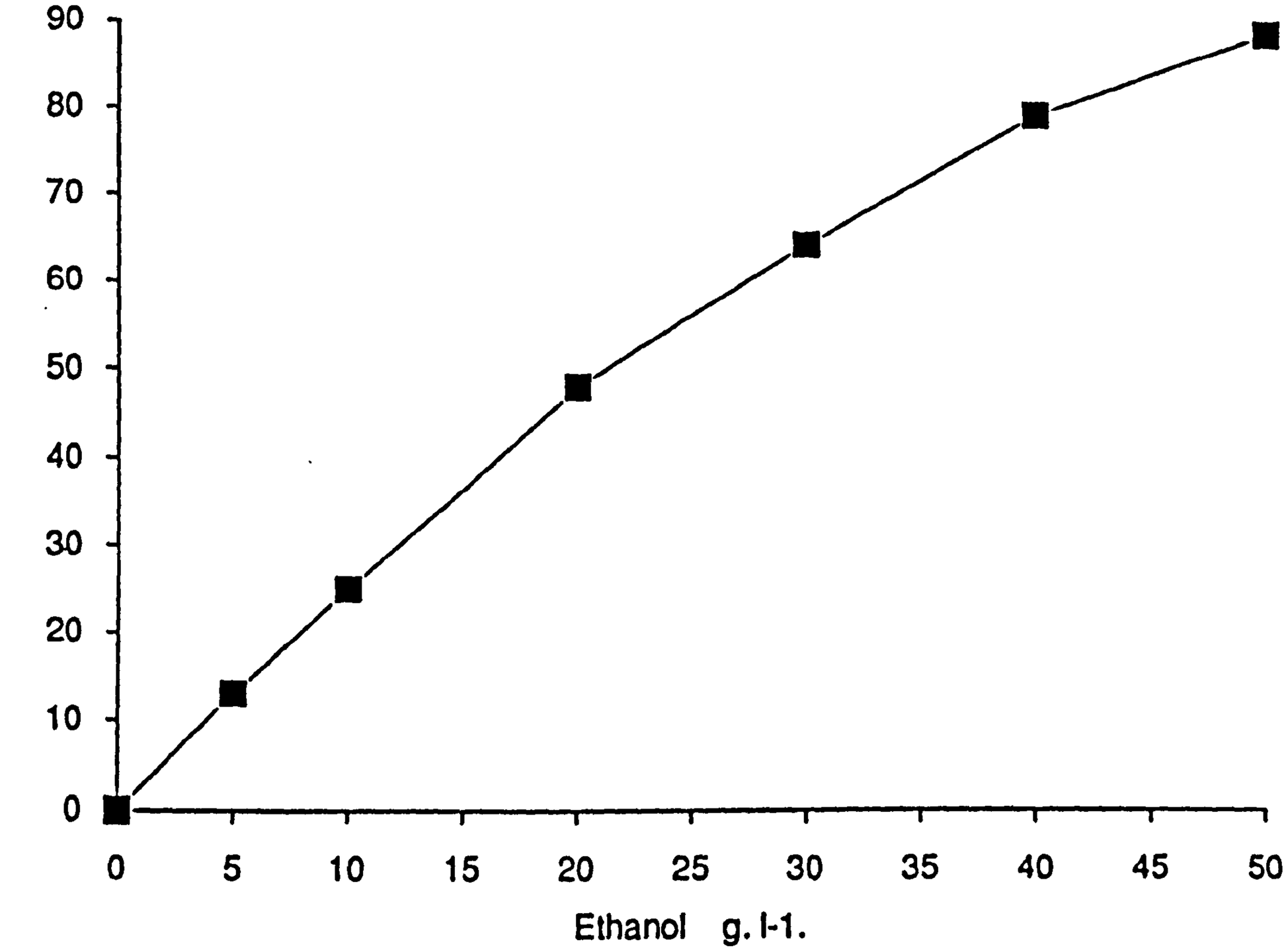
2.H.2. Dialysis Probe. Use in fermentors.

The dialysis probe was used to follow the consumption of glucose

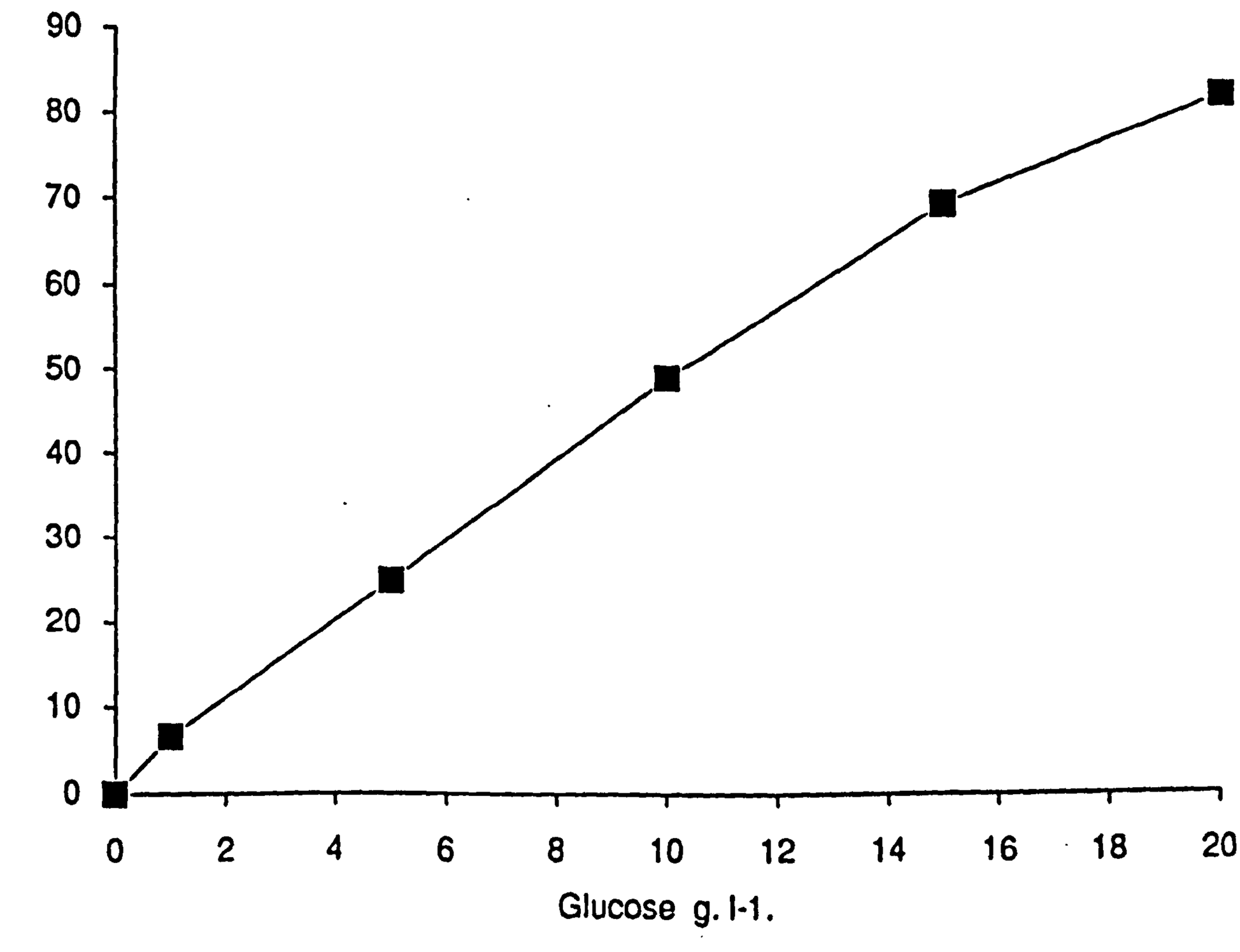
FIG.11 DIALYSIS PROBE.



Graph 15. DIALYSIS PROBE. Ethanol Standard Curve.



Graph 16. DIALYSIS PROBE. Glucose Standard Curve.



and the production of ethanol in model fermentations. Two commercial home brew kits, (Tom Caxton Bitter and Pains) were used. Aliquots were diluted to 1.21 volumes and added to a 21 Applikon fermentor, 1.0g of yeast slurry was added and sampling was started using isosmotic sucrose solution as carrier, flow rate 0.26ml.min^{-1} or 1.0 ml.min^{-1}. The medium was stirred with minimal aeration,(>20ml.min^{-1}) and sampled intermittently over a 48 - 72 hour period using an auto sampler, (Technicon Instruments) fitted with a time switch. After each 24 hour period, 100ml glucose, (200g.l^{-1}) was added to prolong the fermentation.

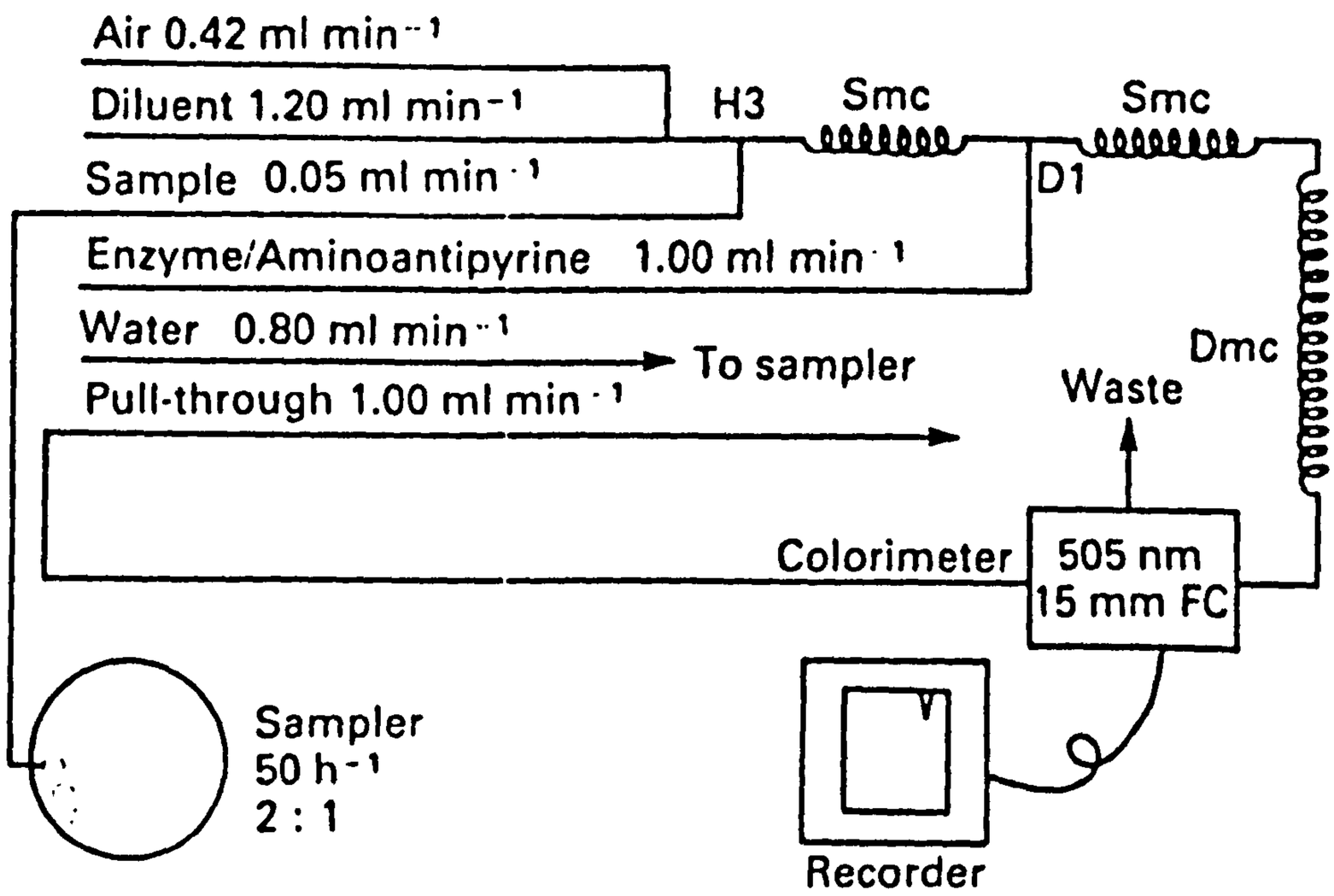
In addition to these fermentations, a model bacterial fermentation, (S.Lactis) was set up according to section 2.A.2. The fermentor containing medium and the dialysis probe was sterilised in an autoclave at 121°C for 20 minutes. The medium was inoculated with an actively growing culture of S.Lactis, stirred with no aeration and diacetyl production and glucose consumption were measured using a flow system, (sections 2.I.2 and 2.I.3).

2.I.1. Segmented Flow Analysis. Soluble Enzymes.

The manual method for ethanol, (section 2.F.5) was adapted for a Technicon Auto Analyser Mark II system. The reagent compositions and the flow chart are shown in fig. 12.

The sample was aspirated from the sampler, mixed with diluent and the enzyme/aminoantipyrine reagent and the developed colour measured in a flow through optical cell at 505nm, the peak height being directly related to concentration. Diluent 1 was used for a range of 50-500mg.l^{-1} ethanol with a sample flow rate of 0.32ml.min^{-1} and 0.5g.l^{-1} to 5.0g.l^{-1} ethanol with a sample flow rate of 0.05ml.min^{-1}. Diluent 2 was used to measure ethanol between 5mg.l^{-1} to 100mg.l^{-1}

FIG.12 SEGMENTED FLOW ANALYSIS of ETHANOL. SOLUBLE METHOD.



Enzyme/Aminoantipyrine: sodium phosphate buffer — 100 mmol l⁻¹ pH 7.0
Alcohol oxidase — 76 units l⁻¹
Peroxidase — 2 300 units l⁻¹
4-Aminoantipyrine — 187 mg l⁻¹ — 0.92 mmol l⁻¹

Ranges 50–500 mg l⁻¹ EtOH, 0.5–50 g l⁻¹ EtOH

Diluent 1. Sodium phosphate buffer — 100 mmol l⁻¹ pH 7.0
4-Hydroxybenzene sulphonic acid sodium salt — 47.9 mmol l⁻¹
Tween 20 — 1.0 ml l⁻¹

Range 5–100 mg l⁻¹ EtOH

Diluent 2. Sodium phosphate buffer — 100 mmol l⁻¹ pH 7.0
3,5-Dichloro-2-hydroxy-benzene sulphonic acid, sodium salt — 9.25 mmol l⁻¹
Tween 20 — 1.0 ml l⁻¹

with a sample flow rate of 0.42ml.min^{-1} and the developed colour was measured at 523nm.

2.I.2. Segmented Flow Analysis. Immobilised Enzymes.

The nylon tube immobilised enzymes, (section 2.G.1) were incorporated into a flow system and used to measure their respective analytes. The reagent compositions and the flow diagram are shown in fig. 13.

The reactions and concentration ranges for ethanol with the two colour reagents were the same as for the soluble method, with the exception of a less sensitive response at low levels of ethanol, (10-120mg.l^{-1}). Also the number of samples analysed per hour was reduced from 50 to 30. Analysis of glucose was identical to that of ethanol using colour reagent 1 over a range of 1 - 5g.l^{-1}. Cholesterol was analysed over the range of 0 - 2.0g.l^{-1} using cholesterol diluent and colour reagent 1.

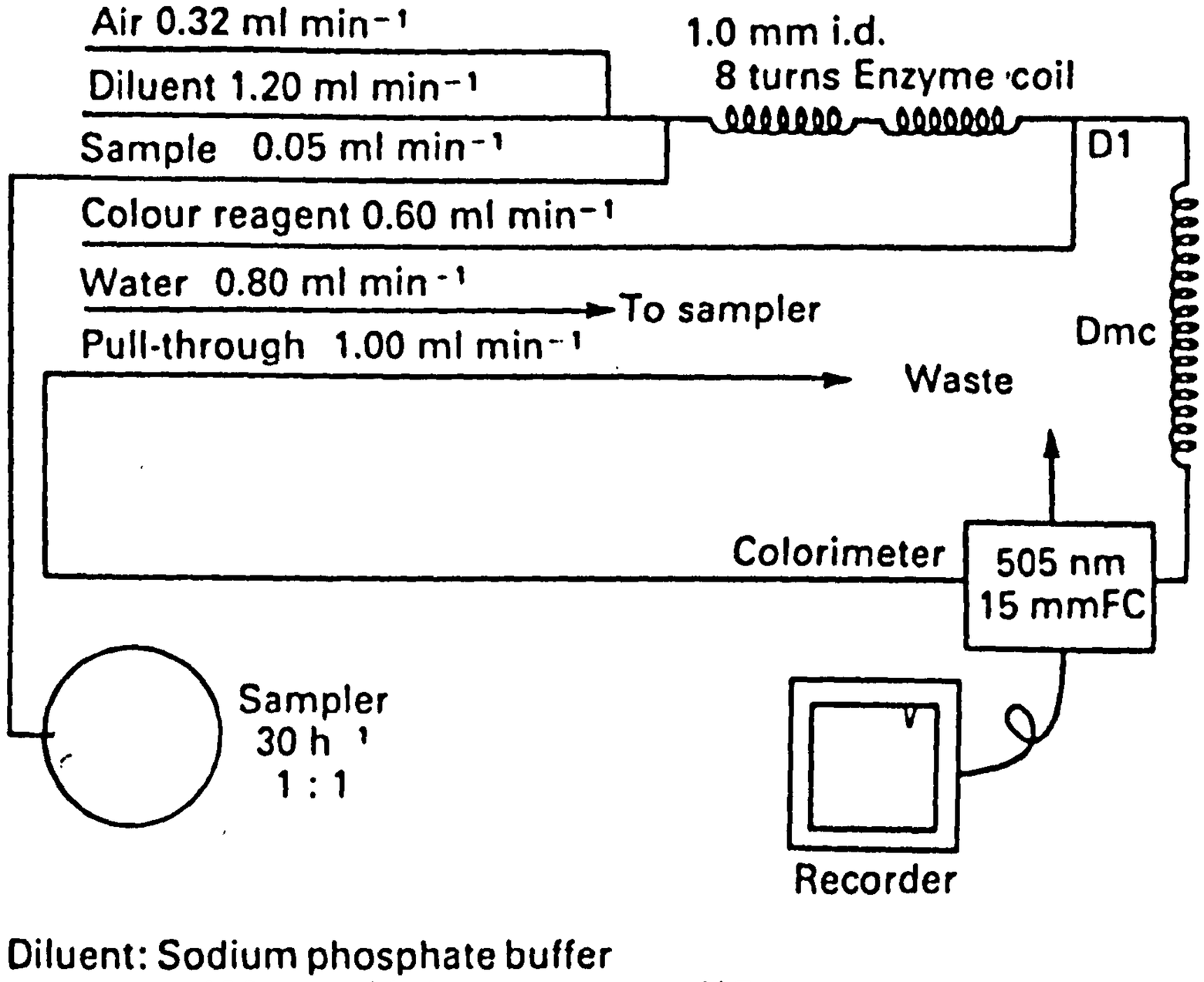
2.I.3 Segmented Flow Analysis. Chemical Diacetyl Method.

The manual 1-naphthol/creatine method for diacetyl, (section 2.F.1) was adapted for flow analysis. The reagents are identical to those in section 2.F.1. and the flow diagram is given in fig.14. Diacetyl may be measured at 1 - 10mg.l^{-1} using this system. Adjusting the sample flow rate to 1.0ml.min^{-1} and omitting the water line enables diacetyl concentrations of 0.1 - 1mg.l^{-1} to be measured.

2.I.4. Flow Injection Analysis. Soluble enzymes.

Flow injection analysis was carried out using the flow system and reagents shown in fig.15. The apparatus used included a Gilson Minipuls 3 peristalic pump, an Anachem valve switching module fitted with a low pressure Rheodyne injector valve, a Jencons colorimeter with an 18 µl, 10mm pathlength Hellma glass flowcell and a Gallenkamp

FIG.13 SEGMENTED FLOW ANALYSIS of ETHANOL. IMMOBILISED . METHOD.



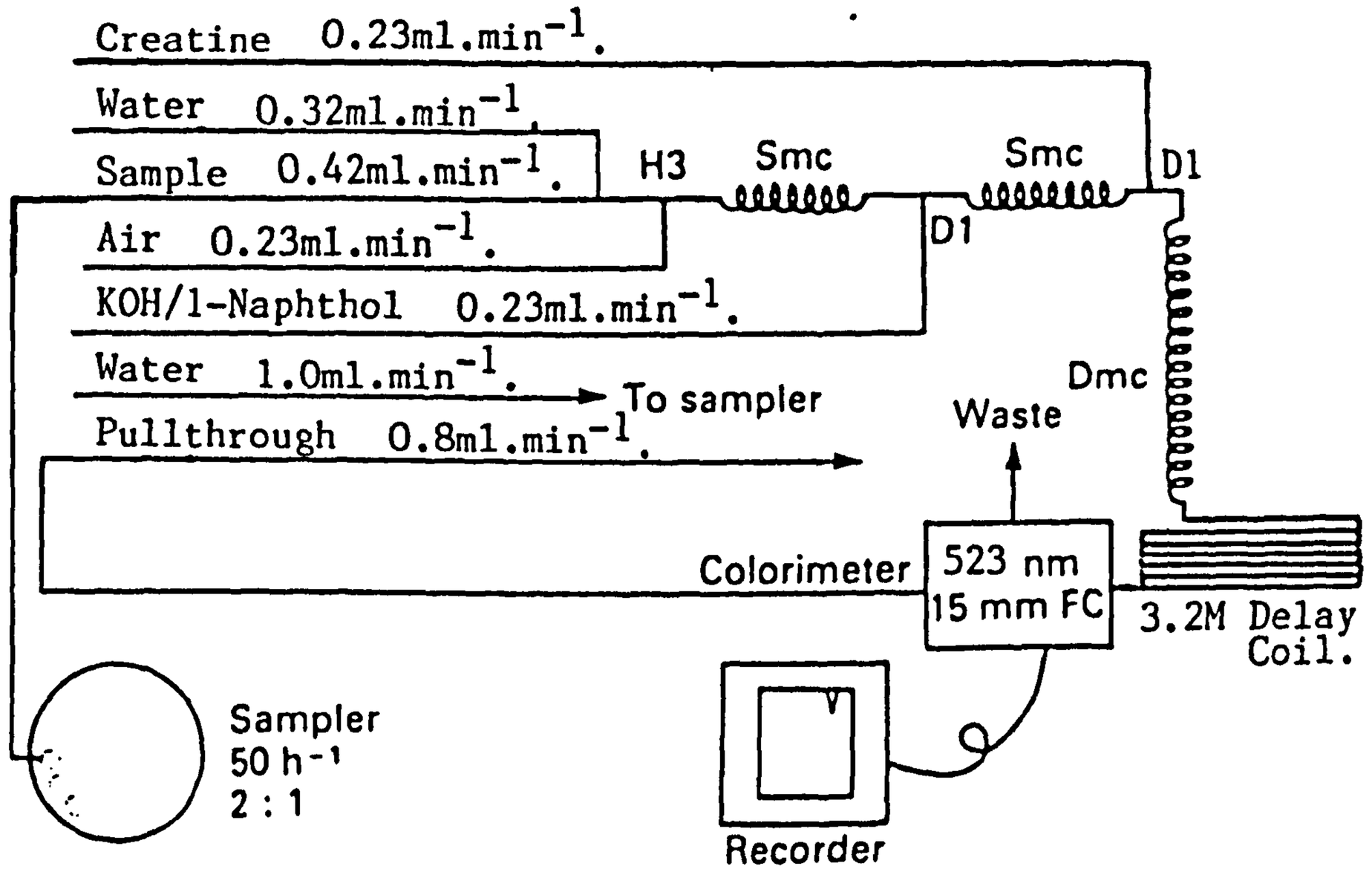
Diluent: Sodium phosphate buffer
100 mmol l^{-1} pH 7.0
4-Aminoantipyrine
(125.2 mg l^{-1}) 0.62 mmol l^{-1}
Tween 20

Enzyme coil 0.5 m, Nylon 6, i.d. 1.0 mm
containing 0.454 units alcohol oxidase

Range 50–500 mg l^{-1} EtOH
0.5–5.0 g l^{-1} EtOH
Colour reagent: Sodium phosphate
buffer 100 mmol l^{-1} pH 7.0
4-Hydroxybenzene
sulphonic acid
sodium salt 73.9 mmol l^{-1}
Peroxidase 3080 units l^{-1}

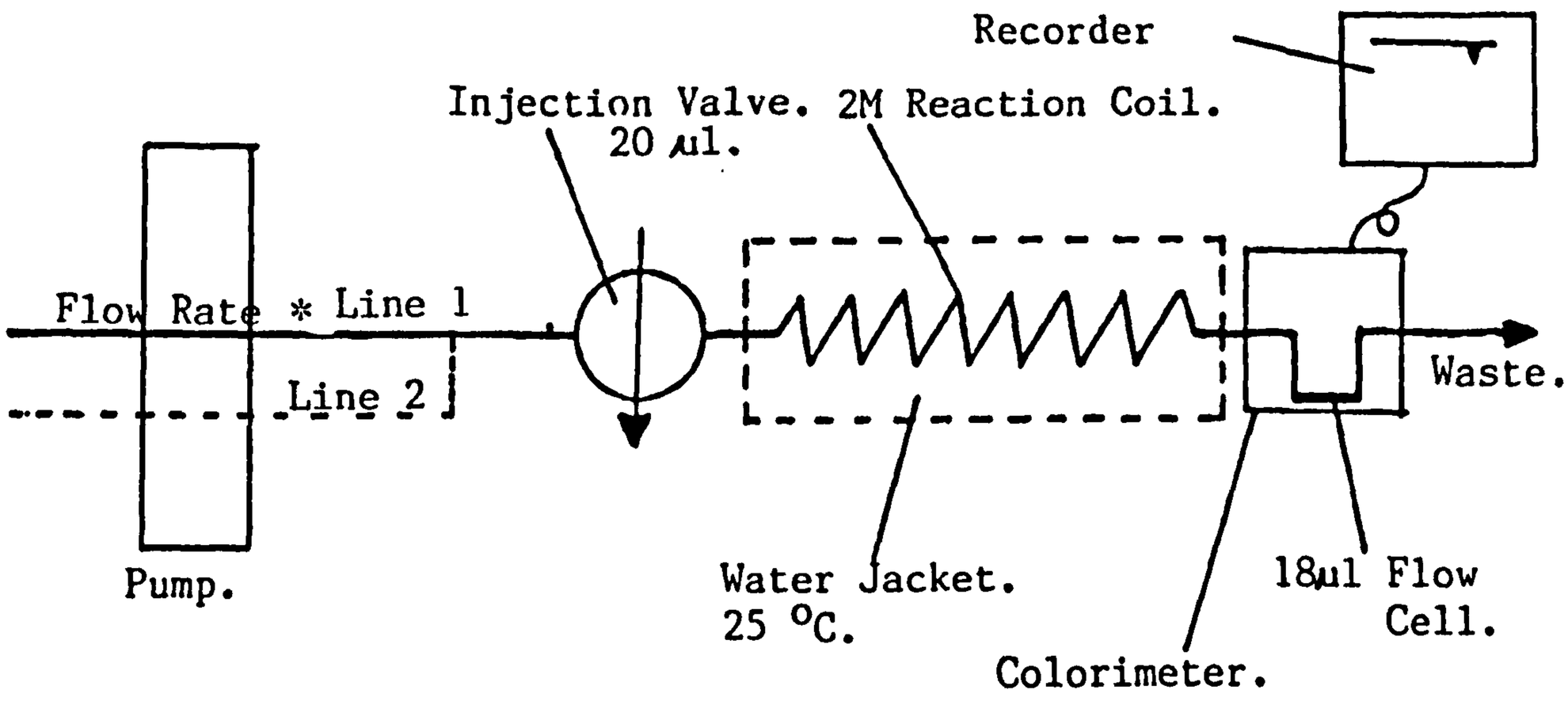
Range 10–120 mg l^{-1} EtOH
Sodium phosphate buffer 100 mmol l^{-1} pH 7.0
3,5-Dichloro-2-hydroxybenzene sulphonic
acid sodium salt 15.4 mmol l^{-1}
Peroxidase 3080 units l^{-1}

FIG.14 SEGMENTED FLOW ANALYSIS of DIACETYL.



Creatine.	1.0% w/v in distilled water.
KOH/1-Naphthol	5.0% w/v 1-Naphthol in 2.5M Potassium hydroxide in distilled water.

FIG.15 FLOW INJECTION ANALYSIS of ETHANOL.
SOLUBLE METHOD.



* The same system is used for both single reagent and double reagent methods.

Single Line.	Flow Rate	$1.90 ml.min^{-1}$
Double Line.	Flow Rate 1.	$0.95 ml.min^{-1}$
	Flow Rate 2.	$0.95 ml.min^{-1}$

Single Reagent.

Alcohol Oxidase	$5000\ U.l^{-1}$
(or Glucose Oxidase	$10,000\ U.l^{-1}$)
Peroxidase	$2000\ U.l^{-1}$
4-Aminoantipyrine	$81.3 mg.l^{-1}$(0.4mM)
Phenol Sulphonic Acid	$5.80 g.l^{-1}$ (25mM)

100mM Na Phosphate buffer pH 7.0 up to 1.01.

Double Reagent.

Line 1.	Alcohol Oxidase	$10,000 U.l^{-1}$
	(or Glucose Oxidase	$20,000 U.l^{-1}$)
	Peroxidase	$4000 U.l^{-1}$
	4-Aminoantipyrine	$162.6 mg.l^{-1}$(0.8mM)

100mM Na Phosphate buffer pH 7.0 up to 1.01.

Line 2. Phenol Sulphonic Acid $11.6 g.l^{-1}$(50mM)

100mM Na Phosphate buffer pH 7.0 up to 1.01.

Datatrace flat bed recorder. Tubing was 0.8 mm ID teflon, (Omnifit) and connections were made using 1/4 - 28 thread low pressure HPLC unions, nuts and ferrules, (Upchurch). The mixer blocks were made out of perspex by the Biophysics workshop. Pump tubing was accurated auto analyser tubing, (Elkay).

2.I.5. Flow Injection Analysis. Immobilised Enzyme.

The flow system and reagents are shown in fig.16. The glass immobilised enzyme, (section 2.G.2) was packed in a microcolumn, (2mm ID x 20-80mm) which was inserted between the two mixing coils.

2.J.1. Preparation of Dry Alcohol Oxidase. Vacuum Drying.

Alcohol oxidase, (40 units, 4-5mg protein) in buffered solution was mixed with stabiliser. The resulting solution was sub-aliquotted, (100 ul) into disposable polystyrene macro-cuvettes using a Gilson adjustable pipette or a Gilson repetman pipette. The water was removed under vacuum in a heated vacuum oven, (Gallenkamp) at 30°C, 0.1mbar for 4 hours, with silica gel as desiccant. The resulting dry enzyme films were then stored desiccated over silica gel and reconstituted with colour reagent before assay by the standard technique described in section 2.F.5.(ii).

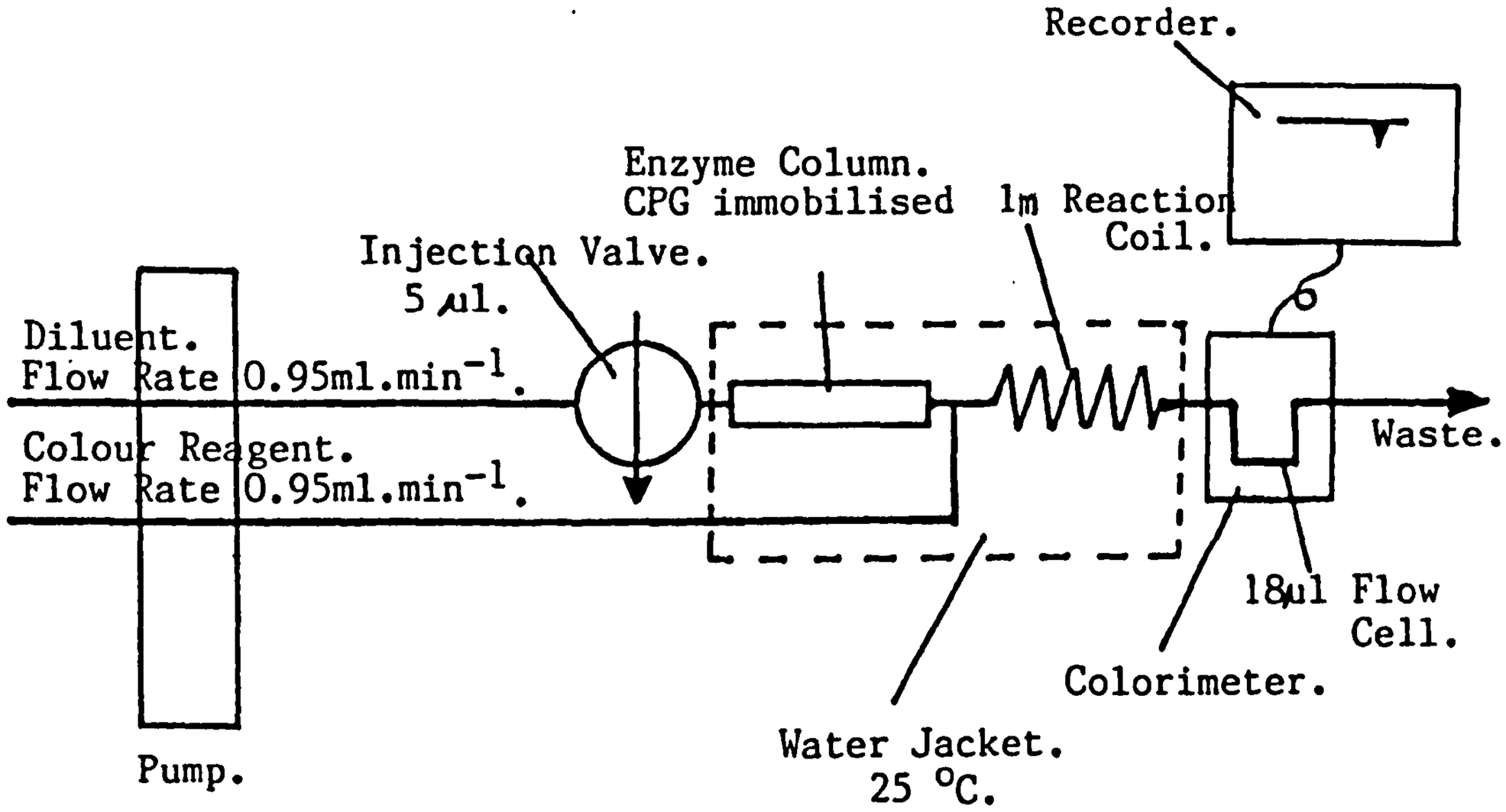
This basic procedure was employed using a range of compounds as stabilisers, table 11

Bulk preparation of the dry enzyme was carried out using the same drying procedure with enzyme / stabiliser solution being spread evenly on a glass plate. The dry film produced was ground to powder using a mortar and pestle and stored dry as before.

2.J.2. Preparation of Dry Alcohol Oxidase. Freeze Drying.

Enzyme / stabiliser solutions were prepared as before and frozen using dry ice. The frozen solutions were then dried in a Chemlab

FIG.16 FLOW INJECTION ANALYSIS of ETHANOL. IMMOBILISED METHOD.



Diluent.

Phosphate buffer (Na)	100mM, pH 7.0.

Colour Reagent.

Peroxidase	4000U.l^{-1}
Phenol Sulphonic Acid	11.6g.l^{-1} (50mM).
4-Aminoantipyrine	162.6mg.l^{-1}(0.8mM).
100mM Na Phosphate buffer	pH 7.0 up to 1.01.

Table 11. Potential Stabilisers for Dry Alcohol Oxidase.

Compounds.	Source.	Concentration.	Type of drying.*
Monosaccharides.			
Glucose	BDH	10% w/v	V
Fructose	SIGMA	10% w/v	V
Galactose	SIGMA	10% w/v	V
Xylose	SIGMA	10% w/v	V
Sorbose	SIGMA	10% w/v	V
Disaccharides.			
Sucrose	SIGMA	10% w/v	F/V
Lactose	BDH	10% w/v	V
Maltose	May & Baker	10% w/v	V
Trehalose	SIGMA	5% w/v	V
Cellobiose	SIGMA	5% w/v	V
Trisaccharides.			
Raffinose	BDH	5% w/v	V
Sugar Alcohols.			
Mannitol	SIGMA	10% w/v	F/V
Dulcitol	SIGMA	5% w/v	V
Sorbitol	SIGMA	10% w/v	V
Lactitol	BIO-KITS,CORTECS Ltd.	1%-10% w/v	F/V
Maltitol	SIGMA	5% w/v	V
Cyclic Polyalcohol.			
Inositol (Meso)	BDH	1% - 10% w/v	F/V
Neutral Polymers.			
Dextran Mol wt. 500,000	SIGMA	1% w/v	V
Dextran Mol wt. 150,000	SIGMA	1% w/v	V

Table 11, CONTINUED.

Compound	Source.	Concentration.	Type of Drying*
Dextran Mol wt. 17,900	SIGMA	1% w/v	V
Dextran Mol wt. 5-40 x 10^6	Sigma	1% w/v	V
Dextran T10	Pharmacia	1-5% w/v	F/V
Dextran T40	Pharmacia	1% w/v	V
Dextran T70	Pharmacia	1% w/v	V
Dextran T500	Pharmacia	1% w/v	V
Dextran T2000	Pharmacia	1% w/v	V
Polyethylene glycol Mwt 6000	BDH	10% w/v	V
Polyethylene glycol Mwt 10000	BDH	10% w/v	V
Polyethylene oxide	BDH	1% w/v	V
Polyvinyl pyrollidone	Sigma	5% w/v	V
Ficoll 400	Pharmacia	5% w/v	V
Dextrin Type 1	Sigma	5% w/v	V
Dextrin Type III	Sigma	5% w/v	V
Soluble starch	Sigma	Saturated	V
B-Cyclodextrin	Sigma	1% w/v	V
Xylan	Sigma	0.5% w/v	V
<u>Cationic Polymers.</u>			
DEAE dextran	Pharmacia	1% w/v	V
Polyethyleneimine	Sigma	1% w/v	V
Chitosan	Protan	0.5% w/v	V
<u>Anionic Polymers.</u>			
Carboxymethyl cellulose	Sigma	1% w/v	V
Dextran sulphate	Pharmacia	1% w/v	V
Sodium alginate	BDH	0.5% w/v	V

Table 11. CONTINUED.

Compound.	Source.	Concentration.	Type of Drying*
Fucan A	Dr.W.Mackie	0.5% w/v	V
Fucan B	Dr.W.Mackie	0.5% w/v	V
Combinations.			
Lactitol / DEAE dextran		0.1 - 10% /0.01-1% w/v	F/V
Lactose / DEAE dextran		5% / 1% w/v	V
Sucrose / DEAE dextran		5% / 1% w/v	V
Maltose / DEAE dextran		5% / 1% w/v	V
Inositol / DEAE dextran		5% / 0.01-1% w/v	V
Maltitol /DEAE dextran		5% / 1% w/v	V
Lactitol/Carboxymethyl cellulose		2.8-5% /0.6-1% w/v	V
Lactitol / Sodium al ginate		2.8% / 0.24% w/v	V
Lactitol / Dextran sulphate		5% / 1% w/v	V
Lactitol / Chitosan		5% / 0.01 - 1% w/v	V
Lactitol / Polyethyleneimine		5% / 0.01 - 1% w/v	V

V = Vacuum drying.

F = Freeze drying.

freeze drier. When dry, the resulting powdery product was stored over silica gel until use.

Bulk freeze dried preparations were prepared in a similar fashion. Typically, 1000 units of alcohol oxidase was mixed with 5.0ml of stabiliser solution, (20% w/v) and frozen. The mixture was then freeze dried and thoroughly powdered using a thick glass rod. 10mg samples were weighed out and reconstituted in 1.0ml of 100mM phosphate buffer pH 7.0. 40 µl aliquots were then assayed using standard assay techniques described.

2.K.1. Stability of Enzymes. Dry Preparations.

The stability of dry enzyme preparation was estimated using the technique of elevated temperature testing. The dry preparations produced by vacuum or freeze drying were stored desiccated over silica gel at 37°C. Samples were removed, (individual cuvettes or 10mg samples from bulk enzymes), at various timed intervals usually over a period of days and assayed using standard assay techniques, (sections 2.D.2, 2.D.4. or 2.F.5). The residual enzyme activity measured was taken as an indication of the stability of the enzyme in the dry form.

2.K.2. Stability of Enzymes. Wet Stabilisation.

Wet stability of enzymes was carried out using elevated temperature testing as before. Enzyme solutions were incubated with stabilisers at a range of temperatures, (37, 50 or 60°C) and the residual activity measured as before. Enzymes and stabilisers are shown in table 12.

Table 12. Wet Stabilisation of Enzymes.

Enzyme	Stabiliser	Concentration
Alcohol oxidase H.Polymorpha.	Lactitol	Saturated
Alcohol oxidase H.Polymorpha.	Sorbitol	Saturated
Cholesterol oxidase Nocardia	Lactitol	Saturated
Cholesterol oxidase Nocardia	Sorbitol	1M to Saturated
Cholesterol esterase Pancreatic	Sorbitol	1M

2.K.3. Stability of Immobilised Enzymes.

Nylon or glass immobilised enzymes "in - use" in flow systems, (sections 2.I.2 and 2.I.5) were continually perfused with buffer solution over a period of days. Standards were assayed intermittently and the residual activity of the enzyme detected by the reduced recorder response.

2.L.1. Dry Enzyme Tests. Paper Supports.

Dry reagent analytical strips employing all buffers, enzymes, colour reagents, stabilisers and additives were fabricated by both single saturation or multiple saturation of various grades of filter papers with a cocktail of pre-mixed ingredients. The following typical procedure will illustrate the process.

Filter paper. Whatman 3MM Chr 5cm x 5cm.

1.0ml Enzyme cocktail.

Alcohol oxidase	200 Units.
Peroxidase (Sigma or Biozyme)	100 Units.
4-Aminoantipyrine (Sigma or Aldrich)	20 mM.
N,N Bis(hydroxyethyl)aniline (ICI)	25 mM.
Inositol (BDH)	5% w/v.
Bovine serum albumin (Sigma)	3% w/v.

Cysteine (Sigma)	4.2 mg.
MOPS buffer pH 7.9 (BDH)	50 mM.

The alcohol oxidase was freshly prepared by dialysis against 10mM MOPS buffer, pH 7.9. The enzymes, inositol, BSA, cysteine and buffer were mixed well and the colour reagents added. The cocktail was quickly mixed, poured on a clean glass plate and absorbed thoroughly onto the filter paper, making sure the paper was evenly saturated. Excess liquid was drained onto soft tissue and the saturated filter paper was placed flat in a vacuum oven at 30 - 35°C where it was dried at 0.1mbar for 30 minutes, with silica gel as desiccant. The dry paper was then stored over silica gel. In use, 4 - 5 mm diameter discs were cut from the paper using a paper punch and developed with aqueous ethanol solutions.

Multiple saturation of paper supports was carried out when one or more of the colour reagents or additives were insoluble in aqueous solvents. e.g. The dry enzyme paper was prepared as before but omitting the N,N,bis(hydroxyethyl) aniline. A 10mM solution of 2,4,6-triiodophenol, (Sigma) in 1,1,1-trichloroethane, (BDH) was then poured over the enzyme paper in a fume cupboard and the excess liquid blotted off. The solvent was then evaporated in an air stream and the paper stored and developed as before.

Microporous films of polymer solutions were also applied to the paper supports using a similar solvent coating technique. Ethyl cellulose, (Sigma) was dissolved in toluene, (BDH) to 1% w/v. The dry enzyme paper was dipped in this solution, the excess was blotted off and the solvent evaporated in an air stream. The dry paper was then stored and developed as before. Variations in cocktail composition and technique will be discussed in more detail in chapter 6.

2.L.2. Dry Enzyme Tests. Gel Films.

Multiple layer dry enzyme tests were constructed using dry enzyme papers prepared as described below, laminated onto a film of gelatine containing the colour detection chemistry.

Dry paper layer.	Whatman 4 Chr.	5cm x 7cm
1.0ml Enzyme cocktail	Alcohol oxidase	200 units
	Inositol	5% w/v
	MOPS buffer pH 7.9	100 mM
	Cysteine	4.2 mg
	BSA	3% w/v

The paper is saturated, drained and dried as before.

1.0 ml Gel layer.	Gelatine 300 bloom (Sigma)	15% w/v
	4-Aminoantipyrine	20 mM
	N,N Bis(hydroxyethyl) aniline	25 mM
	Peroxidase	100 Units
	Cysteine	4.2 mg
	Mops buffer pH 7.9	100 mM

The gelatine is prepared by dissolving 3g of solid in 10 ml of hot distilled water at 55°C to give a 30% solution, this is kept molten at 55°C. The peroxidase, colour reagents and buffer were made up to double concentration and heated to 40°C just before use. Equal volumes of gelatine and colour reagents were then mixed and poured as a thin band onto one edge of a piece of gel bond transparent membrane, (Sigma). A grooved metal rod, so constructed to have an isosceles triangular thread profile of known thread depth was then quickly drawn across the molten gel mixture to produce a thin film. This technique was a variation of the Meyer Rod method for producing

thin films, (Walter 1988). The film was allowed to become tacky, (1-2 minutes) and the dry enzyme paper prepared previously was laid on the gel and rolled flat on a silicon rubber surface using an artists rubber roller. This procedure caused the gel layer to laminate evenly with the enzyme paper. The composite element is air dried at 20-35°C, with the paper side uppermost and developed as before. The test is illustrated in fig 17. The developed test was observed from the back through the transparent support. Variations in this process are discussed in chapter 6,section 6.C.

2.L.3. Dry Enzyme Tests. Stability.

The storage stability of dry enzyme tests was estimated by the same type of elevated temperature testing as for the dry enzyme preparations. Enzyme papers were stored over silica gel at 37°C and small samples were developed using aqueous standards. The time taken to produce a detectable response was used as indication of the stability of the test paper.

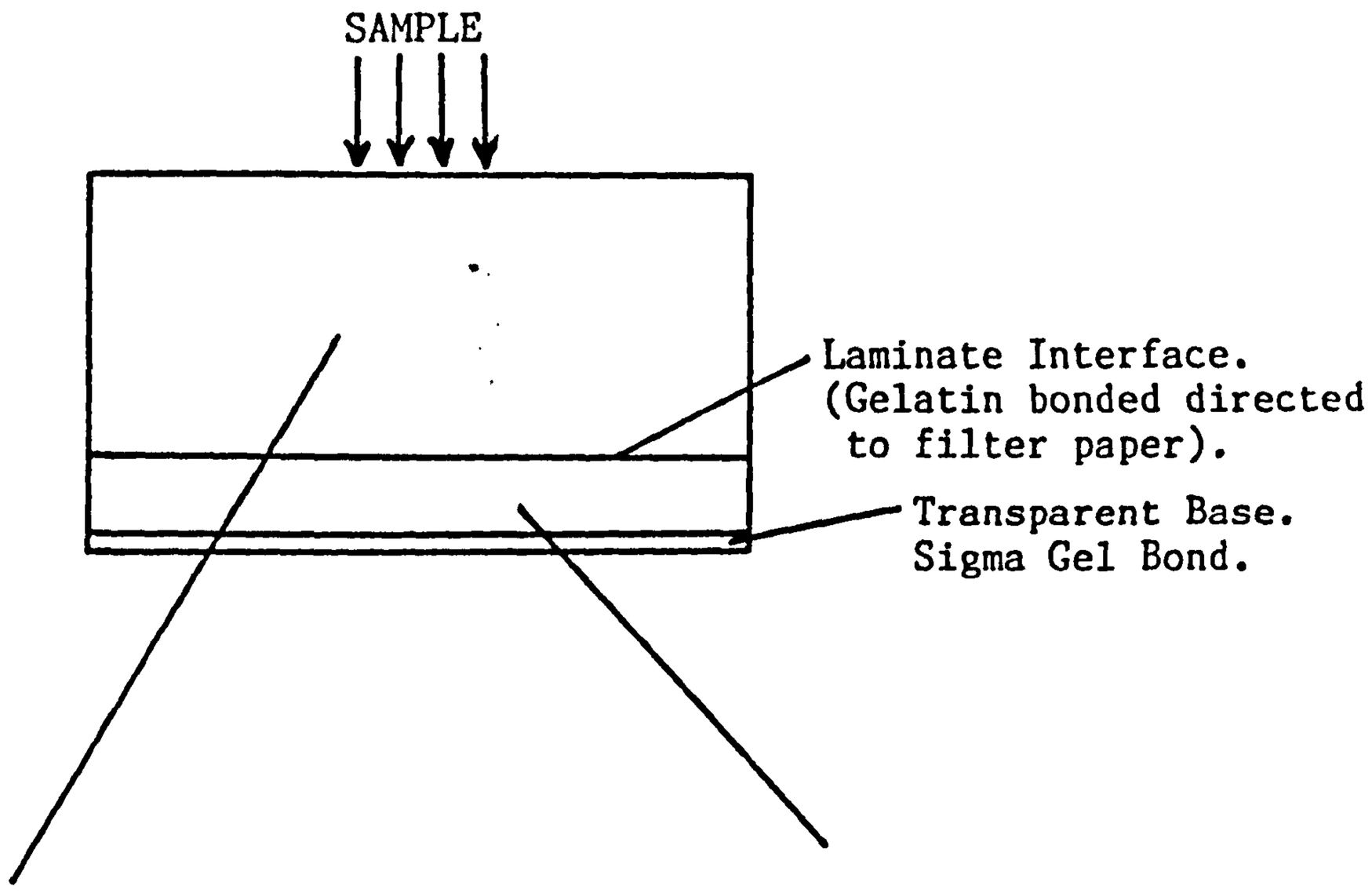
2.L.4. Dry Enzyme Tests. Reflectance Measurement.

Dry enzyme paper or gel laminate test strips were punched out to give either 5 or 7 mm discs using a hole punch. Paper discs were mounted on pieces of white card using a solution of plastic in 1,1,1-trichloroethane as a glue, (plastic glue) or double sided tape, (Sellotape). Gel laminates were developed upsidedown on a white card backing and the colour viewed through the transparent base. 5 to 10 ul of solution, (standard or sample) were applied to the discs using a Gilson micro pipette and the development of colour recorded using a Dr.Lange Microcolor reflectometer.

2.L.5. Dry Enzyme Tests. Formats.

(1). A dry alcohol test card prototype was constructed using

FIG.17 DRY PHASE MULTILAYER TESTS.



Filter Paper Layer,containing:-

(a) Enzymes/Buffers/Stabilisers/ Mediators.

(b) Enzymes/Buffers/Stabilisers/ Coupler/Mediators.

(c) Enzymes/Buffers/Stabiliers/ Developer/Mediators.

Gelatin Layer,containing:-

(a) Developer/Coupler/Mediators.

(b) Developer/Mediators.

(c) Coupler/Mediators.

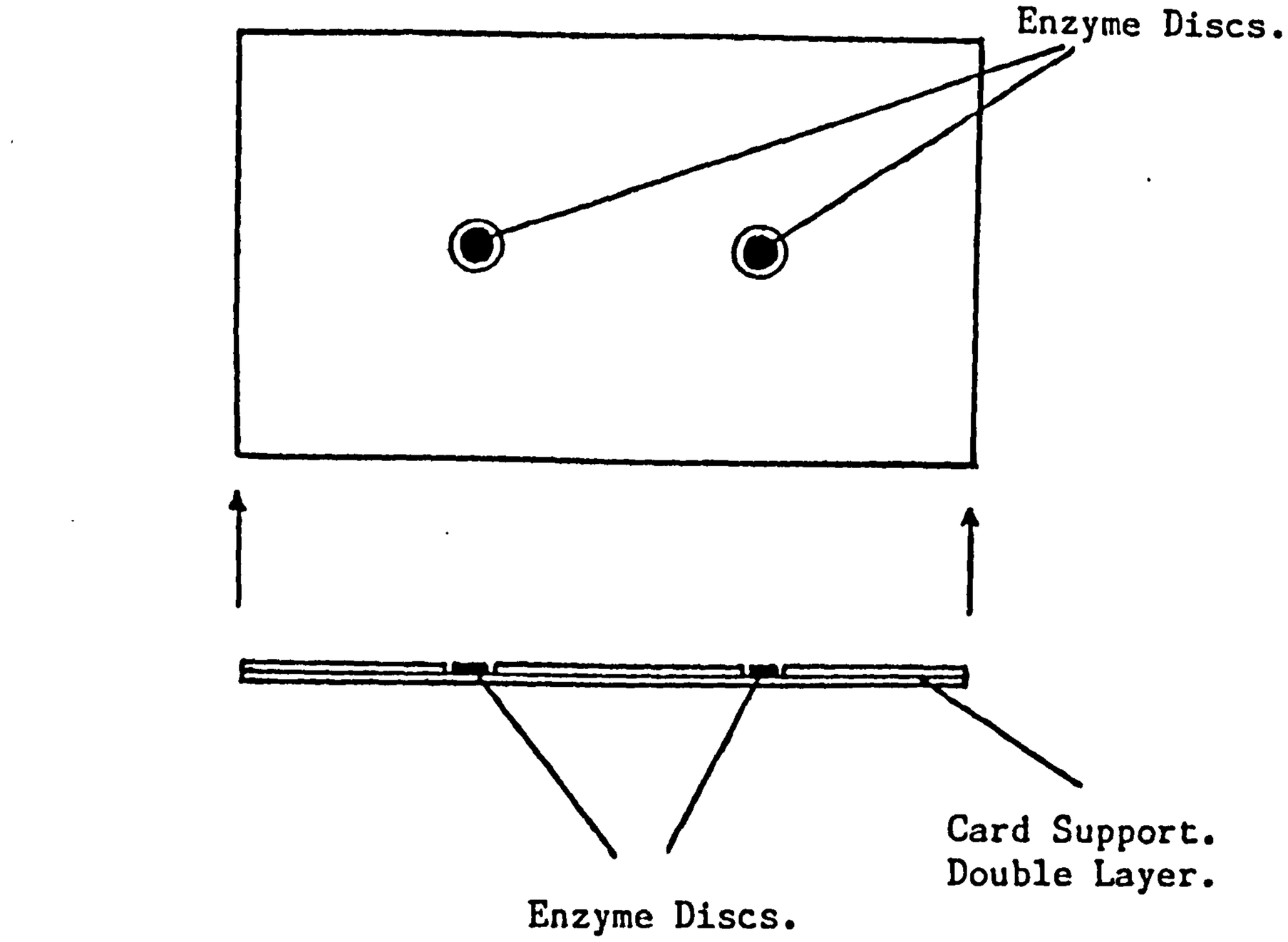
Multilayer tests formed from laminates of filter paper and gelatin allow combinations of sensitive reagents / enzymes to be used in a single test format,thereby largely eliminating unwanted interactions. Addition of sample caused solubilisation of the components of the system and subsequent reaction,producing colour formation in the gel layer,which was then viewed through the base.

multisaturated paper supports on Whatman 3MM Chr filter paper. One paper was prepared as described in section 2.L.1, with 4.2 - 4.5 $mg.ml^{-1}$ cysteine and another using 6.2 - 6.5$mg.ml^{-1}$ cysteine, with all other components being the same.

The dry papers were overlaid with 1% ethyl cellulose in toluene as described. 5mm discs were cut and mounted using plastic glue into a dry, double laminate card, fig 18a. The top layer contained two wells in which to mount the discs. The card was absorbent in character. The completed tests were foil packed with silica gel sachets and heat sealed to produce a moisture free environment. The dry tests when developed with ethanol standards or saliva samples, gave a purple colour above $500mg.l^{-1}$ ethanol for the first disc and above $800mg.l^{-1}$ ethanol for both discs.

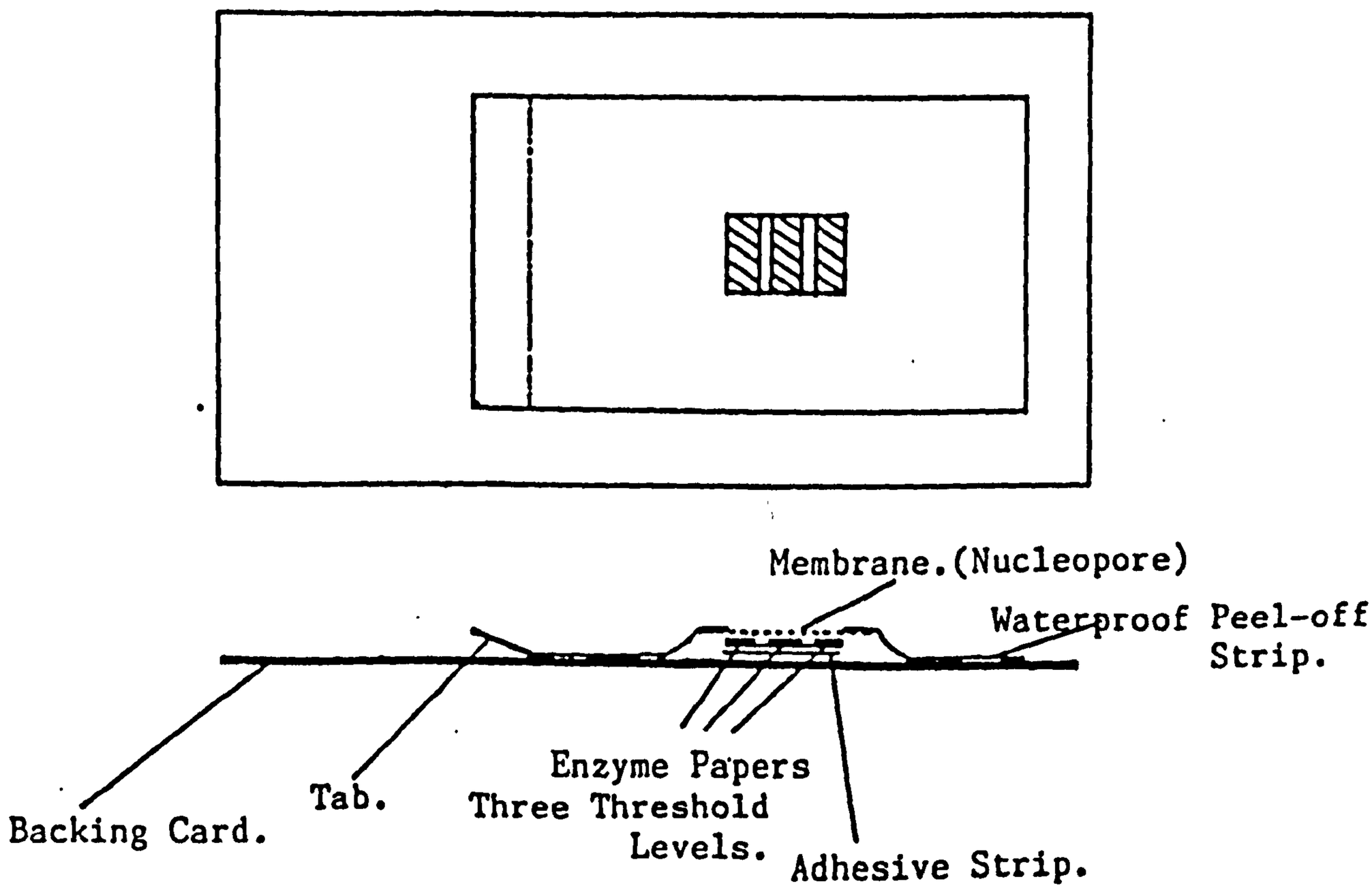
(2). A second type of test was constructed using the same multiple saturation technique. 2,4,6-Tribromo 3-hydroxy benzoic acid at a final concentration of 10mM replaced the N,N Bis(hydroxyethyl) aniline as chromagen and the cysteine concentration was varied to give three levels of $5.0mg.ml^{-1}$, $7.25mg.ml^{-1}$ and $11.15mg.ml^{-1}$. The filter paper used was Whatman 541 grade or Whatman 54 grade and the resulting dry papers were overlaid with 1% ethyl cellulose in toluene as before. The dry papers were then cut into 1.5 mm strips using a new scalpel blade and mounted to a strip of double sided tape, (Sellotape) which in turn was mounted onto a white adhesive label, (Lakeland Plastics). Three enzyme paper strips each of the different cysteine levels comprised the analytical element. This was then cut across the strips to give rectangular pads of 4mm x 7.5mm which were mounted on printed card backings, the lowest cysteine concentration at the top. Each whole pad was then covered by a 10mm square of

FIG.18a DRY ALCOHOL TEST. ORIGINAL FORMAT.



The enzyme discs were mediated to give a positive response at ethanol levels of $500mg.l^{-1}$. and $800mg.l^{-1}$.
The card support was absorbant in nature and was designed to remove excess sample away from the enzyme discs.

FIG.18b DRY ALCOHOL TEST. PEEL OFF FORMAT.



Sample was applied to the membrane surface and after development (2 mins.) the tab was peeled off. Development of colour on one bar indicates an ethanol concentration of $300mg.l^{-1}$, two bars $500mg.l^{-1}$ and three bars $800mg.l^{-1}$ and above.

polycarbonate membrane containing 0.4 μ pores, (Nucleopore) mounted over a window, (5mm x 8mm) cut in the centre of a white adhesive label, fig 18b. Light pressure on the membrane covered pad ensured an intimate contact between the membrane and the analytical element underneath. The completed tests were foil packed with a silica gel sachet and heat sealed.

The tests were developed by application of ethanol standards or saliva samples to the membrane area. After 2 minutes the top adhesive label was peeled off. The results were interpreted by the number of red coloured bars present as follows, top only 300mg.l^{-1} ethanol, top and middle bars 500mg.l^{-1} ethanol and all three bars 800mg.l^{-1} ethanol and above.

2.L.6. Dry Enzyme Tests. Artificial Saliva Standards.

Ethanol standards used to develop and pre-set the threshold levels on the dry ethanol tests were prepared by adding set volumes of 10g.l^{-1} ethanol to a modified artificial saliva preparation, (Wiesenfield et al 1983). The composition is given in table 13.

2.M.1. Synthesis of N-Ethyl, N-Sulphopropyl 3-Toluidine.

The method of Tamaoku et al (1982) was followed with slight modifications. 12.2g, (0.1mol) of 1,3-propane sultone in 50 ml of propan-2-ol was added to a solution of 13.5 g, (0.1mol) of N-ethyl 3-toluidine in propan-2-ol and refluxed for 3 hours. The mixture was cooled, neutralised with sodium hydroxide solution and evaporated to dryness over gentle heat. The dry pale grey residue was dissolved in a minimum of water and acetone was added. The mixture was allowed to recrystallize and the off white residue was filtered off and dried in a vacuum oven.

Table 13. Artificial Saliva. Composition.

Potassium chloride	0.624g
Sodium chloride	0.865g
Magnesium chloride 6 H_2O	0.059g
Calcium chloride 2 H_2O	0.166g
Di-Potassium hydrogen orthophosphate	0.804g
Potassium di-hydrogen orthophosphate	0.326g
Methyl p-hydroxybenzoate	1.000g
Sorbitol	29.950g
Sodium carboxymethylcellulose	10.000g
Water	up to 1.0 l

2.M.2. Synthesis of N-Alkyl N-(2-Hydroxy 3-Sulphopropyl) Aniline Derivatives.

The method of Tamaoku et al (1982) was followed. N-ethyl, N-(2-hydroxy 3-sulphopropyl) 3-toluidine; N,n-butyl, N-(2-hydroxy 3-sulphopropyl) aniline; N-2 hydroxyethyl, N-(2-hydroxy 3-sulphopropyl) aniline and N-2-aminoethyl, N-(2-hydroxy 3-sulphopropyl) aniline were prepared by the following general method. 50 millimoles of sodium hydroxide, (2.0g) was added to 50 millimoles of sodium 3-chloro 2-hydroxy propane sulphonate, (9.8g) in 100ml of distilled water. This was added to 50 millimoles of the N-alkyl aniline, (or toluidine) in propan 2-ol. The mixture was heated during the addition and then refluxed for 3 hours. The majority of the propan-2-ol was then distilled off and the residual solution was cooled and extracted twice with diethyl ether. The water was then evaporated off in a shallow crystallization dish in an air stream and the damp residue dried in a vacuum oven for 2-3 hours.

2.M.3. Synthesis of Dialdehyde Dextran.

The method of Onyezili and Onitiri (1981) was followed with modifications. 8g of Sigma dextran D-4626, mol.wt. 17,900 was dissolved in 250 ml distilled water and cooled in ice. 200 ml of 5.25% w/v sodium periodate, (BDH) was added dropwise with stirring the temperature being maintained at 25°C or less. The mixture was stirred overnight, (16 hours) in the dark and the mixture was added to 500 ml of ethanol and shaken vigorously to precipitate the dialdehyde dextran formed. This was redissolved in distilled water and reprecipitated with acetone, (1.01) and collected by filtering through two layers of muslin. The precipitate was washed with 0.1M NaCl, redissolved and reprecipitated twice more with acetone to give

an off white amorphous solid. This was redissolved in a minimum of water and vacuum dried to give a white powder.

2.M.4. Synthesis of Aminodextran.

Two methods were used to synthesise aminodextran.

(i) The method of Onyezili and Onitiri (1981) was followed with modifications. One gramme of dialdehyde dextran prepared as in section 2.M.3. was dissolved in 200mM borate buffer pH 8.45, and 1.75g of diaminoethane, (Sigma) was added and the mixture stirred for 2 hours at 20°C. The aminodextran was precipitated with propan-2-ol as for the dialdehyde dextran to give an off white solid which was dried in a vacuum oven to give a white powder.

(ii) The method of Jellum et al (1973) was followed with modifications. 10g of Sigma dextran D-4626 mol.wt. 17,900 was dissolved in 23.0ml of distilled water containing 9.23g sodium hydroxide and 3.07g 2-aminoethyl hydrogen sulphate,(Sigma) to give a thick yellow syrup. This was placed in a shallow glass or ceramic dish and heated at 115°C in an oven overnight. The dry residue was dissolved in a minimum volume of hot distilled water, (50-60°C) with stirring and added to 900ml of propan-2-ol in a wide mouthed bottle which was vigorously shaken. The aminodextran precipitated as a yellowish sticky mass. This was redissolved in a minimum of water and the process repeated to give a creamy white sticky precipitate which was dissolved in a minimum of water and vacuum dried to give a creamy white amorphous solid.

2.M.5. Synthesis of Mercaptodextran.

The method of Jellum et al (1973) was followed with modifications, 10g of aminodextran, (section 2.M.4, method ii) was dissolved in 200ml of distilled water and the pH was adjusted to 7-8

with glacial acetic acid. 1.92g of N-acetyl homocysteine thiolactone was added and 2.0g silver nitrate in 25ml of distilled water was added dropwise with stirring. The pH was maintained at 7.6-8.0 by concomitant addition of 1M sodium hydroxide solution. After addition of the silver nitrate the reaction mixture was stirred for a further 2 hours at room temperature. The pH was maintained at 7.8 with 1M sodium hydroxide solution. Thiourea was then added to saturation, (40g) and the pH adjusted to 1-2 with concentrated nitric acid. The mixture was then filtered through a column, (4cm x 50cm) of coarse sephadex G-25, (Pharmacia) pre-equilibrated with distilled water. The fraction containing mercaptodextran appeared in the void volume and the solid product was precipitated by addition of 9 volumes of acidified, (HCl) propan-2-ol and vigorous shaking. The white sticky precipitate was redissolved in a minimum of distilled water and dried in a vacuum oven at 30°C to give a white crystalline solid.

2.M.6. Synthesis of Pectinamine.

1.0g pectin, (methoxy content approx. 60%) was dissolved in 50 ml distilled water to give a 2% w/v solution and cooled to 4°C. 25 ml of 4M, 1,3-diamino 2-hydroxy propane, (9.03g) was added slowly with stirring and the solution was stirred for 4 hours at 4°C. The pectinamine was precipitated from solution by pouring into ice cold acetone, (9 volumes) and the jelly like precipitate was filtered off using muslin, redissolved and reprecipitated as before. Excess liquid was removed from the precipitate by squeezing between layers of filter paper and then drying in a vacuum oven for several hours at 35°C. The grey, brown flaky product weighed 1.037g.

CHAPTER 3.

MANUAL ASSAY DEVELOPMENT using ALCOHOL OXIDASE and GLUCOSE OXIDASE.

3.A.1. Alcohol Oxidase.

Alcohol oxidase is a large flavoprotein of about Mr 600,000 composed of eight subunits. It is produced in several species of methylotrophic yeasts including Hansenula, Pichia, Torulopis and Candida. The enzyme accepts short chain aliphatic alcohols as substrates, utilising molecular oxygen to oxidise them to the corresponding aldehydes and hydrogen peroxide. The enzyme has been used to assay alcohols, produce hydrogen peroxide "in situ" in detergents and has been proposed as the active agent in antimicrobial preparations. For a detailed account of the biochemistry and applications of the enzyme see Woodward (1990).

3.A.2. Alcohol Oxidase. Commercial Availability.

Commercially available alcohol oxidase is isolated from Pichia or Candida species. Provesta, a subsidiary of Phillips Petroleum, manufacture and market an enzyme isolated from Pichia pastoris, which is supplied frozen in concentrated sucrose solution or as an isoelectric precipitate in concentrated sucrose. Both forms of the enzyme are azide treated and are bright red in colour. Freeze dried preparations are available from a number of suppliers including Boehringer and Sigma. Usually they are stabilised with an excess of a non-volatile peroxide scavanger such as glutathione, as the enzyme has a tendency to spontaneously generate hydrogen peroxide, probably from formaldehyde which is bound as an adduct to the free amino groups of the enzyme, (Hopkins and Mueller 1987). Hansenula alcohol oxidase is not available commercially.

3.A.3. Alcohol Oxidase. Hansenula Polymorpha.

The enzyme prepared from Hansenula Polymorpha, (section 2.B.1) was yellow to deep orange red in colour depending on the

concentration. It had a high activity over a broad pH range from about 6.0 to 10.5 and was maximally stable above pH 7.5. The substrate specificity was found to be relatively narrow being in the order of methanol › ethanol › propan-1-ol › butan-1-ol, table 14.

Table 14. Substrate Specificity. Hansenula Alcohol Oxidase

Substrate.	% Activity relative to Methanol
Methanol	100
Ethanol	55.2
Propan-1-ol	20.7
Butan-1-ol	12.1
Propan-2-ol	-
Allyl alcohol	31.0
Benzyl alcohol	-
2 Methyl butan-1-ol	-
3 Methyl butan-1-ol	-
2 Methyl propan-1-ol	-
2 Phenylethanol	-
Ethyl acetate	-
Isoamyl acetate	-
Acetaldehyde	-
Formaldehyde	5.2
Acetic acid	-
Acetone	-

The temperature profile was found to be different from other sources the enzyme being maximally active at 40-42°C. Also the enzyme

is less susceptable to inhibition by halides than those from other sources, (Woodward 1990).

The enzyme obtained by ion exchange chromatography was sufficiently pure to use in the alcohol detection assays that form the basis of this work. SDS electrophoresis of the purified protein showed one major band corresponding to the subunit of Mr 73-74 x 10^3. Further purification using gel filtration chromatography on Pharmacia CL-4B sepharose and Pharmacia sephacryl S-200 columns did not improve the purity appreciably, fig. 19. The major contaminant was catalase, however this was present at levels less than 1U.ml^{-1} in fresh preparations, which decreased rapidly on storage.

3.A.4. Ethanol Assay. ABTS.

This assay system was very sensitive and tended to develop high backgrounds, probably due to low basal rate of hydrogen peroxide production from the alcohol oxidase. Absorbance was linear up to 200 mg.l^{-1} at 650nm or 75mg.l^{-1} at 410 nm. Limit of sensitivity was about 2mg.l^{-1}, graph 11.

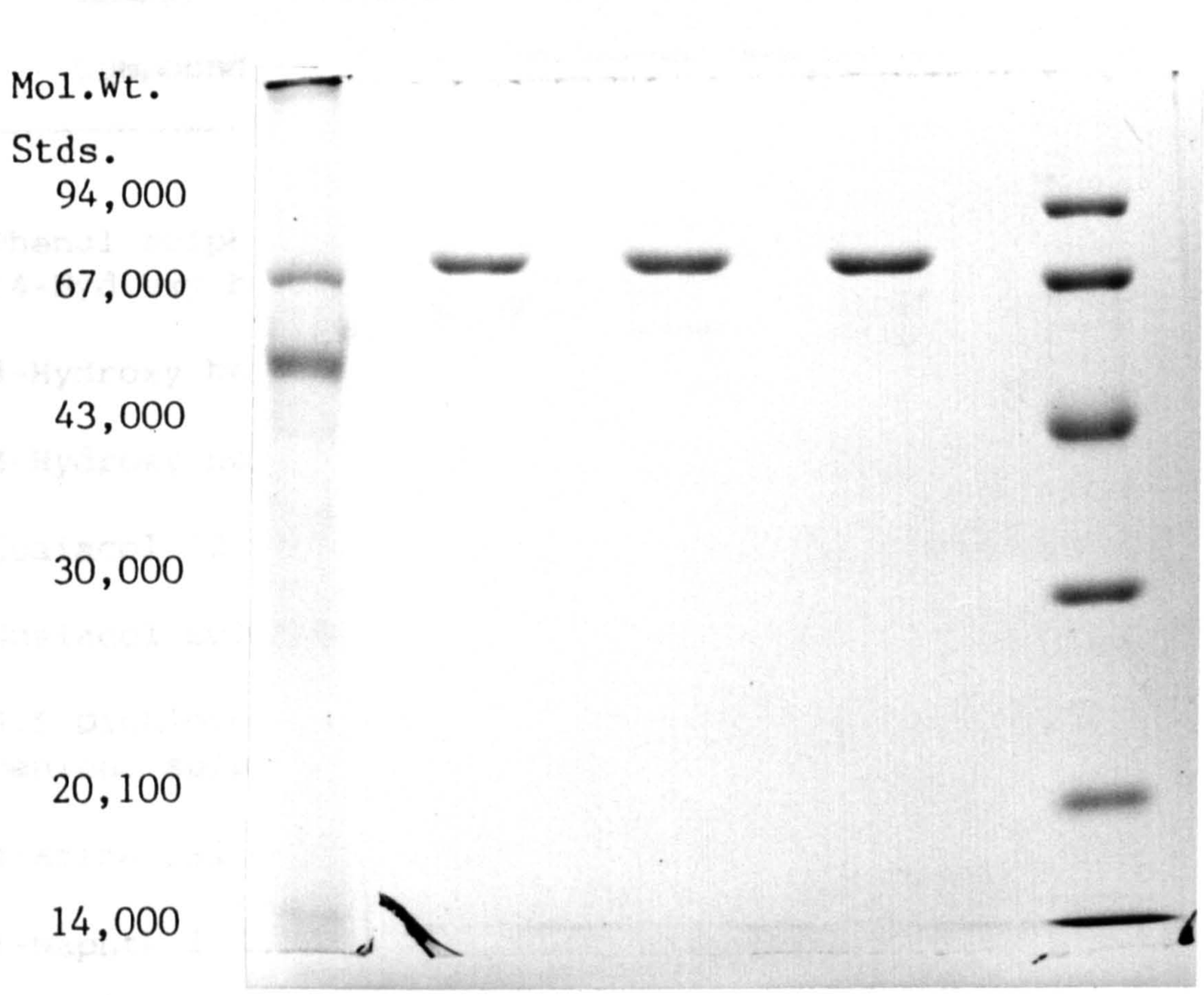
3.A.5. Ethanol Assay. MBTH and Colour Couplers.

Attempts to use the colour development system of Ngo and Lenhoff (1980) comprising MBTH and 3-dimethylamino benzoic acid to assay ethanol were unsuccessful. The colour regents were so sensitive that upon addition of the enzymes a deep purple colour resulted. The use of this method in wet tests for ethanol was rejected.

3.A.6. Ethanol Assay. 4-Aminoantipyrine and Colour Couplers.

The assay system of 4-aminoantipyrine and phenol was one of the first to be used to measure oxidative enzyme assays by Trinder (1969). Since phenol is very toxic, several substituted hydroxy aromatics were tested for use as colour couples, table 15.

FIG.19 SDS ELECTROPHORESIS ALCOHOL OXIDASE.



LANE 1. Crude Cell Free Extract. 10 µg Protein.

LANE 2. Fraction off DEAE Sepharose Fast Flow Column.

LANE 3. Fraction off Sepharose CL-4B Column.

LANE 4. Fraction off Sephacryl S-200 Column.

The fractions from CL-4B and S-200 steps represent further purification steps of the enzyme obtained from DEAE Fast Flow.

LANE 5. Low Molecular Wt. Standards.(Pharmacia). Weights given at left of gel.

(Lanes 2-4 run with 5.0 µg Protein).

Table 15. Colour Couplers for Ethanol Assay.

Compound.	Wavelength of Measurement.nm.	Colour.
Phenol sulphonic acid (4-Hydroxy benzene sulphonic acid)	500	Red
4-Hydroxy benzoic acid	500	Red
3-Hydroxy benzoic acid	490	Orange-Red
Guaiacol (2-Methoxy phenol)	500	Red
Guaiacol sulphonic acid	500	Red
3,5-Dichloro 2-hydroxy benzene sulphonic acid	520	Pink-Red
4-Amino salicylic acid	470	Orange
1-Naphthol 4-sulphonic acid	500	Red
1-Naphthol 3,6-disulphonic acid	550	Purple
3-Hydroxy 2-naphthoic Acid	410	Green
8-Hydroxy quinoline 5-sulphonic acid	500	Red

The sodium salt of phenol sulphonic acid, (4-hydroxy benzene sulphonic acid) was chosen as the preferred reagent on the basis of low backgrounds, high stability of the assay system, purity, availability and cost.

The red quinoneimine dye formed has an absorption maximum at 500nm, and appears to be identical to that produced from phenol and 4-AAP in character, (Artiss et al 1981).

3.A.7. Ethanol Assay. Optimisation of Conditions.

(a) Alcohol Oxidase.

The initial aim of the wet assay system was to produce a linear response to ethanol concentrations up to $5g.l^{-1}$ within 5 minutes incubation time, between 20-25°C, (rapid assay). An enzyme concentration of between 0.5 - $1U.ml^{-1}$ was found to achieve this. For lower concentrations of ethanol using an end point assay up to $5U.ml^{-1}$ was used, graph 17.

(b) Peroxidase.

Peroxidase concentrations between 0.1 to $3.0U.ml^{-1}$ were tested and reaction rates were measured, graph 18. Peroxidase concentrations were set at $1.0U.ml^{-1}$.

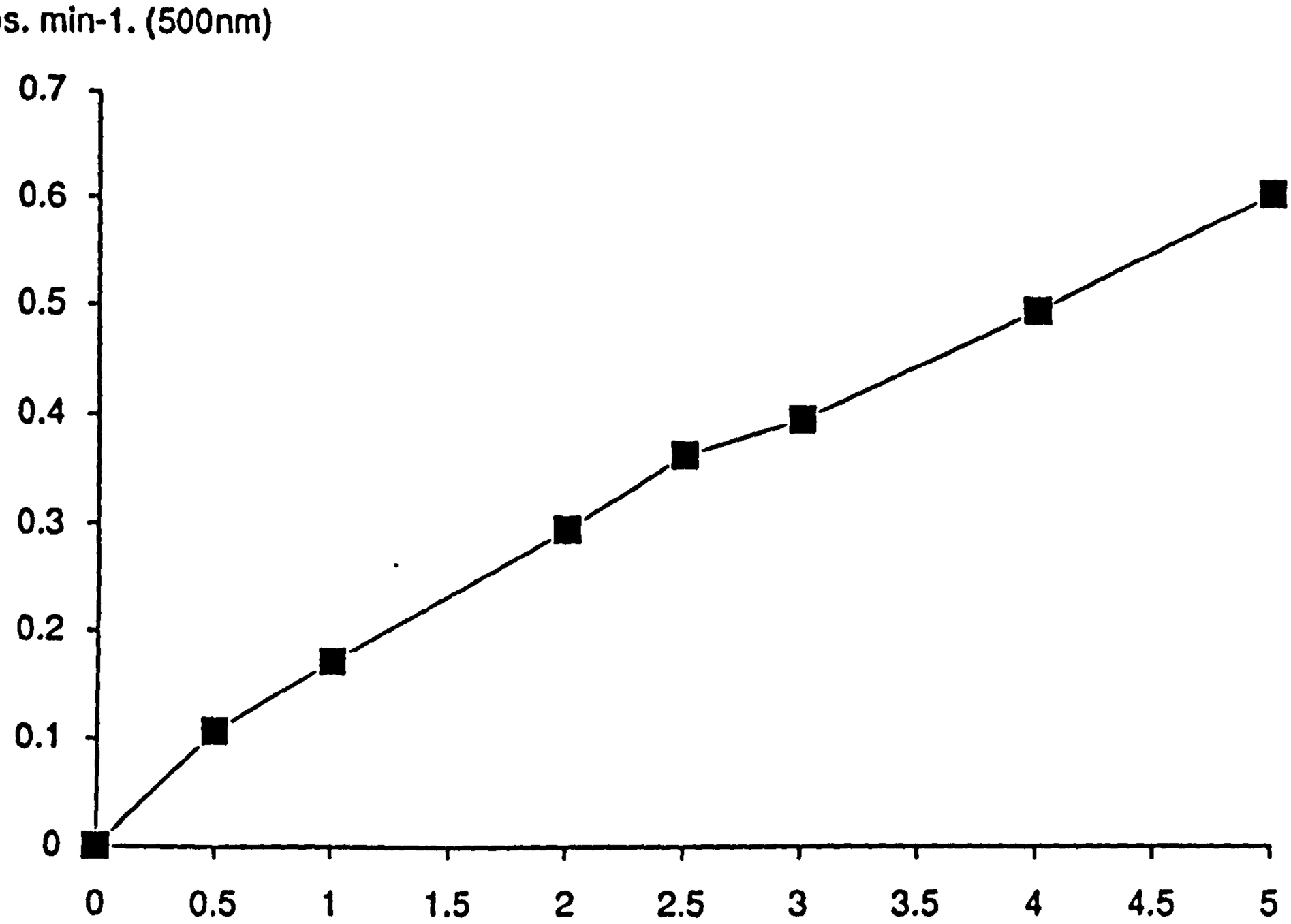
(c) 4-Aminoantipyrine.

The concentration range of 4-AAP tested in the assay was between 0.1 to 4.0mM, with a range of ethanol concentrations between 1 to 5 $g.l^{-1}$, graph 19. 4-AAP concentration was standardised at 0.4mM.

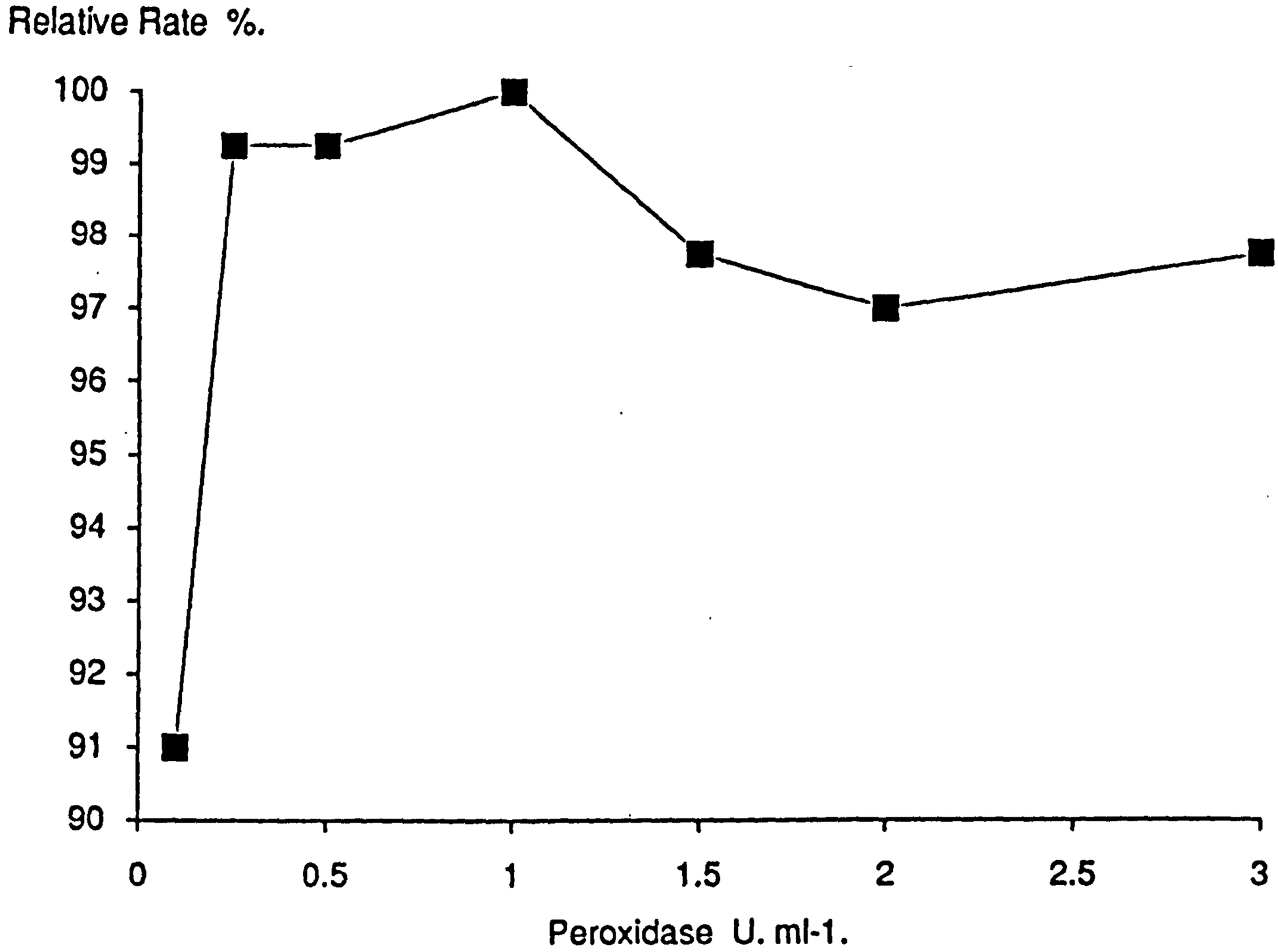
(d) Phenolsulphonic Acid. Na Salt.

Concentrations between 1 to 50mM were tested with a range of ethanol concentrations between 1 to $5g.l^{-1}$, graph 20. Phenolsulphonic acid concentration was standardised at 25mM.

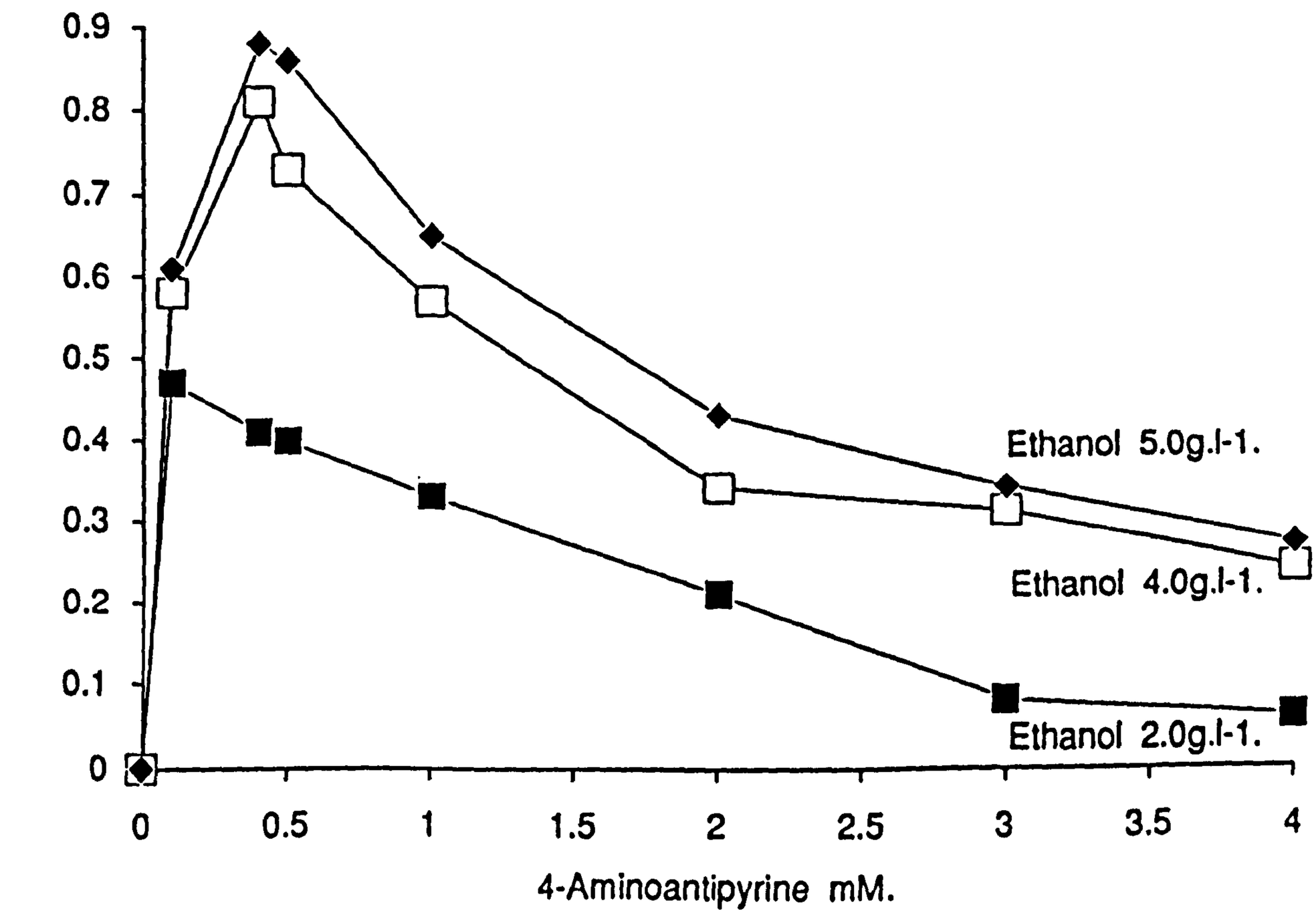
Graph 17. ETHANOL ASSAY OPTIMISATION. Vary Alcohol Oxidase.



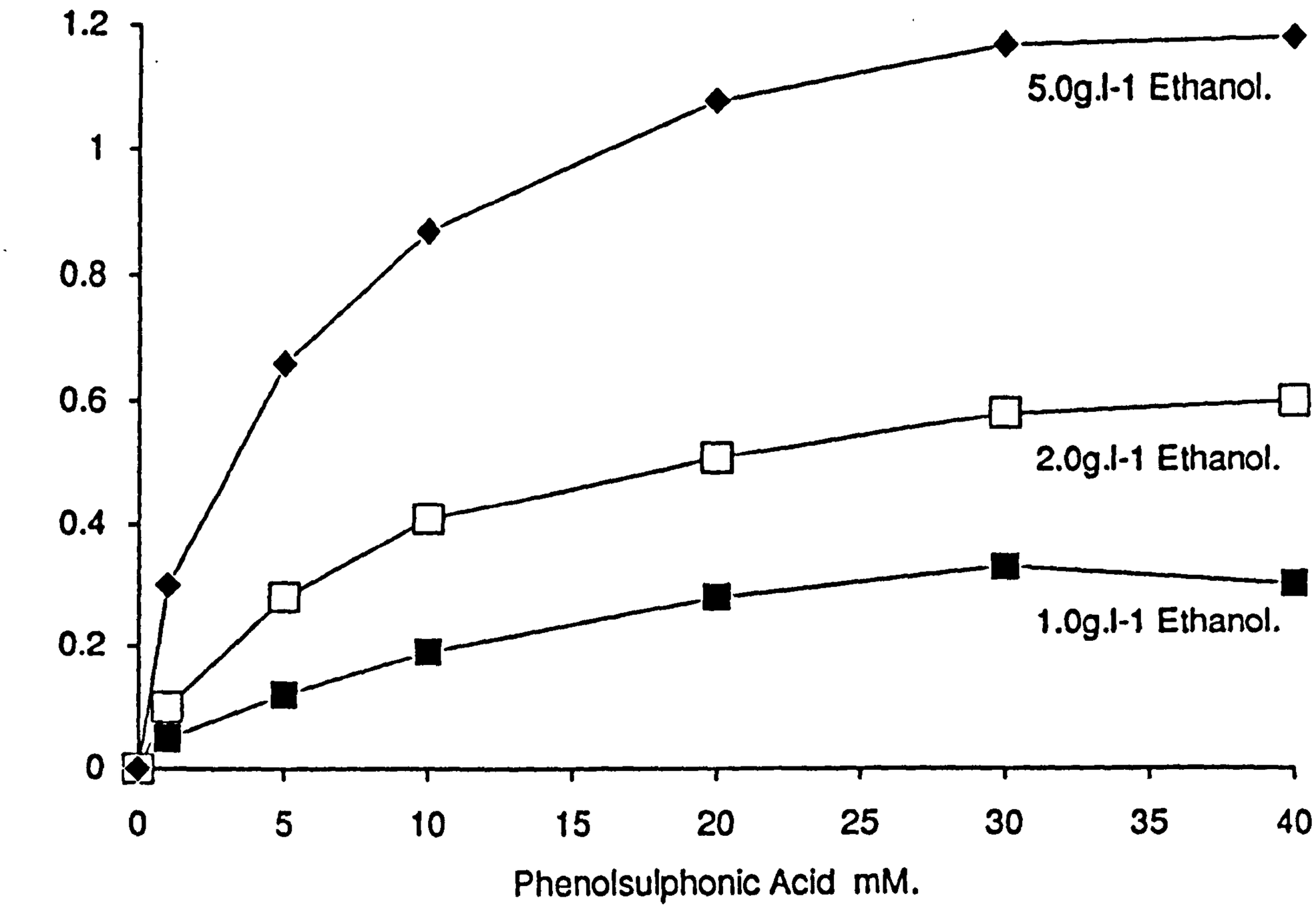
Graph 18. ETHANOL ASSAY OPTIMISATION. Vary Peroxidase.



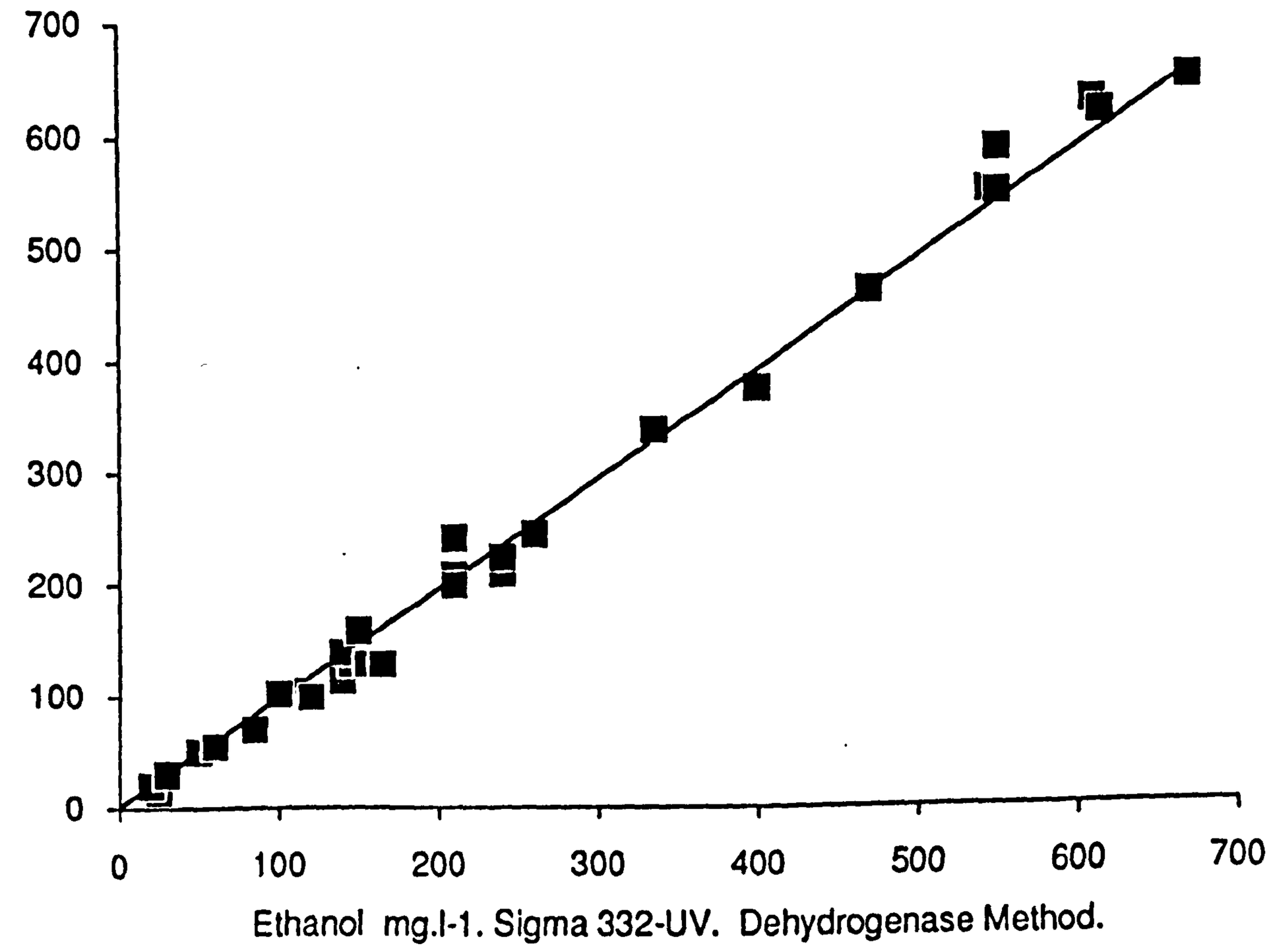
Graph 19. ETHANOL ASSAY OPTIMISATION. Vary 4-Aminoantipyrine.



Graph 20. ETHANOL ASSAY OPTIMISATION. Vary Phenolsulphonic Acid.



Graph 21. ETHANOL. Regression Curve.



Standard wet assay reagent. Rapid ethanol assay.

Alcohol oxidase (Hansenula) *	0.5-1.0U.ml^{-1}
Peroxidase. (Horseradish)	1.0U.ml^{-1}
Phenolsulphonic acid. Na salt.	25.0mM.
4-Aminoantipyrine.	0.4mM.

in phosphate buffer 100mM. pH 7.0.

* Up to 5U. ml^{-1} for the end point assay.

3.A.8. Ethanol Assay.Comparison with Standard Assay.

The standard wet assay reagent, (up to 700mg.l^{-1} ethanol) was compared to a standard alcohol dehydrogenase assay kit obtained from Sigma, (No.322-UV). Aqueous ethanol samples were analysed by both methods. The regression line is shown in graph 21. The correlation coefficient (r) was 0.993 and the slope was 0.994.

3.A.9. Application of Ethanol Assay to Beer Analysis.

The standard assay method was used to analyse samples taken from an alcoholic fermentation using a home brew kit, (Tom Caxton Lager). Samples were withdrawn at intervals, diluted if necessary and assayed. The results are shown in table 16.

Table 16. Analysis of Home Brew Fermentation.

Time (hours)	Dilution	Absorbance	Ethanol.g.L
0	-	0.052	-
3.42	-	0.280	0.90
6.17	-	0.620	3.00
24.00	x10	0.490	25.30
29.00	x10	0.540	29.00
47.83	x20	0.400	41.00
54.34	x20	0.470	48.00
79.17	x20	0.500	55.00

3.A.10. Alcohol Oxidase. Analysis of Methanol.

The standard assay method may be used to assay methanol using a sample volume of 25 ul. The results are shown in graph 22.

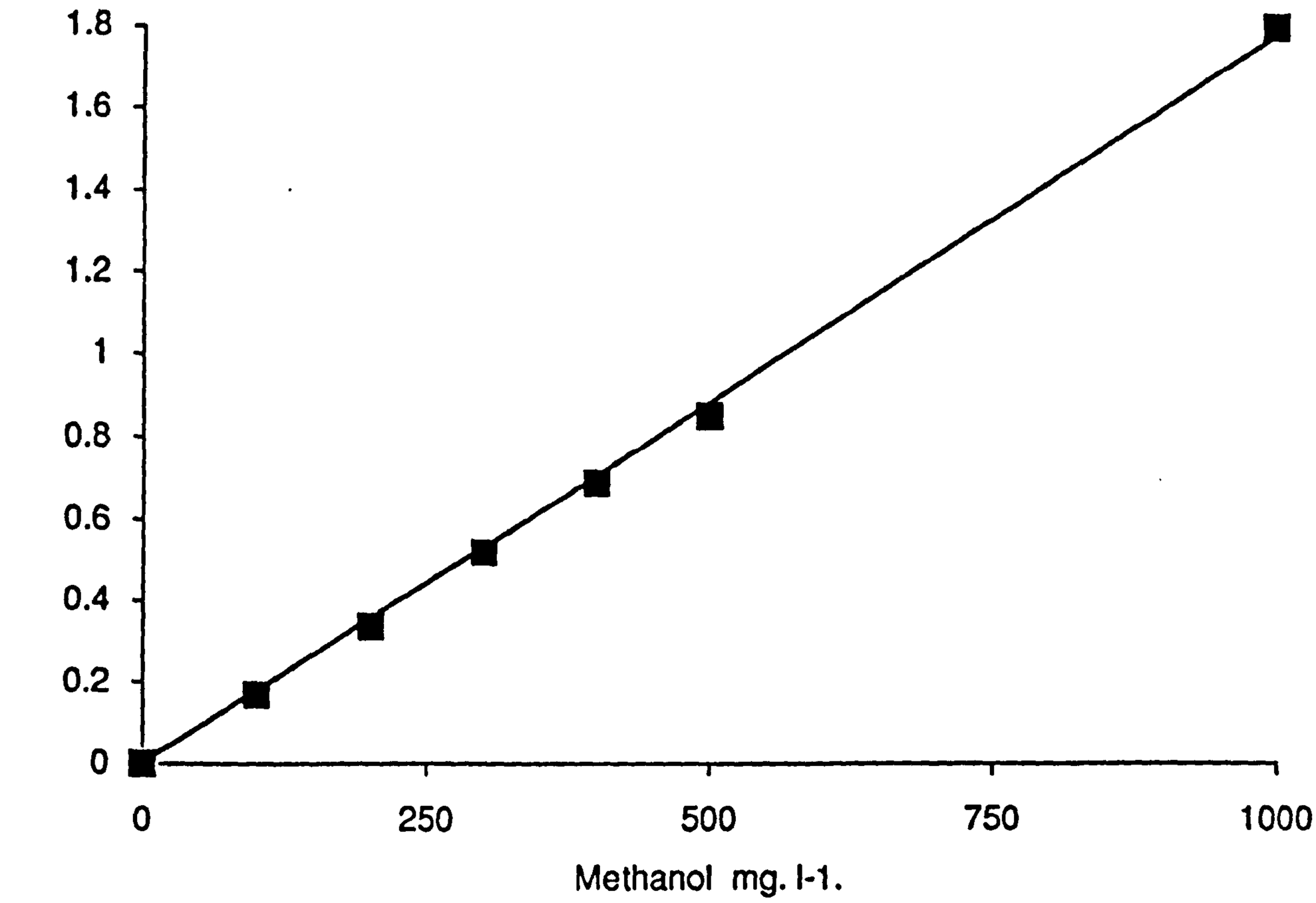
3.B.1. Glucose Oxidase.

Glucose oxidase and its use in analytical assays is well documented, (Trinder 1969, Barham and Trinder 1972, Bergmeyer and Bernt 1973, Carey et al 1974). Standard glucose assays typically measure clinical levels of glucose, 2.5mM - 5.3mM (0.45g.l^{-1} - 0.95g.l^{-1}), however higher levels usually require dilution. The following section reports an attempt to use glucose oxidase to measure levels of glucose up to 25g.l^{-1} without dilution.

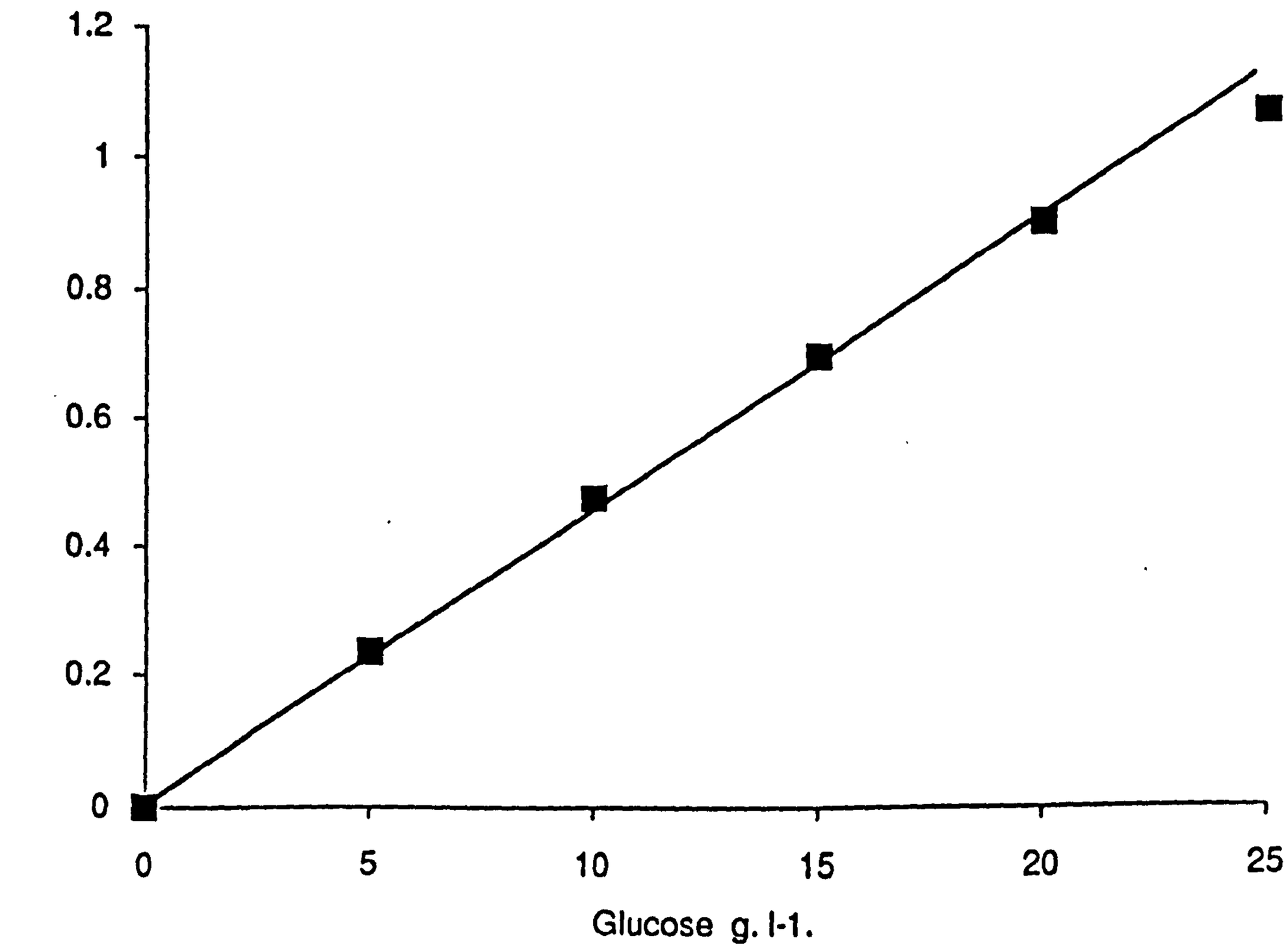
3.B.2. High Concentration Glucose Assays.

The components of the standard ethanol reagent were used with 100mM phosphate buffer, pH 6.0 and alcohol oxidase being replaced by glucose oxidase at 1U.ml^{-1}. Aqueous standards of glucose, (100 µl) were added to 5.0 ml of reagent at 25°C, incubated for 5 minutes and the absorbance read immediately at 500nm, graph 23. The same reaction mix could be used to measure clinical levels of glucose, however enzyme levels up to 10U.ml^{-1} were preferable to reduce the reaction time. Use of the redox indicators ABTS and sodium diphenylamine sulphonate were useful for clinical levels, (graphs 11 and 12) but were too sensitive for high levels of glucose.

Graph 22. METHANOL. 4-Aminoantipyrine/Phenolsulphonic Acid Detection.



Graph 23. GLUCOSE. High Concentration Method.



CHAPTER 4.

AUTOMATED ASSAYS using OXIDASE ENZYMES.

4.A.1. Ethanol Analysis using Segmented Flow. Soluble Enzyme Method.

The wet standard assay for ethanol was adapted for the Technicon AutoAnalyzer as described in materials and methods 2.I.1. Typical traces are shown in figs 20-22. Successive aspirations of 2g.l^{-1} standards, (n = 16) gave a coefficient of variation of 1.52%. The reagents were stable for 1 month if stored at 4°C , protected from light.

4.A.2. Ethanol Analysis using Segmented Flow. Immobilised Method.

The immobilised enzyme flow system gave results similar to using soluble enzyme, figs 23-25. The advantage was primarily in terms of the smaller amount of enzyme used, (between 0.46 and 3.25 units against 255 units for 1600 assays). Loading was between 0.92 - 7.21 U.m^{-1} of tubing depending on which spacer molecule was used. The highest activities were recorded with polyamine dextran and pectinamine spacers followed by adipic acid dihydrazide and 1,2-diamino ethane. Also the stability of the immobilized enzyme was higher, (a minimum of 2 months at 4°C) provided the coil was stored wet and the buffer changed periodically. " In use " stability for various amine spacer molecules is shown in graph 24. The effect of ascorbic acid on the response was also estimated, table 17. Successive aspirations of 2g.l^{-1} standards, (n = 16) gave a coefficient of variation of 1.20%.

4.B.1. Glucose Analysis using Segmented Flow. Immobilised Method.

Glucose oxidase, (Sigma Type X) immobilised onto nylon was used to assay glucose using the same reagents and the same flow chart as the alcohol method in 4.A.2. A linear response up to 3.0g.l^{-1} was observed using this system. The stability of the immobilised enzyme was very high, no activity loss being observed after 12 days

FIG.20 SEGMENTED FLOW ANALYSIS TRACE.
ETHANOL 0.5-5.0g.l^{-1}.

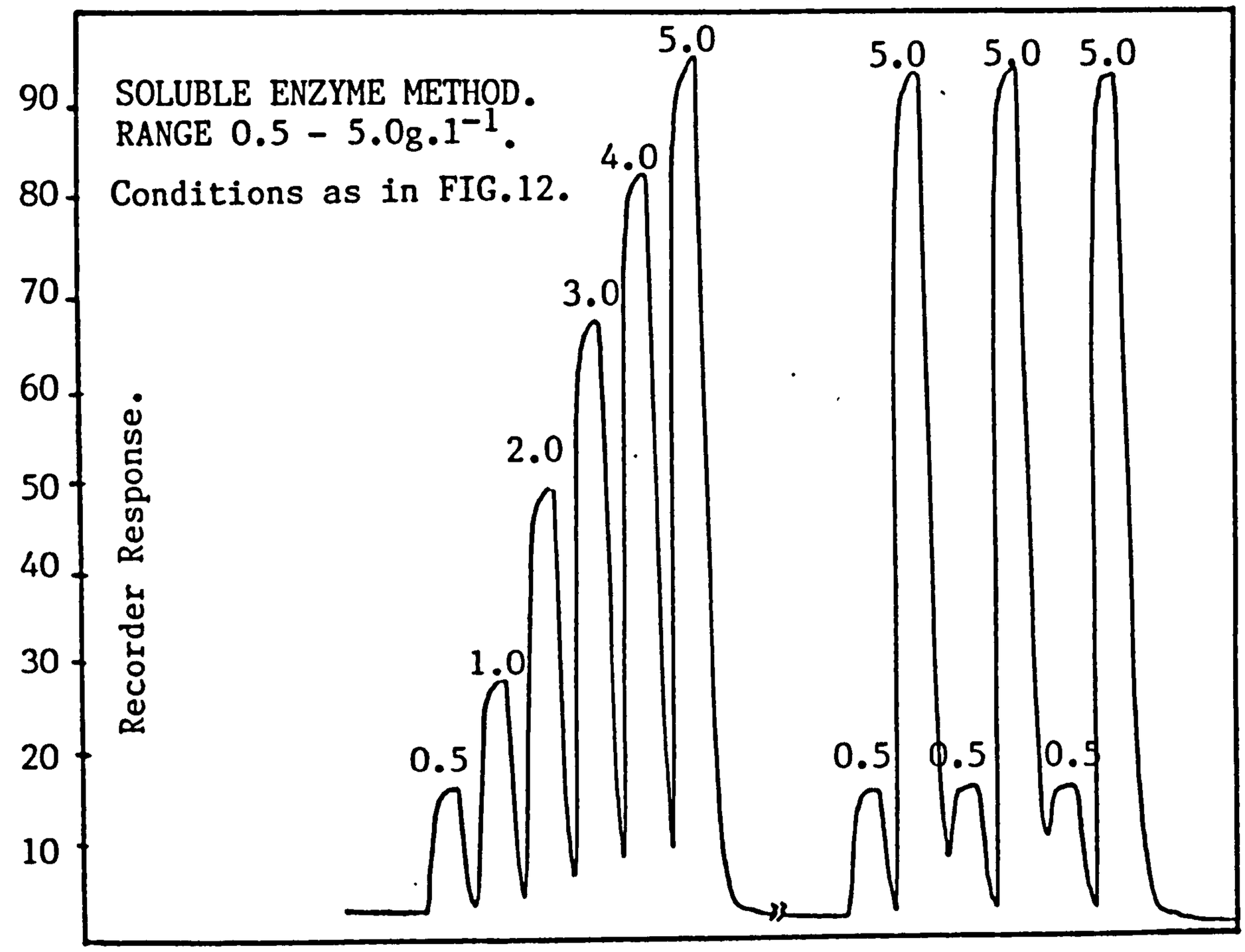


FIG.21 SEGMENTED FLOW ANALYSIS TRACE.
ETHANOL 50-500mg.l^{-1}.

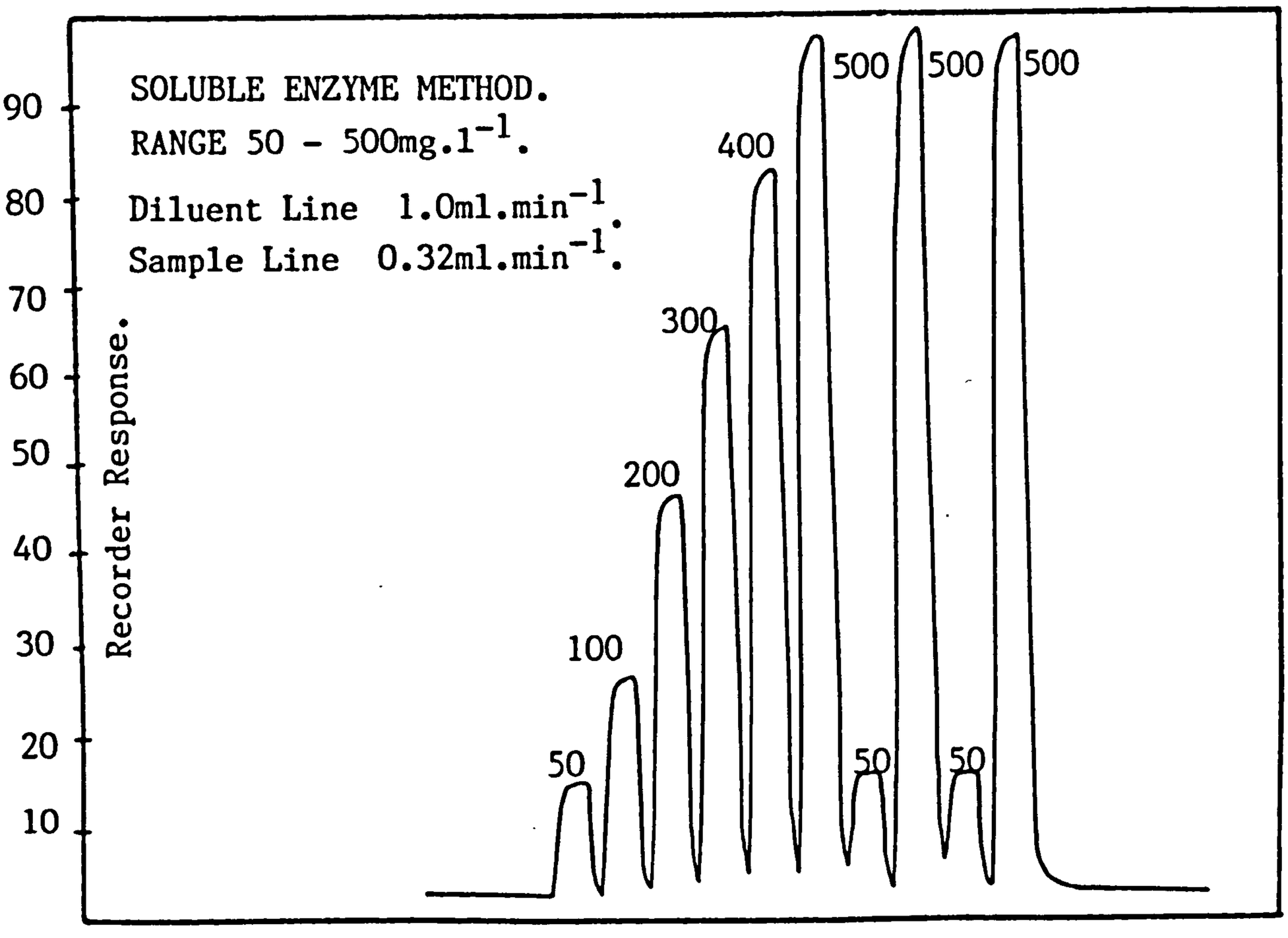


FIG.22 SEGMENTED FLOW ANALYSIS TRACE.
ETHANOL 5-100mg.l^{-1}.

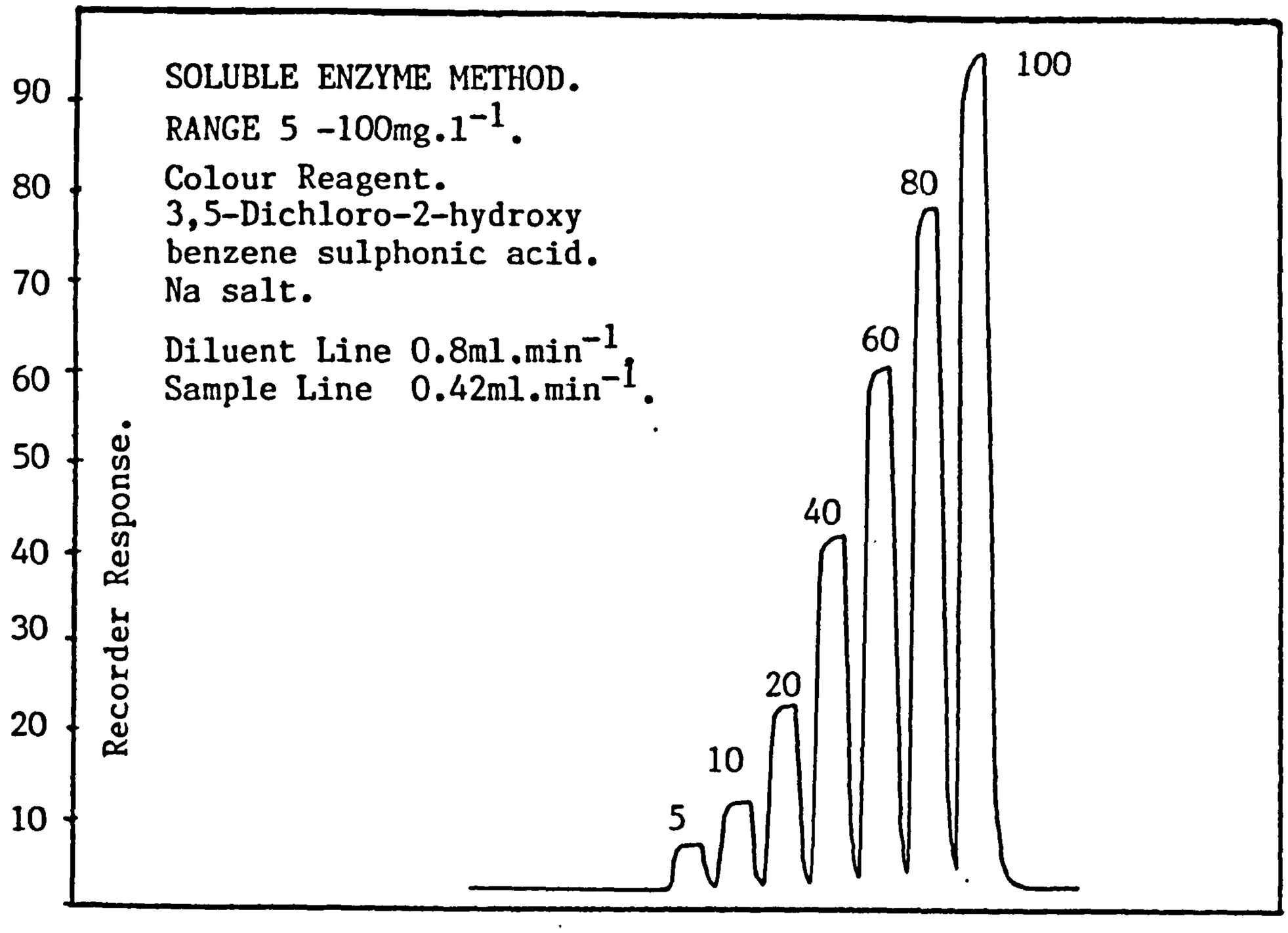


FIG.23 SEGMENTED FLOW ANALYSIS TRACE.
ETHANOL 0.5-5.0g.l^{-1}.

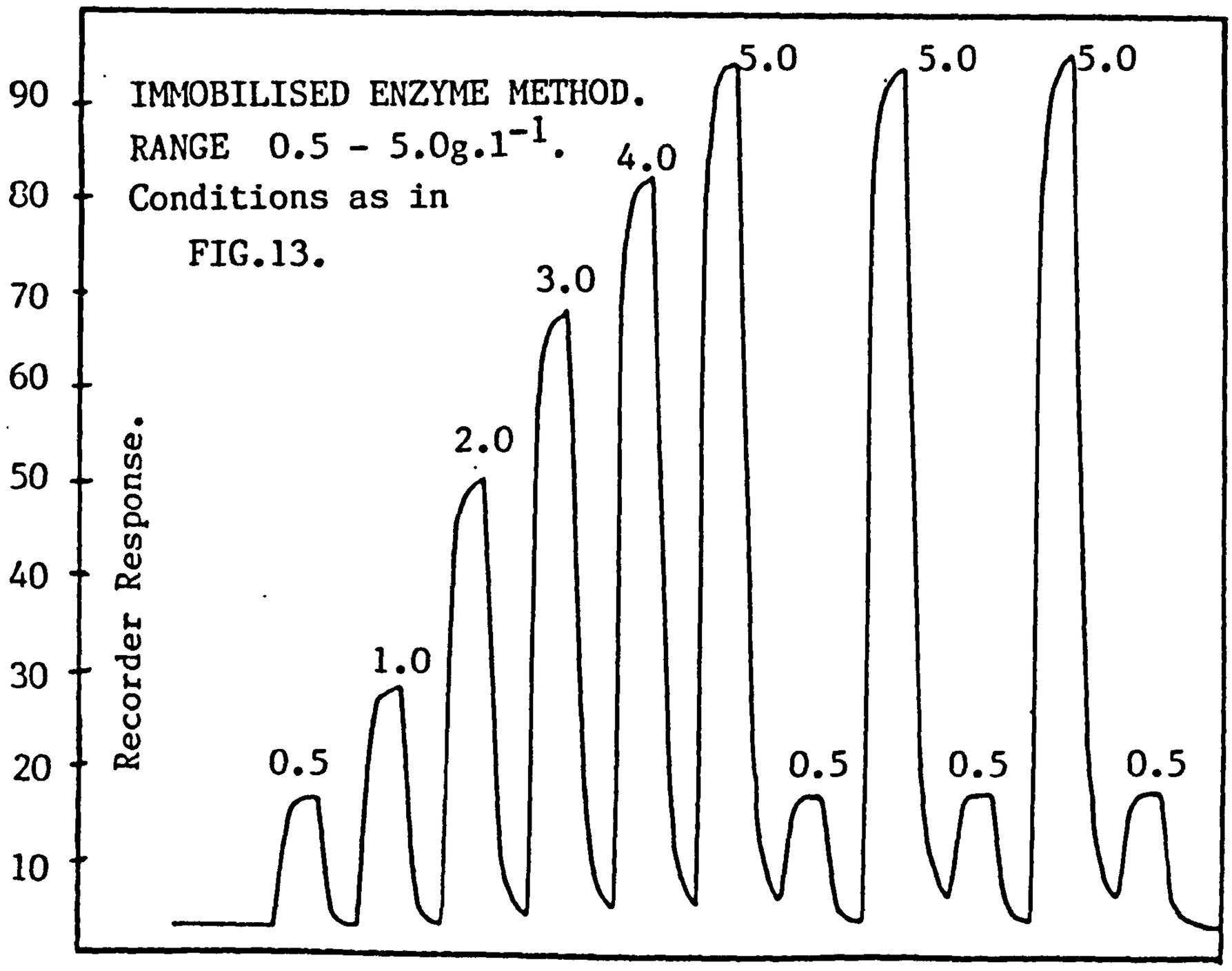


FIG.24 SEGMENTED FLOW ANALYSIS TRACE.
ETHANOL 50-500mg.l^{-1}.

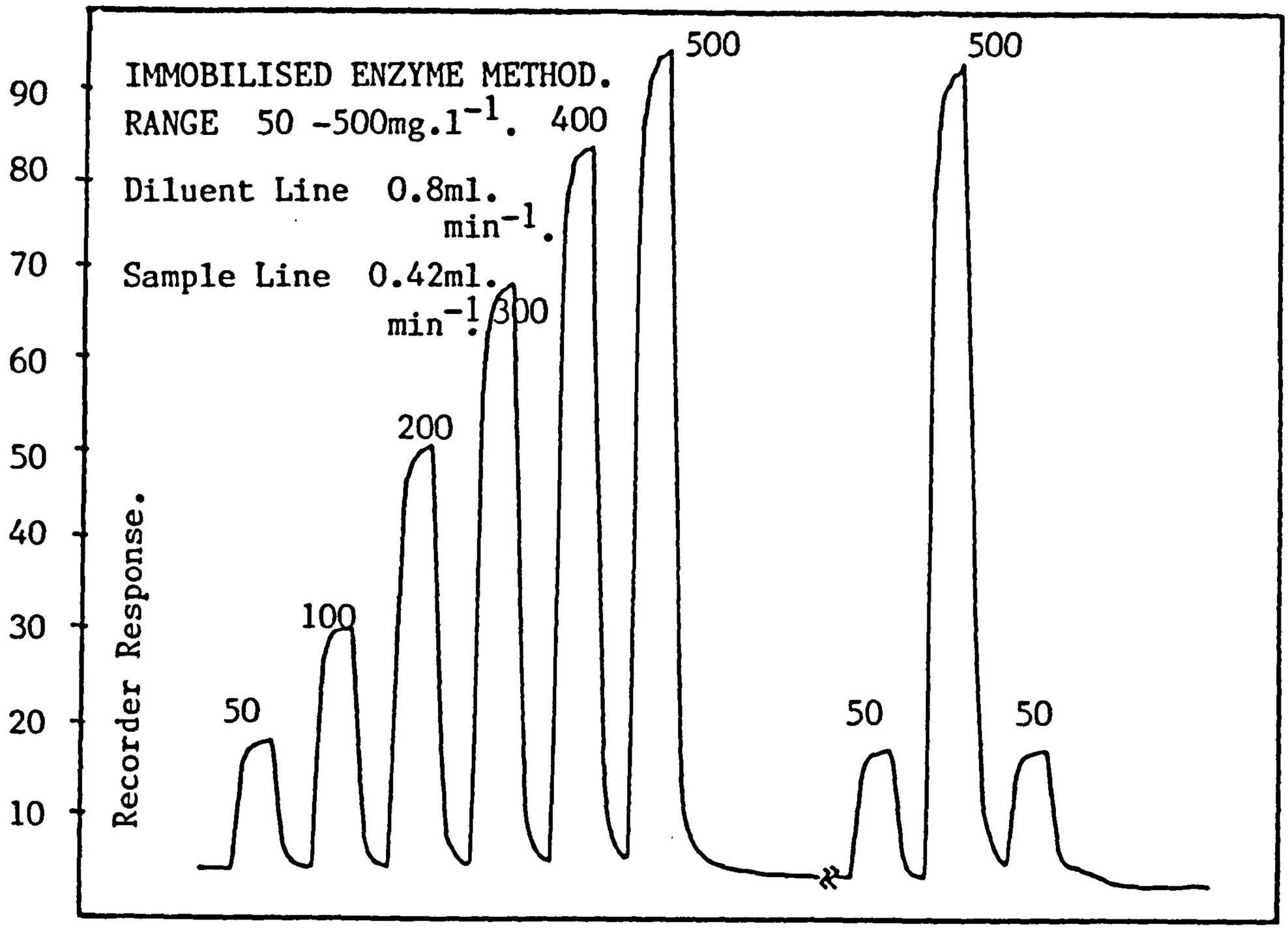
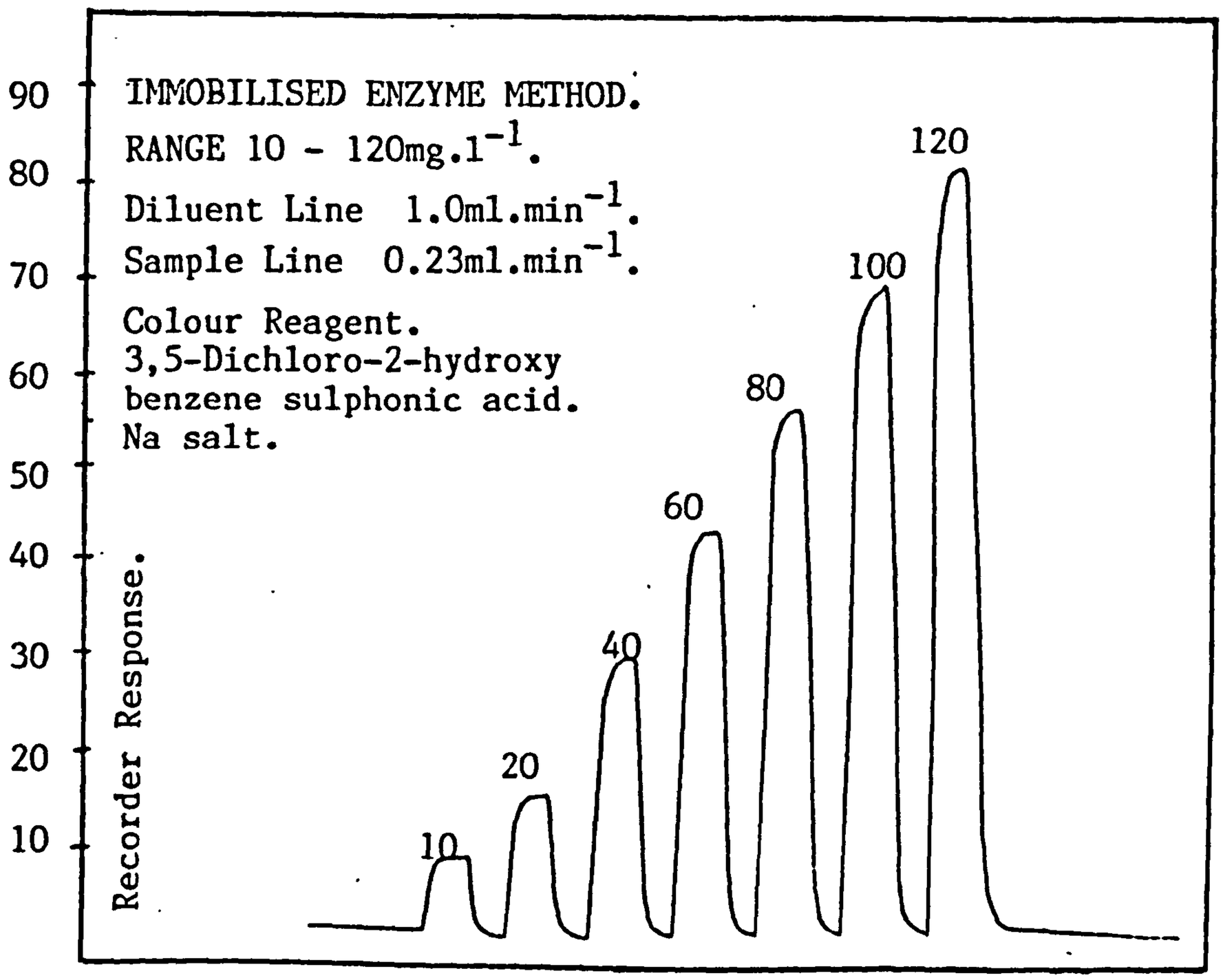


FIG.25 SEGMENTED FLOW ANALYSIS TRACE.
ETHANOL 10-120mg.l^{-1}.



continual use, graph 24. Loading was 4.7U.m^{-1} of tubing.

Table 17. The Effect of Ascorbic Acid.

Segmented Flow: Immobilised Method.

Ascorbic acid mg.l^{-1}	/	mM.	Ethanol g.l^{-1}	% Decrease
0	/	0	2.00	0
5.0	/	0.028	1.98	1.0
10.0	/	0.057	1.97	1.5
25.0	/	0.142	1.73	13.5
50.0	/	0.284	1.41	29.5
100.0	/	0.568	0.80	60.0
200.0	/	1.136	0.18	91.0
500.0	/	2.84	0	100.0

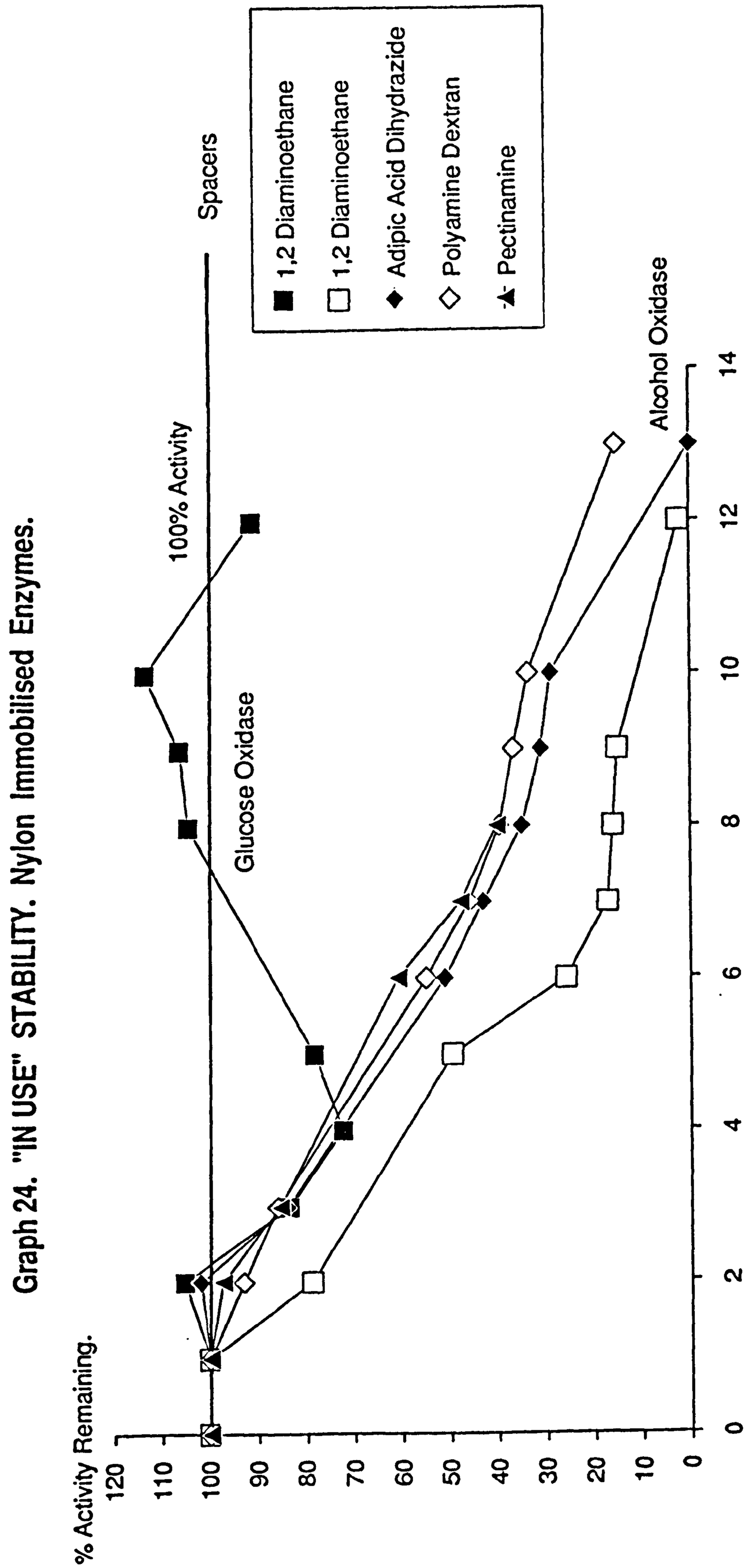
4.C.1. Cholesterol Analysis using Segmented Flow. Immobilized Method.

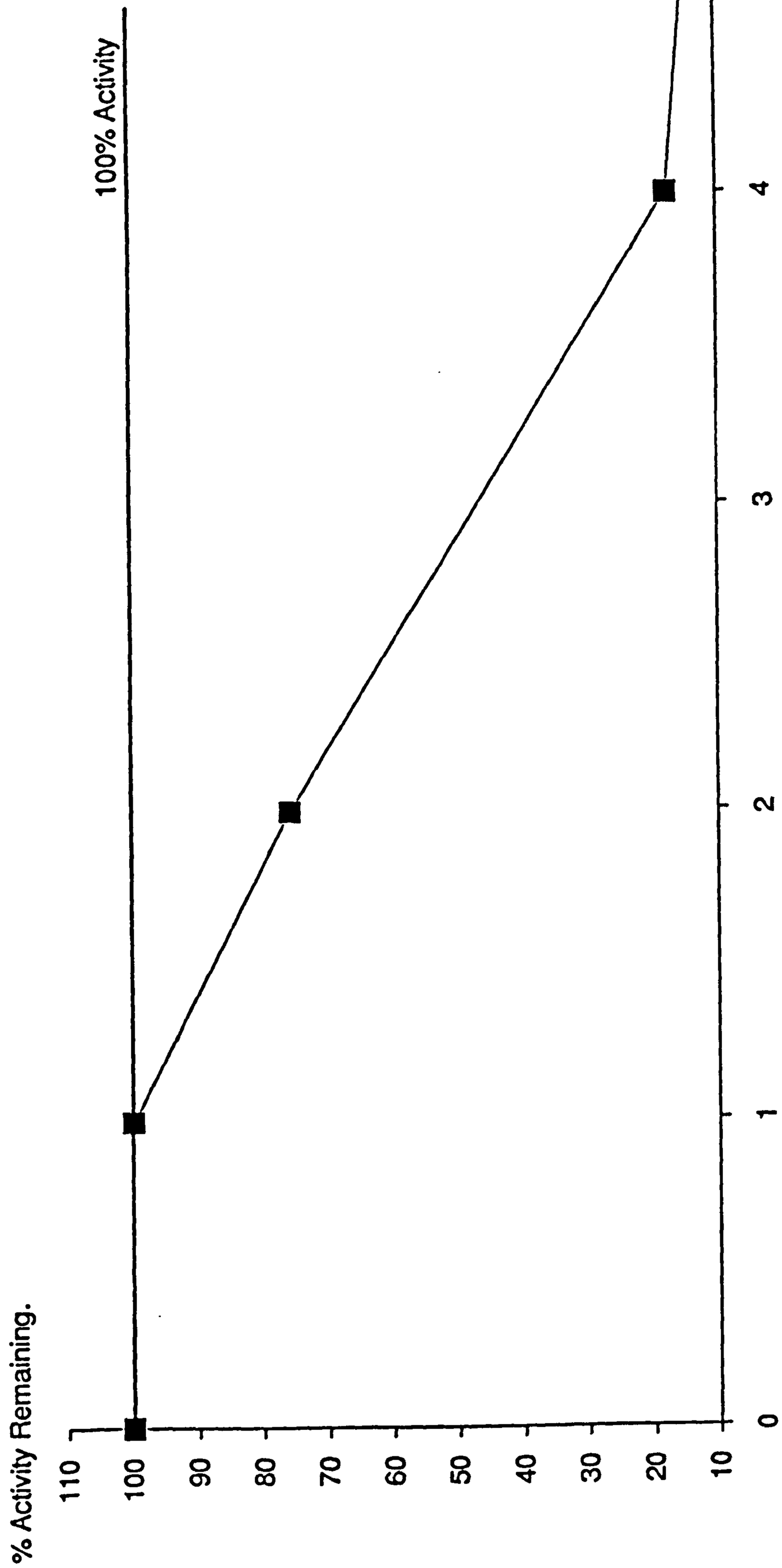
Cholesterol oxidase and cholesterol esterase in a 2:1 ratio were co-immobilised on nylon using adipic acid dihydrazide as the spacer arm. Initial oxidase activities of 9.06 and 11.55U.m^{-1} of tubing were measured. Cholesterol esterase activity was not measured. Immobilisation of cholesterol oxidase alone resulted in lower enzyme activities of 4.46-6.3U.m^{-1} of tubing. The coil was used in the same reaction system as 4.A.2. The response was linear up to 2.0g.l^{-1} cholesterol, however the stability was poor, graph 25.

4.D.1. Ethanol Analysis using Flow injection. Soluble Enzyme Method.

The results for the single and dual reagent systems using the flow chart in fig 15 were essentially the same. The method gave linear results up to 500mg.l^{-1} ethanol. The advantage of the dual reagent was that no background colour develops with time. The method

Graph 24. "IN USE" STABILITY. Nylon Immobilised Enzymes.




Graph 25. "IN USE" STABILITY. Nylon Immobilised Cholesterol Oxidase.
% Activity Remaining.
110
100
90
80
70
60
50
40
30
20
10
0
100% Activity
1
2
3
4
5
Days in use. Room Temp. (21oC).

was fast, (100 samples.hr^{-1}) and accurate in use.

4.D.2. Ethanol Analysis using Flow Injection. Immobilised Enzyme Method.

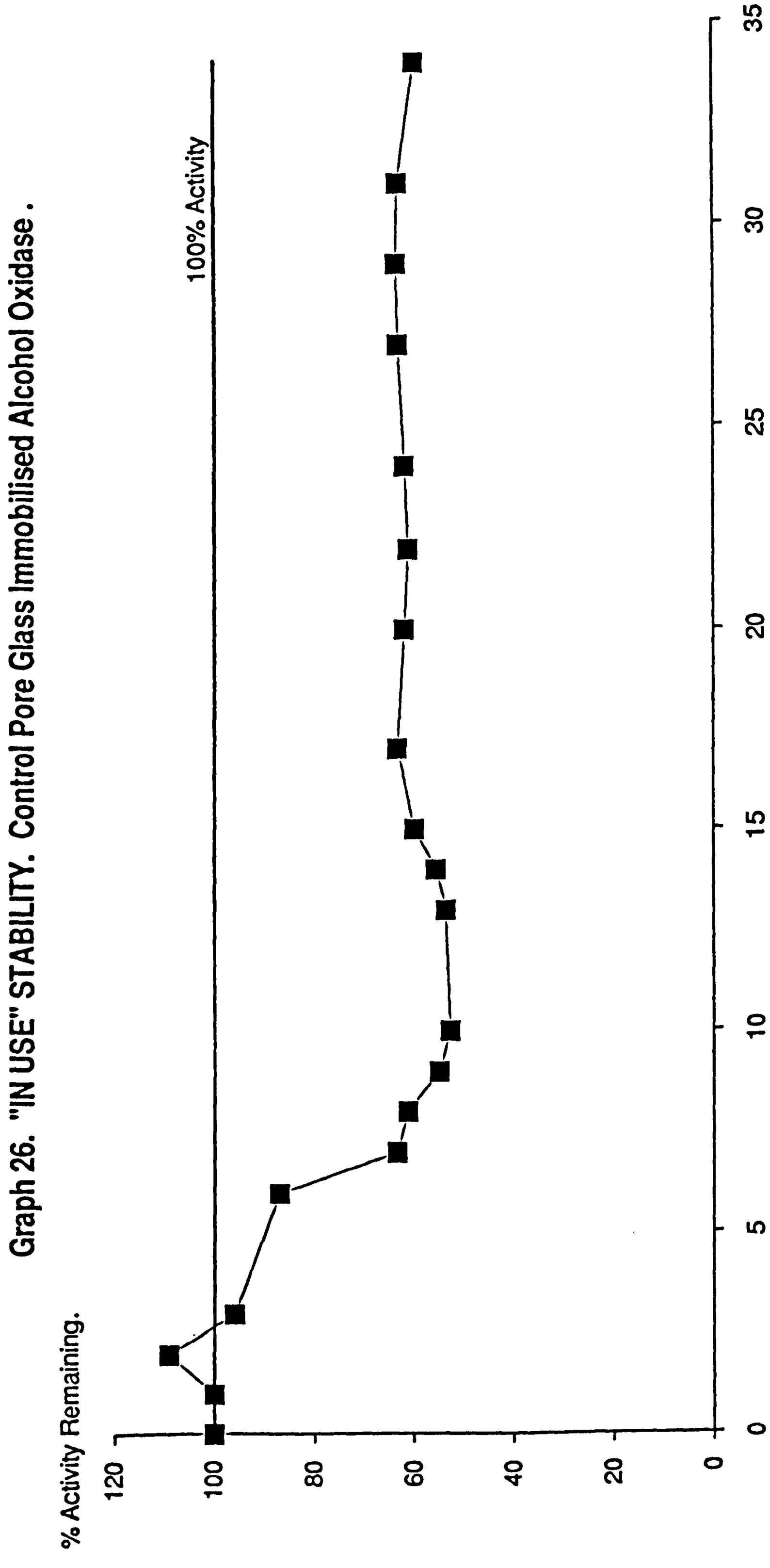
This method offered the advantage of a reduction in enzyme used and very high stability of the immobilised enzyme. Glass bound enzyme, stored for over 12 months at 4°C in buffer, shows minimal loss of activity. In use, the enzyme exhibited a half life of 10 days, the activity loss after this time was negligible, graph 26. The enzyme-glass was kept at room temperature throughout the whole period of analysis. Typical traces of both the soluble and immobilised methods are shown in figs 26-27. The method gives a linear response up to 400mg.l^{-1} ethanol.

4.E.1. Glucose Analysis using Flow Injection. Immobilised Enzyme Method.

F.I.A. methods for glucose have been described, (Gorton and Ogran 1981, Ho and Asouzu 1984). The method used the same colorimetric system as for ethanol and was used to estimate glucose concentrations in the dialysis probe tests. The method is linear up to 800mg.l^{-1} glucose.

4.F.1. Automated Analysis Application. Dialysis Probe Characterisation.

The dialysis probe described in materials and methods, section 2.H.1. was designed to measure analyte concentrations in fermentors in conjunction with some form of automated analytical method. Initial standardisation was carried out using segmented flow analysis using apparatus described in section 2.H.1. Discrete samples were analysed to produce standard curve responses shown in graphs 15 and 16. The rapidity of the F.I.A technique allowed measurement of the time



Graph 26. "IN USE" STABILITY. Control Pore Glass Immobilised Alcohol Oxidase .

FIG.26 FLOW INJECTION ANALYSIS TRACE.
ETHANOL SOLUBLE METHOD.

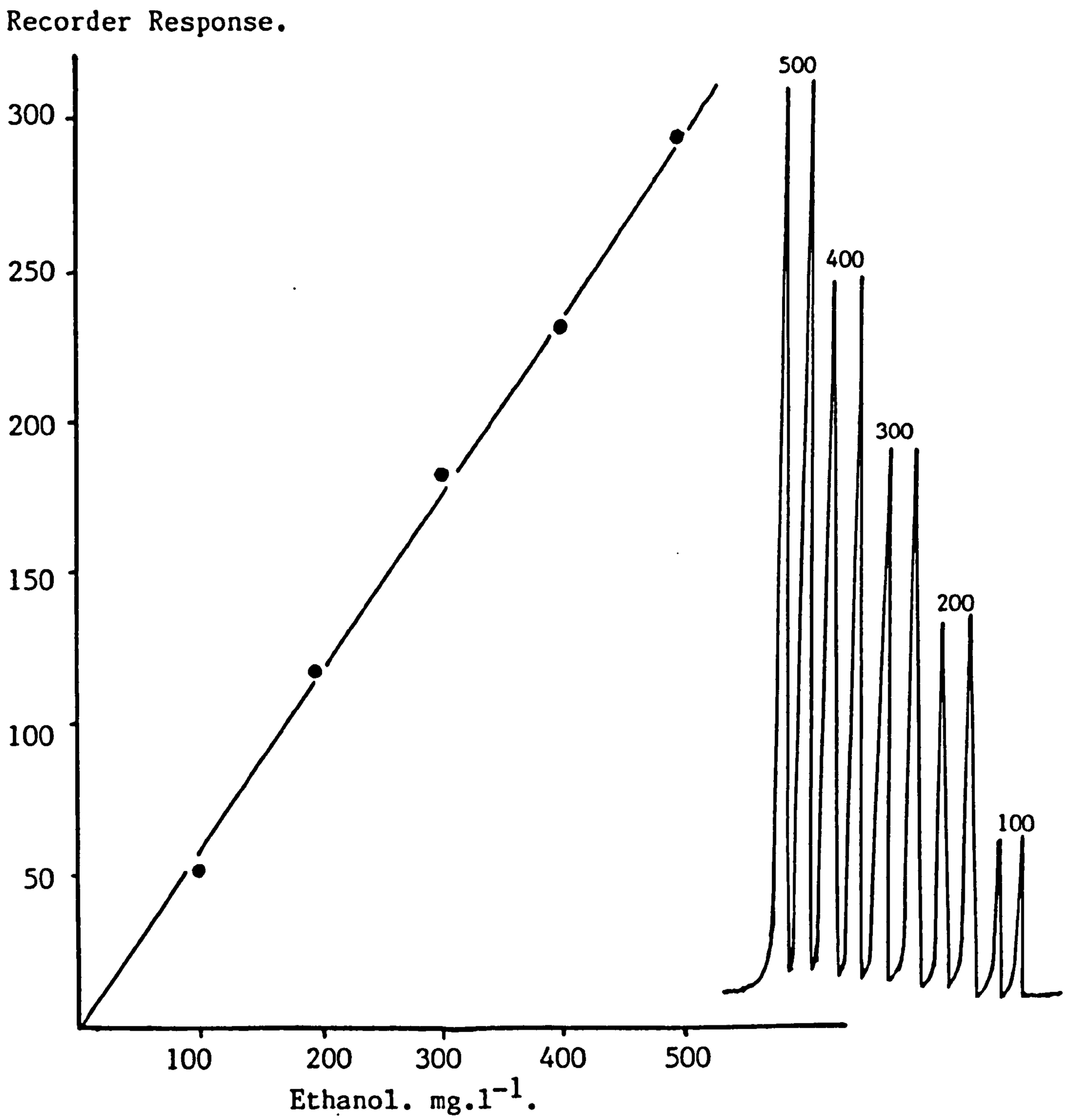
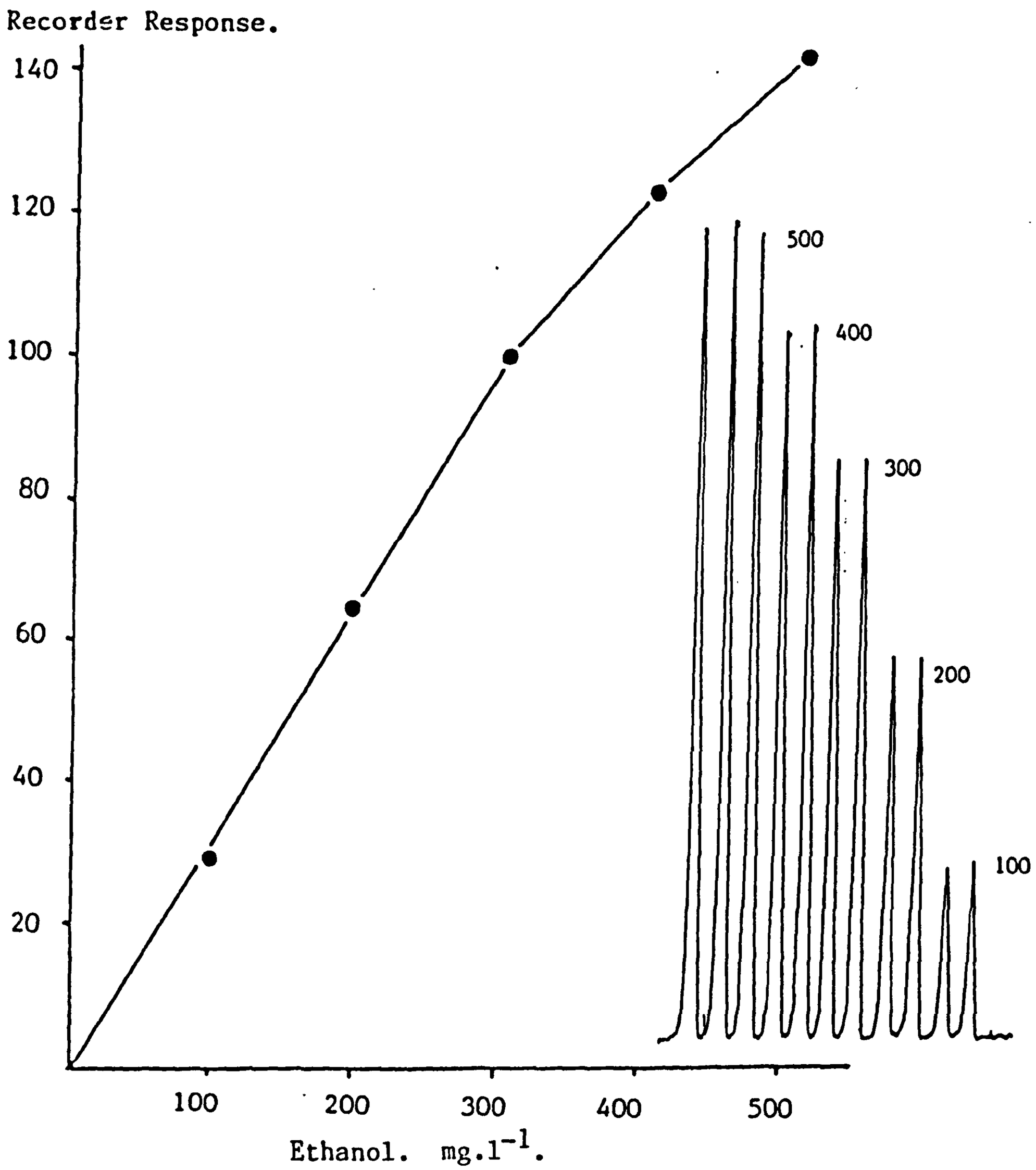


FIG.27 FLOW INJECTION ANALYSIS TRACE.
ETHANOL IMMOBILISED METHOD.



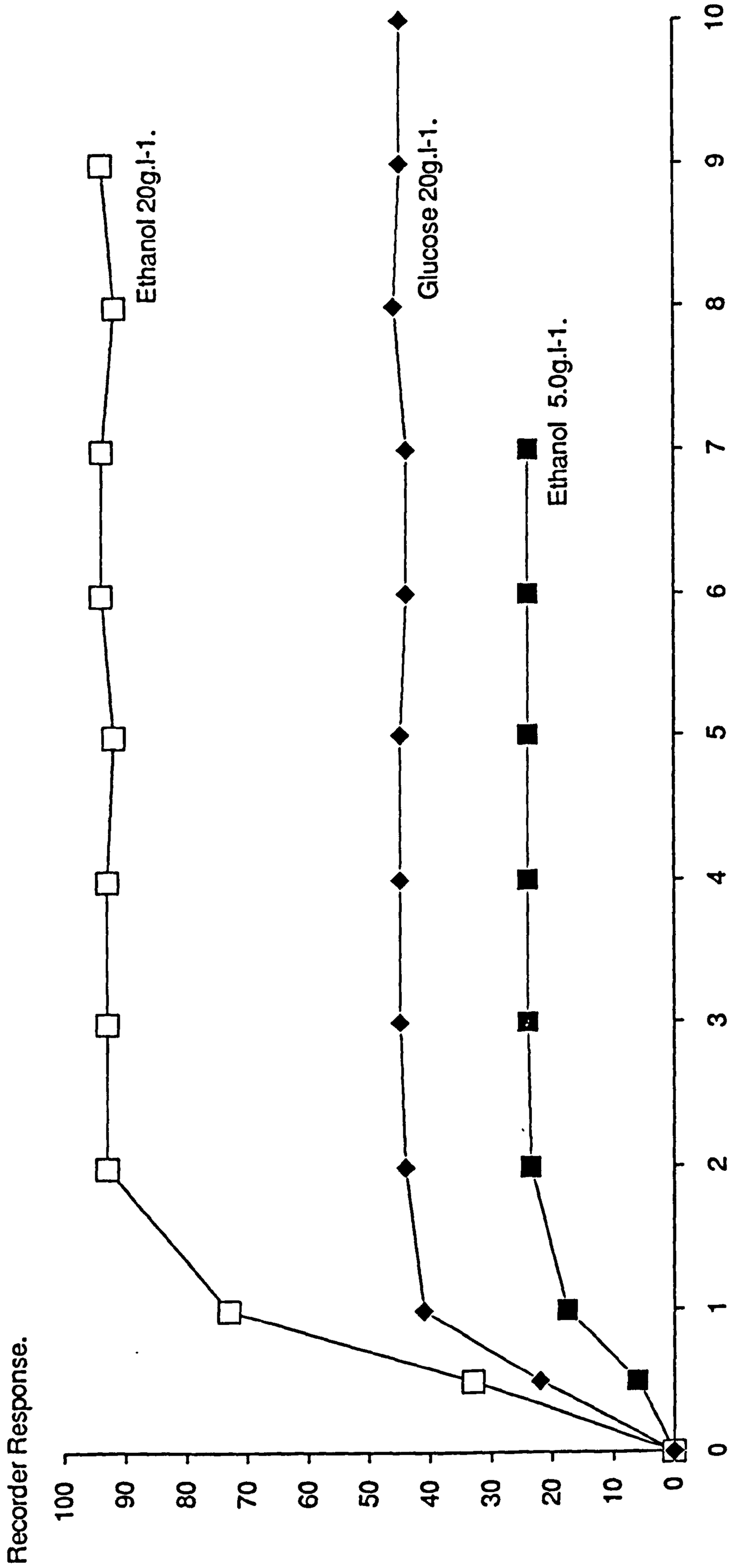
dependence of the probe to reach a steady state response when exposed to solutions of various concentrations of ethanol and glucose. Direct injections of the carrier solution were analysed using the soluble enzyme system at set times after exposure of the probe surface to the standard solutions. The results are shown in graph 27. F.I.A was also used to measure the temperature dependance of the probe, graph 28.

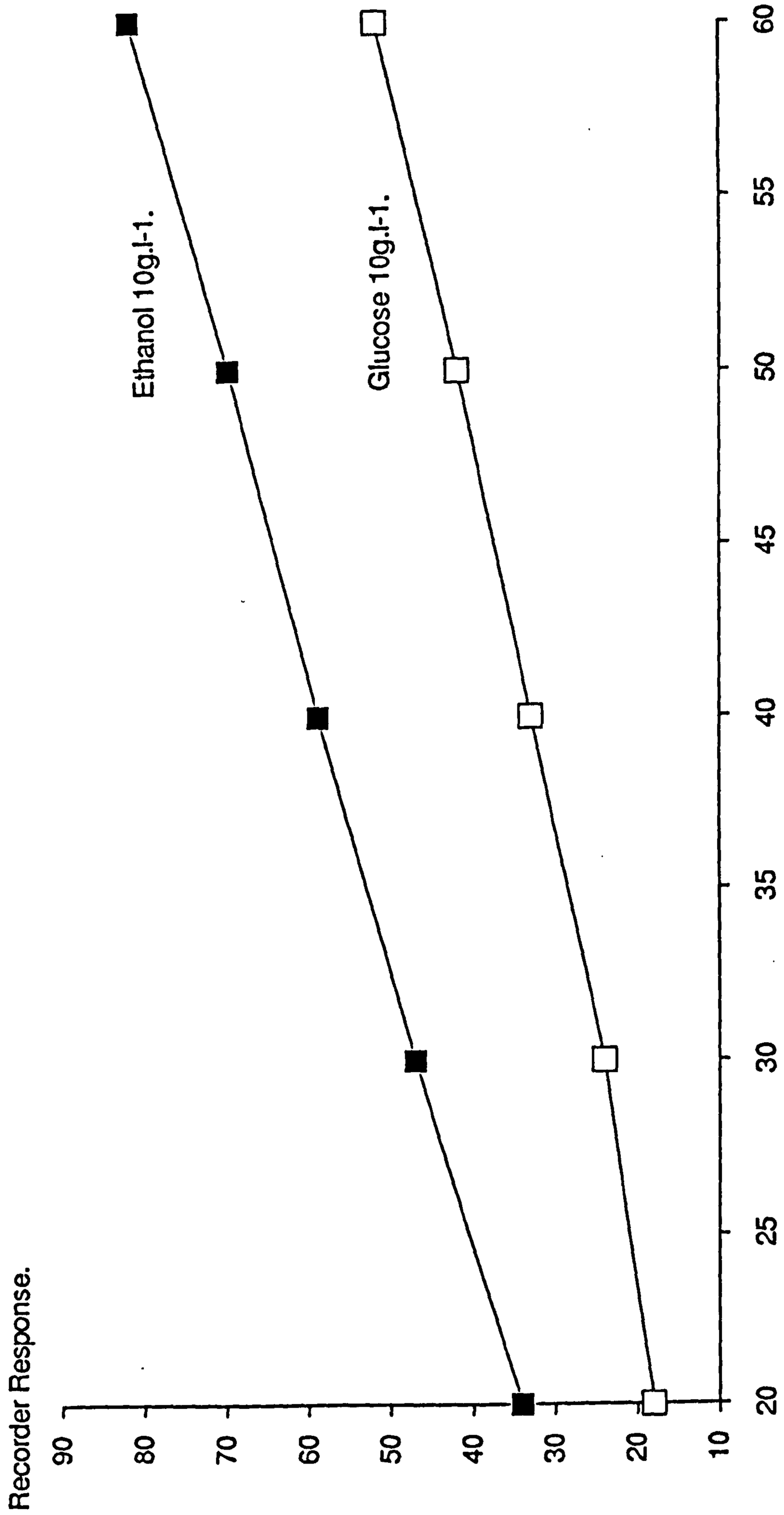
There was no effect in the response of the probe to variations in the pH or to the presence of solid material, (microcrystalline cellulose) in the samples analysed. Also, the viscosity of the standard solutions analysed was varied using differing concentrations of high molecular weight dextran, (5-40 x 10^6). No effect was noticed on the response of the probe.

4.F.2. Automated Analysis Application. Fermentation Experiments.

Both segmented flow and F.I.A. systems were used to follow model fermentations of brewers yeast with malt extract substrates. Glucose and ethanol levels were monitored using immobilised enzyme methods in segmented flow systems, (section 2.I.2) and soluble enzyme methods in F.I.A. systems, (section 2.I.4). The soluble and the immobilised enzyme methods for ethanol gave reproducible, consistent results, however some loss of activity of the glucose oxidase was noticed in the immobilised glucose method. This was especially apparent on analysis of the samples of fermentation media taken by direct sampling. A comparison was made between direct sampling of the media, followed by dilution if necessary, and samples obtained using the dialysis probe. Colorimetric detection was used as the standard technique however, in a separate experiment, incorporation of a Yellow Springs Instruments peroxide electrode into the system was also investigated. This was fitted with, (a) a glucose oxidase

Graph 27. DIALYSIS PROBE. Time for Maximum Steady-State Response.



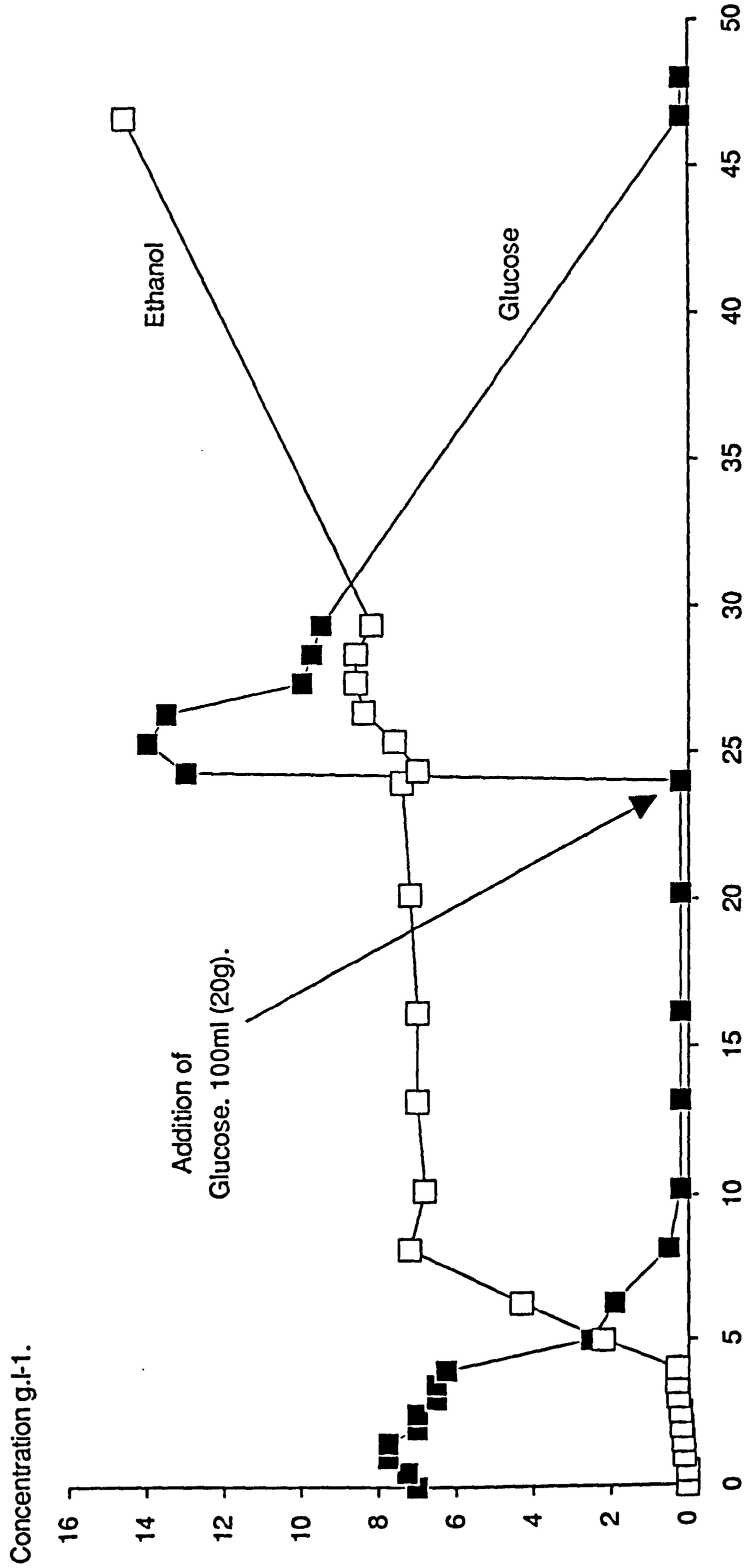


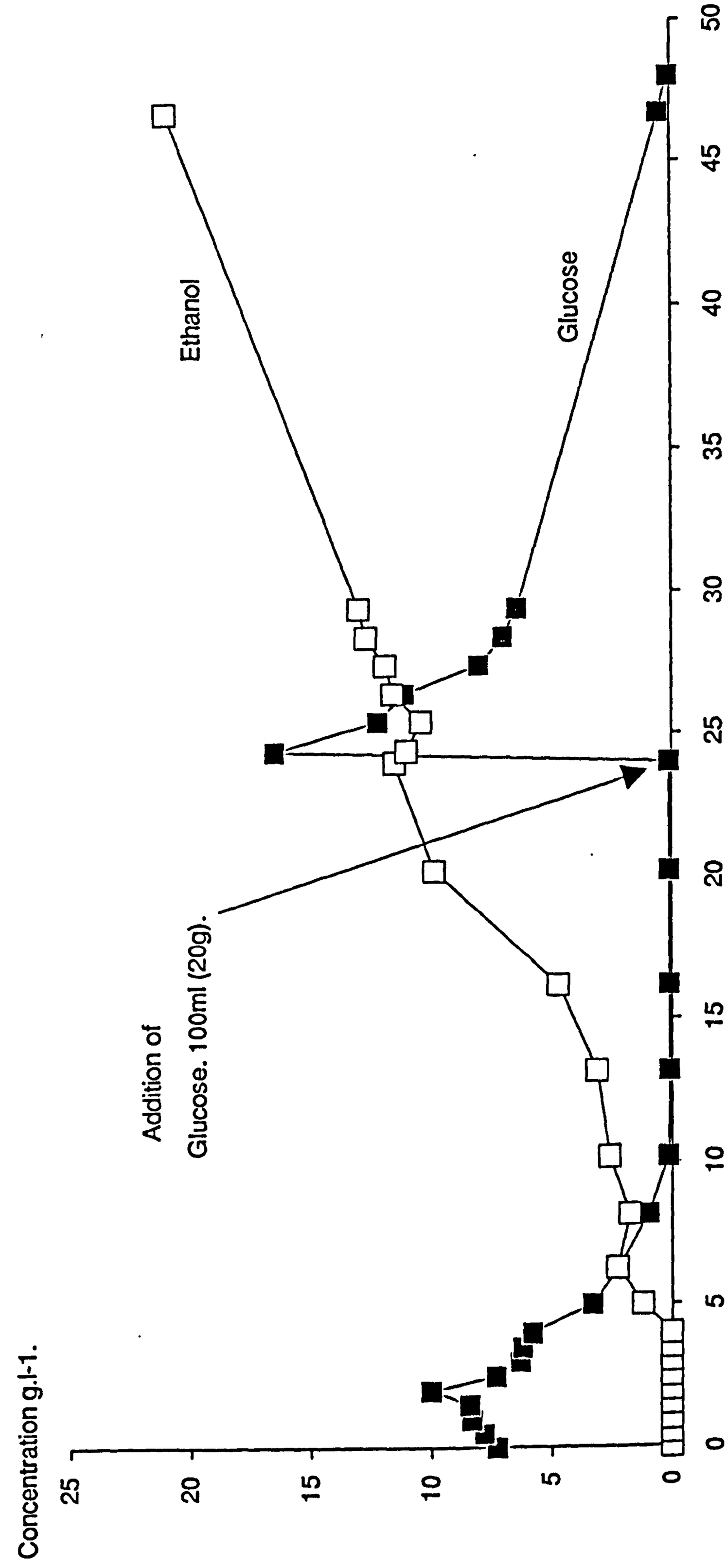
Graph 28. DIALYSIS PROBE. Temperature Dependance.

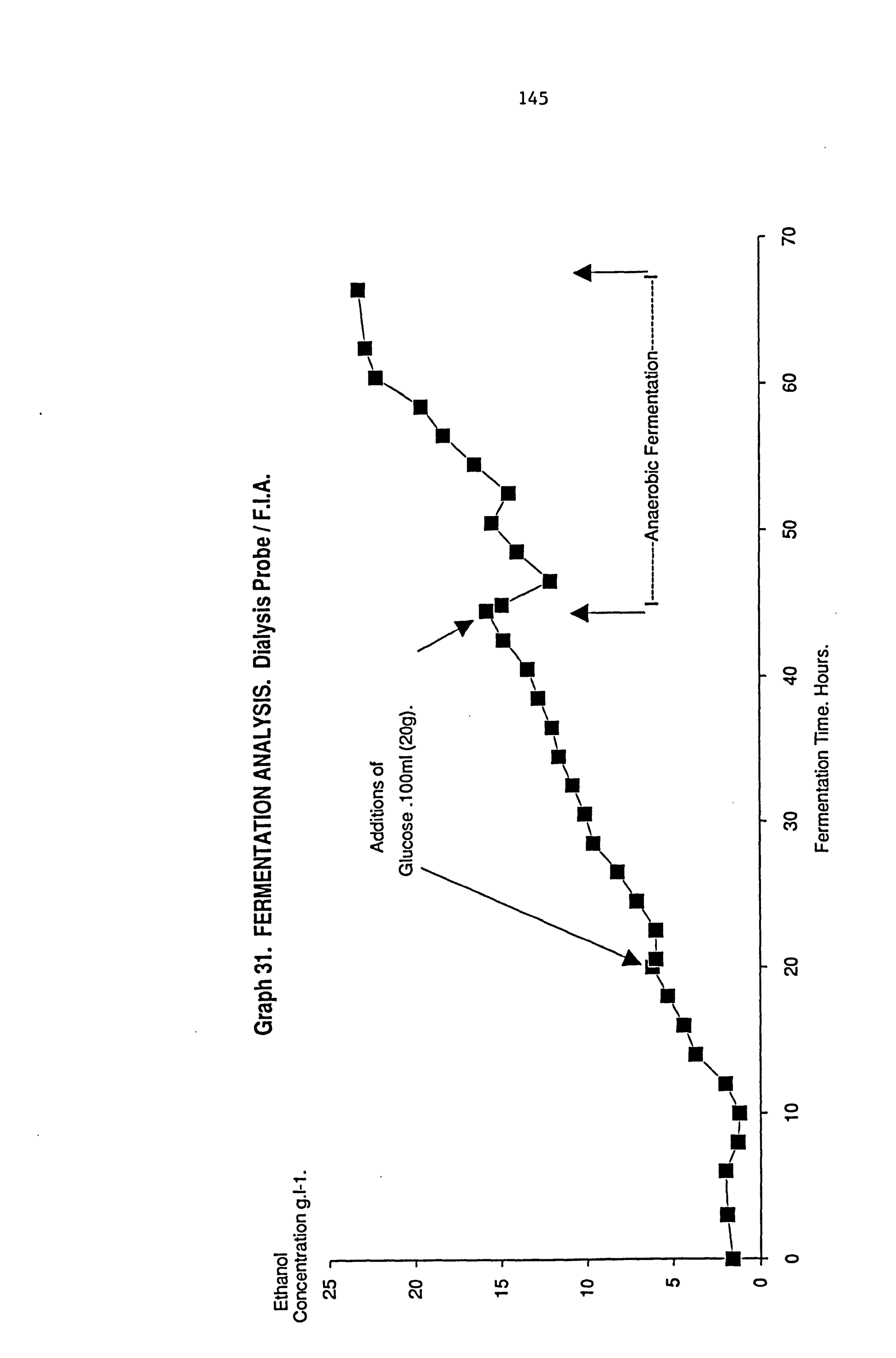
membrane to measure glucose and, (b) an alcohol oxidase membrane to measure ethanol. The results are shown in graphs 29 - 32.

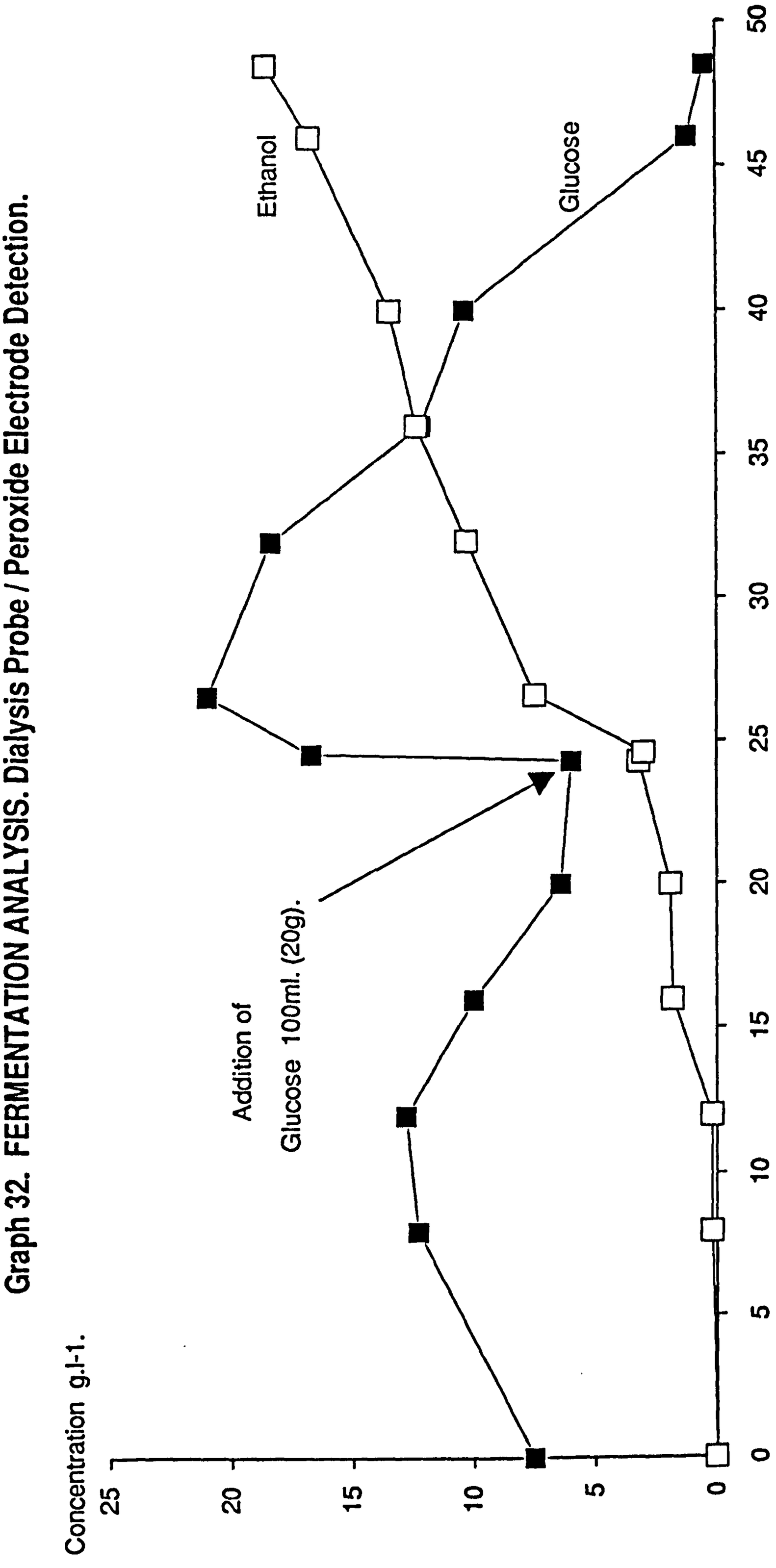
In addition to the model fermentations a bacterial culture of S.Lactis 18-16 was prepared. The dialysis probe was pre-standardised and sterilised at 121°C for 20 minutes in the medium. Diacetyl and glucose levels were measured using segmented flow analysis, graph 33.

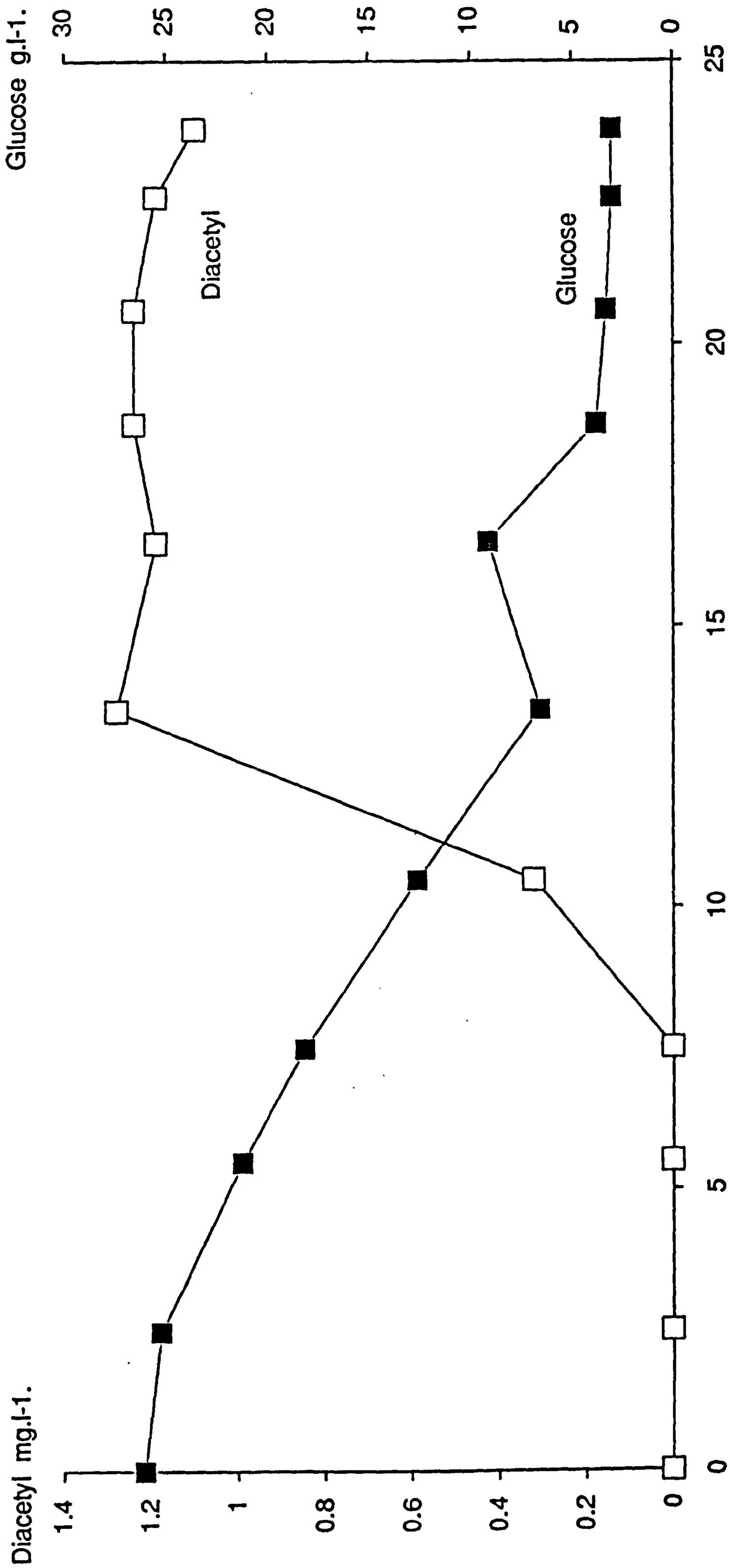
Graph 29. FERMENTATION ANALYSIS. Direct Sampling /S.F.A.




Graph 30. FERMENTATION ANALYSIS. Dialysis Probe / S.F.A.
Ethanol
Glucose
Addition of
Glucose. 100ml (20g).
Concentration g.l-1.
Fermentation Time .Hours.
0
5
10
15
20
25
30
35
40
45
50


Graph 31. FERMENTATION ANALYSIS. Dialysis Probe / F.I.A.
Ethanol Concentration g.l-1.
25
20
15
10
5
0
0
10
20
30
40
50
60
70
Fermentation Time. Hours.
Additions of Glucose .100ml (20g).
I--------Anaerobic Fermentation--------I


Graph 32. FERMENTATION ANALYSIS. Dialysis Probe / Peroxide Electrode Detection.
Ethanol
Glucose
Addition of
Glucose 100ml. (20g).
Concentration g.l-1.
Fermentation Time. Hours.
0
5
10
15
20
25
30
35
40
45
50



Graph 33. BACTERIAL FERMENTATION ANALYSIS. Dialysis Probe / S.F.A.

CHAPTER 5.

STABILISATION of ALCOHOL OXIDASE and OTHER ENZYMES.

5.A. Hansenula Alcohol Oxidase.

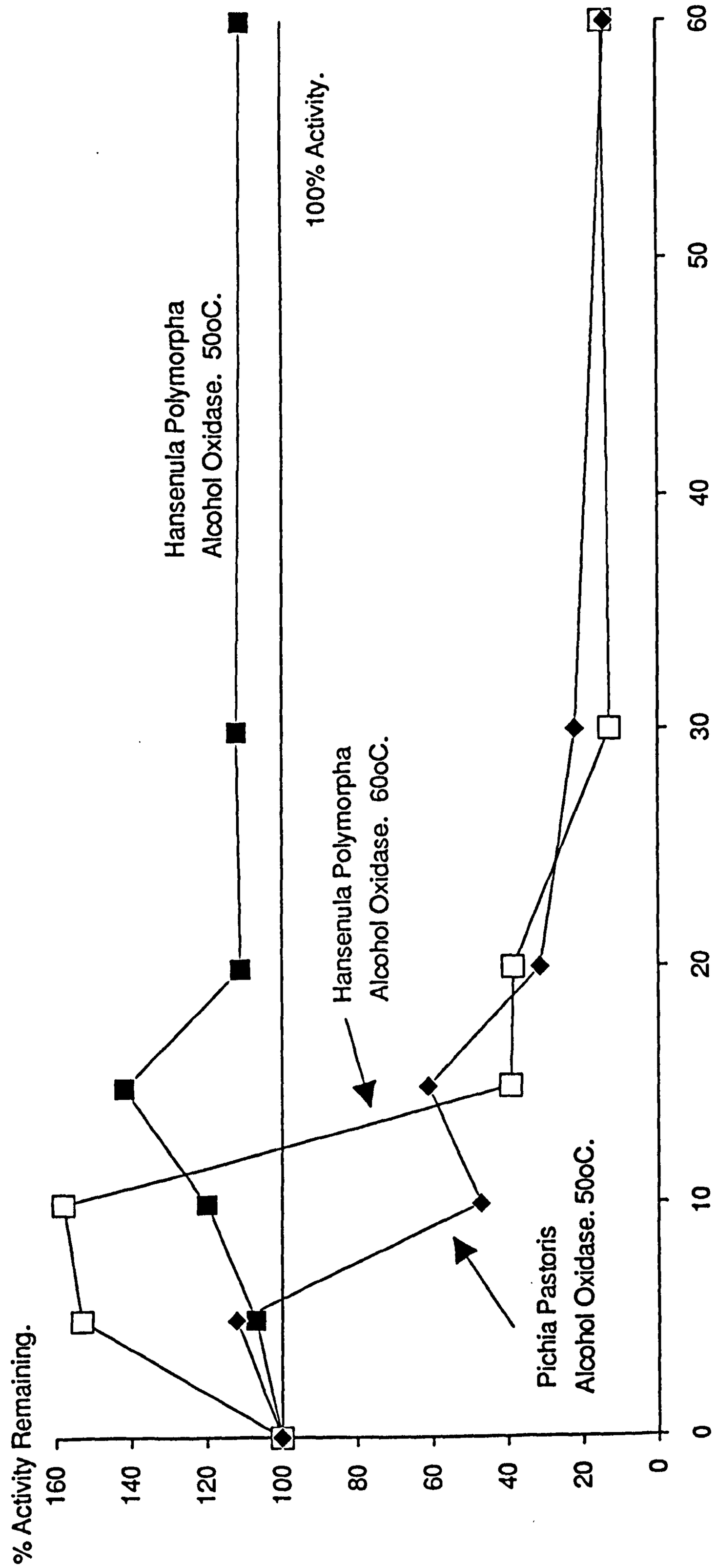
5.A.1. Stability of the Native Enzyme.

Native alcohol oxidase was found to be very stable when stored as a precipitate in ammonium sulphate solution. Fully active enzyme was recovered after 2 years storage at 4°C in this form. Frozen enzyme, (-20°C) was also relatively stable, however buffered solutions of the enzyme deteriorated over a period of several days, usually with the formation of an insoluble precipitate, which was probably aggregated and inactive protein, (chapter 8, section 8.C). Precipitate formation may be due to spontaneous formation of low levels of hydrogen peroxide, which was more apparent in aged solutions of enzyme. When present in fairly dilute concentrations as part of an analytical reagent the enzyme was fairly stable, (up to 1 month at 4°C and 1 week at room temperature), however high background values occurred, presumably from the peroxide liberated.

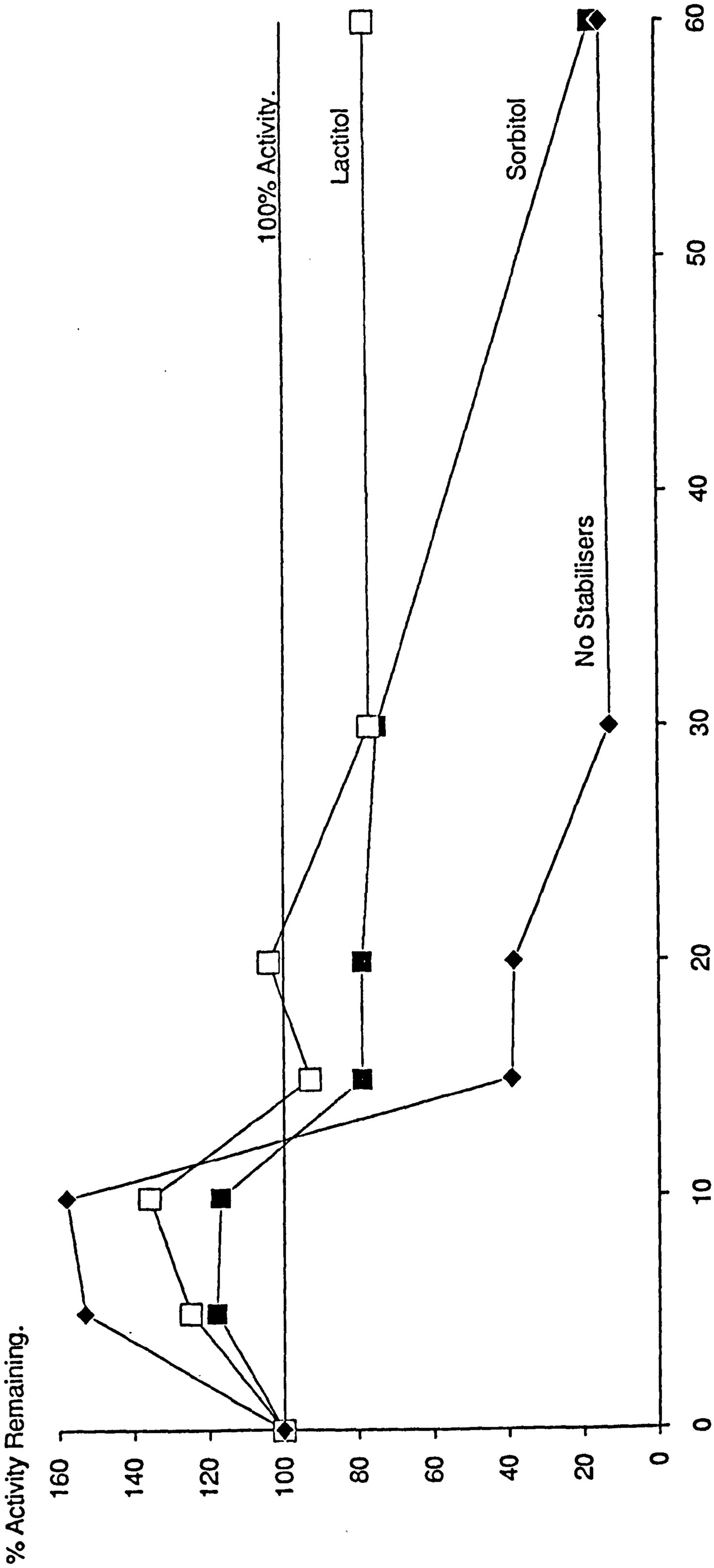
Freeze dried native enzyme was difficult to prepare and was very labile, the activity remaining was usually lost within a few days. Air or vacuum dried native enzyme was somewhat more stable, however an unacceptable loss of activity occurs on drying and over the subsequent weeks of storage. Accelerated degradation testing shows this clearly, graph 36.

5.A.2. Stabilisation Using Additives. "Wet" enzyme.

Solutions of alcohol oxidase could be partially stabilised by addition of high concentrations of lactitol and sorbitol to the solution. Graph 34 shows the degradation of the native enzyme at 50°C and 60°C with respect to time. The initial rise after 5 minutes of incubation was probably due to the destruction of small amounts of


Graph 34. WET ENZYME STABILITY. Alcohol Oxidase.
% Activity Remaining.
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Incubation Time. Minutes.
Pichia Pastoris
Alcohol Oxidase. 50oC.
Hansenula Polymorpha
Alcohol Oxidase. 60oC.
Hansenula Polymorpha
Alcohol Oxidase. 50oC.
100% Activity.

Graph 35. WET ENZYME STABILISATION. Hansenula Polymorpha Alcohol Oxidase. 60oC.



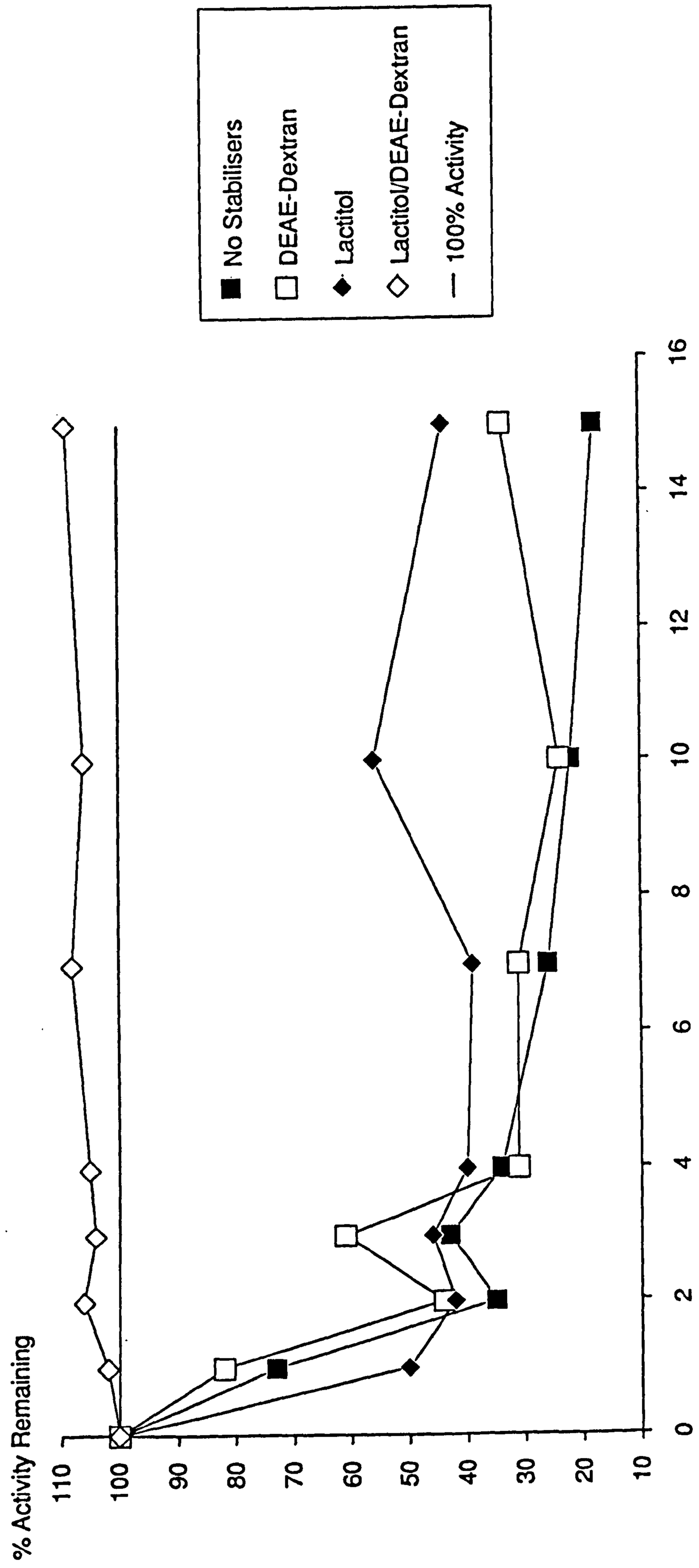
catalase, this effect was smaller when the stabilisers were present, graph 35.

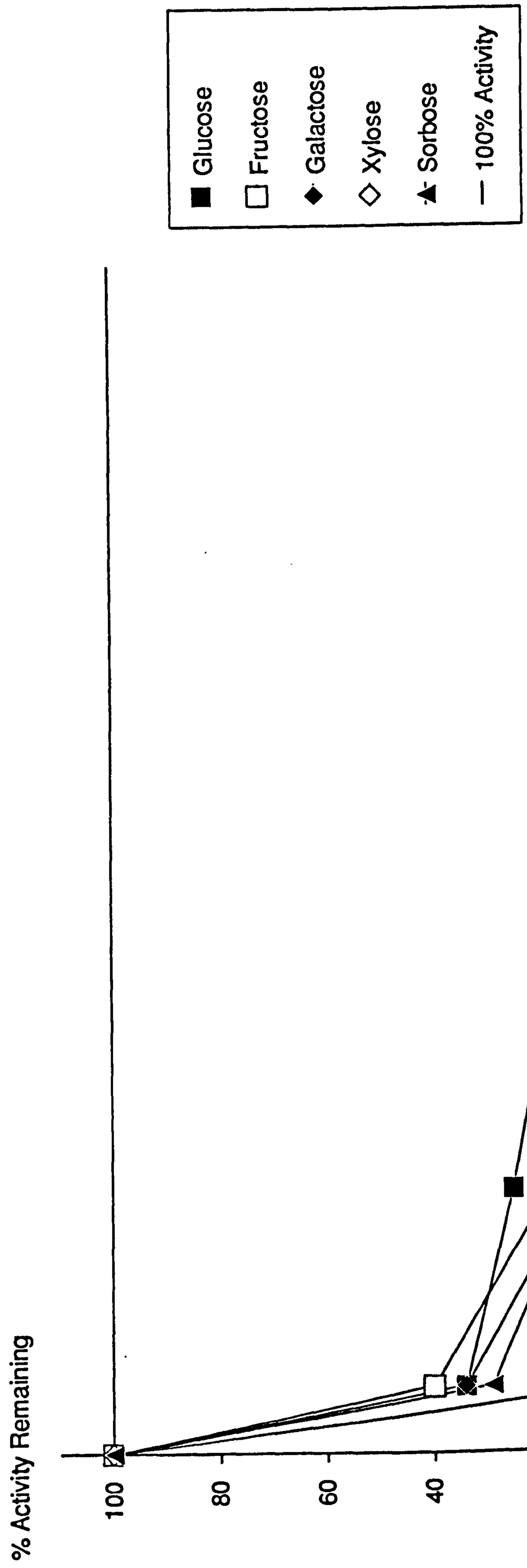
5.A.3. Stabilisation Using Additives. Dry Enzyme.

Dry enzyme powders of alcohol oxidase were prepared using a large range of different potentially stabilising compounds listed in table 11. Because of the observation that vacuum dried native enzyme retained higher activity than freeze dried preparations, the initial trials were carried out using this method. The effects of the various stabilisers were determined by accelerated degradation testing of the dry enzyme. The results were plotted, using the initial dry activity as the 100% reference value, graphs 36 - 47.

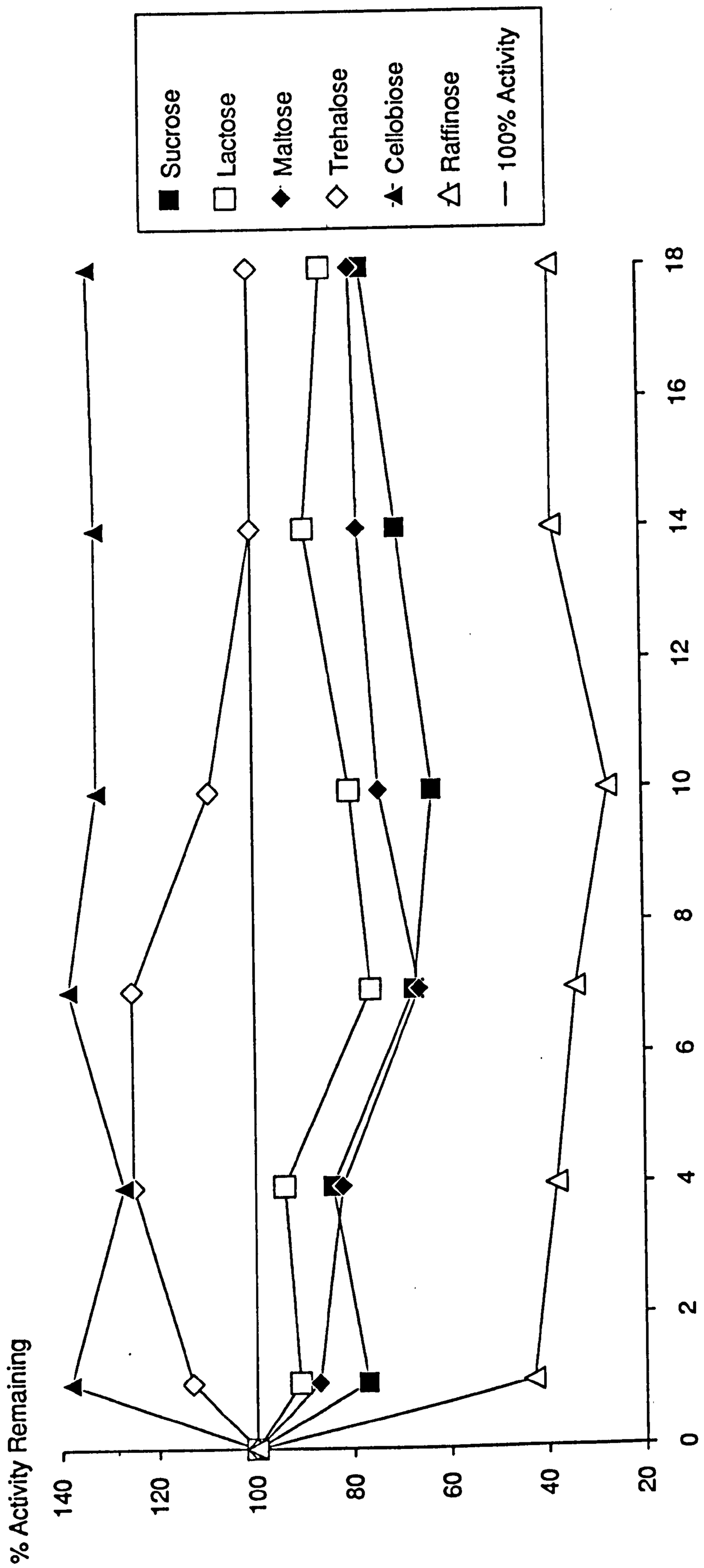
As can be seen from the graphs, various compounds have a beneficial effect on enzyme stability, whilst others actually destabilise. Disaccharides and their alcohol derivatives predictably enhanced stability of the enzyme, however destabilisation occurred with monosaccharides, possibly due to the reducing power of the molecules. Trehalose in particular enhanced stability confirming the claims of Roser, (1986). The cyclic polyalcohol inositol was exceptional in its ability to stabilise the enzyme, unlike linear polyhydroxyl compounds such as mannitol and sorbitol. These compounds did not stabilise alcohol oxidase efficiently, contrary to the claims of Phillips (1985). Dextrans, which are polymers of glucose with predominantly α-1,6 linkages are also good stabilisers, however the sulphate and diethylaminoethyl derivatives appear to lose this ability to some extent.

The most unexpected discovery was to find that combinations of such polyelectrolyte derivatives and related molecules such as chitosan, alginate, carboxymethyl cellulose and polyethyleneimine

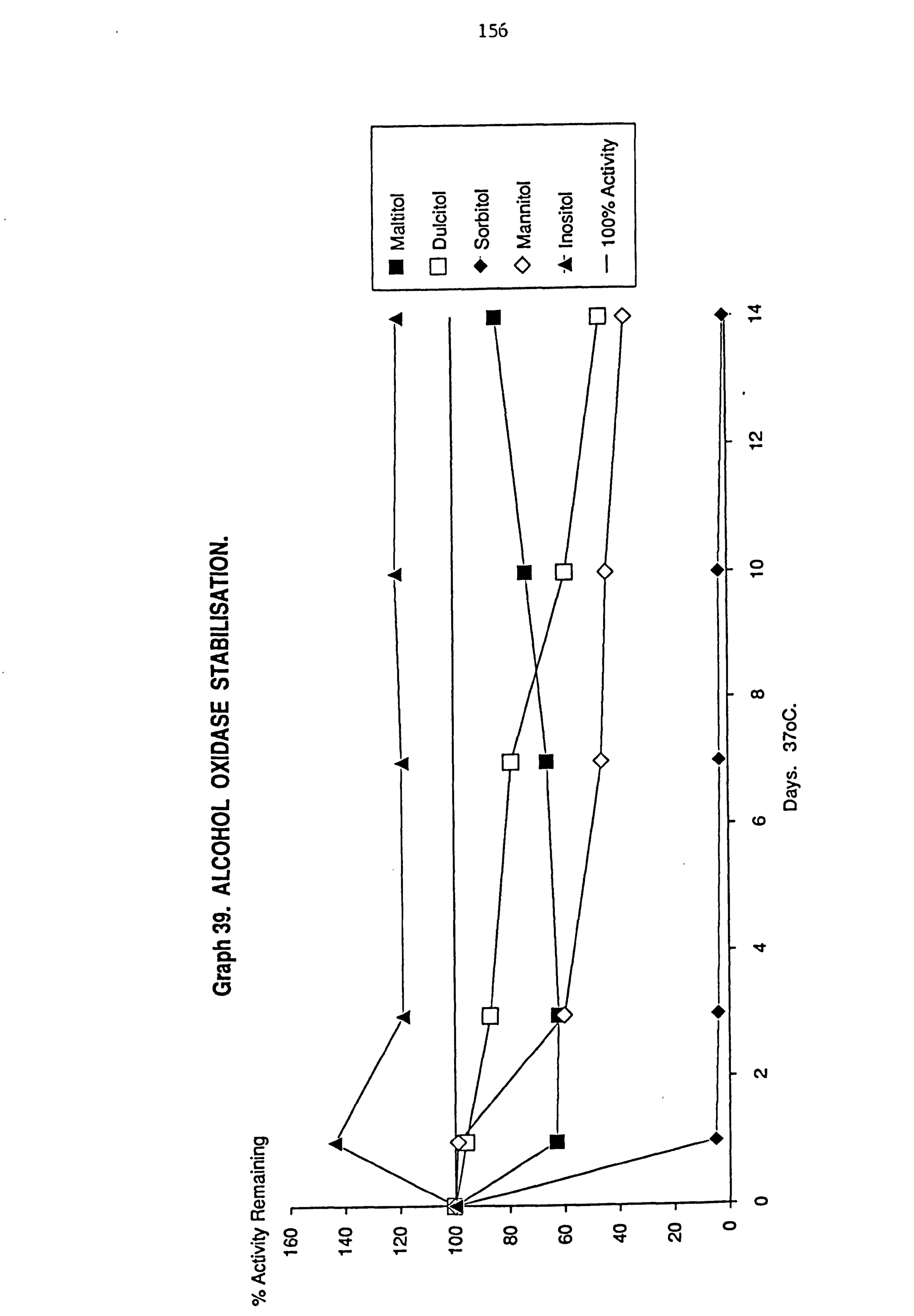

No Stabilisers
DEAE-Dextran
Lactitol
Lactitol/DEAE-Dextran
100% Activity
Graph 36. ALCOHOL OXIDASE STABILISATION.
% Activity Remaining
110
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
Days. 37oC.



Graph 37. ALCOHOL OXIDASE STABILISATION.

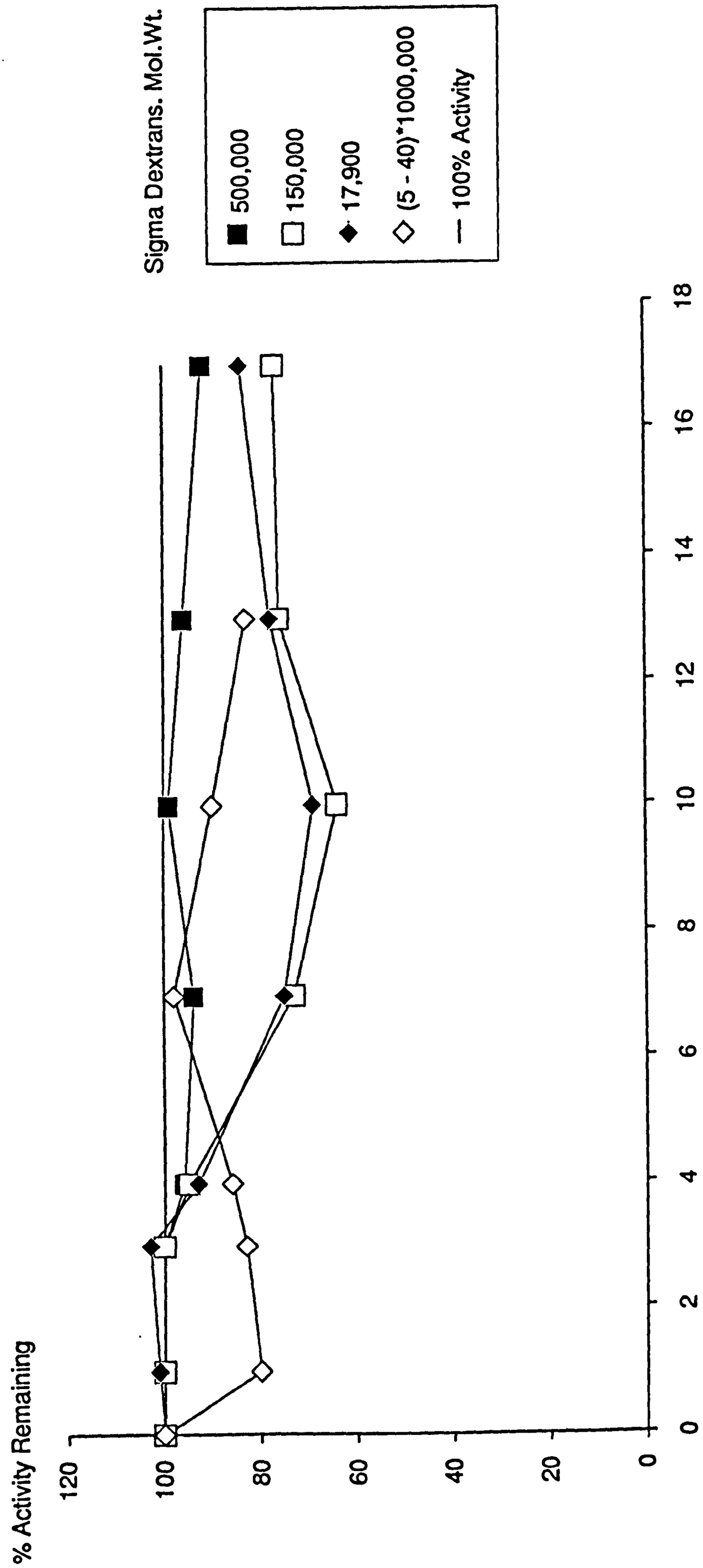


Graph 38. ALCOHOL OXIDASE STABILISATION.

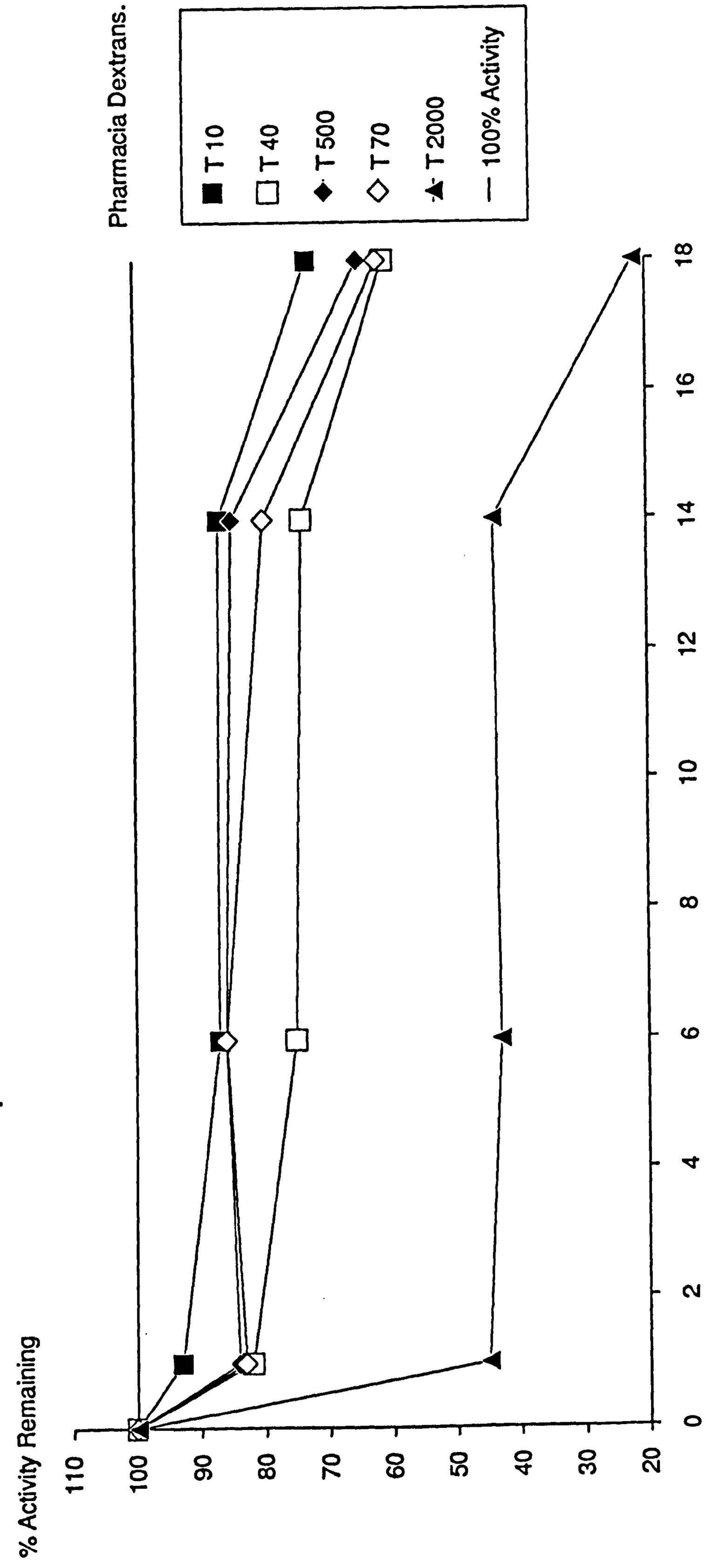


Graph 39. ALCOHOL OXIDASE STABILISATION.

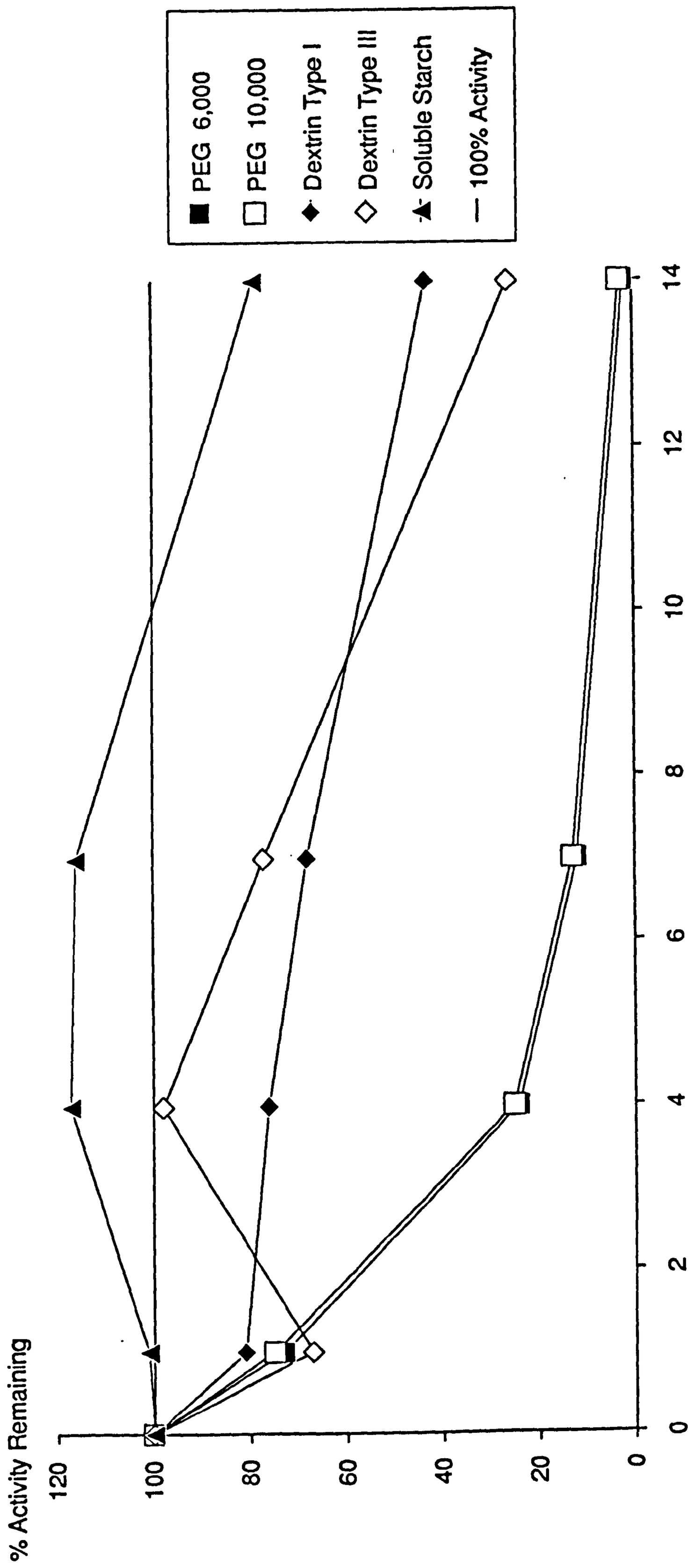
Graph 40. ALCOHOL OXIDASE STABILISATION.

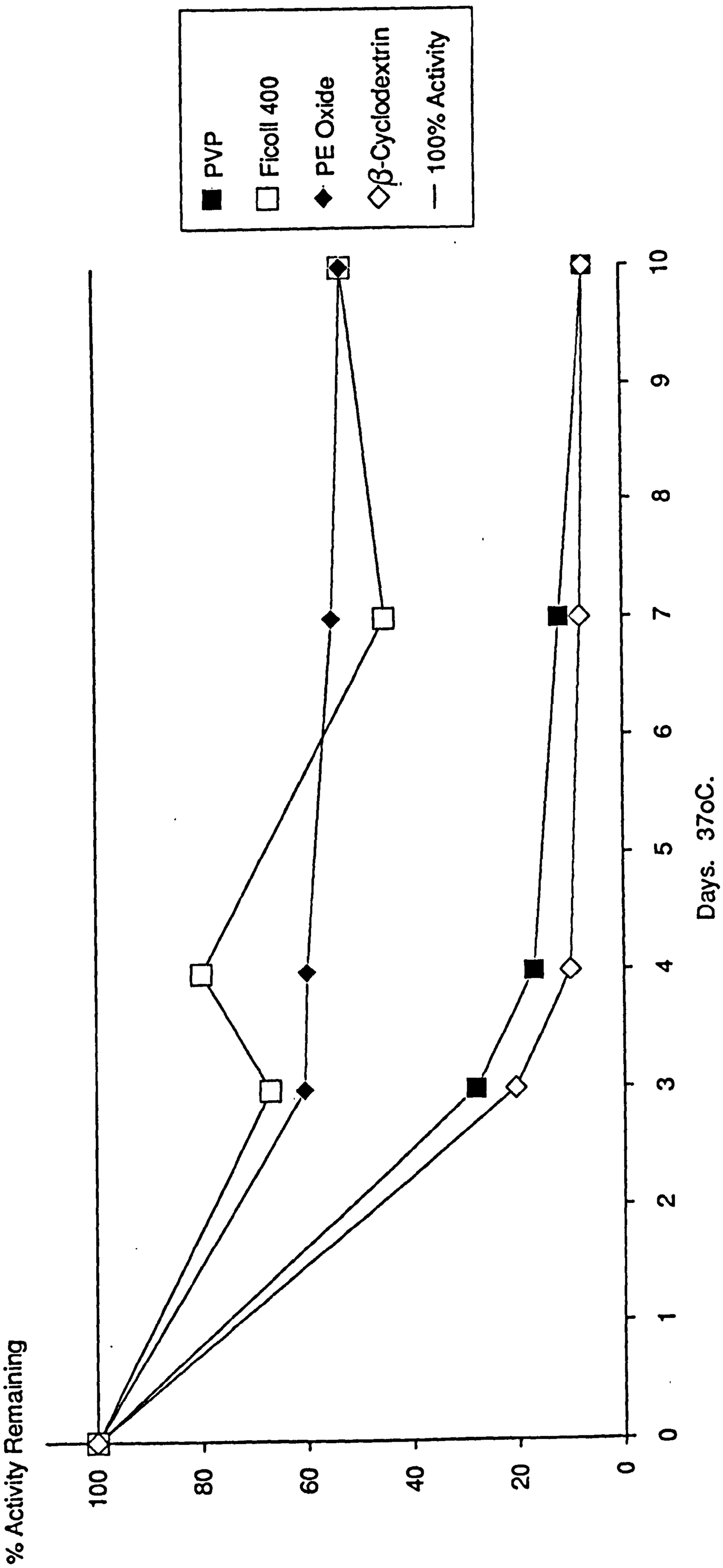


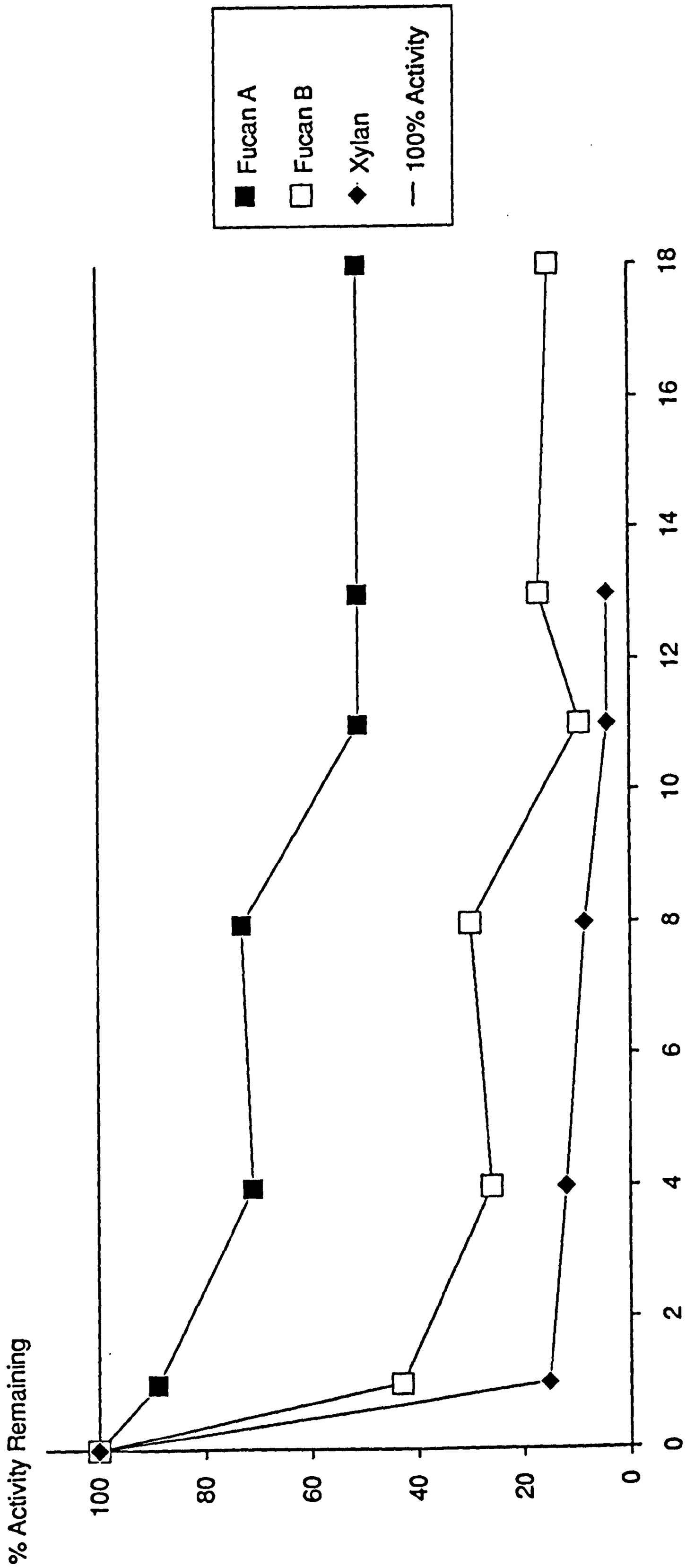
Graph 41. ALCOHOL OXIDASE STABILISATION.



Graph 42. ALCOHOL OXIDASE STABILISATION.

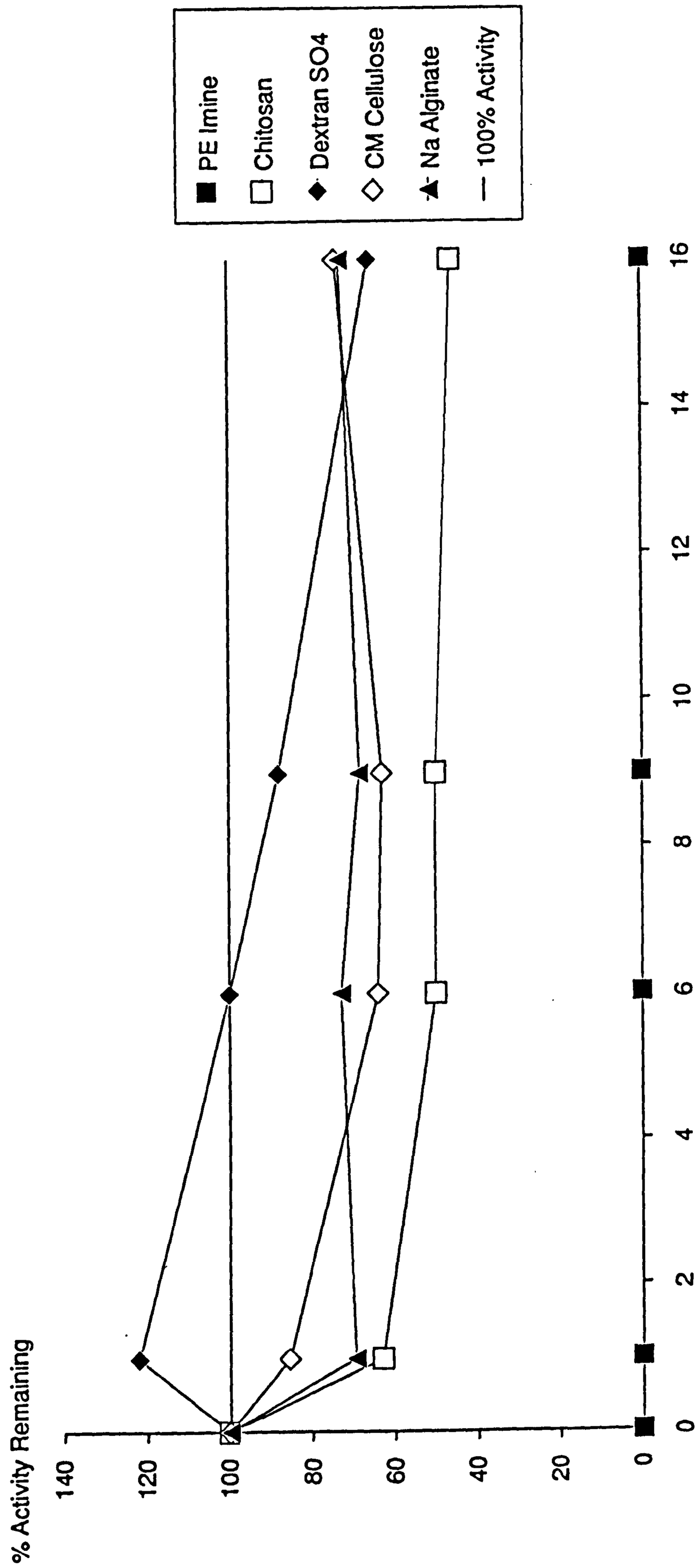


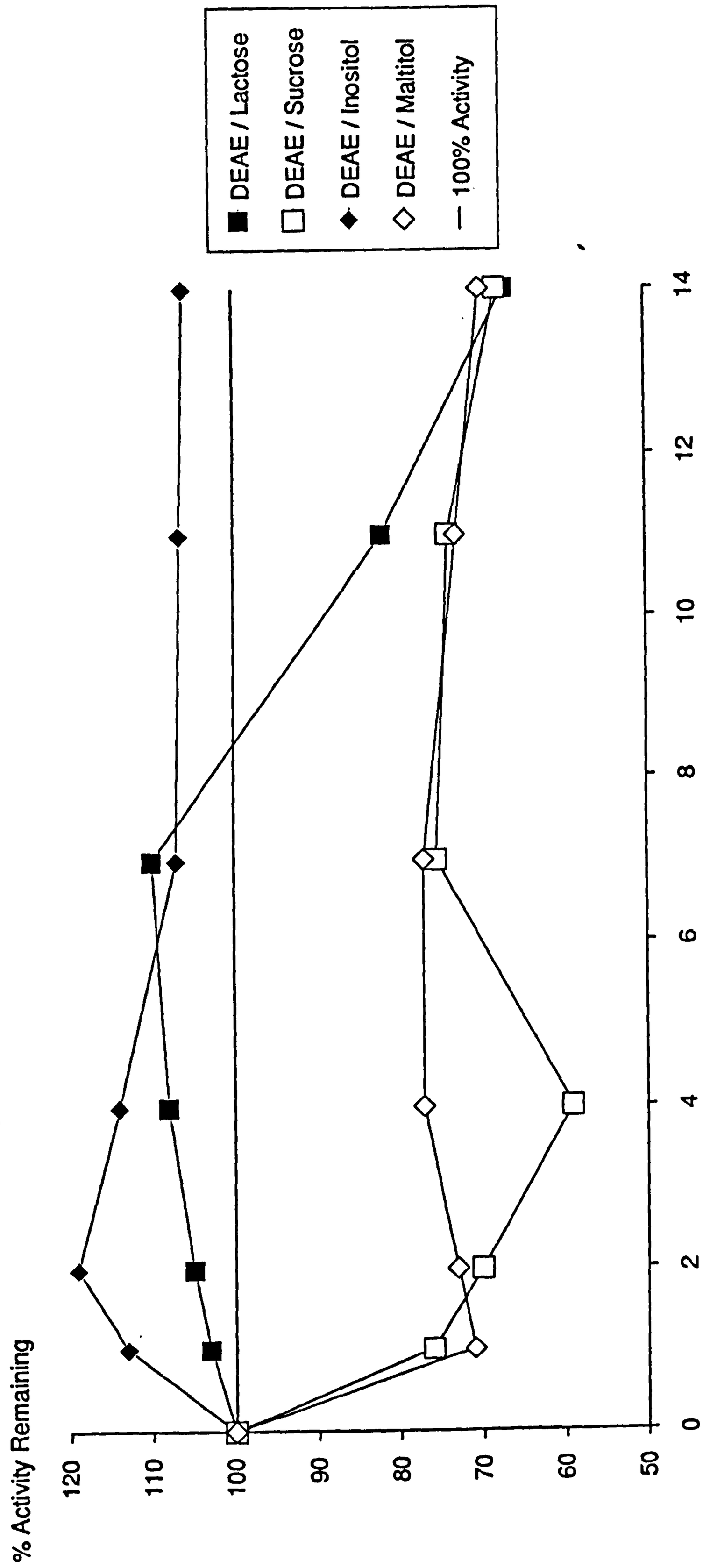

■ PVP
□ Ficoll 400
◆ PE Oxide
◇β-Cyclodextrin
— 100% Activity
Graph 43. ALCOHOL OXIDASE STABILISATION.
% Activity Remaining
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
Days. 37oC.



Graph 44. ALCOHOL OXIDASE STABILISATION.

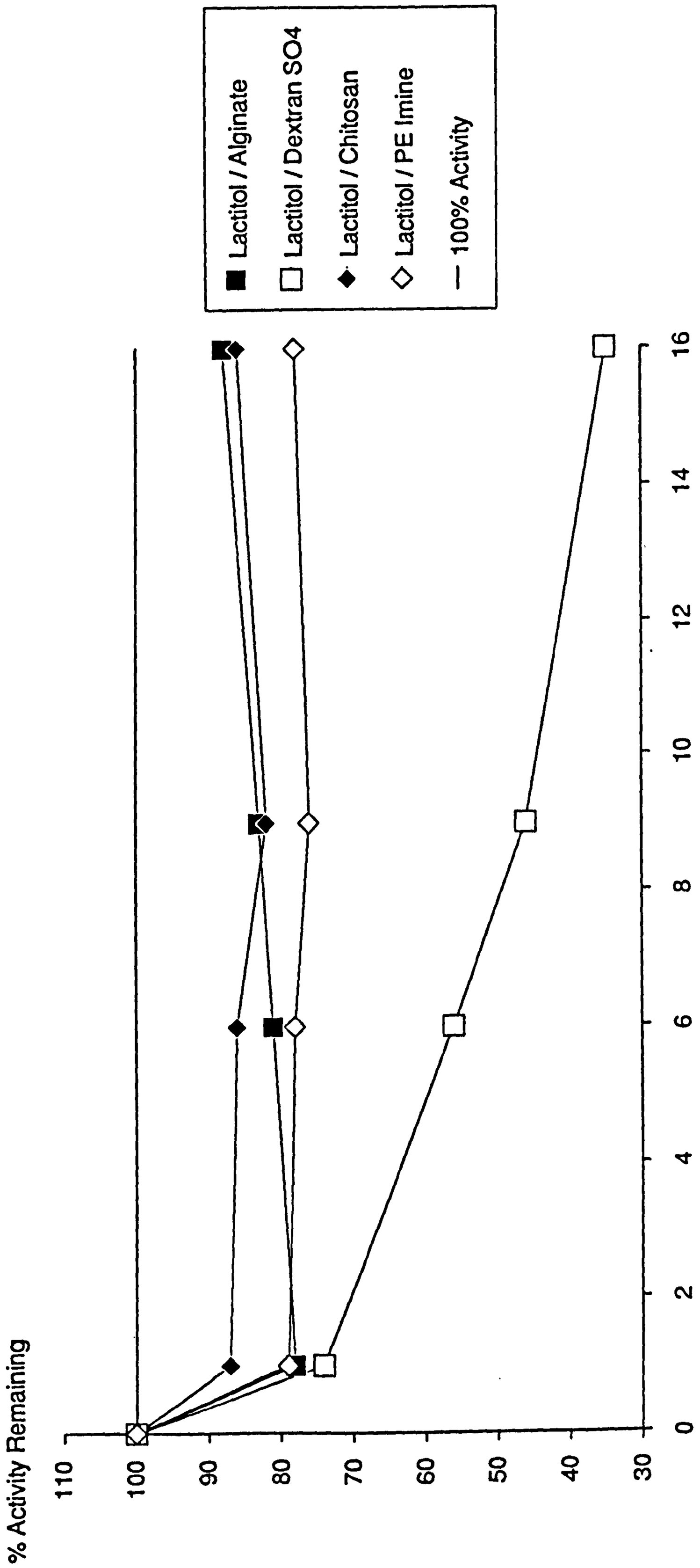
Graph 45. ALCOHOL OXIDASE STABILISATION.





Graph 46. ALCOHOL OXIDASE STABILISATION.

Graph 47. ALCOHOL OXIDASE STABILISATION.



with disaccharides or sugar alcohols produced almost total stabilisation. Extended tests on such combinations have shown that no loss of activity occurred even after 1-2 months at 37°C and only 15-20% loss after 11.5 months at 37°C.

During this work it was noticed that large losses in enzyme activity were sometimes noticed immediately after the drying step. Such initial losses were decreased on adding the stabilisers but not eliminated. Subsequent experiments revealed the pH of the enzyme solution to be dried was extremely important, (section 5.A.4). A comparison of freeze drying to vacuum drying was also carried out in the presence of stabiliser, (section 5.A.5).

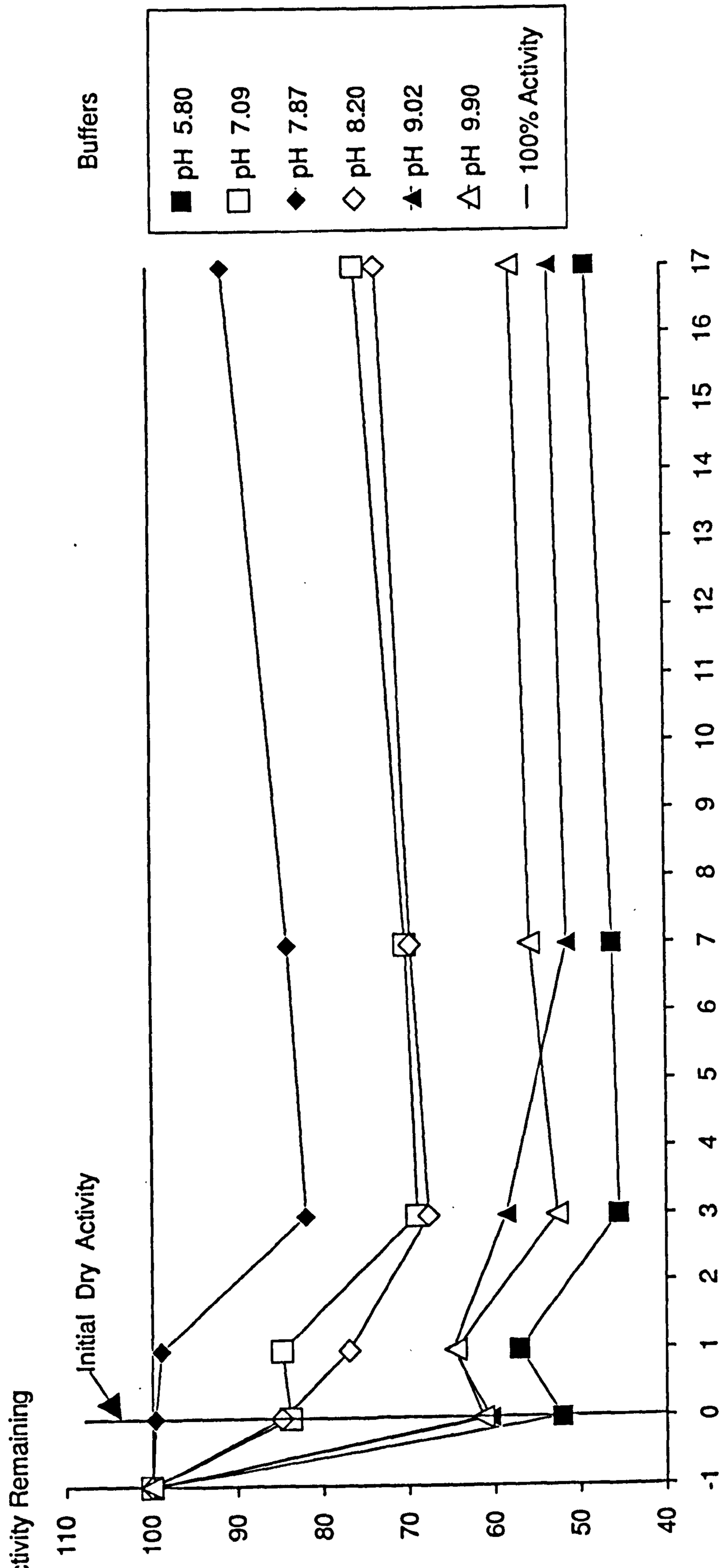
5.A.4. Effect of pH on Stability of Dry Enzyme.

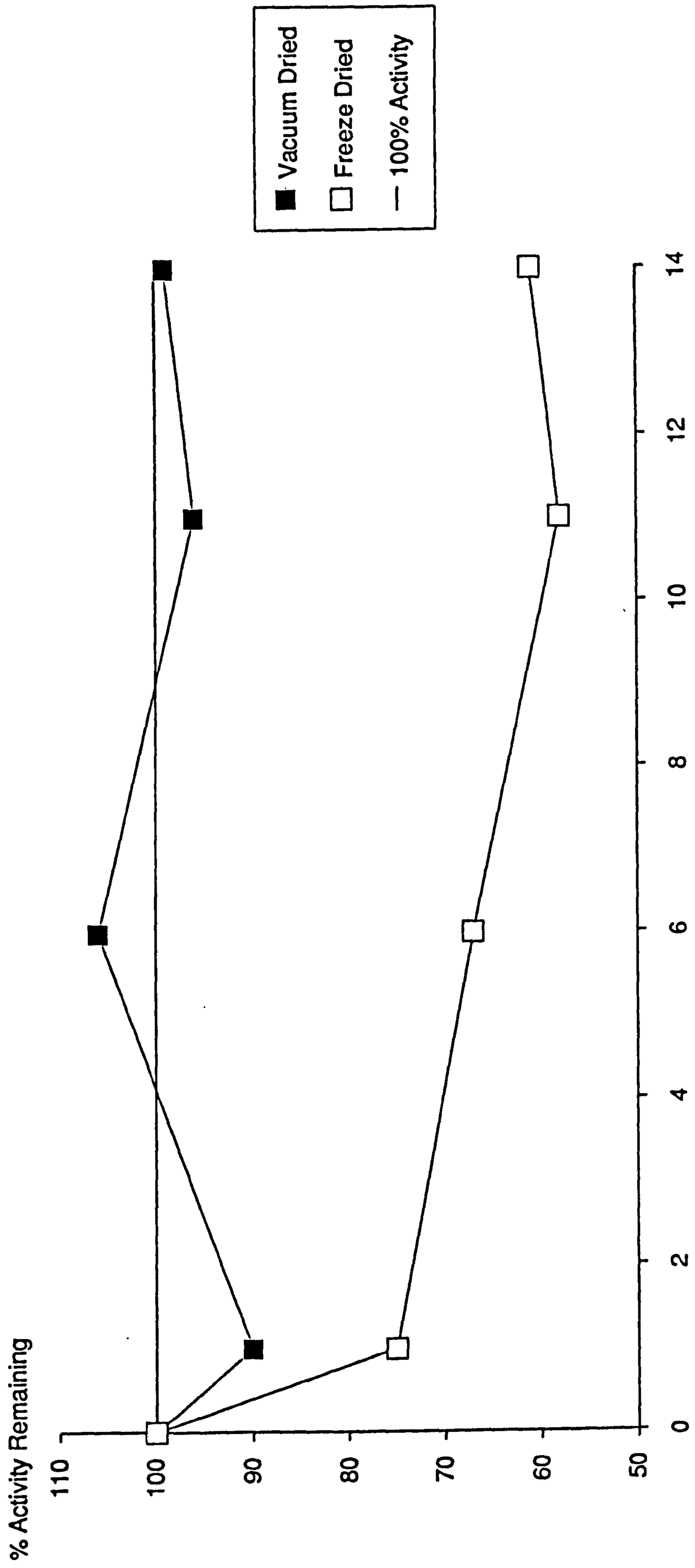
Variation of the pH of the buffer system affected the activity of the dry enzyme produced. The main effects appeared to be on the initial loss of activity on drying. However, some effects were seen on the overall stability of the dry enzyme produced. The results were plotted with the initial activity of the wet enzyme cocktail, (lactitol / DEAE-dextran stabilisers) taken as 100% activity, graph 48. In this way the initial loss of activity on drying was seen clearly. The optimum pH to reduce loss of activity on drying was found to be 7.87 for Hansenula alcohol oxidase.

5.A.5. Comparison of Vacuum vs Freeze Drying.

Both type of drying techniques were carried out on the enzyme in the presence of stabilisers. The results were plotted using initial dry activity as 100% reference value, graph 49. Stabilised freeze dried enzyme exhibited some loss of activity over the time period of the experiment, whereas vacuum dried enzyme retained full activity. Earlier attempts at freeze drying the unstabilised preparations of

Graph 48. ALCOHOL OXIDASE STABILISATION. pH Effect on Activity Loss.





Graph 49. ALCOHOL OXIDASE STABILISATION. Comparison of Vacuum vs Freeze Drying.

enzyme resulted in 92% inactivation. Vacuum drying unstabilised enzyme gave fully active preparations and in some cases slight stimulation of activity, however the activity decreased rapidly on storage, graph 36.

5.B. Pichia Alcohol Oxidase.

5.B.1. Characteristics and Stability of Native Enzyme.

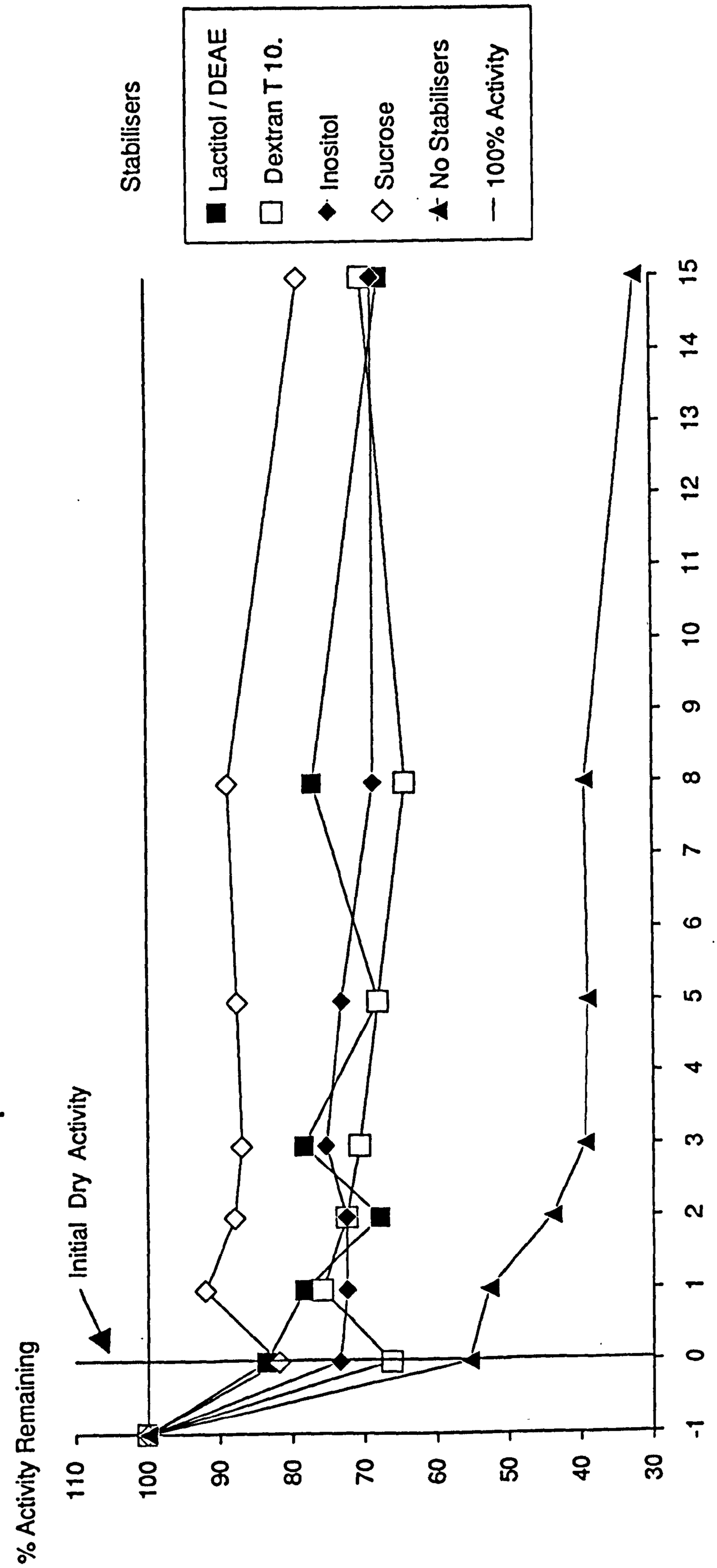
Pichia Pastoris alcohol oxidase was found to be similar to the Hansenula enzyme however it differed in its pH and temperature ranges. Also it differed in its solubility characteristics in that at low ionic strength it precipitated from solution, (Hopkins and Mueller 1987). The enzyme has also been found to be very sensitive to halide ion inhibition, much more so than the Hansenula enzyme, (Woodward 1990).

The stability of the native enzyme in "wet" preparations was poor, over 50% being inactivated within 10 minutes at 50°C and nearly 90% inactivated after 1 hour. Hansenula enzyme under the same conditions retained full activity, (Woodward 1990), graph 34. Stabilisation of the Pichia enzyme in solution was not attempted. Similarly dry preparations of the enzyme exhibited 40-45% loss of activity on drying with a further loss of 23% activity after 15 days at 37°C, graph 50.

5.B.2. Stabilisation using Additives. Dry Enzyme.

Pichia alcohol oxidase was supplied from the Provesta coporation as a gift. Two preparations were tested. One was an isoelectric precipitate slurry and the other the conventional solution of enzyme. Both were in 50mM phosphate buffer and preserved with sodium azide, (0.02%) and sucrose, 35% w/v for the slurry and 60% w/v for the soluble enzyme. The results obtained for both forms of enzyme were

Graph 50. PICHIA ALCOHOL OXIDASE STABILISATION.



identical, so no distinction between the original preparations was made in the data shown.

The enzyme was dialysed exhaustively against 200mM phosphate buffer, pH 7.0 to produce enzyme solutions free from sucrose and azide, before stabilisers were added. Only the stabilisers which produced stable preparations of Hansenula enzyme were tested, with the exception of sucrose. The buffer was phosphate at pH 7.0. The results were plotted using the activity of the wet enzyme as 100% reference value, graph 50.

5.B.3. Comparison of Vacuum vs Freeze drying.

Identical samples of Pichia alcohol oxidase were either vacuum dried or freeze dried with and without stabilisers. The results were plotted using the activity of the wet enzyme as 100% reference value, graph 51. Both types of drying inactivate the enzyme to some degree, however freeze drying retains higher initial activities overall than vacuum drying. This is particularly noticeable in the unstabilised enzyme, with a 24% loss in the case of the freeze dried enzyme and a 44.5% loss on vacuum drying.

5.C. Stabilisation of other enzymes.

The discovery of successful stabiliser combinations and the technique to produce a dry stable preparations of alcohol oxidase led to the attempt to stabilise five other enzymes, using the same techniques and stabilisers. These are shown as the top five enzymes in table 18. Also included in this table are results obtained by workers other than myself, using the same stabilisation techniques with a range of other enzymes. The results clearly indicate enhancement of enzyme stability in nearly all cases.

Graph 51. PICHIA ALCOHOL OXIDASE STABILISATION. Comparison of Vacuum vs Freeze Drying.

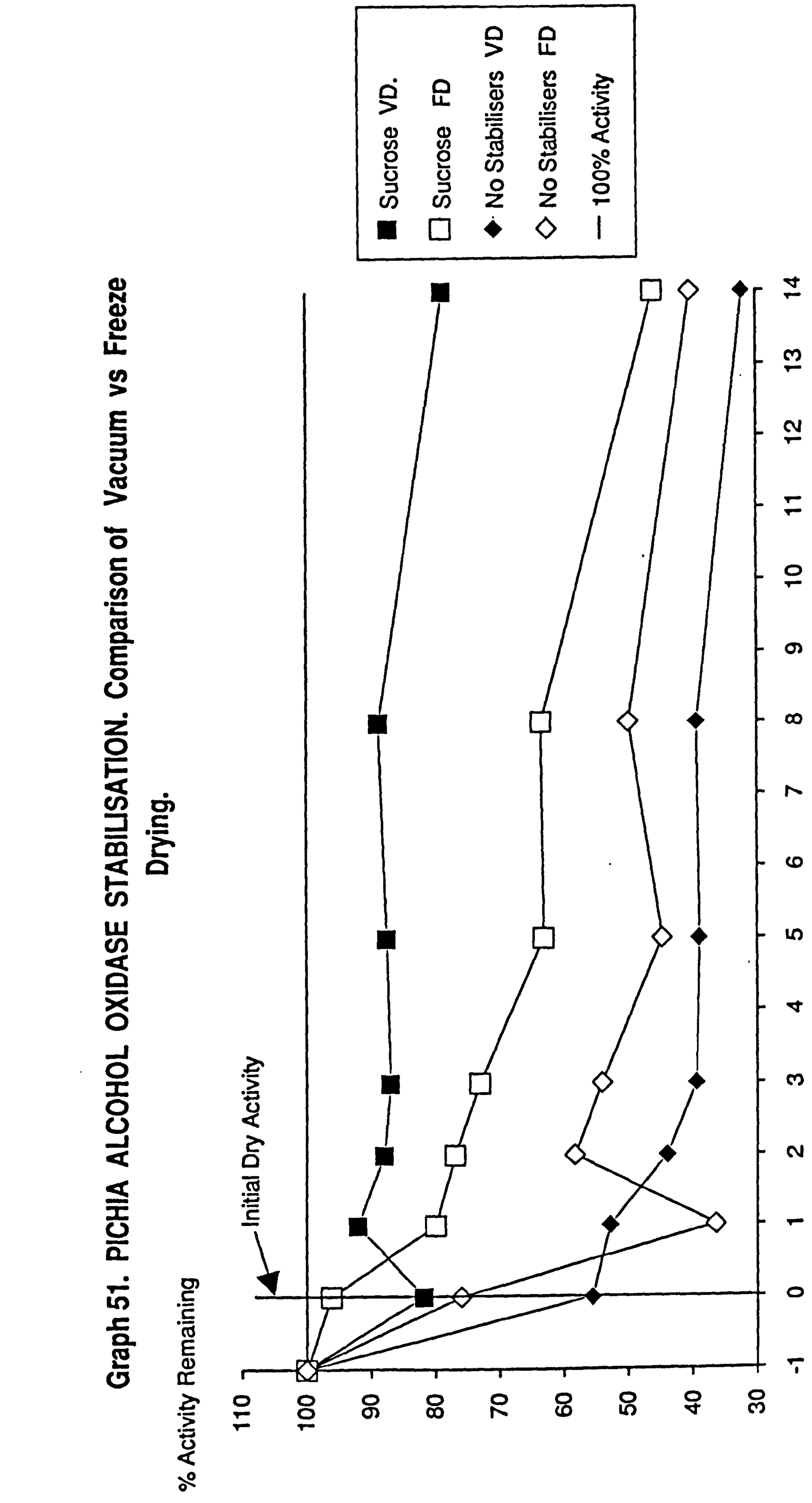


Table 18. STABILISATION OF OTHER ENZYMES.

ENZYME	SOURCE	STABILISER/CONCENTRATION	% ACTIVITY REMAINING AFTER DRYING	% ACTIVITY REMAINING AFTER INCUBATION	DAYS 37oC
Cholesterol Oxidase	Sigma	Lactitol 10% w/v : DEAE Dextran 1% w/v	93.7	92.5	21
Choline Oxidase	Sigma	Lactitol 10% w/v : DEAE Dextran 1% w/v	97.7	80.6	15
		None	63.3	3.9	15
Glycerol 3-Phosphate Oxidase	Sigma	Lactitol 10% w/v : DEAE Dextran 1% w/v	98.2	111.3	15
		None	110.7	59.7	15
Lactate Oxidase	Sigma	Lactitol 10% w/v : DEAE Dextran 1% w/v	90.0	91.6	15
		None	77.1	91.1	15
Galactose Oxidase	Dr.P.F.Knowles	Inositol 5% w/v	86.0	63.0	10
	Biophysics Dept.	Dextran (T 10 Phamacia) 5% w/v	84.0	69.0	10
Uricase	Enzymatix Ltd.	Lactitol 5% w/v : DEAE Dextran 1% w/v + EDTA 25mM	91.0	100.0	10
		None	82.0	26.0	15

Table 18. CONTINUED.

ENZYME	SOURCE	STABILISER/CONCENTRATION	% ACTIVITY REMAINING AFTER DRYING	% ACTIVITY REMAINING AFTER INCUBATION	DAYS 37oC
Cholesterol Oxidase	Genzyme	Lactitol 5% w/v : DEAE Dextran 1% w/v	36.0	93.0	17
		Dextran (T 10 Phamacia) 5% w/v	64.0	70.0	17
		Lactitol 5% w/v	67.0	100.0	17
		Sorbitol 5% w/v	85.0	79.0	17
		None	78.0	38.0	17
Galactose Oxidase	Dr.P.F.Knowles	Maltitol 5% w/v : DEAE Dextran 1% w/v	78.0	101.0	16
	Biophysics Dept.	Sucrose 5% w/v : DEAE Dextran 1% w/v	120.0	121.0	16
		Inositol 5% w/v : DEAE Dextran 1% w/v	125.0	117.0	16
		None	117.0	38.0	16
Peroxidase	Sigma	Lactitol 5% w/v : DEAE Dextran 1% w/v	117.4	100.0	15
		Lactitol 2.7% w/v : CM Cellulose 0.56% w/v	102.4	73.1	15
		Lactitol 2.7% w/v : Dextran SO_4 0.71% w/v	102.3	73.1	15
		Lactitol 5% w/v	79.4	66.7	15
		None	106.0	31.3	15

Table 18. CONTINUED.

ENZYME	SOURCE	STABILISER/CONCENTRATION	% ACTIVITY REMAINING AFTER DRYING	% ACTIVITY REMAINING AFTER INCUBATION	DAYS 37oC
Peroxidase	BioZyme.HRP-4b	Lactitol 5% w/v : DEAE Dextran 1% w/v	76.0	49.0	17
		Lactitol 5% w/v : Dextran SO_4 1% w/v	71.0	56.0	17
		Lactitol 5% w/v : CM Cellulose 1% w/v	80.0	35.0	17
		None	52.0	7.0	9
Peroxidase	BioZyme.HRP-5	Lactitol 5% w/v : DEAE Dextran 1% w/v	53.0	29.0	17
90% Isoenzyme C		Lactitol 5% w/v : Dextran SO_4 1% w/v	87.0	60.0	17
		Lactitol 5% w/v : CM Cellulose 1% w/v	78.0	65.0	17
		None	51.0	17.0	17
Alkaline Phosphatase	Sigma	Lactitol 5% w/v : DEAE Dextran 1% w/v	115.0	113.0	15
Type 1-S		Lactitol 2.7% w/v : CM Cellulose 0.56% w/v	100.0	92.0	15
		Lactitol 2.7% w/v : Dextran SO_4 0.71% w/v	83.0	95.0	15
		Lactitol 5% w/v	102.0	117.0	15
		None	112.0	64.0	15

Table 18. CONTINUED.

ENZYME	SOURCE	STABILISER/CONCENTRATION	% ACTIVITY REMAINING AFTER DRYING	% ACTIVITY REMAINING AFTER INCUBATION	DAYS 37oC
Diacetyl Reductase Chicken Liver	In house prep.	Lactitol 5% w/v : DEAE Dextran 1% w/v	59.0	40.0	8
		Lactitol 5% w/v : Dextran SO_4 1% w/v	216.0	144.0	8
		Lactitol 5% w/v : CM Cellulose 1% w/v	64.0	36.0	8
		Lactitol 5% w/v : Na Alginate 0.5% w/v	68.0	60.0	8
		Lactitol 5% w/v	82.0	58.0	8
		None	79.0	9.0	8
Lactate Dehydrogenase Type II	Sigma	Lactitol 5% w/v : DEAE Dextran 1% w/v	81.0	69.0	54
		Lactitol 5% w/v : Dextran SO_4 0.7% w/v	96.0	88.0	54
		Lactitol 5% w/v	85.0	88.0	54
		Dextran (T 10 Phamacia) 5% w/v	79.0	69.0	54
		None	64.0	46.0	54
				Temp. 50oC	
β - Galactosidase	Sigma	Lactitol 5% w/v : DEAE Dextran 1% w/v	115.0	103.0	23
		Lactitol 5% w/v : Dextran SO_4 1% w/v	109.0	81.0	36

Table 18. CONTINUED.

ENZYME	SOURCE	STABILISER/CONCENTRATION	% ACTIVITY REMAINING AFTER DRYING	% ACTIVITY REMAINING AFTER INCUBATION	DAYS 37oC
Temp.50oC					
β- Galactosidase	Sigma	Lactitol 5% w/v : CM Cellulose 1% w/v	114.0	72.0	36
		None	119.0	52.0	36
Cholesterol Esterase	Genzyme	Lactitol 5% w/v : DEAE Dextran 1% w/v	98.4	93.5	19
Pancreatic		None	98.5	109.3	19
Maleate Dehydrogenase	Sigma	Lactitol 5% w/v : DEAE Dextran 1% w/v	114.0	96.0	20
		Lactitol 5% w/v : CM Cellulose 1% w/v	77.0	89.0	20
		Lactitol 5% w/v	60.0	61.0	20
		None	14.0	2.0	20
Hexokinase	Genzyme	Lactitol 5% w/v : DEAE Dextran 1% w/v	71.0	71.1	21
		None	57.1	38.9	21
Glucose 6-Phosphate Dehydrogenase	Genzyme	Lactitol 5% w/v : DEAE Dextran 1% w/v	94.1	100.4	16

5.D. Summary of effective stabilisers.

The compounds which stabilise activity in the enzymes tested were predictably rich in hydroxyl groups. Sugars, sugar alcohols and other polyhydroxyl compounds are well known for their effect of protein stabilisation, (Back et al 1979, Combes and Monsan 1984, Ye et al 1988).

The enhancement of such stabilising effects by the inclusion of polyelectrolytes such as DEAE - dextran, chitosan, dextran sulphate, sodium alginate, sodium carboxymethyl cellulose and polyethylene imine, (PE imine) was unexpected and appears to be effective for a wide range of enzymes. The stabilisers used in further work on the preparation of dry phase diagnostic tests were routinely classified into three groups.

(1) Polyelectrolyte / sugar or sugar alcohol combinations
e.g. DEAE-dextran / lactitol, sodium alginate / lactitol.

(2) Cyclic polyalcohols or sugars,(particularly disaccharides)
e.g. inositol, trehalose.

(3) Neutral polymers
e.g. dextrans from various sources.

The results of this work form the basis of two patent applications on enzyme stabilisation.

CHAPTER 6.

The DEVELOPMENT of DRY PHASE ALCOHOL TESTS and COLOUR THRESHOLD SYSTEM.

6.A. Dry Phase Tests: Overview.

Dry phase or dry reagent tests are basically complete analytical systems in a discrete single use format. Sometimes they incorporate all the necessary chemical reagents and / or enzymes in a single format such as a dry filter paper disc. Such tests require only the addition of the sample to be analysed to produce a result. Other tests incorporate part of the analytical chemistry and require sequential additions of reagents for analysis. The former type of test is becoming much more widespread and more generally accepted as a valid means of analysis.

The earliest reported dry test was the glucose oxidase - peroxidase reaction for blood glucose (Free et al 1957). Many variations of this type of test are cited in the patent literature, the majority using highly visible chromagens as the detection method. Table 19 lists a selection of applications of such techniques.

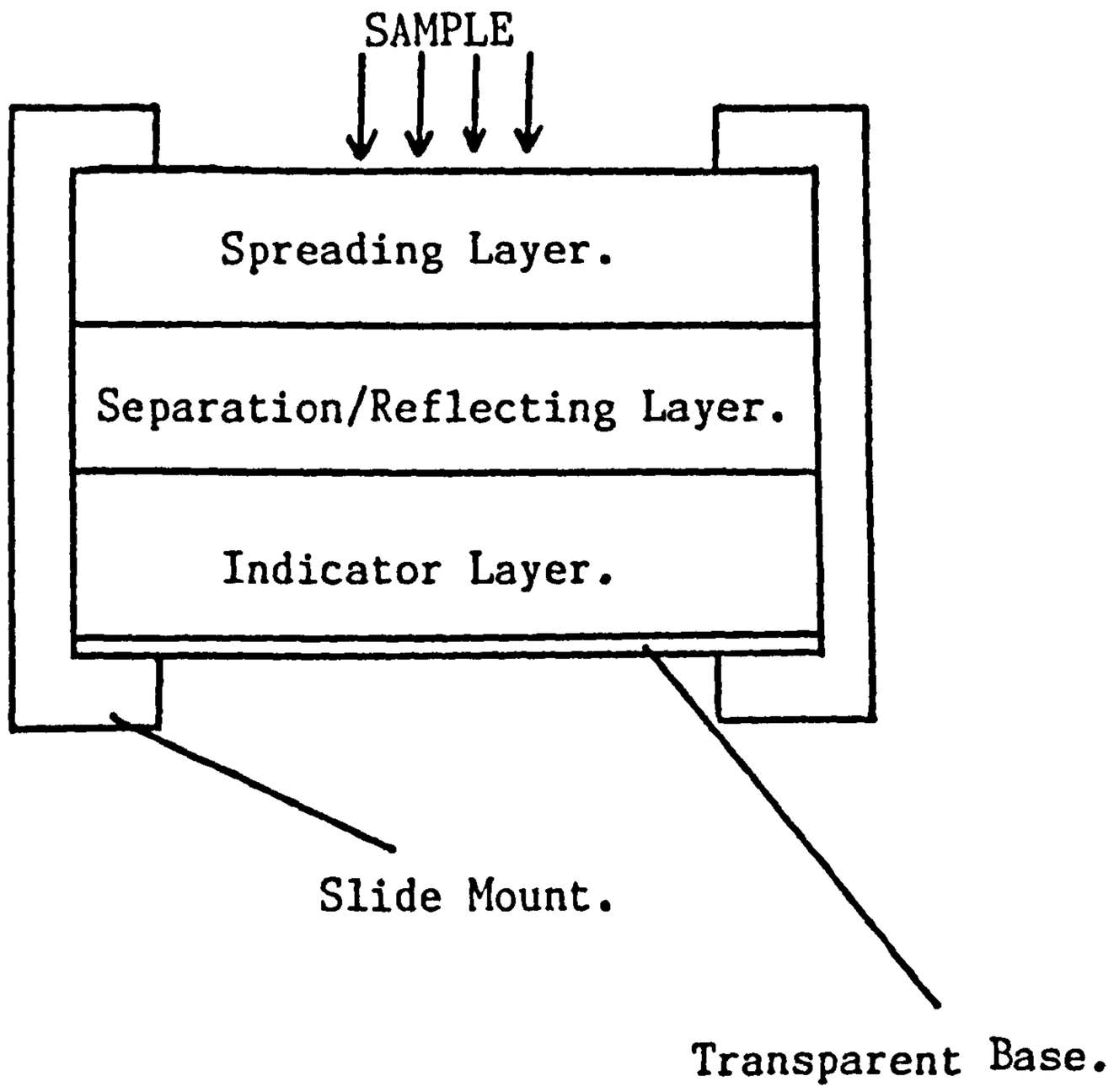
More complex single use dry tests have since been developed, particularly using the photographic technique of producing thin reactive films or emulsions. The "Kodak" Ektachem system is a product of such technology, where multiple layers are sequentially built up on a transparent base each containing specific components required for the final analytical reaction, fig 28a. One advantage of such a system is that controlled micro environments may be produced, suitable for the individual reaction components of the test. Mixing and subsequent reaction only occurs during the actual analysis, thus enhancing storage stability.

The "Boehringer Mannheim" Reflotron system is similar in that the analytical element is a composite of several discrete layers of material which are saturated and compressed together during analysis,

Table 19. Analysis Using Dry Tests.

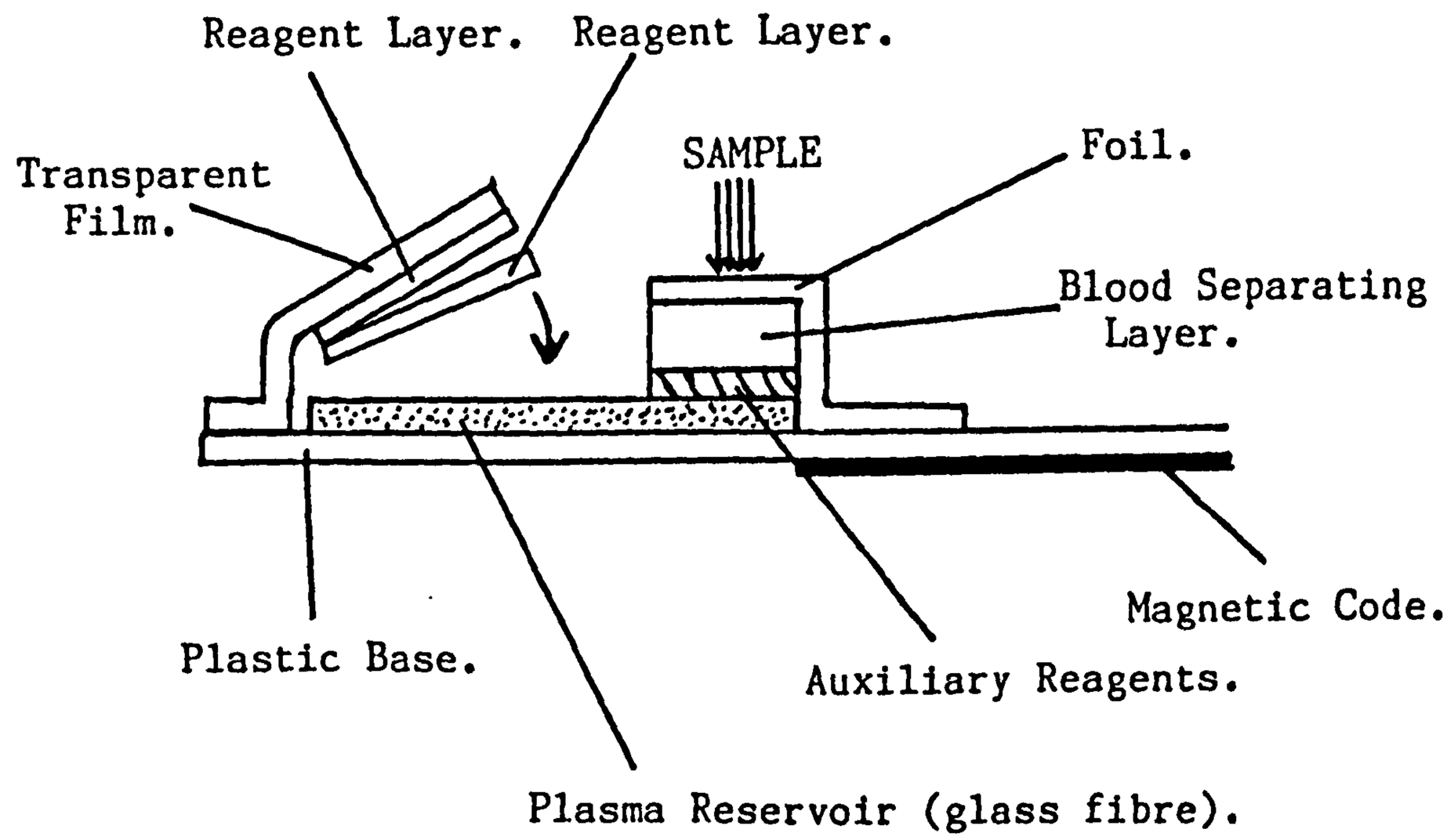
Analyte	Enzymes	Author	Patent No	Date
Cholesterol	Cholesterol Oxidase Lipase Peroxidase	Goodhue C.T et al	U.S.3,983,005	1976
Glucose	Glucose Oxidase Peroxidase	Genshaw M.A.and White W.I.	U.S.4,211,845	1980
Digoxin	Tyrosinase Laccase	Duffy P.	W.081/00725	1981
Occult Blood Haemoglobin	-	Burkhardt A.E. and Tideman A.M.	EP. 0,041,188	1981
Glucose Cholesterol	Glucose Oxidase Cholesterol Exterase Cholesterol Oxidase Peroxidase	Shuenn-Tzong C.	U.S.4,36,648	1982
Phenytoin	Apoglucose Oxidase Peroxidase	Tabb D.L. Tyhach R.J.	U.S.4,362,697	1982
Glucose	Glucose Oxidase Peroxidase	Shuenn-Tzong C. et al.	U.S.4,427,770	1984
Glucose Alcohol	Glucose Oxidase Alcohol Oxidase	Phillips R.C. et al.	EPA.0,110,173	1984
Cholesterol	Cholesterol Esterase Cholesterol Oxidase Peroxidase	Arai F. and Kondo A.	EPA.0,256,562	1988

FIG.28a KODAK EKTACHEM DRY PHASE TEST.



The Kodak Ektachem multilayer system incorporates a series of layers each with its own specific function.The spreading layer delivers the sample evenly to the underlying reactive layers which are formulated to detect and quantify specific analytes. The reflectance is measured through the back of the slide.

FIG.28b BOEHRINGER REFLOTRON DRY PHASE TEST.



The Reflotron system incorporates a blood separation layer which removes the erythrocytes and delivers the plasma via the plasma reservoir to the reagent layers.During analysis these are crushed by the reflectometer onto the plasma. This is indicated by the curved arrow. The indicator reaction takes place in the reagent layers and the reflectance is measured through the top of the transparent film.

fig 28b.

Quantification of analyte concentration using these dry phase systems may be carried out by using fairly sophisticated instruments specifically designed to measure reflected light, these are known as reflectometers. Recently, a simple hand held reflectometer has been introduced, which in conjunction with specific test strips, provides an accurate measurement of glucose concentration in blood, (Brodrick et al 1987, Begley and Forrest 1988). In the absence of such instrumentation, semi-quantitative analysis can be performed by visual comparison of the developed test with a colour bar chart, which is usually supplied printed on the test strip container. Accurate comparison using such a technique is highly operator dependent, thus producing large errors.

Certain tests, such as pregnancy tests, which utilise a positive or negative end point are definitive and largely free from operator error, if they are made in the single addition analytical format. e.g. "Clearblue" from Unipath, (May et al 1988).

A recently published series of patents, (Palmer and Timmerman 1988, Palmer et al 1989, Palmer and Timmerman 1989) disclosed diagnostic systems based on a range of enzymes which give a clear cut positive or negative end point. The concentration of analyte may be quantitated using such a system by varying the concentration of the components during manufacture to internally set the end point required.

The work reported here has developed from early trials in 1986 and the observance that peroxide scavengers such as ascorbate, cysteine and other reducing agents, destroyed the colour formed in enzyme / chromagen systems during drying procedures using cellulose

filter papers as supports. This phenomenon was reported earlier by Phillips (1985). Some of the reducing agents have also been found to be substrates in the peroxidase reaction, (Randell 1946, Stonier and Yang 1972).

The inclusion of this type of reaction into dry enzyme - chromagen systems, in conjunction with the effective stabilisers found earlier, produced an analytical test system which could be pre-set to develop colour at a range of analyte concentrations. This type of detection system produces definitive yes/no results thereby largely eliminating operator error.

The prototype systems developed were based on the Hansenula alcohol oxidase - peroxidase coupled reaction with ethanol as the variable analyte.

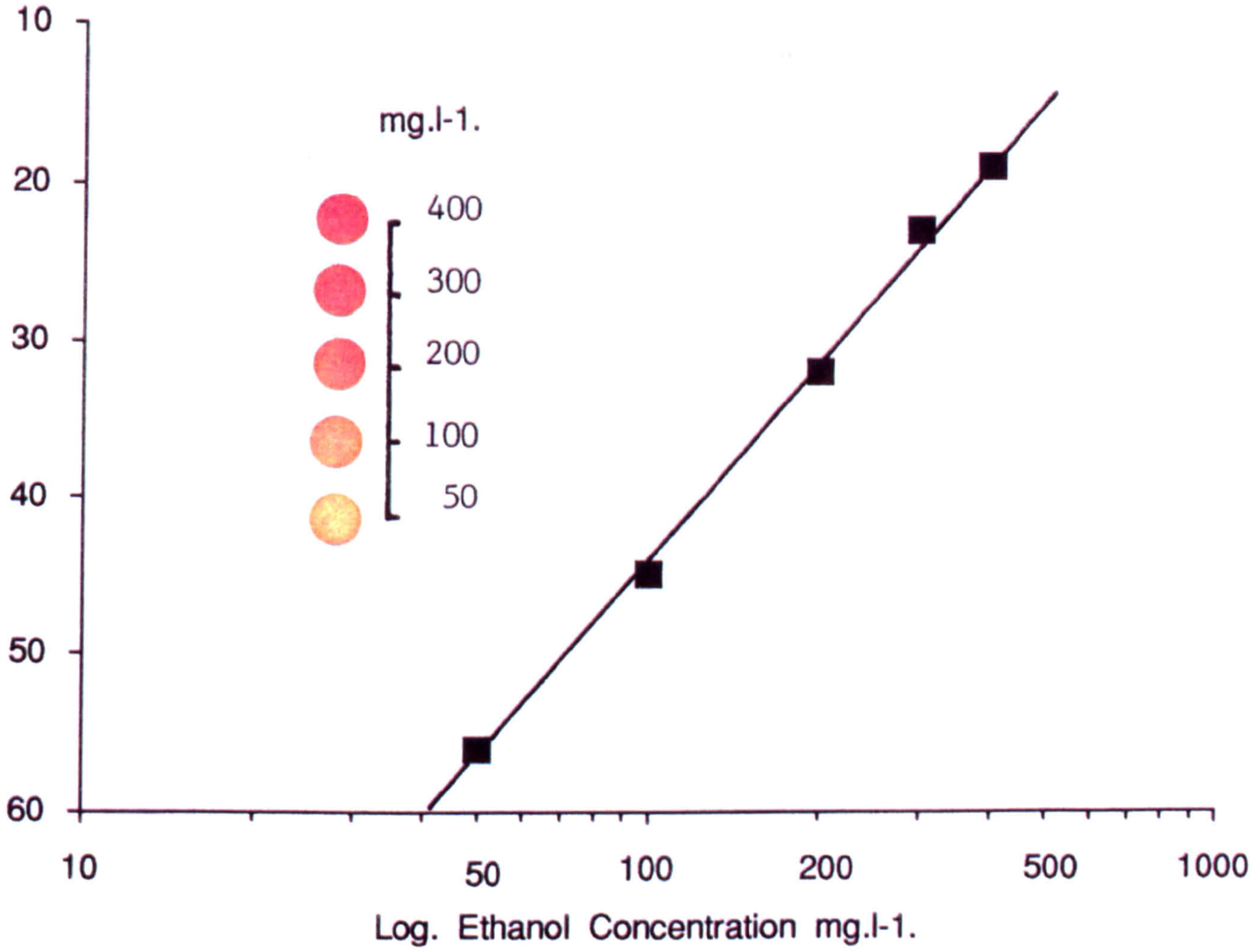
6.B.1. Dry Alcohol Tests. Early Models.

The earliest model systems fabricated, were designed to produce a graduation of colour when developed with ethanol concentrations of 100 $mg.l^{-1}$ up to 1000$mg.l^{-1}$. Graph 52 shows a reflectance plot of the colour bars as a function of ethanol concentration up to 400$mg.l^{-1}$. The graduation was improved if ascorbate was added to the system and in some cases, no colour was formed due to an excess of ascorbate being added. The stability of the enzymes was poor, as the effective stabilisation systems had not yet been discovered.

The next series of trials involved incorporation of the stabilisers into the enzyme system, with a variation in ascorbate concentration. The formulation, (1) is shown below:

Hansenula alcohol oxidase	40$U.ml^{-1}$
Peroxidase	25$U.ml^{-1}$
Lactitol	5% w/v

Graph 52. DRY PHASE GRADUATED ETHANOL TEST.



COLOUR REAGENTS.

4-AMINOANTIPYRINE

3-HYDROXY 2,4,6-TRIBROMO BENZOIC ACID.

DEAE-dextran	1% w/v
N,ethyl N,sulphopropyl 3-toluidine	10.0 mM.
4-Aminoantipyrine.	1.0 mM.
Phosphate buffer, pH 7.0	approx. 100 mM.
Ascorbic acid	variable see table 24.

This cocktail was absorbed on Whatman 3MM Chr paper and dried in a vacuum oven at 30°C for 30 minutes. The results on development with aqueous ethanol solutions are shown in table 20. Full development of the deep purple colour is indicated by + , partial development by +- and no colour by - .

Table 20. Colour Development. Ethanol Dry Phase Test. Ascorbate Concentration Varied.

	Ascorbic Acid $mg.ml^{-1}$							
Ethanol $mg.l^{-1}$	0.2	0.4	0.6	0.8	1.0	1.2	1.4	2.25
100	-	-	-	-	-	-	-	-
200	+	+-	-	-	-	-	-	-
300	+	+	+	-	-	-	-	-
400	+	+	+	+-	+-	-	-	-
500	+	+	+	+	+-	+-	-	-
800	+	+	+	+	+	+-	-	-
1000	+	+	+	+	+	+	+-	-
2000	+	+	+	+	+	+	+	+-
4000	+	+	+	+	+	+	+	+
Time for Full Colour	2min			5min				10min

Further trials using the same formulation, with the exception of an increase in alcohol oxidase concentration up to $60U.ml^{-1}$ and 100

U.ml^{-1}, met with little success in reducing the time taken to develop full colour. Replacement of the ascorbate with cysteine produced an almost identical result. The development time was 5-10 minutes at an alcohol oxidase concentration of 60U.ml^{-1}, table 21.

Table 21. Colour Development. Ethanol Dry Phase Test
Cysteine concentration varied.

	Cysteine mg.ml^{-1}								
Ethanol mgl^{-1}	0.5	1.0	1.5	2.0	2.5	4.0	4.5	7.0	8.0
200	+-	-	-	-	-	-	-	-	-
300	+	+-	-	-	-	-	-	-	-
400	+	+	+	+-	-	-	-	-	-
500	+	+	+	+	+	-	-	-	-
800	+	+	+	+	+	+	+-	-	-
1000	+	+	+	+	+	+	+	+-	-
2000	+	+	+	+	+	+	+	+	+-

Replacement of the Hansenula alcohol oxidase with the enzyme from Pichia supplied by Provesta Corporation in a similar system, (colour coupler, N,N bis(hydroxyethyl)aniline) gave similar results. However the system was more sensitive to the mediator, giving no colour at concentrations of 3.5mg.ml^{-1} cysteine.

The susceptibility of the Pichia enzyme to chloride inhibition and its relative instability led to its use being discontinued in the dry test development.

6.B.2. Dry Alcohol Tests. Mediation of the Colour Reaction.

From earlier observations, mediators, such as cysteine and ascorbate which react readily with hydrogen peroxide, (being oxidised to cystine and dehydroascorbate) may be used to inhibit colour

formation in dry phase tests which rely on hydrogen peroxide detection for the final colour reaction. The sequential increase in concentration of the mediator appeared to offer a means of quantitation of the analyte being detected, by generating a concentration threshold. Above this threshold colour developed rapidly, whereas below the threshold, no colour was formed. An example of this effect in the dry phase threshold test for ethanol is given in fig 29. The colour coupler was 10mM 2,4,6-tribromo 3-hydroxy benzoic acid and the mediator was cysteine at concentrations of 1.0, 2.0, 3.0, 4.0, 5.0 $mg.ml^{-1}$. The support was Whatman 3MM Chr paper.

A series of potential mediators were tested in the basic formulation (2) given below, to ascertain their effectiveness in generating a concentration threshold.

Alcohol oxidase	100-200$U.ml^{-1}$
Peroxidase	100$U.ml^{-1}$
Inositol	5% w/v
N,N,Bis(hydroxyethyl) aniline	25 mM
4-Aminoantipyrine	20 mM
N-(Morpholino) propane sulphonic acid, (MOPS) buffer, pH 7.9	100 mM

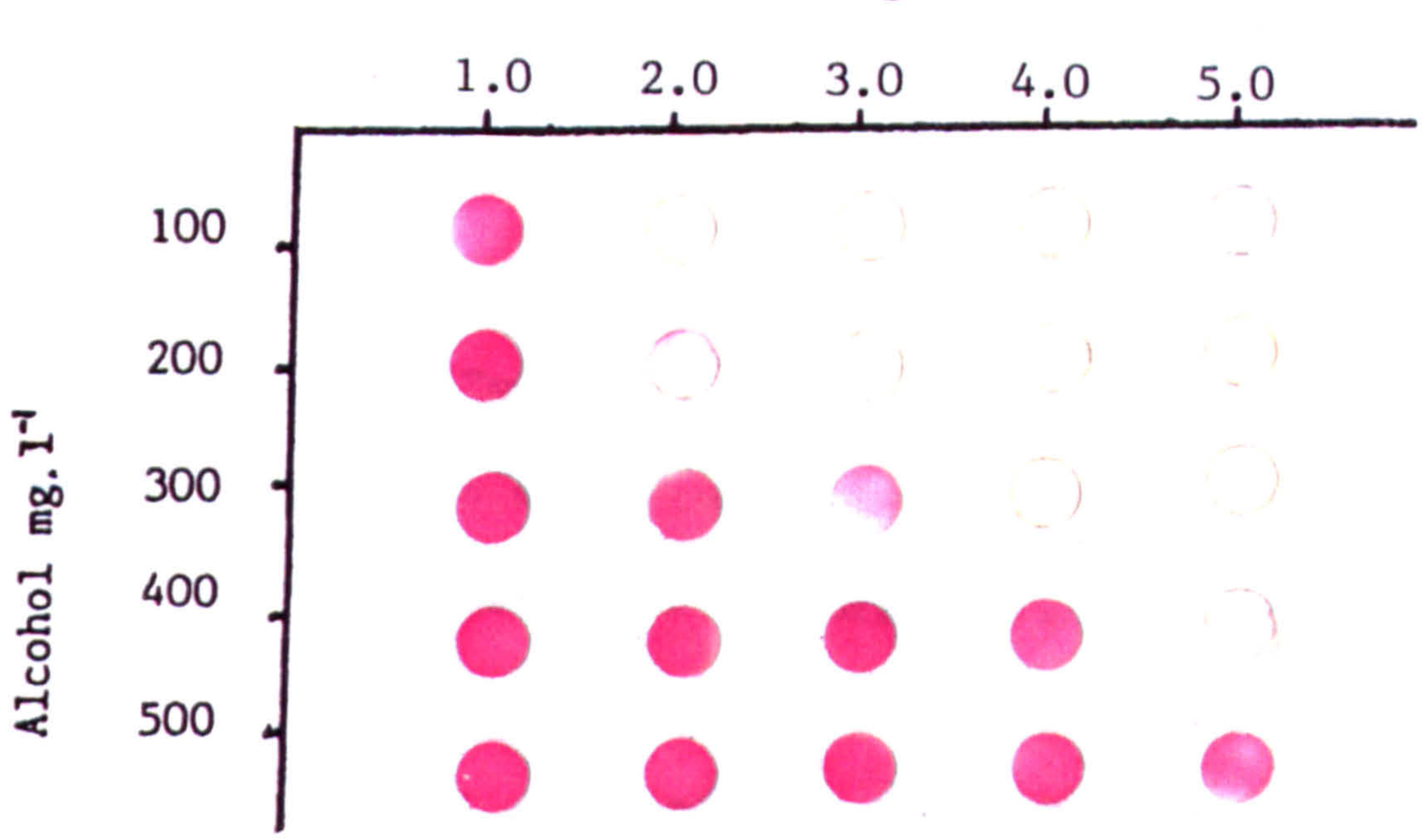
The improvements made to the earlier enzyme / colour reagent cocktail involved various stages:-

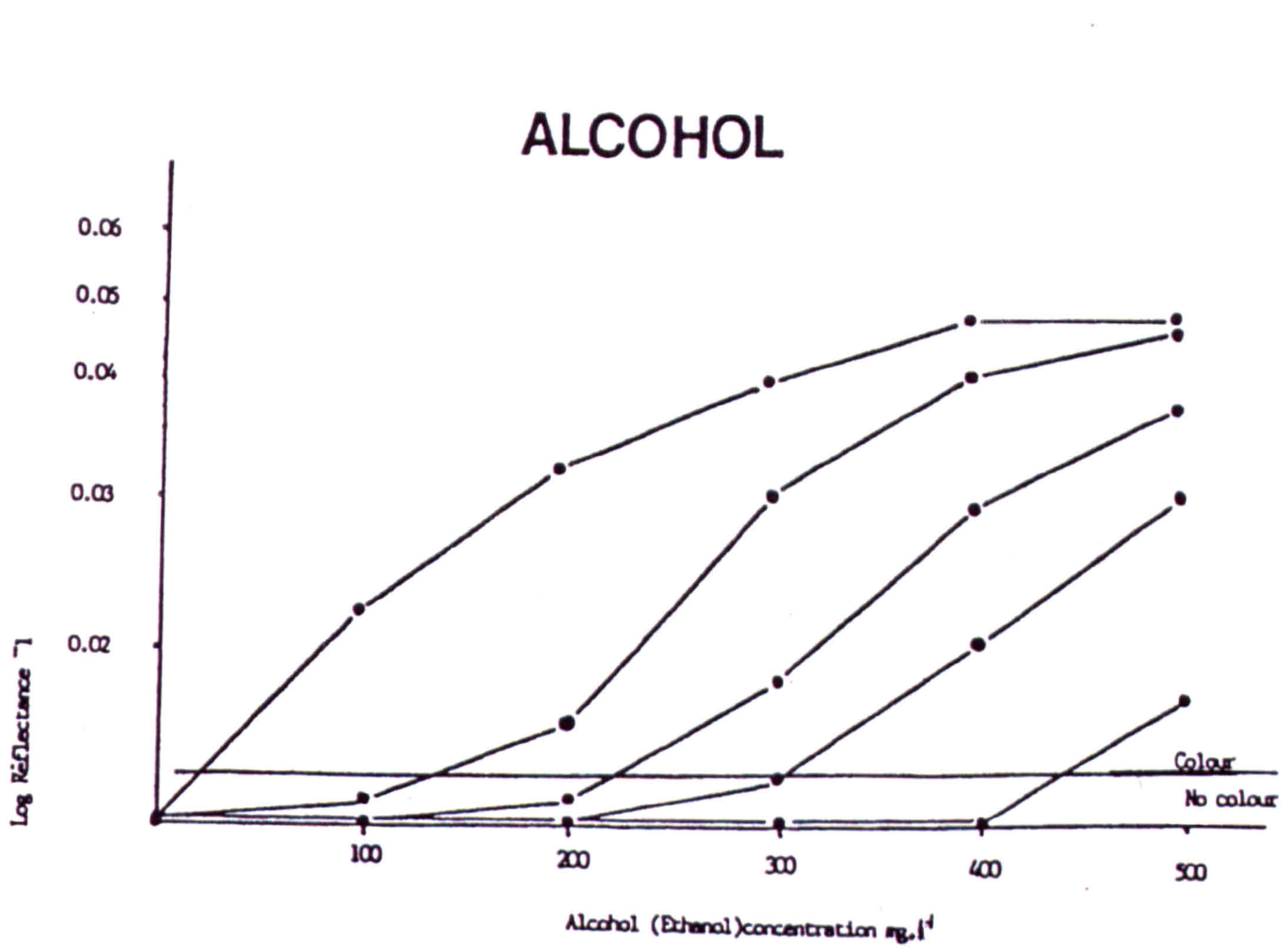
1) Higher enzyme levels to reduce reaction times and give rapid results.

2) Replacement of lactitol / DEAE-dextran by inositol to avoid the precipitation of the enzymes.

3) Alternative colour couple which was; (a) less soluble to

FIG 29. DRY PHASE ALCOHOL THRESHOLD TEST.





prevent excessive leaching, (b) sensitive to mediation, (c) produces a very intense purple colour on development and (d) was cheap and readily available.

4) Phosphate buffer was replaced by MOPS to ensure complete and almost instantaneous solubilisation of the buffer system. Also enhanced enzyme activity was noticed in this buffer and at this pH.

The mediators used and their effects are listed in table 22. Ascorbate and cysteine are included in the table for completeness.

6.B.3. Dry Alcohol Tests. Colour Reagents.

A large selection of colour reagents were tested in the dry phase system. An overview of the colours and susceptibility of the dyes produced to bleaching by ascorbate or cysteine is reported in appendices II and III. In addition to this, general observations and rationales are reported below for the various classes of chromogenic systems tested.

(a) Redox dyes.

Benzidine type dyes are used in many dry enzyme systems especially 3,3',5,5'-tetramethyl benzidine (TMB), which is reported to be non-carcinogenic, (Holland 1974, Liem et al 1979, Josephy et al 1982). The colour change is from colourless through to green-blue with a further brown-orange oxidation state, fig 3. Most tests are fabricated to stop at the green-blue stage, the chromagenic species being stabilised by some sort of additive e.g. pyridine N-oxides, (Magers and Tabb 1981).

Ascorbate or cysteine may be used to mediate the colour development of TMB. The main drawback using TMB was its very low solubility in water, which necessitated preparation of the enzyme paper plus mediators, stabilisers, buffers, etc. which was then dried

Table 22. POTENTIAL MEDIATORS. COLOUR THRESHOLD DRY PHASE TESTS.

MEDIATOR.	CONCENTRATION RANGE mg.ml-1	MEDIATION.	SOLVENT.	SOLUBILITY.	STABILITY. WET	STABILITY. DRY	COMMENTS
Ascorbate	0.1 - 2.5	Good	Water	V Soluble	L	A	Sensitive to light and air.
Palmitoyl Ascorbate		Good	Ketones e.g.Butanone	Soluble	NK	A	Sensitive to light and air. Patchy Development.
Cysteine Free Base	0.5 - 20.0	V Good	Water	Soluble	L	A	Sensitve to air.Stable in acidic solution.
Cysteine HCl	0.5 - 20.0	Good	Water	V Soluble	L	A	Hygroscopic. Sensitive to air.
Glutathione	0.1 - 2.0	Good	Water	V Soluble	L	F	Sensitive to air and heat.
Dithioerythritol	0.1 - 2.0	V Good	Water	Soluble	L	A	Sensitive to air and heat.
Dithiothreitol	0.1 - 2.0	V Good	Water	Soluble	L	A	Sensitive to air and heat.
Thioglycollic Acid Na Salt	0.5 - 3.0	Good	Water	V Soluble	VL	NK	Sensitive to air and heat. Stench. Hygroscopic.
Thiouracil	0.5 - 3.0	None	Water	Poor	A	A	No apparent mediation.
Mercaptobenzo-thiazoline	0.5	Poor	Water	Poor	NK	NK	Small mediation effect.

Table 22. CONTINUED.

MEDIATOR.	CONCENTRATION RANGE mg.ml-1	MEDIATION.	SOLVENT.	SOLUBILITY.	STABILITY. WET	STABILITY. DRY	COMMENTS
Sodium Mercapto-ethane Sulphonate	0.5 - 3.0	V Good	Water	V Soluble	NK	NK	Very effective mediation. Stability not yet tested.
Cysteamine	0.5 - 3.0	Good	Water	V Soluble	L	NK	Sensitive to air. Hygroscopic.
Cysteine Ethyl Ester	0.5 - 3.0	Good	Water	Soluble	NK	NK	Sensitive to air.
Thiosalicylic Acid	0.5 - 3.0	Poor	Water	Soluble	NK	NK	Small mediation effect.
Thiourea	0.5 - 3.0	Fair	Water	Soluble	A	A	Toxic.
2-Mercapto-Pyridine	0.5 - 3.0	Good	Water	Soluble	NK	NK	Sensitive to heat.
Sodium Sulphite	0.5 - 3.0	Good	Water	Soluble	L	NK	SO_2 evolved in solution.
Sodium Thiosulphate	0.5 - 3.0	Fair	Water	V Soluble	L	NK	Sensitive to air.
Potassium Iodide	0.5	None	Water	Soluble	A	A	No mediation effect.
Potassium Ferricyanide	0.5 - 3.0	None	Water	Soluble	A	A	No mediation effect.
Potassium Ferrocyanide	0.5 - 3.0	None	Water	Soluble	A	A	No mediation effect.
Sodium Metabisulphite	0.5 - 3.0	Good	Water	Soluble	L	NK	SO_2 evolved in solution.

Abbreviations. L Labile. A Acceptable. NK Not Known. F Fair. V Very.

and overlaid with a solution of TMB in 1,1,1-trichloroethane and allowed to air dry. The water soluble hydrochloride salt of TMB proved difficult to mediate and invariably the colour development continued to the brown-orange state. For these reasons, TMB was not used routinely in the threshold development system.

ABTS proved unsuitable as a chromagen in the dry phase system as instead of the blue-green radical formed in solution, a pale purple grey colour resulted on development.

Sodium diphenylamine sulphonate was also tested. This compound gave a dirty, brown-grey colour on development, which was not suitable for routine use.

(b) Condensation reactions.

The majority of colour producing reactions tested belong to the class where two separate intermediate molecules form a dye molecule under oxidative conditions. These conditions are provided by a peroxidase catalysed reaction of one of the intermediates with H_2O_2, thus giving a reactive species. Subsequent colour generation occurs as the second intermediate reacts, producing the final dye. This type of reaction is analogous to colour generation in photographic films and in peroxide catalysed hair dyes.

Four types of reactive compounds which, for ease of identification, are termed developers, are listed below. These, in combination with various other coupling compounds, produced a wide range of dyes, differing in stability and spectral properties. The dyes so formed were produced in a "wet" enzyme system and are listed in appendix II. A variety of these systems have been tested in a dry format.

(i) 4-Aminoantipyrine (4-AAP).

This compound was a very soluble pale straw coloured hygroscopic solid. It gradually oxidised in air, with subsequent generation of a deep yellow colour. It appeared to be compatible with all the enzyme systems used, producing a variety of red, purple, and violet colours. The colour systems based on this compound were the most stable tested and gave very pale background colours with a sharp clear cut off at the threshold. Colour generation tended to be slow, such as in the case of cholesterol and at low enzyme concentrations.

Stoichiometrically the reaction requires two molecules of hydrogen peroxide to form one molecule of dye, fig 5. This was an advantageous feature of the colour reactions based on 4-AAP, because threshold generation was dependent on the amount of hydrogen peroxide present. An analogous non-hygroscopic compound, 4-dimethyl-aminoantipyrine was also tested in the dry phase system. This gave a similar response to 4-AAP, however the dyes formed were not as intensely coloured and the reactions were much slower.

(ii) 3-Methyl 2-Benzothiazolinone Hydrazone (MBTH).

The hydrochloride salt of MBTH was a pale cream, aromatic crystalline solid. It was soluble in water, but not very soluble in the common buffer systems used in the dry phase tests. Addition of MOPS buffer to a solution of MBTH in water produced a copious crystalline precipitate. For this reason alone it was not used very frequently in dry phase tests. The dyes produced using MBTH were very intensely coloured and resistant to bleaching. Some of these dyes were sparingly soluble and precipitated out of aqueous solution on reaction. Mediation of MBTH reactions was found to be fairly difficult, with higher concentrations of mediators being required,

the best results being obtained using ascorbate.

MBTH itself could be used to produce a blue/green coloured dye on development in the ethanol system. This was presumably due to self-coupling as reported by Capaldi and Taylor (1983), fig 7. Dry test cards containing MBTH were air and light sensitive, darkening from a pale straw colour to orange yellow or green yellow over a period of time. Background development of dye in the absence of analyte was also noticed, particularly in the test cards stored over long periods.

(iii) N,N, Disubstituted 1,4 Phenylene Diamines.

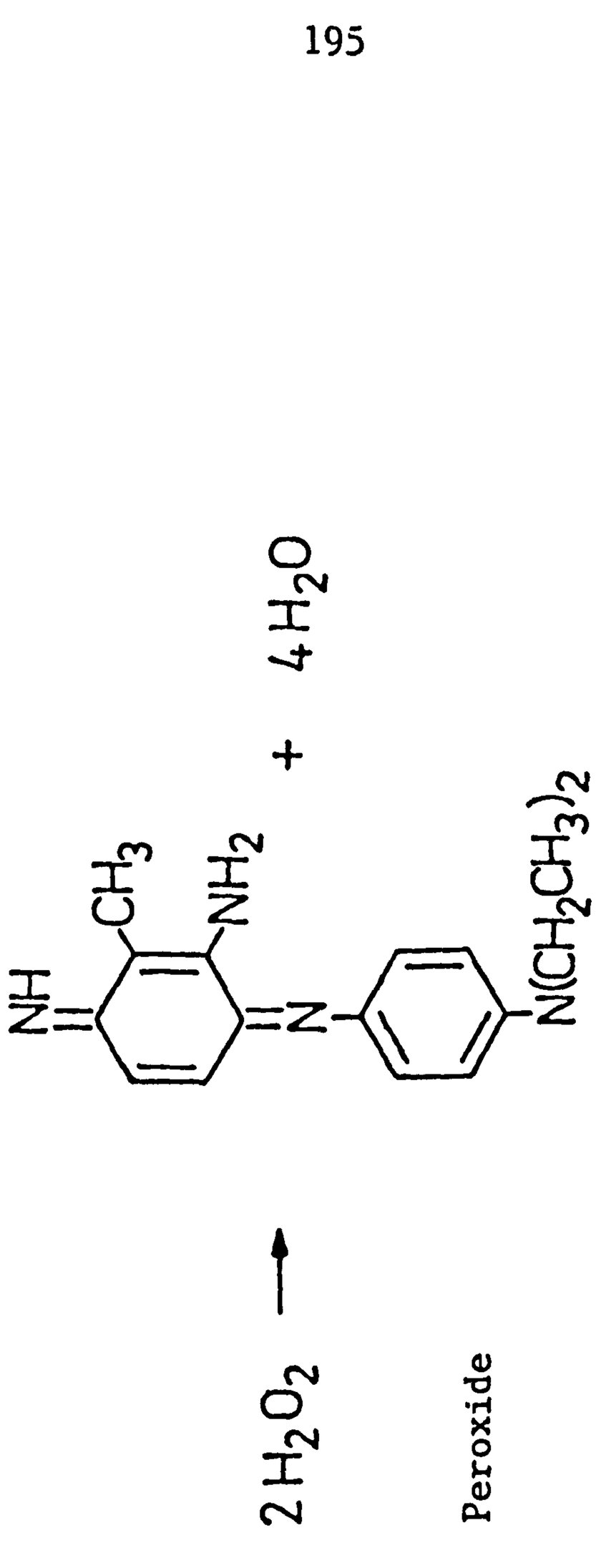
These compounds form the basis of the colour photography industry, being used as colour developers with a variety of colour couplers. The synthesis and toxicity of a large number of such compounds has been investigated in detail, (Bent et al 1951). A typical reaction is shown in fig 30. A variety of compounds from several different sources were tested in dry phase tests, these are listed in table 23. The dyes produced were very intensely coloured and stable. Mediation of the colour reaction was good, however, development of background colour was a serious problem. The reactivity of the compounds was very high and they were also air and light sensitive. A typical example of such compounds is N,N diethyl 1,4-phenylene diamine sulphate (Fluka). This was used to produce the data in appendix II.

An advantage of compounds of this type is the rapidity of the colour reaction on development. The extreme sensitivity of the system precluded its use routinely.

(iv) Substituted Pyrimidines.

These are listed in table 24. Their reaction with a range of

FIG.30 1,4 PHENYLENE DIAMINE CONDENSATION REACTION.



Oxidative condensation reactions of substituted 1,4 phenylene diamines are well known in photography and in hair dying. Such enzymereactions may also be used to detect phase enzyme systems.For a comprehensive overview of colour reactions see appendix II.

Table 23. N,N Dialkyl Substituted Phenylene Diamines.

Compound	Source
N,N,Dimethyl 1,4 phenylene diamine sulphate.	Lowenstein & Son Inc.
N,Phenyl 1,4 phenylene diamine sulphate.	Lowenstein & Son Inc.
N,N Bis(2-hydroxyethyl) 1,4 phenylene diamine sulphate.	Lowenstein & Son Inc.
N,N Diphenyl 1,4 phenylene diamine.	Aldrich.
N,N Diethyl 1,4 phenylene diamine sulphate.	Fluka.
N Ethyl,N(2-hydroxyethyl) 1,4 phenylene diamine sulphate.	Fluka.
2-Amino,5-diethylamino toluene HCl (CD-2).	Kodak.
4-Amino, N,ethyl, N,(methane sulphamido ethyl) 3-toluidine sesquisulphate (CD-3).	Johnson.
4-(N,Ethyl N, 2-hydroxyethyl) 2-methyl phenylene diamine sulphate (CD-4).	Kodak.
4-(N,Ethyl N, 2-methoxyethyl) 2-methyl phenylene diamine di p-toluene sulphonate.	Kodak.

Table 24. Substituted Pyrimidines.

Compound.	Source.
2,4,5,6 Tetra-amino pyrimidine sulphate.	Sigma
2,5 Diamino 4,6 dihydroxy pyrimidine hemisulphate.	Sigma.
5,6 Diamino 2,4 dihydroxy pyrimidine.	Sigma.
6 Hydroxy 2,4,5 triamino pyrimidine sulphate.	Sigma.

couplers produced dyes which were easily mediated, the exception being the reaction with 3-aminophenols which produced very intense, stable violet-red dyes, (appendix III) for which mediation was found to be difficult. This was thought to be due to the formation of a three membered heterocyclic ring system on reaction.

The major drawback of these compounds was their solubility and the sensitivity of the colour reaction, which produced high background colours in some cases. 2,4,5,6-Tetraamino pyrimidine has itself, an intense yellow colour which produced a yellow background in dry phase tests.

(c) Miscellaneous reagents.

Histological reagents, used to demonstrate peroxidative activity were also tested in dry phase enzyme systems. 4-Chloro 1-naphthol gave a deep blue black dye when oxidised and mediates very well. However, the reagent was air and light sensitive and discoloured badly on storage.

Colour couplers which were insoluble in water were dissolved in a suitable solvent and overlayed on enzyme/developer loaded paper. One advantage of this method was the dye formed was non-leaching, however such systems were slow to develop colour, presumably due to the partitioning between the soluble and insoluble phases. Examples include; tribromophenol, triodophenol, 3-diethylaminophenol and several substituted pyrazolones, (appendix II). Leuco crystal violet dissolved in 1,1,1-trichloroethane and overlayed on enzyme paper proved insensitive on development, giving only a very pale lilac colour at ethanol levels above 800mg.l^{-1}.

Attempts to produce a threshold test for uric acid using uricase were complicated by the fact that uric acid itself has a

tendency to bleach chromagen systems, (Phillips 1985). A suitable dye system that was resistant to the uric acid and yet sensitive to mediation was not identified. Most attempts resulted in colour graduation rather than a definite threshold response.

6.B.4. Dry Alcohol Tests. Miscellaneous Effects.

(a) Buffers.

Buffers were primarily included in enzyme based systems to provide a constant pH which maintained enzyme activity, usually at an optimum level. Variations in buffer composition and pH markedly affected the enzyme activity retained on drying, which has been discussed in chapter 5, (section 5.A.4). In colorimetric tests the buffers also influenced the colorimetric reaction in some cases. Acidic pH was necessary to produce dyes from certain aniline derivatives and 4-AAP, (Tamaoku et al 1982) whereas alkaline pH favoured the condensation of phenols with 4-AAP. In this case acidic pH destroyed the colour formed, (Michal et al 1984).

In dry phase tests it was also important to use a buffer system which was very soluble to promote rapid dissolution and subsequent pH control. Phosphate buffer was used in early experiments, however it became clear, enhanced reaction rates could be obtained using a range of substituted organic sulphonic acid derivatives, the so called "Good" buffers, (Good et al 1966, Good and Izawa, 1968, Ferguson and Good 1980). These were very soluble in water and covered a wide range of pH, table 25.

Routinely, MOPS buffer, pH 7.9 was used for the alcohol dry tests. This pH gave maximum enzyme activity retention, the enzymes were sufficiently active in use, the colour reaction was rapid and the buffer was almost instantaneously soluble.

Table 25. "Good" Biological Buffers.

Compound.	Abbreviation	pKa	pH range
2-(N-Morpholino) ethane sulphonic acid	MES	6.1	5.5-6.7
3-(N-Morpholino) propane sulphonic acid	MOPS	7.2	6.5-7.9
N-(2-Hydroxyethyl) piperazine -N-(2-ethanesulphonic acid)	HEPES	7.5	6.8-8.2
3-[(1,1-Dimethyl 2-hydroxy ethyl) amino]-2-hydroxy propane sulphonic acid	AMPSO	9.0	8.3-9.7
2-(N-Cyclohexylamino)-ethane sulphonic acid	CHES	9.3	8.6-10.0
3-(Cyclohexylamino)-1-propane sulphonic acid	CAPS	10.4	9.7-11.1

Table 26. Whatman Filter Papers.

Grade.	Thickness(mm).	Filtration Rate	Surface.
3MM Chr.	0.34	4.3mm.min-^{1}	Medium.
4 Chr.	0.21	6.0mm.min-^{1}	Smooth.
31 ET. Chr.	0.50	7.5mm.min-^{1}	Smooth.
SG - 81	0.27	3.7mm.min-^{1}	Medium.
(Silica Gel Loaded)			
52	0.18	25.5ml.min-^{1}	Smooth.
54	0.19	153.9ml.min-^{1}	Smooth.
541	0.16	176.5ml.min-^{1}	Smooth.
542	0.15	2.4ml.min-^{1}	Smooth.

(b) Support Matrix.

The majority of the matrices used in the production of dry phase tests were cellulose based filter materials. Whatman filter papers of various types were chosen as readily available supports and the grades commonly used are listed in table 26.

The thickness and type of paper used affected the mediation of the colour reaction. A formulation absorbed onto 3MM Chr paper would generate a threshold at a different ethanol level to the same formulation absorbed on 542 grade. The thickness and texture of each grade influenced the amount of components absorbed and retained on the support and the subsequent effects noticed when the tests were developed.

Special grades of paper containing reactive groupings, (DEAE,-carboxymethyl- or phosphate-) appeared not to influence the development of colour appreciably. Such papers may have some influence on enzyme stability but this was not tested.

Glass based matrices were not used as supports, mainly due to uneven absorption of the reaction components and subsequent uneven development of colour. They were also very fragile when wet.

Particulate material such as cellulose or silica gel which is used to make supports for thin layer chromatography may also be used to produce mediator generated colour thresholds.

Gel layers produced from gelatine or similar gel forming compounds have also been tested. This is reported in section 6.C.

(c) Enzyme Concentration.

The concentration of the enzyme used influenced the rate of development of the colour and consequently the rapidity of the test. Low levels of alcohol oxidase produced prolonged reaction times

whereas increasing the concentration reduced the reaction time to a finite level, table 27. Variation in the peroxidase level from 6.25 $U.ml^{-1}$ up to $100U.ml^{-1}$ did not influence development time appreciably, however colour intensity and evenness of development were superior at the higher enzyme levels, table 27.

(d) Colour Reagent Concentration.

Variations in colour reagent concentrations influenced the intensity of the colour produced and also the eveness of the colour formed on the dry phase. Initially concentrations of 1mM 4-AAP and 10-25mM coupler were used which approximated to the "wet" analytical reagent concentrations. Development of colour tended to be patchy at these concentration ratios.

Increasing the 4-AAP to 20mM and holding the coupler concentration at 10-25mM resulted in more intense colour development and also a more even colour throughout the matrix. This increase in the ratio of developer to coupler is common practice in dry phase tests, (Phillips 1985).

(e) Microporous Overlay.

Certain polymeric materials such as ethyl cellulose (Sigma) or cellulose acetate (BDH) were dissolved in organic solvents and applied to dry phase enzyme tests and dried. This produced a microporous layer surrounding the fibres of the support matrix which contained the enzyme based colorimetric test. This had the effect of rendering the test impermeable to blood cells and large macromolecules and so prevented interference from such substances when the dry test was developed, (Phillips 1985).

Application of a 0.5% w/v solution of ethyl cellulose in toluene (GPR,low sulphur grade, BDH), to dry phase tests containing $100U.ml^{-1}$

Table 27. Enzyme Concentrations.Influence on Dry Test.*

Alcohol Oxidase. $U.ml^{-1}$.	Peroxidase. $U.ml^{-1}$.	End Point. min.	Colour.
40	25	5 - 10	Purple
60	25	5 - 10	Purple
100	25	5 - 6	Purple
150	25	3 - 5	Purple
200	25	3 - 4	Purple
250	25	3 - 4	Purple
300	25	3 - 4	Purple
100	6.25	5 - 6	Blue
100	12.50	5 - 6	Blue/ Purple
100	25.00	5 - 6	Purple
100	100.00	5 - 6	Deep Purple

* This was carried out using formulation (1) described in section 6.B.1 , absorbed onto 3MM Chr. paper and developed with aqueous ethanol solutions at 21°C (+/- 2°C).

alcohol oxidase prepared as formulation (2) and absorbed onto 3MM Chr paper, gave tests which reacted fully in 2 minutes instead of the 3-4 minutes of untreated cards. Later tests with 1.0% w/v ethyl cellulose and 200U.ml^{-1} enzyme, consistantly produced tests which developed fully in 1.5-2 minutes.

A more rapid reaction time and enhanced colour formation was also noticed, when a microporous overlay was applied to a test made with a thin layer of particulate cellulose as the support matrix.

(f) Bovine Serum Albumin (BSA).

When BSA was added to formulation (2) to a concentration of 3% w/v it was found that a much more even and rapid colour development ensued. This, in conjunction with the ethyl cellulose overlay, gave a threshold test of even colour, very clear cut off points on the threshold and a rapidity of reaction which was acceptable as a rapid on/off semi-quantitative dry phase test for ethanol in saliva.

(g) Standardisation of the test.

Threshold levels of mediator were set using either human saliva or artificial saliva ethanol standards, (table 13) rather than aqueous ethanol standards. Colour development was consistent using the former, whereas aqueous ethanol standards tended to produce patchy and variable colour development.

6.B.5. Dry Alcohol Tests. Stability.

The stability of the compounds of the dry phase test with respect to time of storage is particularly important for the use of such a system to measure ethanol accurately.

The enzymes themselves remain stable for weeks at 37°C individually and in combination, in the presence of the stabilisers reported in chapter 5. In the presence of 4-AAP they are also

stable, graph 53. The full dry phase system retained enzyme stability for 21 days at 37°C when stored desiccated in the dark. Inositol at 5% w/v and 10% w/v was used as stabiliser in formulation (2) absorbed on 3MM Chr paper. The development time was 5 minutes, both for the fresh and the incubated tests. The threshold was set at 500mg.l^{-1} and 800mg.l^{-1} ethanol. Incubation of the same tests for a further two months at 37°C increased the development time to 10 minutes indicating an approximate 50% loss of enzyme activity.

Mediator stability appeared to be mainly dependent on the humidity under which the tests were stored. Damp conditions caused marked deterioration within several hours, indicated by dissappearance of the threshold. Incubation under inert gas, (N_2) as opposed to air appeared to offer no advantages. Foil sachets gave the best overall protection against photodeterioration of mediators such as ascorbate and sensitive colour reagents. However,the majority of the colour reagents used were stable in all conditions tested.

6.C. Multilayer Tests.

Gelatine films produced by the Meyer Rod technique, (chapter 2, section 2.L.2) were made containing the colour reagents and the mediators. Dry phase enzyme paper, (4 Chr grade paper) was laminated to the upper surface, as in fig 17. Development of such a system gave a threshold effect within the gel layer when viewed through the transparent base, table 28.

The development time was slow, (5-10 minutes) possibly due to the relatively slow diffusion of oxygen into the gel layer.

This type of system was tested for sensitive colour reagents such as those of the photographic class, (table 23). The developer was incorporated in the gel layer and the enzyme / coupler / stabiliser

Graph 53. ALCOHOL OXIDASE STABILISATION.

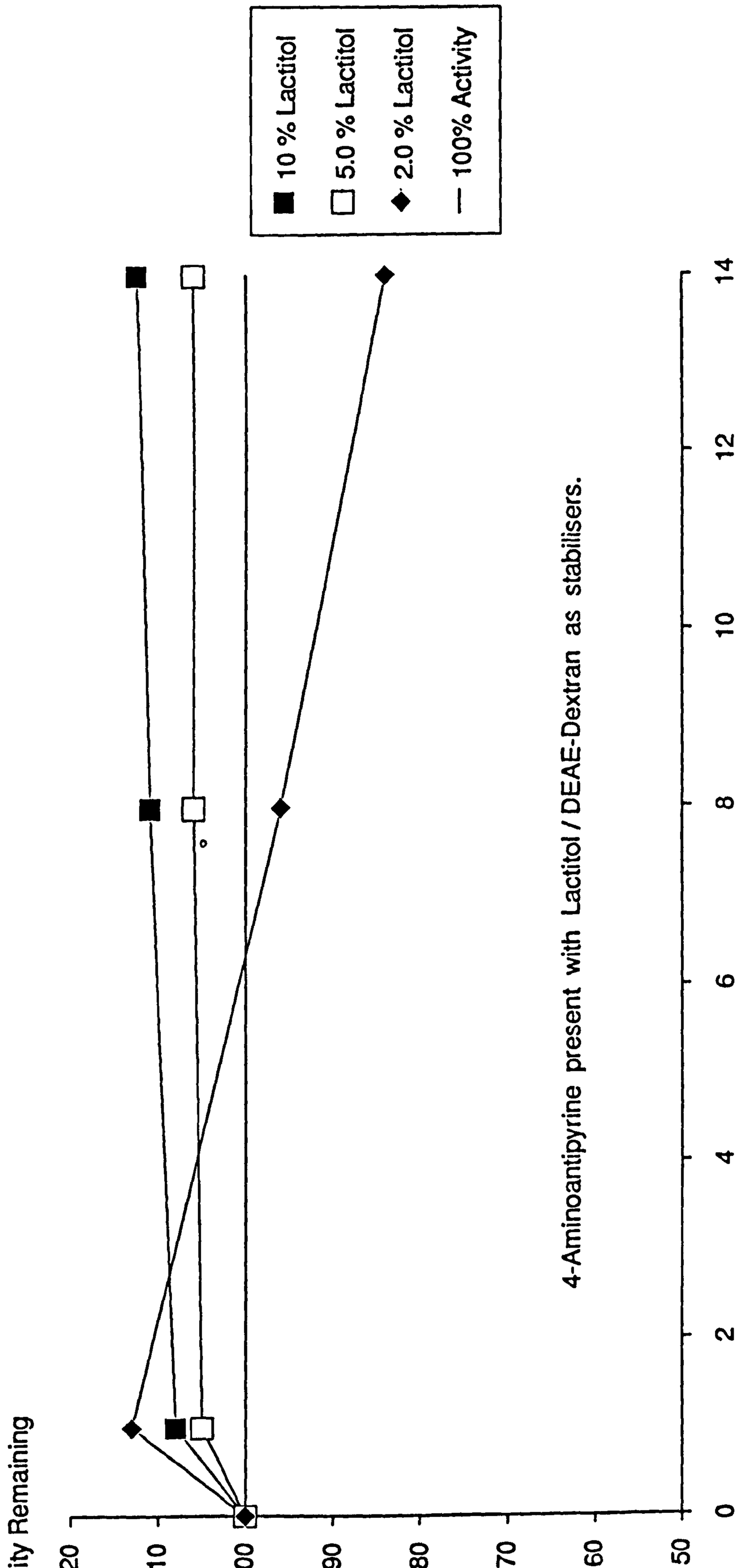


Table 28. Gel Threshold Alcohol Test.

Ethanol mg.l^{-1}	Cysteine mg.ml^{-1} 1.0	2.0	3.0	4.0	5.0	6.0
100	+-	-	-	-	-	-
200	+-	+-	-	-	-	-
300	+	+-	+-	-	-	-
400	+	+	+-	+-	-	-
500	+	+	+	+-	+-	-

mixture on 4 Chr paper was laminated on the top surface. Also the reverse system was tried with colour coupler in the gel layer and developer / enzyme / stabiliser in the paper. In both cases the mediator used, (cysteine or ascorbate) was present throughout and discolouration of the dry test was noticed on storage. However, this effect took significantly longer, (7-14 days) than when the whole enzyme/coupler/developer/stabiliser system was only present in the paper, (1-2 days) under the same storage conditions. If both developer, (CD-2, table 23) and coupler, (2,6-diaminotoluene) were present in the gel layer and the enzymes / stabilisers were loaded and dried onto 4 Chr paper which was then laminated on top, no appreciably discolouration appeared for several weeks and the test was still active after 5 months at room temperature.

Two disadvantages of these type of tests were the inability to overlay using ethyl cellulose, as the gel base does not hydrate on development, and the long development times, (5 minutes or more).

Two distinct advantages were the ability to use the same gel layer for different enzyme loaded papers, which allowed a variety of analytes to be determined using the same colour system, and the

separation of the colour detection layer from the enzyme layer which may enhance stability of the enzyme and/or colour reagents.

6.D.1. Alternative Analytes. Dry Phase Tests.

Dry phase tests using many different enzymes to assay many different analytes have been reported widely in the literature, (see table 19). The application of the basic dry system devised for ethanol analysis was then adapted for analysis of cholesterol, uric acid, hydrogen peroxide and glucose using the corresponding enzymes and buffers. Reflectance plots of graduated enzyme tests are shown in graphs 54-57.

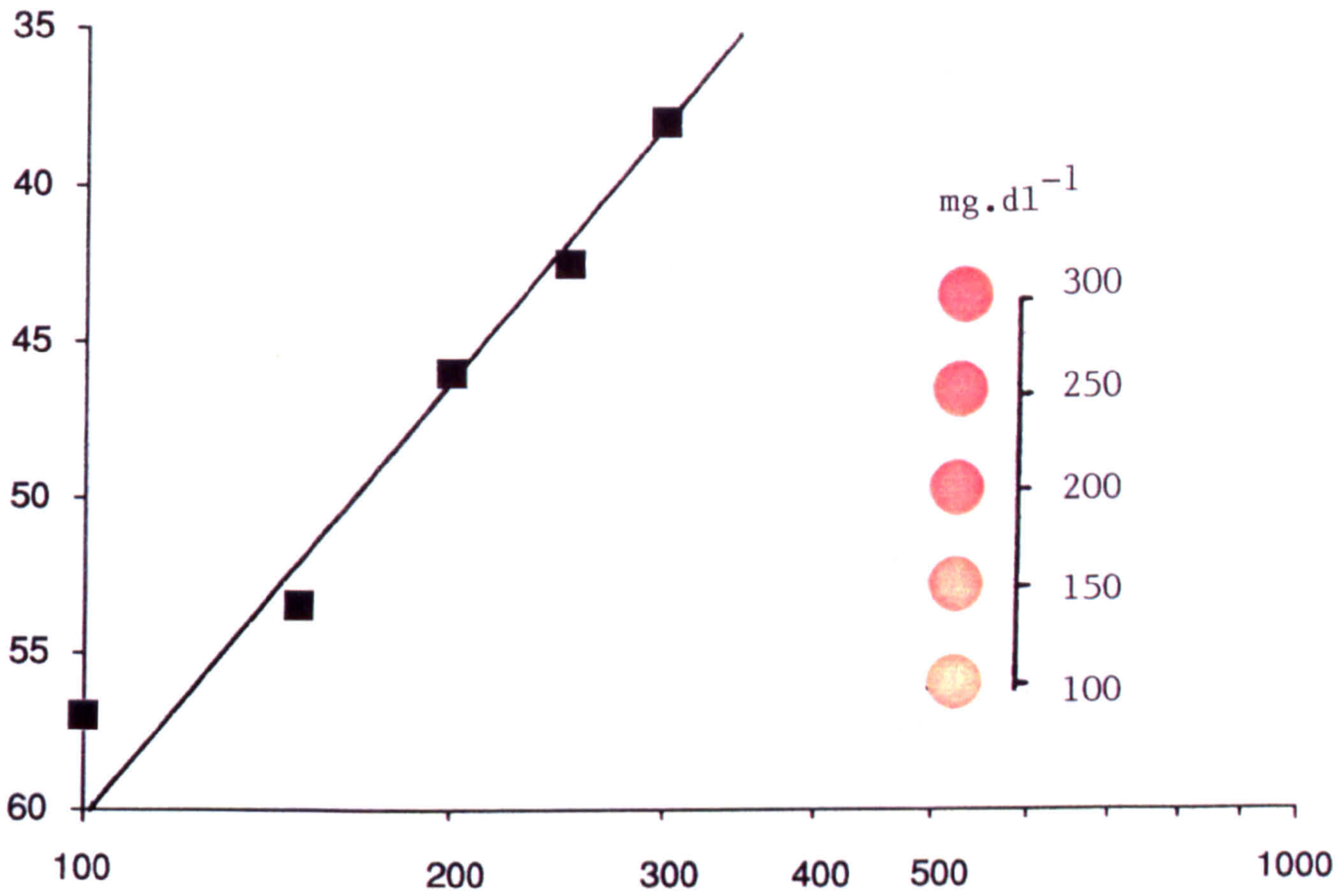
6.D.2. Alternative Analytes. Threshold Tests.

Replacement of alcohol oxidase with other oxidase enzymes in the threshold system described, allowed the detection and quantitation of alternative analytes. The specificity of the test is dependent on the oxidase enzyme used. The threshold generation is a function of the hydrogen peroxide detection reaction catalysed by peroxidase. This may be seen to be the case in fig. 31 which shows the threshold test for hydrogen peroxide alone. Peroxidase was the only enzyme present and the dry system was developed by aqueous hydrogen peroxide solutions.

The formulation was as follows:-

Peroxidase	100 $U.ml^{-1}$
Lactitol	5% w/v
DEAE-dextran	1% w/v
4-Aminoantipyrine	20 mM
N,N, Bis(hydroxyethyl) aniline	25 mM
MOPS buffer pH 7.0	> 100 mM
Cysteine	1, 2, 3, 4. mg ml^{-1}

Graph 54. DRY PHASE GRADUATED CHOLESTEROL TEST.

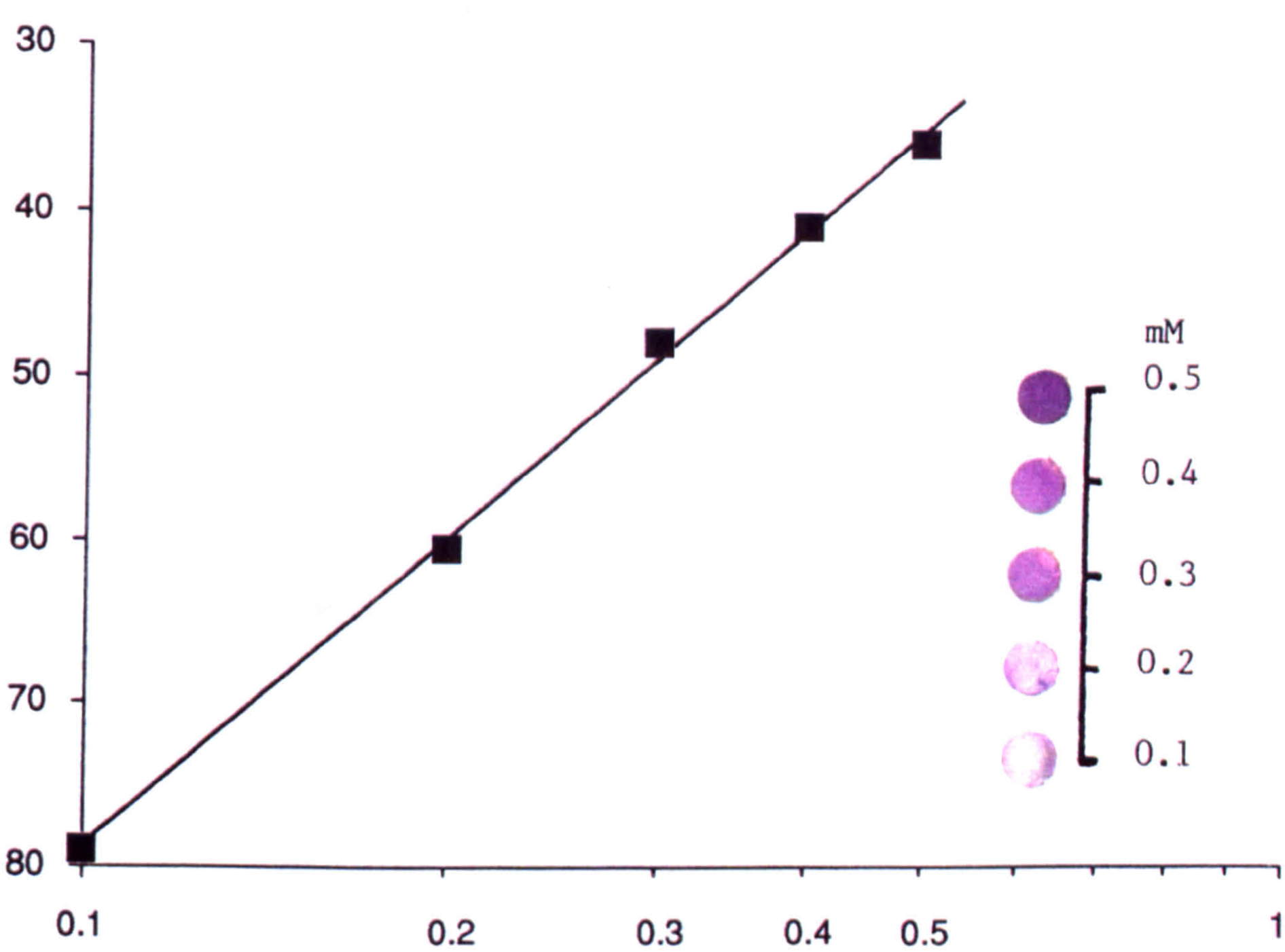


COLOUR REAGENTS.

4-AMINOANTIPYRINE

GUAIACOL 6 SULPHONIC ACID.K Salt.

Graph 55. DRY PHASE GRADUATED URIC ACID TEST.

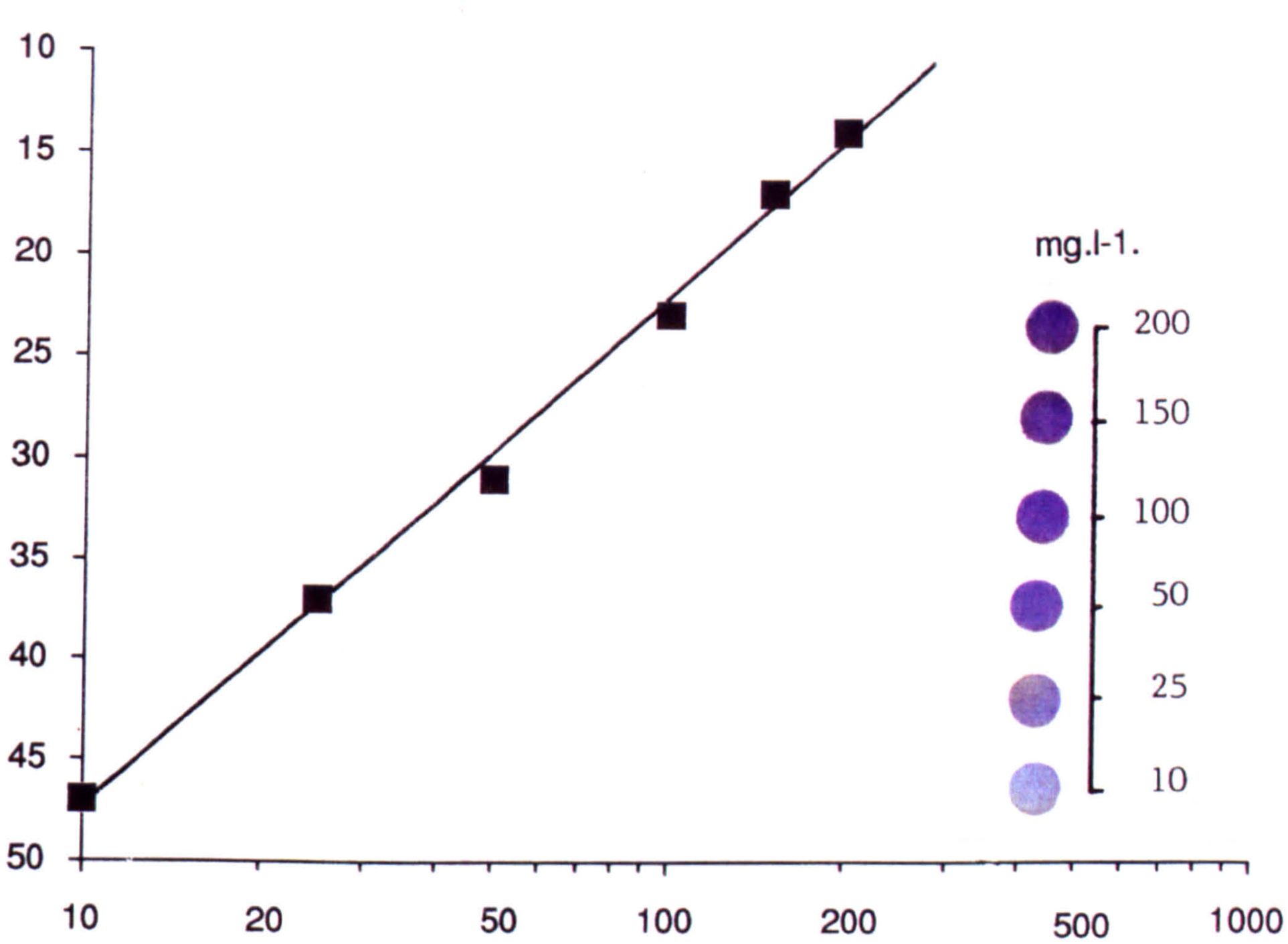


COLOUR REAGENTS.

4-AMINOANTIPYRINE

N,N BIS(2-HYDROXYETHYL) ANILINE.

Graph 56. DRY PHASE GRADUATED PEROXIDE TEST.

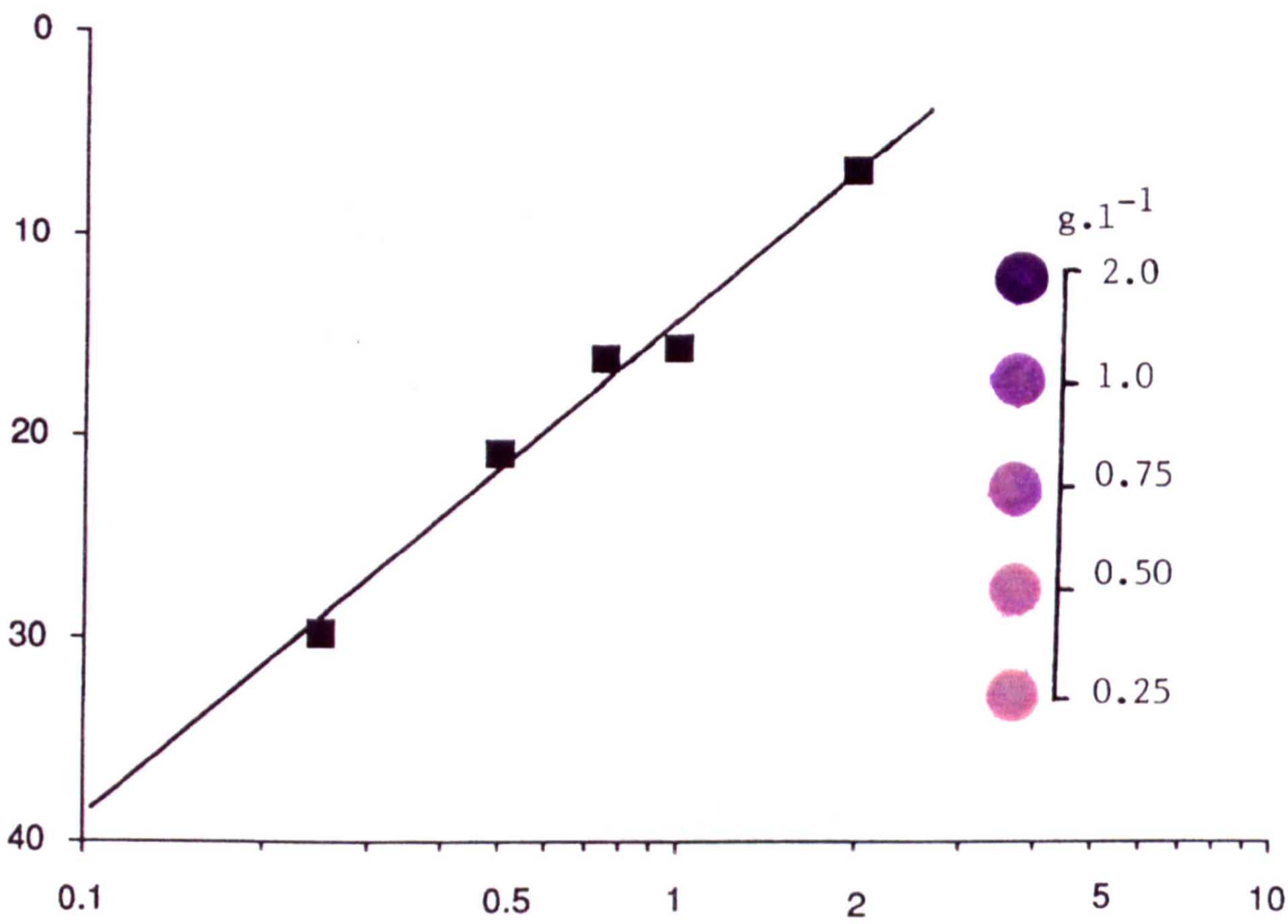


COLOUR REAGENTS.

3-METHYL 2-BENZOTHIAZOLINONE HYDRAZONE

3-DIMETHYLAMINO BENZOIC ACID.

Graph 57. DRY PHASE GRADUATED GLUCOSE TEST.

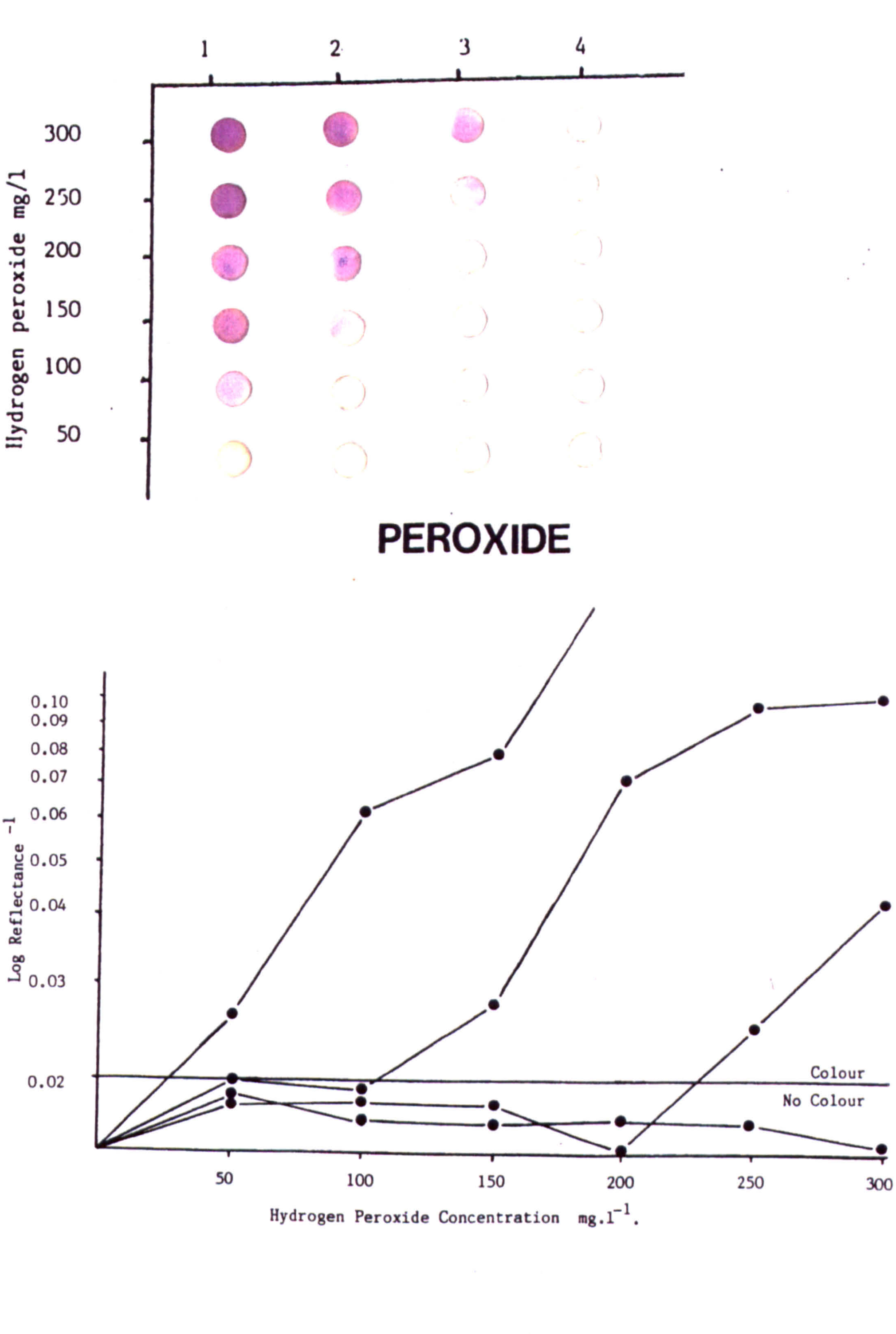


COLOUR REAGENTS.

4-AMINOANTIPYRINE

N,N BIS(2-HYDROXYETHYL) ANILINE.

FIG 31. DRY PHASE PEROXIDE THESHOLD TEST.



The cocktail was absorbed onto 4 Chr filter paper and vacuum dried at 35°C for 30 min.

Dry tests containing cholesterol oxidase, (Enzymatix Ltd, Nocardia Species) using ascorbate as mediator and a colour developer /coupler combination of MBTH and 3-dimethylamino benzoic acid gave the results depicted in fig 32. The test was rather slow to develop taking 10 minutes at 22°C. This was thought to be due to; (1) low enzyme activity and; (2) the limitation of oxygen solubility in the system, (Palmer et al 1989). The formulation for the cholesterol test was as follows:-

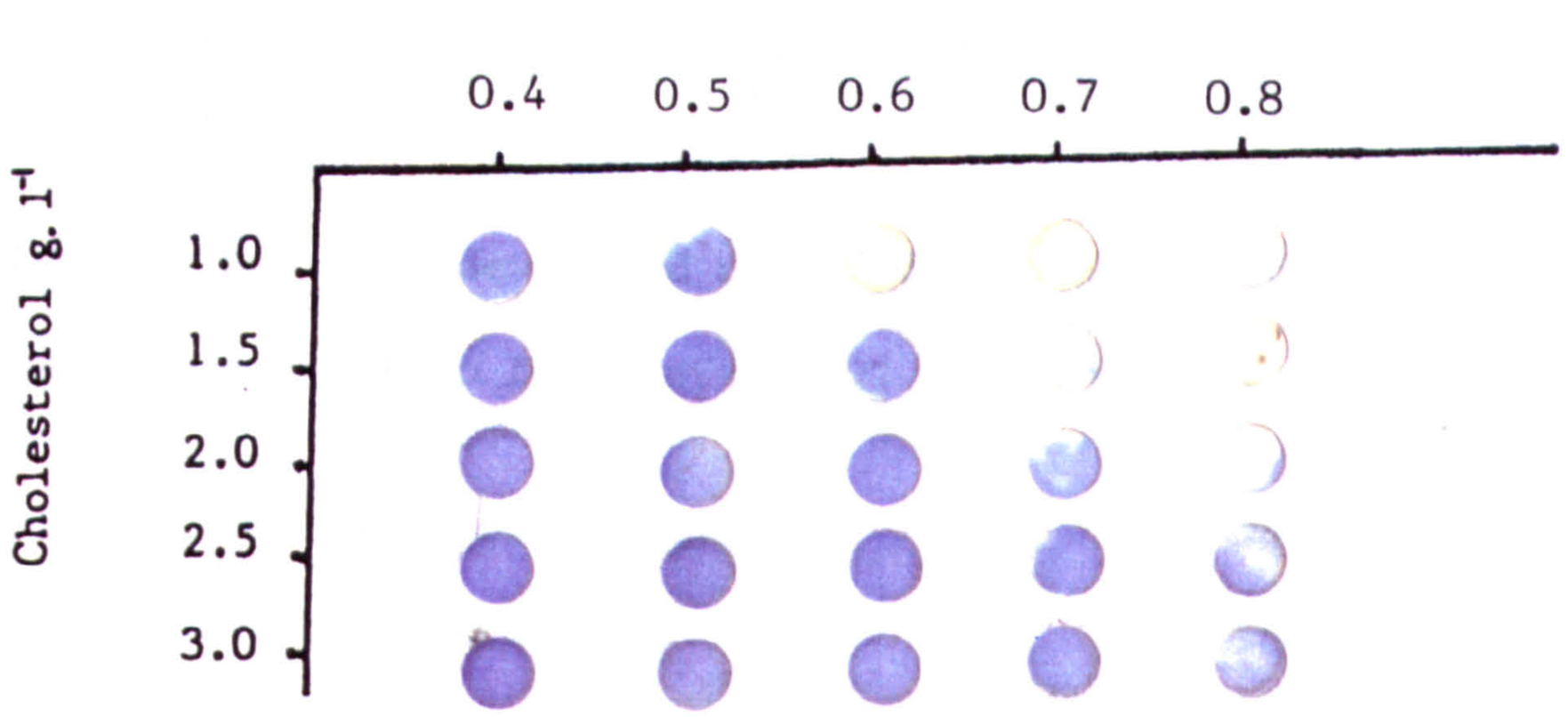
Cholesterol oxidase	50U.ml^{-1}
Peroxidase	100U.ml^{-1}
4-Aminoantipyrine	20mM
3-Dimethylamino benzoic acid	10mM
Triton X-100	1% w/v
AMPSO buffer pH 9.0	› 100mM
Lactitol	5% w/v
DEAE-dextran	1% w/v
Ascorbate	0.4, 0.5, 0.6, 0.7, 0.8 mg.ml^{-1}

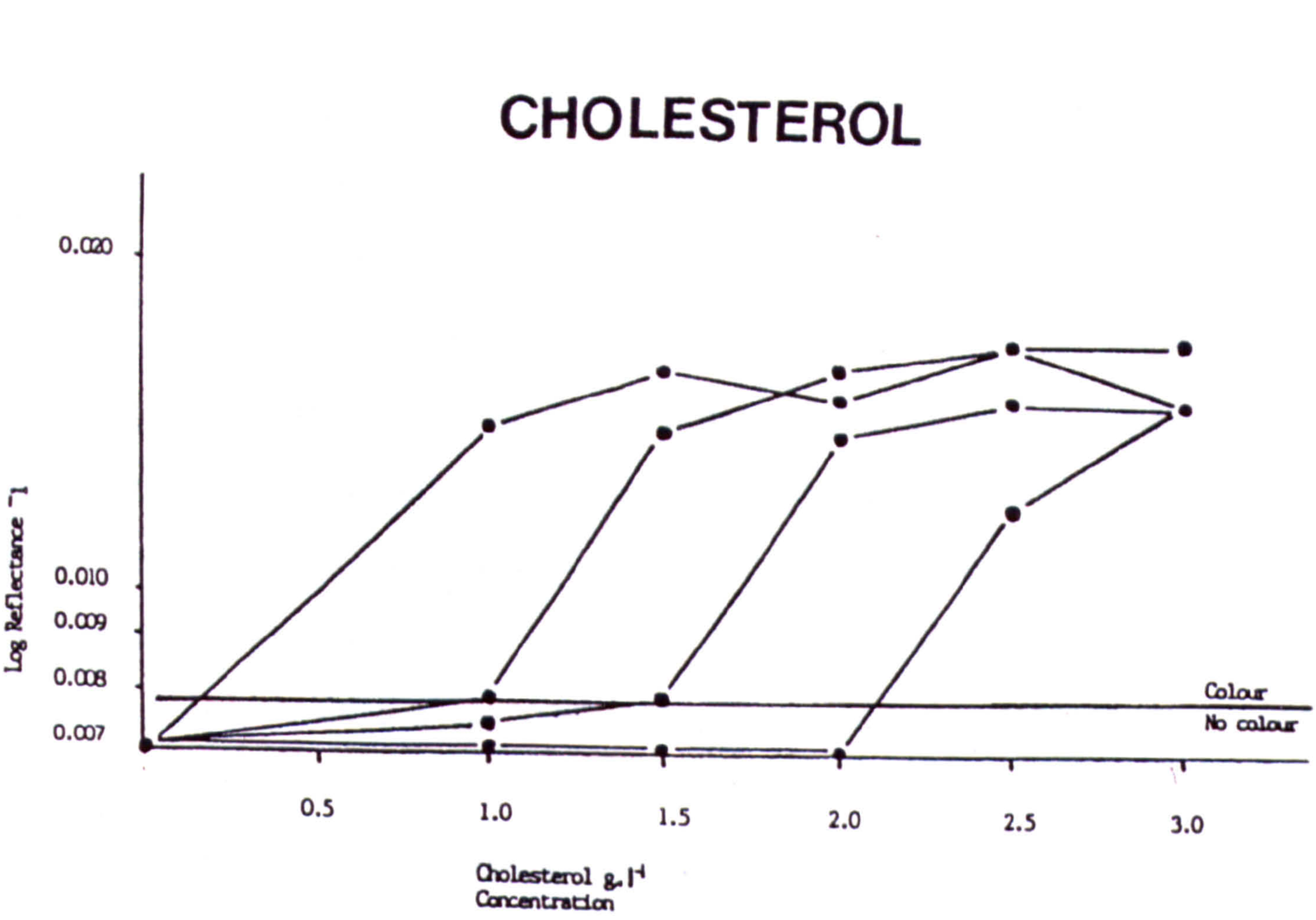
The cocktail was absorbed onto Whatman 4Chr filter paper and vacuum dried at 35°C for 30 minutes.

Similarly a threshold test for glucose was formulated using glucose oxidase.

Glucose oxidase	200U.ml^{-1}
Peroxidase	100U.ml^{-1}
4-Aminoantipyrine	20mM
2,4,6-Tribromo 3-hydroxy benzoic acid	10mM

FIG 32. DRY PHASE CHOLESTEROL THRESHOLD TEST.





MOPS buffer pH 7.0	› 100mM
Inositol	5% w/v
Cysteine	1.0, 4.0, 6.0, 8.0 $mg.ml^{-1}$

The cocktail was absorbed onto Whatman 4 Chr filter paper and vacuum dried at 35°C for 30 minutes, the results are shown in fig 33.

6.E.1. Application of the Threshold Reaction. Alcocard 1

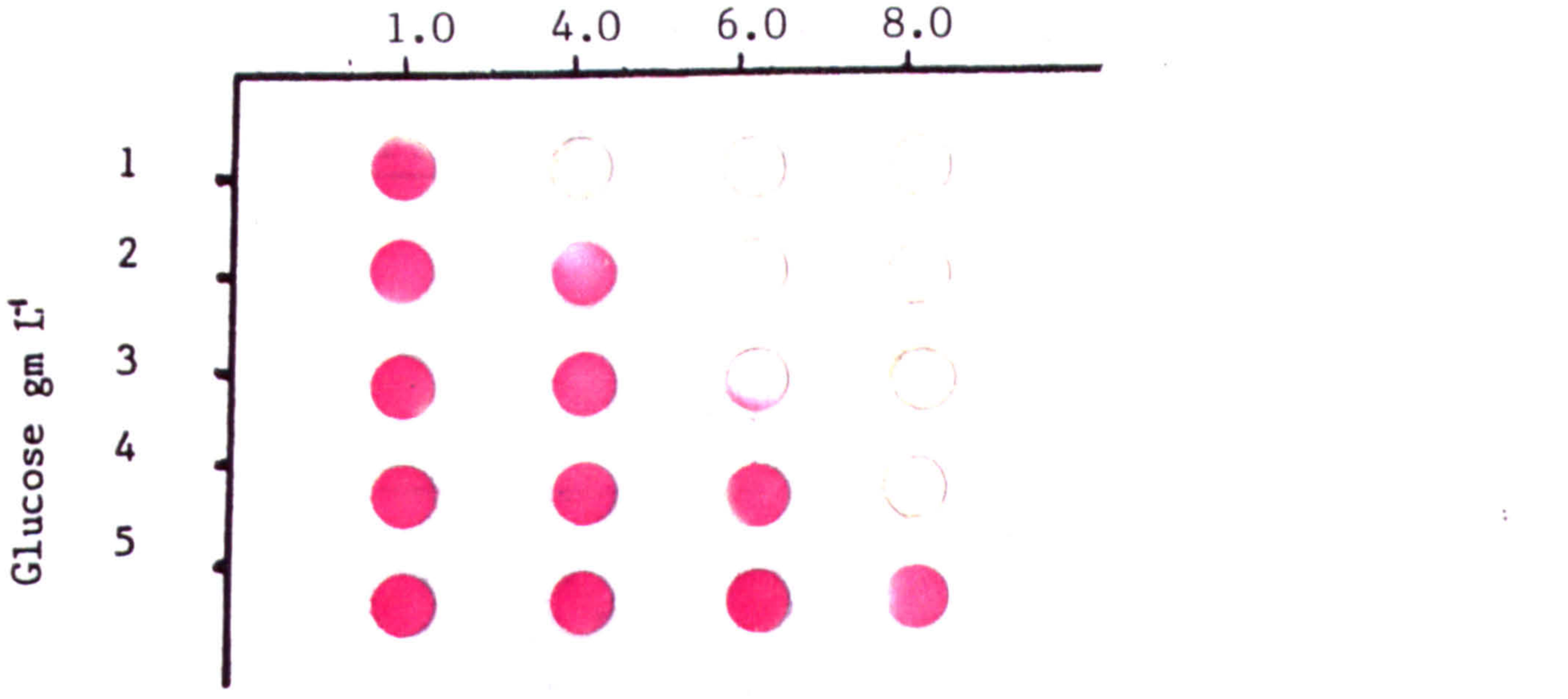
The formulation for the enzyme discs used in the first saliva alcohol test card developed using the threshold generation system was almost identical to formulation (2), but with the addition of BSA.

Alcohol oxidase	200$U.ml^{-1}$
Peroxidase	100$U.ml^{-1}$
4-Aminoantipyrine	20mM
N,N,Bis(hydroxyethyl) aniline	25mM
Inositol	5% w/v
BSA	3% w/v
MOPS buffer pH 7.9	› 100 mM
Cysteine	4.2 or 6.2$mg.ml^{-1}$

The cocktail was absorbed onto Whatman 3MM Chr filter paper and vacuum dried at 35°C for 30 minutes. The dry paper was then overlaid in 1% w/v ethyl cellulose in toluene and air dried. The fabrication of the test was described in detail in section 2.L.5. The developed test gave a positive purple response at saliva ethanol concentrations of 500$mg.1^{-1}$ and above for the first disc and 800$mg.1^{-1}$ and above for the second disc, fig 34.

Comparison of the response of the alcocard using saliva samples from 10 individuals who had been drinking, with the ethanol concentration determined by a "wet" alcohol dehydrogenase method

FIG 33. DRY PHASE GLUCOSE THRESHOLD TEST.



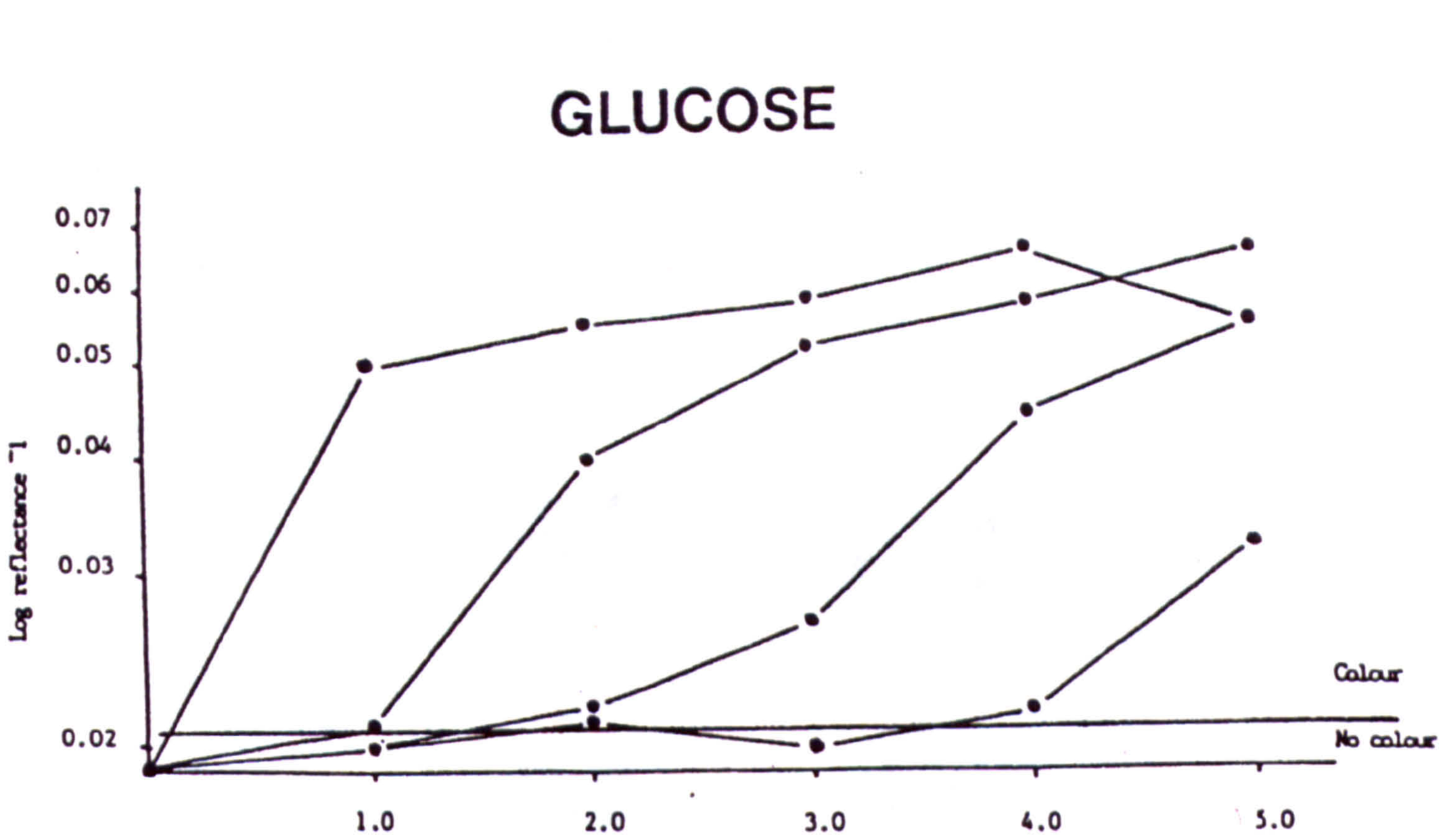
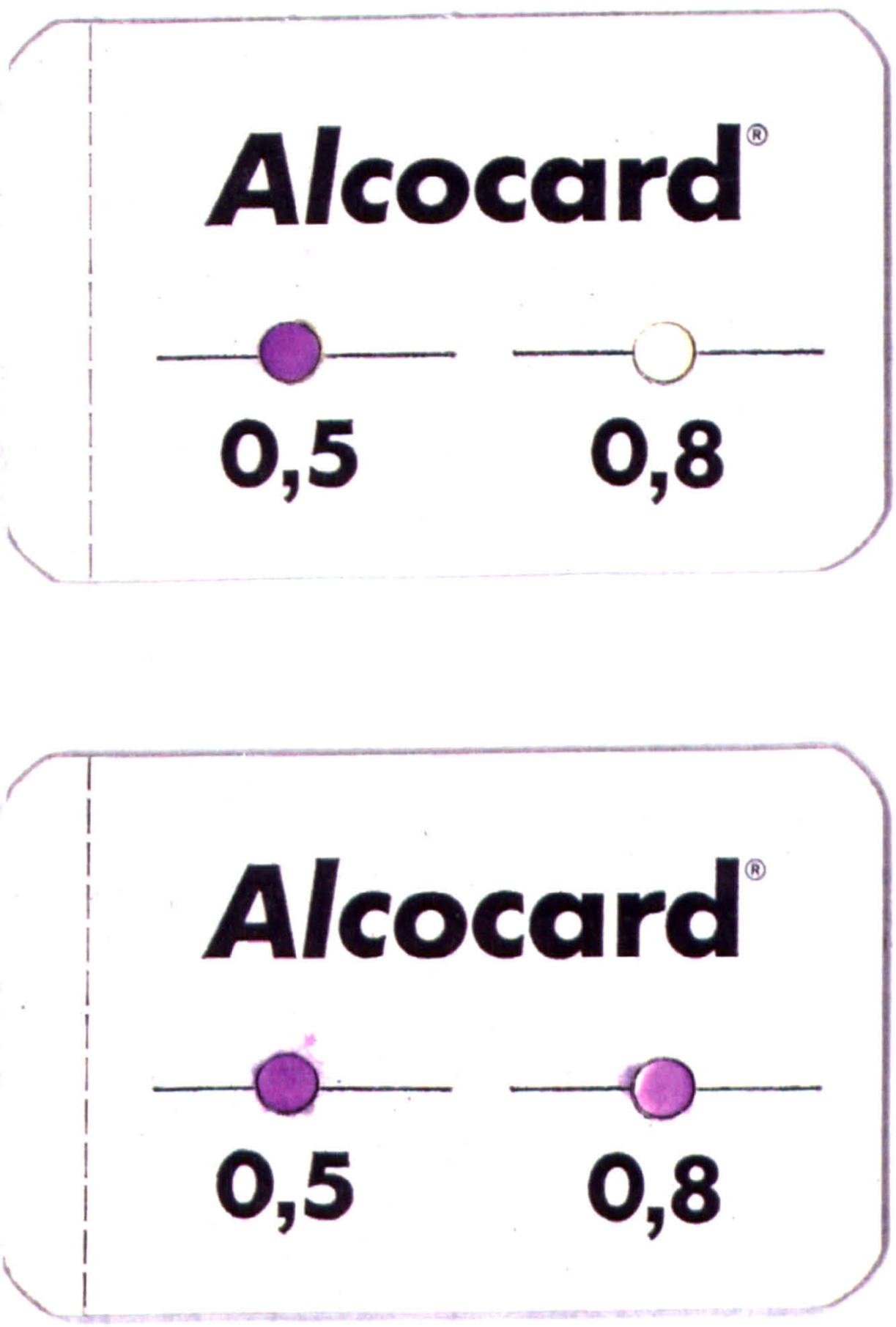


FIG. 34. ALCOCARD 1



(Sigma Ethanol 332-UV) gave an 86% correlation of positive results on the card, corresponding to ethanol concentrations above the lower threshold level set at 500mg.l^{-1}, (the first disc). Only one subject gave a positive response on both threshold levels at the correct concentrations. When using this test format lower concentrations of ethanol tended to give positive responses on both discs, which was especially noticeable when large sample volumes were used. This indicated a dose dependent response of the test, which was found to be the main drawback for this type of test format. However, provided a metered dose was applied to the discs, (usually 10 µl), the response was very reproducible giving virtually 100% correct results. If a larger volume was applied, (20 µl and above) the response was almost random in nature. Attempts were made to adjust this defect by mounting the enzyme discs on absorbent cards. This produced the correct response, however obtaining the necessary reproducibility was difficult.

The stability of the test cards was estimated by accelerated degradation testing as before. 100% of cards, (n = 25) retained full activity after incubation for 7 days at 37°C, giving correct threshold responses in two minutes at 21°C. Longer incubation times, (up to several weeks at 37°C) tended to inactivate the enzymes, which gave longer development times of up to 5 minutes.

6.E.2. Application of Threshold Reaction. Alcocard 2.

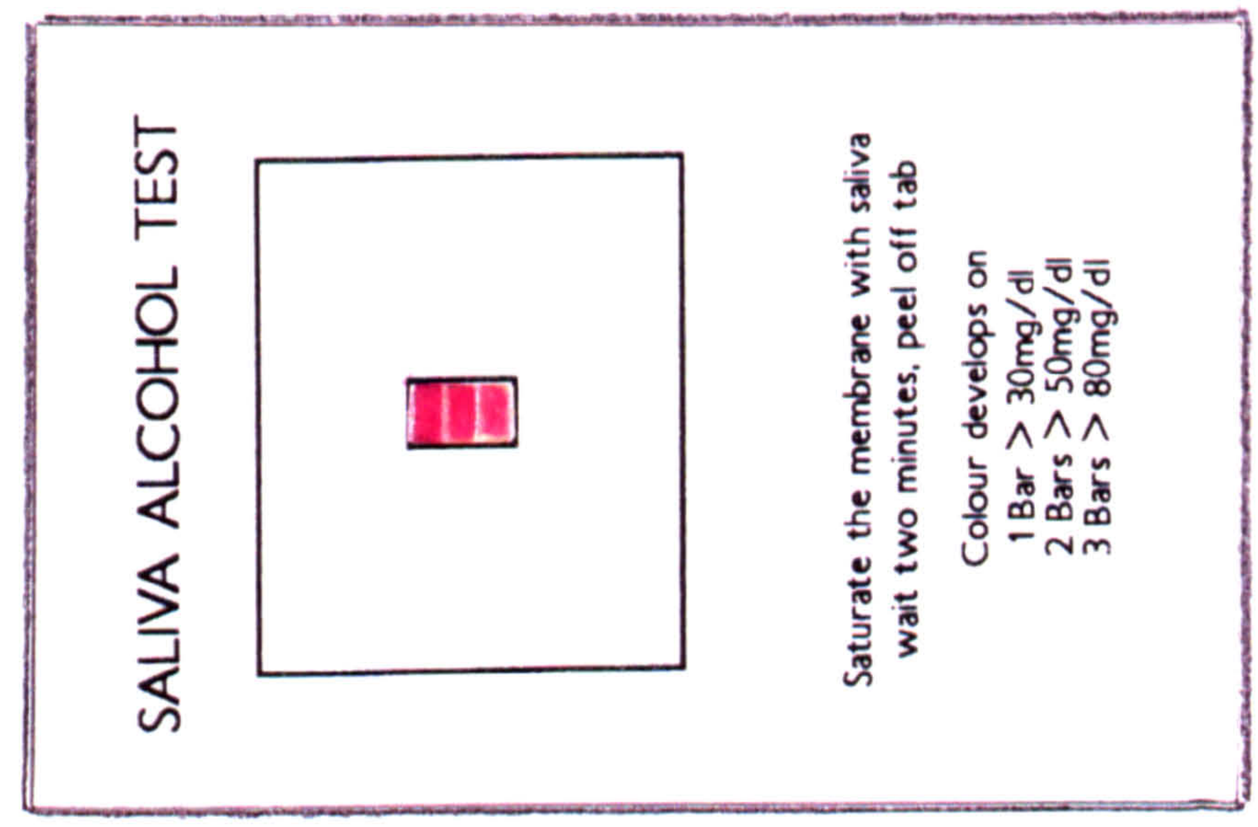
To overcome the problem of metering a controlled dose onto the enzyme discs a second format was devised and employed. This is described in detail in chapter 2, section 2.L.5, part 2 and illustrated in fig 18b.

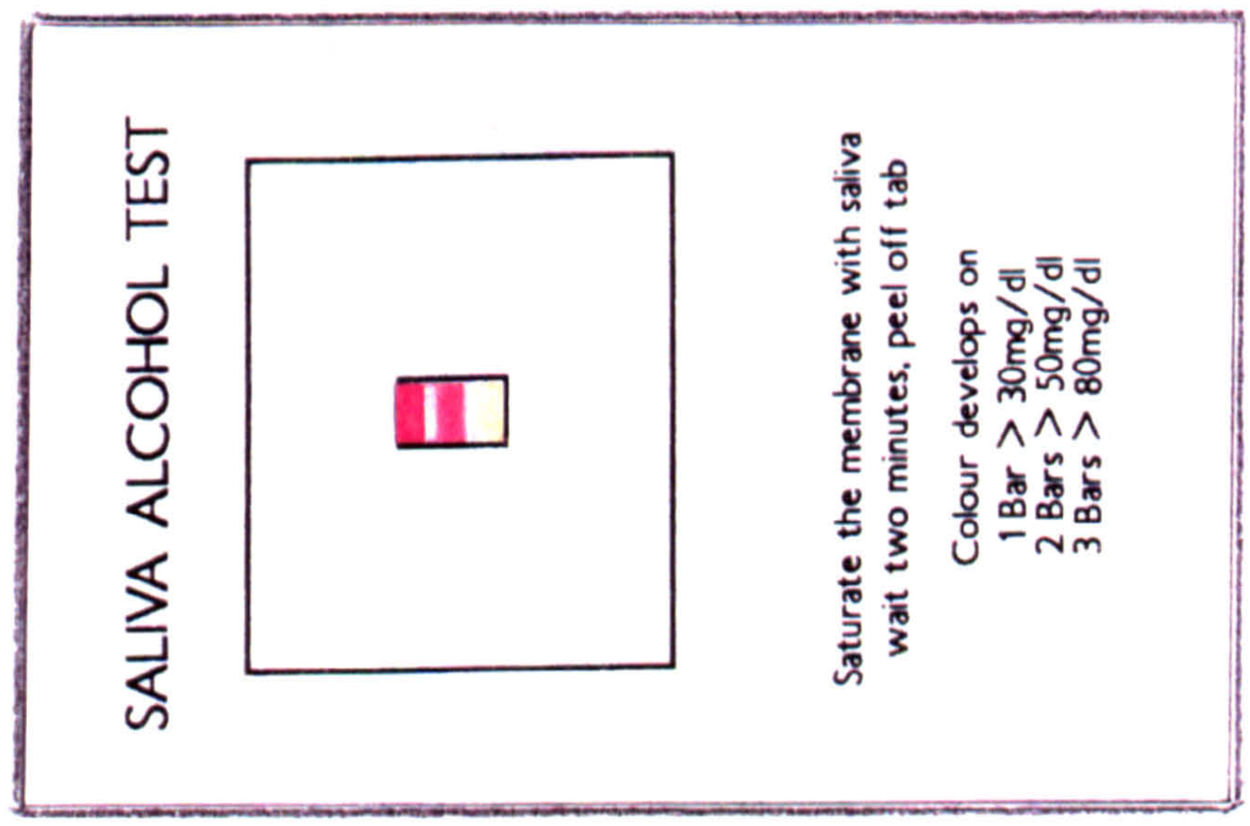
The second format consisted of a base card, three absorbent

rectangles of filter paper, (Whatman grade 54 or 541) of an average area of 8-12 square mm. The filter papers were loaded with enzyme cocktail set to respond at 300, 500 and 800 $mg.l^{-1}$ ethanol. The rectangles were mounted underneath a peel off strip containing a membrane. The membranes chosen were nucleopore and more recently, isopore from the Millipore Corporation. The membrane allowed a finite loading of saliva onto the enzyme loaded papers, which produced a dose independent system, provided a minimum amount, (20-30 µl) of saliva was applied to the top surface of the peel off membrane. The completed tests were sealed in foil sachets containing desiccant for storage. Peel off membranes of this type have been reported before, particularly in the area of blood separation, (Kennedy et al 1987, Kennedy et al 1989).

A series of developed tests are shown in fig 35. This format of test card is currently undergoing modification with regard to chromagens and the supports used for the enzyme elements.

Application of the enzyme / stabiliser / colour reagent / mediator cocktail onto microcrystalline cellulose thin layers and subsequent drying and overlaying with 1% ethyl cellulose in toluene produced the characteristic, mediator concentration dependent, threshold response. Further work is continuing in this area to establish the correct parameters for such a system, which will be used in the commercial production and exploitation of the threshold generated response.





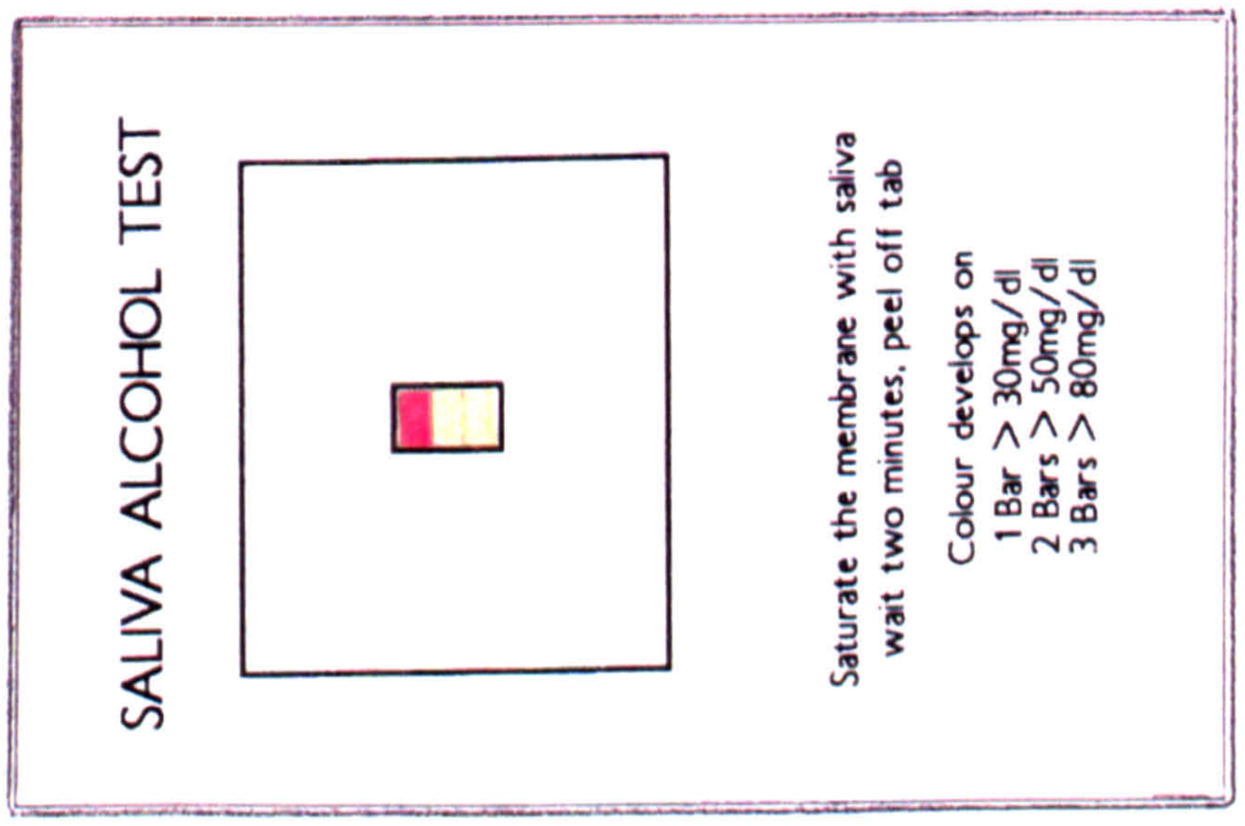


FIG. 35. ALCOCARD 2

CHAPTER 7.

DIACETYL ANALYSIS using DIACETYL REDUCTASE ENZYMES.

7.A. Diacetyl and Diacetyl Reductase. Overview.

Diacetyl, (2,3 butanedione) is a volatile potent flavouring and aroma substance in foods and beverages. It imparts a buttery or toffee like flavour, and is one of the most important aroma substances in butter and fermented milk products such as yoghurt, (Scherrer 1972). However, above a certain level of concentration, diacetyl imparts an undesirable flavour to beers and lagers, producing an unpleasant aroma and taste, the so called "Sarcina sickness" of beer.

Diacetyl is produced in bacteria and yeast both enzymatically and spontaneously by various metabolic pathways associated with amino acid biosynthesis, particularly valine, (Seitz et al 1963, Inoue et al 1968, Speckman and Collins 1968, Lopez and Fortnagel 1972, Scherrer 1972, Wainwright 1973).

The analysis of diacetyl has been carried out by physical methods such as headspace gas liquid chromatography (GLC). Chemical analysis, using a variety of methods, were routinely used before GLC became widely available, table 29.

These methods have been used extensively to quantify diacetyl in various foodstuffs and beverages, usually after some sort of separation step such as distillation or gas stripping of the volatile substances from the sample. (Pack et al 1964, Ault 1968). There is evidence that such procedures influence the final diacetyl concentration, as it is spontaneously formed from α-acetolactate during sample preparation,(Inoue and Yamamoto 1970, Wainwright 1973).

Enzymes that metabolise diacetyl are found in various organisms such as bacteria, yeasts and animal tissues. These are listed in table 30. The reaction mechanism is almost identical in all organisms

Table 29. Analysis of Diacetyl.

Method.	Reference.
Headspace G.L.C.	Scherrer. 1972
Flow through G.L.C.	Rayner et al 1980
Colorimetric using 1-naphthol/ creatine	Westerfield 1945
U.V. using Girard T reagent	Mitchel and Birnboim 1977
Colorimetric using urea/ hydroxylamine	White et al 1945
Colorimetric using hydroxy-lamine / ferrous ions	Owades and Jakovac 1963
U.V. using thiosemicarbazide	Olea et al 1979

with diacetyl being reduced to acetoin in all cases.

(1) $CH_3.C(=O).C(=O).CH_3 + NADH + H^+ \longrightarrow CH_3.C(=O).CH(OH).CH_3 + NAD^+$

Diacetyl Acetoin

The enzymes which catalyse this irreversible reaction are systematically known as acetoin:NAD oxido reductases EC.1.1.1.5. or, colloquially, diacetyl reductase. A further reversible reduction step converting acetoin to 2,3 butanediol is catalysed by some bacterial diacetyl reductases. (Bryn et al 1971, Gibson et al 1990).

(2) $CH_3.C(=O).CH(OH).CH_3 + NADH + H^+ \rightleftharpoons CH_3.CH(OH).CH(OH).CH_3 + NAD^+$

The application of such enzymes has been limited to the enzymic removal of excess diacetyl in beers, (Bavisotto et al 1964, Tolls et al 1970, Thompson et al 1970). Enzymic analysis of diacetyl using diacetyl reductase has not been reported to date.

Table 30. Occurrence of Diacetyl Reductase.

Organism.	Reference.
Bacteria.	
Aerobacter aerogenes	Bryn et al 1971
Klebsiella pneumoniae	Shimiza et al 1977
Bacillus polymyxa	Ui et al 1987
Escherichia coli	Silber et al 1974
Lactobacillus casei	Branen and Keenan 1970
Staphylococcus aureas	Strecker and Harery 1954
Streptococcus diacetylactis	Seitz et al 1963
Streptococcus diacetylactis	Cogan 1981
Various other species.	Seitz et al 1963
Yeast.	
Saccharomyces cerevisiae	Gupta et al 1973
Mammalian Tissue.	
Beef liver	Burgos and Martin 1972
Rat liver	Gabriel et al 1971
Hamster liver	Sawada et al 1985
Avian Tissue.	
Pigeon liver	Diez et al 1974

7.B. Bacterial Diacetyl Reductases.

Two organisms which produced diacetyl reductase were used, Klebsiella aerogenes, strain FG9. and Streptococcus lactis subspecies diacetylactis 18-16. These correspond to the organisms used by Bryn et al (1971) and Seitz et al (1963). Partial purification was initially carried out in each case, however the streptococcus enzyme was later purified to homogeneity by another member of the unit.

7.B.1. Purification of Klebsiella Aerogenes Diacetyl Reductase.

The preparation is described in detail in chapter 2, section 2.B.2. The column elution profile is shown in graph 58. The preparation gave a clear solution which was enzymically active with diacetyl as substrate. Little work was carried out using this enzyme except to test the feasibility of diacetyl analysis using enzymes. Substrate specificity tests concurred with the findings of Bryn et al (1971), that acetoin was also accepted as a substrate.

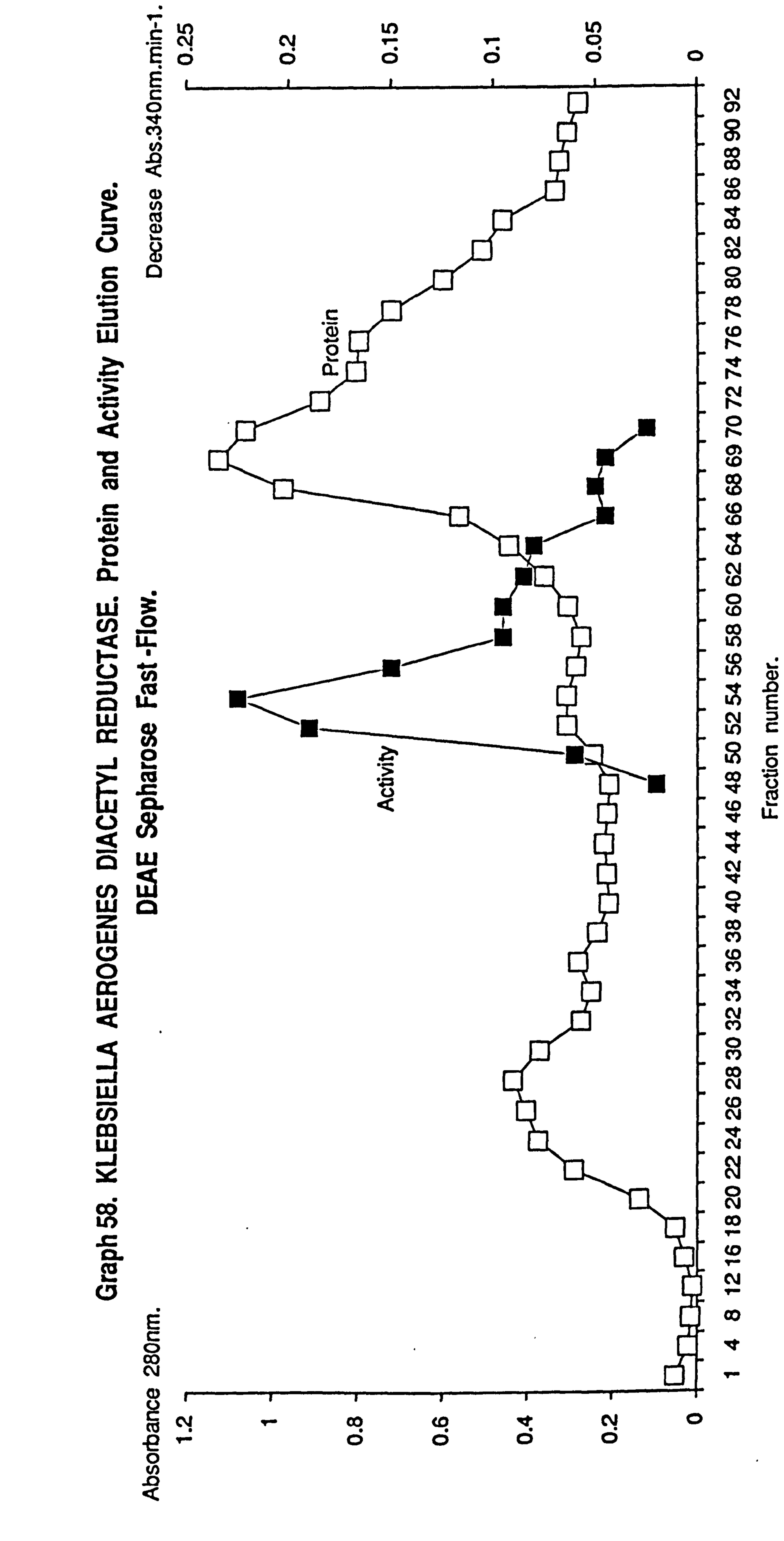
7.B.2. Purification of Streptococcus Lactis subsp. Diacetylactis 18-16 Diacetyl Reductase.

The preparation is described in detail in chapter 2, section 2.B.3. The column elution profiles are shown in graphs 59 and 60. Again a clear solution of enzyme was obtained, which was active with diacetyl, 2,3 pentanedione and acetoin as substrates. This activity was shown to be associated with the same enzyme by further purification of this preparation as mentioned in section 7.B. The additional purification procedure and the characterisation of the enzyme was reported in Gibson et al, (in press).

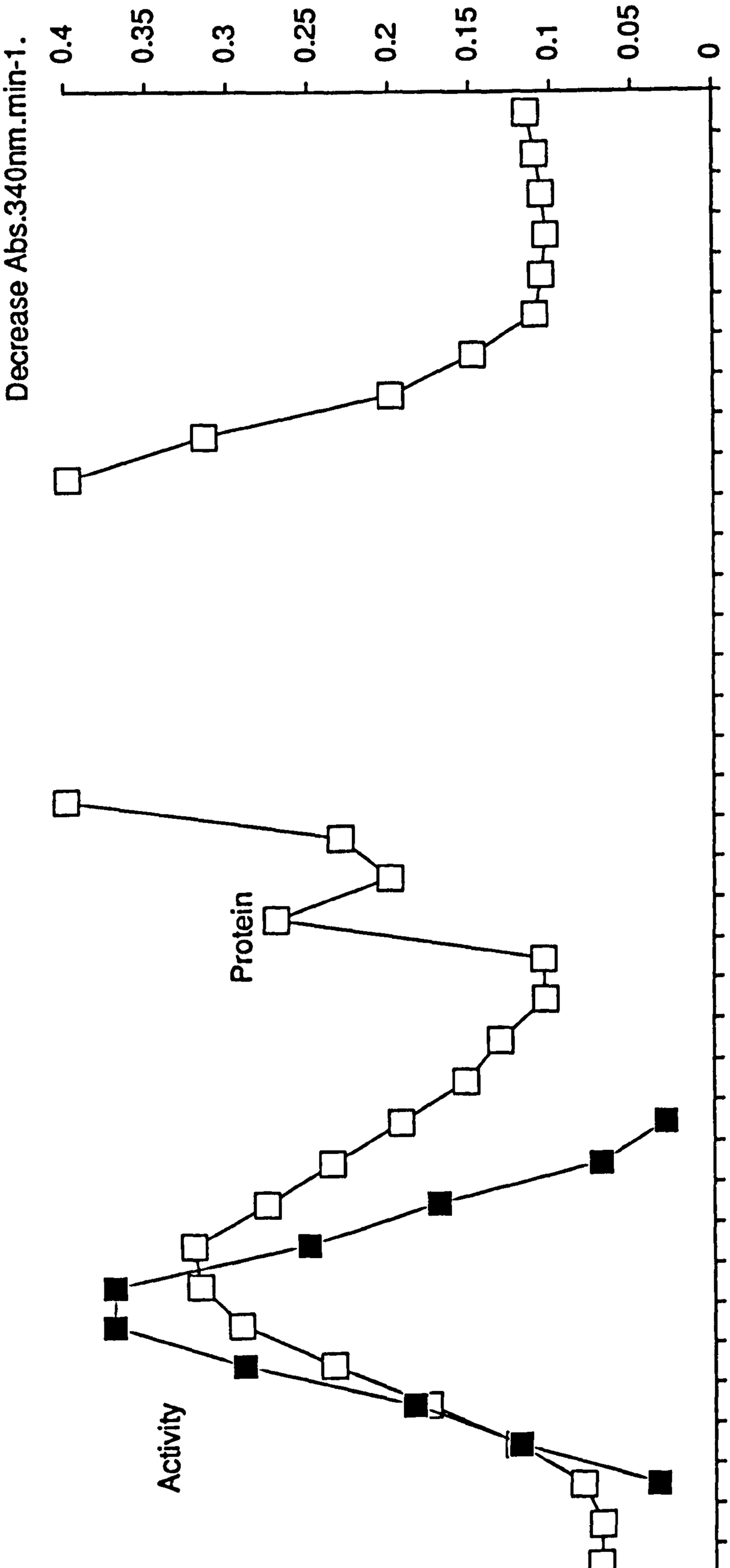
7.B.3. Use of Bacterial Enzymes in the Analysis of Diacetyl.

Both bacterial enzymes were tested in an quantitative assay system for diacetyl, similar to the one described in chapter 2,

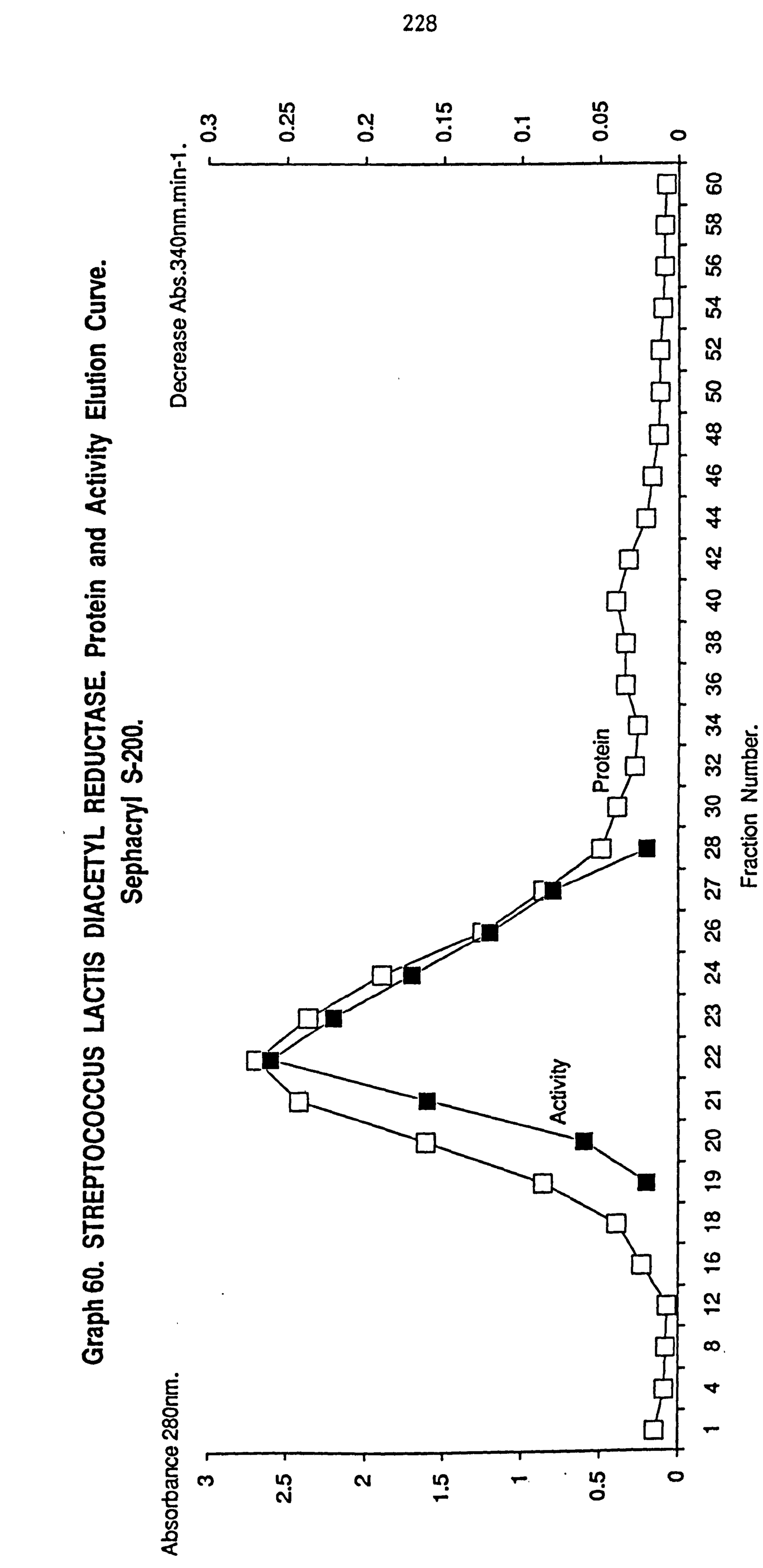
Graph 58. KLEBSIELLA AEROGENES DIACETYL REDUCTASE. Protein and Activity Elution Curve.
DEAE Sepharose Fast-Flow.



Graph 59. STREPTOCOCCUS LACTIS DIACETYL REDUCTASE. Protein and Activity Elution Curve.
DEAE Sepharose Fast-Flow.



Graph 60. STREPTOCOCCUS LACTIS DIACETYL REDUCTASE. Protein and Activity Elution Curve.
Sephacryl S-200.



section 2.F.2. Larger volumes of enzyme solutions were used,(100 ul) to obtain acceptable reaction rates. The pH of the reaction was held at 7.0, the time of the reaction was 20 minutes and the temperature was held at 30°C. The results obtained were similar to the standard curves shown in graphs 6-9.

The principle in using the enzyme reaction to assay diacetyl was the same in all cases. The NADH remaining after enzymic reaction was inversely related to the original amount of diacetyl in the sample. These conditions hold true when the enzyme used catalyses a single reaction and that reaction is essentially irreversible. In such cases one substrate (the analyte) is catalysed to give one product and no further reaction occurs, which allows quantitation of the original concentration of analyte. In the cases of the bacterial enzymes the results obtained were not accurate as the product of the first reaction shown in section 7.A, (acetoin) was also a substrate for the enzymes, (reaction 2 in section 7.A). Consequently the amount of NADH remaining after reaction was a measure of both enzymic steps and could not be directly related to the original diacetyl concentration.

Also due to the rather non-specific nature of the bacterial enzymes, analysis of diacetyl in "real" samples was inaccurate. This was especially noticeable in samples containing high levels of acetoin. For these reasons the bacterial enzymes were considered unsuitable for enzymic diacetyl analysis.

7.C. Liver Diacetyl Reductases.

Diacetyl reductases isolated from mammalian or avian liver have been reported to have no specificity for acetoin as a substrate and appear only to react with diketones and certain other dicarbonyl

compounds. (Burgos and Martin 1972, Diez et al 1974, Martin-Sarmiento and Burgos 1982, Provecho et al 1984, Bernardo et al 1984). For this reason the isolation and purification of two liver enzymes was undertaken.

Chicken was chosen as a readily available source of avian liver, and fresh beef liver as the source of a mammalian liver enzyme.

7.C.1. Purification of Beef Liver Diacetyl Reductase.

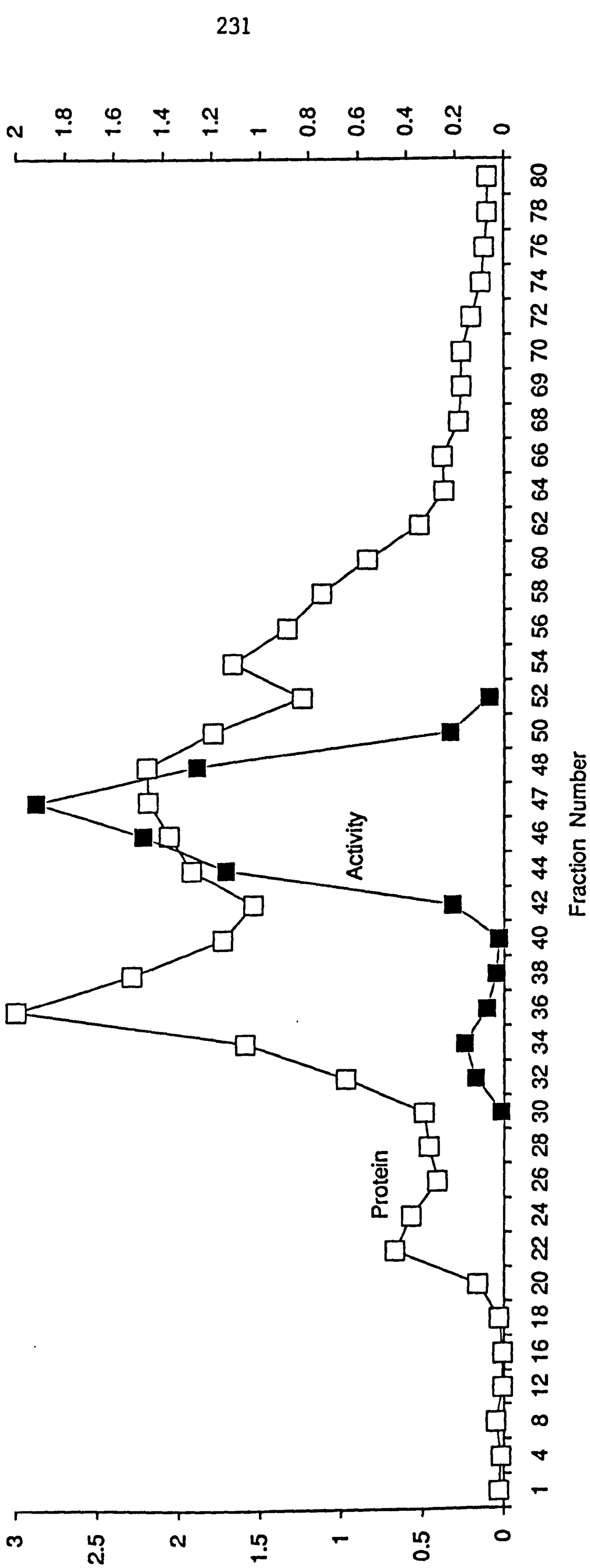
The purification procedure carried out according to the method of Burgos and Martin (1972) was described in chapter 2, section 2.B.4. The column elution profiles are shown in graphs 61 and 62. The final preparation after the S-200 step gave a series of bands on an SDS gel, fig 36. The enzyme was not homogeneous. This was similar to the result reported in Provecho et al (1984), where final purification was attained using a preparitive electro-focusing column.

In the preparation so obtained, no activity could be detected using acetoin as substrate, however activity was found using ethyl pyruvate, diacetyl and 2,3 pentanedione as substrates. These results were entirely consistent with those reported previously, (Provecho et al 1984).

7.C.2. Purification of Chicken Liver Diacetyl Reductase.

The purification procedure carried out according to the method of Bernardo et al (1984) was described in chapter 2, section 2.B.5. The column elution profiles are shown in graphs 63-65. The enzyme was purified to electrophoretic homogeneity as can be seen in fig 37. Activity staining on a non-denaturing gel or after subunit reconstitution on a modified SDS gel,(chapter 2,section 2.C.3) showed one band of activity associated with one band of protein, fig 38.

Graph 61. BEEF LIVER DIACETYL REDUCTASE. Protein and Activity Elution Curve. DEAE Sepharose.



Graph 62. BEEF LIVER DIACETYL REDUCTASE. Protein and Activity Elution Curve. Sephacryl S-200.

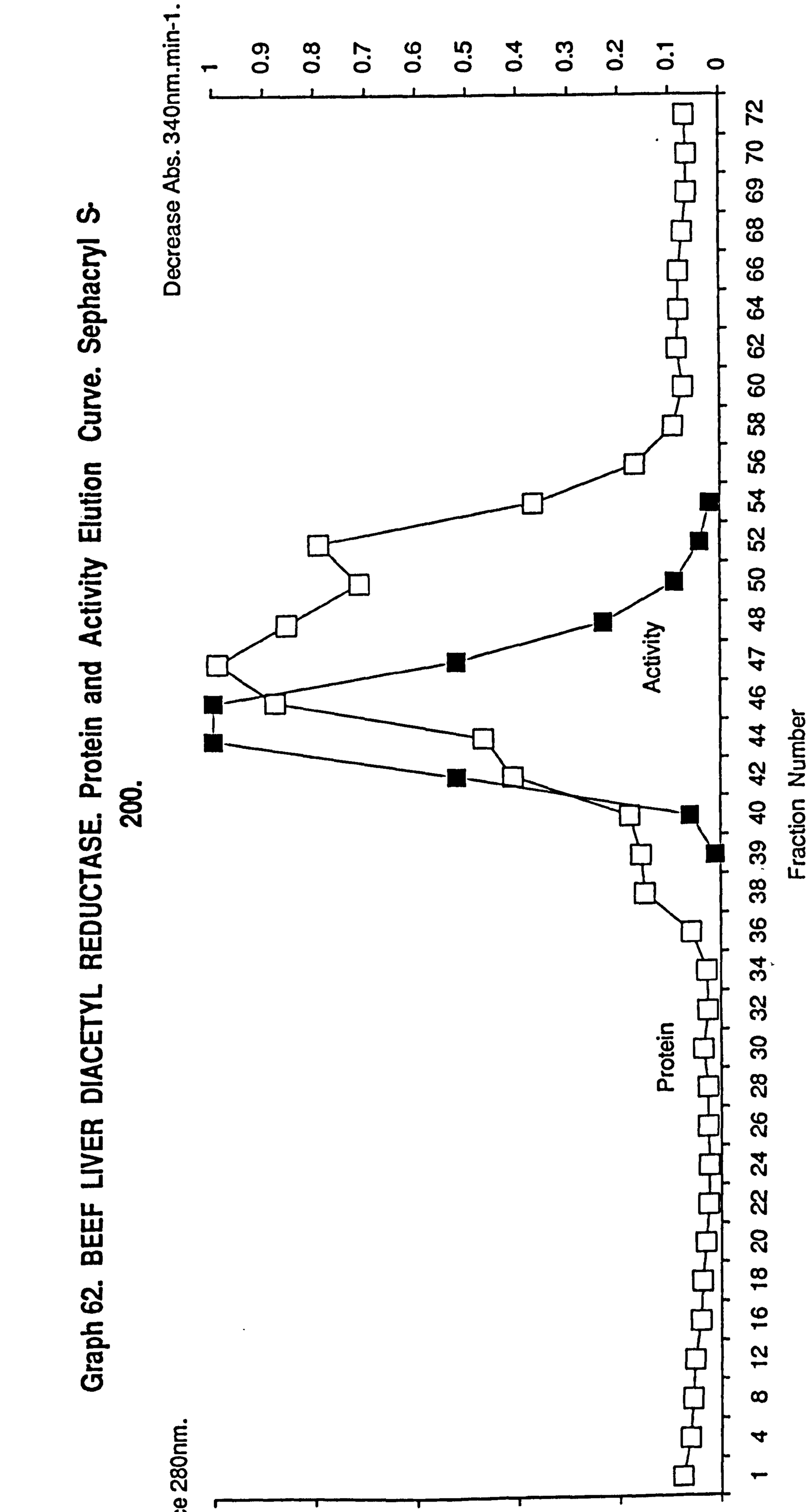
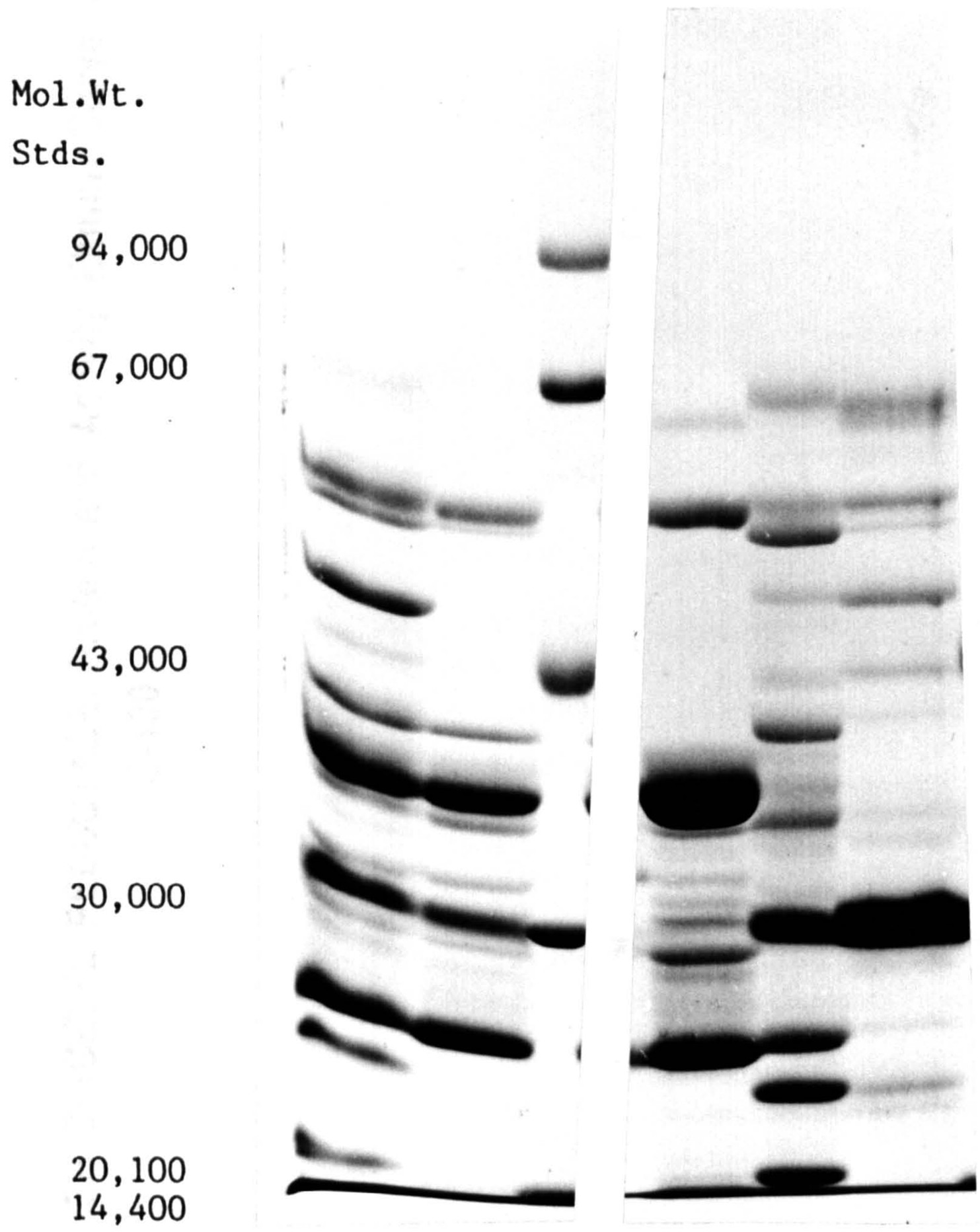


FIG.36 SDS ELECTROPHORESIS DIACETYL REDUCTASE. BEEF LIVER.



LANE 1. Acetone Precipitate,Crude Enzyme.15-20 µg Protein.

LANE 2. Eluate from DEAE Cellulose column,(as comparison).

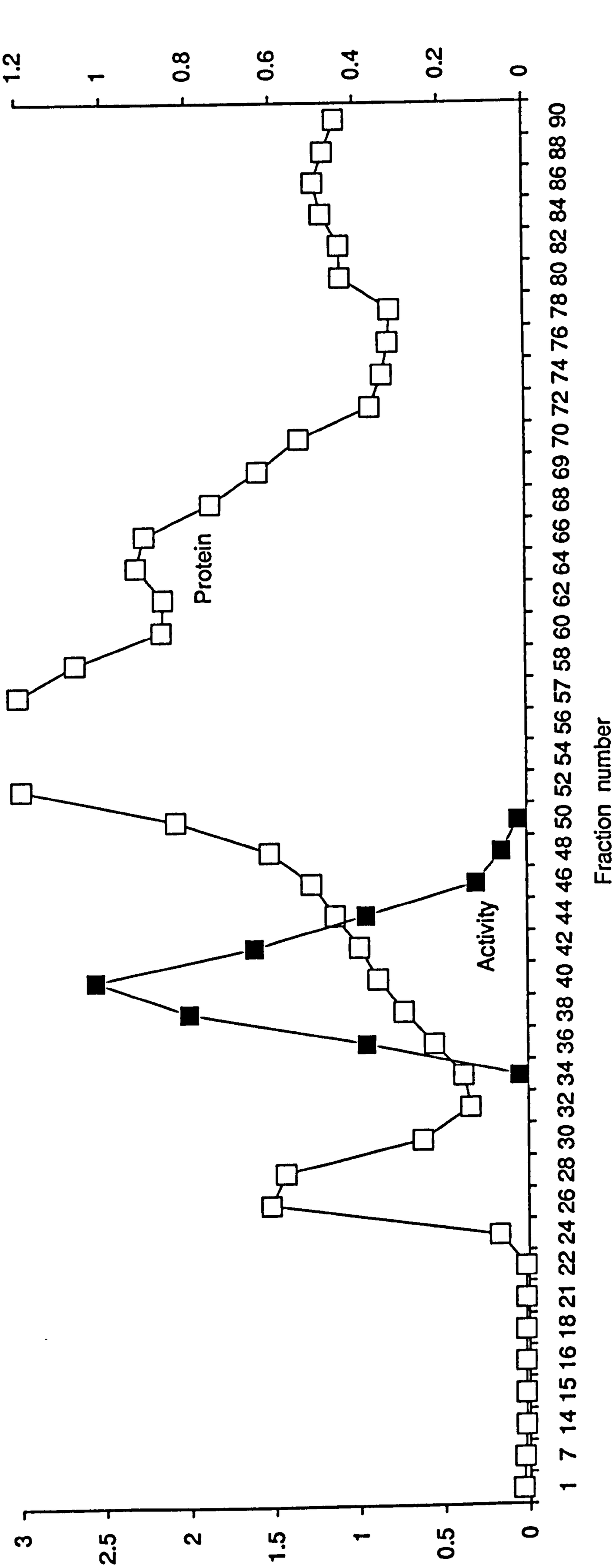
LANE 3. Low Molecular Wt. Standards (Pharmacia).
Weights given at left of gel.

LANE 4. Pooled Fractions 31-36 off DEAE Sepharose.
10 µg Protein. (Graph 61).

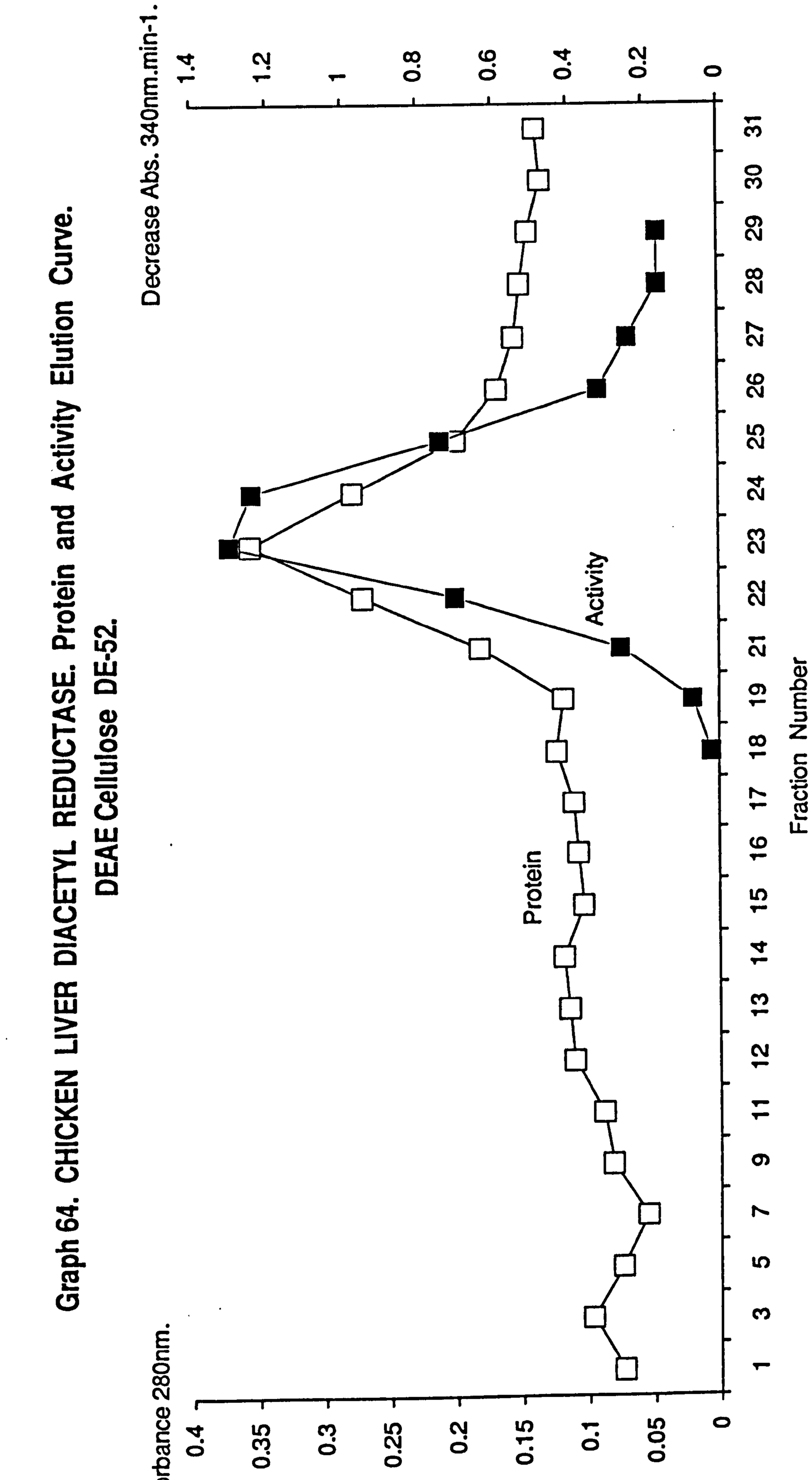
LANE 5. Pooled Fractions 41-51 off DEAE Sepharose.
10 µg Protein. (Graph 61).

LANE 6. Pooled Fractions 41-44 off Sephacryl S-200
10 µg Protein. (Graph 62).

Graph 63. CHICKEN LIVER DIACETYL REDUCTASE. Protein and Activity Elution Curve. Sephadex G-100.



Graph 64. CHICKEN LIVER DIACETYL REDUCTASE. Protein and Activity Elution Curve.
DEAE Cellulose DE-52.



Graph 65. CHICKEN LIVER DIACETYL REDUCTASE. Protein and Activity Elution Curve.
Chromatofocusing. PBE-94.

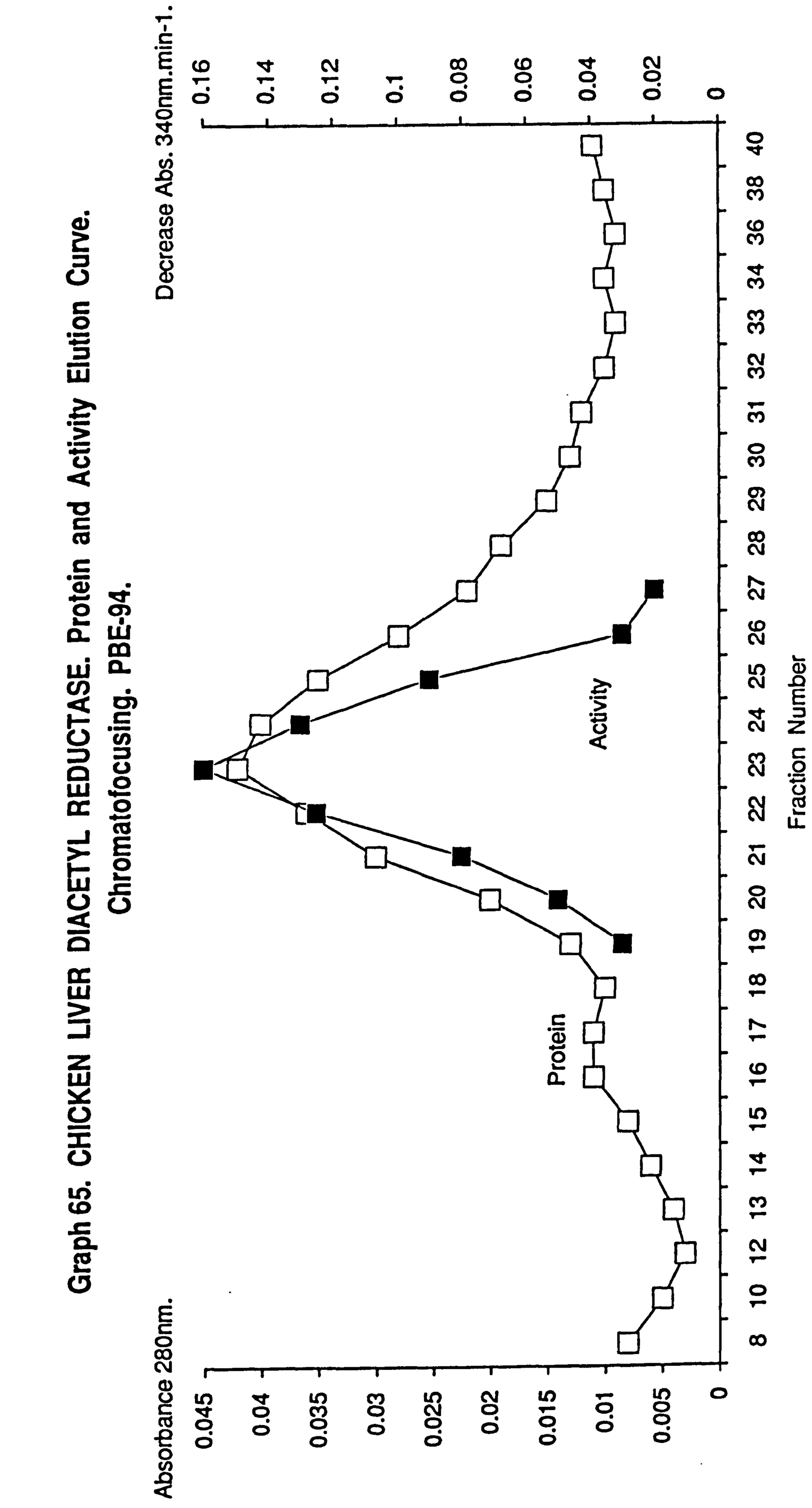
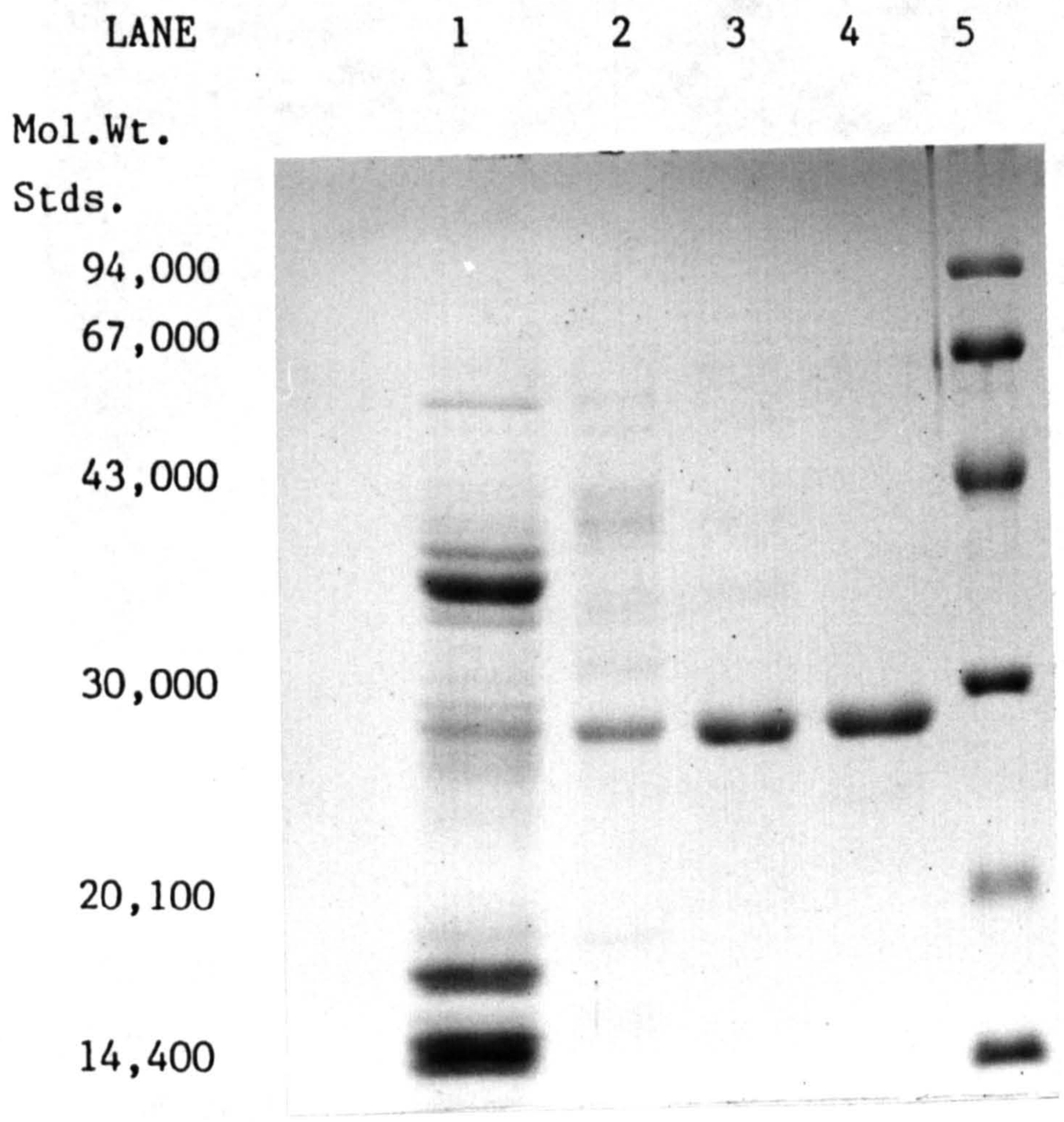


FIG.37 SDS ELECTROPHORESIS DIACETYL REDUCTASE.

CHICKEN LIVER.



LANE 1. Acetone Precipitate of Aqueous Liver Extract.

LANE 2. Pooled Fractions 34-48 off Sephadex G-100
4.0 µg Protein.(Graph 63).

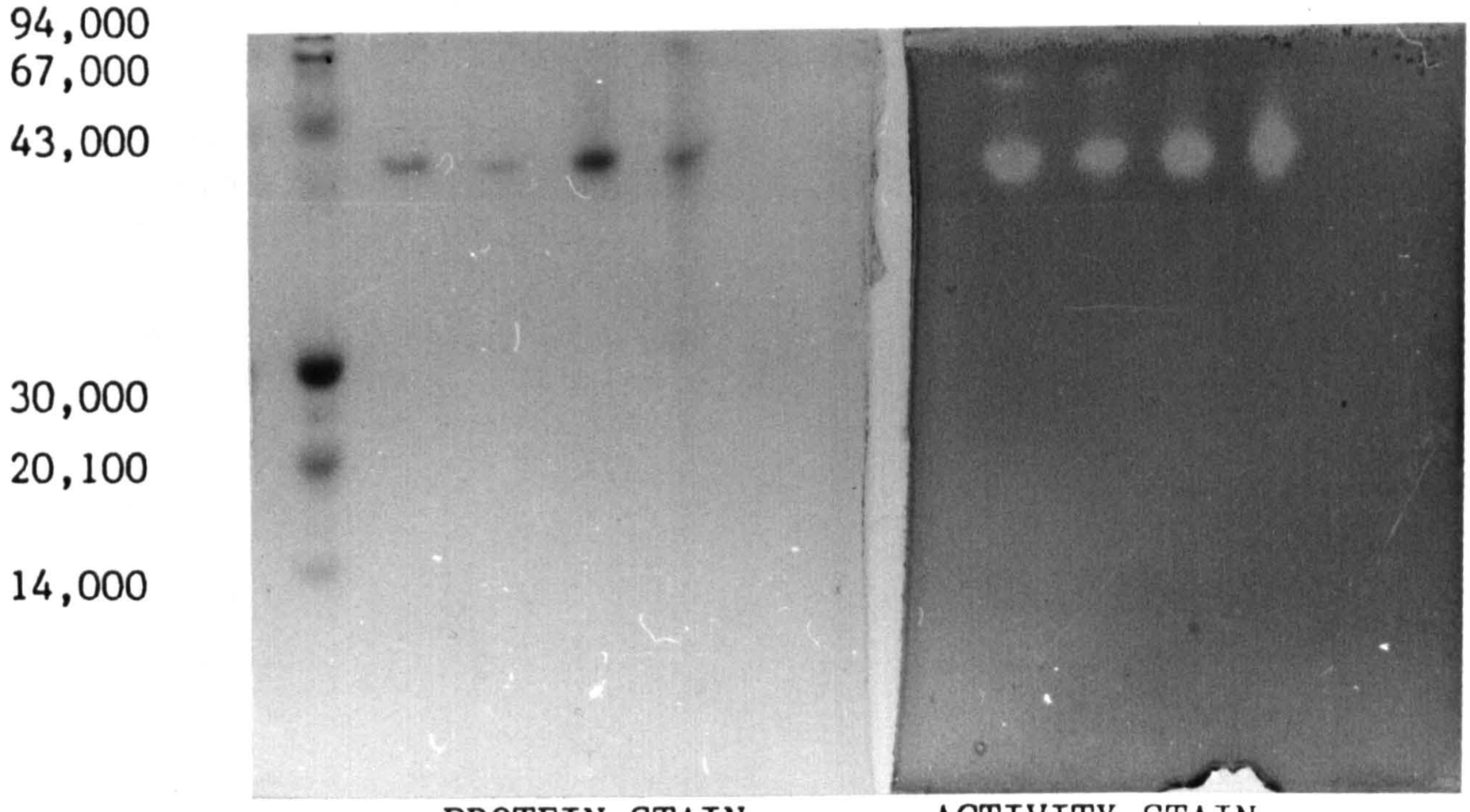
LANE 3. Pooled Fractions 21-26 off DEAE Cellulose,
2.5 µg Protein. DE-52. (Graph 64).

LANE 4. Pooled Fractions 19-26 off Chromatofocusing
2.4 µg Protein. PBE-94. (Graph 65).

LANE 5. Low Molecular Wt. Standards.(Pharmacia).
Weights given at left of gel.

FIG.38 NATIVE GEL ACTIVITY STAINING.

CHICKEN LIVER DIACETYL REDUCTASE.



LANE 1. Low Molecular Wt. Standards. (Pharmacia).

LANES 2,3,6 and 7. Pooled Fractions off Chromatofocusing.
PBE-94,(Graph 65). Lanes 2 and 6. 0.6 µg Protein.
Lanes 3 and 7. 0.3 µg Protein.

LANES 4 and 8. Pooled Fractions 21-26 off DEAE Cellulose.
DE-52,(Graph 64). Lane 4. 1.65 µg Protein.
Lane 8. 0.83 µg Protein.

LANES 5 and 9. Pooled Fractions 34-48 off Sephadex G-100.
(Graph 63). Lane 5. 3.0 µg Protein.
Lane 9. 1.0 µg Protein.

The purification procedure is summarised in table 31.

Table 31. Chicken Liver Diacetyl Reductase. Purification.

Step.	Total Activity. (units)	Total Protein. (mg)	Specific Activity. (units.mg^{-1})	Purif- ication. (2)	Yield. (%)
(1) Supernatant 50min 20,000g	891.0	33,550	0.027	1.2	58.0
Crude acetone precipitate	788.0	511.0	1.54	70.0	51.0
Sephadex G-100	384.0	30.5	12.3	560.0	25.0
DE-52 cellulose	239.6	7.3	35.3	1604.0	15.6
Chromato-focusing	50.7	1.2	42.2	1918.0	3.3

1/ Preparation from 300g liver.

2/ Relative to aqueous extract of liver, 1g to 4ml H_2O.

7.C.3. Characterisation of Chicken Liver Diacetyl Reductase.

(a) Isoelectric Focusing. (IEF)

IEF of the purified protein gave one major band at pI 6.2 and a very faint minor band at pI 6.05, (chapter 2, section 2.C.4). Activity staining of IEF gels was unsuccessful due to broad diffuse bands developing during the staining technique.

(b) Molecular Weight Determination.

The molecular weight of the native protein was found to be Mr 81,000, (chapter 2, section 2.E.4).

The molecular weight of the subunit was found to be Mr 28,000, (chapter 2, section 2.E.4). The ratio of these estimated weights suggests that the enzyme occurs as a trimer.

(c) Effect of Temperature on Activity.

The activity curve relative to temperature is shown in graph 66. The enzyme is maximally active at 35°C, (chapter 2, section 2.E.6).

(d) Effect of pH on Activity.

The activity curve relative to pH is shown in graph 67. The enzyme is maximally active at pH 6.1, (chapter 2, section 2.E.5).

(e) Reversibility of Reaction.

Addition of NAD to 0.2mM and acetoin to 11.3mM to the purified enzyme followed by incubation at both pH 6.1 and pH 8.8 at 35°C for up to five hours gave no evidence of the reversibility of reaction 1 shown in section 7.A. of this chapter.

(f) Co-factor and Substrate Specificity.

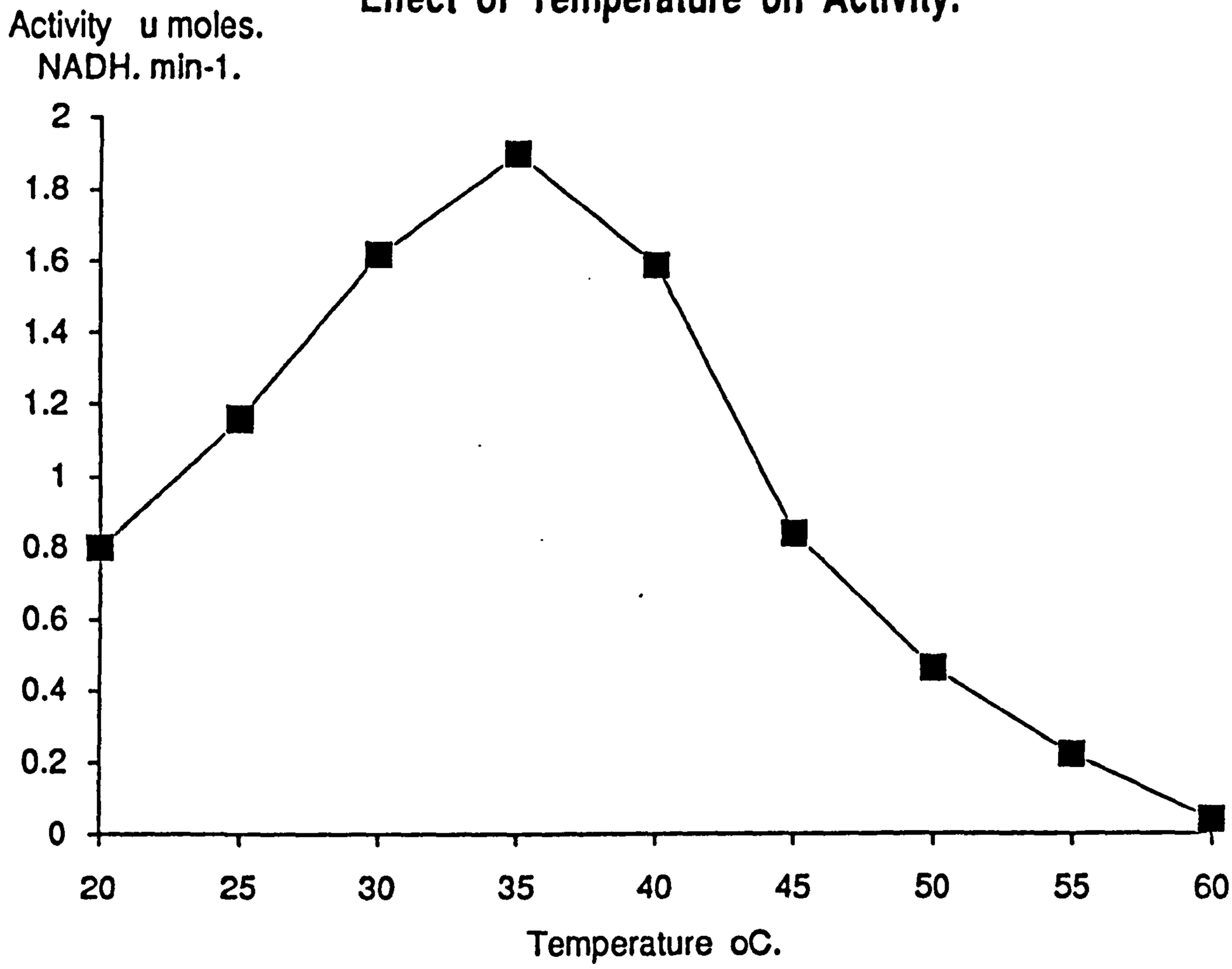
The purified enzyme accepted both NADH and NADPH as co-factors. Higher activities were seen with NADPH. A range of potential substrates was incubated with the enzyme and both co-factors, (chapter 2, section 2.E.2). The results are shown in table 32.

The purified enzyme has no activity with acetoin as substrate however it did react with both methyl and ethyl pyruvates and 2,3 pentanedione as well as diacetyl. Low activity was noted with methyl glyoxal and glyceraldehyde.

(g) Kinetic Affinity Studies.

The purified enzyme was incubated with various concentrations of co-factors or substrates as described in chapter 2, section 2.E.3. The results are expressed in terms of the apparent affinity constant, (Km^{APP}) in table 33. The purified enzyme had an affinity for NADPH which was some 29 times higher than that for NADH. Also the best substrate for the enzyme appeared to be 2,3 pentanedione. Diacetyl itself was the second most favoured substrate.

Graph 66. CHICKEN LIVER DIACETYL REDUCTASE. Effect of Temperature on Activity.



Graph 67. CHICKEN LIVER DIACETYL REDUCTASE. Effect of pH on Activity.

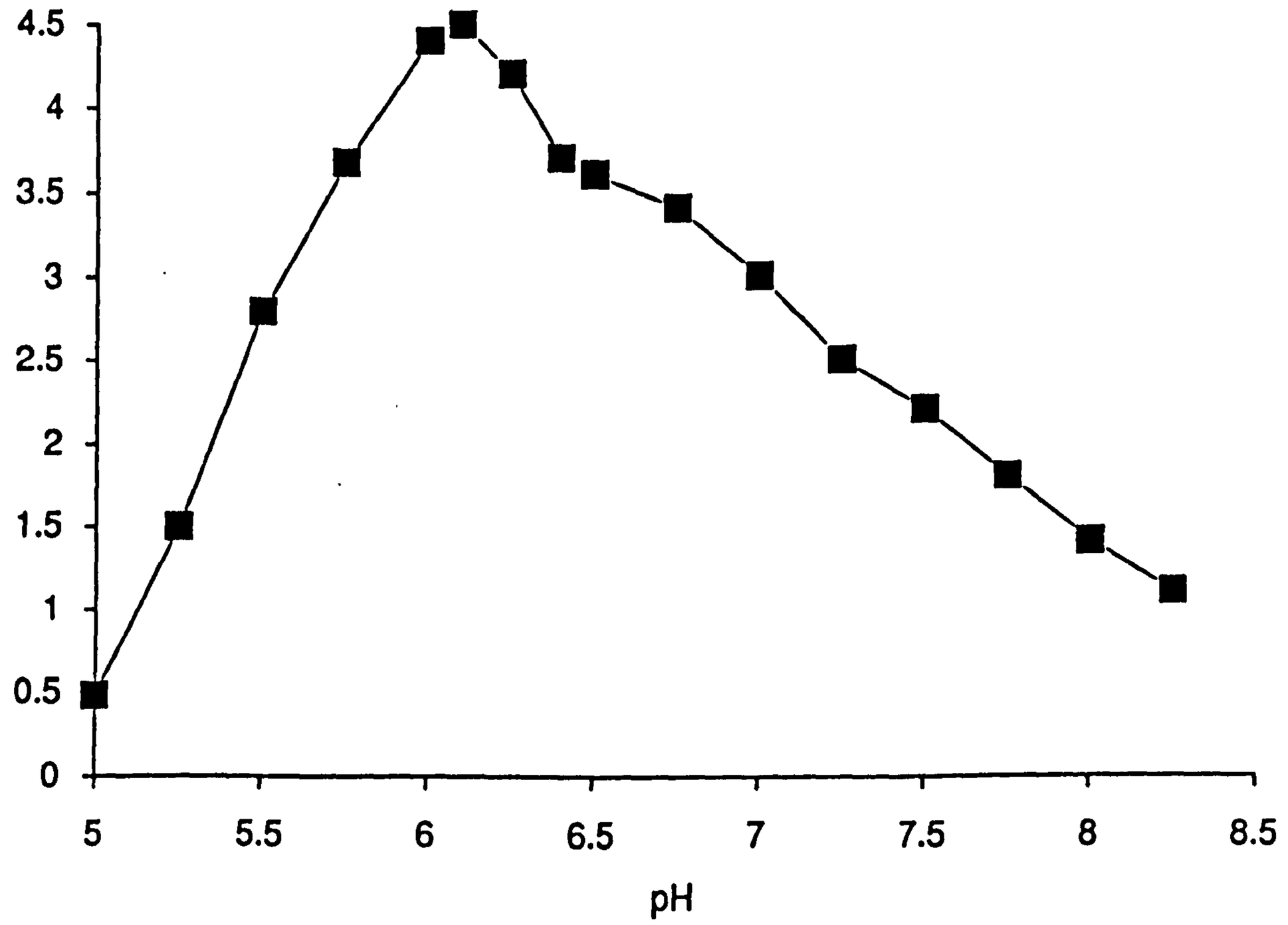


Table 32. Chicken Liver Diacetyl Reductase.

Substrate and Coenzyme Specificity.

Substrates 10mM.	NADH	NADPH
Acetone	0	0
Pyruvic Acid	0	0
2,4 Pentanedione	0	0
Glyoxal	0	0
Methylglyoxal	13	95
Diacetyl	1000	1594
2,3 Pentanedione*	369	413
Glyceraldehyde	4	191
Acetoin	0	0
Methyl pyruvate	527	953
Ethyl pyruvate	650	905
Ethyl acetoacetate	0	0

The results are expressed in n moles of substrate reduced per unit enzyme under standard assay conditions, (chapter 2, section 2.D.4).

* Substrate concentration of 10mM inhibitory. Gave higher activity at lower concentrations.

Table 33. Chicken Liver Diacetyl Reductase.

Kinetic Affinity Studies.

Variable Substrate	Fixed Substrate	Km^{app} mM.
NADH	Diacetyl ($10mM.l^{-1}$)	0.501
NADPH	Diacetyl ($10mM.l^{-1}$)	0.017
Diacetyl	NADH ($0.2mM.l^{-1}$)	1.8
2,3 Pentanedione	NADH "	0.29
Methyl pyruvate	NADH "	4.6
Ethyl pyruvate	NADH "	6.5
Methyl glyoxal	NADH "	22.1
Glyceraldehyde	NADH "	42.6

7.C.4. Use of Liver Enzymes in Analysis of Diacetyl.

The lack of purity of the beef liver enzyme preparation precluded its use in the enzymic analysis of diacetyl.

Initial results using the enzyme in the recycle assay described in chapter 2, section 2.F.4 and shown in fig 39, indicated that diacetyl levels down to $0.2mg.l^{-1}$ could be detected, however the assay was not reproducible. The use of this enzyme was discontinued in the analytical system.

The chicken liver enzyme was used to analyse aqueous diacetyl standards. The results of these analyses are shown in chapter 2, graphs 6-10.

The responses to aqueous diacetyl solutions were linear in all cases, however the sensitivity varied considerably, being dependent on the type of detection method used for the residual NADH, or in the case of the recycling method, the NAD produced.

The purified chicken liver enzyme preparation gave reproducible

FIG.39 RECYCLING ASSAY. DIACETYL ANALYSIS.

and representative results for the aqueous standards used, provided care was taken in analytical technique and the standards were freshly prepared. Aged standards showed a variation in results due to the volatility and instability of diacetyl in dilute solution.

Routine determination of the residual NADH after enzymic reaction was attempted using the methods described in chapter 2, section 2.F.3. The method using 2,2-dipyridyl or ferrozine and ferrous ions was found to be impractical due to the instability of the working reagents. This effect gave spontaneous colour generation in the solutions used, which resulted in high background readings.

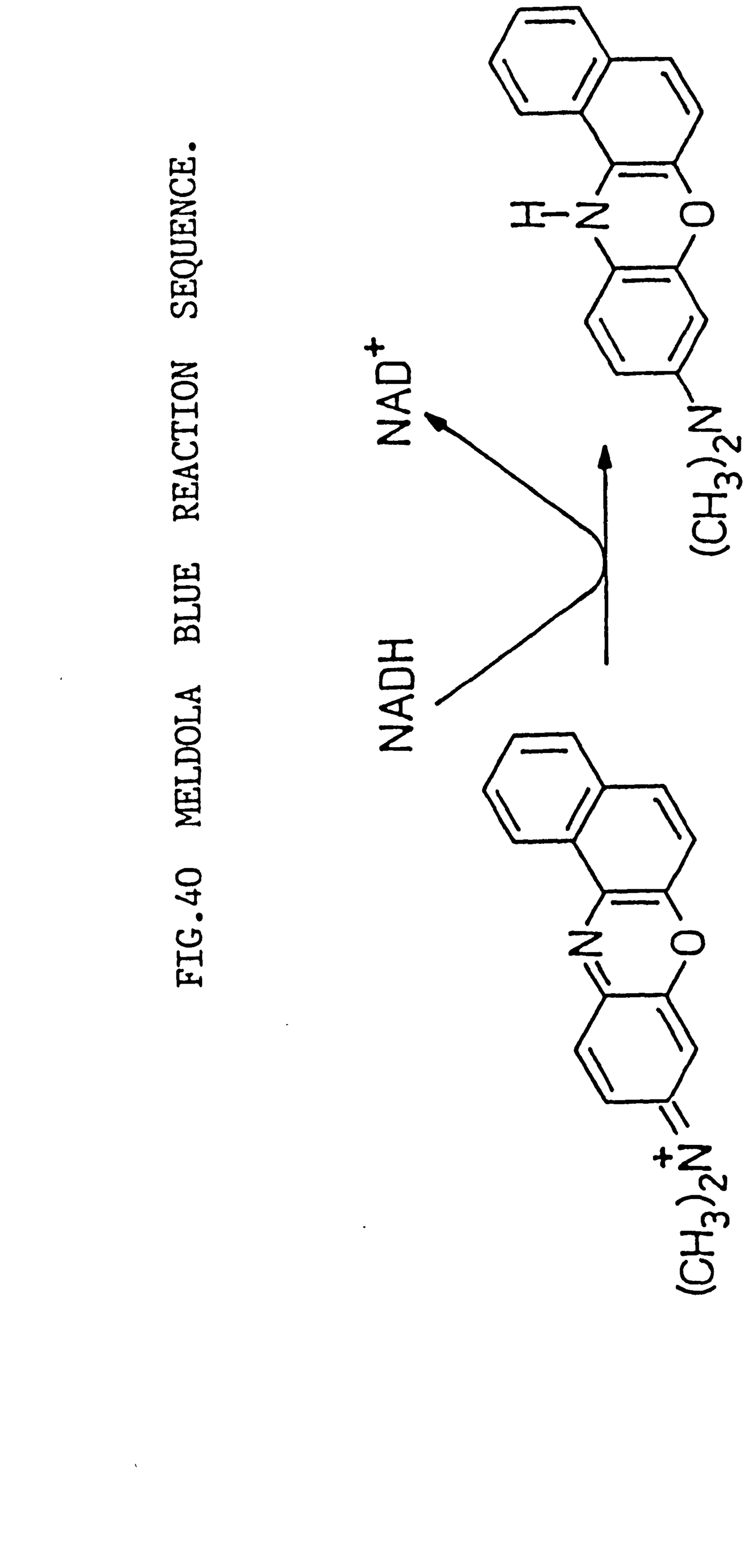
Detection of NADH using formazan production was a trouble free method, however the standard curve produced had a negative slope and the sensitivity was fairly low, graph 8.

Decolourisation of meldola blue resulted in a positive response curve. High diacetyl concentrations gave correspondingly low residual NADH concentrations, resulting in a high absorbance. The opposite being true for low diacetyl standards. Also, assays carried out in pH 7.1 buffer gave superior sensitivity to those incubated in pH 6.1 buffer, graph 9. This method also required fewer additional steps than the previous methods described and was the method of choice for diacetyl concentrations between 5-50mg.l^{-1}. The reaction sequence is shown in fig 40.

The use of chicken liver diacetyl reductase in the recycling method, (chapter 2, section 2.F.4) for detection of the NAD produced from the reduction of diacetyl gave consistent and reproducible results for diacetyl concentrations between 0.1-1.5mg.l^{-1}, graph 10.

Because of the sensitivity of the assay accurate pipetting was essential to ensure errors were minimised.

FIG.40 MELDOLA BLUE REACTION SEQUENCE.



MELDOLA BLUE,Reduced Form.
(Colourless.)

MELDOLA BLUE,Oxidised Form.
(Blue 565nm.)

The reaction of meldola blue with NADH (or NADPH) in the presence of Triton X-100 and in acid solution produces a stoichiometric amount of the colourless reduced form of the dye. Estimation of the reduction in absorbance gives a direct measurement of the initial NADH (or NADPH) concentration.Linking this reaction into an enzymic reaction gives a direct measurement of the substrate concentration,(Orsonneau et al 1982).

Also, thorough mixing was necessary, particularly after the addition of the HC1 so that the residual NADH was completely destroyed. The method was more accurate and sensitive around pH 7.0 than at pH 6.1, (the pH at which the enzyme was maximally active). It was thought that at acid pH's some non-enzymatic destruction of NADH occurred which affected the enzymic reaction and produced erroneous results.

Direct estimation of diacetyl in real samples which usually contain low levels, (milligrams per liter or less) has only been possible so far by using GLC methods. The sensitivity of the recycling asssay is sufficient to measure such levels of diacetyl accurately.

One disadvantage of the liver enzyme was the lack of absolute specificity for diacetyl. Interference by ketoesters, (e.g. methyl and ethyl pyruvate) could be removed by pre-incubation with esterase. However, the interference produced by other diketones such as 2,3 pentanedione could not be removed. Because of this, the enzyme method may be more accurately described as a diketone assay, rather than a diacetyl assay.

7.D.1. Application of Enzymic Assay to Analysis of Diacetyl in Fermentation Media.

Samples of dialysate obtained from the bacterial fermentation set up as described in section 2.H.2 were analysed by the meldola blue decolorisation method and the results compared to those obtained by the automated chemical method described in section 2.I.3. The results are shown in table 34. The enzyme method consistently gave lower results than the Westerfield method. This was found to be due to high levels of acetoin in the samples, (determined by an acetoin

Table 34. Comparison of Diacetyl Estimation in Fermentation Broth Dialysate Using Chemical and Enzymic Analysis.

Sample	Fermentation Time (hrs.)	Concentration Diacetyl ($mg.l^{-1}$) Westerfield Method	Enzyme Method Method (b) Meldola Blue
Blank	0	0	0
1	2.5	0	0
2	5.5	0	0
3	7.5	0	0.7
4	10.5	5.9	N/A
5	13.5	23.7	4.2
6	16.5	22.0	5.6
7	18.6	23.0	1.2
8	20.6	23.0	1.9
9	22.6	22.0	N/A

N/A not assayed due to lack of sample.

accepting enzyme) which reacted in the Westerfield method in the same way as diacetyl but at a reduced rate, (Speckman and Collins 1982). The rapidity of the automated method only allowed a proportion of the acetoin present to react, so giving an apparent high diacetyl content.

The enzyme method does not detect acetoin and the results obtained reflected a truer measure of the diketone content of the samples.

7.D.2. Application of Enzyme Assay to Analysis of Diacetyl in Beer.

Several attempts to analyse beer samples, (lager, bitter, brown ale and stout) for diacetyl by the enzyme method resulted in failure.

Both the meldola blue and the recycling assays were used and in both cases large false positive values were recorded. These results only occurred in the presence of both enzyme and NADH. No false positive values occurred using enzyme in the absence of NADH or using NADH in the absence of enzyme.

It was concluded that the probable reason for such false positives was interference in the enzyme reaction itself. Substances such as glyceraldehyde are present in beer in relatively large amounts compared to diacetyl and even though the reaction rate is slow, (section 7.C.3, table 33) the interference would be significant. Ketoesters are also present in beer, but although treatment of beer samples with esterase, (to destroy such esters) tended to reduce the interference, it did not eliminate it.

For enzymic analysis of diketones in beer, it will be necessary to use some sort of separation to provide a suitable aqueous sample, thereby removing the interference effects. In this respect, no advantage will be gained over conventional analytical methods.

CHAPTER 8.

DISCUSSION.

8.A. Alcohol Oxidase as an Analytical Enzyme.

Enzymes which are used for analytical purposes are required to possess several characteristics before they are generally considered acceptable. These include the specificity of the reaction catalysed, the irreversibility or apparent irreversibility of the reaction catalysed, the stability of the purified enzyme, the purity of the enzyme, the availability and the subsequent cost of the enzyme.

Specificity of Reaction.

The specificity of any particular enzyme is of major importance to the accuracy of the analysis being carried out. The ideal situation is where only one specific substrate is recognised by the one enzyme. In practice this is not always the case, as many enzymes have some activity on other substrates, albeit at a reduced rate to the preferred substrate.

e.g. Alcohol dehydrogenase is not specific for ethanol, it also accepts a wide range of other compounds, (Sund and Theorell 1963).

However, the usual finding in such cases is that the alternative substrates recognised by the enzyme are not present in biological or real samples, thus rendering an effective 100% specificity for the required substrate.

In the case of alcohol oxidase the specificity range is narrow, (section 3.A.3, table 14). Methanol, propan-1-ol, butan-1-ol, formaldehyde and allyl alcohol are not present in body fluids to any appreciable extent and so any enzyme reaction is specifically due to ethanol, giving a quantitative test for ethanol in body fluids.

Similarly alcoholic beverages are relatively free of large quantities of the interfering substrates and are usually very high in ethanol content. Dilution of such solutions is usually necessary

before analysis; again causing a specific response for ethanol. Where other types of sample are analysed, e.g. bacterial ferments, etc. care is needed to ensure the response is due to a specific substrate.

Reversibility of Reaction.

The reaction catalysed by an enzyme needs to be irreversible in nature to give accurate results, as reversible reactions usually do not go to completion and are in an equilibrium state. Some reversible reactions may be made apparently irreversible by altering the pH. e.g. Lactate dehydrogenase at pH 7.0 specifically catalyses the conversion of pyruvate to lactate. However, if the pH is adjusted to 8.9 and above the enzyme specifically converts lactate to pyruvate.

Alternatively, removal of one of the products formed by chemical or enzymatic means can have the same effect. e.g. The determination of ethanol using the enzyme, alcohol dehydrogenase produces acetaldehyde, which can be removed from solution using semicarbazide and in the lactate to pyruvate reaction above at pH 8.9, the pyruvate produced can be removed by its reaction with L-glutamate, catalysed by the enzyme, alanine aminotransferase.

In the case of alcohol oxidase and oxidases in general, the reaction is essentially irreversible and proceeds to completion. This gives the ideal type of reaction for an analytical enzyme.

The detection reaction for ethanol using alcohol oxidase was optimised using standard reagents developed from the basic "Trinder" assay, graphs 17-20, (Trinder 1969). The responses of the optimised assay system are shown in graphs 13-14 and are linear over the concentration ranges shown. Phenolsulphonic acid was chosen in preference to phenol itself as it is far less toxic and much more

stable. The reagents may, therefore, be packaged as an analytical enzyme kit for ethanol. The correlation of the oxidase method using Trinder detection chemistry compares very favourably to the standard alcohol dehydrogenase method marketed by Sigma. The correlation coefficient (r) was 0.993 with a slope of the regression line of 0.994, graph 21.

Stability of Enzyme in use.

The stability of enzymes is of prime importance in their use as analytical reagents. This is arguably the major area of difficulty regarding the use of enzymes as analytical reagents. The stability of the enzyme used often dictates the effective shelf life of the analytical system.

Alcohol oxidase is very stable in conditions of low water activity, (Woodward 1990) hence the storage of the enzyme as a precipitate in ammonium sulphate solution. Dilute solutions of the enzyme are fairly stable for short periods, however there is a slow production of hydrogen peroxide presumably from formaldehyde, which appears to be bound as an adduct onto free amino groups, (Hopkins and Mueller 1987). Hydrogen peroxide appears to have a particular effect on the enzyme, causing aggregation and subsequent inactivation of the enzyme upon storage.

It is not clear whether the loss of activity is due to enzyme destruction or just the production of an insoluble precipitate from the enzyme solution thus reducing the concentration of the soluble enzyme. Also the aggregation and subsequent precipitation of protein occurs more rapidly in more concentrated solutions of enzyme in buffer and is more noticeable in aged enzyme preparations.

Hopkins and Mueller (1987) reported aged preparations to be more

completely succinylated than fresh enzyme when reacted with succinic anhydride, indicating a removal of formaldehyde from the free amino groups. If more free formaldehyde is produced in aged samples, it is reasonable to assume a higher concentration of peroxide is produced, which in turn promotes a higher degree of aggregation. This may be due to the formation of disulphide bridges between the free sulphydryl groups of the enzyme, as peroxide will spontaneously oxidise such reducing groups to produce the more stable disulphides. (This has been tested with some of the sulphydryl containing mediators listed in table 22 and is found to be the case). This theory of aggregation is supported by the fact that incubation of precipitated aged enzyme solutions with 2-mercaptoethanol and sodium mercaptoethane sulphonate results in complete dissolution of the precipitate to give clear solutions. No enzyme activity could be demonstrated in the solutions so prepared, which may indicate inactivation occurs as a result of precipitation in aged solutions.

Solutions of alcohol oxidase may be stabilised by the addition of high concentrations of polyhydroxyl compounds such as lactitol or sorbitol, graph 35. This is discussed more thoroughly in section 8.2. For storage purposes, such concentrated solutions may be of use provided dilution of the enzyme is carried out prior to assay. Assays are difficult in high concentrations of stabilisers usually due to viscosity effects. Long term stability studies of solutions of alcohol oxidase have not been carried out but incubations of enzyme solutions for 5 days at 37°C indicated enhancement of enzyme activity retention in the presence of some polyhydroxyl compounds.

The stability of alcohol oxidase in a dry state, as in a reagent/enzyme dipstick format has long been a major source of

difficulty. Similarly, dry lyophilised preparations of the enzyme from various sources are notoriously unreliable for retention of activity. Various stabilised preparative techniques have been claimed, (Phillips 1985, Hopkins 1988, Adams 1988) however the source of the enzyme used was Pichia pastoris and the same stabilising techniques appear to be ineffective for the Hansenula enzyme. Dry stabilisation is discussed in detail in section 8.C.

It may be concluded then, that whilst solutions of alcohol oxidase may have a reasonable working life, long term storage of the enzyme is a major drawback in its use as an analytical enzyme. The enzyme must be stored frozen or as an ammonium sulphate precipitate or may be stabilised as described in chapter 5, to retain enzyme activity. Commercially available dry preparations are unreliable and are not to be trusted.

Enzyme Purity.

The purity of the enzyme is important in determining the specificity of the analytical reaction. Associated enzyme activities in any analytical enzyme assay are undesirable. The major contaminant of alcohol oxidase is catalase. This may be seen clearly in fig 19, lane 1. The purification steps carried out give an enzyme of sufficient purity to use in analytical methods, fig 19, lane 2.

Availability of Enzyme.

The cost of the commercially produced enzyme from the Provesta Corporation, (a subsidiary of Phillips Petroleum), varies from $8 to $20 per 1000 units depending on the amount bought. Other sources include Sigma at £29 for 1000 units and Boehringer at £26 for 50 units, (the enzyme from Hansenula is not commercially available). As can be seen, the enzyme is relatively expensive, which may be one

reason why the enzyme is not widely used in analytical techniques. Also the quality and stability of the commercial enzyme is not adequate for routine enzymic analysis.

Conclusions.

The overall conclusions that may be drawn from the above discussion are that the purity, specificity and type of reaction catalysed are adequate for the Hansenula enzyme to be used as an analytical tool. The availability and subsequent cost are fairly high, but with careful analytical design are not excessive. The stability of the enzyme is poor and on this point alone the enzyme fails in producing an acceptable, widespread, analytical method for ethanol.

8.B. Immobilised Enzyme and Use in Flow Systems.

Immobilising enzymes onto insoluble supports, offers certain advantages over soluble enzymes, which include increased stability, multiple use of the same enzyme for many assays and thus a saving in cost of the enzyme. Co-immobilisation of two or more enzymes together also allows multiple reaction sequences to take place in one "enzyme reactor".

Nylon Immobilisation.

Alcohol oxidase immobilised on the internal wall of nylon tube using the reaction sequence in fig 9, gave a usful method to analyse ethanol using a flow system. The responses of the nylon immobilised enzyme reaction in a segmented flow analytical system are shown in figs 23-25. These are comparable to those obtained from the soluble enzyme systems, figs 20-22. The amount of enzyme used was very small indeed, being less than 0.5 units. The enzyme coils were stable for 1 month's use provided they were well washed and stored in phosphate

buffer, pH 7.0 at 4°C. after each days work, (average running time per day 5-6 hours). Continuous perfusion over several days at an average room temperature of 21°C, reduced the activity of the same coils by half in 5 days, (half life). An increase in stability was noticed when other amino group containing spacer arms were used in place of 1,2 diaminoethane, graph 24. Polymeric amino compounds gave the highest stability under continuous flow conditions, with a half life of 7 days.

Nylon immobilised enzymes are subject to nucleophilic cleavage of the co-valent bond formed between the nylon imidate and the amino spacer molecule, (Sunderam 1979). The reduction of activity under continuous flow conditions may be partly due to removal of bound enzyme by this process, however the retention of glucose oxidase activity under the same conditions, (having been immobilised by the same reaction sequence), points away from this explanation. Inactivation of the enzyme during the prolonged conditions of the test is a more likely possibility.

It was noticed that the enzyme exhibited a better stability profile when itwas exposed to substrate. Enzyme coils which were continuously used for assays and then washed and stored overnight in buffer at 4°C before being used again, retained activity longer than those perfused continuously with few estimations of substrate. Newly prepared enzyme coils also exhibited an increase in activity after exposure to substrate. This normally reached a plateau after the first days use and from then on activity gradually decreased.

The probable explanation of these phenomena is the orientation of the immobilised enzyme with respect to the solid support and the maximum availability of "active" enzyme molecules for the substrate.

On immobilisation a proportion of the enzyme may be bound in such a way as to become unavailable for catalysis to take place. In the presence of substrate some of the bound, unavailable enzyme may be able to reorientate itself to become available and thus apparent enzyme activity increases. This also appears to be true for pyruvate oxidase in membrane entrapped systems and glucose oxidase immobilised on nylon, (personal communications from Dr.R. Spokane Y.S.I.Inc. and ChemLab. Instruments Ltd.).

Cholesterol esterase and cholesterol oxidase co-immobilised on nylon, exhibited a half life of about 2.5 days in continual use with cholesterol as substrate. The total activity of the coil made, (section 2.G.1) was fairly low and was found to be unsuitable for routine use. Higher activity enzyme reactors have been made using controlled pore glass as the solid support, (Tabata et al 1981).

Glass Immobilisation.

Alcohol oxidase immobilised on control pore glass was used to make enzyme columns for use in flow injection systems. The response of the glass immobilised enzyme is shown in fig 27 and is linear to 300mg.1^{-1}. For comparison the soluble method is shown in fig 26 and is linear to 500mg.1^{-1}. Further work to optimise the immobilised system may extend the linearity of the method, however, due to time and lack of suitable controlled pore glass samples, this was not carried out.

The stability of such columns is remarkable. The enzyme half life was about 10 days, (graph 26) but thereafter no loss in activity was noticed during 35 days continuous use at room temperature, graph 26.

The essential loss of activity has been attributed to much the same process as that of the nylon immobilised enzyme, however the

residual enzyme is far more stable than that immobilised on nylon.

It is probable that the enzyme activity remaining after the initial loss is due to enzyme bound in the pores of the glass. This would probably result in multiple attachments between the enzyme and the activated glass support, fig 10.

As it has already been reported by Martinek et al (1977), multipoint attachment of enzymes to supports markedly enhance their stability. The result observed with glass immobilised alcohol oxidase becomes even more remarkable, when it is considered that the data obtained in graph 26 was from enzyme that had first been immobilised 12 months previously and stored in phosphate buffer pH 7.0 at 4°C. Recent tests have shown the same glass immobilised enzyme column has retained the same residual activity for a further 2 months at room temperature.

Use in Flow Systems.

Both the nylon and glass immobilised enzymes have been used to quantify ethanol in fermentation experiments using flow systems, figs 13 and 16. The main development for such measurements was the use of a dialysis probe as a sampling device in the fermentation, fig 11.

The characterisation of the probe was carried out using the flow injection soluble method for glucose and ethanol, (fig 15) to determine the temperature dependence, graph 28 and the maximum steady state response of the probe with respect to time, graph 27. Because the probe gave a dynamic response, the equilibration time of the analyte across the dialysis membrane, between the bulk solution and the carrier solution was relatively short. For ethanol this was approximately 2 minutes and for glucose approximately 3 minutes,

graph 27. The size and shape of the molecule determined the speed of dialysis. The rapidity of equilibrium was much shorter for calcium ions being approximately 1 minute. Also the flow rate of the carrier solution influenced the equilibration time, a higher flow rate gave a shorter time and a slower flow rate gave a higher one, (unpublished results).

Using the probe in actual fermentation systems and analysing the effluent carrier stream using the enzyme flow systems, gave the results obtained in graphs 30-33. In graph 33 the diacetyl was estimated using the chemical method flow system, fig.14. In the case of graph 32 the H_2O_2 produced by the immobilised enzyme was detected electrochemically rather than spectrophotometrically using a Yellow Springs Instruments Inc. peroxide probe.

As a comparison to the results obtained using the dialysis probe shown in graph 30, direct sampling of the same fermentation gave the results shown in graph 29. It as noticed that at higher analyte concentrations interference in the colour reaction occurred. Dilution of these samples gave an apparent increase in the observed values for ethanol produced towards the end of the fermentation, indicating a diluting out effect of the interfering compounds in the fermentation broth. It was also noticed that nylon immobilised glucose oxidase was subject to irreversible inactivation after several consecutive analyses of the fermentation broth obtained by direct sampling. The activity decreased approximately 70-80% over the course of one experiment, (48hrs). The reason for this inactivation was not elucidated. Alcohol oxidase coils were unaffected by the same samples.

8.C. Stabilisation of Alcohol Oxidase and Other Enzymes.

One major problem in using enzymes as reagents for quantitative estimation of analytes is their lack of stability and consequently the effective long term storage, (shelf life) of enzyme based diagnostic tests is often fairly short and may require specialised storage conditions. Also the stability of the enzyme component within such a test system will tend to dictate the types of analytical tests that become available commercially. Many tests that are suitable for a laboratory environment become unusable when taken into the field. The effective stabilisation of the active components of such tests would be of great advantage in the production and shelf life of the tests produced.

The stabilisation of alcohol oxidase was carried out using a variety of techniques and then identifying those which were effective. Further development on the effective methods produced the results that form the basis of the patent application on enzyme stabilisation, (Gibson and Woodward 1989).

Wet Stabilisation.

It was noticed early on in the work that alcohol oxidase when bound electrostatically to DEAE-sepharose, (or DEAE derivatised supports) became very stable with respect to activity retention with time. Storage of bound enzyme for up to 2 years with 100% retention of activity was common.

As has already been mentioned, free enzyme solutions form a precipitate on aging with subsequent partial loss of activity. Elevated temperature testing of alcohol oxidase solutions from Hansenula polymorpha and Pichia pastoris showed a marked difference in the stability of the two enzymes, graph 34. At a temperature of

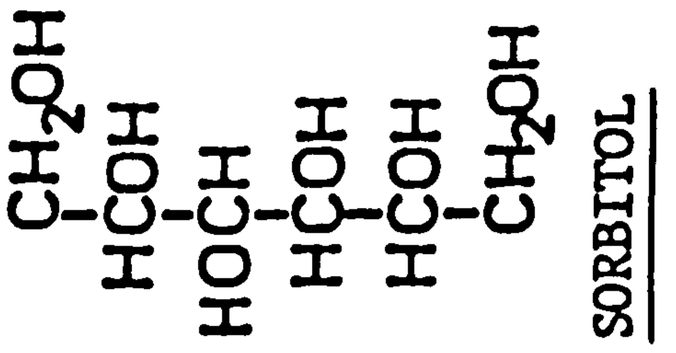
50°C Pichia enzyme was 80% inactivated after 30 minutes whilst Hansenula enzyme was stable for many hours. Elevating the temperature to 60°C inactivated the Hanensula enzyme, the curve was similar to that of the Pichia enzyme at 50°C, graph 34.

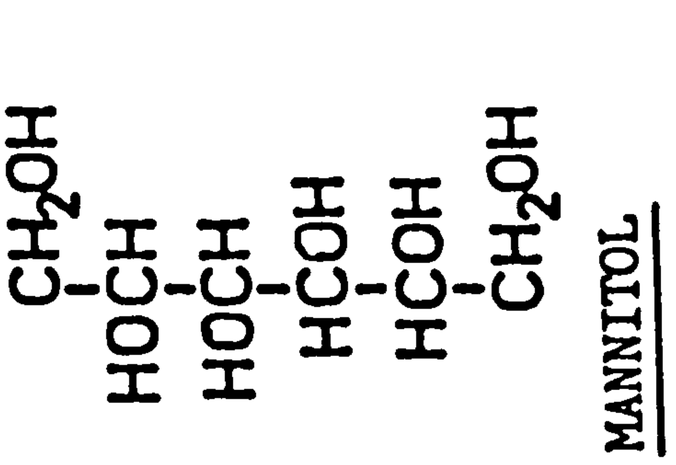
Effect of Additives.

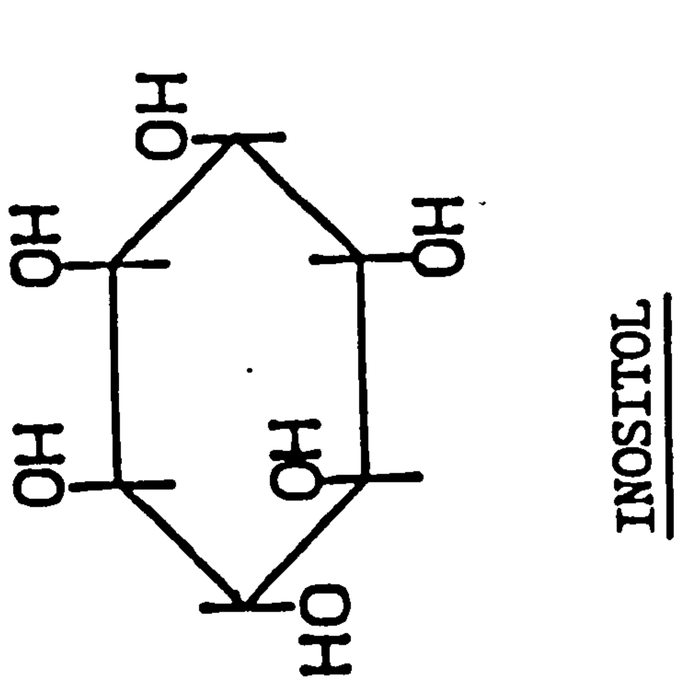
Addition of polyhydroxyl compounds to enzyme solutions have been shown to increase the stabilities of enzymes, (Back et al 1979, Arakawa and Timasheff 1982, Fujita et al 1982, Monsan and Combes 1984, Combes and Monsan 1984, Ye et al 1988). This is thought to be due to the interaction of the polyhydroxyl compound, (e.g. sucrose, polyethylene glycols, sugar alcohols, etc), with water in the system. This effectively reduces the protein - water interactions as the polyhydroxyl compounds become preferentially hydrated and thus the hydrophobic interactions of the protein structure are effectively strengthened. This leads to an increased resistance to thermal denaturation of the protein structure, and in the case of enzymes, an increase in the stability of the enzyme, shown by retention of enzymic activity at temperatures at which unmodified aqueous enzyme solutions are deactivated.

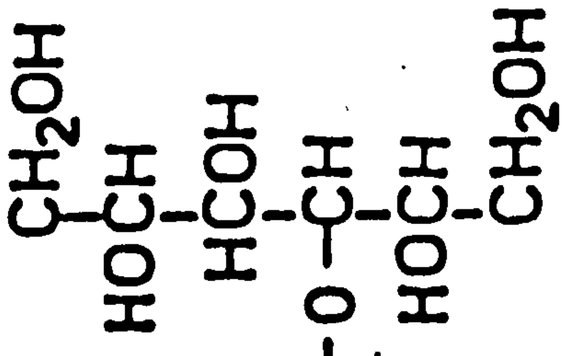
This effect of polyhydroxyl compounds may not be quite as simple as it has been described, as the structure of the polyhydroxyl compound may play some part in effective stabilisation of enzymes in "wet" systems. Thus Fujita et al, (1982) reported that inositol was more effective than sorbitol in stabilising lysozyme in aqueous solutions. Both compounds contain six hydroxyl groups, but inositol is cyclic in structure whereas sorbitol is linear, fig 41. The interaction of polyhydroxyl compounds with water promotes a change in the molecular structure of water. Inositol was reported to have a

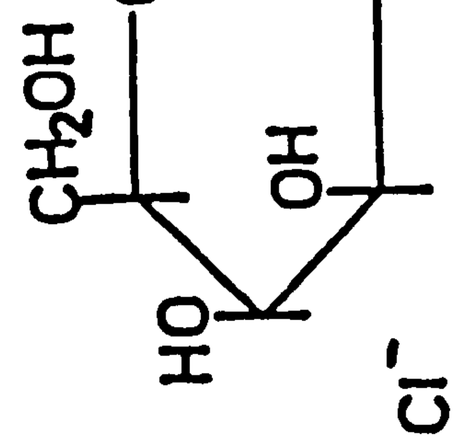
FIG.41 MOLECULAR STRUCTURES of STABILISERS.

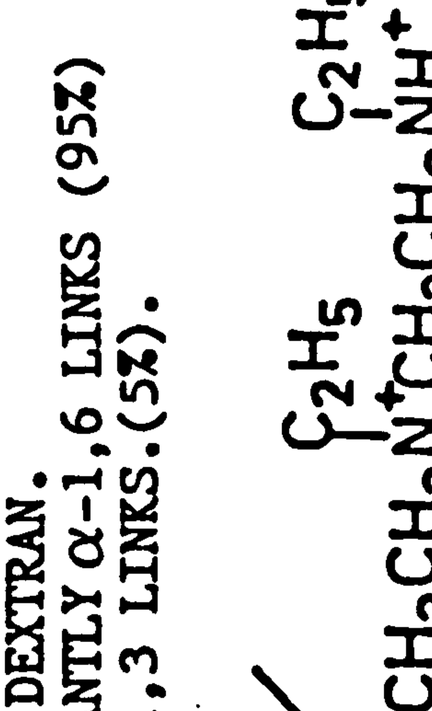

SORBITOL

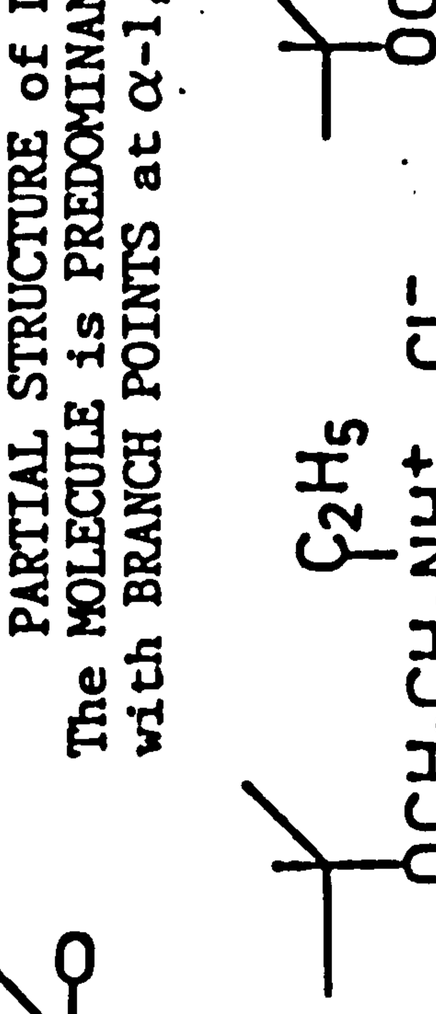

MANNITOL

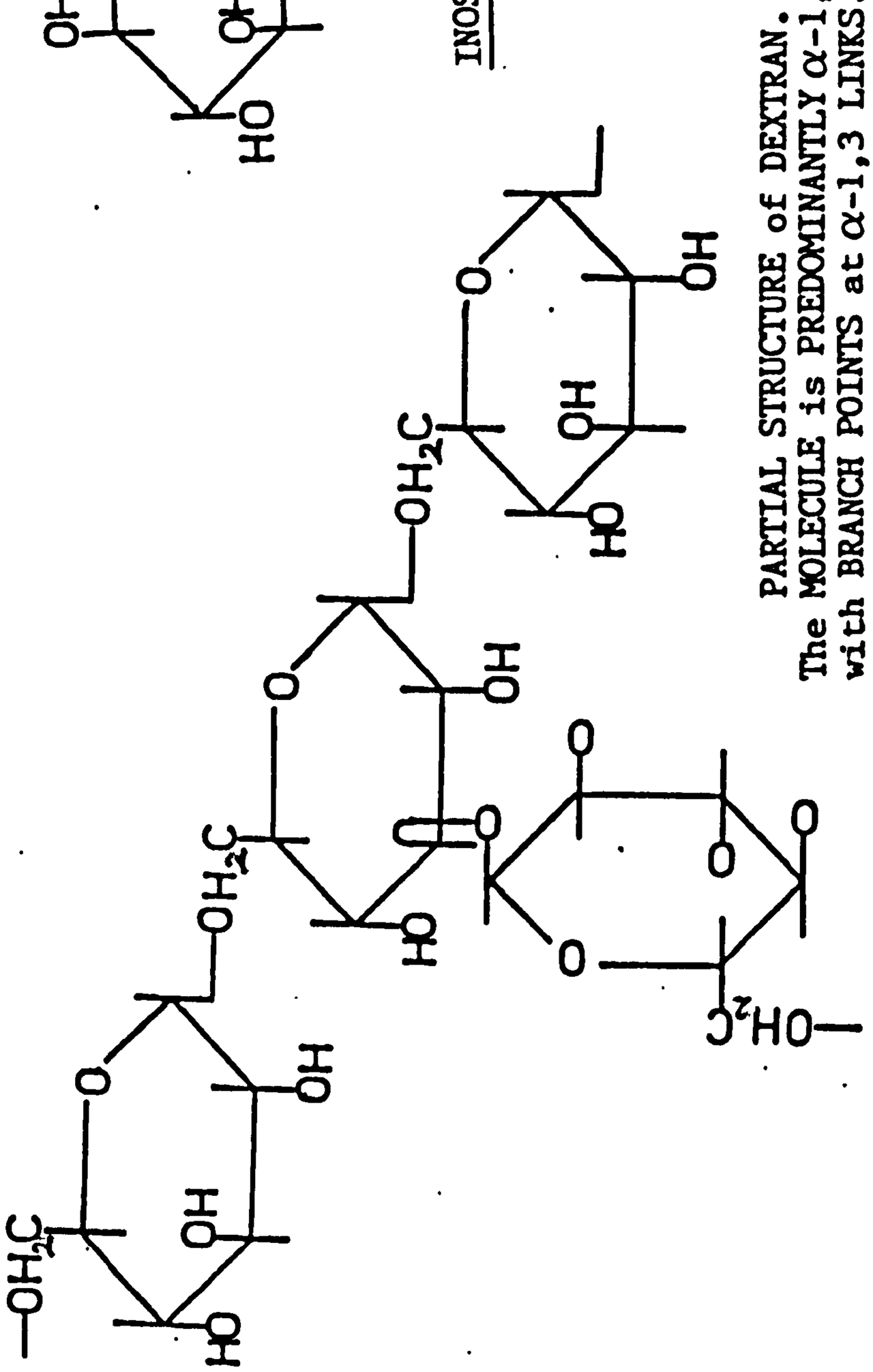

INOSITOL


LACTITOL


PARTIAL STRUCTURE of DEXTRAN. The MOLECULE is PREDOMINANTLY α-1,6 LINKS (95%) with BRANCH POINTS at α-1,3 LINKS.(5%).


THE TWO GROUPINGS of DEAE DEXTRAN. SUBSTITUTED on the 2 POSITION of the GLUCOSE RING.


DEXTRAN SULPHATE. 2 POSITION SUBSTITUTED.



larger structure-making effect than sorbitol, which accounted for the greater stabilisation effect of this compound.

Similarly Ye et al, (1988) reported that glucose oxidase was stabilised in solution by a variety of compounds which included polyhydroxyls, (xylitol being the most effective), polyethylene glycols, (PEGs) and inorganic salts, all of which showed the ability to affect the structure of water by making it more "organised". This effect was not purely dependent on the hydroxyl content of the additive used, as PEG molecules only have two free hydroxyls and inorganic salts have none, yet both gave stabilisation effects. Also solutions of glucose oxidase in D_2O, which was reported to be a more structured solvent than water, showed enhanced thermal stability when compared to aqueous solutions.

The conclusion that was drawn for the stabilisation of protein structure in aqueous solvent systems was as follows: the type of protein, the hydrophilic or hydrophobic character and thus the subsequent interaction of the protein with, (i) water and, (ii) the additives themselves, all play an important part in the stabilisation process.

Aqueous stability of enzymes was not of great interest to this work, so no exhaustive stability trials were conducted on aqueous enzyme solutions. However in short trials using alcohol oxidase, both sorbitol and lactitol showed a distinct effect on the "wet" stability of the enzyme, graph 35. Lactitol showed a greater stabilisation effect than sorbitol. This was thought to be due to the larger number of hydroxyl groups present, (fig 41) and consequently the interaction of the molecule with water should be of a greater magnitude than that of sorbitol. Lactitol may also interact with the enzyme

in some way, as an enhanced stabilisation effect was noticed on drying the enzyme with lactitol, but not with sorbitol, see below and graph 39.

It was also noticed that using cholesterol oxidase and cholesterol esterase, sorbitol appeared to stabilise aqueous solutions more effectively than lactitol. This possibly reflects some sort of specific interaction of the enzymes with sorbitol.

Dry Stabilisaton. General.

The preparation of dry enzymes is of great commercial use, in that dry systems are generally much more stable and consequently much easier to store. The increase in stability associated with drying of proteins may be predicted from the understanding of the interaction of water with the protein molecules. As mentioned earlier reducing the interaction of water with proteins effectively stabilises them by strengthening the intermolecular forces associated with protein structure. The removal of water from a protein should therefore have much the same effect as addition of polyhydroxyls to an aqueous solution. Generally this is found to be true. Removal of water, particularly by techniques that do so under conditions of low temperature, such as freeze drying, often result in very high enzyme activity retention in the dry product produced, which remains for extended periods of storage, (Potthast 1978).

The removal of water from purified enzymes by similar techniques is usually accompanied by some loss in activity, probably due to denaturation of the protein molecule. However, the activity remaining is usually quite stable to thermal denaturation and may be stored for extended periods of time with minimal loss of activity. The product from the drying procedure used is almost always

associated with water molecules which are "bound" to the protein. Such "bound" water appears to be vital for the activity to be preserved, presumably by stabilising the structure of the protein into the configuration required for activity. The losses of activity noticed on drying may therefore be associated with removal of "bound" water, with the end result of producing dentured, inactive protein. This effect has been noticed in many cases, (Hellman et al 1983, Carpenter et al 1986, Crowe et al 1987).

Addition of sugars and other agents to enzyme solutions before the drying step usually enhances the stability, which is reflected in the retention of activity of the enzyme, (Phillips 1985, Kishore 1985, Crowe et al 1987, Roser 1987, Hopkins 1988). This area of additive inclusion to stabilise proteins in the dry state is a large and growing field, particularly in the patent literature, for reviews see Barker (1978) and Schmid (1979).

Dry Stabilisation of Alcohol Oxidase.

All previous literature reporting the production of dry stable preparations of alcohol oxidase was based on the enzyme isolated from Pichia pastoris. Additives such as mannitol and other polyhydroxyl compounds were reported to stabilise the enzyme in a dry chemistry format, (Phillips 1985). Also algin, (sodium alginate) has been used, (Adams 1988). Alternative non-carbohydrate stabilisers selected from the group; peroxidase, catalase, cytochrome C or myoglobin have been claimed, (Hopkins 1988). In all reported cases the enzyme / stabiliser mixture was dried as a liquid preparation by water removal in an air stream or under low pressure conditions using a vacuum oven. Freeze drying was not used.

Attempts to reproduce the stabilisation using mannitol, sorbitol,

peroxidase or alginate with Hansenula alcohol oxidase met with limited success, graphs 39 and 45. The enzyme was vacuum dried as described in section 2.J.1. The test system with peroxidase, (100 fold excess) was not plotted graphically. The alcohol oxidase retained 50% activity after 1 day at 37°C decreasing to 37.3% after 9 days incubation. This approximates to the values for unstabilised enzyme over the same time interval, graph 36. Inositol, however, was found to stabilise the enzyme considerably, graph 39.

From these results it became clear that Hansenula alcohol oxidase differed in some respects to Pichia alcohol oxidase and a more comprehensive survey of potential stabilising compounds was neccesary. These are listed in table 11.

Effective Stabilisation of Hansenula Alcohol Oxidase.

It was thought initially that a soluble DEAE-derivatised polymer might produce a similar stabilising effect on the enzyme as that seen using DEAE-Sepharose. When DEAE-dextran was tested, however, the stabilising effect was insignificant as compared to an unstabilised control, graph 36. Various other polyhydroxyl compounds were tried including sugars, sugar alcohols, neutral polymers, cationic and anionic polymers. Various degrees of success were found using these compounds, graphs 36-45.

Monosaccharides were unsuitable, possibly due to their reducing action, graph 37.

Disaccharides were found to be generally effective particularly trehalose and cellobiose, graph 38.

Sugar alcohols partially stabilised in most cases, with the exception of inositol and sorbitol. The former conferred high stability, the latter destabilised the enzyme, graph 39.

Dextrans tended to stabilise quite well, graphs 40 and 41, whereas synthetic polymers tended to have little effect, graphs 42 and 43.

Other charged polymers had various effects when tested, graphs 44 and 45.

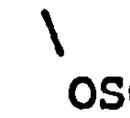
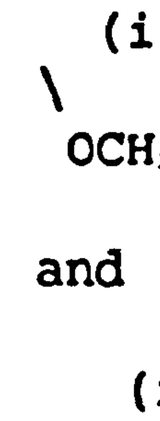
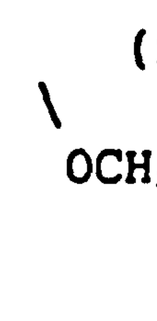
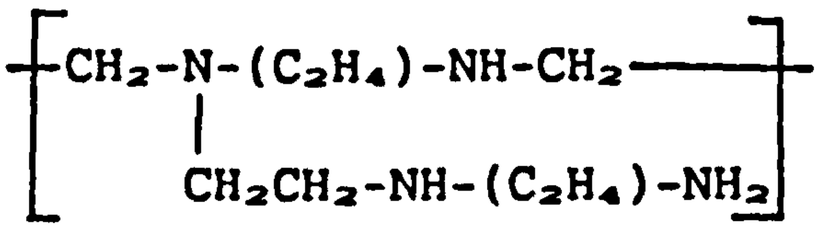
The most unexpected discovery was found when combinations of charged polymers and sugar alcohols or sugars were tested. The combination of DEAE-dextran and lactitol produced a stabilisation effect exceeding 100%. Graph 36 shows activity up to 16 days incubation at 37°C. Further incubation of such preparations has been carried out. No loss of activity was found up to 2 months incubation at 37°C and only 15-20% loss on 11.5 months incubation at 37°C. Other combinations of cationic or anionic polymers and polyhydroxyls have been tested, graphs 46 and 47, but as yet a thorough detailed study has not been carried out. In most cases tested to date, effective stabilisation with respect to blank values has been noticed.

As may be expected in dealing with charged species the effectiveness on the stabilisation seen is dependent of the pH of the buffer system in which the enzyme is dried, graph 48. This effect is probably due to the ionisation changes of the protein rather than the polymers used, as in most cases the pKa values of the charged species on the polymer surface are outside of the range of buffer conditions tested, thus the polymer groups remain ionised at all times. Table 35 lists the structure and the pKa values of the polymers used.

<u>Possible Mechanism. Role of the Charged Polymer.</u>

The overall surface charge of a protein is determined by the pH of the buffer environment in which it is dissolved and the isoelectric point or pI of the protein. For buffers whose pH is above

Table 35. Charged Groups in Cationic and Anionic Polymers

Structure	Group	Polymer	pKa
$-NH_3^+ Cl^-$	Amino	Chitosan	7 - 8
$-COO^- Na^+$	Carboxyl	Alginic acid	3 - 3.5
$-O-CH_2COO^- Na^+$	Carboxymethyl	CM cellulose	3.5 - 4
	Sulphate	Dextran sulphate	> 2.0
 and 	(3 Group Types) Diethyl amino ethyl (DEAE)	DEAE dextran	(i) 9.2 (ii) 14.0 (iii) 5.5
	Polyethylene - imine	Overall value	8.9 - 9.5

3 Amino group types in ratio.

(i) Primary	1
(ii) Secondary	2
(iii) Tertiary	1

the pI, the protein becomes negatively charged overall and similarly, for buffers whose pH is below the pI the overall charge is positive. This effect forms the basis of protein separation by ion exchange chromatography, (Pharmacia Technical Booklet, Ion Exchange Chromatography).

For alcohol oxidase the pI is about 5.7-6.2 depending on the source of the enzyme, so at pH's above this, the enzyme is negatively charged overall and interacts electrostatically with the DEAE groups on the soluble polymer. This interaction may be demonstrated to occur by adding DEAE-dextran to alcohol oxidase solution in low ionic strength, (20mM) phosphate buffer at pH 7.0, when a precipitate forms. Raising the ionic strength above 100-200mM produces a clear solution. The electrostatic interaction of the negatively charged protein and the positively charged polymer produces ionic complex formation and subsequent aggregation, causing precipitation to occur. Increasing the ionic strength weakens the electrostatic interaction, dissociates the ionic complexes and resolubilisation occurs. This ionic interaction is analogous to the precipitation of certain lipoproteins using polyanions, (Burstein et al 1970). Similarly precipitation of negatively charged nucleic acids using polycations, such as protamine, is another example of electrostatic interactions inducing ionic complexes. Many such ionic interactions occur with biological molecules including proteins and nucleic acids. This area of polyelectrolyte interaction with macromolecules has been reviewed by Elbein (1974).

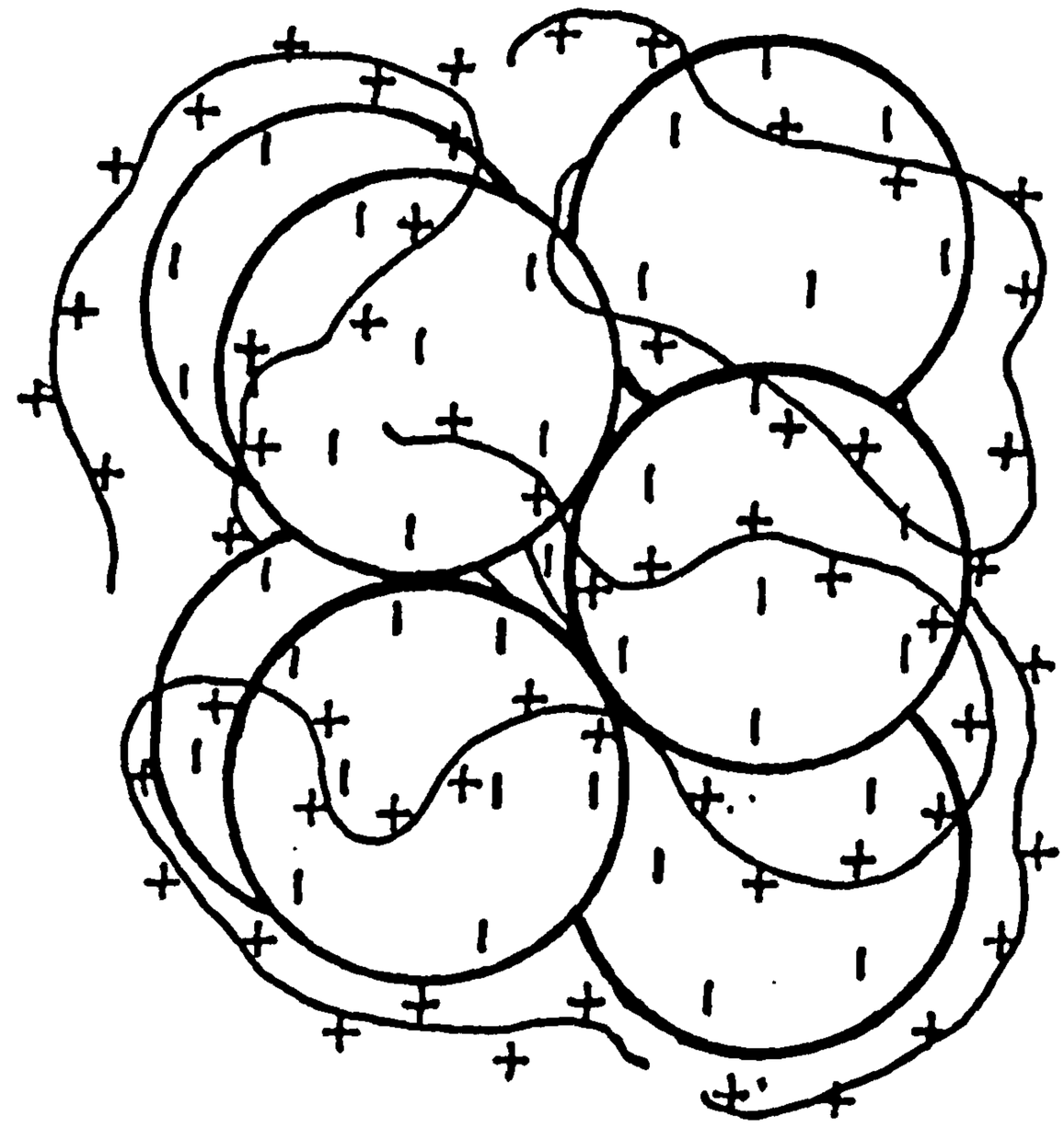
The increase in ionic strength of the medium affects the nature of the structure of the charged polymers in solution, (Pharmacia Technical Booklet on Dextrans). At low ionic strengths the charges

interact minimally with counter ions in solution and therefore repel one another strongly, forming long chain like molecules. Addition of such a molecule to a protein solution would favour electrostatic crosslinking and subsequent aggregation and precipitation. At higher ionic strengths the charged groups dynamically interact with ions in solution and the polymer chain tends to be more random in nature, (Katchalsky 1964).

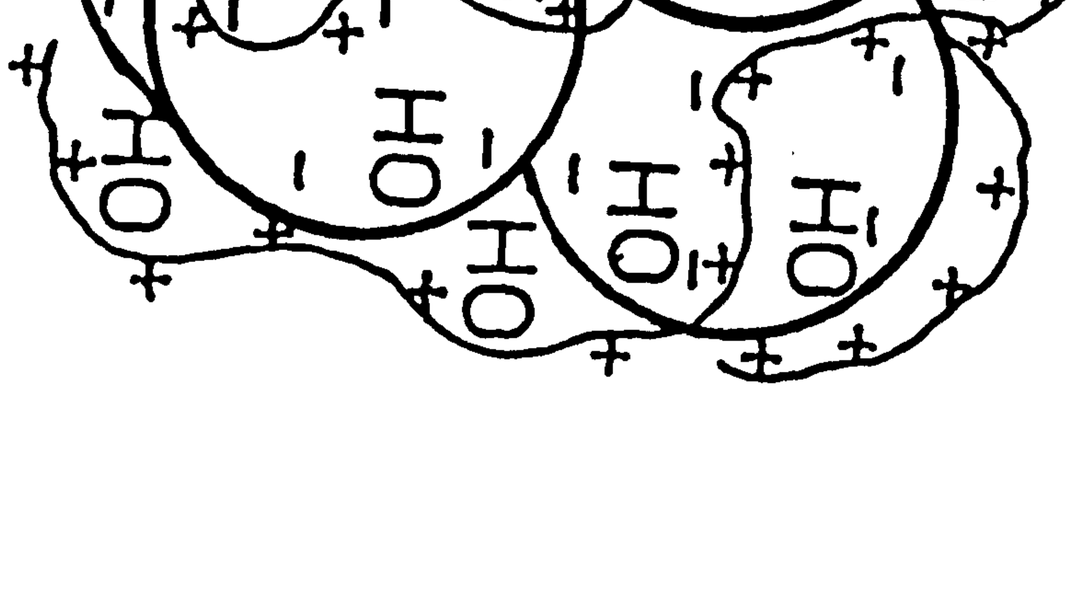
In such solutions, interaction of the charged polymer with the protein would tend to be limited and formation of large electrostatically bound aggregates would be less favourable, thus no precipitation is seen. Where a precipitate has been formed at low ionic strength, increasing the ionic strength causes redissolution to take place, probably due to the smaller molecular shape of the polymer and the dynamic interaction of counter ions in solution on the protein-polyelectrolyte complex. This is similar to the release of electrostatically bound proteins on ion exchange gels by exposure to high salt media. However, it would be expected some electrostatic interaction between charged polymer and protein will take place, even at higher ionic strengths, as multiple positive groups on the surface of the polymer will associate with multiple negative charges on the protein surface.

It is postulated that the soluble alcohol oxidase becomes enveloped or "caged in" by one or a number of positively charged polymer molecules, fig 42. The type of interaction which is suggested here has been observed to occur at the surface of ultrafiltration membranes where combinations of enzyme and polyelectrolyte appear to form a type of polymeric network similar to that of used in gel entrapment techniques, (Gianfreda et al 1989 and

FIG.42 ALCOHOL OXIDASE. INTERACTION of POLYELECTROLYTES/POLYHYDROXYLS.



(a) ALCOHOL OXIDASE with DEAE-DEXTRAN.



(b) ALCOHOL OXIDASE with DEAE-DEXTRAN/POLYHYDROXYL.

The diagrams above represent the postulated interaction of Alcohol Oxidase with (a) DEAE -Dextran and (b) the same interaction in the presence of polyhydroxyl compounds. The structure of the enzyme was taken from Woodward 1990.

section 1.G.6). Drying the enzyme in this state, however, gradually removes the aqueous environment necessary for such electrostatic interactions to take place with the subsequent effect that no real stabilisation of the enzyme is seen, graph 36.

Possible Mechanism. Role of Polyhydroxyl Compounds.

Polyhydroxyl compounds when dried with enzymes tend to stabilise activity. This is thought to be due to the hydroxyl groups holding or substituting for the "bound" water which is necessary for the retention of the tertiary structure of the protein and the subsequent activity of the molecule, (Roser 1987). Similarly removal of the carbohydrate, (polyhydroxyl) side chains of certain fungal enzymes rendered them susceptible to the effects of dehydration, in that enzyme activity was lost compared to untreated controls with intact carbohydrate side chains. The water - carbohydrate interaction was thought to be necessary for stability of the enzymes, (Darbyshire 1974). When drying a protein in the presence of polyhydroxyl compounds which tend to interact with and order the structure of water, it may be envisaged that the molecules may "coat" the surface of the protein with a layer or layers of polyhydroxyl - water complexes. The polyhydroxyl compounds used are usually small molecules and as such, will probably penetrate into the protein structure.

Such infiltration of the protein structure and subsequent drying may account for the increase in stability with certain small polyhydroxyl molecules such as inositol. The efficiency of this proposed process may depend on molecular size, molecular shape, charge and chemical properties of the molecule. This may account for the fact that linear polyhydroxyl compounds, (e.g. sorbitol,

mannitol) do not stabilise to the same extent as the cyclic molecule of inositol.

If the association of the polyelectrolyte with the enzyme occurs as suggested, then it may be reasonable to assume that the protein molecule is held by a fairly rigid electrostatic interaction, allowing a greater degree of infiltration of the polyhydroxyl compounds present in solution, fig 42. Drying such a mixture produces a high stabilisation effect on the enzyme. This may reflect on a more efficient "layering" of the polyhydroxyl compounds within the three dimensional structure of the protein molecule, which is able to take place as a result of the molecule being "anchored" to some extent by the polyelectrolyte. (This effectively replaces the "free" water within the protein structure). Dehydration of the protein-polyelectrolyte-polyhydroxyl-water complex so formed, effectively removes the electrostatic interaction between the protein and polyelectrolyte as described before, but now the protein is packed internally with polyhydroxyl compounds and retains a stable configuration.

If this mechanistic theory is correct, at least in part, then other polyelectrolyte / polyhydroxyl compound combinations should also stabilise alcohol oxidase. This appears to be the case, graphs 46 and 47.

<u>Stabilisation of Other Enzymes.</u>

This type of electrostatic interaction of enzymes with charged molecules is not restricted to alcohol oxidase. Many enzymes are purified by ion exchange chromatography and therefore it would be expected that they will interact with soluble polyelectrolytes also. If this is the case, it may be expected that stabilisation of the

enzyme structure and consequent retention of activity in the dry enzyme may be achieved.

The effect of four stabilisers on the activity of dried Pichia alcohol oxidase is shown in graph 50. Stabilisation was observed in all cases, however it would seem that in the case of this enzyme, sucrose was found to be a more efficient stabiliser. Vacuum drying is less effective than freeze drying in the case of the Pichia enzyme graph 57, whereas the converse is true for the Hansenula enzyme. Such results indicate the two enzymes though similar are not identical in structure or biochemistry.

Testing several enzymes using various combinations of polyelectrolytes and polyhydroxyl compounds gave the data recorded in table 18. In the majority of cases a positive stabilisation effect on enzyme activity was observed, indicating a general effect of the stabilisers on a variety of enzymes.

This principle of stabilisation using combinations of polyelectrolytes and polyhydroxyl compounds would appear to apply to a large range of enzymes. Recent work indicates that immunoglobulins are also stabilised with retention of antigenic properties.

Further work to discover the nature of the electrostatic interactions with respect to a larger range of charged polymers of known structure and properties, and the use of purified proteins with well defined properties would be of great interest. An investigation of the effect of structure, charge, molecular shape, hydroxyl content and water interaction of polyhydroxyl compounds would also be necessary to give understanding of dry enzyme stabilisation and its mechanism of action. Understanding the mechanism of stabilisation would enable confident prediction of which stabiliser combination to

use with which protein/enzyme system.

8.D. Dry Phase Enzyme Detection Systems.

Graduated Tests.

Effective stabilisation of the enzyme component of any analytical system is a great advantage in the production and storage of such a system. In the area of analysis the basic requirement is to detect and quantify the analyte of interest. Dry chemistry tests where all the necessary reactants are present in a dry, ready to use format usually meet this need by some sort of comparison method, where an unknown response is related to responses given from calibrated values, thus giving the concentration of analyte in the original sample.

Comparison by eye of a colour produced by the unknown against a colour chart, has been used frequently in this type of dry test, (e.g. pH papers, Merck inorganic test papers, glucose dipsticks, etc). This method is particularly open to operator error and depending on the colour response of the test and the colour vision of the operator, may be of no use whatever, (e.g. in cases of colour blindness). Reflectometers, which measure reflected light from the surface of the developed test strips and electronically interpret the result to give a measured concentration, are now available for use. However, they are expensive or limited to one or two particular tests. They do give accurate results when used with care and the range of analytes being estimated using these machines is growing quickly. Alcohol and other analytes may be estimated in a dry phase format using such methods, (sections 6.B.1 and 6.D.1) and linear reflectance plots for ethanol, uric acid, glucose, cholesterol and hydrogen peroxide in dry test cards have been constructed as examples

of the feasibility of such a system, graphs 52 and 54-57.

Threshold Systems.

Alternative dry phase systems using chemical or enzymic techniques to produce a definite positive signal or a definite negative signal have been devised during this work. The development of this method has been reported in chapter 6, and the principle is illustrated in figs 29 and 31-33.

The method was originally thought to work by means of a competition reaction between the mediator present and the colour reagents. This explanation appears to be basically correct in the light of several recent experiments carried out, (see below) but also a direct effect on the dye produced has been observed in some cases. This type of effect has also been observed in a similar threshold generating system using different mediator substances, (Hochstrasser 1977, Palmer and Timmerman 1989).

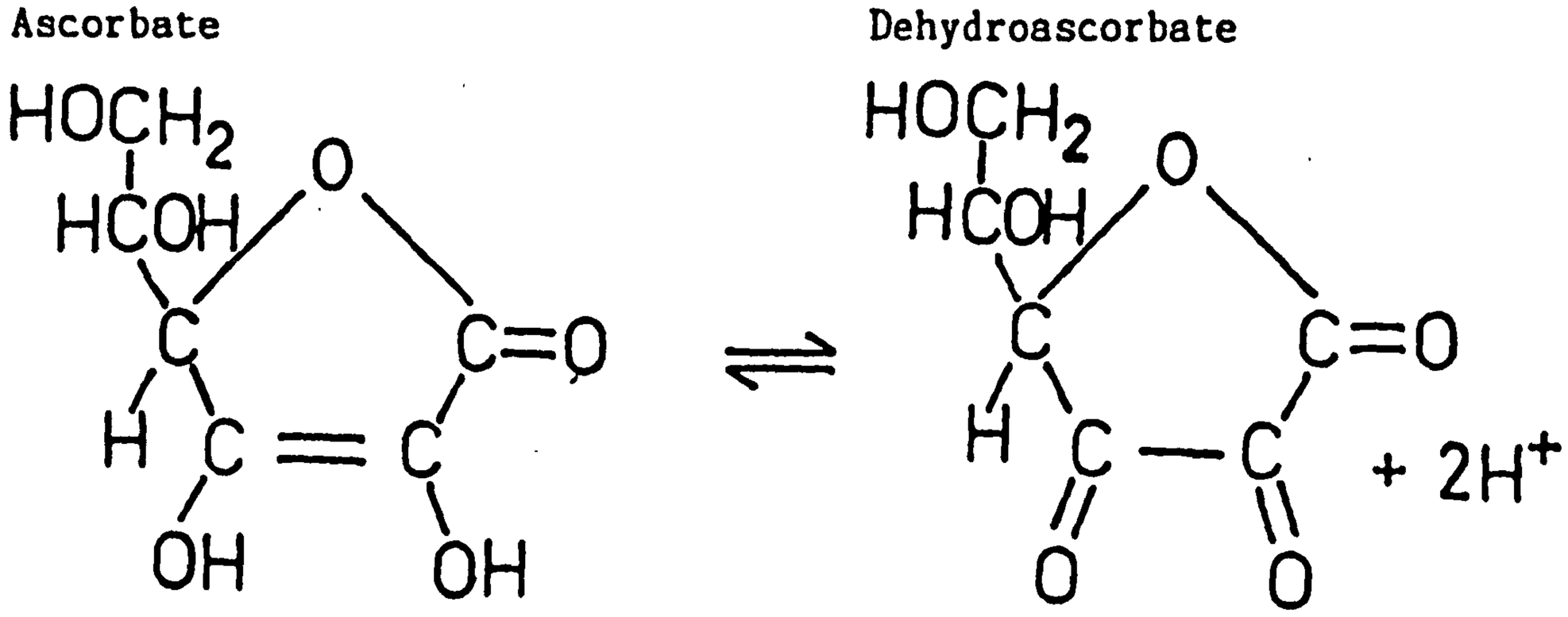
The mediators used in generating colour thresholds are listed in table 22. All the compounds listed that are effective in generating a colour threshold response have a fairly high reducing capacity.

In the case of sulphydryl compounds and ascorbate this may be seen as the capacity to donate protons, fig 43. Inorganic sulphites and related salts are strong reducing agents in solution. The chemistry is fairly complex, with several different ionic species being present in solution, (Cotton and Wilkinson 1966).

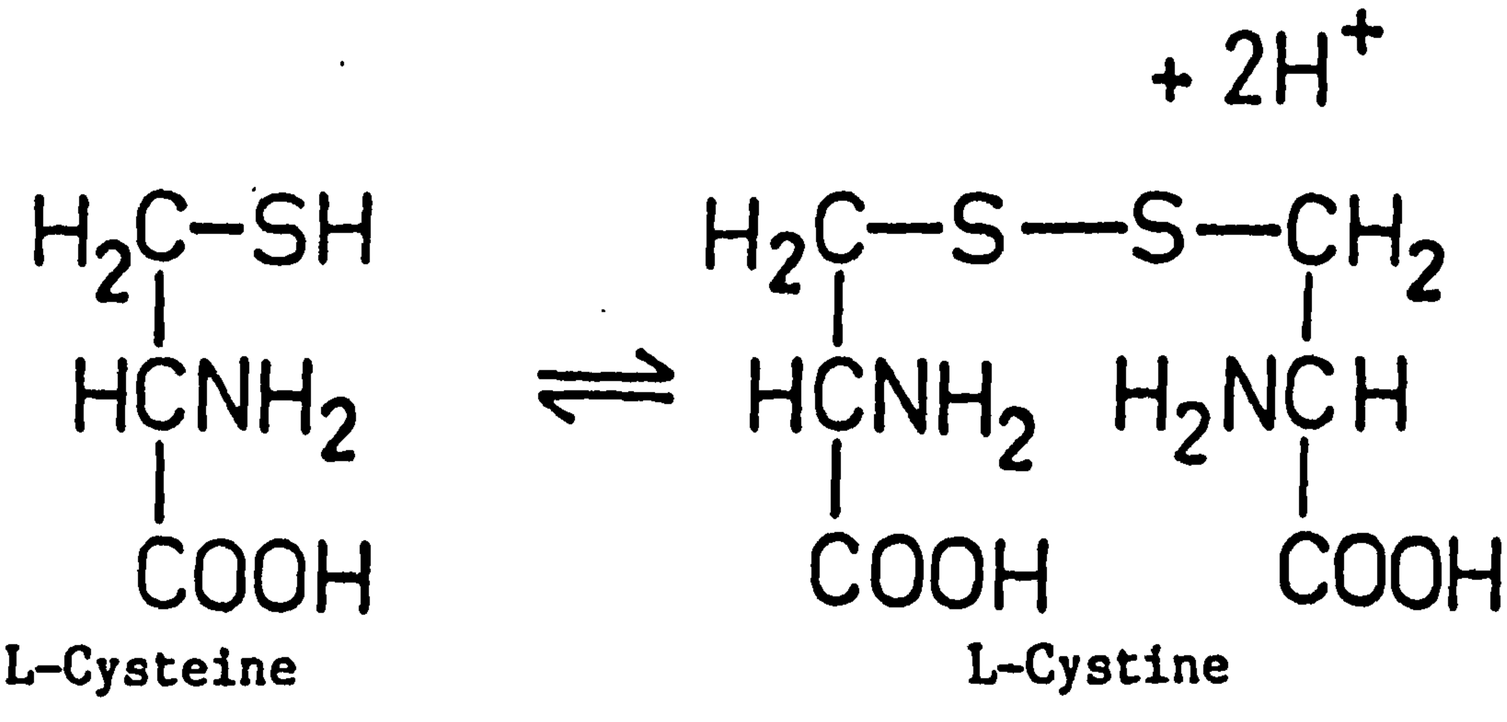
Probable Mechanism for Colour Threshold Generation.

Based on laboratory observations and reference to the literature it would seem that at least four interacting effects occur in generating a threshold response.

FIG.43 REACTIONS of MEDIATORS.



The reaction of ascorbate shown above involves the donation of protons with the subsequent formation of Dehydroascorbate. This type of reducing reaction was utilised in the generation of colour thresholds in dry phase tests,chapter 6.



The reaction of sulphydryl compounds is illustrated by that of cysteine. This type of reaction was the same for other sulphydryl compounds such as dithiothreitol, glutathione, etc.

The reaction generates protons with the subsequent formation of a disulphide bridge.

(i) Direct Chemical Interaction of Mediator with Peroxide.

This has been demonstrated in the laboratory recently by incubation of mediators, particularly sulphydryl containing compounds, with hydrogen peroxide solutions. Residual estimation of mediator concentrations, using a specific assay procedure for sulphydryl groups, (Ellman et al 1961) indicated a direct chemical reaction had taken place. When cysteine was tested, a fine white precipitate of cystine was formed, (The Merck Index, nº 2775). This confirmed the findings of Randell (1964), where thiourea and dithiouracil were shown to react with hydrogen peroxide.

(ii) Enzymatic Enhancement of Mediation / Peroxide Reaction.

Thio compounds were also shown by Randell to be substrates in the peroxidase catalysed breakdown of hydrogen peroxide. The basal rate of peroxide removal due to chemical interaction could be stimulated by adding peroxidase to the system. This type of reaction was also reported by Stonier and Yang (1972), where glutathione was oxidised rapidly by peroxidase in the presence of manganese ions and dichlorophenol. Non-enzymatic reduction of glutathione was minimal in this system.

Olsen and Davis (1976) reported that dithiothreitol was oxidised in the presence of peroxidase and hydrogen peroxide and Brooks (1983) also reported that dithiothreitol was oxidised using peroxidase and hydrogen peroxide and that this reaction was stimulated by phenols and hydroxamates.

This type of reaction has been demonstrated with cysteine, cysteine ethyl ester, sodium 2-mercaptoethane sulphonate and dithioerythritol. The rate of reaction of mediators and hydrogen peroxide in the presence of peroxidase was approximately twice the

rate when no enzyme was present.

(iii) Mediators as Substrates for Peroxidase in the Absence of Hydrogen Peroxide.

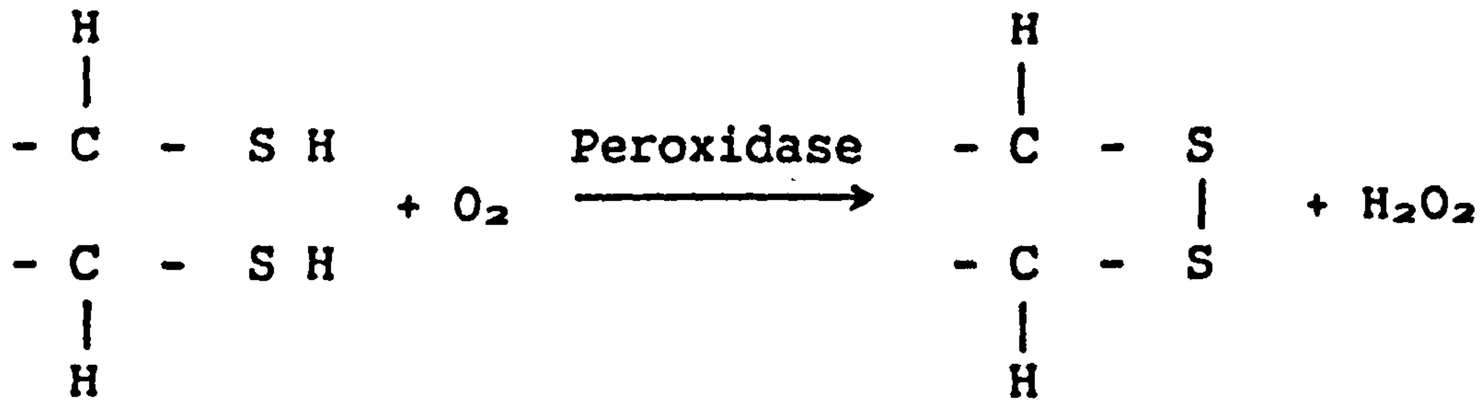
Peroxidase will react with dithioerythritol, cysteine and glutathione in an oxygen consuming reaction in the absence of hydrogen peroxide, (Olsen and Davis 1976). This has been recently confirmed using an oxygen electrode with peroxidase in phosphate buffer. Several mediators were tested and the results are shown in table 36.

Table 36 . Reaction of Mediators with Oxygen Catalysed by Peroxidase.

Mediator.	Reaction Rate μMole.O_2.min^{-1}.
Cysteine	16.07
Cysteine ethyl ester	36.54
Cysteamine	10.08
Ascorbic acid	0
Dithioerythritol	11.34
Glutathione	1.58
Sodium mercaptoethane sulphonate	2.21
Thiourea	0
2-Thiouracil	0

1. Peroxidase concentration constant at 10U.ml^{-1}.
2. Mediator concentrations constant at 1mg.ml^{-1}.

Sulphydryl compounds were proposed to produce hydrogen peroxide in an oxidase type reaction, (Olsen and Davies 1976) shown in the following reaction sequence.



The production of hydrogen peroxide in this system was likened to the intial reaction of dihydroxyfumaric acid with peroxidase. Direct evidence for the production of hydrogen peroxide in this reaction has not been reported, however stoichiometric measurements using dithiothreitol as substrate indicates an initial 1:1 ratio of dithiothreitol oxidised to oxygen consumed. This ratio changes as the reaction proceeds probably due to the reaction of the peroxide produced with dithiothreitol as described previously, section 8.D.ii and Olsen and Davis (1976).

(iv). Direct Reaction of the Mediator with the Dye Molecules.

Appendix II lists over 200 compounds used to form dye molecules with 4 different developers. The dyes produced were treated with both cysteine, (5mg.ml^{-1}) and ascorbate, (1.0mg.ml^{-1}). Direct reaction of the mediators with the dye molecules, shown by decolourisation of the dye, was seen in several cases.

It was noted that particular combinations of couplers and developers were more susceptible to decolourisation than others, e.g. dyes formed from 4-AAP and anilines show particular susceptibility to decolourisation, whereas MBTH and anilines are very resistant to decolourisation.

Redox dyes which are not listed in appendix II also exhibit rapid decolourisation with the mediators used. The choice of dye precursors will influence the participation of the dye molecule in the overall

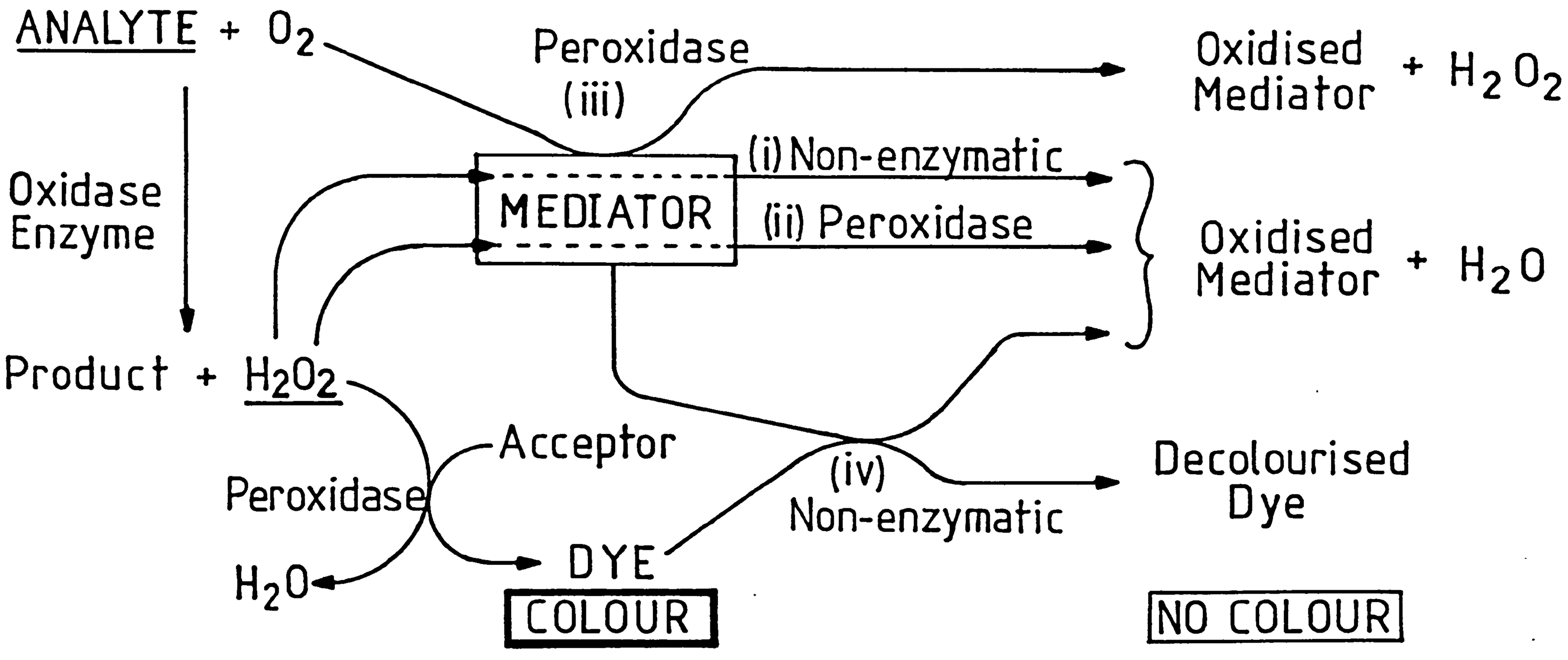
threshold generation reaction.

The overall mechanism of threshold generation would appear to be quite complex depending on the mediators, dyes and corresponding interaction of the enzymes present. The reactions thought to be occurring are summarised in fig 44. Not all mediators tested take part in all the reactions shown, e.g. ascorbate does not appear to react with oxygen in the presence of peroxidase and resistant dye molecules do not directly react with mediator.

The direct reaction of mediators with oxygen introduces a competition reaction for the available oxygen present, as the oxidase enzyme also requires oxygen as a substrate. Avoidance of the use of mediators which react with oxygen may increase the efficiency and rapidity of the oxidase reaction and promote a more rapid response of the complete system, thus lowering incubation times of dry phase threshold tests.

Also it has been noticed that ascorbate and sodium mercaptoethane sulphonate mediate efficiently at lower final concentrations than other mediators such as cysteine. This may be explained by, (a) little or no reaction with oxygen and peroxidase, (b) a more rapid reaction with hydrogen peroxide and, (c) the solubility of the molecules. Point (a) is a fact, (table 36), point (b) may be true, experimental work is in progress to verify this possibility and point (c) is very likely. Cysteine does not dissolve easily, whereas both ascorbate and sodium mercaptoethane sulphonate dissolve very rapidly. In a support matrix certain effects were noticed in the threshold system using buffers of various solubilities, chapter 6, section 6.B.4. It was found that MOPS was a more effective buffer in the dry phase tests than phosphate. This was thought to be due to its

FIG.44 OVERVIEW of THRESHOLD GENERATION.



The diagram above represents an overall series of reactions believed to be taking place in the generation of the threshold response in dry phase tests. Reaction (i) occurred with the majority of the mediators used and was potentiated in the presence of peroxidase, reaction (ii). Reaction (iii) has been reported to occur with thiol, (sulphydryl compounds). This has been confirmed in the laboratory, table 41. Direct bleaching of the dye, reaction (iv) was seen in many cases and was thought to be a function of the dye molecule, see appendices II and III.

superior solubility, which enabled more rapid dissolution and subsequent pH control.

It would be reasonable to suppose that a mediator of higher solubility would dissolve more rapidly on application of the sample to be analysed, and thus would be more effective in generating a threshold response than one of lower solubility, because of the higher initial concentration of mediator in the developing test.

Effect of Microporous Overlay.

The main effect noticed using overlays such as ethyl cellulose or cellulose acetate was a decrease in the development time of the test. This was especially noticeable using microcrystalline cellulose as the support for threshold generated tests and is probably due to an increased ratio of oxygen to hydrated support.

This explanation is based upon the observation that developed tests having a microporous overlay show colour only at the upper surface of the support where the sample was applied, indicating that only the surface layers of the test became hydrated. Consequently the reaction volume is smaller and the relative amount of oxygen available to the system is increased.

Also the microporous layer may hinder the passage of large macromolecules into the test which tend to inhibit the reaction, (e.g. proteins present in saliva). Certainly such a layer excludes cells, (Phillips 1985) so oral bacteria would be excluded from the test.

Application of the Threshold System.

In practice two types of threshold test format have been produced, figs 18a and 18b. The completed tests are shown developed in figs 34 and 35. The major area of difficulty in the development

of these tests was in the regulation of a metered dose of sample to the reactive element of the system. Too large a sample volume caused a leaching effect of the soluble components of the system and also an oxygen depletion effect. Too small a sample does not solubilise the components sufficiently for reaction to take place.

The first format of Alcocard, (fig 34) was very susceptible to overloading with sample. Some protection was afforded using absorbent card, but saliva tended to "sit" on the upper surface of the card and prevent colour development. This was probably due to oxygen starvation of the oxidase reaction, because when the sample was wiped off, very rapid colour development took place. This occurred at ethanol levels below the pre-set values. To overcome this problem the second format incorporated a thin, (10 μ) porous polycarbonate membrane such as nucleopore, to deliver a set sample volume to the enzyme cards. Also an extra set concentration level was added and the colour reagent was changed to produce a dye which was more resistant to decolourisation, fig 35.

The stability, reproducibility and speed of the second test format is as yet unsuitable for a commercial product. The area of difficulty was found to be mainly due to aged preparation of the alcohol oxidase used in the card. This is currently being rectified and a series of trials of a modified format of Alcocard 2 will soon be made.

Also mass production of this format, using filter paper strips as supports for the mediated enzyme reaction is impractical. Direct deposition of the enzyme cocktail onto a particulate support material is under investigation as a viable alternative to the original method.

Threshold tests are currently being developed for cholesterol, HDL cholesterol, lactate and glucose. Such tests will form a range of simple, user friendly assays, the results of which may be interpreted rapidly without the use of sophisticated instrumentation.

8.E. Diacetyl Analysis and Diacetyl Reductases.

The original connection of diacetyl analysis and the development of stabilised enzyme based analyses was from the estimation of both alcohol and diacetyl in beer samples. The occurrence of diacetyl and diketones in beer is required by law to conform to minimum allowable levels, therefore the analysis of diacetyl is very important in this industry. The various methodologies are discussed in chapter 7 and the final conclusions drawn from the various analytical formats using diacetyl reductase are that:-

(i) The enzyme method is not specific but measures total diketones in the sample.

(ii) The enzyme method is a viable method if it is presented with suitable aqueous samples. Beer and other complex mixtures of compounds contain substances which interfere with the enzyme assay.

(iii) The enzyme recycling system is quite sensitive enough to estimate the low concentration of diketones normally present in beer.

(iv) The method is reproducible and applicable to multiple samples if care is taken with the analysis.

The major problem associated with the enzymic assay technique is that of providing a suitable aqueous sample, largely free from interfering contaminants of the enzyme reaction. This has been overcome for G.L.C. methods by sampling the headspace of the sample, diacetyl and related diketones being volatile in nature. During a recent conference, (ANABIOTECH '90) a technique was disclosed to

selectively dialyse diacetyl from the gas phase of a sample into a smaller volume of carrier liquid, using special membranes. This had the effect of concentrating the sample and also it produces an aqueous solution free from the major contaminant species. Subsequent analysis of the dialysate was by F.I.A. techniques using diacetyl reductase.

The example shown in section 7.D.1, using the dialysis probe to produce samples was analogous to the above procedure, however the dialysis was performed in the aqueous phase which allows other non-volatile small molecules to dialyse and also the sample obtained was diluted rather than concentrated. These two effects render the aqueous dialysis method unsuitable for beer samples.

Diacetyl Reductase.

The preparation of the various diacetyl reductases and the subsequent evaluation of the enzymes, alongside the published results from other workers revealed several interesting observations concerning diacetyl reducing enzymes in general.

They would all appear to be diketone reductases accepting a variety of uncharged dicarbonyl compounds as substrates using NADH or NADPH or both NADH and NADPH as cofactors. The molecular weights of the enzymes range from Mr 10,000 for the E.Coli enzyme, (Silber et al 1974) up to Mr 120,000 for the enzyme from Bacillus Polymyxa, (Ui et al 1987). Liver enzymes were between Mr 78,000 for beef, (Provecho et al 1984) and Mr 110,000 for pigeon, (Diez et al 1974). The larger molecular weight enzymes are all reported to consist of subunits around Mr 23-28,000, however the enzymes differ from one another in the number of subunits present.

The enzymes from S. Lactis, A. Aerogenes, pigeon liver and

hamster liver are all reported to be tetramers, whilst the enzymes from beef liver and chicken liver appear to be trimers. The enzyme from beef liver is found largely associated with subcellular particles, (Martin and Burgos 1970). Sedimentation studies and activity estimation on the chicken liver enzyme also indicated that the enzyme was associated with subcellular particles. The pigeon liver enzyme is, however, reported to be predominantly cytosolic in nature. Perhaps the structural differences noticed in terms of subunit composition are related in some way to their association or lack of association with subcellular particles. This possibility may only be resolved by further work on various diacetyl reductases to confirm their association with particulate material and the molecular subunit composition.

8.F. Conclusions.

The overall nature of the work reported here leads to yet more questions that remain to be answered. The continuation of the work is probably best centred on two main areas: (a) The understanding of the molecular basis for the stabilisation of proteins and other biological materials with a view to efficiently predict a regime of stabilisation for any given biological molecule and, (b) The continuing development of novel detection systems for detection and quantitation of analytes of particular interest.

Enzyme based tests remain a very important area of analysis, yet many enzymes which have been reported in the literature have not been exploited, particularly in the analytical field. The use of new techniques to produce stabilised enzyme based diagnostics is necessary to expand the range of analysis and also to simplify existing technology. For progress to be made in any field it is not

enough merely to know about something, it is necessary to apply the knowledge to make it work in practice. The work reported here has attempted to do just that.

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APPENDIX I

AMMONIUM SULPHATE SATURATION TABLE.

AMMONIUM SULPHATE SATURATION TABLE.

Final Concentration of Ammonium Sulphate, % Saturation at 0 °C

Initial Concentration of Ammonium Sulphate, % Saturation at 0 °C	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Grammes of Solid Ammonium Sulphate to Add to 100ml of Solution.																
0	10.6	13.4	16.4	19.4	22.6	25.8	29.1	32.6	36.1	39.8	43.6	47.6	51.6	55.9	60.3	65.0	69.7
5	7.9	10.8	13.7	16.6	19.7	22.9	26.2	29.6	33.1	36.8	40.5	44.4	48.4	52.6	57.0	61.5	66.2
10	5.3	8.1	10.9	13.9	16.9	20.0	23.3	26.6	30.1	33.7	37.4	41.2	45.2	49.3	53.6	58.1	62.7
15	2.6	5.4	8.2	11.1	14.1	17.2	20.4	23.7	27.1	30.6	34.3	38.1	42.0	46.0	50.3	54.7	59.2
20	0	2.7	5.5	8.3	11.3	14.3	17.5	20.7	24.1	27.6	31.2	34.9	38.7	42.7	46.9	51.2	55.7
25		0	2.7	5.6	8.4	11.5	14.6	17.9	21.1	24.5	28.0	31.7	35.5	39.5	43.6	47.8	52.2
30		0	2.8	5.6	8.6	11.7	14.8	18.1	21.4	24.9	28.5	32.3	36.2	40.2	44.5	48.8	
35			0	2.8	5.7	8.7	11.8	15.1	18.4	21.8	25.4	29.1	32.9	36.9	41.0	45.3	
40				0	2.9	5.8	8.9	12.0	15.3	18.7	22.2	25.8	29.6	33.5	37.6	41.8	
45					0	2.9	5.9	9.0	12.3	15.6	19.0	22.6	26.3	30.2	34.2	38.3	
50						0	3.0	6.0	9.2	12.5	15.9	19.4	23.0	26.8	30.8	34.8	
55							0	3.0	6.1	9.3	12.7	16.1	19.7	23.5	27.3	31.3	
60								0	3.1	6.2	9.5	12.9	16.4	20.1	23.9	27.9	
65									0	3.1	6.3	9.7	13.2	16.8	20.5	24.4	
70										0	3.2	6.5	9.9	13.4	17.1	20.9	
75											0	3.2	6.6	10.1	13.7	17.4	
80												0	3.3	6.7	10.3	13.9	
85													0	3.4	6.8	10.5	
90														0	3.4	7.0	
95															0	3.5	
100																0	

APPENDIX II

DEVELOPER / COLOUR COUPLER OXIDATIVE CONDENSATION REACTIONS.

Key to symbols.

P	Pink	Pl	Purple
R	Red	Gr	Grey
V	Violet	Br	Brown
G	Green	Bg	Beige
B	Blue	Gd	Gold
O	Orange	Sg	Sage Green
S	Sand	N	No Colour
Y	Yellow		
T	Turquoise		
L	Lilac		

dp	Deep		
vdp	Very Deep		
t	Tinge	+	No Decolourisation
pl	Pale	+-	Partial Decolourisation
dt	Dirty	-	Decolourisation
bt	Bright		
ppt	Precipitate		

The appendix is arranged by class of compound with the names of compounds in the first column.Subsequent columns show the colour produced with four different developers given below.The columns are divided into three,the first letters show the colour produced according to the symbols given.The two following symbols indicate the dyes susceptibility to decolourisation by (a) cysteine (C) 5.0 $mg.ml^{-1}$ and (b) ascorbate (A) 1.0 $mg.ml^{-1}$.

DEVELOPERS.

4 - AAP	4 - Aminoantipyrine
MBTH	3 - Methyl Benzothiazolinone Hydrazone
NNPD	N,N Diethyl 1,4 Phenylene Diamine
TAP	2,4,5,6 Tetraamino Pyrimidine

PHENOLS.

Coupler	4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
3-Hydroxy 2,4,6-Tribromo Benzoic Acid	dpR	+-	+-	N	-	-	BG	-	-	TB	-	-
3-Hydroxy Benzoic Acid	OR	-	-	tP	+	-	plGrB	+-	+-	tYG	+	+
4-Hydroxy Benzoic Acid	P	-	-	tP	+	-	PlVGr	-	-	tYG	-	-
Guaiacol Sulphonic Acid K Salt	R	-	-	plO	+	-	B	-	-	tGr	plO	plO
3,5-Dichloro 4-Hydroxy Benzoic Acid	PR	-	-	OBrppt	+	+	VGr	plB	plB	plBG	-	-
3-Choro 4-Hydroxy Benzoic Acid	dpGrP	-	-	P	+	+	VGr	+-	+-	plBG	plO	plO
3,5-Dibromo 4-Hydroxy Benzoic Acid	dtP	-	-	OBrppt	+	+	GrV	plB	plB	plBGppt	+-	+-
3,5-Dichloro 2-Hydroxy Benzene Sulphonic Acid	dpVR	-	-	PPl	-	+-	dpBG	+-	+-	GB	Y	Y
2,4-Xylenol	tBg	-	-	plGrVppt	+	+	BV	-	-	tGY	-	-
2,6-Xylenol	plR	-	-	Oppt	+	+	V	-	-	GrB	-	-
3,5-Xylenol	plP	-	-	RO	+-	+-	plV	-	-	YG	-	-
3,5-Dihydroxy Benzoic Acid	GrP	-	+-	Oppt	+	+	Gr	+-	+-	btO	RO	+-
4,5-Dihydroxy 1,3-Benzene Disulphonic Acid	plGrP	Y	+	tBg	+	+	S	+	+	plO	+-	+-
Methylene Bis(2-Hydroxy 5-Chloro Benzene Sulphonic Acid Na Salt)	plP	-	-	N	-	-	V	+-	+-	tG	-	-
2,3-Dihydroxy Bezaldehyde	SYppt	+	+	BrPlppt	+	+	dtSGBr	+-	+-	SY	+-	+-
Resorcinol Monoacetate	O	Y	+-	BrO	+-	+	plGrPl	+	+	OBr	+-	+-
3-Hydroxy Benzaldehyde	dtSg	+-	+-	dpBr	+-	+-	GGrBr	+-	+-	dtSg	+-	+-

PHENOLS.

Coupler		4 -AAP		MBTH		NNPD		TAP	
		C	A	C	A	C	A	C	A
Pyrogallol	Y	+	+	SO	+	+	plY	+-	+-	tBr	+-	+-
Catechol	GbrR	S	S	dtBrO	+	+	dtGrV	+-	+-	tBr	-	+-
Resorcinol	O	S	+-	O	+-	+-	dtGrPl	+	+	plPO	+-	+-
4,6-Dinitro O-Cresol	N	-	-	N	-	-	BrV	G	G	N	-	-
4-Chloro Resorcinol	BrO	+-	+-	RO	+-	+-	dtGrV	+-	+-	plR	+-	+-
4-Chloro Salicylic Acid	N	-	-	tG	-	-	GrPl	-	plB	tO	-	-
Vanillin	tGr	-	-	N	-	-	GrPl	-	-	N	-	-
2,3-Dihydroxy Benzoic Acid	GrR	-	-	plGrR	+-	-	GrV	-	-	GrG	-	-
2,5-Dihydroxy Benzoic Acid Na Salt	GrP	-	-	tP	-	-	GrPl	plSg	-	N	-	-
3,4-Dihydroxy Benzoic Acid	GBrR	+-	+-	plOBr	+-	+-	GrV	-	+-	tO	+-	+-
2,4-Dihydroxy Benzaldehyde	S	+-	+-	tP	-	-	GGr	+-	+-	plGrPlBr	+-	+-
2,6-Dihydroxy Benzoic Acid	SO	+-	+-	PGr	+-	+-	GrPl	+-	+-	plPO	+-	+-
2,4-Dihydroxy Bezophenone	GrBrRppt	-	-	tP	+	+	GrV	GrB	GrB	GrP	+-	+-
4-Bromo 3,5-Dihydroxy Benzoic Acid	GrP	-	+-	btO	+-	+-	PlGr	-	+	btR	+	+

ANILINES.

Coupler	4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
3-Dimethylamino Benzoic Acid	Pl	-	-	VB	+	+-	plG	-	-	tY	-	-
N,Phenyl Anthranilic Acid	tP	-	-	TB	+-	+-	GrV	-	-	tY	-	-
4-(Dimethylamino) Benzene Sulphonic Acid	N	-	-	V	+	+	RVGr	-	-	tY	-	-
N,Ethyl N,Sulphopropyl 3-Toluidine	dpV	-	-	dtGrPl	+	+	btG	-	-	plG	-	-
N,N ,Bis(2-Hydroxyethyl) Aniline	V	-	-	dpBV	+	+	dpG	-	-	plBG	-	-
N,N ,Bis(2-Hydroxyethyl) 3-Toluidine	dpV	-	-	vdpVB	+	+	BG	-	-	plGB	-	-
N,Benzyl Aniline	plP	-	-	dpV	+	+	dtPl	tS	tS	tY	-	-
4-(N,N,Dimethylamino)2-Hydroxy Benzaldehyde	S	Y	+	S	+	+	dtGrPl	SGr	SGr	plP	+-	+-
N,Ethyl N,2-Hydroxyethyl Aniline	BV	-	-	vdpV	+-	+-	G	-	-	plG	-	-
N,Methyl N,2-Hydroxyethyl Aniline	V	-	-	dpV	+	+	BG	-	-	plG	-	-
N,N,Bis(2-Hydroxyethyl)3-Chloro Aniline	tPl	-	-	V	+	+	GrV	-	-	N	-	-
N,Benzyl N,Ethyl Aniline	tP	-	-	plB	+	+	PR	-	-	N	-	-
N,2-Cyanoethyl N,Ethyl Aniline	plV	-	-	V	+	+	GrV	-	-	tG	-	-
N,Phenyl Ethylenediamine	tY	-	-	plL	+	+	GrV	tS	tS	N	-	-
N,Phenyl Ethanolamine	VP	+-	-	dpPl	+	+	GB	-	-	plG	-	-
N,Ethyl N,2-Hydroxyethyl 3-Toluidine	dpV	-	-	Pl	+	+	plG	-	-	btG	-	-
2-Amino 4-Methyl Benzoic Acid	V	-	-	GrRPl	+-(ppt)	+-	dpGB	-	-	N	-	-
N,2-Hydroxyethyl N,2-Hydroxysulphopropyl Aniline	L	+-	-	dpBV	+	+	BG	-	-	plG	-	-

ANILINES.

Coupler	4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
N,2-Aminoethyl N,2-Hydroxysulphopropyl Aniline	plPlR	-	-	GrV	+	+	GrGB	+-	+-	N	-	-
N,n-Butyl Aniline	Pl	+-	-	Pl	+-	+-	plBG	-	-	Y	-	-
N,N,Dibutoxyethyl Anilne	plPlP	-	-	Pl	+-	+-	plGrPl	-	-	Y	-	-
N,Acetoxyethyl N,2-Cyanoethyl Anilne	tO	-	-	VB	+	+	GrP	-	-	Y	-	-
N,Benzyl N,Ethyl 3-Toluidine	Pl	-	-	BV	+	+	GrP	-	-	Y	-	-
N,n-Butyl N,2-Hydroxysulphopropyl Aniline	dpVB	+-	-	G	plB	-	plG	-	-	plGY	-	-
3-Aminobenzene Sulphonic Acid	tO	-	-	Pl	+	+	PR	-	-	Y	-	-
3-Chloro Aniline	tP	-	-	LPl	+-	+-	PlP	-	-	Y	-	-
Diphenylamine 4-Sulphonic Acid Na Salt	tGd	-	-	plGY	+	+	RVGr	-	-	tY	-	-
2-Amino Benzene Sulphonic Acid	tP	-	-	Plppt	+	+	PPl	-	-	N	-	-
Anthranilic Acid	Pl	-	-	Pl	+	+	TB	-	-	tY	-	-
2-Amino 3,5-Dimethyl Benzene Sulphonic Acid	N	-	-	tY	+	+	RPl	-	-	N	-	-
2-Amino Benzene 1,4-Disulphonic Acid	tY	-	-	tO	+-	+-	GrPl	-	-	tY	-	-
N,N Diethyl Aniline 3-Sulphonic Acid	plPlP	-	-	plB	+-	+-	GS	-	-	N	-	-
4-Dimethylamino Benzoic Acid	V	-	-	plLV	-	-	PGr	tS	-	plG	-	-

NAPHTHOLS.

Coupler		4 -AAP		MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
1-Naphthol 4-Sulphonic Acid	R	-	+-	dpBrppt	+	+	btB	+-	+-	dpGrppt	RS	S
1-Naphthol 5-Sulphonic Acid	VR	-	-	PR	+	+	dpGB	+-	+-	GGd	+-	+
1-Naphthol 3,6-Disulphonic Acid	V	-	+	RV	+	+-	plGr	-	-	btY	+-	+
1-Hydroxy 2-Naphthoic Acid	P	-	+-	Vppt	+	+	dtVB	-	+-	Y	+-	+-
4-Chloro 1-Naphthol	R	+-	+-	dpR	+-	+-	LB	+-	+-	dpB	plB	plB
1,5-Dihydroxy Naphthalene	R	+	+	dtGrVR	+	+	GrB	+	+	Sppt	+-	+-
1,6-Dihydroxy Naphthalene	GrBrP	+-	+	dtBrR	+	+	dpGrB	+	+	dtSgppt	+-	+-
1,7-Dihydroxy Naphthalene	plVP	+-	+	dtV	+	+	GrB	+	+-	plSg	dtY	dtY
3-Hydroxy 2-Naphthoic Acid	plG	-	-	dtP	+	-	plGrPl	-	-	Y	+-	+-
4,5 Dihydroxynaphthalene 2,7-Disulphonic Acid	GrG	-	-	vdpPl	+	+	N	-	-	Gd	+-	+-
2-Hydroxynaphthalene 6,8-Disulphonic Acid Na Salt	BGr	-	+	PR	-	-	VP	-	-	Y	+-	+-
2-Hydroxynaphthalene 6-Sulphonic Acid Na Salt	GBGr	-	-	RV	+	+	BrP	-	-	Gd	+-	+-
2-Hydroxynaphthalene 3,6-Disulphonic Acid Na Salt	tY	-	-	N	-	-	GrRV	-	-	Y	+-	+-
2,3,4-Trichloro 1-Naphthol	PlGr	-	-	P	+-	+-	BGppt	-	-	dtBGppt	Pl(ppt)	dtGr
BCF 1089 (Kodak)	tP	-	-	plG	P	-	T	+	+	Y	-	-
1,3-Dihydroxy Naphthalene	GrBr	S	S	S	+	+-	GrBr	+-	+-	OBr	+-	+-
2,6-Dihydroxy Naphthalene	BG	+-	+-	tP	-	-	GrG	+-	+-	tS	+-	+-

AMINO-NAPHTHALENES.

Coupler	4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
1-Aminonaphthalene 8-Sulphonic Acid	S	+-	+-	V	+-	+-ppt	BGr	+-	+-	GY	+-	+-
1-Aminonaphthalene 5-Sulphonic Acid	RBr	-	-	dpV	+	+	Gr	+-	+-	Y	+-	+-
1-Aminonaphthalene 7-Sulphonic Acid	O	-	P	Plppt	+(ppt)+		dtB	-	-	Y	+-	+-
1,8 Diamino Naphthalene	tY	-	-	plG	-	-	PV	-	-	N	-	-
1,5 Diamino Naphthalene	Br	-	+-	BV	+-	-	dtV	-	-	S	+	+
N,Phenyl 1-Naphthylamine	plO	+-	P	S	+	+	dtPV	-	-	Y	+-	+-
2-Aminonaphthalene 4,8-Disulphonic Acid	plP	-	-	V	+-	-	VR	-	-	Y	+-	+-
2-Aminonaphthalene 6-Sulphonic Acid	tY	plP	-	OBrppt	+(ppt)+		P	+-	+-	Y	+-	+-
2-Aminonaphthalene 1-Sulphonic Acid Na Salt	tGY	-	-	plVPppt	+(ppt)+		dtV	plG	Gr	Y	+-	+-
2-Aminonaphthalene 6,8-Disulphonic Acid	tO	-	-	V	+-	-	dtV	-	-	Y	+-	+-
4-Aminonaphthalene 1-Sulphonic Acid Na Salt	S	-	+-	dtVppt	+(ppt)+		dtGrV	+-	+-	S	+-	+-
1-Dimethylamino Naphthalene 5-Sulphonic Acid	GrR	+-	+-	BGr	+-	+-	Br	+-	+-	tY	-	-

AMINO-PHENOLS.

Coupler	4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
6-Amino 2,4-Dimethyl Phenol	RBr	Y	Gd	Br	+	+	Gppt	+(ppt)+		SO	RS	+-
4-Acetamido Phenol	tY	-	-	VB	+-	+-	VR	-	-	Y	+-	+-
6-Amino 3-Cresol	Y	+-	+-	OBr	Br	+	G	Y	-	O	RO	+-
3-Amino Phenol HCl	SO	+-	+-	dpR	+	+	dtGrB	+-	+-	dpRn	+-	+-
2,6-Dichloro 4-Amino Phenol	GrG	S	GrBr	GrPl	+	+	S	+-	+-	GrY	+-	+-
2-Amino 4-Cresol	O	Y	+-	S	+	+	GrG	+-	+-	dtP	+-	+-
4-Amino Salicylic Acid	O	+-	+-	LPlppt	+(ppt)+		GrV	Gr	Gr	GrP	+-	+-
3-Dimethylamino Phenol	R	tO	+-	RBr	+	+	B	-	-	vdpRV	+	+
3-Diethylamino Phenol	RP	tY	+-	R	S	+-	VB	plBGr	plBGr	vdpRV	+	+
2-Dimethyl 1-Aminomethyl 4-Xylenol HCl	R	-	-	SOBr	+	+	plB	-	-	plG	Y	Y
2-Aminophenol N,N,Dimethyl 4-Sulphonamide	dtSGd	+	+	BrO	+-	+-	Gr	+-	+-	PY	+-	+-
3-Dimethylamino Anisole	dpPl	-	-	plPl	+-	+	btG	-	-	G	-	-
3-Hydroxy Diphenylamine	dtRGr	+-	+-	GrRV	+	+	GrB	+-	+-	vdpV	+-	+-
3-Amino 4-Hydroxy Benzene Sulphonic Acid	YO	+-	+-	BrO	+	+-	dtGrSg	+-	+-	plO	+	+
2-Aminophenol 4-Sulphonic Acid	YO	+-	+-	RO	+-	+-	dtGGr	+-	+-	O	+-	+-
4-Dimethylamino Phenol Sulphate	dtRBr	SGr	SGr	plGrBr	+-	-	Gr	+-	+-	GrR	-	-

AMINO-NAPHTHOLS.

Coupler	4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
1-Amino 7-Naphthol	Gr	+-	+-	BV	+	+	dtGrV	+-	+-	plGrBr	+-	+-
1-Amino 2-Hydroxynaphthalene 4-Sulphonic Acid	Y	+-	+-	dtP	+	Br	dtP	Gr	Gr	S pldtP		+
1-Amino 8-Naphthol 4-Sulphonic Acid	GrPO	-	-	dtplVB	+-	+-	PlR	plGrB	plGrB	dtGd	+	+
2-Amino 3-Hydroxy Naphthalene	BrGppt	S	+-	S	Br	+	dtBGr	dtG	+	GrB	+-	+-
4-Amino 3-Hydroxynaphthalene 1-Sulphonic Acid	Gd	+-	+	GrV	+	+	dtS	+-	+-	BrO	R	+
5-Amino 2-Naphthol 6-Sulphonic Acid	OBr	Y	P	BrPlppt	+	+	plBG	plY	-	plYG	+-	+-
6-Amino 1-Naphthol 3-Sulphonic Acid Na Salt	Br	Y	+-	SBr	+-	+-	plGrGB	plGr	plGr	dtBrY	+	+
8-Amino 1-Naphthol 3,6-Disulphonic Acid	GrSg	GY	+-	GrV	+	+-	S	-	-	btO	+	+
8-Amino 1-Naphthol 5-Sulphonic Acid	BGr	GrG	GrBr	dpdtBGr	+	+	dpGB	Sg	Sg	GBr	+-	+-
8-Amino 1-Naphthol 5,7-Disulphonic Acid	dpPl	+-	+-	dpPl	+	+	dpGB	Sg	plB	OGd	+-	+-
1-Amino 4-Naphthol	dtRGrppt	S(ppt)	+-	plGrP	+-	+-	dtBGr	+-	+-	GrBr	+-	+-

DIAMINES.

Coupler	4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
2,6-Diamino Toluene	R	plO	tO	OR	+-	+	btPlB	-	-	dpBG	Y	Y
2,4-Diamino Phenol	dpBr	+-	+-	GrRBr	+-	+-	dpBr	+-	+	dpBr	+-	+-
4-Chloro 1,2-Phenylene Diamine	dtSg	+	+	dpBr	+-	+-	GrGBr	+-	+-	dtSg	+-	+-
1,2-Phenylene Diamine HCl	dpGrG	+	+-	dpRPl	+-	+-	plBGr	+-	-	dtY	+	+
3-Acetamido Aniline HCl	dpVR	-	-	dpRV	+	+	GB	-	-	tG	-	-
3,4-Diamino Toluene	O	Gd	Y	dtY	+	+	tY	+	+	btYGd	+	+
4-Amino Acetanilide	tGrO	+-	-	dtGrP	+	+	dtPl	-	-	Y	-	-
1,3-Phenylene Diamine HCl	OR	+-	+-	btO	+-	+	dpB	-	-	BG	Y	Y
2,5 Diamino Toluene Sulphate	BrGr	S	S	dpVR	+-	+-	plGr	S	S	GrY	-	-
3,4-Diamino Benzoic Acid	tO	-	-	SY	+	+	dtVGr	GGr	GGr	tG	+-	+-
3,5-Diamino Benzoic Acid	dpV	-	+-	btORppt	+	+	GrB	P	P	btGdY	+	+
1,4-Phenylene Diamine Sulphonic Acid	GdBr	+-	+-	SgGr	Y	PR	B	GBrY	GBrY	Y	-	-
Tetramethyl Diamino Benzophenone	tY	-	-	N	-	-	GrRV	tS	-	tY	-	-
N,N,Diethyl 1,3-Phenylene Diamine HCl	dpR	+-	+-	plPlP	+	+	dpB	-	-	GrB	-	-
2,4-Diamino Toluene	dpOR	+	+-	dtP	+-	+	dpB	-	-	vdpBG	Y	Y

ANILIDES.

Coupler	4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
α-Benzoyl Acetanilide	tYppt	+	(ppt)+	plGY	+	+	OY	+	+	Y	+	+
Acet 3-Toluidide	tY	-	-	N	-	-	PR	-	-	N	-	-
Acet 2-Toluidide	tY	-	-	tG	-	-	PR	-	-	N	-	-
Acet 4-Toluidide	tY	-	-	tG	-	-	PR	-	-	N	-	-
Carbanilide	tY	-	+	tG	-	-	PR	-	-	N	-	-
4-Nitro Acetanilide	tY	-	+	tG	-	-	PR	-	-	N	-	-
Acetanilide	tY	-	-	tG	-	-	PR	-	-	N	-	-
2-Chloro Acetanilide	tY	-	-	tG	-	-	PR	-	-	N	-	-
4-Bromo 2-Chloro Acetanilide	tY	-	-	tG	-	-	PR	-	-	N	-	-
Benzanilide	tY	-	-	tG	-	-	PR	-	-	N	-	-
4-Hydroxy Acetanilide	tY	-	-	dtBV	+-	-	PR	-	-	N	-	-
Benz 2-Toluidide	tY	-	-	N	-	-	PR	-	-	N	-	-
3-Nitro Acetanilide	tY	-	-	N	-	-	PR	-	-	N	-	-
3-Dimethylamino 4-Methoxy Acetanilide	plVR	+-	-	pldtL	-	-	GrGB	-	-	tGrG	-	-
N,N,Diethyl 3-Amino Acetanilide	dpRP	-	-	N	-	-	BG	-	-	G	-	-
Propionanilide	tY	-	-	N	-	-	PR	-	-	N	-	-
Aceto-Acet 4-Anisidide	tYG	-	-	plYG	+	+	Y	-	+	GY	-	-

AMINO-HETEROCYCLICS.

Coupler		4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
8-Amino Quinoline	OR	Y	+-	RV	+	+	plGr	-	-	S	+	+
Primaquine	BV	G	G	vdpR	+	+	dtGrG	YGr	YGr	O	+-	+-
2,6-Diamino Pyridine	OR	VP	+-	GrRV	+-	+-	T	-	-	TG	+-	+-
2-Amino 6-Methoxy Benzothiazole	tY	-	-	N	-	-	VR	Gr	Gr	N	-	-
2-Aminomethyl Benzimidazole DiHCl	plS	+-	+-	GrV	-	-	plPR	YGr	YGr	N	-	-
2-Amino Pyridine	tY	-	-	N	-	-	PR	-	-	N	-	-
2-Amino 4-Methyl Pyridine	tY	-	-	N	-	-	PR	-	-	N	-	-
2,6 Diacetylamino Pyridine	tY	-	-	N	-	-	PR	-	-	N	-	-
3,5-Diamino 1,2,4-Triazole	tGY	-	-	tG	+	+	PR	GrG	-	N	-	-

HYDROXY-HETEROCYCLICS

Coupler	4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
7-Iodo 8-Hydroxyquinoline 5-Sulphonic Acid	ORppt	+-	+-	plOBgppt	+	+	Pl	Gr	plBG	plGppt	-	-
8-Hydroxyquinoline 5-Sulphonic Acid	R	+-	+-	RBr	+	S	dpBG	YG	YG	dpS	O(ppt)O	
3-Hydroxy 2-Methyl 4-Pyrone	Y	-	-	btY	+	+	SY	tY	tY	N	-	-
2-Hydroxy Pyridine	tY	-	-	tG	+	+	PR	-	-	Y	-	-
3-Hydroxy Pyridine	BrO	Y	+-	N	-	-	GrV	+-	+-	SO	+-	-
3-Hydroxy γ-Pyrone	Y	+-	+	dtGSY	+-	+-	Tppt	+-(ppt)+		btY	+-	+-
1-(3-Dimethylaminopropyl) 3-Cyano 6-Hydroxy 4-Methyl Pyridine-2-one.	Pl	Y	+	dtBr	btO	btO	plB	-	-	Y	+	+
1-(3-Methoxypropyl) 3-Cyano 6-Hydroxy 4-Methyl Pyridine-2-one.	dpPl	+-	+-	plTB	L	tL	GrV	-	-	Yppt	+(ppt)+	

PYRAZOLONES.

Coupler	4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
1-Phenyl 3-Pyralozidinone (Phenidone)	tO	-	-	plPL	+	+	plGrG	-	-	tP	-	-
3-Methyl 1-Phenyl 2-Pyrazolin-5-one	PR	-	+	dpGd	+	+	dtGrLppt	+-	+-	Oppt	+(ppt)+	
2,3-Dimethyl 1-Phenyl 3-Pyrazolin-5-one	tY	-	tP	plGd	+-	+-	PR	Gr	Gr	N	-	-
3-Amino 1-Phenyl 2-Pyrazolin-5-one	Gd	tY	+	SY	+	+	dtGrVppt	+-	+	RO	+-	+-
1-(4-Nitrophenyl) 3-Methyl 2-Pyrazolin-5-one (CF 251)	P	+-	+-	plY	-	-	PlR	+-	+-	Gd	+-	+-
1-Phenyl 2,3-Dimethyl 4-Nitroso Pyrazolone	plG	+-	+	plSY	+-	+-	dtR	plGrY	plGrY	plGY	+-	+-
1-(4-Sulphophenyl) 3-Carboxy 5-Pyrazolone	PlR	-	+	btOGd	+	+	TB	-	-	dpVR	plBrP	plBr
1-Phenyl 3-Methyl 5-Chloro Pyrazol-2-Chloromethylate	tGGd	-	-	plGrY	+-	+-	P	-	-	Y	-	-
M-Sulphamido Pyrazolone	plOP	+-	+	Y	+	+	GrPl	-	-	SOppt	+(ppt)+	
Oxyethyl Sulpho Pyrazolone	plOP	+-	+	Y	+	+	GrPPl	-	-	plSO	+	+
P-Tolyl Methyl Pyrazolone	OP	+-	+	plY	+	+	GrRPl	+-	+-	Sppt	-(ppt)-	
(4-Sulphophenyl) Pyrazolone	plO	+	+-	Y	+	+	GrV	plY	plY	plO	+	+
(2,5-Dichloro,4-Sulphophenyl)Pyrazolone	plO	+-	+	plSY	+	+	btV	plY	plY	S	+-	+-
Phenyl Pyrazolone Carboxilic Acid Ethyl Ester	PR	+-	+-	N	-	-	VB	-	-	plBrSppt	+(ppt)+	
2-Chloro(5-Sulphophenyl) Pyrazolone	O	+-	+	plSY	+	+	V	plY	plY	SO	+-	+-
(2-Chlorophenyl) Pyrazolone	BrO	+-	+	plY	+	tP	btV	-	+-	O	+-	+
(3-Chlorophenyl) Pyrazolone	SO	+-	+	plS	+	+	RV	+-	+	Sppt	+(ppt)+	
(3-Sulphophenyl) Pyrazolone	BrS	+-	+	plY	+	+	dpV	Y	plGrY	SO	+-	+

PYRIMIDINES.

Coupler	4 -AAP			MBTH			NNPD			TAP		
		C	A		C	A		C	A		C	A
6-Hydroxy 2,4,5-Triamino Pyrimidine	N	-	-	Y	+	+	plL	+	+	N	-	-
2,4,6-Triamino Pyrimidine	plO	-	-	Gd	+	+	L	-	-	Yppt	+(ppt)	+
4,5,6-Triamino Pyrimidine	N	-	-	N	-	-	PR	-	-	N	-	-
2-Hydroxy Pyrimidine	tY	-	-	tG	-	-	PR	Gr	Gr	N	-	-
2-Amino Pyrimidine	tY	-	-	tG	-	-	VR	Gr	Gr	N	-	-
4,6 Diamino 2-Hydroxy Pyrimidine Sulphate	tP	-	-	Gd	+	+	GrV	S	S	N	-	-
5,6-Diamino 2,4-Dihydroxy Pyrimidine	N	-	-	plY	+-	+-	plL	+-	+	Y	-	-
2-Amino 4,6-Dimethyl Pyrimidine	tY	-	-	N	-	-	P	-	-	Y	+	+
2,4 Diamino 6-Hydroxy Pyrimidine	RV	+-	+-	tY	-	-	vdpB	plS	-	tY	-	-
4,6-Diamino 2-Thio Pyrimidine	tGY	-	-	N	-	-	btO	-	-	N	-	-

APPENDIX III

COLOUR REACTIONS using SUBSTITUTED PYRIMIDINES as COLOUR DEVELOPERS.

Key to Symbols as APPENDIX II

DEVELOPER. 2,5-DIAMINO 4,6-DIHYDROXY PYRIMIDINE.

COLOUR COUPLER		C	A
3-Dimethylamino Phenol	plRPl	-	+-
3-Diethylamino Phenol	GrP	GY	+-
6-Hydroxy 2,4,5-Triamino Pyrimidine SO_4	Pl	-	+
2,4,6-Triamino Pyrimidine	plPl	-	+
4,5,6-Triamino Pyrimidine SO_4	tY	+	+
2,6-Diamino Toluene	plYBr	Y	+-
2,6-Diamino Pyridine	PGr	Y	tGr
2,4-Diamino Phenol	dpBr	+	+
3-Acetamido Aniline	plPl	-	-
N,N,Diethyl 3-Amino Acetanilide HCl	N	-	-
1,3-Phenylene Diamine HCl	plBr	-	+-
2,4-Diamino Toluene	BrY	Y	+-
3,5-Diamino Benzoic Acid	plP	tY	+-
4,6-Diamino 2-Hydroxy Pyrimidine SO_4	plPPl	-	+-
3,5-Diamino 1,2,4-Triazole	plPl	-	+-
3-Dimethylamino Anisole	plPl	-	+-
3-Hydroxy Diphenylamine	plPl	-	+
Resorcinol	Gr	+-	+-

DEVELOPER. 5,6-DIAMINO 2,4-DIHYDROXY PYRIMIDINE.

COLOUR COUPLER		C	A
3-Dimethylamino Phenol	RPl	+-	+-
3-Diethylamino Phenol	RV	+-	+-
6-Hydroxy 2,4,5-Triamino Pyrimidine SO_4	N	-	-
2,4,6-Triamino Pyrimidine	Yppt	+(ppt)	+
4,5,6-Triamino Pyrimidine SO_4	N	-	-
2,6-Diamino Toluene	dtPlppt	+	+
2,6-Diamino Pyridine	RVppt	+	+
2,4-Diamino Phenol	Br	+	+
3-Acetamido Aniline	Y	-	-
N,N,Diethyl 3-Amino Acetanilide HCl	plB	-	-
1,3-Phenylene Diamine HCl	BrPlppt	V(ppt)	+
2,4-Diamino Toluene	Brppt	plBr(ppt)	+
3,5-Diamino Benzoic Acid	btY	+	+
4,6-Diamino 2-Hydroxy Pyrimidine SO_4	plY	-	-
3,5-Diamino 1,2,4-Triazole	plY	-	-
3-Dimethylamino Anisole	B	-	-
3-Hydroxy Diphenylamine	dtV	+-	+-
Resorcinol	O	+-	+-

DEVELOPER. 6-HYDROXY 2,4,5-TRIAMINO PYRIMIDINE.

COLOUR COUPLER		C	A
3-Dimethylamino Phenol	vdpPl	+-	+-
3-Diethylamino Phenol	vdpPl	+-	+-
6-Hydroxy 2,4,5-Triamino Pyrimidine SO_4	plP	-	-
2,4,6-Triamino Pyrimidine	Yppt	+	+
4,5,6-Triamino Pyrimidine SO_4	plP	-	-
2,6-Diamino Toluene	Plppt	+	+
2,6-Diamino Pyridine	btVppt	+	+
2,4-Diamino Phenol	dpBrppt	+	+
3-Acetamido Aniline	plBrP	-	-
N,N,Diethyl 3-Amino Acetanilide HCl	plB	plP	plP
1,3-Phenylene Diamine HCl	dtPlppt	+	+
2,4-Diamino Toluene	RPl	+-	+-
3,5-Diamino Benzoic Acid	dpOY	+-	+-
4,6-Diamino 2-Hydroxy Pyrimidine SO_4	plP	-	-
3,5-Diamino 1,2,4-Triazole	plY	-	-
3-Dimethylamino Anisole	vdpB	PlP	PlP
3-Hydroxy Diphenylamine	dpV	+-	+-
Resorcinol	dpR	+	+