The construction of enzyme-coenzyme conjugates: with a view to their use in enzyme electrodes

Phillips, R. O.

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in Enzyme Electrodes

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R O Phillips

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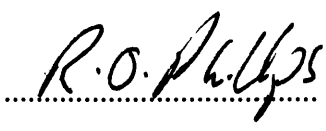

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SUMMARY

Recent improvements in biosensor technology have led to the development of novel enzyme electrodes. However, the potential application of many enzymes to be applied for use in enzyme electrodes has been severely restrained because of their absolute requirement for the coenzyme NAD. One solution to the problem is to covalently bind NAD to the surface of these enzymes to form intrinsically active enzyme-coenzyme conjugates. These conjugates could then be applied for use in reagentless enzyme electrodes.

This report investigates the application of established enzyme-coenzyme conjugation techniques for a range of dehydrogenases. NAD conjugates were formed using glutaraldehyde and carbodiimide coupling techniques, with lactate dehydrogenase, glutamate dehydrogenase and alcohol dehydrogenase. The amount of coenzyme incorporated per enzyme subunit was determined, along with the activity of the conjugates in the absence of exogenously added coenzyme (intrinsic activity). An intramolecular reaction mechanism was determined for a lactate dehydrogenase-NAD conjugate coupled using glutaraldehyde. An interesting inhibitory effect by glutaraldehyde on alcohol dehydrogenase was observed and terephthalaldehyde was used successfully as an alternative coupling reagent.

A novel coenzyme conjugation method was investigated: thiolated NAD derivatives were synthesised, characterised and then coupled to thiol groups present on the surface of lactate dehydrogenase to form disulphide linked enzyme-coenzyme conjugates.

The conjugates showed varying degrees of coenzyme incorporation and intrinsic activity, although the latter failed to approach the activity in the presence of exogenous coenzyme in all cases.

Lactate dehydrogenase-NAD was incorporated into an enzyme electrode for lactate to demonstrate the potential of enzyme-coenzyme conjugates for use in enzyme electrodes. Although the biosensor was unstable, a linear response was obtained between 0.5 and 10 mM lactate.

CONTENTS

ABBREVIATIONS

ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
DCC	dicyclohexylcarbodiimide
DCPIP	dichlorophenolindophenol
DHBT	3,3'-(1,6-dioxo-1,6-hexanediyl)bis-2-thiazolidinethione
DPDS	dipyridyldisulphide
DTT	dithiothreitol
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	ethylenediaminetetraacetic acid
FDH	formate dehydrogenase
G-3-PDH	glyceraldehyde-3-phosphate dehydrogenase
GDH	glutamate dehydrogenase
GlcDH	glucose dehydrogenase
HPLC	high performance liquid chromatography
2-IT	2-iminothiolane
k_{CAT}	catalytic constant
K_d	dissociation constant
K_M	Michaelis constant
LADH	liver alcohol dehydrogenase
LDH	lactate dehydrogenase
MDH	malate dehydrogenase
MTT	3-[4,5 dimethylthiazolyl-2-yl]-2,5 diphenyltetrazolium bromide
N^6AHCM-NAD	N^6[aminohexyl(carbamoylmethyl)]-NAD
N^6CM-NAD	N^6carboxymethyl-NAD
$NAD^{(+)}$	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
Nm	nicotinamide
NMN	nicotinamide mononucleotide
ODS	octadecylsilane
PDE	phosphodiesterase
PDS	pyridyldisulphide
PEG	polyethyleneglycol
PEI	polyethyleneimine
PES	phenazine ethosulphate
PESK	phosphate-EDTA-sodium/potassium buffer
SPDP	N-succinimidyl-3-(2-pyridyldithio)propionate
TEA	triethanolamine
TLC	thin layer chromatography
YADH	yeast alcohol dehydrogenase

CHAPTER 1

General Introduction

Enzymes make excellent analytical tools. By measuring the initial rate of the reaction that takes place between an enzyme and its corresponding substrate, a means of specific and quantitative detection of a vast range of biological molecules is provided. Enzymes can be used in this way for the efficient detection of substrates, cofactors, activators, inhibitors and even some synthetic molecules (Buckmann and Carrea, 1989). For many years now, the initial rate of an enzyme-catalysed reaction has most commonly been measured by monitoring a change in optical absorbance between a reactant and its corresponding product (Chaplin and Bucke, 1990). For example, the reactions of the NAD-dependent dehydrogenase enzymes have been monitored by following the change in concentration of the coenzyme NADH at 340nm using a spectrophotometer. Unfortunately, the spectrophotometric method becomes undesirable when a large number of repetitive assays need to be performed because valuable enzyme, and in many cases coenzyme, must be added each time an assay is performed. In addition, the spectrophotometric assay is time-consuming and must often be performed 'off-site' in a suitably equipped laboratory. For the many situations where the routine on-site analysis of biological substrates is required - for example, for clinical diagnosis, for 'real time' monitoring in the food and drink industries or for the collection of environmental data - such disadvantages must be overcome. Fortunately, the development of the biosensor has provided a very attractive alternative to the spectrophotometric assay. Ideally, a biosensor is a portable, user-friendly device which can provide selective, rapid and repeated measurements of a biological substrate without the addition of exogenous reagents. The structure and function of a typical biosensor will now be considered.

1.1 Biosensors

A biosensor is an analytical device which can convert a biological response into an electrical signal. The generalised construction of a biosensor is shown in *Figure 1.1*, which also illustrates how a biosensor works:

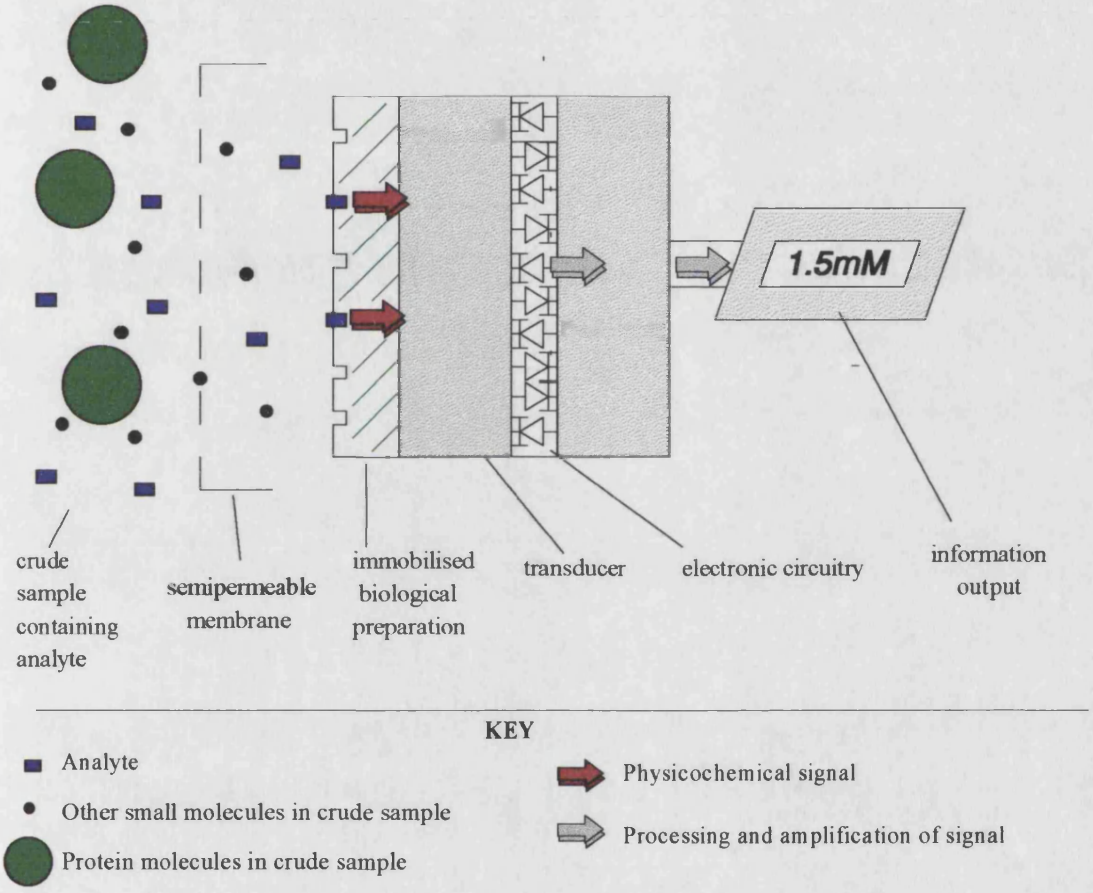

***Figure 1.1:** Representaion of a generalised biosensor*

The substrate to be analysed (the analyte) diffuses through pores in an outer membrane, which serves to exclude interfering substances, and into the vicinity of an immobilised biological preparation. The analyte reacts with the selective biological material and a product is formed. A physicochemical signal is emitted as a result of this

reaction and it is detected by a suitable transducer which then converts it into an electrical signal.

An immobilised enzyme preparation is often used as the biological component of a biosensor. In addition to enzymes, the immobilised biological preparation can also consist of antibodies, whole cells (tissues) or even organelles (Hall, 1990; Turner *et al*, 1987) but, so far, most biosensor research has concentrated upon the development of enzyme-based devices (Lowe, 1985; Mascini, 1993; Gronow, 1985).

Upon reaction with its substrate, an enzyme yields several powerful physicochemical signals. One of these signals may take the form of a change in the redox state of the solution immediately surrounding the enzyme and it may be detected by an amperometric* electrode, which can therefore act as a transducer (Schumann and Schmidt, 1992). Alternatively, potentiometric** electrodes can be employed to act as transducers to detect changes in the ion concentration of a solution brought about by an enzyme-catalysed reaction. The physicochemical signal emitted by an enzyme reaction may also take the form of a change in the optical properties of the enzyme solution; this signal can be detected by an optical fibre (Scheper and Buckmann, 1990). An enzyme-catalysed reaction may often be characterised by a change in the acoustic properties of the solution in the vicinity of the enzyme, and piezoelectric crystals have recently been used to detect this change (Griffiths and Hall, 1993). Nearly all enzyme reactions are, to varying extents, exothermic and small changes in the temperature of a solution can be detected by thermistors (Gronow *et al*, 1985).

The development of biosensors has been made possible due to technological advances in the field of microelectronics which have allowed the response of the transducers

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* An amperometric electrode operates at a fixed potential and directly monitors any change in current brought about by a change in the redox state of the solution in the direct vicinity of the electrode.

** A potentiometric operates at a constant current and monitors the change in potential between two half cells.

described above to be converted into an electrical signal which can then be output as a digital display (*Figure 1.1*). With the possible exception of optical fibres, transducers other than electrodes have had limited research activity, although there is now an increasing interest in the use of more unconventional transducers for biosensor construction. At the present moment enzyme electrodes are, by far, the most actively researched biosensor devices (Mascini, 1993). Despite this fact, the development of enzyme electrodes is being restrained. In order to help explain why this should be so, the origins of the enzyme electrode will now be described.

1.2 Enzyme electrodes and enzyme immobilisation

The story of the biosensor began in the 1950s when the voltage bias of a platinum electrode was set so that the rate of current flow depended on the rate at which oxygen diffused through a surrounding plastic membrane (Lessler and Brierly, 1969). In 1962 glucose oxidase was immobilised onto the surface of this oxygen electrode, and the first enzyme electrode was formed (Updike and Hicks, 1966) (*Figure 1.2*).

$$\text{Glucose} + O_2 \xrightleftharpoons{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

The enzyme electrode was sealed with a semipermeable membrane, so that the analyte, glucose, could diffuse in and the non-dialysable enzyme could not diffuse out. As illustrated in the above equation, any decrease in the concentration of oxygen could be directly related to the concentration of glucose in solution - a glucose-sensitive electrode had been constructed. However, as had been hoped, the electrode could not be used to measure blood sugar levels because it was seriously affected by fluctuations in the oxygen tension of sample solutions that were not related to the glucose concentration. It was nevertheless a very important discovery which is still finding applications today (Atkin, 1992).

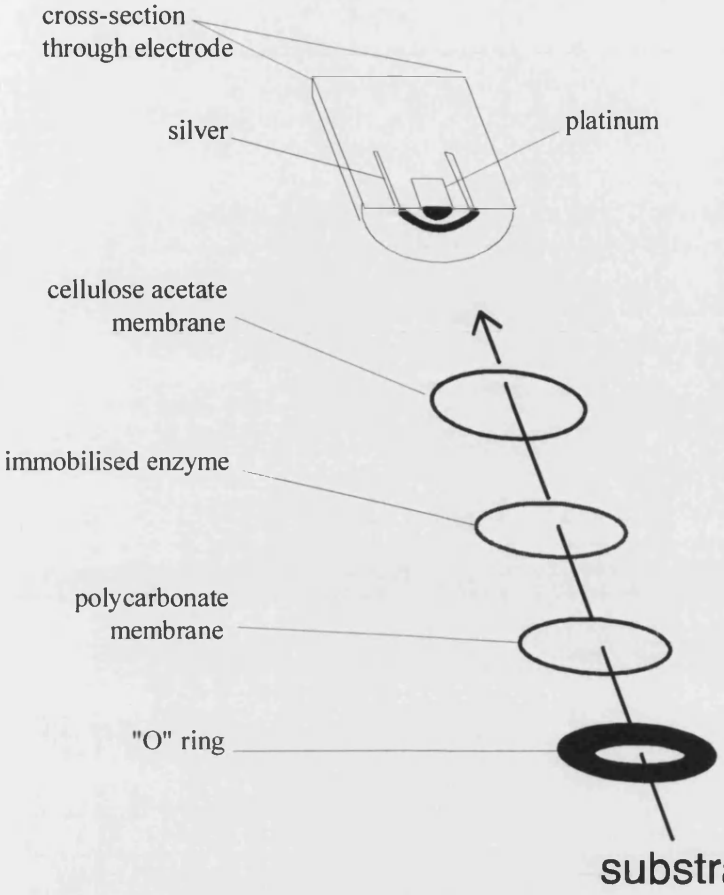
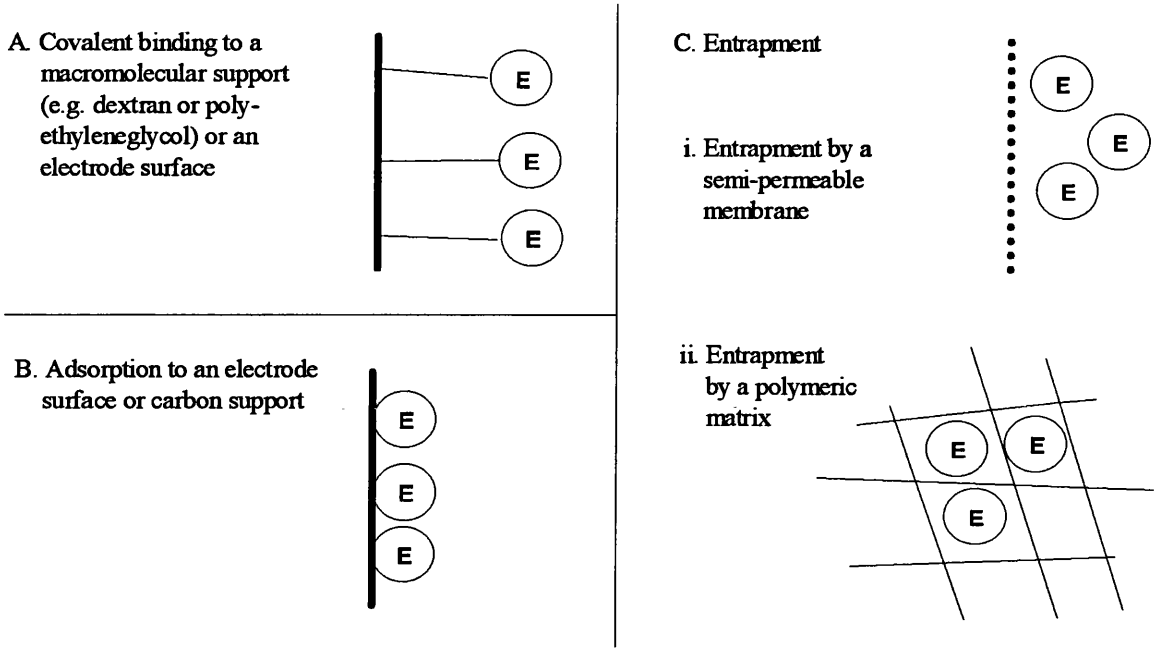


Figure 1.2: *Assembly of a simple glucose oxidase enzyme electrode*

Enzyme immobilisation

Immobilisation of the enzymic component is essential to the formation of a successful enzyme electrode because it allows repeated use of the enzyme preparation, thereby increasing the productivity of the enzyme by separating the enzyme from the substrate/product phase. Immobilisation also allows the enzyme electrode to be 'reagentless' because fresh enzyme need not be added each time the electrode is used. Finally, in many cases immobilisation helps to increase the stability of the enzyme molecules (Guilbault, 1984). Several successful methods of enzyme immobilisation are known:



Covalent binding

The covalent binding of an enzyme to a soluble or insoluble support (see diagram above) ensures a strong anchorage of the enzyme and decreases the possibility of the enzyme leaching into free solution. This method does, however, suffer from relatively poor enzyme loading, averaging 0.02g enzyme / (g matrix) (Chaplin and Bucke, 1990), although it has been used successfully in the construction of dehydrogenase-based enzyme electrodes. For example, Laval and his co-workers immobilised lactate dehydrogenase onto a vitreous carbon electrode using carbodiimide-mediated coupling, resulting in the formation of a lactate dehydrogenase-modified electrode for lactate (Laval *et al*, 1984). Glutaraldehyde cross-linking and cyanogen bromide activation methods have also been used (Miyamoto *et al*, 1991; Guilbault, 1984). The possibility of covalent immobilisation inhibiting the enzyme in some way must be kept in mind using this sort of immobilisation technique (see *Section 5.1.3*).

Entrapment

Because of their large molecular size, enzymes can often be entrapped by cross-linking into the internal structure of a polymeric membrane. This method has also been used successfully in the construction of dehydrogenase-based enzyme electrodes. In one application lactate dehydrogenase was entrapped in a gelatin membrane along with oxalate oxidase and combined with an oxygen electrode, forming a bienzyme electrode for glyoxalate detection (Schubert *et al*, 1990). The cross-linking method leads to a high density of immobilised enzyme activity but complicates the construction of the enzyme electrode, leading to increased manufacturing problems and extra expense.

A simpler method is to entrap the enzyme molecules behind a dialysis or cellulosic membrane. The pore size of the membranes used is smaller than the molecular size of the enzyme molecules to be immobilised.

Adsorption

This is the most established and most basic method of enzyme immobilisation with a wide applicability to many enzymes. It involves the adsorption of enzyme molecules onto an insoluble support, typically made of a carbon material. Adsorption occurs upon the formation of several salt links between the enzyme and the support, although hydrophobic forces are also involved (Guilbault, 1984). High loading of up to 1g enzyme / (g matrix) is possible (Chaplin and Bucke, 1990), although desorption sometimes occurs, especially upon variations in the surrounding conditions such as pH, temperature and solvent (Gronow *et al*, 1985).

1.3 Application of enzyme electrodes

Since the glucose oxidase enzyme electrode was discovered, and increasingly with the development of immobilisation techniques such as those described above, and micro-electronic techniques (Penner and Lewis, 1991), enzyme electrodes have been discovered for a number of substrates. Enzyme electrodes have been marketed for

use in the medical industry (for example, for the *in vivo* treatment of diabetics) (Turner, 1985), in the food and fermentation industry (for example for the detection of sucrose, lactose, lactate and alcohol) (Williams, 1992), in microbiology (Mullen and Vadgama, 1986), in pharmaceutical manufacture and in environmental control (Hall, 1990). Although several enzyme electrodes are now commercially available, they usually exploit the reactions of hydrolases, isomerases, oxidases (oxygen dependent) and other enzymes not requiring dissociable organic coenzymes. Application of organic coenzyme-dependent enzymes, particularly oxidoreductases and ATP-dependent phosphotransferases, has been more limited. The development of enzyme electrodes incorporating these enzymes has been restricted because the resulting biosensors are not 'reagentless': they require the addition of coenzyme each time a sample is measured. Not only does this decrease the user-friendliness of the biosensor, it also adds considerably to the cost of its use, with NAD, for example, costing around £1,100 / mol* . Unfortunately NAD- and ATP-dependent enzymes comprise more than a third of all known enzymes, there being over 250 NAD-dependent enzymes alone (Mosbach, 1978). The potential for the specific detection of important analytes such as lactate, ethanol and glutamate cannot be ignored. The demand for the rapid, on-site determination of these particular substrates will be discussed - firstly, to illustrate the need for commercially available enzyme electrodes for the substrates of coenzyme-dependent dehydrogenases and, secondly, because lactate dehydrogenase, alcohol dehydrogenase and glutamate dehydrogenase are of particular interest to this study.

Lactate is an important analyte in many fields. In medicine, quick and frequent determination of lactate is often required: a change in serum lactate levels is indicative of anoxia which can be caused by heart disease, coronary artery disease or pneumonia; lactate levels are also related to muscle disease, lymphomas, overinsulation in diabetics, and levels of shock (Mizutani *et al*, 1983). Lactate is an important ingredient in the production of food and can be monitored in order to follow fermentation

* Sigma Chemical Co. catalogue, 1993

processes (Adamowicz and Berstein, 1987). Even though lactate dehydrogenase is readily available, very selective for lactate and is independent of fluctuations in oxygen concentration, it is not yet available as part of a biosensor because of its dependence on NAD (Schumann and Schmidt, 1992).

Ethanol is also a very important analyte, being the most common toxic substance in legal cases (Turner *et al*, 1987). The accurate, rapid, on-site analysis of ethanol is vital in drink-drive cases. Acute alcohol toxication also requires fast and reliable analysis of ethanol. Ethanol is also an important analyte for the fermentation industry. NAD-dependent alcohol dehydrogenase - potentially an excellent enzyme for the detection of ethanol in a biosensor, being relatively cheap and readily available - has yet to be incorporated into a successful biosensor. In addition, the different specificities found between mammalian and yeast alcohol dehydrogenases could be exploited (Branden *et al*, 1975): a biosensor for a whole range of alcohols could be made by incorporating horse liver alcohol dehydrogenase, whilst an ethanol-specific biosensor could be made using baker's yeast as a source of the enzyme. Finally, although glutamate, too, is of great importance as an analyte it has usually been detected by biosensors that are only generally specific for amino acids (Turner *et al*, 1987). Glutamate is of great import in the food industry and in medicine for the detection of amino acid synthesis disorders and myocardial infarction (Henry *et al*, 1974). Altered glutamate levels are also indicative of hepatic and jaundiced diseases which are becoming increasingly common in industrialised countries (Turner *et al*, 1987). Glutamate dehydrogenase is readily available from several sources (Smith *et al*, 1975) but, again, because it is dependent upon the coenzyme NAD for activity it has not been incorporated into an enzyme electrode for the determination of glutamate.

Although biosensors are now commercially available for lactate and ethanol (Scheller *et al*, 1985) and have been researched for glutamate (Kauffmann and Guilbault, 1992), they exploit the reactions catalysed by oxygen-dependent oxidases and so are subject

to variations in surrounding oxygen levels, as described above for the glucose oxidase enzyme electrode. It is now known that systems dependent on oxygen are unstable and are not versatile (Huck *et al*, 1984). If improved biosensors are to be constructed for substrates such as those described above and a whole range of others, a way must be found to incorporate coenzyme-dependent enzymes into reagentless biosensors.

The above examples clearly illustrate the great potential for the use of NAD-dependent dehydrogenases in biosensors. If compact probes based on coenzyme-dependent dehydrogenases are to be commercially available in the future, the coenzyme problem must be solved in such a way as to maintain the 'reagentless' state and low cost of the biosensor. The most effective way to achieve this would be to retain and regenerate the highly dissociable coenzyme within the biosensor, alongside the enzyme. There have been several attempts at accomplishing this, with varying degrees of success. In order to consider fully the possibilities that are available, methods of coenzyme immobilisation and retention used for applications other than enzyme electrodes will also be discussed.

1.4 Coenzyme immobilisation

In order to construct a successful NAD-dependent dehydrogenase-based enzyme electrode, the coenzyme must be retained in a way that maintains its substrate activity and does not interfere with its mobility or mediator properties. At the same time a high concentration of coenzyme is required and 'leaching' of the coenzyme must be limited in order to generate a strong physicochemical signal (Miyamoto *et al*, 1991). A number of different coenzyme immobilisation strategies have been studied. The immobilisation of NAD usually involves derivatisation of the coenzyme, although some attempts have been made to immobilise the unmodified coenzyme.

1.4.1 Physical entrapment of NAD

The method of using membranes to entrap coenzyme molecules alongside enzyme molecules is limited because the small coenzyme molecules must be retained and yet the substrate and product molecules, whose size is often of a similar order of magnitude to the coenzyme, must be allowed to diffuse freely to and from the sample solution. However, a reagentless electrode for lactate was constructed by immobilising unmodified NAD along with lactate dehydrogenase using a chemically modified dialysis membrane (Blaedel and Engstrom, 1980). The membrane was acetylated so that NAD molecules could not pass through, but the substrate and product molecules could freely diffuse through the modified membrane, reportedly due to their slightly smaller size. The resulting enzyme electrode showed poor sensitivity to lactate although the response time was rapid, if rather erratic. In a more recent study NAD was sandwiched between a layer of ADH which was fixed by glutaraldehyde in the direct vicinity of the electrode surface, and an outer layer of BSA, also fixed with glutaraldehyde and which was permeable to ethanol but not to NAD. The electrode was described as sufficiently stable, but with quite low sensitivity (Miyamoto *et al*, 1991). Despite these attempts, a method has yet to be established whereby free NAD can be totally retained by a suitable semipermeable membrane.

Free NAD has been immobilised by physical entrapment, along with ADH, into the pores of a polyethyleneimine matrix in the construction of a biosensor for ethanol (Dominguez *et al*, 1993b). The cationic network present on the surface of polyethyleneimine matrix reportedly attracted the overall negative surface charge of the ADH molecules by electrostatic attraction. The positively charged polymer was also thought to act as an electron transport network, enhancing the transfer of electrons from reduced coenzyme to the electrode surface. The resulting electrode showed a linear response and was sensitive to 2μM ethanol. Unmodified NAD has also been immobilised into the pores of a collagen membrane, but with limited success (Morikawi *et al*, 1978).

1.4.2 Covalent binding of NAD to a macromolecular support

The effective size of a coenzyme molecule can be increased by covalent linkage to a water-soluble or -insoluble macromolecular support such as dextran or agarose (Mosbach *et al*, 1976). These enlarged coenzyme molecules are non-dialysable and can therefore be easily entrapped by a semipermeable membrane. NAD is usually derivatised via its catalytically insensitive adenine moiety (*Figure 1.3a*) and is attached to the support via a molecular spacer arm (Schmidt and Dolabdjian, 1980; Mansson and Mosbach, 1987). For example, the NAD derivative N^6[(aminohexyl)carbamoylmethyl]-NAD (**N^6AHCM-NAD**) (*Figure 1.3b*) contains an aliphatic amine function attached to the adenine ring via a long hexamethylene chain. This particular derivative has proved to be a popular choice for the covalent immobilisation of NAD because its long spacer arm allows the NAD moiety to protrude outwards from the macromolecular support, where it is accessible to enzymes.

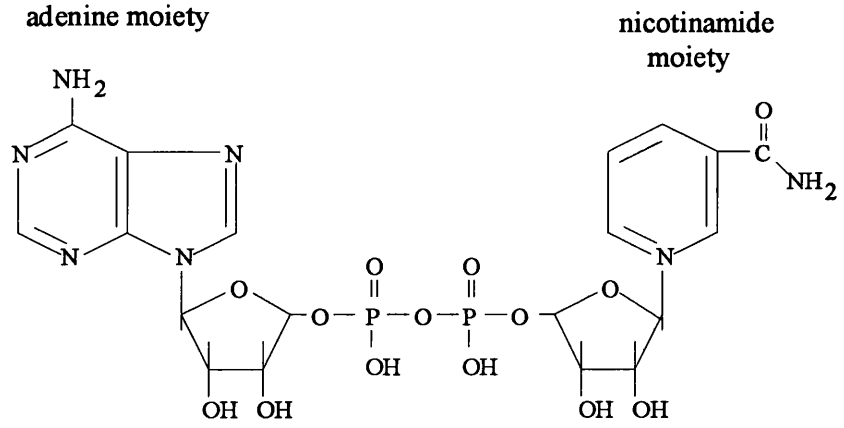


Figure 1.3a: *The structure of NAD.*

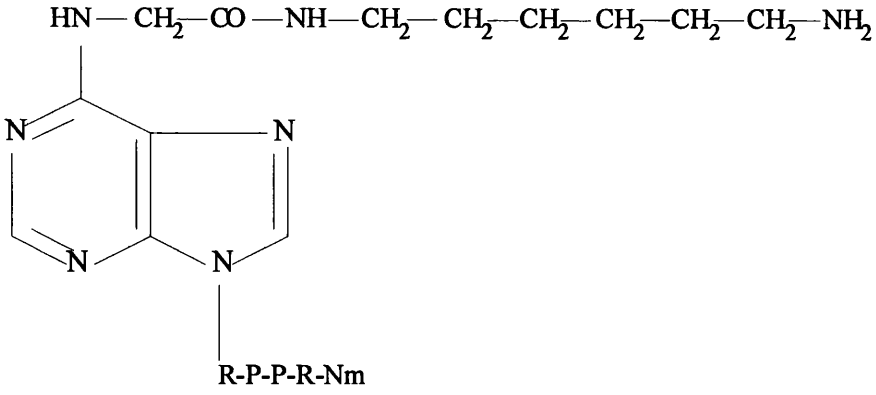


Figure 1.3b: *The structure of N^6[(aminohexyl)carbamoylmethyl]-NAD*
R= ribose; P = phosphate group, Nm = nicotinamide group

Immobilisation of NAD onto water-insoluble supports has proved to be far from ideal (Schmidt and Grenner, 1976). For example, the efficiency of the bound NAD moiety of Sepharose-N^6AHCM-NAD, designed chiefly for use in affinity chromatography, is very low when used as a coenzyme with alcohol dehydrogenase or lactate dehydrogenase (Mosbach, 1978; Ukeda *et al*, 1989a). The NAD derivative is over 70% inaccessible because of steric hindrance by the matrix backbone of the insoluble support. Despite this drawback, an agarose-NAD preparation was used in the construction of a reagentless electrode for lactate by constraining the macromolecular coenzyme alongside lactate dehydrogenase in the direct vicinity of the electrode surface using a cellulosic membrane. The electrode showed low sensitivity and a slow response time to lactate (Blaedel and Jenkins, 1976).

When NAD is immobilised onto soluble supports the coenzyme is largely accessible: 80% of the NAD moieties of dextran-N^6AHCM-NAD are enzymically reducible (Mansson and Mosbach, 1987). Dextran-enlarged NAD molecules have been succesfully immobilised, usually by entrapment with a dialysis membrane, in both the construction of enzyme reactors (Fu Gu and Chang, 1987) and enzyme electrodes. For example, N^6AHCM-NAD has been covalently bound onto dextran and coentrapped with lactate dehydrogenase and glutamate dehydrogenase within a dialysis bag

containing an ammonia-sensitive electrode. The resulting device was sensitive to glutamate (Mosbach, 1978).

In the synthesis of another macromolecular coenzyme, NAD was coupled to alginic acid using carbodiimide-mediated coupling (Aizawa *et al*, 1976a). The resulting macromolecular NAD was actually more stable than free NAD and could be made soluble or insoluble by adjustment of the pH. Alginic acid-bound NAD was successfully electrocatalytically regenerated but it has yet to find an application in enzyme electrodes.

A popular method by which to synthesise a water-soluble molecular weight-enhanced NAD derivative is by coupling an NAD derivative that usually contains an aliphatic amine attached at the adenine ring of NAD (e.g. N^6AHCM-NAD) to polyethyleneglycol, or PEG (Okada and Urabe, 1987; Ottalina *et al*, 1990; Yomo *et al*, 1989; Kulys *et al*, 1991). With most dehydrogenases, there is only a small loss in the substrate activity of the coenzyme and this method has become widely used in the immobilisation of NAD, especially with recent improved methods for synthesising PEG-NAD (Buckmann, 1987; Buckmann and Carrea, 1989). However, the application of PEG-NAD will probably be restricted for use in enzyme reactors, as it has not proved to be suitable for use in the construction of reagentless biosensors. For example, a fibre-optic biosensor was formed using PEG-NAD which was entrapped by a dialysis membrane in direct vicinity of the tip of a fluorometer sensor, alongside either alcohol dehydrogenase or formate dehyrogenase. The electrode showed poor stability and a limited accessibility of the NAD moiety of PEG-NAD was reported (Scheper and Buckmann, 1990). In another study, PEG-NAD was entrapped alongside glucose-6-phosphate dehydrogenase by a cellulosic membrane in the construction of a reagentless enzyme electrode using glucose-6-phosphate dehydrogenase. The electrode was again unstable, in this case due to a reported loss of coenzyme; the electrode also demonstrated a slow response time. In this study it was suggested that most of the

PEG-bound NAD molecules could not reach the electrode surface and that the presence of the PEG polymer restricted the transfer of electrons from the NAD molecules to the electrode surface (Skoog *et al*, 1991). These suggestions were echoed in a recent attempt to form an enzyme electrode using physical entrapment of PEG-NAD alongside one of several dehydrogenases: the resulting electrode demonstrated poor activity and stability and a non-linear, unstable response. It was found that the viscosity of the PEG molecule slowed the transfer of electrons from the enzymically reduced coenzyme molecules to the electrode surface (Schumann and Schmidt, 1992). Thus, although PEG-NAD is a very useful non-dialysable NAD derivative, it cannot provide the high concentrations of accessible coenzyme with unhindered mobility properties that are required for the construction of successful enzyme electrodes. Other water-soluble macromolecular NAD derivatives include polyethyleneimine-NAD and polylysine-NAD, both of which have yet to find successful applications (Wykes *et al*, 1972; Zappelli *et al*, 1975).

NAD has also been immobilised by copolymerisation (Fuller *et al*, 1980). An acrylic derivative of NAD was made and subjected to radical copolymerisation in the presence of acrylic monomers. A polyacrylamide was formed with NAD molecules covalently bound onto the matrix. The enlarged NAD molecules were soluble and enzymically active. This method was used to construct an enzyme reactor by entrapping the enzyme molecules in pores of the matrix (Yamazaki and Maeda, 1987; Yamazaki and Maeda, 1982). However, this method has not been widely applied, probably because of problems associated with enzyme leakage. This particular coenzyme immobilisation technique would probably suffer similar drawbacks to PEG-NAD and agarose-NAD if applied to the construction of enzyme electrodes.

A variation on the theme of the molecular weight enhancement of NAD in order to ease its immobilisation is to attach NAD covalently to a membrane. In an attempt to fix free NAD in the vicinity of an electrode surface, Blaedel and Jenkins (1976)

immobilised NAD alongside LDH by covalently linking both enzyme and coenzyme to a cellulosic membrane using glutaraldehyde. Unfortunately, the electrode showed poor sensitivity. NAD has also been covalently immobilised, along with alcohol dehydrogenase, onto the inside of a nylon tube. However, this form of immobilised NAD will probably not be used in the formation of enzyme electrodes because of the physical restraints imposed by the presence of the nylon tube (Laidler and Mazid, 1987).

1.4.3 Other methods of coenzyme immobilisation

In previous constructions of enzyme electrodes, the coenzyme has been immobilised onto the actual electrode surface. In one study, NAD was immobilised onto a platinum electrode surface with the help of a urethane polymer. The resulting enzyme electrode demonstrated a low specificity for its substrate (Phadke *et al*, 1991). This method of coenzyme immobilisation must be carefully considered as the immobilisation of both enzyme and coenzyme at the electrode surface is not easy and, with certain electrode materials, can lead to the decomposition of both enzyme and coenzyme. The drawbacks of this method are further discussed in *Section 5.1.2.*

Finally, a unique method in which NAD can be *supplied* to the electrode surface has been developed: pores were bored into a graphite rod electrode, filled with a solution of 10mM NAD and pressurised (Skoog and Johansson, 1991). In this way NAD was supplied to glucose dehydrogenase at the electrode surface. The electrode was sensitive to 1μM of glucose and used only 11μl of NAD per hour - a 500-fold saving of coenzyme compared to using a bulk solution of 10mM NAD. However, this technique only optimised conditions of NAD supply, and, because it did not adhere to the principle of a reagentless biosensor, will not be further considered here.

An alternative and attractive way with which to immobilise a coenzyme alongside the enzyme that requires it for catalysis is to attach it covalently to the surface of the

enzyme molecule. In this way a holoenzyme can be formed which should be able to catalyse the NAD-dependent reaction in the absence of free coenzyme. Before the advantages of using this coenzyme immobilisation technique are considered, the reasons why the enzyme-coenzyme method is also attractive with respect to coenzyme regeneration will first be explained. This will be discussed after a consideration of the methods of coenzyme regeneration that are available.

1.5 Coenzyme regeneration techniques

There are three main ways in which NAD can be regenerated with respect to the construction of an enzyme electrode: firstly, enzyme-catalysed regeneration, whereby the reduced or oxidised coenzyme can be regenerated by an appropriate NAD-dependent coenzyme-recycling enzyme (*Figure 1.4*); secondly, NAD can be regenerated by a redox chemical with a suitable redox potential, such as phenazine ethosulphate (*Figure 1.4*); lastly, the electrode itself can act as a coenzyme regenerator, usually by oxidation of the reduced coenzyme, NADH. The problem with using redox mediators is that they are often unstable and may denature the enzyme component of the enzyme electrode (Schumann and Schmidt, 1992; Huck *et al*, 1984). Until recently, the most desirable method of coenzyme regeneration was enzyme-catalysed regeneration because of the specificity and efficiency of enzyme reactions. However, this method requires the immobilisation of a second enzyme in the enzyme electrode which complicates construction of the enzyme electrode and can also add to instability. This method also requires the presence of substrate for the second enzyme and hence is not reagentless. In addition, if an oxygen-dependent coenzyme-recycling enzyme such as NAD oxidase is used, the enzyme electrode will also suffer from oxygen-dependency, as described previously (Huck *et al*, 1984).

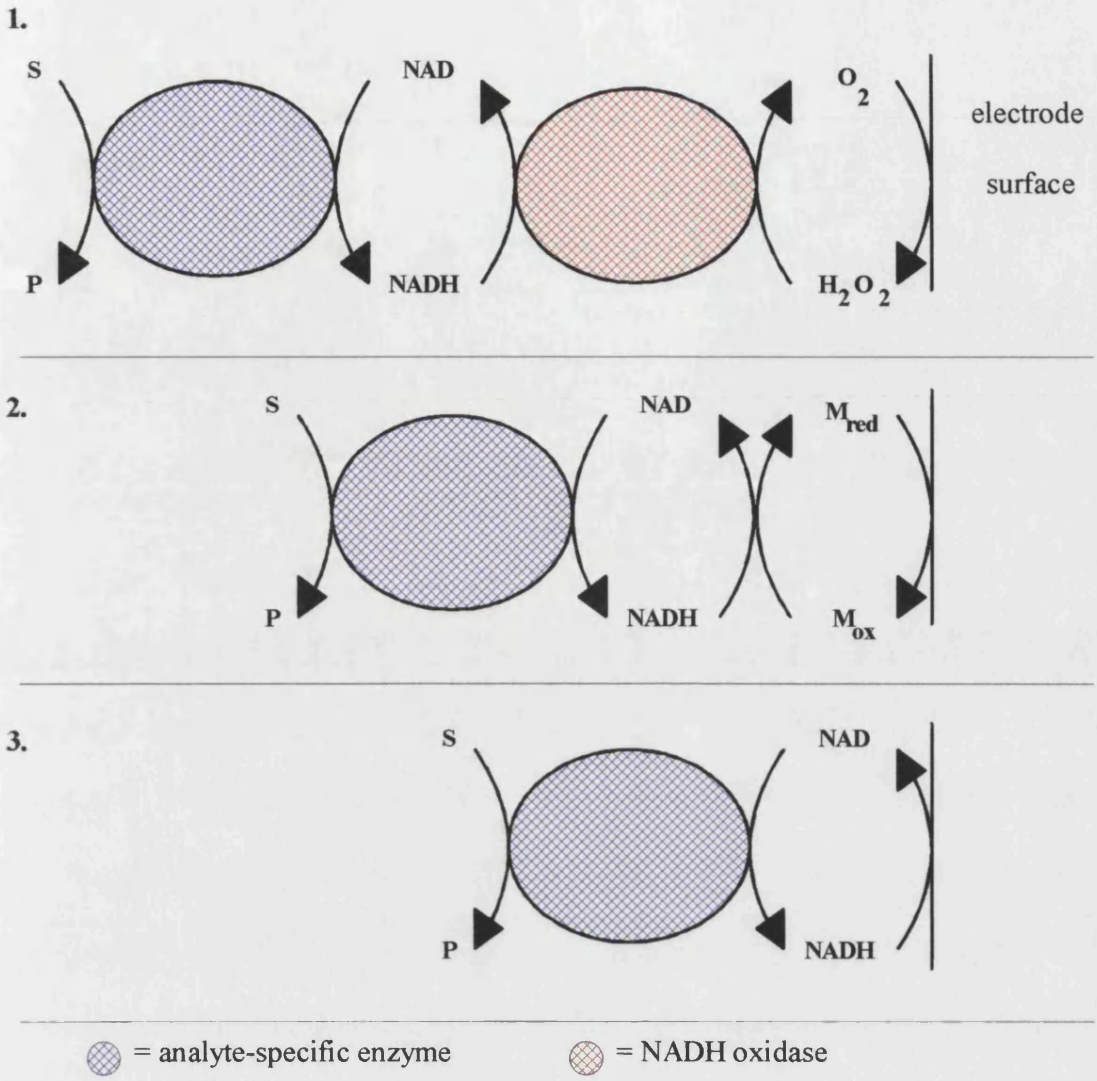


***Figure 1.4**: Three methods of coenzyme regeneration in enzyme electrodes*
1. Enzymic regeneration by, for example, NADH oxidase. The substrate of the coenzyme-recycling enzyme is usually regenerated by the electrode. If oxygen-dependent coenzyme-recycling enzymes are used, the enzyme electrode becomes unreliable due to its dependence upon the oxygen tension of the sample.
2. Regeneration by a redox mediator (M), for example, phenazine methosulphate or ferrocene. The redox mediator must have a redox potential that allows it to oxidise NADH spontaneously. The oxidised mediator (M_{ox}) is regenerated at the electrode surface.
3. Electrocatalytic regeneration at the electrode surface.

Electrocatalytic regeneration of NAD is fastly becoming the more popular regeneration method; this is largely due to recent improvements in electrode surfaces enabling NADH to be oxidised at a reduced overpotential (*Section 5.1.1*). Electrocatalytic regeneration is, ultimately, the more desirable method of coenzyme regeneration because enzyme electrodes can be constructed using a minimum number

of components, as is now explained. When the coenzyme, say NAD, is regenerated by oxidation of NADH at an amperometric electrode surface, a flow of electrons passes from NADH to the electrode surface; this eventually leads to a finite current which is passed through the electrochemical cell: the electrode acts as both a transducer and a coenzyme regenerator. At the same time, NAD acts as both a coenzyme for the enzyme and a mediator of the physicochemical signal between the enzyme and the transducer. NAD can do this because it is a highly dissociable, non-integrative cofactor unlike, say, FAD.

A contribution to the electrode current is only made by NADH molecules in the direct vicinity of the electrode surface (Nernst layer). Therefore, the response time of the electrode is linked to the probability that NADH molecules reach the electrode surface. The strategy of NAD-dependent electrode studies has consequently been to immobilise the coenzyme alongside the enzyme at the electrode surface. In order to avoid coenzyme "leaching" the coenzyme must be covalently bound but, as described previously, the use of macromolecular supports for such coenzyme binding has led to interference in enzyme electrode operation and added to its instability. The simplest answer would be to bind the coenzyme to the surface of the enzyme. The enzyme could then be immobilised at the electrode surface by one of a number of established techniques (*Section 1.3*), and the coenzyme should be able to interact with both the active site of the enzyme and the electrode surface (*Figure 1.5*).

In summary, the formation of enzyme-coenzyme conjugates could provide an effective way to solve the problem of coenzyme-dependency of enzyme electrodes incorporating NAD-dependent dehydrogenases. The potential advantages of forming such conjugates are now summarised:

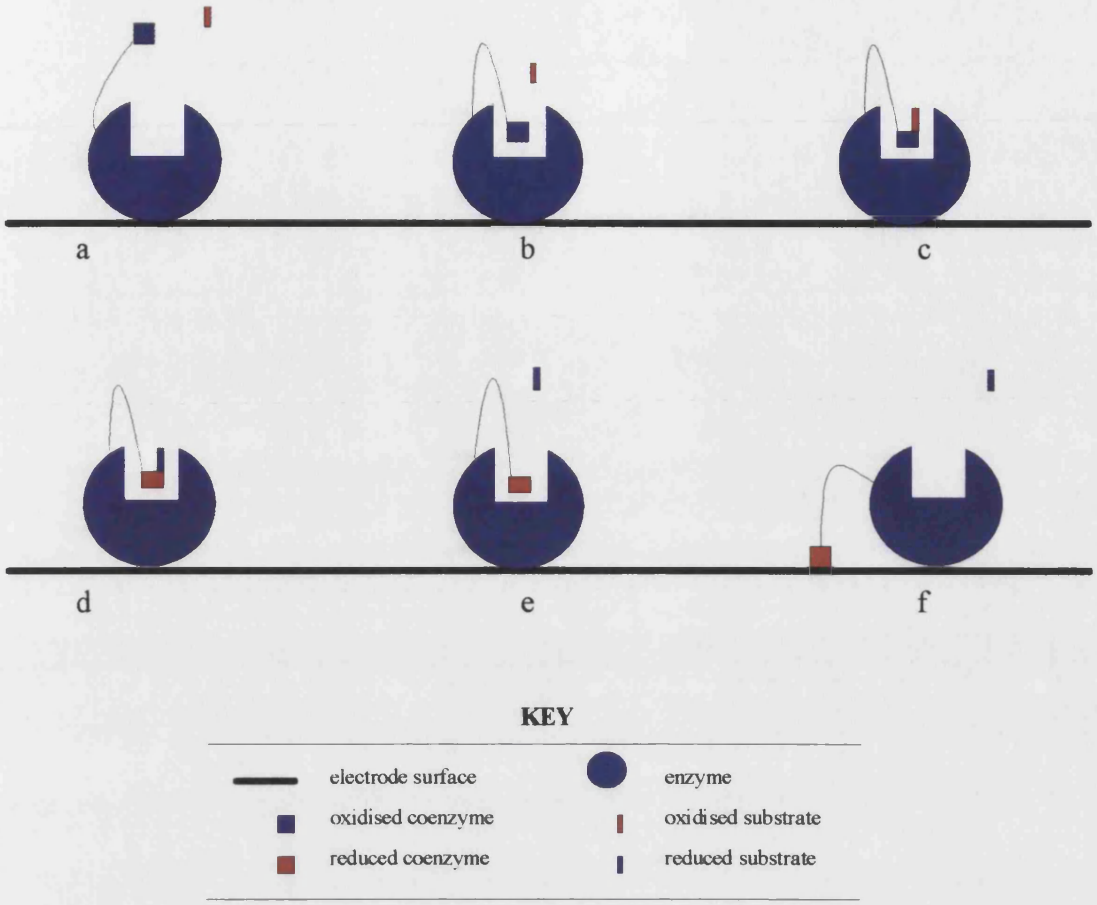


Figure 1.5: *Diagram showing how an enzyme covalently bound to an enzyme via a long spacer arm can interact with both the active site and an electrode surface.*

a. Enzyme-coenzyme conjugate immobilised in direct vicinity of electrode surface
b. Spacer arm allows NAD interact with enzyme active site resulting in the formation of a binary complex.
c. In the presence of reduced substrate a ternary complex is formed between NAD, substrate and enzyme.
d. After catalysis NAD oxidises the substrate molecule and NADH is formed.
e. The oxidised substrate dissociates from the active site
f. The spacer arm allows NADH to swing out of the active site and come into contact with the area of potential gradient at the electrode surface. The overpotential of the electrode is set to oxidise NADH, resulting in the formation of NAD (a.)

1. The covalent fixation of coenzyme would ensure strong immobilisation and the "leaching" problems that are associated with physical entrapment or adsorption methods would not occur.
2. A macromolecular NAD derivative is formed that does not require the presence of polymers that can hinder electron transfer processes and be generally detrimental to the functioning of the electrode.
3. The enzyme-coenzyme conjugate could be immobilised as one catalytic unit in the construction of an enzyme electrode without the need for addition of coenzyme

immobilisation supports. This both simplifies electrode construction and minimises problems that may occur during manufacture.

4. Perhaps one of the most attractive advantages is that an increased interaction between enzyme and coenzyme may actually lead to an increased stability of both coenzyme and enzyme. For example, water has been shown to degenerate organic coenzymes, but in the active site of a dehydrogenase enzyme water is excluded (Lowry and Passoneau, 1972). In addition, the binding of NAD to the active site of an enzyme has been shown to contribute to the increased stability of the enzyme during catalysis (Villaume *et al*, 1990). It can be concluded that if the normally dissociable coenzyme, NAD, were to spend more of its time in contact with the enzyme binding site, the stability of both enzyme and coenzyme would be increased and an enzyme electrode constructed using an enzyme-coenzyme conjugate would have greater stability than a similar enzyme electrode constructed using a more conventional coenzyme immobilisation technique.
5. Immobilisation of coenzyme onto the surface of an enzyme present at the electrode surface ensures optimisation of the proximity of the physicochemical signal generated by the enzyme (i.e. reduced coenzyme) to the electrode surface.
6. An enzyme-bound coenzyme should be able to interact with other enzyme active sites and redox compounds as well as the active site of the enzyme molecule it is bound to, and so it could be used as a self-contained NAD regeneration unit in other applications.
7. The effects of covalently binding an enzyme to a coenzyme would, if nothing else, be interesting from a theoretical point of view, for example, to observe the effects of conjugate formation upon the kinetics and stability of the enzyme involved.

The aim of this project was to investigate the possibility of using the enzyme-coenzyme method of coenzyme immobilisation to construct improved enzyme electrodes for substrates of NAD-dependent dehydrogenases.

Although research into this novel immobilisation technique is still in its embryonic stage, a number of studies involving enzyme-coenzyme conjugates have been carried out.

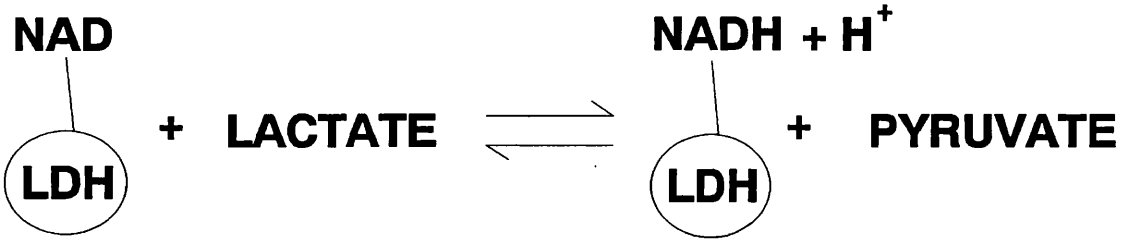
1.6 Previous studies involving enzyme-coenzyme conjugates

As *Table 1.1* shows, there has been an increasing interest in enzyme-coenzyme conjugates. Conjugates have been constructed using several different enzymes but so far only NAD has been used as a coenzyme. The covalent linkage formed between the enzyme and coenzyme has proved to be stable in all of the studies attempted so far. Covalent modification of enzymes can lead to a loss of enzyme activity and this must be considered before the construction of a conjugate. In fact, the earliest attempts at forming enzyme-coenzyme conjugates failed because of a serious inhibition of enzyme activity and they are not reported here (Woenckhaus *et al*, 1983). Reactive amino acid side chains present on the surface of enzymes have been used as anchorage points; the side chains of the acidic amino acids, glutamate and aspartate, and those of lysyl residues have been used. There has been only one study where cysteine, generally the most reactive enzyme residue, has been used as an anchorage point for the covalent attachment of coenzyme (Persson *et al*, 1991). During conjugate construction, molecular spacers between bound coenzyme and the enzyme surface have been used with the accessibility of the bound coenzyme to the active site in mind. The length of spacer arms used has varied around 1nm apart from attempts where a polymer was employed as the spacer arm, the length of which was 25nm. There now follows a discussion of the properties of each enzyme-coenzyme conjugate constructed to date; they are classified according to the constituent enzyme of the enzyme-coenzyme conjugate. Terms pertaining to the study of enzyme-coenzyme conjugates will firstly be defined:

The **intrinsic activity** of an enzyme-coenzyme complex is defined as the catalytic activity present in an enzyme-coenzyme complex in the absence of exogenously added coenzyme. During an assay non-coenzymic substrate is present at saturating concentrations.

Specific activity is defined as the catalytic activity generated by an enzyme or enzyme-coenzyme complex in the presence of exogenously added coenzymic and non-coenzymic substrate, both of which are present at saturating concentrations.

*1.6.1 Lactate dehydrogenase (LDH)**



The first successful LDH-coenzyme conjugate prepared comprised 0.8 molecules of NAD coupled per LDH subunit (M.W. 35,000) and used glutaraldehyde as a cross-linking reagent (*Section 3.4.1*) (Venn *et al*, 1977). A condensation reaction between the amino group of NAD and a terminal aldehyde group of glutaraldehyde produced a Schiff base linkage (Gacesa and Whish, 1977), whilst the other terminal aldehyde reacted with ε-amine groups of lysyl residues. In total, there are 23 lysyl residues on pig heart LDH (Klitz *et al*, 1977) and so not many of these were accessible during coupling. Attempts were also made to couple the NAD derivative N^6AHCM-NAD to LDH. 2.2 molecules of the NAD derivative were coupled per LDH subunit. The hexamethylene spacer of N^6AHCM-NAD was thought to increase the availability and reactivity of the coenzyme amine function which has low reactivity due to electron delocalisation and low accessibility for reaction due to steric hindrance by the purine ring. The results showed no evidence that the hexamethylene spacer gave LDH-bound

* The pig heart isoenzyme of LDH was used, unless stated otherwise.

NAD a better chance of interacting with an active site. The intrinsic activity of the LDH-N^6AHCM-NAD conjugate was 0.8% of the specific activity of the conjugate, whereas the intrinsic activity of the LDH-NAD conjugate was 0.6% of its specific activity despite there being less than half as many bound coenzyme molecules per subunit. The specific activity of each conjugate was between 24 and 28% of the specific activity of unmodified LDH. The complex was only partially stable due to a loss of covalently bound coenzyme but in a subsequent study a modification of the method was designed. The Schiff base linkages formed between glutaraldehyde and both enzyme and coenzyme were stabilised by reduction wth potassium borohydride and, as a consequence, there was no loss of conjugated coenzyme (Gacesa and Venn, 1979) (*Table 1.1*). The latter study confirmed the results of Venn *et al* and it was established that the length of spacer arm attached to the coenzyme was important during coupling between enzyme and coenzyme. These studies describe the only enzyme-coenzyme conjugates to date that have been prepared using unmodified NAD. In a later study, NAD was derivatised with various spacer arms of length between 0.4 and 1.0 nm, each one possessing a terminal aromatic amino group. Upon reaction with nitrous acid these groups formed diazonium functions which are suitable for reaction with a variety of amino acids present on the surface of an enzyme with the resulting formation of azo bridges (*Figure 1.6a & b*) (Woenckhaus *et al*, 1983). During the coupling reaction, a dead-end ternary complex was formed between LDH, NAD and a pseudosubstrate, oxalate. This complex served to stabilise the interaction between the NAD derivative and the active site; it was thought that the NAD derivative, with the spacer pointing out of the binding site, was more likely to become anchored to an amino acid residue that was near the coenzyme binding site and consequently, more likely to be available for interaction with the active site after covalent binding. The dead-end ternary complex was also formed to protect "sensitive" active site residues from unwanted modification. Up to 1.0 molecule of substrate-reducible coenzyme was incorporated per LDH subunit, comparable to the glutaraldehyde

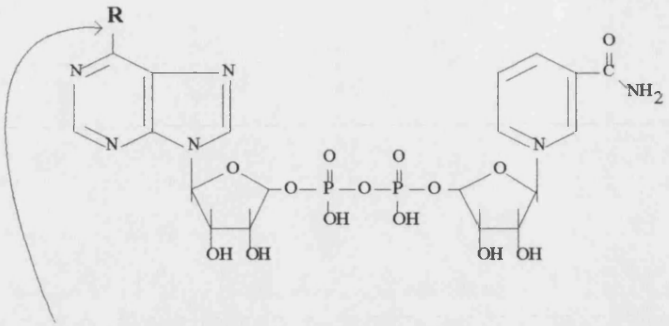
Table 1.1: *Comparison of properties of enzyme-coenzyme conjugates prepared to date*

Enzyme	**LDH**			**ADH**		**GlcDH**		**MDH**	**FDH**
Coenzyme	i. NAD ii.N^{6}AHCMNAD (Fig 1.3b)	Imidoester, acyl azide and carbodiimide NAD derivatives (Fig 1.6 e & f)	Diazonium and imidoester NAD derivatives (Fig 1.6 f, g & h)	N^{6}AHCM-NAD (Fig 1.3b)	N^{6}ADCM-NAD (Fig 1.6d)	PEG-NAD (Fig 1.6c)	N^{6}AHCM-NAD (Fig 1.3b)	PEG-NAD (Fig 1.6c)	N^{6}AHCM-NAD (Fig 1.3b)
Spacer length (nm)*	i. 0.8 ii. 2.2	0.3-1.7	1.4-1.8	1.4	1.7	25	2.4	25	1.4
NAD/subunit (molar ratio)	i. 1.7 ii. 0.9	0.8-2.1	0.4-0.9	1.6	0.5	2.1	1.0	1.2	0.2
Specific Activity (%)**	i. 24 ii. 28	50-90	80-90	37	52	92	5	80	116
Intrinsic activity (%)***	i. 0.6 ii. 0.8	----	40-50	40	53	2	82	0.1	13
Author & Year	Gacesa & Venn 1979	Warth *et al* 1989	Schafer *et al* 1986	Mansson *et al* 1978	Goulas 1987	Nakamura *et al* 1986	Persson *et al* 1991	Eguchi *et al* 1986	Kato *et al* 1987
Coupling Method	Glutaraldehyde reacts with amine of coenzyme and ε-amine of enzyme lysines	Imidoester and acyl azide functional groups react with ε-amine of enzyme lysines. Carbodiimide -see Mansson *et al*	Imidoesters react with ε-amine of enzyme lysyl residues	Carbodiimide activation of enzyme acidic residues for reaction with amine of coenzyme	Carbodiimide activation of enzyme acidic residues in presence of pseudosubstrate	DHBT activates reacts with amine groups of PEG and ε -amine of enzyme lysyl residues	SPDP reacts with amine of coenzyme derivative and ε-amine of enzyme lysyl residues	DHBT activates reacts with amine groups of PEG and ε -amine of enzyme lysyl residues	Carbodiimide activation of enzyme acidic residues for reaction with amine of coenzyme

* Length includes distance from N^6 amine of NAD to point of attachment of original enzyme. Note: cross linkers will be included.
** Expressed as a percentage of the specific activity of the unmodified enzyme.
*** Expressed a a percentage of the specific activity of the modified enzyme.
Abbreviations: SPDP = N-succinimidyl 3-(2-pyridylthio) propionate; PEG = polyethyleneglycol; DHBT = 3,3'-(1,6-dioxo-1,6-hexanediyl) bis-2-thiazolidinethione.
LDH = lactate dehydrogenase; GlcDH = glucose dehydrogenase; MDH = malate dehydrogenase; ADH = alcohol dehydrogenase; FDH = formate dehydrogenase

coupling method described above, which also used lysyl residues as coenzyme attachment sites. The loss in specific activity due to chemical modification of LDH was between 0 and 20%. Although it was reported that the bound coenzyme molecules could be reduced, no results for intrinsic activity were given. Woenckhaus's group has carried out further coupling experiments using LDH (Schafer *et al*, 1986). NAD was derivatised to form N^6-[(aminobutyl)carbamoylmethyl]-NAD and N^6-[aminobutyl]-NAD, and these spacer arms were then activated with an aromatic imidoester group (*Figure 1.6f & g*), the resulting spacer arms being 1.4 and 1.8nm in length, respectively. Imidoesters react with ε-amine groups of lysyl residues to form stable amidinium bonds. Only 10-20% of the specific activity of LDH was lost upon modification using the coenzyme derivatives, although the K_M value of LDH for free NAD was significantly increased: between ten and twenty times the normal amount of NAD was required to achieve maximum velocity. It was clear that the presence of bound coenzyme had interfered with the reaction between enzyme and free coenzyme,suggesting that the association between enzyme and coenzyme was increased by covalent fixation of the coenzyme to the enzyme. Up to 0.9 NAD derivatives were incorporated per LDH subunit, but only 40-60% of these were reducible as determined by the increase in absorbance at 340nm upon coenzyme reduction. The values for intrinsic activity were good, being between 40 and 50% of the specific activity of the modified enzyme.

More recently, the Woenckhaus group attempted to determine the molecular spacer length that was required between covalently-bound coenzyme and enzyme in order to achieve an optimum interaction between the coenzyme and the active site (Warth *et al*, 1989). It must, however, be pointed out that this would also depend upon the method of coupling used, *i.e.* which amino acids are modified and the chemical nature of the spacer arm involved. During their study, Woenckhaus and his co-workers proposed that if a coenzyme is bound far from the enzyme active site (labelled non-specific incorporation) the rate of its reduction will be slower than that



The spacer arms shown below were attached at the N^6adenine position of NAD

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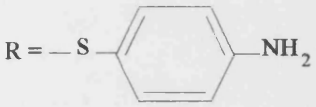
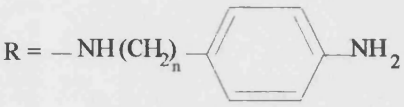
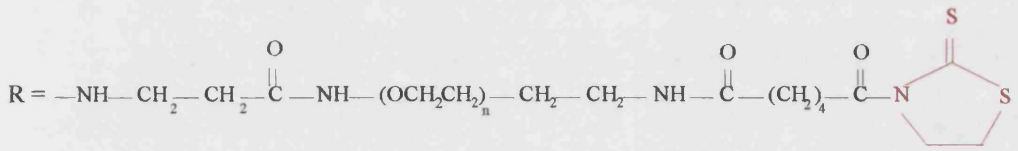
a.



b.

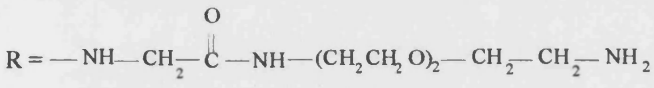


c.

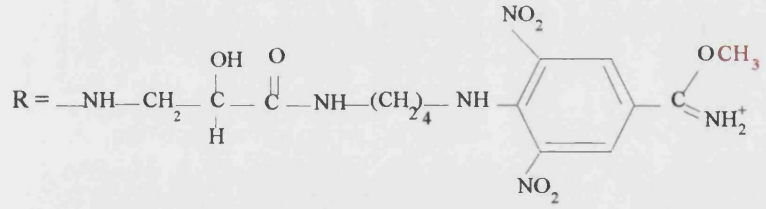


n = ~70

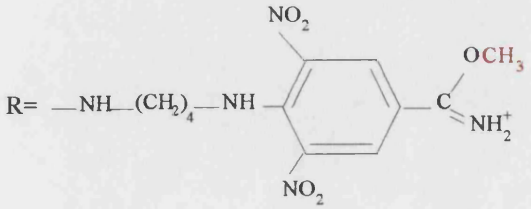
d.



e.

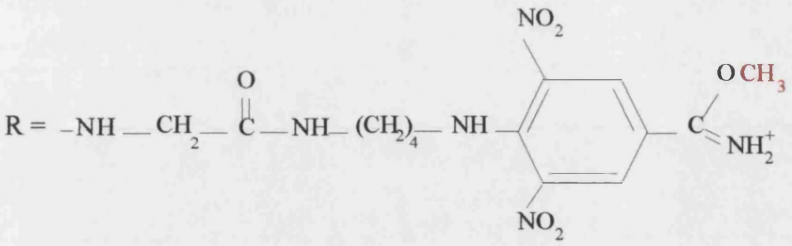


f.

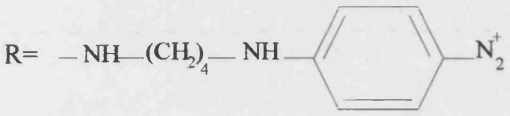


over/

g.



h.



i.

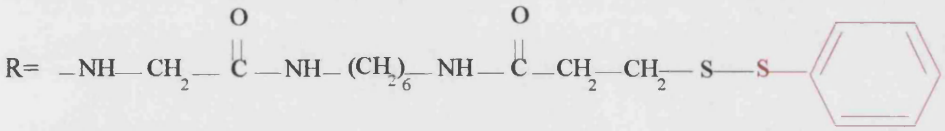


Figure 1.6: *Coupling chemistry used in past studies in the formation of enzyme-coenzyme conjugates.* Red colouring shows terminal groups that were not included in the final spacer arm structure

of a coenzyme bound in a greater proximity to the active site (specific incorporation) but the study assumed that the interaction between coenzyme and enzyme was predominantly intramolecular and that bound coenzyme molecules would not interact with the active sites of neighbouring LDH molecules. NAD derivatives with spacer arms of length between 0.3 and 1.7nm were investigated. A number of different reactive functional groups were involved: NAD containing acyl azide groups formed amide bonds with ε-amine groups of lysyl residues; carboxyl-containing NAD-derivatives were coupled to lysyl residues by carbodiimide-activation, or else amine-containing NAD derivatives were coupled to acidic enzyme residues using the same coupling reagent (*see Section 3.4.2*). Finally, aromatic imidoester NAD derivatives were used as described above (*Figure 1.6f & g*). It was found that the optimum spacer length of the NAD derivatives used was between 1.2 and 1.7nm, as determined by the

reducibility of the bound derivatives. Hence, a lower limit on the optimum spacer length was defined for these couplings. Between 50 and 90% of specific activity of native LDH was retained after modification, and the incorporation of coenzyme was comparable to previous studies with between 1.4 and 2.1 molecules incorporated per LDH subunit. Imidoester-coupled derivatives were reported to have a higher specific incorporation than carbodiimide coupled derivatives, but this was probably because the imidoester derivatives contained longer spacer arms. In an extension of this study, bis-NAD, an NAD dimer formed by treatment of N^6CM-NAD with adipic acid dihydrazide (*Figure 1.7*) (Mosbach, 1979; Siegbahn *et al*, 1986) was used to orientate the active sites of alanine dehydrogenase and lactate dehydrogenase so that they were opposite each other in order to create a more efficient coupled enzyme regenerating system (*Figure 1.8*). The enzyme molecules were fixed in this position by cross-linking using dimethyl suberimidate so that an NAD derivative that was covalently bound to the LDH molecule could go from the active site of one enzyme to another. The NAD derivative used contained an imidoester group (spacer length 1.7nm) and because it could interact with active sites of both enzymes it was self-regenerating (*Figure 1.8*).

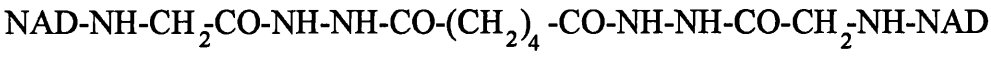


Figure 1.7: *The structure of bis-NAD*

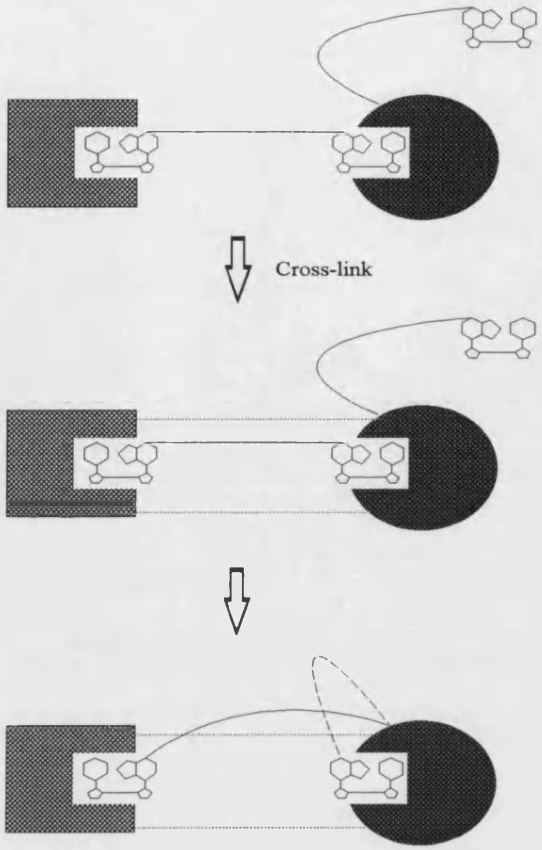


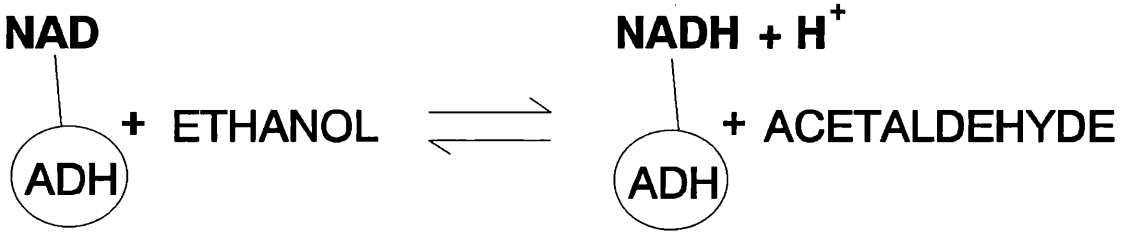
Figure 1.8: *Preparation of a self-contained coenzyme-regenerating enzyme reactor.* The active sites of two enzymes (alanine dehydrogenase and LDH) are orientated using bis-NAD so that they are opposite each other. The enzymes are cross-linked in this position and the bis-NAD removed. An NAD molecule covalently bound to one of the enzymes (LDH) now has access to both active sites and by passing from one to the other can be continually self-regenerated.

Recently, a self-contained semi-synthetic lactate oxidase was constructed by covalently binding NAD to LDH* along with the redox mediator 5-ethylphenazine (Yomo *et al*, 1992). 5-Ethylphenazine was oxidised in the presence of oxygen and it, in turn, oxidised NADH, regenerating the coenzyme which had been reduced in the presence of lactate. Both NAD and 5-ethylphenazine were first covalently bound to PEG (MW 3000) which was itself then bound to LDH using the spacer 3,3'-(1,6-dioxo-1,6 hexanediyl) bis-2-thiazolidinethione (DHBT). Incorporation of coenzyme was quite low at 0.3 molecules of PEG-NAD per subunit, but almost all of these coenzyme molecules were enzymically reducible. A low interaction between bound NAD and the redox mediator was reported because the covalently-bound NAD molecule was "hiding" in the active site of the enzyme. 80% of the specific activity of the LDH was maintained after modification and again the K_M value of the enzyme for free coenzyme

* From rabbit muscle

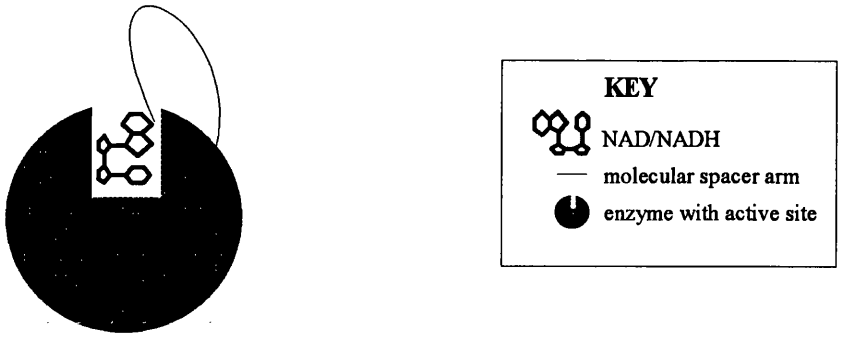
increased upon modification, due to competitive inhibition with bound NAD. This method was not considered for use in enzyme electrode experiments because of the undesirable involvement of PEG (*section 1.4.2*).

1.6.2 Alcohol dehydrogenase (ADH)[**]

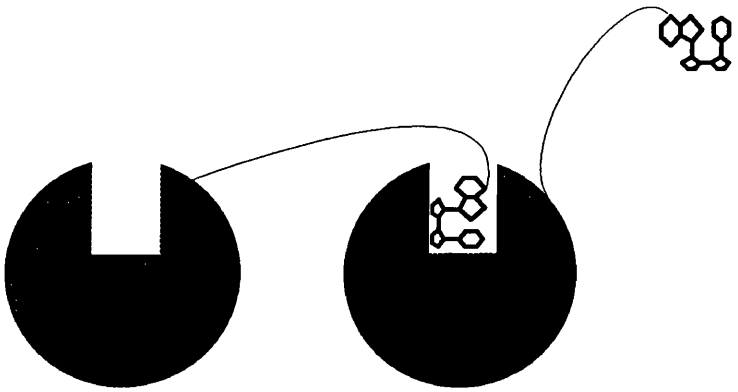


The first enzyme-coenzyme conjugate study using ADH involved the covalent fixation of the amino group of N^6AHCM-NAD to carbodiimide-activated acidic residues present on the surface of the enzyme, resulting in the formation of amide linkages (Mansson *et al*, 1978; Mansson *et al*, 1982). Of the 31 acidic amino acid groups in horse liver ADH (Branden *et al*, 1975) only 1.6 were modified with coenzyme per ADH subunit (M.W. 40,000). As was the case with LDH it seemed that very few of the reactive surface groups were accessible for coupling with coenzyme. One quarter of the bound NAD derivatives were deemed enzymically reducible with a second enzyme. Modification of ADH molecules led to a 73% reduction in specific activity but the intrinsic activity was relatively large at 16% of total specific activity of the native enzyme. In this study, Mansson and his co-workers showed how the mechanism of intrinsic activity could be determined. It was argued that if an intramolecular mechanism was predominant (*Figure 1.10*), the rate constant of the intrinsic reaction would be independent of the enzyme concentration and the reaction would therefore be of the first order. However, if an intermolecular reaction prevailed

[**] All attempts to covalently bind NAD to ADH so far have used the horse liver enzyme.



intramolecular activity



intermolecular activity

Figure 1.10: *Representation of the two mechanisms by which intrinsic activity can be generated by an enzyme-coenzyme conjugate*

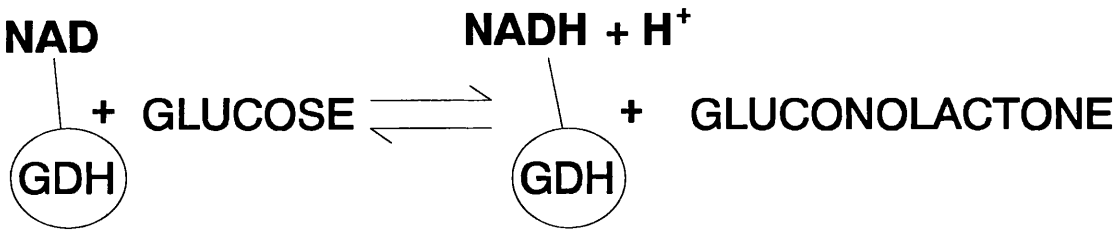
the rate constant would be dependent on the enzyme concentration and the reaction would be of the second order. This theory is explained in more detail in *Section 3.13.1.4.* The intrinsic activity generated by the ADH-N^{6}AHCM-NAD conjugate was due to an intramolecular mechanism. Evidence for a closer enzyme-coenzyme interaction upon covalent binding of coenzyme was again provided: the inhibitory effect of AMP was reduced markedly after enzyme modification, probably because of increased competition of the covalently bound coenzyme with the inhibitor. An attempt was initially made to perform the coupling reaction during the formation of a dead-end ternary complex between ADH, N^{6}AHCM-NAD and the pseudosubstrate pyrazole. However, during ternary complex formation no intrinsically active coenzyme became coupled to the enzyme. It was thought that the ternary complex was sterically blocking

an acidic amino acid residue, so that the coenzyme derivative could not become coupled near the active site. In a later study an NAD derivative with a similar, but slightly longer spacer arm than N^6AHCM-NAD (1.4nm), N^6[N-(8-amino-3,6-dioxaoctyl)carbamoylmethyl]-NAD (1.7nm) (*Figure 1.6d*), was also coupled to ADH using carbodiimide during the formation of a ternary complex with pyrazole (Goulas, 1987). In this case, the bound coenzyme derivative *was* intrinsically active, probably because the longer spacer arm allowed it to reach the active site when anchored to an acidic residue that was accessible during ternary complex formation. The incorporation of coenzyme was lower than the value reported by Mansson and his co-workers (*Table 1.1*) at 0.5 molecules per subunit and the specific activity was slightly higher at 52%. The difference of these values reflects the blockage of an accessible acidic residue by the ternary complex. This study emphasised the importance of spacer length of the coenzyme to be coupled and also showed that the use of ternary complex formation may block the optimum site of covalent binding of a coenzyme with respect to its active site interaction.

The ADH-N^6AHCM-NAD conjugate described above has become the most thoroughly investigated enzyme-coenzyme conjugate, and the results reported by Mansson and his co-workers have become established. The enzyme-bound coenzyme was shown to be enzymically active with LDH and MDH (Mansson *et al*, 1979; Mansson *et al*, 1982). In 1980, the conjugate was incorporated into an enzyme electrode for ethanol in the only study of its kind known to date (Torstensson and Johansson, 1980). Kinetic studies on the conjugate revealed that the coenzyme was bound in an open form (coenzyme not associated with coenzyme-binding-site) and available to interact with other active sites only 5% of the time when reduced and 30% of the time when oxidised. This study demonstrates that, when bound, the dissociation constant, K_d, for the oxidised and reduced coenzyme reflects that of free NADH and NAD (Kovar and Klukanova, 1984).

In another coenzyme-coupling approach using ADH, NAD derivatives were attached to residues on the surface of the enzyme via azo-bridges, as described in *Section 1.6.1* (Woenckhaus *et al*, 1983). Up to 1.0 NAD moiety was incorporated per enzyme subunit and between 30 and 80% of the specific activity of native ADH remained after modification. Quantitative values for intrinsic activity were not reported but the bound coenzyme molecules were enzymically reducible. Woenckhaus's group also fixed N^6-[(4-aminobutyl)-carbamoylmethyl]-NAD to ADH, but the conjugate was not well characterised: 0.3 molecules of coenzyme were incorporated per ADH subunit and the bound coenzyme was reducible.

1.6.3 Glucose dehydrogenase (GlcDH)



The first study on GlcDH-coenzyme conjugates involved the covalent binding of PEG-NAD to the surface of GlcDH from *Bacillus megaterium* (Nakamura *et al*, 1986). The cross-linker 3,3'-(1,6-dioxo-1,6 hexanediyl) bis-2-thiazolidinethione (DHBT) (*Figure 1.6c*) was used to activate amino groups of the linear hydrophilic polyethyleneglycol (MW 3000) molecule to react with ε-amine groups of surface lysyl residues of glucose dehydrogenase. The spacer arm (DHBT-PEG) was around 25nm long, and 2.1 PEG-NAD molecules were incorporated per GDH subunit (MW 30,000) ; 80% of these bound NAD moieties were enzymically reducible. Only 8% of the specific activity of GlcDH was lost after modification. The intrinsic activity was calculated as 2% of the specific activity of the conjugate. A remarkable improvement in the interaction between PEG-NAD and GlcDH was demonstrated upon formation of the GlcDH-PEG-NAD conjugate. Although free PEG-NAD is, in general, a good

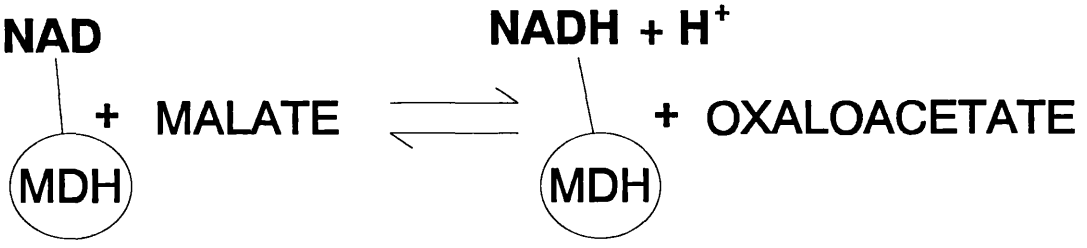
substrate for dehydrogenases its activity with glucose dehydrogenase is only 0.08% of that of free NAD (Nakamura *et al*, 1986). However, it was found that upon formation of a glucose dehydrogenase-PEG-NAD conjugate the bound coenzyme showed an astonishing 10,000-fold improvement in substrate activity; in fact enzyme-bound PEG-NAD was a more active substrate than free NAD. The reason for the increase in substrate activity of the coenzyme was put down to an apparent increase in concentration upon the covalent binding of the coenzyme near the enzyme active site - a so-called "anchimeric" assistance effect where the enzyme "feels like it's in a more concentrated coenzyme solution". This study greatly demonstrated the potential of enzyme-coenzyme conjugates. The GlcDH-PEG-NAD conjugate was subsequently used as an NAD regeneration unit in enzyme reactors for several dehydrogenases (Nakamura *et al*, 1988). However, this conjugate is probably unsuitable for use in enzyme electrodes because of the unsuitability of the PEG molecule to electrocatalytic regeneration of coenzyme (*Section 1.4.2*).

Recently, genetic engineering techniques were applied to the formation of an enzyme-coenzyme conjugate (Persson *et al*, 1991). Native GlcDH from *Bacillus subtilis* contains no cysteine residues. A three-dimensional computer model of the structure of GlcDH was used to find the most suitable area on the surface of the enzyme to introduce a single cysteine residue which could then be specifically coupled to a thiol-containing coenzyme. An aspartate residue of the native enzyme was replaced by a cysteine residue using the technique of site-directed mutagenesis. A thiol-containing NAD-derivative was made by attaching *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) to N^6AHCM-NAD (*Figure 1.6i*). The advantage of this technique was that the site of coenzyme coupling was precisely defined. The molecular ratio of coenzyme to GlcDH subunit was 1.0, confirming that all of the introduced cysteines had reacted with the coenzyme derivative. The intrinsic activity of the conjugate was 82% of the specific activity of the modified enzyme. However, the specific activity of the modified enzyme was only 5% of that of native GlcDH with free NAD because of the

poor reactivity of the thiol-containing NAD derivative. An intramolecular reaction mechanism was responsible for the intrinsic activity generated by the conjugate. The bound coenzyme could successfully interact with LDH in an enzyme reactor, and lactate and gluconic acid were produced from pyruvate and glucose. The disadvantage of this approach is that its success can not be predicted for the majority of NAD-dependent enzymes that inherently possess several cystyl residues.

Finally, a semisynthetic glucose oxidase was recently constructed by covalently binding 5-ethylphenazine-PEG-NAD to GlcDH (Yomo *et al*, 1991) as described for LDH in *Section 1.6.1*. 1.2 NAD molecules were bound per subunit, and the bound coenzyme was reported to be enzymically reducible.

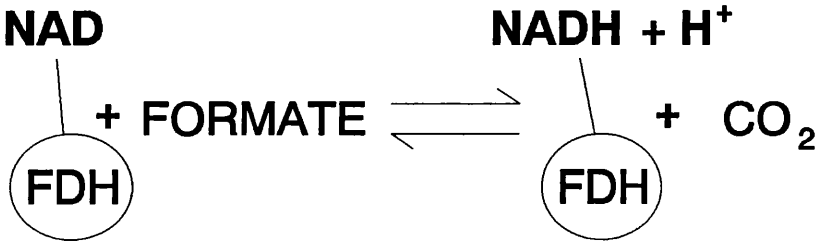
1.6.4 Malate dehydrogenase (MDH)



Only one enzyme-coenzyme conjugate study involving MDH is known: MDH from *Thermus thermophilus*, a heat-stable enzyme, was linked to NAD via a long PEG spacer (MW 3000) using DHBT (see above) (Eguchi *et al*, 1986). The high molecular weight of PEG-NAD allowed the degree of chemical modification to be detected by electrophoresis and the randomness of the chemical modification approach was demonstrated. The MDH-PEG-NAD preparation was quite heterogeneous: there were four different bands present after electrophoresis of the conjugate. Two of the bands possessed 1.2 PEG-NAD molecules bound per enzyme subunit (native subunit MW = 35,000), whilst the other two bands possessed 0.8 and 0.5 molecules of PEG-NAD bound per subunit. One of the fractions containing 1.2 PEG-NAD molecules per

subunit also contained the highest amount of protein and this fraction was used for further investigations. Over 80% of the bound NAD molecules were reducible. The specific activity of the modified enzyme was over 80% of the native enzyme but the intrinsic activity was only 0.07% of the specific activity. Enzyme-bound NAD was calculated to be dissociated from the active site 36% of the time. This figure was slightly higher than the 30% obtained for the ADH-N^{6}AHCM-NAD conjugate, probably because of the larger length of the polyethyleneglycol spacer arm involved (25nm). Again, the K_M of the modified enzyme for free NAD was increased after modification, this time by three-fold.

1.6.5 Formate dehydrogenase (FDH)



There has been only one enzyme-coenzyme conjugate study involving FDH: N^{6}AHCM-NAD was bound to FDH using the method of Mansson *et al* (1978) (*Section 1.6.2*). The amine function of the coenzyme was coupled directly to acidic residues of FDH by carbodiimide activation (Kato *et al*, 1987). 0.2 molecules of substrate-reducible NAD were bound per FDH (M.W. 36,000) and the specific activity of the modified enzyme was 16% greater than that of the native enzyme. This was a marked difference to 63% inhibition of ADH reported by Mansson *et al* (1978) using the same coupling technique (*Table 1.1*). There was also a difference in the intrinsic activity, it being 12.9% of the specific activity of the FDH-N^{6}AHCM-NAD conjugate compared to 40% for the ADH-N^{6}AHCM-NAD conjugate. This serves to illustrate that the success of a particular coupling technique will depend to a large extent on the constituent enzyme of the enzyme-coenzyme conjugate.

One may conclude that past studies on the preparation of enzyme-coenzyme conjugates have proved that the interaction between a covalently bound coenzyme and enzyme is feasible and is worth investigating as a favourable alternative to other methods of coenzyme retention and regeneration in enzyme electrodes. The length of the molecular bridge between enzyme and bound coenzyme appears to be important for an effective conjugate preparation. Although a maximum limit on the optimum length of such a bridge has not yet been set it has been shown that a spacer length of over 1.2nm is desirable for the coupling of certain coenzyme derivatives with LDH (Warth *et al*, 1989). The presence of a covalently linked coenzyme in the active site has been supported by increased K_M values of the modified enzymes for free coenzyme (Yomo *et al*, 1992; Schafer *et al*, 1986), the reduced effects of an inhibitor upon a coenzyme-modified enzyme (Mansson *et al*, 1978), a weakened interaction of a coenzyme-modified enzyme with affinity ligands (Eguchi *et al*, 1986) and increased thermostability of modified enzymes (Schafer *et al*, 1986). It has been shown that an intramolecular reaction prevails upon the binding of coenzyme to enzyme, although this may not necessarily be true for all conjugates studied in the future (Mansson *et al*, 1978; Persson *et al*, 1991; Eguchi *et al*, 1986 and Nakamura *et al*, 1986). Although all but one of the enzyme-coenzyme conjugates synthesised have involved the use of pre-derivatised NAD, the attraction of using unmodified NAD must not be discounted. Not only would the use of unmodified NAD reduce costs and simplify the manufacture of future applications of enzyme-coenzyme conjugates, but the derivatisation of NAD usually leads to a certain loss of biological activity of the coenzyme.

A quick glance back at *Table 1.1* shows that the approach to enzyme-coenzyme conjugate formation has been quite random in nature. There have been recent calls for more research to be carried out into forming enzyme-coenzyme conjugates in order to improve the knowledge of coenzyme coupling effects, to optimise the spacer lengths and to find the best coupling chemistries that can be used to form the link between

enzyme and coenzyme (Buckmann and Carrea, 1989). Cysteine, the amino acid that is generally the most reactive present on the surface of an enzyme, has only been exploited in one rather complex study that required the use of genetic engineering techniques. A simple chemical modification approach may be just as effective, if not more so, and would apply to a wider range of NAD-dependent dehydrogenases. There is also a need for further construction of enzyme-coenzyme conjugates using more established coupling techniques to include a range of dehydrogenases in order to establish which of these methods is more generally applicable.

It is clear then that more work needs to be carried out before enzyme-coenzyme conjugation can be fully exploited to make possible the construction of commercial enzyme electrodes for substrates of NAD-dependent dehydrogenases. There has only been one study involving the incorporation of enzyme-coenzyme conjugates into enzyme electrodes (Torstensson and Johansson, 1980). An ADH-N^6AHCM-NAD conjugate synthesised via carbodiimide coupling (*Section 1.6.2*) was used to prepare a reagentless electrode for ethanol by the immobilisation of the conjugate onto the surface of an amperometric electode. The success of the study was limited: the bound coenzyme was catalytically active for only one cycle, reportedly due to degeneration of the coenzyme at the graphite electrode surface. It seems that this study may have caused a loss of interest in the use of enzyme-coenzyme conjugates in enzyme electrodes. However, new electrode materials are now available and electrocatalytic coenzyme regeneration has since been greatly improved (*Section 5.1.1*). There is now a need for fresh electrode studies in order to demonstrate the potential of enzyme-coenzyme conjugates using up-to-date technologies.

The aims of this study are therefore laid out as follows:

1.7 The aims of this study

1. To use coupling methods that have been successful in the past for selected enzyme-coenzyme couples to investigate their applicability to a wider range of dehydrogenases. As discussed above, past investigations have been rather randomly executed and a more organised approach is required to increase the knowledge of a wide range of enzyme-coenzyme conjugates before their potential for use in enzyme electrodes can be fully realised. Those coupling techniques involving polyethyleneglycol were not considered because of their inapplicability to enzyme electrodes (*Table 1.1*). Because the complex methods of Woenckhaus and his co-workers (Woenckhaus *et al*, 1983; Schafer *et al*, 1986; Warth *et al*, 1989) did not convey any real advantages over the simpler, more established techniques of glutaraldehyde or carbodiimide-mediated coupling, these latter techniques were used to find out whether they were generally applicable (*Table 1.1*). Both of these techniques also had their individual advantages. The carbodiimide-coupling technique developed by Mansson *et al* (1978) gave rise to high intrinsic activities in both FDH- and ADH-coenzyme conjugates and would therefore be likely to generate a large physicochemical signal in the presence of substrate. However, the use of carbodiimide to couple unmodified NAD to enzymes has yet to be reported. In fact, the glutaraldehyde coupling method of Venn *et al* (1977) is the only method so far reported that is able to form conjugates from unmodified NAD. Both glutaraldehyde and EDC have proved to be stable when used in electrode studies (Aizawa *et al*, 1976; Miyamoto *et al*, 1991).

The enzymes chosen for use in coupling experiments were pig heart LDH, ADH from yeast, and bovine liver glutamate dehydrogenase, partly because of the commercial interest for the detection of lactate, ethanol and glutamate, respectively (*section 1.3*). LDH has been used in both enzyme electrode and enzyme-coenzyme conjugate experiments with success. LDH is in several ways a 'typical' dehydrogenase and was used as a model dehydrogenase in this study. It is also a relatively inexpensive enzyme,

as is yeast ADH. YADH was also chosen to compare its coenzyme conjugates to those of liver ADH. These two enzymes differ in many ways: perhaps most importantly, YADH is more specific for, and has a higher activity with, ethanol and could therefore be used to form a more specific and more sensitive enzyme electrode for ethanol. The yeast enzyme is also less expensive as it is easily obtained from baker's yeast. A glutamate dehydrogenase-coenzyme conjugate has not yet been constructed despite the interest in its substrate.

The coenzyme derivatives N^6AHCM-NAD and N^6CM-NAD were synthesised for coupling to the above enzymes because they have spacers of different length and chemical nature, but are on the same synthetic pathway starting from NAD (Mosbach *et al*, 1976). Attempts were also made to couple unmodified NAD.

2. To perform novel coupling experiments in an attempt to exploit the reactivity of cysteine residues as anchorage sites for coenzymes. BSA was used as a 'template' of a typical protein surface to achieve successful protein-coenzyme conjugates before LDH was used as a model dehydrogenase to demonstrate the potential of this approach. Extra thiol groups were introduced onto the surface of BSA and LDH to investigate the effect of increased incorporation of coenzyme. Unmodified NAD and the NAD derivatives described above were further derivatised to form thiol-containing coenzymes which would readily form disulphide bridges with cysteine residues. As a result, novel NAD derivatives were synthesised that may be of interest for future applications involving immobilised coenzymes

3. To synthesise and characterise the LDH-NAD and LDH-N^6AHCM-NAD conjugates described by Gacesa and Venn (1979) (*Table 1.1*) and incorporate them into an enzyme electrode for lactate in an attempt to demonstrate the potential of enzyme-coenzyme conjugates for use in reagentless biosensors. Improved electrode materials

were used and the enzyme electrode constructed involved a minimal number of components.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 General materials

All chemicals used in the preparation of buffers and solutions, along with all solvents and assay reagents, were from BDH Laboratory Supplies (Dorset, UK), Sigma Chemical Co. (Dorset, UK) or Fisons Scientific Equipment (Loughborough, UK) and were of AnalaR grade or equivalent. Gases were supplied by BOC (London, UK). Bovine serum albumin (fraction V) was from Sigma Chemical Co. (Dorset, UK). GFC Millipore and Whatman No.1 filter papers were from Whatman (Kent, UK). Optiphase "Safe" scintillation cocktail was obtained from FSA Laboratory Supplies (Loughborough, UK).

2.1.2 Enzymes and enzyme purification

Lactate dehydrogenase, E.C. 1.1.1.27 (pig heart, 480 U* /mg or bovine heart, 600 U/mg), malate dehydrogenase, E.C. 1.1.1.37 (pig heart, 480 U/mg) and alcohol dehydrogenase, E.C.1.1.1.1 (horse liver, 1-2 U/mg or baker's yeast, 320 U/mg) were obtained from Sigma Chemical Co. (Dorset, UK). Glutamate dehydrogenase, E.C. 1.4.1.3 (bovine liver, 170 U/mg) and phosphodiesterase, E.C. 3.1.4.1 (*Crotalus durissus* venom, 1.5 U/mg) were from Boehringer Mannheim UK (East Sussex, UK).

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* lactate dehydrogenase U/mg = μmol of pyruvate reduced /min/mg
malate dehydrogenase U/mg = μmol of oxaloacetate reduced /min/mg
alcohol dehydrogenase U/mg = μmol of ethanol oxidised /min/mg
glutamate dehydrogenase U/mg = μmol of oxoglutarate reduced /min/mg
phosphodiesterase U/mg = μmol of phosphate hydrolysed /min/mg

Charcoal (activated for adsorption, granular, 10-18 mesh) was obtained from BDH Laboratory Supplies (Dorset, UK).

Dialysis membrane (visking tubing size 3-20/32") was from Medicell International (London, UK).

2.1.3 Cross-linking reagents

25% Glutaraldehyde (Grade 1), 1-ethyl-3-(dimethylaminopropyl) carbodiimide, 1,6-diaminohexane and 2-aminoethanethiol were from Sigma Chemical Co. (Dorset, UK). *N*-hydroxysuccinimide and terephthalaldehyde were from Aldrich Chemical Co. (Dorset, UK), and 2-iminothiolane ("Traut's reagent") was from Pierce Chemical Co. (USA) c/o Life Science Laboratories (Luton, UK)

2.1.4 Column chromatography

All chromatography columns and Sephacryl S-100 were from Pharmacia (Uppsala, Sweden). Dowex 1X8-400 (Cl^-) and Dowex 50W-400 (H^+) were from Sigma Chemical Co. (Dorset, UK).

2.1.5 Synthesis of thiol-agarose

Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden), whilst epichlorohydrin, 2,2-dipyridyldisulphide ("Aldrithiol") and dithiothreitol were from Aldrich Chemical Co. (Dorset, UK).

2.1.6 Thin layer chromatography

PEI-Cellulose and Cellulose-F precoated thin layer plates (20 x 20cm) were from Camlab (Cambridge, England)

2.1.7 High Performance Liquid Chromatography

The ODS Hypersil™ column (4.6mm x 15cm) was from Anachem (London, UK) and was run using LDC/Milton Roy HPLC equipment.

2.1.8 Electrode materials

The electrode materials were kindly donated by Cambridge Life Sciences (Ely, UK). Activated carbon (5% Pt on Vulcan XC27R) manufactured by E-Tek is available from Mastik, Massachusets, USA.

2.2 Methods

2.2.1 Standard spectrophotometric dehydrogenase assays, carried out by monitoring the oxidation or reduction of NADH at 340nm

All enzymes and reagents were kept on ice and allowed to warm to assay temperature when required. Each sample was analysed in triplicate.

Lactate dehydrogenase

0.10ml of 23mM sodium pyruvate and 0.05ml of 12mM NADH were added to 2.83ml of 100mM potassium phosphate buffer, pH7.0, and mixed. The enzyme sample (0.02ml) was then added to start the reaction.

Alcohol dehydrogenase

0.1ml of 95% ethanol (w/v), 0.1ml of 2.2M semicarbazide, pH 6.5, 0.2ml of 28mM NAD and 0.1ml of 1mg/ml BSA were added to 2.5ml of 75mM glycine-sodium pyrophosphate buffer, pH 9.0 and mixed. The enzyme sample (0.02ml) was then added to start the reaction.

Glutamate dehydrogenase

0.2ml of 0.2M 2-oxoglutarate, 0.05ml of 12.8M ammonium acetate, 0.03ml of 12mM NADH, 0.10ml of 25mM EDTA and 0.05ml of 0.1mM ADP were added to 2.5ml of 0.1M imidazole.HCl buffer, pH7.9, and mixed. The enzyme sample (0.02ml) was then added to start the reaction.

Malate dehydrogenase

0.1ml of 15mM oxaloacetate and 0.05ml of 12mM NADH were added to 2.83ml of 100mM potassium phosphate buffer, pH 7.5, and mixed. The enzyme sample (0.02ml) was then added to start the reaction.

2.2.2 Coenzyme recycling assays using phenazine ethosulphate (PES) as a redox mediator and either diclorophenolindophenol (DCPIP) or 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) as colourimetric redox indicators

The DCPIP-PES chemical recycling assay for dehydrogenase activity.

This assay was based on the method of Venn *et al*, 1977. *Specific activity:* 0.05ml of 1mM dichlorophenolindophenol, 0.05ml of 40mM phenazine ethosulphate, 0.05ml of 60mM NAD and 0.05 ml of either 1M lactate (for lactate dehydrogenase assay) or 0.5M glutamate (for glutamate dehydrogenase assay) were added to 0.70-0.79ml of ice-cold 100mM potassium phosphate buffer, pH 7.5, and mixed. The reaction was kept in the dark and was initiated by the addition of 0.01-0.10ml of enzyme sample. The reaction was followed by monitoring the change in absorbance of the solution at 610nm in a spectrophotometer and each sample was assayed in triplicate. *Intrinsic activity:* The assay was carried out as above, except for the omission of NAD, the volume of which was made up with buffer.

The MTT-PES chemical recycling assay for dehydrogenase activity

This assay was adapted from the method of Nisselbaum and Green (1969). *Specific activity:* 0.05ml of 12mM 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide. 0.8ml of 3mM phenazine ethosulphate and 0.05ml of 100mM NAD were added to ice-cold buffer, pH 7.4, made up of 100mM triethanolamine, 100mM nicotinamide and either 500mM ethanol (for alcohol dehydrogenase assays) or 20mM glutamate (for glutamate dehydrogenase assays). The buffer was added so that the volume of the assay was between 2.90 and 2.99ml before the addition of 0.01-0.10 ml of enzyme sample. The reaction was mixed and kept in the dark and the change in absorbance at 556nm recorded between 5 and 30 minutes after the start of the reaction. *Intrinsic activity:* The assay was carried out as above, except for the omission of NAD, the volume of which was made up with buffer.

The MTT-PES chemical recycling assay for the measurement of NAD concentration.

This assay was based on the method of Nisselbaum and Green (1969): 0.05ml of 12mM 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide, 0.80ml of 3mM phenazine ethosulphate and 0.10ml of a sample of NAD or NAD derivative were added to 100mM triethanolamine/ 100mM nicotinamide/ 500mM ethanol buffer, pH7.4, to a final volume of 2.90ml and mixed. 0.10ml of yeast alcohol dehydrogenase (1mg/ml) was added to initiate the reaction, which was kept in the dark, and the rate of the reaction was calculated from the change in absorbance at 556nm between 5 and 15 minutes after the reaction start. Each sample was assayed in triplicate. NAD solutions of concentration beween 0 and 0.05mM were used as samples from which to prepare a calibration curve. In cases where alcohol dehydrogenase-coenzyme conjugates were assayed, lactate dehydrogenase was used as the assay enzyme: 100mM triethanolamine/ 100mM nicotinamide buffer, pH 7.4 was used and lactate was present at 50mM in the assay. 0.10ml of lactate dehydrogenase (1.5 mg/ml) was added to initiate the reaction.

2.2.3 Measurement of K_M

0.1ml of 1.2M lactate and 0.1ml of a coenzyme sample (0.01 - 1mM) were added to 2.78 ml of 0.1M sodium pyrophosphate buffer, pH 7.0 and mixed. Finally, 0.02ml of freshly dialysed beef heart LDH (0.02mg/ml) were added to start the reaction which was followed at 340nm in a spectrophotometer. The rate of change in absorbance at 340nm was plotted against the concentration of coenzyme in the assay to obtain a substrate saturation curve. Perpendicular lines were drawn from the x and y axes to each point on the saturation curve, and the extrapolation described by Eisenthal and Cornish-Bowden (1974) was used to determine the K_M value of the enzyme for the coenzyme.

2.2.4 Preparation of the NAD derivatives N^6CM-NAD and N^6AHCM-NAD

The synthesis of the above derivatives was based on the method of Mosbach *et al* (1976).

N^6CM-NAD

15g of iodoacetic acid was dissolved in a minimum amount of double-distilled water and the solution neutralised using 2M lithium hydroxide. NAD (5g) was added and the pH adjusted to pH 6.5 using 2M lithium hydroxide. The pH was maintained twice daily and the reaction kept in the dark at room temperature. The conversion was followed using TLC and HPLC, and when at least 90% of the NAD had reacted, the pH was lowered to pH 3.0 using 6M hydrochloric acid. Two volumes of ice-cold 95% ethanol were added and the whole solution poured into 10 volumes of vigorously stirred ice-cold 95% ethanol before the mixture was left overnight at 4^{o}C. The precipitate was filtered and washed with ethanol and ether (250ml of each) and dissolved in 150ml of 0.1M triethanolamine buffer, pH 8.5. Sodium dithionite (2.5g) was added to the solution which was then left for 2 hours in the dark at room temperature. The reduction was terminated by bubbling a stream of oxygen through the solution for 20mins, followed by 1min of a stream of nitrogen. 2M

sodium hydroxide was added to the solution until the pH reached pH 11.5. The alkaline solution was heated at 75°C for 60mins, and then cooled on ice. The reduced coenzyme was enzymically oxidised at room temperature by adjusting the pH of the solution to pH 7.5, and adding 10ml of 2M Tris.HCl, 2.5ml of fresh acetaldehyde and 500 units* of yeast alcohol dehydrogenase. The oxidation reaction was followed by monitoring the decrease in absorbance at 340nm in a spectrophotometer. After the oxidation was complete the solution was acidified to pH 3.5 using 6M hydrochloric acid and 1 volume of 95% ethanol was added. The solution was poured into 10 volumes of vigorously stirred ice-cold 95% ethanol, left at 4°C overnight, filtered, washed with ethanol and ether, and dried in a vacuum dessicator. The N^6CM-NAD was used in this form in coupling experiments, but the kinetic properties of the coenzyme were determined after further purification, as follows: a chloride-charged Dowex 1-X8 (200-400 mesh) anion exchange column (2.5 x 30cm) was loaded with a solution that contained the crude precipitate of N^6CM-NAD (40ml, in water, pH 8.0). The column was washed with 250ml of double-distilled water, followed by 5mM calcium chloride, until the pH of the effluent had decreased to pH 2.8. A step gradient of 500ml each of 5, 10, 20, 30, 40 and 50mM of calcium chloride was applied. The effluent between 1.6 and 2.5 litres was collected and neutralised with calcium hydroxide solution. The purified N^6CM-NAD solution was concentrated to 5ml on a rotary evaporator and freeze-dried. The pure N^6CM-NAD obtained was stored at -20°C in a sealed container, containing a packet of hygroscopic silica gel, before analysis.

N^6AHCM-NAD

2.25g of crude N^6CM-NAD was dissolved in 30ml of 2M 1,6-diaminohexane dihydrochloride, prepared by lowering the pH of 1,6-diaminohexane to pH 5.0 using hydrochloric acid. EDC (0.7g) was dissolved in 2.5ml of water and added to the solution; the pH was kept at pH 4.8 by adding 1M lithium hydroxide or 1M hydrochloric acid. After

* one unit will reduce 1μmol acetaldehyde per minute at pH 7.0 and 25°C

4 h, the pH of the solution was increased to pH 6.5 and 1 volume of 0.5M lithium chloride in ethanol was added; a precipitate was obtained using ethanol, as described above. The precipitate, which contained a strongly red by-product, was dried in a vacuum. N^6AHCM-NAD was used in this form in coupling experiments, but was purified further before kinetic characterisation, as follows. The crude N^6AHCM-NAD precipitate was dissolved in 40ml water (pH 8.0) and passed through a lithium-charged Dowex 50W-X8 (200-400 mesh) cation exchange column (2.5 x 30cm). The effluent was adjusted to pH 6.0 with hydrochloric acid and then passed through a chloride-charged Dowex 1 X8 (200-400 mesh) anionic exchange column (2.5 x 30cm) and the final effluent adjusted to pH 6.8. The pure N^6AHCM-NAD was concentrated on a rotary evaporator to 5ml, before freeze-drying, and storage at -20^{o}C in a sealed container containing a packet of hygroscopic gel.

2.2.5 Preparation of dehydrogenase-coenzyme conjugates coupled using glutaraldehyde.

This preparation was based on the method of Gacesa and Venn (1979). Solid coenzyme (0.075mmol) was dissolved in a solution containing 2ml of 50mM sodium acetate buffer, pH 5.7 and 3ml of 25% glutaraldehyde. The mixture was left at room temperature for 4 h before 10 volumes of chilled 95% ethanol were added. The solution was then left in a freezer for 5 hr, and the resulting precipitate was spun down in a Sorvall RC-5B centrifuge at 35,000g at a tube temperature of -10^{o}C. The pellet was thoroughly washed in ice-cold 95% ethanol and redissolved in 1ml of 0.1M potassium phosphate buffer, pH7.5. 1.5ml of dehydrogenase (1.4mg/ml) which had been freshly dialysed against 3 x 2 litre portions of 0.1M phosphate buffer, pH 7.5, for a total of 18 hr, was added to the modified coenzyme solution and left for 24min at 4^{o}C. 4M ethanolamine.HCl (0.05ml) was added and left for 30 minutes to stop the reaction. The pH was adjusted to pH 8.5 using 0.2M sodium hydroxide, and solid potassium borohydride was slowly added to a final concentration of 25mM. The solution was left for 20 minutes before the pH was reduced to pH 7.0. The solution was left to stand for 2 hours, or until the effervescence had stopped, before it

was applied to a Sephacryl S-100 gel filtration column (80 x 1.5cm), with 0.1M potassium phosphate, pH7.5, as running buffer. Any remaining non-covalently bound coenzyme was removed using charcoal (*Section 2.2.10*)

2.2.6 Preparation of dehydrogenase-coenzyme conjugates coupled using terephthaladehyde

7.5μmol of coenzyme was added to a solution of yeast alcohol dehydrogenase (8mg in a total of 1.6ml) which had been freshly dialysed against 3 x 2 litre volumes of 50mM sodium phosphate buffer, pH 7.5, for a total of 18hrs. 100% formamide was added to 5%. 0.2ml of 0.5% terephthalaldehyde in 10% formamide / water (v/v), heated to 40°C in a water bath, was added to the solution which was then left for 12 hours at room temperature. 4M ethanolamine.HCl (0.05ml) was added and mixed periodically for 30 mins to stop the reaction. The conjugate was then reduced and purified as described above for glutaraldehyde-coupled conjugates.

2.2.7 Preparation of dehydrogenase-coenzyme conjugates coupled using carbodiimide

Carbodiimide coupling of amine-containing coenzyme was carried out according to the method of Mansson *et al* (1978). 5μmol of coenzyme, NAD or N^6AHCM-NAD, was added to enzyme (50nmol with respect to subunit concentration), in 1ml of 50mM triethanolamine buffer, pH 7.5. The enzyme was dialysed against 3 x 2 litre portions of 0.1M triethanolamine buffer, before use. EDC (0.025mmol) and *N*-hydroxysuccinimide (0.012mmol) were supplied in four or two equal portions, respectively, at 12 hour intervals. The pH was maintained at pH7.5 in a Radiometer pH-stat using sodium hydroxide. After 48 hours, the reaction was stopped by adding 2M glycine buffer, pH7.5, to a final concentration of 0.1M. The coupling solution was then applied to a Sephacryl S-100 gel filtration column (1.5 x 80cm), using 0.05M sodium bicarbonate, pH7.5, as

running buffer (section *2.2.19*). Any remaining non-covalently bound coenzyme was removed by charcoal adsorption (*section 2.2.10*).

Carbodiimide coupling of carboxyl-containing coenzymes was carried out as follows. 0.01mmol of coenzyme was dissolved in 1ml of water and brought to pH7.5 using 1M sodium hydroxide in a Radiometer pH-stat. 0.1mmol of EDC and 0.06mmol of *N*-hydroxysuccinimide were added and the pH maintained at pH7.5. After 4 hrs at room temperature, the mixture was added to 50nmol enzyme (with respect to subunit concentration) in 1ml 0.1M potassium phosphate buffer, pH7.0 (LDH) or triethanolamine buffer, 50mM, pH7.5 (ADH and GDH). After 48 hrs the enzyme-coenzyme conjugate was purified as described above.

2.2.8 Synthesis of thiolated nucleotide derivatives

a.) Thiolated derivatives of NAD and adenosine

7.5, 15, 75, or 225 mg of solid 2-iminothiolane was added to 1ml of triethanolamine buffer, 50mM, pH 8.0 containing 6µmol of adenosine or NAD. The reaction was carried out at 4°C or 24°C for up to 96 hours and was also attempted at pH 8.5 (50mM sodium pyrophosphate), pH 10 (borate) or pH 7.0 (sodium phosphate buffer). The thiolated derivative was purified as described below.

b.) Thiolated derivatives of N^6AHCM-NAD

6µmol of the NAD derivative was added to 1ml of 50mM triethanolamine buffer, pH 8.0 or pH 9.0, followed by 15mg of Traut's reagent. The reaction was left at 4°C for 2 hours. The thiolated derivative was purified as described below.

c.) Thiolation of N^6CM-NAD

0.014mmol of N^6CMNAD was added to 0.3mmol of 2-aminoethanethiol in 1.5ml of water and the pH was adjusted to 4.5. 0.1mmol of EDC and 0.06mmol of *N*-hydroxysuccinimide were added and the pH maintained at 4.5 using 0.1M hydrochloric acid or 0.1M lithium chloride in a Radiometer pH-stat and left for 2hrs at room temperature. The solution was purified as follows.

The aqueous coenzyme solution was dissolved in 10 volumes of chilled 95% ethanol and left at -20°C until a precipitate had formed. The precipitate was centrifuged, washed with ethanol and this precipitation and purification process repeated several times. Finally, the precipitate was dissolved in 0.5ml of double distilled water and freeze-dried before analysis.

***2.2.9** Measurement of protein concentration using Coomassie Brilliant Blue G250*

Coomassie reagent was prepared according to the method of Sedmak and Grossberg (1977). To 1.00ml of reagent, 0.90-1.00ml of saline were added and the solution was mixed. Protein sample was added to a final volume of 2.00ml, the solution mixed and left for 5 mins before the absorbance was read at 620nm. The A_{620} value was compared with a calibration curve prepared using known concentrations of samples of the protein under assay.

***2.2.10** Removal of traces of non-covalently bound coenzyme from protein solutions using activated charcoal*

Solid charcoal, activated for adsorption, was added to a gel-filtrated protein solution in a ratio of 1 part charcoal to 1 part protein (w/w) in an eppendorf tube, at room temperature. The solution was agitated briefly in a whirlimixer at intervals of 5 mins for a total time of

30mins, before the solution was spun in a microfuge for 5mins. The supernatant was taken for analysis, and the pellet discarded.

2.2.11 Protein concentration using polyethyleneglycol

The dilute protein solution was concentrated against polyethyleneglycol (M.W. 20,000) by placing the solution in a length of sealed dialysis tubing, and then covering the tubing with flakes of solid polyethyleneglycol, in a tray. The outside of the tubing was thoroughly washed with distilled water to remove all traces of polyethyleneglycol before the protein solution was extracted from the dialysis tubing.

2.2.12 Thiolation and activation of protein surfaces using Traut's reagent, 2-iminothiolane and dipyridyldisulphide

This method was based on that of Jue *et al* (1978). 0.1ml of Traut's reagent (4mg/ml) was added to 0.9ml of dialysed BSA (2mg/ml) or LDH (1.2mg/ml) in 50mM triethanolamine buffer, pH 8.0. The reaction was left for 2 hrs before gel filtration at 4°C using a Sephacryl S-100 column (1.5 x 60cm).

Activation 0.05ml of dipyridyldisulphide (1mg/ml for BSA activation or 0.6mg/ml for LDH activation), in 50% acetone/water (v/v), was added to 0.95ml of BSA (1mg/ml) or LDH (1mg/ml) and the reaction was followed to completion by monitoring the release of pyridine-2-thione at 343nm in a spectrophotometer. The DPDS-thiolated protein was purified by gel filtration on a Sephacryl S-100 column (1.5 x 60cm).

2.2.13 The effect of dipyridyldisulphide on the specific activity of dehydrogenases

0.00-0.20ml of dipyridyldisulphide (1mg/ml) in 50% acetone/water (v/v) was added to 1ml of dialysed enzyme (0.2mg/ml) in 50mM triethanolamine buffer, pH 8.0 and left for 3 hours at 4°C. Each enzyme was then dialysed against 3 x 2 litres of 50mM phosphate buffer, pH 7.5 before a standard assay for residual specific activity at 340nm.

2.2.14 Formation of protein-coenzyme conjugates linked via a disulphide bond.

0.3μmol of thiolated coenzyme derivative was added to 1ml of 0.70 mg/ml DPDS-activated LDH solution or 0.5μmol thiolated coenzyme was added to 0.70mg/ml DPDS-activated BSA in 50mM triethanolamine buffer, pH 8.0. The mixture was agitated with an overhead stirrer at room temperature and the reaction was followed intermittently by observing the change in absorbance of the solution at 343nm. The reaction was complete after 2 hrs and was left for a further hour at room temperature. The protein-coenzyme conjugate was purified on a Sephacryl S-100 gel filtration column (1.5 x 80cm) at 4°C, concentrated using polyethyleneglycol, washed with activated charcoal (1:1 [w/w]) and stored at 4°C for no longer than 12 hrs before analysis.

2.2.15 Preparation of activated thiol agarose

Thiol agarose was prepared as according to Dean *et al* (1986): 100g of 6% Sepharose 4B beads were thoroughly washed with water and collected on a sintered glass funnel under suction. The gel was suspended in 80ml of 1M sodium hydroxide and 2.5ml of epichlorohydrin was added. The suspension was gently agitated using an overhead stirrer for 15mins at room temperature and then for 4hrs at 60°C in a water bath. The resulting epoxy beads were washed with water until neutral, followed by 5 volumes of 0.5M sodium phosphate, pH 6.2. The beads were immediately filtered under suction - no air was allowed to pass through the beads - and 100ml of 2M sodium thiosulphate added. The beads were stirred in the thiosulphate for 8hrs at room temperature before they were washed in 15ml of 8mg/ml DTT in 1mM EDTA. After 30mins at room temperature the beads were removed from the reduction solution and filtered under suction, making sure that no air passed through the beads. The thiol agarose was washed with 1 litre each of i.) 0.1M sodium bicarbonate containing 1M sodium chloride ii.) 1mM EDTA, and iii.) 10mM sodium acetate, pH4.0, containing 1mM EDTA. The beads were not stored in their reduced state, but immediately activated as follows: The beads were washed with 50%

solution of acetone/water (v/v) before suspension in 50% acetone/water (v/v) containing dipyridyldisulphide (100mg). The suspension was agitated for 2 hrs using an overhead stirrer. The DPDS-activated thiol agarose beads were washed with several volumes of 50% acetone/water (v/v) followed by several volumes of 1mM EDTA, pH7.0. They were stored at 4°C in degassed phosphate buffer, 0.1M, pH 7.0 containing 1mM EDTA.

2.2.16 Activated thiol agarose column

A small graduated plastic column (0.5x10cm) fitted with a reservoir was packed with 5ml of swollen, activated thiol agarose beads. The gel was thoroughly washed with several volumes of sodium pyrophosphate, 0.1M, pH 8.0 (running buffer), and checked to see that the absorbance of the eluant at 343nm was zero. The sample was carefully loaded using a pipette, onto the top of the gel bed and allowed to run in. Running buffer was then carefully added until the reservoir was loaded. The column was run under gravity with a flow rate of 5ml per hr. The sample was eluted and washed through until the absorbance of the eluent at 343nm remained at zero. Any material that had bound to the activated thiol ligands was then eluted using a solution of 3mM DTT in running buffer. The concentration of bound sample eluted was calculated using A_{343} values against a blank solution containing 3mM DTT. The molar extinction coefficient of pyridine-2-thione was 8100/M/cm at 343nm. Each time the gel had been used it was discarded and a fresh column made up using stored DPDS-activated thiol agarose.

2.2.17 Measurement of radioactivity

Acid-insoluble radioactivity was measured as follows. To the ice cold protein sample, ice-cold 100% trichloroacetic acid was added to a final concentration of 20%. If the sample volume was below 1ml, it was made up to 1ml before the addition of trichloroacetic acid using 0.2 mg/ml BSA in water as a co-precipitate. The sample was left on ice for at least 1 hr, before the precipitate was passed through a GFC filter disc

(previously washed with 2ml of ice-cold 5% trichloroacetic acid) in an ultrafiltration tower, under suction. The disc was washed with 10ml of 5% trichloroacetic acid, before it was dried in a warm oven. The disc was then placed in a scintillation vial and 2ml Optiphase "Safe" scintillation cocktail was added. The whole vial was shaken before it was assayed for radioactive counts as described below.

Total radioactivity was measured as follows: At least 0.2ml of sample solution was added to Optiphase "Safe" scintillation cocktail in a scintillation vial, 10 parts of scintillant to 1 part of sample solution, mixed thoroughly and counted for radioactivity. The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer for at least 5mins with the ^{3}H and ^{14}C "windows" open. The counting efficiency of the spectrometer was determined as 60% for ^{3}H and 98% for ^{14}C using respective dated samples of known count values given in disentegrations per minute.

2.2.18 Thin layer chromatography

All thin layer chromatography experiments were performed at room temperature using 0.1M, 0.3M and 0.5M lithium chloride on PEI-cellulose pre-coated plastic sheets, or 1M isobutyric acid : aqueous ammonia (5:3 v/v), saturated with EDTA, on Cellulose-F pre-coated plastic sheets. Before use, the thin layer sheets were washed by soaking in 2M sodium chloride for 60mins, rinsing with distilled water, soaking with distilled water for 60mins, and drying at room temperature. 95% ethanol was added to each nucleotide sample to a final concentration of 50%, before it was applied at the origin, 1.0cm from the labelled bottom of the thin layer sheet using a 10-100µl capillary tube. Each spot was dried using a stream of cool air. The thin layer sheet was placed in a glass tank containing solvent to a height of 0.5cm, and the tank was sealed. When the solvent front had reached at least 4cm from the top of the sheet, the sheet was extracted from the tank and left to

dry at room temperature. The sheet was analysed for nucleotide spots using an ultra-violet lamp set at 250-280nm.

2.2.19 Gel filtration chromatography

Gel filtration was performed at 4°C using a Sephacryl S-100 column, bed volume 1.5cm x 80cm (*Chapter 3 and Chapter 4*) or 1.5 x 60cm (*Chapter 4*), with a flow rate of 0.2ml/min. In all cases the gel was swollen overnight in running buffer, before the column was packed. The fractions were collected in a dropwise manner, under gravity using an LKB Redirac fraction collector. Each sample (1-3ml) was carefully applied to the top of the gel bed using a pipette, and allowed to run in. The buffer was then carefully applied to the top of the bed until the rest of the column was filled, and the buffer reservoir was attached to the column. Before use, each freshly-packed column was calibrated using Blue Dextran 2000 and riboflavin. The eluted fractions were stored at 4°C or taken for immediate analysis.

2.2.20 Ion exchange chromatography

A Pharmacia ion exchange column (2.5 x 30cm) was packed with Dowex 1-X8 (200-400 mesh; chloride charged) or Dowex 50W-X8 (200-400 mesh; lithium charged). The Dowex beads were thoroughly washed with double-distilled water before use. Each column was run under gravity at a flow rate of 0.5ml/min; the sample (40ml) was applied and collected as described for gel filtration chromatography.

2.2.21 High performance liquid chromatography (HPLC)

HPLC was carried out at room temperature using an ODS Hypersil™ column (0.48 x 15cm) at 1800 lb/in^2 with a flow rate of 1ml/min. All buffers and solvents were filtered through a millipore filter and then degassed by bubbling helium through them for 5mins. Before use, the column was left to equilibrate with 10mM of potassium dihydrogen

phosphate buffer, pH6.8, (running buffer) for at least 3min using a flow rate of 1ml/min. 0.5ml of nucleotide sample (~0.2mg/ml), in either water or running buffer, was injected into the loading chamber. The sample was loaded, and a linear gradient of 0-30% methanol in running buffer (v/v), over 30 min, was applied to the column simultaneously. The eluted nucleotide material was analysed using an ultraviolet meter, set at 260nm, connected to a chart recorder. The purity of the applied sample was calculated from the area under each eluted peak on comparison with a standard sample.

2.2.22 Enzyme-electrode preparation and operation

Electrode preparation and operation was carried out at room temperature but all solutions used were stored at 4°C. 0.25ml of enzyme preparation (1-2mg/ml) in 0.1M potassium phosphate buffer, pH 7.5, was mixed with 25 mg of adsorptive carbon in an eppendorf tube; the suspension was agitated occasionally over a period of 60 min. The eppendorf tube was microfuged for 5 min, or until a clear supernatant was obtained. The supernatant was discarded and the carbon pellet was mixed with 40µl of a commercial teflon binder solution using a microspatula, until a paste formed. The paste was compressed onto a $3cm^2$ layer of conductive carbon paper by firmly drawing the edge of a glass slide over the carbon paste. The electrode preparation was left to dry for 15 min and stored, if necessary, in PESK buffer, pH 7.4 at 4°C; PESK buffer comprised 100mM sodium dihydrogen phosphate, 50mM EDTA (disodium salt) and 100mM sodium chloride. Discs of 2mm diameter were cut out of the enzyme-electrode surface using a hole-punch, and inserted into the linings of one of two titanium-lined working electrodes, with the enzyme-carbon layer facing upwards (see *Figure 5.5*). The electrodes were covered with a polycarbonate membrane (pore size 50nm) held in place with a rubber "O" ring. The half-cell was connected to a Ag/AgCl reference electrode and the whole cell screwed into place at the bottom of a solution reservoir. The reservoir was supplied with PESK buffer, pH7.4, and an overpotential of 150mV applied to the cell using a potentiometer. The enzyme

electrode was left until a steady current was flowing through each working electrode, the current being measured using a twinned-pen chart recorder.

Electrode operation

PESK buffer was removed from the solution reservoir using a plastic pipette and excess liquid was removed from the outer surface of the polycarbonate membrane using a cotton bud. The sample, containing the analyte, was pipetted into the solution reservoir (1.5ml) and mixed using an overhead stirrer. The electrode response due to the change in current was measured on the chart recorder. The solution chamber was rinsed out with PESK buffer, pH 7.4 and each electrode allowed to reach a steady current, before the application of the next sample.

CHAPTER 3

Preparation of dehydrogenase-coenzyme conjugates coupled via amine- and carboxyl- functional groups

Introduction

The topics dealt with in the first part of this chapter are outlined in *Figure 3.1*.

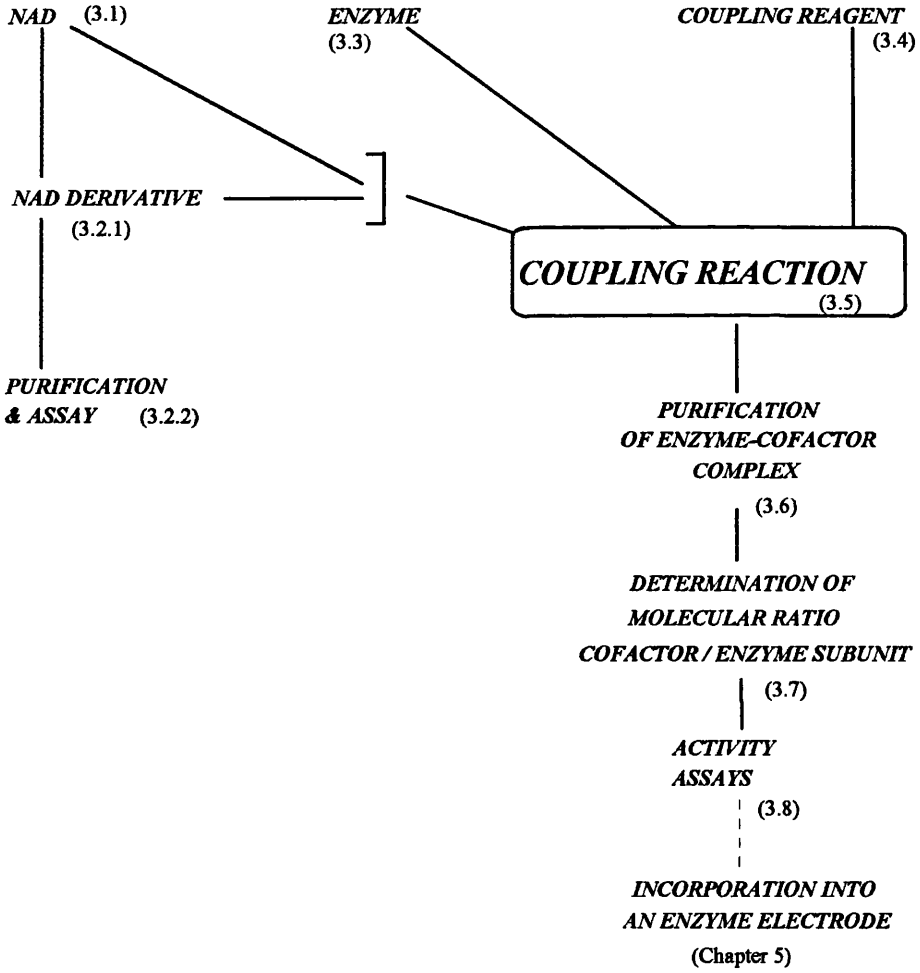


Figure 3.1: *An overview of the approach used in the synthesis and characterisation of dehydrogenase-coenzyme conjugates coupled via amine- and carboxyl- groups.* The numbers in brackets relate to sections of the first part of this chapter.

Figure 3.1 also serves as an overview of the approach used to synthesise and study enzyme-coenzyme conjugates synthesised via carboxyl and amine groups in this study.

A number of different dehydrogenases, coenzymes and coupling reagents were used to prepare a variety of dehydrogenase-coenzyme conjugates. As can be seen in *Figure 3.1*, each dehydrogenase was coupled to its natural coenzyme, NAD, or else the nucleotide was first modified to form a derivative of NAD which was then used for coupling. Each NAD derivative was characterised before use in order to validate its synthesis and to ensure that it had significant biological activity as a coenzyme.

Two coupling methods were used. The first method involved the use of a homobifunctional dialdehyde to link an amine group present on the coenzyme to one of the several amine groups found on the surface of the protein. The second method employed a carbodiimide reagent to link either an amine group present on the coenzyme to one of several carboxyl groups of the protein, or a carboxyl group present on the coenzyme to one of several amine groups of the protein.

Three different dehydrogenases were used in these modification studies: lactate dehydrogenase, alcohol dehydrogenase and glutamate dehydrogenase. The relevant properties of each enzyme, along with the reasons for using them, can be found in the second part of this chapter which also describes the properties of the dehydrogenase-coenzyme conjugates that were synthesised.

Meanwhile, the first part of this chapter describes the individual steps of the approach used to generate these conjugates and presents the preparative results that arose from the use of this approach.

PART ONE: The approach used to synthesise enzyme-coenzyme conjugates

3.1 NAD

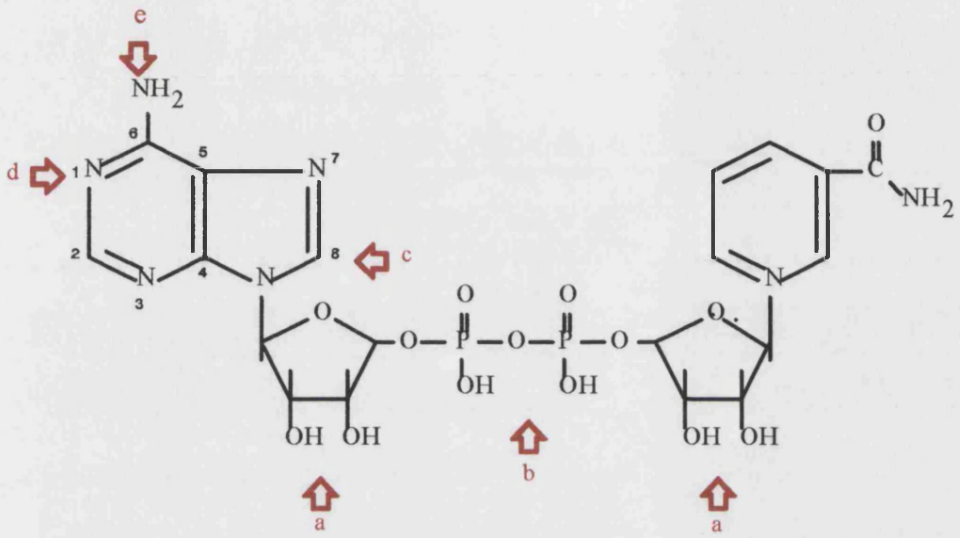


Figure 3.2: *The structure of NAD, showing suitable points for derivitisation.* The nicotinamide moiety is involved in catalysis and the ribose and phosphate moieties are involved in enzyme recognition. Although the adenine moiety is also involved in enzyme recognition, chemical modification at this position is least likely to lead to a serious loss in substrate activity of the coenzyme. The points that may be easily derivatised are shown: (a) ribosyl linkage, (b) phosphate linkage, (c) linkage at C-8 of adenine moiety, (d) linkage at N-1 of adenine moiety, (e) linkage at N^6 of adenine moiety.

The structure of NAD is illustrated in *Figure 3.2* Although several potential modification sites for linkage to an enzyme molecule are evident, NAD modification studies have shown that very few sites are acceptable for modification if the biological activity of the coenzyme is to be maintained (Lowe *et al*, 1980).

From NAD-binding studies with lactate dehydrogenase (Holbrook *et al*, 1975), alcohol dehydrogenase (Branden *et al*, 1975) and glyceraldehyde 3-phosphate dehydrogenase (Rossman *et al*, 1974) the following conclusions were drawn:

1. The pyridine nucleus is the active centre of the NAD molecule and so it is very sensitive to modification.
2. The phosphate moieties are involved in generating the active conformation of the NAD molecule, and their modification results in a total loss of biological activity.
3. The ribose moieties bring about orientation of the coenzyme by hydrogen bonding to enzyme side chain residues in the active centre, and so they are also sensitive to modification.
4. The adenine nucleus is important in coenzyme binding, although substitution here results in NAD derivatives with relatively high biological activity.

The adenine moiety has, in fact, been established as the most desirable moiety for derivatisation. The most reactive sites on the adenine ring are the N-1, N^6, and C-8 positions (*Figure 3.2*).

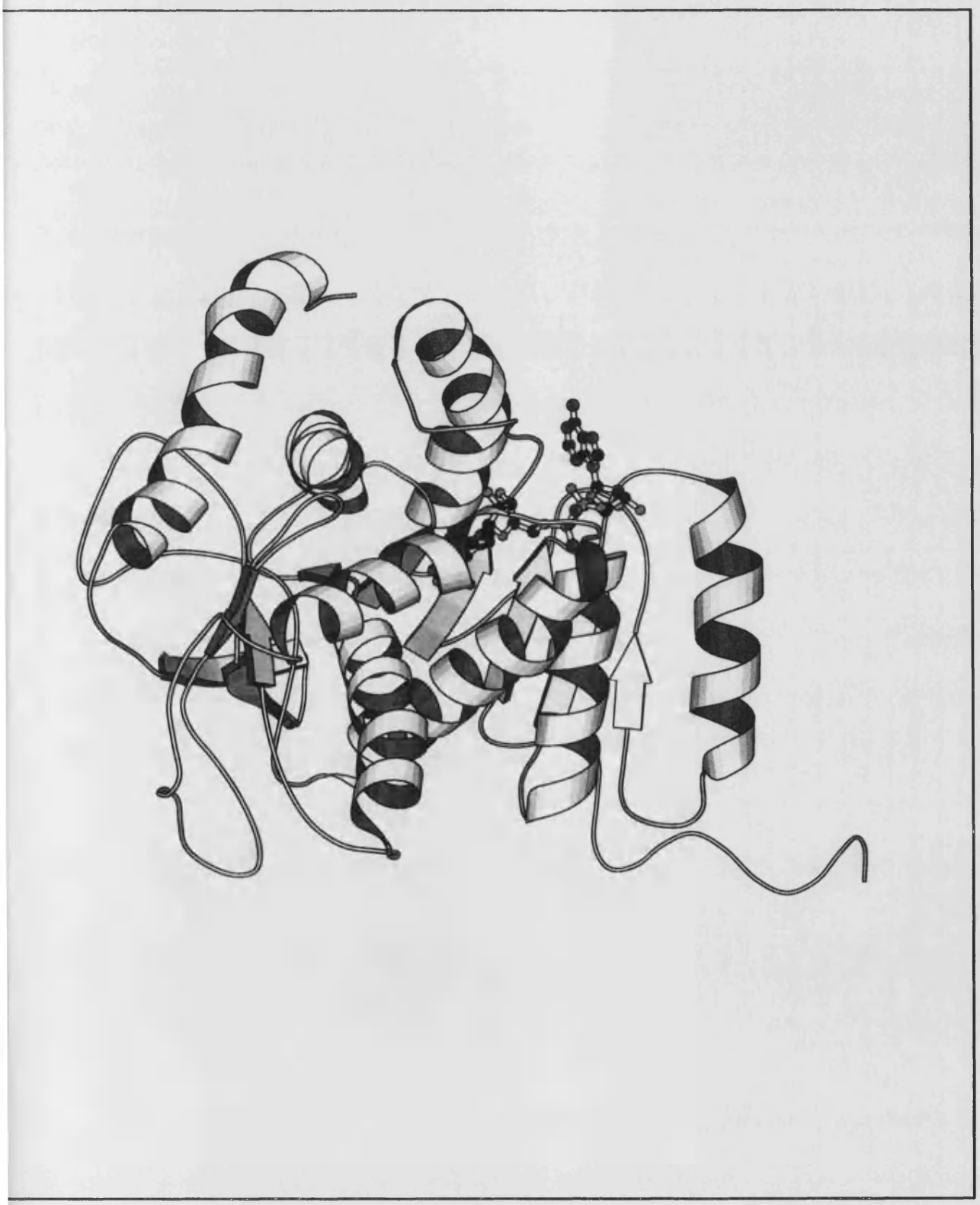
During NAD binding, the adenine moiety is buried in a hydrophobic pocket found in the active centre of the enzyme (for an example of this see *Figure 3.19*). Within this hydrophobic pocket, the N-1 position of the adenine ring is involved in hydrogen bonding with side chain residues of the enzyme active centre. Derivatives formed by substitution at this position are generally not good substrates. The effects of modification at the N-1 and N^6 positions upon the substrate activity of the coenzyme have been studied in detail (Hendle *et al*, 1993). It was found that NAD derivatives formed by modification at the N-1 position of NAD had K_M values with lactate dehydrogenase that were between 10 and 40 times larger than the values of similar derivatives formed by modification at the N^6 position. Moreover, the k_{CAT}/K_M ratios were 25-250 times lower for the N-1 modified derivatives, making the N^6 position the more desirable position for modification. X-ray diffraction techniques confirmed the suitability of the N^6 amine position as a site for modification, by showing that it pointed away from the active centre and into the surrounding solution, making it

easily available for reaction with an approaching molecule (Rossman *et al*, 1974; Grau *et al*, 1981) (*Figure 3.3*).

The other suitable position for modification of the adenine nucleus is the C-8 position (Mosbach, 1978). In theory, several types of modification reaction could occur here because it is relatively electrophilic in nature, but at the same time is susceptible to electrophilic attack (Lister, 1971)

Indeed, it would appear that modification of the adenine nucleus at the N^6 position, and to a lesser extent at the C-8 position, preserves the orientation of NAD in the active centre (Schmidt and Dolabdjian, 1980). Upon modification of these positions the substrate activity of the coenzyme is not seriously affected (Buckmann and Carrea, 1989). Consequently, the C-8 and N^6 positions of the adenine ring have been modified in several studies. Comparing the two positions, it generally follows that those NAD derivatives formed by modification of the N^6 position bind to dehydrogenases with greater affinity than similar derivatives formed by modification of the C-8 position (Mosbach, 1978).

Figure 3.3: *Representation of the solvent accessibility of the N^6 amine group of an NAD molecule when bound at the active centre of pig H_4 LDH.* NAD and lysine residues are shown as ball and stick models.
Key for atoms: blue = nitrogen, red = oxygen, grey = carbon, pink = phosphate (hydrogens not shown).
Key for surrounding protein: coiled ribbons = α-helix, arrows = parallel β-sheet.
The picture was prepared using the data of Grau *et al* 1981, on a Molscript™ package. The bound NAD molecule is actually a molecule of *S*-lac-NAD which was designed to simulate the ternary complex structure formed between LDH, NAD and lactate.



Thus, the N^6 amine group of the adenine ring was chosen as the site of NAD modification in these studies. In fact, the most desirable scenario was to couple unmodified NAD directly to dehydrogenases, via the N^6 group of the adenine ring. However, in the past, the introduction of a bifunctional spacer arm attached to the N^6-amine group appeared to increase the reactivity of the coenzyme towards coupling reagents. This increase in reactivity probably resulted because the spacer arm effectively moved the amine functional group away from the undesirable effects of the adenine ring (*Figure 3.4*) which may hinder the coupling reaction in two ways (Gacesa and Venn, 1979). Firstly, it may sterically hinder the approach of the coupling reagent, and secondly, the resonance effects of the ring may reduce the nucleophilic character of the N^6 amine group, and so explain its poor reactivity towards coupling reagents (Mosbach, 1978). In this study, coupling reactions were performed using both natural NAD and NAD derivatives, which had functionalised spacer arms attached to the N^6 amine group.

The modified coenzymes were included, then, in an attempt to improve the properties of NAD as a coupling ligand. The following section describes the NAD derivatives that were synthesised, and also compares their coenzymic properties to those of their parent nucleotide, NAD.

3.2 NAD derivatives

The NAD derivatives N^6[(aminohexyl)carbamoylmethyl]-NAD (N^6AHCM-NAD) and N^6carboxymethyl-NAD (N^6CM-NAD) (*Figure 3.4*) were synthesised according to Mosbach *et al* (1976). An overview of the synthesis of each derivative is shown in *Figure 3.5*. The reasons for modification of NAD at the N^6 position are described in the previous section.

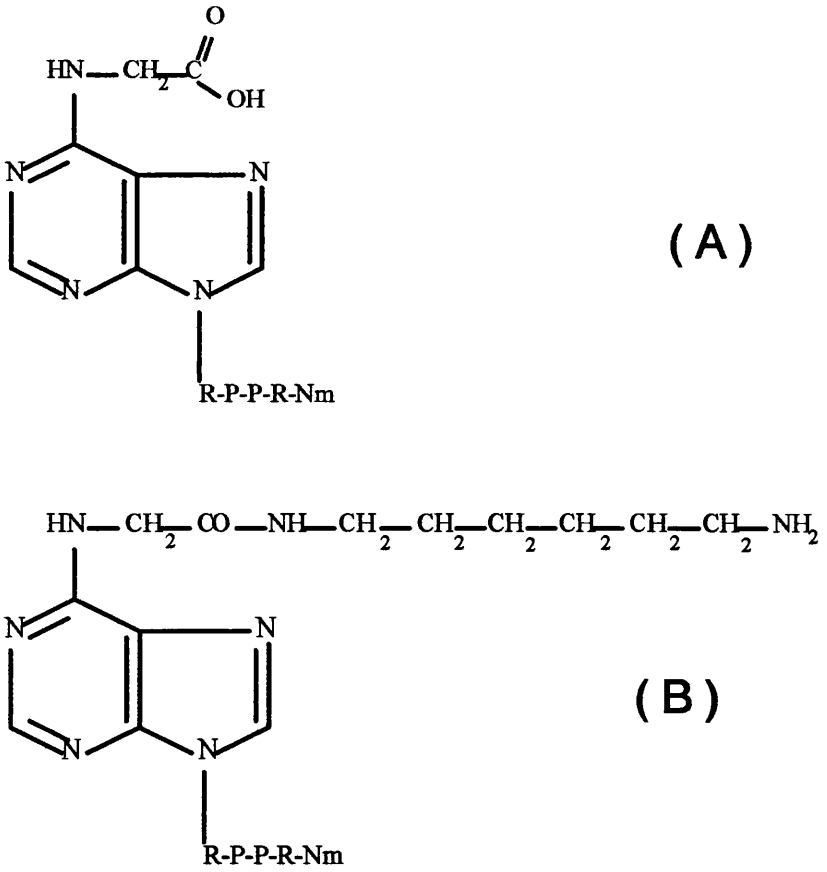


Figure 3.4: *The structure of (A) N⁶carboxymethyl-NAD and (B) N⁶[(aminohexyl)-carbamoylmethyl]-NAD.* R= ribose; P = phosphate group; Nm = nicotinamide moiety. Both derivatives possess reactive aliphatic functional groups (shown in colour).

N^6AHCM-NAD has been extensively used as a precursor for the synthesis of immobilised NAD preparations (Lindberg *et al*, 1973; Schmidt and Dolabdjian, 1980; Lowe, 1978; Mosbach *et al*, 1976). It introduces to NAD a spacer arm attached at the N^6 position of the adenine ring. The arm includes a hydrophobic region and a terminal amine group which may be easily derivatised. The alkyl amine group has a pK_a value of 11.5, substantially improving the basic properties and the nucleophilic character of the original coenzyme, which possesses an aromatic amine group with a pK_a below 5.0 (Streitwiesser and Heathcock, 1985).

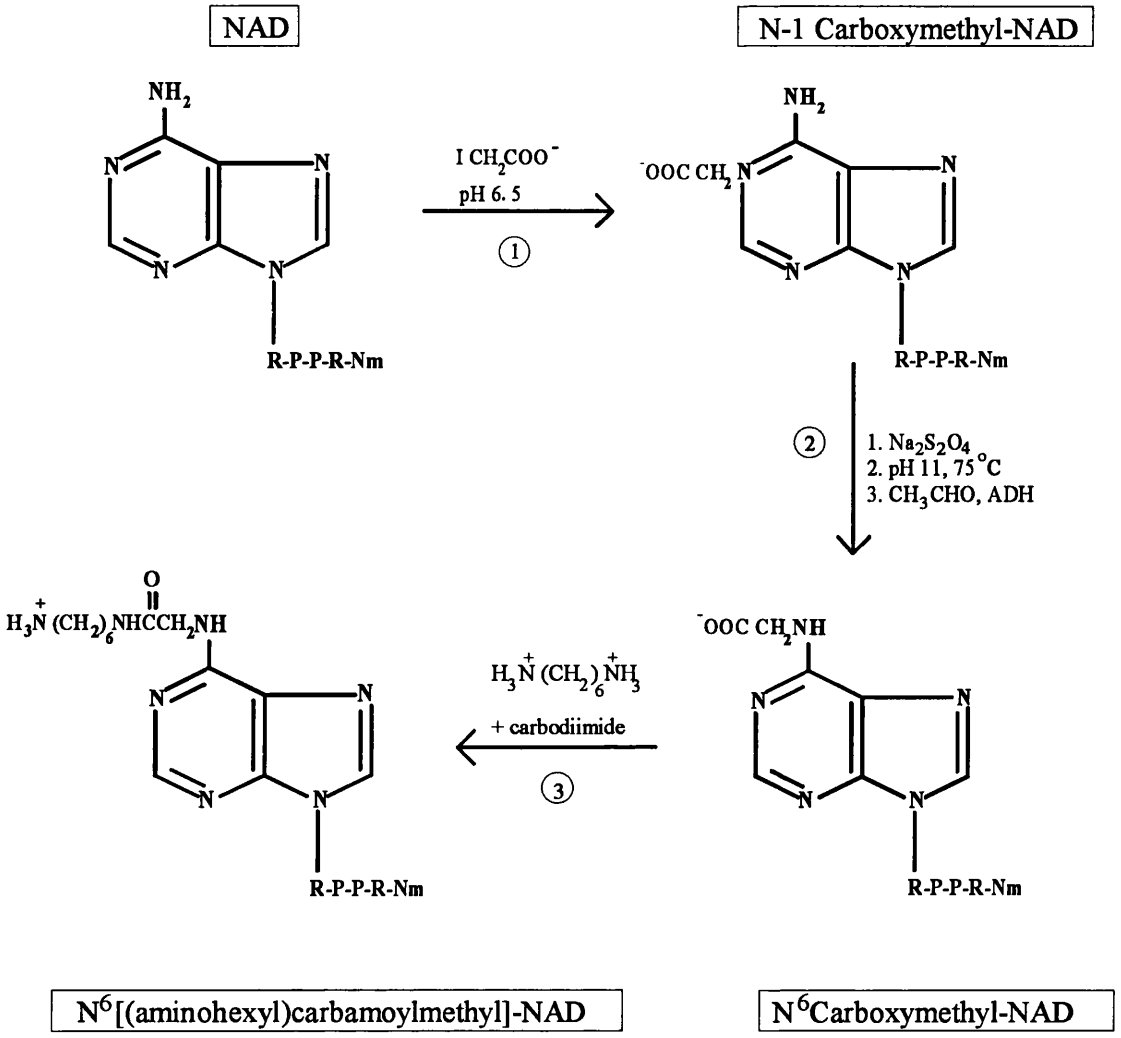


Figure 3.5: *The synthesis of N⁶carbamoylmethyl-NAD (N⁶CM-NAD) and N⁶[(aminohexyl)carbamoylmethyl]-NAD (N⁶AHCM-NAD) starting from NAD*
R= ribose; P= phosphate; Nm = nicotinamide moiety.

N⁶CM-NAD is conveniently found on the synthetic route going from NAD to N⁶AHCM-NAD (*Figure 3.5*). It also introduces to NAD a reactive group which is a suitable site for further derivatisation, although it has been less widely used as a precursor for immobilised NAD. In contrast it has a spacer arm which is short and hydrophilic in nature. The carboxyalkyl group carries a negative charge at neutral pH, the pK_a value being slightly less than 4.0 (the pKa of ethanoic acid) due to the inductive effect of the purine ring (Streitwiesser and Heathcock, 1985).

Upon the study of several different spacer arms attached to the N^6-adenine position of NAD, Hendle *et al* (1993) observed that a negative charge and a short spacer was of minor importance with respect to substrate activity of the coenzyme, whereas the introduction of positive groups resulted a slight increase in the K_M value of the coenzyme. Conversely, Schmidt and Dolabdjian (1980) suggested that a hydrophobic spacer arm extended the hydrophobic binding region of NAD, improving the affinity of the enzyme for the coenzyme, and hence improved the substrate properties of the coenzyme.

Thus, the two NAD derivatives described above were chosen because of the contrasting properties of their spacer arms. The derivatives were used in coupling experiments, along with unmodified NAD to investigate which characteristics of a spacer arm, if any, are more favourable towards coupling reactions with an enzyme.

3.2.1 The synthesis of NAD derivatives

A drawback in the method of synthesis used by Lindberg *et al* (1973) with modifications by Mosbach *et al* (1976) (*Figure 3.5*) is that there is a 70-90% loss of nucleotide material. A large amount of material is lost during the final purification step *i.e.* during ion exchange chromatography. The total purification of N^6CM-NAD requires passing the crude derivative through two ion exchange gradients, and a further two such ion exchange steps are required to purify N^6AHCM-NAD. In order to avoid these laborious and inefficient purification steps in this study, ion exchange chromatography was omitted during the preparation of coenzyme derivatives for use in enzyme-coupling experiments. Each of the crude derivative preparations was considered adequate for use in coupling experiments because the only impurities would be nucleotides and they would not interfere with the coupling reactions. Mosbach *et al* (1976) showed that alkylation of the phosphate entities of NAD did not occur during the preparation of N^6CM-NAD (*Figure 3.5, step 1*), and that during the preparation of N^6AHCM-NAD the carbodiimide-promoted attachment of the 1,6-diaminohexane

spacer did not result in phosphorimidate formation (*Figure 3.5, step3*). It therefore follows that in a crude preparation the reactive carboxyl- or amine- functional groups will only be present on either N^6CM-NAD or N^6AHCM-NAD, respectively. During HPLC analysis of final "pure" derivative preparations analysed after the ion exchange steps, Kahle *et al* (1981) observed that ion exchange purification had been successful only to a certain extent and the nucleotide impurities still prevailed.

3.2.1.1 HPLC analysis of NAD derivatives

The purity of each derivative preparation taken for use in coupling experiments in this study was estimated using Reverse Phase Liquid Chromatography (RPLC), a form of High Performance Liquid Chromatography (HPLC), in order to determine the actual percentage purity of the crude preparations.

In RPLC, the stationary phase, typically hydrocarbonaceous in nature, is inert and is less polar than the mobile phase which is usually a non-u.v.-absorbing organic solvent such as methanol or acetonitrile. It is the polarity of the mobile phase which determines the elution time of the material under investigation. Although the mechanism of RPLC is not completely understood, it is known that under a mobile phase of decreasing polarity (or an increasing concentration of organic solvent), substances are eluted in order of increasing hydrophobicity (Cooke and Olsen, 1980).

The hydrophobic properties of N^6CM-NAD and N^6AHCM-NAD are different because of the different spacer arms attached at the adenine moiety. RPLC of these derivatives has been investigated by Kahle *et al* (1981) who noted that HPLC performed better than TLC, showing impurities that TLC did not detect. Kahle *et al* used a strongly acidic mobile phase (pH 2.0-2.5) to elute the derivatives. A milder mobile phase is reported here, with a good separation being achieved at neutral pH (pH6.8). An octadecylsilane (ODS) material was chosen as the stationary phase, and a 0-30% methanol gradient as the mobile phase, (personal communication, Dr. A. Mayes) in

order to elute nucleotide material in order of increasing hydrophobicity. Commercial preparations of N^6CM-NAD and N^6AHCM-NAD were also subjected to RPLC, to confirm the elution times of the corresponding derivatives present in the crude preparations. The elution times of each coenzyme derivative and unmodified NAD are shown in *Table 3.1.*

Table 3.1

Purity of NAD derivatives used in coupling experiments

Coenzyme	Elution time / min.	Purity (%)[a]
NAD[b]	(10.8)[c]	97
N^6CM-NAD	9.1 (9.1)	60
N^6AHCM-NAD	16.7 (17.1)	68

[a] Purity measured using RPLC: 500µl nucleotide material (0.2mg ml^{-1}) was applied to an ODS Hypersil column equilibrated with KH_2PO_4 buffer (10mM, pH 6.8) and eluted with a 0-30% methanol gradient over 30 min; flow rate = 1ml min^{-1}; elution profile scanned at 260nm and purity calculated from the area under each peak.

[b] NAD was purchased from Sigma Chemical Co. who estimate its purity to be ~98%

[c] Figures in brackets represent elution times of commercially prepared derivatives, obtained from Sigma Chemical Co.

Elution time is given as the time the peak appeared after the application of sample (t=0)

The longer elution time of 16.7 mins for N^6AHCM-NAD compared with 10.8 mins for NAD would seem to be due to the hydrophobic property imposed on the structure of NAD by the addition of a "hexyl" spacer arm. Similarly, the shorter elution time of 9.1 min. for N^6CM-NAD confirms the more hydrophilic nature of its spacer arm.

Each crude derivative preparation was 60-70% pure, indicating that each derivative was present in substantial proportions before ion exchange purification, and that the preparations were suitable for use in coupling experiments.

RPLC proved to be a convenient and effective way to analyse NAD derivative preparations and it had excellent separation properties. It would appear that substitutions at the adenine N^6 amine position of NAD dramatically altered its elution

time, making a rapid analysis of the purity and hydrophobicity of each structure possible.

The next step was to confirm the correct synthesis of the desired derivatives using TLC and to ensure that each derivative prepared had siginificant biological activity by using them as cofactors in dehydrogenase-catalysed kinetic assays. Before such characterisation, however, the derivatives were fully purified using ion exchange chromatography (Mosbach *et al*, 1976) to remove any traces of unmodified NAD from the derivative preparations.

3.2.2 Characterisation of NAD derivatives

The NAD derivatives were characterised by measuring their R_f values using thin layer chromatography (TLC) and by assaying them for their activity as substrates for dehydrogenases.

3.2.2.1 Thin layer chromatography

During TLC, the R_f value of a nucleotide, as opposed to nucleosides and bases, depends largely upon the salt concentration of the solvent (Randerath and Randerath, 1967). Its mobility with respect to other nucleotides is determined by its pH-dependent net negative charge, whilst the arrangement of non-ionisable groups is also important. Both of these properties differ between NAD and its N^6-modified derivatives, N^6CM-NAD and N^6AHCM-NAD, and subsequently a succesful separation of these nucleotides has been achieved using TLC (Lindberg et al, 1973). The R_f values for N^6CM-NAD and N^6AHCM-NAD have been published (Mosbach *et al*, 1976), providing a convenient way with which to confirm the synthesis of these derivatives.

Therefore, after purification with ion exchange chromatography, each NAD derivative was subjected to TLC on polyethyleneimine-cellulose (PEI-cellulose) sheets as described in *Materials and Methods.*

Table 3.2
Thin layer chromatography of NAD derivatives

	R_f values in chromatography systems	
Coenzyme	0.1M LiCl	0.5M LiCl
NAD	0.5 [a](0.51)	0.7 (0.74)
N^6CM-NAD	0.2 (0.17)	0.8 (0.81)
N^6AHCM-NAD	>0.9[b] (>0.95)	>0.9 (>0.95)

[a] Figures in brackets denote values recorded by Mosbach *et al* (1976)

b R_f values above 0.90 could not be recorded accurately due to spot diffusion
TLC was performed on PEI-cellulose coated plates at room temperature. 10µl of each coenzyme (1 mg/ml) was spotted onto a plastic sheet pre-coated with PEI-cellulose using a 10µl capillary tube, 1.5 cm from the labelled bottom of the sheet.The sheet was then placed upright in a glass tank containing lithium chloride solution to a height of 0.5cm. After the solvent front had reached 2-4cm from the top of the sheet, the sheet was removed from the tank and dried using a stream of cool air. The sheet was then analysed for nucleotide spots under ultra-violet light.

Table 3.2 shows that the R_f value for each derivative was in good agreement with values reported previously.

3.2.2.2 Substrate activity

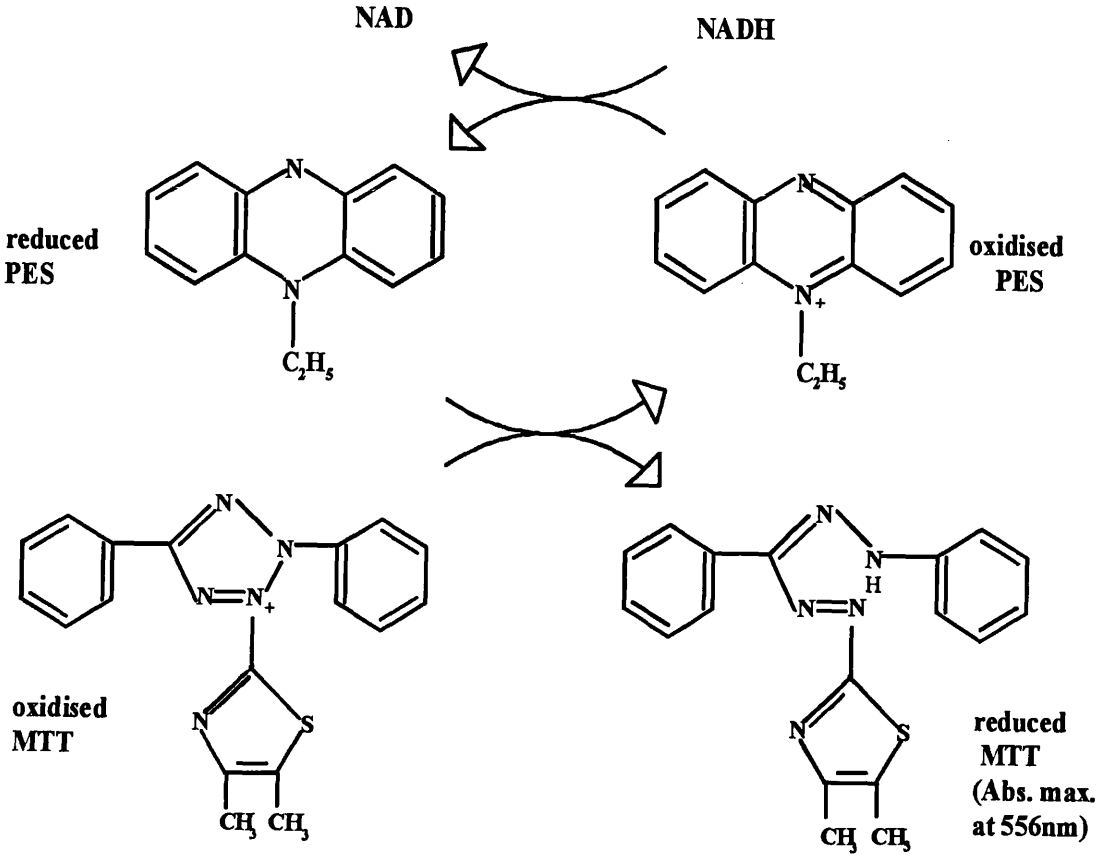
Each derivative was assayed for its activity as a reducible coenzyme using two types of assay.

1. The rate of reduction of the coenzyme by a dehydrogenase was monitored at 340nm in a **standard assay**. If the derivatives were enzymically reducible, they would show an absorbance maximum at 340nm due to the addition of a hydride ion to the nicotinamide moiety upon reduction altering the absorption properties of the pyridine nucleus. The concentration of the coenzymes in assays with lactate dehydrogenase (pig heart) and alcohol dehydrogenase (yeast) was $1x10^{-4}$M, the K_M values of the respective enzymes for NAD being $2.7x10^{-4}$ and $7.5x10^{-5}$M (Schafer *et al*, 1986). It

was anticipated that if the derivatives were conjugated to enzyme molecules they would not be present in a large excess of their K_M values and so assaying them at this concentration would provide conditions similar to those prevailing in their practical use. However, this concentration was primarily chosen to give a convenient rate of change in absorbance at 340nm.

2. A **coenzyme-recycling assay** was used, again employing alcohol dehydrogenase and lactate dehydrogenase. This assay also depends upon the enzymic reduction of the coenzyme, but here the reducing equivalents are chemically reoxidised by phenazine ethosulphate (*Figure 3.6*). The reoxidised coenzyme units are then re-available for catalysis. Such recycling of coenzyme units makes it possible to assay very low concentrations of coenzyme but more importantly it provides a method with which to assay the activity of the enzyme-coenzyme conjugates in the absence of exogenous coenzyme. The problem with assaying enzyme-coenzyme conjugates is that the formation of product will be limited by the amount of fixed coenzyme unless the reduced cofactors can be reoxidised by a second process. Chemical reoxidation by phenazine ethosulphate is one such process. The redox process can be monitored by following the change in the spectrophotometric absorbance of a redox indicator such as DCPIP or MTT. In this way the substrate activity and recyclability of each coenzyme is monitored indirectly (*Figure 3.6*). Two redox indicators were used: 3(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was used in assays with alcohol dehydrogenase, and dichlorophenolindophenol (DCPIP) was used in assays with lactate dehydrogenase; the dyes have absorbance changes which can be monitored at 556nm and 610nm, respectively. The free coenzyme derivatives were assayed using the coenzyme-recycling assay to ensure that they were recyclable and also to ensure that their activity as substrates in a recycling assay was substantial compared to that of NAD. The results from both assays are given in Table 3.3.

a. MTT/PES



b. DCPIP/PES

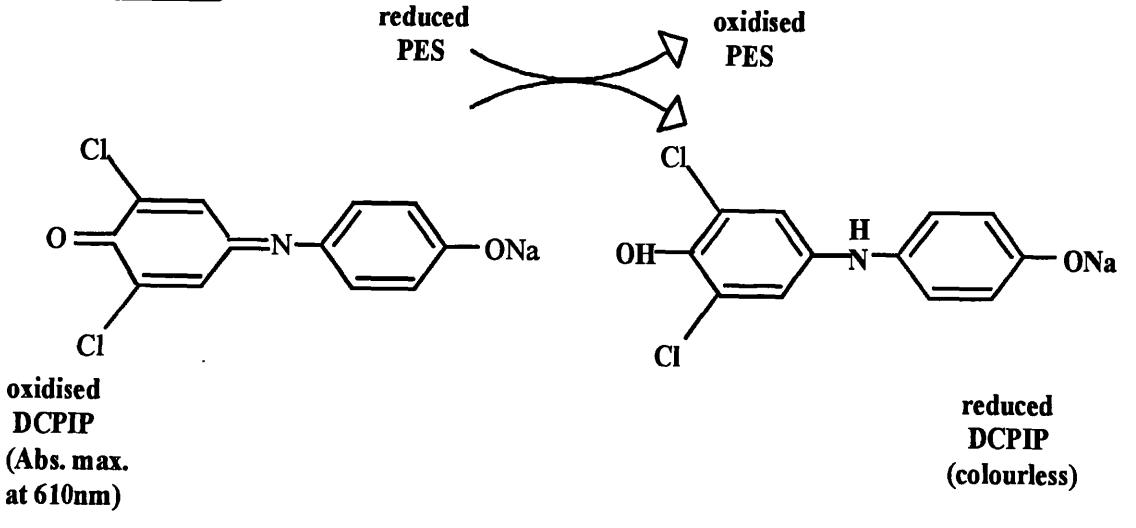


Figure 3.6: *Recycling assay for intrinsic activity of enzyme-coenzyme conjugates based on recycling of NADH.* NADH is generated in the presence of susbstrate. PES- phenazine ethosulphate, MTT = 3 (4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide. DCPIP = dichlorophenolindophenol

Table 3.3

NAD derivatives as coenzymes for alcohol dehydrogenase and lactate dehydrogenase

	Reduction rate with ADH				Reduction rate with LDH				
	standard assay[a]		chemical recycling assay[b]		standard assay[a]		chemical recycling assay[c]		K_MLDH[d]
Coenzyme	$\mu mol\ min^{-1}mg^{-1}$	(%)	$A_{556}min^{-1}$	(%)	$\mu mol\ min^{-1}mg^{-1}$	(%)	$A_{610}min^{-1}$	(%)	$(x10^{-4}M)$
NAD	79	100	0.40	100	12	100	0.90	100	2.7
N^6CM-NAD	42	54	0.31	77	8.0	67	0.51	57	7.9
N^6AHCM-NAD	69	88	0.23	58	6.2	52	0.47	52	6.6

Substrate activities of the derivatives are also given in % activity relative to NAD (100).

[a] Coenzyme reduction rates were determined at 340nm with yeast alcohol dehydrogenase and porcine heart lactate dehydrogenase at pH 9.0 and pH 7.0, respectively. Coenzyme concentration was 0.10mM.

ADH assay solution: 2.60ml glycine-sodium pyrophosphate buffer (75mM), 0.1ml ethanol (95%); 0.1ml semicarbazide (2.2M), 20µl YADH ($5\mu gml^{-1}$), 0.1ml cofactor sample (0.10mM).

LDH assay solution: 2.78ml sodium phosphate buffer (100mM), 0.1ml lactate (1M), 20µl pig heart LDH ($20\mu gml^{-1}$), 0.1ml cofactor sample (0.10mM).

[b] The cycling experiment was carried out at pH 7.4 using a MTT/PES redox couple with yeast alcohol dehydrogenase. To 1.95ml TEA/nicotinamide/ethanol buffer (100mM / 100mM / 0.5M, respectively), was added 100µl coenzyme present at 0.1µM in the assay solution. 50µl of 12mM MTT, 800µl of 3mM PES and 100µl of yeast alcohol dehydrogenase ($30\mu gml^{-1}$) were also added.

[c] The cycling experiment was carried out at pH 7.5 using a DCPIP/PES redox couple with porcine heart lactate dehydrogenase. To 0.75ml phosphate buffer (100mM) were added 100µl coenzyme present at 10µM in the assay solution, 50µl of 1mM DCPIP, 50µl of 40mM PES, and 50µl of 100mM lactate, and 0.1ml lactate dehydrogenase ($100\mu gml^{-1}$).

[d] K_M was determined with beef heart lactate dehydrogenase at 340nm using a NAD concentration range of $1x10^{-5}$- $1x10^{-3}$M. *Assay solution*: 2.78ml sodium phosphate buffer (pH 7.0, 100mM), 0.1ml lactate (1M), 20 µl beef heart lactate dehydrogenase ($20\mu gml^{-1}$), 0.1ml cofactor sample.

Results from the standard assay show that N^6AHCM-NAD had a very good substrate activity with alcohol dehydrogenase, it being 88% of the substrate activity of the natural coenzyme, NAD. The activity of N^6CM-NAD was somewhat lower at 54%. In contrast, N^6CM-NAD was the better coenzyme for lactate dehydrogenase, its substrate activity being 67% of the substrate activity of NAD, compared to an activity of 52% for N^6AHCM-NAD. Whatever the individual substrate activity, the derivatised NAD molecules seem to make good cofactors for dehydrogenase enzymes, confirming the results of Lindberg *et al* (1973). It was important to know that derivatisation at the N^6 position did not dramatically affect the coenzyme properties of the NAD molecule, so that if a serious loss in substrate activity was found upon immobilisation of a coenzyme, derivatisation of the original NAD molecule could not be to blame.

Results from the recycling assay also illustrate the excellent coenzyme properties of the derivatives. N^6CM-NAD was a slightly better substrate for both dehydrogenases, its activity with lactate dehydrogenase and alcohol dehydrogenase relative to NAD (100%) being 77% and 57%, respectively, compared to values of 58% and 52% for N^6AHCM-NAD. These values serve to demonstrate the excellent recycling properties of each derivative.

The results do not show any strong differences between N^6AHCM-NAD and N^6CM-NAD with respect to their suitabilty as coenzymes with these enzymes. This indicates that a hydrophobic arm does not necessarily convey better coenzyme properties than a hydrophilic arm, in contrast with the results of Lowe (1977) in studies of immobilised cofactors derivatised at the C-8 position. Conversely, the results support the theory of Hendle *et al* (1993) who observed that shorter, negatively charged spacer arms attached at the N^6 position make the least difference with respect to the substrate activity of NAD. However, the results discussed here refer to the analysis of cofactors that are in free solution. Upon immobilisation to an enzyme the character of the spacer arm may prove to have a stronger influence on the substrate activity of the coenzyme.

3.2.2.3 K_M values of LDH for NAD and its derivatives

The K_M values of lactate dehydrogenase for each NAD derivative were also determined to show whether derivatisation of NAD had affected the affinity of the enzyme towards the coenzyme. The direct linear plot was used to estimate the K_M values and an example is shown in *Figure 3.7* (Eisenthal and Cornish-Bowden, 1974).

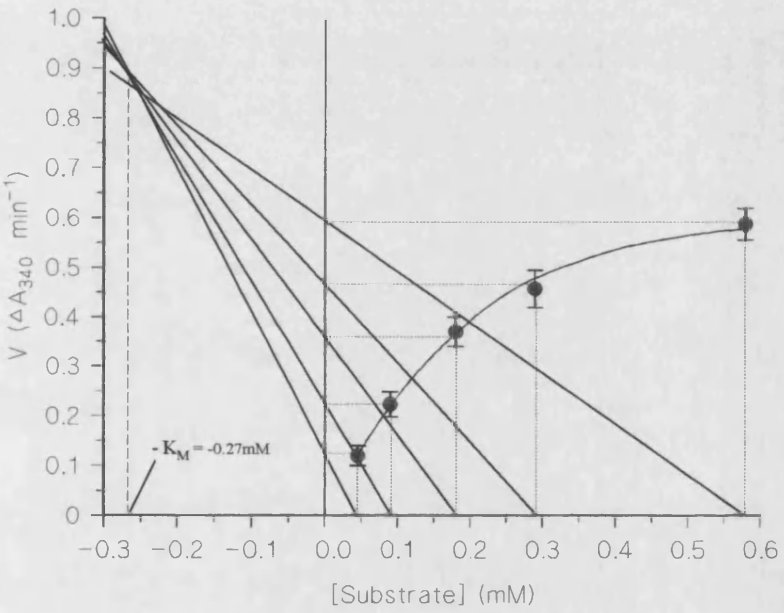


Figure 3.7*: *Estimation of the K_M value of lactate dehydrogenase for NAD using the direct linear plot. K_M was determined with beef heart lactate dehydrogenase at 340nm using a NAD concentration range of $1x10^{-5}$- $1x10^{-3}$M. *Assay solution*: 2.78ml sodium phosphate buffer (pH 7.0, 100mM), 0.1ml lactate (1M), 20μl beef heart lactate dehydrogenase (20μgml^{-1}), 0.1ml NAD sample. The velocity of NAD reduction at 340nm was plotted against [NAD] to obtain a substrate saturation curve; lines are then drawn and extrapolated as shown to give the -Km value of NAD (Eisenthal and Cornish-Bowden, 1974).

The estimated K_M value for each derivative is presented in *Table 3.3*. The K_M values given are actually apparent K_M values because the non-coenzymic substrate (lactate) is present at saturating concentrations. The K_M values of lactate dehydrogenase for N^6CM-NAD and N^6AHCM-NAD are similar, at around three times the K_M for NAD.

Derivatisation of NAD probably resulted in an alteration of its coenzyme binding properties, causing an overall reduced affinity between enzyme and coenzyme, and hence an increase in K_M. Comparing the two NAD derivatives, the enzyme has slightly more affinity for N^6AHCM-NAD, its K_M value being $6.6x10^{-4}$M compared to a value of $7.9x10^{-4}$M for N^6CM-NAD, the value for the natural coenzyme being $2.7x10^{-4}$M. Therefore, although substrate assays show that N^6CM-NAD is reduced by lactate dehydrogenase at a faster rate than the more hydrophobic derivative, the enzyme would appear to have slightly less affinity for it. The hydrophobic arm may convey better enzyme-binding properties than a hydrophilic arm, perhaps acting as an extension of the hydrophobic binding properties of the adenine ring (Schmidt and Dolabdjian, 1980) but at the same time the arm may affect the reduction rate of the bound coenzyme. It must be noted, however, that differences in K_M and substrate activity values between the two derivatives are not at all striking.

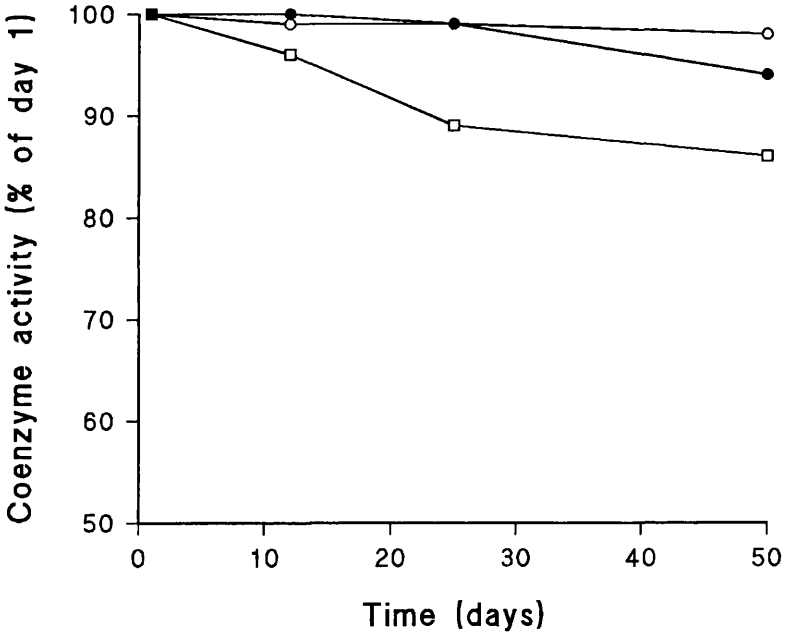
It was next felt necessary to analyse the stability the NAD derivatives to ensure that they were each sufficiently stable for use in further studies.

3.2.2.4 Stability of NAD, N^6CM-NAD and N^6AHCM-NAD

During the characterisation of each NAD derivative, its stability in solution was measured over a two month period. Stability studies were carried out to rule out the possibility that any instability of the enzyme-coenzyme complexes might be inherited from the coenzyme ligand. As the overall aim of this project was to investigate enzyme-coenzyme complexes and their applicability to enzyme electrodes, it was essential that the enzyme-coenzyme complexes were stable, at least for a few weeks, to make their incorporation into an enzyme electrode worthwhile.

The NAD derivatives were stored at a low concentration (0.17mM) in buffered solutions at room temperature and at 4^oC to simulate the environment to which each coenzyme would be subjected after being incorporated into an enzyme-coenzyme

complex. Each derivative was assayed for substrate activity using alcohol dehydrogenase in a coenzyme-recycling assay with MTT and PES; this was the same assay that would be used to measure the substrate activity of each coenzyme derivative after its incorporation into an enzyme-coenzyme complex.



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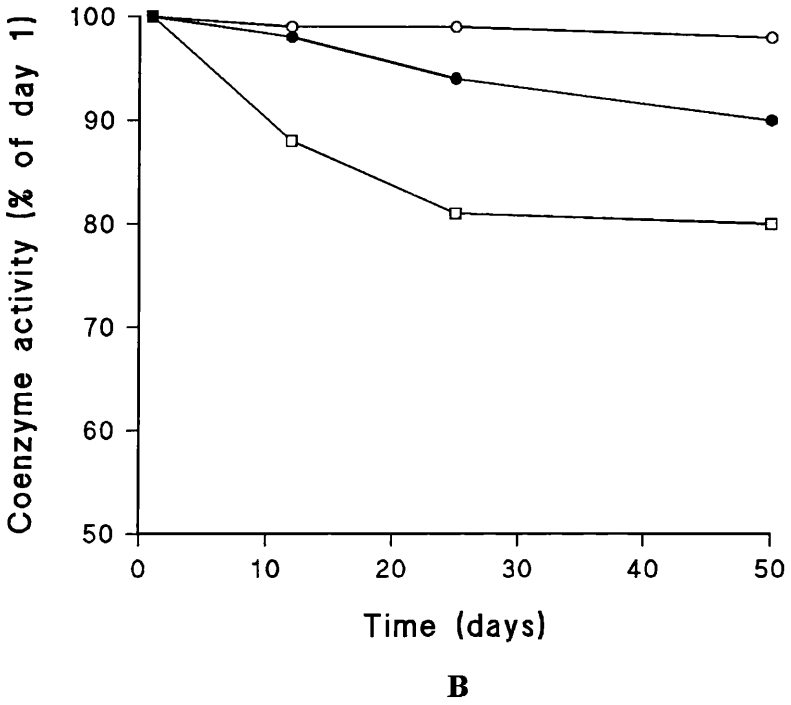

Figure 3.8: *NAD derivative stability at 4°C (A) and at room temperature (B).* • = N^6AHCM-NAD; □ N^6CM-NAD; O = NAD. Coenzyme reduction rates were determined using yeast alcohol dehydrogenase (30μ g/ml) in a coenzyme-recycling assay with MTT and PES, coenzyme being added to 0.5μM. *Assay solution:* to 1.95ml TEA/nicotinamide/ethanol buffer (100mM / 100mM / 0.5M, respectively), pH 7.4, were added 100μl coenzyme present at 0.5μM in the assay solution, 50μl of 12mM MTT, 800μl of 3mM PES and 100μl of yeast alcohol dehydrogenase.

The stability results are presented in *Figure 3.8*. It is clear that NAD and its derivatives are quite stable, with overall activities at room temperature remaining above 80%, even after fifty days. Considering, also, that the immobilisation of biological structures often has a stabilising effect, the stability of each coenzyme was clearly sufficient and the cofactors were now rendered suitable for use in coupling experiments with enzymes.

3.3 Enzymes

The NAD-dependent enzymes, lactate dehydrogenase, alcohol dehydrogenase and glutamate dehydrogenase were used in coenzyme-conjugation experiments with the

coupling reagents glutaraldehyde and carbodiimide. Due to a strong inhibitory effect observed when glutaraldehyde was mixed with alcohol dehydrogenase, terephthal-aldehyde, an aromatic aldehyde, was also studied as a possible replacement cross-linking dialdehyde in studies with this particular enzyme. The enzyme side residues chosen for conjugation reactions were the polar side chains of lysine, glutamate and aspartate. In all three dehydrogenases, these residues are present on the outer surface of the enzyme molecule where they are available for reaction with coupling reagents. Further details of each enzyme, its role in coupling reactions, and the reason for its study are presented in the second part of this chapter, which also describes the results generated from studying enzyme-coenzyme complexes incorporating these enzymes.

Each enzyme was obtained commercially, either in a lyophilised form (yeast alcohol dehydrogenase) or in a concentrated ammonium sulphate solution (pig heart lactate dehydrogenase and beef liver glutamate dehydrogenase). Before use, each enzyme was therefore extensively dialysed against an appropriate buffer to remove unwanted salts and to ensure that the enzyme was buffered at an appropriate pH and that it was present at the correct concentration for use in a coupling reaction.

3.4 Coupling Reagents

Initially, two different coupling reagents were chosen for use in enzyme-coenzyme coupling experiments involving amine and carboxyl functional groups: these were glutaraldehyde and 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide.HCl (EDC). As the studies progressed, terephthalaldehyde was also investigated as a coupling reagent although, for simplicity, its basic coupling mechanism may be regarded as similar to that of glutaraldehyde. More detail of the aromatic dialdehyde will be given in part two of this chapter. The reasons for using glutaraldehyde and EDC and details of their individual coupling mechanisms will be described presently.

3.4.1 Glutaraldehyde

The structure of the homobifunctional reagent, glutaraldehyde, is shown in *Figure 3.9*. It is usually used to cross-link molecules via their primary amine groups. The coupled product incorporates the glutaraldehyde molecule into its structure i.e. glutaraldehyde acts as a molecular spacer between the conjugated molecules.

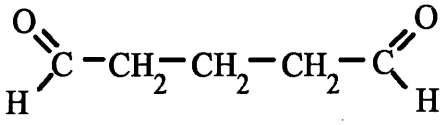

***Figure 3.9:** The structure of glutaraldehyde*

Reaction with proteins

Glutaraldehyde has been widely used in protein cross-linking studies (Lundblad and Noyes, 1984). The chemistry of glutaraldehyde is not simple although it is generally agreed that it reacts with proteins to form Schiff base structures (*Figure 3.10*)

It is thought that glutaraldehyde reacts with the terminal amine groups of lysyl residues to form α,ω-Schiff bases and that this mechanism predominates in the reaction of glutaraldehyde with proteins (Lundblad and Noyes, 1984). The formation of Schiff bases should be readily reversible in the absence of reducing agents such as potassium borohydride. This has not always proved to be the case, however, and in some studies the reaction between glutaraldehyde and proteins was found to be irreverisble without the addition of a reducing agent (Richards and Knowles, 1968). Glutaraldehyde has a tendency to polymerise in solution and in this form it can irreversibly react with primary amines by vinyl addition (*Figure 3.10*). Glutaraldehyde coupling is consequently undefined (Hemeansson *et al*, 1992). Whatever the mechanism of glutaraldehyde, the reaction with amines occurs rapidly at alkaline pH and therefore glutaraldehyde is suitable for use as a coupling reagent with dehydrogenase enzymes which are generally stable between pH 7.0 and 9.0.

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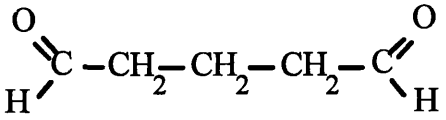

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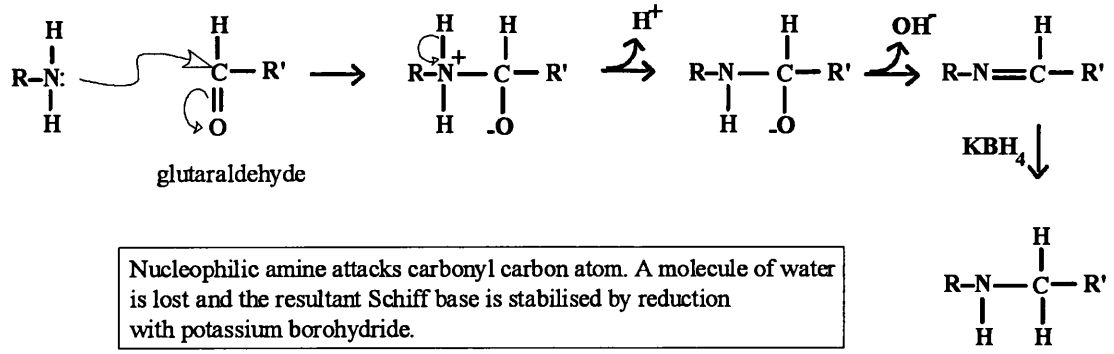
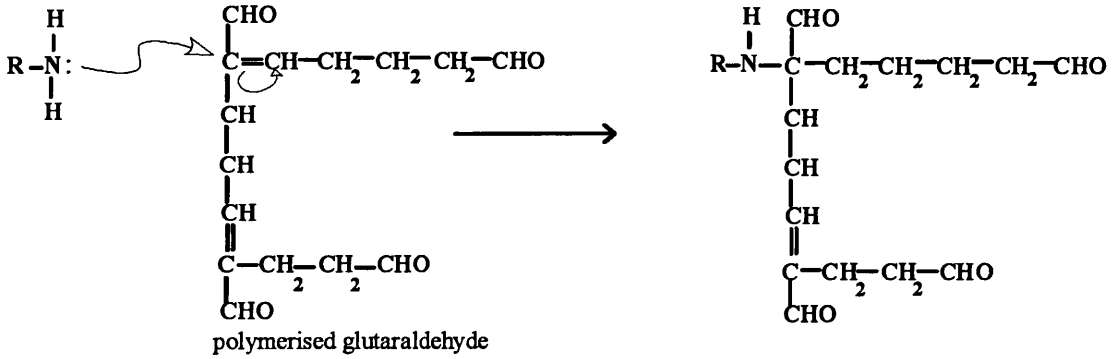
a. Schiff Base formation



Nucleophilic amine attacks carbonyl carbon atom. A molecule of water is lost and the resultant Schiff base is stabilised by reduction with potassium borohydride.

b. Vinyl addition



Nucleophilic addition of amine at a vinyl group of a glutaraldehyde polymer leaves a stable product with multiple formyl groups which may then partake in further coupling reactions with primary amine groups..

Figure 3.10: *Coupling mechanisms of glutaraldehyde*

Glutaraldehyde is fairly specific for the ε-amine groups of Lys residues, although it has also been known to react with other nucleophilic groups present on the surface of proteins such as the cysteine thiol group, the imidazole ring of histidine and the phenolic hydroxyl group of tyrosine residues (Lundblad and Noyes, 1984).

Reaction with nucleotide coenzymes

Glutaraldehyde has been used on several occasions to immobilise adenine nucleotide cofactors. It was used to attach adenosine triphosphate (ATP) to both the insoluble polysaccharide matrix, Sepharose (Gacesa and Whish, 1978) and the soluble

polysaccharide matrix, dextran (Gacesa and Whish, 1977; England *et al*,1979). In these studies a Schiff base mechanism was proposed for the reaction between glutaraldehyde and the N^6-amine group of adenine nucleotides because the immobilisation was reversible in the absence of borohydride reducing agent (Gacesa and Whish, 1978). In another study, in which NAD was coupled to lactate dehydrogenase, the conjugated product was also stabilised by borohydride reduction, which again suggested a Schiff base mechanism as Schiff bases are stabilised in the presence of reducing agents (Gacesa and Venn, 1979, *Section 1.6.1*). However, Ukeda *et al* (1989a) pointed out that there was no direct evidence for an unambiguous structural assignment of the product and therefore the Schiff base mechanism could not be confirmed. They agreed that ATP seemed to react with glutaraldehyde via a Schiff base mechanism, but similar reaction with NAD showed a complicated pH dependence that could not be explained by a simple Schiff base mechanism. Consequently, it was suggested that there was a diversity in the binding mode of NAD to glutaraldehyde. However, the functional groups involved in the reaction were not under question, as Ukeda *et al* also observed that the presence of the N^6 amine group of NAD and the terminal aldehyde groups of glutaraldehyde were both fundamental to the reaction. During their studies, Ukeda *et al* were able to use glutaraldehyde to co-immobilise NAD with alcohol dehydrogenase and diapharase onto Sepharose (Ukeda, 1989b).

The method of Gacesa and Venn (see above) was used to make conjugates coupled via glutaraldehyde in this study. Their results are discussed in detail in *Section 1.6.1* and in the second part of this chapter. Gacesa and Venn used the preassembly approach, where the NAD-glutaraldehyde adduct was first formed before being introduced to the enzyme solution (*Section 3.5*). They observed that 30% of specific activity of lactate dehydrogenase was maintained using this technique, and therefore that conditions of excess glutaraldehyde did not seriously inhibit enzyme activity. N^6AHCM-NAD was also successfully coupled using the same technique. The conjugates formed were stable after reduction with potassium borohydride.

Although the coupling chemistry of glutaraldehyde cannot be clearly defined, its use enables NAD to be coupled directly to an enzyme without a laborious derivatisation process. This in itself, along with the desirable properties described above, merits its inclusion as a coupling reagent in this study.

The other coupling technique used involved the direct conjugation of carboxyl and amine groups by a carbodiimide-mediated reaction.

3.4.2 Carbodiimides

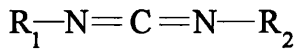


***Figure 3.11** General structure of a carbodiimide.*

Unlike glutaraldehyde, carbodiimide reagents *mediate* coupling reactions and as such they are not incorporated into the final coupled product. They are generally used to link a carboxyl group of one ligand to an amine group of another, forming a conjugate containing a peptide bond. They possess a central carbon atom with twinned double bonds (*Figure 3.11*) and they add carboxylic acids by 1,2-addition to give intermediates from which amides may be formed. The coupling mechanism has been studied in detail (Albertson, 1952) and is shown in *Figure 3.12*.

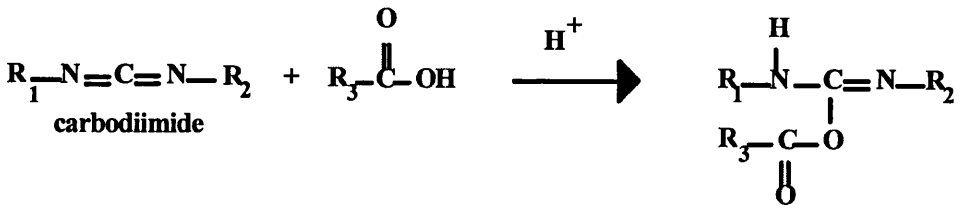
Reaction with proteins

Carbodiimides have been used extensively to modify the carboxyl groups of the aspartyl and glutamyl residues of proteins (Lundblad and Noyes, 1984). The reagent activates a protein-bound carboxyl group for attack by a suitable nucleophile, such as an amine group, as shown in *Figure 3.12* (Hoare and Koshland, 1966). Carboxyl modification requires a protonated carboxyl group and therefore an acidic pH, but the conditions are more amenable to protein stability than other carboxyl- modification

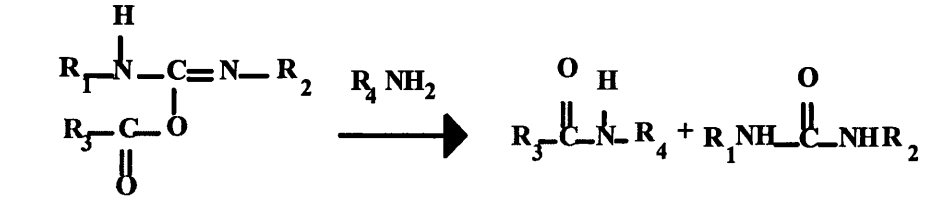
techniques and a carbodiimide reaction at mild pH, although slow, is still possible (Mansson et al, 1978). The yields of carbodiimide couplings can also be enhanced by using *N*-hydroxysuccinimide (*Figure 3.12b*). The *0*-acylurea intermediate formed during carbodiimide coupling (*Figure 3.12a*) is subject to hydrolysis, resulting in decreased coupling efficiencies. The use of *N*-hydroxysuccinimide in conjuction with the carbodiimide reagent enables the formation of active esters that are more resistant to hydrolysis than the *0*-acylurea intermediate, and hence an increase of conjugated material results.

Carbodiimide-mediated reactions have been found to be highly controllable and the selective modification of one residue is often possible. For example, an aspartate residue (Asp 101) found in the active site of lysozyme was selectively modified by using a low excess (five to ten fold) of carbodiimide (Yamada et *al*, 1981). Cysteine and tyrosine have also been known to react with carbodiimide by the same anion attack mechanism as glutamate and aspartate, but these residues are found in special activating environments created by other protein residues (Lundblad and Noyes, 1984). Carbodiimides have also been used to modify the basic amine acid residues of proteins, such as lysine, and to a lesser extent histidine and arginine (Theodoropoulos and Craig, 1955). This sort of modification has been used less often, presumably because an excess of nucleophile was found to promote the carbodiimide reaction (Albertson, 1952). The modification of macromolecular basic residues with nucleotides using carbodiimide reagents has, however, proved feasible (see below).

a. Mechanism of carbodiimide reaction



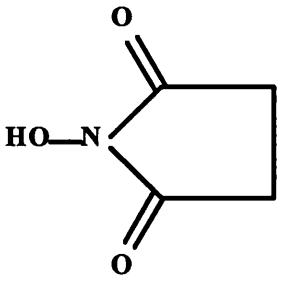
Carbodiimides activate carboxylic acid groups to react with primary amines.



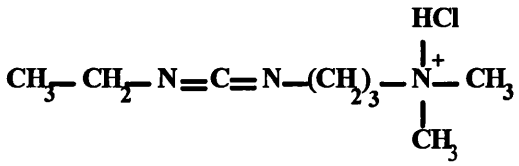
Water-soluble carbodiimides (e.g. EDC) are released as a soluble urea derivative after displacement by a nucleophile, R_4NH_2

b. Role of N-hydroxysuccinimide

The *O*-acylurea intermediate described above is subject to hydrolysis and therefore NHS is used to form active esters of the intermediate which are resistant to hydrolysis. In this way NHS enhances the yields of carbodiimide couplings.



c. Structure of EDC



EDC {1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride}

Figure 3.12: *EDC and carbodiimide coupling of amine- and carboxyl-containing compounds*

The ability of carbodiimide reagents to modify both the acidic and basic residues of proteins allowed the use of both NAD derivatives synthesised in this study. Either the carboxyl group of N^6CM-NAD could be activated for nucleophilic attack by lysyl residues of dehydrogenases, or the carboxyl-containing residues of dehydrogenases could be activated for nucleophilic attack by the terminal amine group of N^6AHCM-NAD.

Reaction with nucleotide coenzymes

In an early study, umodified NAD was coupled to ε-aminocaproic acid ligands on Sepharose beads using dicyclohexylcarbodiimide (Larsson and Mosbach, 1971). However, the coupling reaction took 20 days and the reaction was thought to occur via ester formation with the adenine ribose hydroxyl groups of NAD. Carbodiimide has been used in the popular water soluble form, 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide.HCl (EDC) (*Figure 3.12c.*), to modify both amine and carboxyl containing derivatives of NAD. It has been used in several studies to derivatise carboxyalkyl side chains attached to the amine group of the adenine ring of NAD. In fact, this is the method by which N^6CM-NAD was converted to N^6AHCM-NAD in this study (*section 3.2*) (Lindberg *et al*, 1973). A similar carbodiimide-mediated reaction was employed by Okuda *et al* (1985a&b) to synthesise new derivatives of NADP (again starting from a carboxyalkyl side chain attached to the adenine N^6 position) and also by Okada and Urabe (1987) to attach a vinyl-containing lysine derivative to a carboxyl-containing derivative of NAD, forming a self-polymerisable NAD molecule.

Because the attack of carbodiimide-activated species is promoted by an excess of nucleophilic amine groups, as discussed above, and because N^6CM-NAD is slightly unstable under the acidic conditions of the carbodiimide coupling, it might be expected that carboxyalkylated NAD derivatives are unsuitable for use with EDC. However, carboxyl-containing NAD molecules have been coupled to amine-containing water-

soluble and insoluble macromolecular supports with success. Using EDC, Zappelli *et al* (1975) immobilised an NAD derivative carrying a ω-carboxyalkyl side chain attached to the N^6 adenine position, to polyethyleneimine, polylysine, and aminohexyl-Sepharose. The immobilised NAD derivatives showed a slight decrease in their original substrate activity, each activity being 60% of the corresponding activity in free solution, but a stable amide linkage was effected. Similarly, Okada and Urabe (1987) coupled N^6-(2-carboxyethyl)-NAD to polyethyleneglycol (PEG) using carbodiimide. The resulting PEG-NAD complex performed well as a coenzyme with several dehydrogenases.

NAD derivatives containing aminoalkyl side chains have also been coupled using EDC, mainly for use as affinity ligands (Mosbach, 1978). Carboxy-activated PEG has been linked to N(1) 2-aminoethyl-NAD and similar NAD derivatives have been immobilised to other macromolecular supports (Buckmann and Carrea, 1989). In fact, this sort of coupling has been used to couple NAD derivatives to the surface of dehydrogenases. Mansson *et al* (1978) used carbodiimide to mediate a reaction between N^6AHCM NAD and acidic surface residues of alcohol dehydrogenase. The water soluble carbodiimide, EDC, was again used in excess and the urea by-product washed off with water. The specific activity of the modified enzyme was 37% of that of native alcohol dehydrogenase, indicating that carbodiimide-coupling does not seriously inhibit the catalytic action of dehydrogenases. 0.3-1.6 coenzyme molecules were covalently incorporated per subunit molecule. Carbodiimide was similarly employed by Kato *et al* (1987) to couple N^6AHCM-NAD to formate dehydrogenase (Kato *et al*). They found that the specific activity of the modified enzyme was actually 16% higher than the specific activity of the native enzyme, confirming that carbodiimide reagents were suitable for use with dehydrogenases. A carbodiimide-mediated reaction was again used by Warth *et al* (1989) to link NAD derivatives to lactate dehydrogenase. Several different NAD derivatives were used, all formed by NAD modification at the N^6-adenine position and with either a carboxyl- or amine-containing group attached. The

amine group of the derivative was coupled to carbodiimide-activated carboxyl groups present on the surface of lactate dehydrogenase; alternatively the carboxyl group of N^6CMNAD was activated and then mixed with an LDH solution to react with the enzyme lysyl residues. Each modified lactate dehydrogenase preparation showed only a slight decrease in specific activity - between 30-40% of the specific activity of the original LDH was lost upon modification. Between 1.0 and 2.0 coenzyme molecules were incorporated per LDH subunit and this did not depend upon whether the carbodiimide-activated carboxyl group was present on the enzyme or on the coenzyme (These enzyme-coenzyme conjugates are discussed in more detail in *Section 1.6*).

The NAD derivative-dehydrogenase coupling attempts described above fail to mention any attempt to couple the unmodified coenzyme, NAD, to dehydrogenase enzymes using EDC even though NAD has been successfully coupled to Sepharose using a water-insoluble carbodiimide (Larsson and Mosbach, 1971). No attempts have been made to couple unmodified NAD using the more favourable water-soluble carbodiimide, EDC, and therefore, attempts were made to immobilise unmodified NAD using EDC in this study.

3.5 The coupling reaction

Coenzyme, enzyme and coupling reagent were mixed together in solution. The order of addition and the reaction time varied depending on the method used (*Materials and Methods*).

In this study the preassembly approach to conjugate synthesis was used, whereby the structure of the coenzyme used was defined before its addition to the coupling mixture. The advantages of this method, as opposed to one where the spacer-ligand assembly is built up on the enzyme surface, have been reviewed (Mosbach, 1978). Because the

coenzyme ligand has a defined structure and can be characterised before conjugation, useful information can be obtained about its chemical and kinetic properties. In addition, if a spacer is attached to NAD it can introduce a functional group that is more reactive than any of the functional groups present on the unmodified molecule. This means that there is little doubt as to which group will be involved in the coupling reaction, and the site of linkage to the enzyme can therefore be defined. The preassembly approach also avoids an excess of spacer groups being attached to the protein surface because the reactive NAD ligand can be isolated from excess spacer groups beforehand. An excess of attached spacer groups with hydrophobic or hydrophilic character may interfere with the action of the enzyme involved.

Ternary complex formation

In some previous studies a substrate derivative was added to the coupling mixture so that a "dead-end" ternary complex was formed between the enzyme, its coenzyme and a substrate derivative (*Section 1.6.2*). The presence of coenzyme and substrate bound at the active site is thought to protect 'sensitive' amino acid residues from unwanted modification. These sensitive residues are usually found in or near the active centre, where the coenzyme and substrate binding sites are also found. A ternary complex was formed during the coupling of enzyme-coenzyme conjugates involving lactate dehydrogenase (Woenckhaus *et al*, 1983) and alcohol dehydrogenase (Goulas, 1987; Schafer *et al*, 1986). Oxalate and pyrazole were used, respectively, as substrate analogues.

In our studies a ternary complex was not formed during the coupling reaction. This decision was taken because in enzyme-coenzyme coupling studies carried out in the past without the formation of a "dead-end" ternary complex during coupling, the specific activity has never been seriously affected, the lowest value observed being 30% of the native enzyme for a lactate dehydrogenase-NAD conjugate (Gacesa and Venn, 1979). Moreover, Mansson *et al* (1978) found that the coupling of N^6AHCM-

NAD to LADH was only possible in the absence of pyrazole, and so ternary complex formation was abandoned. It is therefore conceivable that the best position on the enzyme for the covalent binding of a coenzyme, with respect to proximity of the coenzyme binding site, may be unaccessible during ternary complex formation. Goulas (1987) compared different coupling experiments which were carried out either with or without the formation of a ternary complex during the coupling reaction. The results did not appear to be dramatically different. The intrinsic activity of LADH-coenzyme complexes formed during ternary complex formation was 27% of the specific activity of the modified enzyme, whilst that for LADH-coenzyme complexes formed in the absence of pseudosubstrate was 13%.

The case for ternary complex formation during coupling experiments is clearly not strong. In order to simplify the approach, the technique was omitted in this study.

3.6 Purification of prepared enzyme-coenzyme complexes

Once the coupling reaction was complete, the next step was to isolate the enzyme-coenzyme complex from unreacted coenzyme, so that any intrinsic activity detected later could be attributed solely to the presence of covalently-bound coenzyme. Gel filtration has proved to be an effective method for achieving such a separation as long as a "baseline" separation between protein material and free nucleotide material is achieved (Venn *et al*, 1977). However, in one enzyme-coenzyme study, a quantity of free coenzyme was found in the protein fractions after a "baseline" separation by gel filtration (Mansson *et al*, 1978). The impurity made up to a third of the total coenzyme present, although surprisingly this non-specifically bound coenzyme did not appear to have any substrate activity.

In our study a series of experiments was set up to ensure that a negligible amount of free coenzyme would be present in conjugate solutions after gel filtration, as reported in the following section.

3.6.1 Gel filtration chromatography

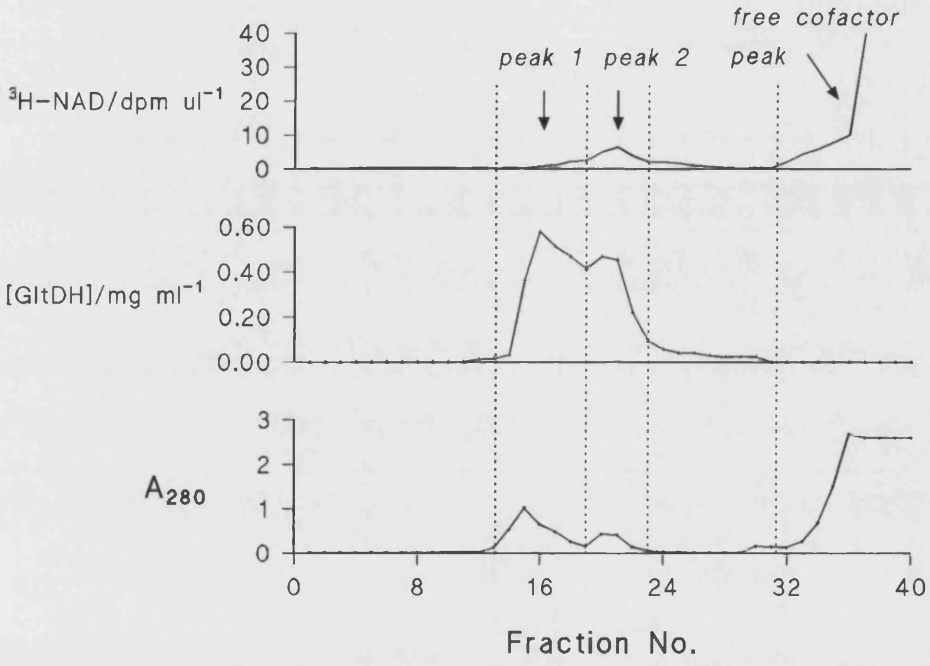


***Figure 3.13**: Elution profile of a typical separation of a dehydrogenase-coenzyme complex from uncoupled coenzyme by gel filtration chromatography.*
GltDH=Glutamate dehydrogenase

A Sephacryl S-100 column (80 x 1.5cm) was used to separate a glutaraldehyde-coupled glutamate dehydrogenase-N^{6}AHCM-NAD complex from uncoupled N^{6}AHCM-NAD. The column was run at 4^{o}C with a flow rate of 0.2ml min^{-1}. Fractions were collected in 2.0ml volumes in a dropwise manner using an automatic fraction collector. The fractions were then immediately taken for analysis to determine the following properties:

protein concentration: measured using Coomassie method; 50µl of each fraction was mixed with 2ml of a 50:50 mixture of saline and Coomassie Brilliant Blue G reagent and left for 5mins. The absorbance of the mixture was then read at 620nm and the protein concentration calculated from a standard curve.

^{3}H-NAD counts: 200µl of each fraction was mixed with 2ml Optiphase "Safe"scintillant in a scintillation vial and counted in a LKB scintillation counter for 15 mins in triplicate, using a ^{14}C window and a ^{3}H window. Counting efficiency was calculated as 60% for ^{3}H and 98% for ^{14}C by calculating the efficency of the machine when counting a dated standard sample of ^{3}H-labelled material. The counts per minute were then adjusted to give disintegrations per minute (dpm).

Figure 3.13 shows a gel filtration elution profile for a glutamate dehydrogenase-coenzyme complex incorporating N^{6}AHCM-NAD coupled via glutaraldehyde. The

elution profile is typical of all the enzyme-coenzyme complexes studied. Other elution profiles can be found in the relevant section for each dehydrogenase in part two of this chapter. The protein-containing fractions had a tendency to separate into two minor peaks upon elution. In most cases, the first peak contained more conjugated coenzyme than the second peak, although this was not always the case. A small amount of free NAD was present in peaks 1 and 2 (*Figure 3.13*), as measured by adding a sample of ^{3}H-NAD to an enzyme-coenzyme conjugate before gel filtration (see below). Because the latter protein fractions (peak 2) contained 80% of the free ^{3}H-NAD found in the protein fractions, only the first half of the protein-containing fractions were used in subsequent studies. This precaution was taken to ensure beyond doubt that the amount of free NAD was always negligible. In fact, the following series of experiments was set up to show that the amount of free coenzyme in both peak1 *and* peak 2 was negligible. The actual values are given below.

3.6.2 Experiments to confirm effective isolation of enzyme-coenzyme complexes from free coenzyme by gel filtration chromatography

1. A known amount of free ^{3}H-NAD was added to a previously coupled enzyme-coenzyme mixture before it was subjected to gel filtration. The total proportion of free coenzyme remaining in the conjugate peak after gel filtration could then be calculated (*Figure 3.13*, top diagram). A small amount of free coenzyme did remain with the protein fractions.

It was calculated that the protein fraction containing the most free coenzyme contained up to 0.01% of the total ^{3}H-NAD added (this was the highest percentage obtained from two separate runs of the above experiment). The highest amount of coenzyme added in a coupling reaction was 10mg. Therefore, 1.0μg (0.01% of 10mg) would be the highest amount of free coenzyme present in any protein-containing fraction. As only a small amount of each fraction was taken for subsequent assay (up to 100μl from

a 2ml fraction volume) then a maximum of only 0.075 nmol of coenzyme would be present duing final assays after gel filtration. This concentration of free coenzyme was considered to be a negligible amount because it was well beyond the detection limits of both the Nisselbaum-Green recycling assay for NAD, and the other less sensitive assays used to measure the endogenous activity present in enzyme-coenzyme conjugates. All assays could not detect below 0.1nmol of NAD.

This result was confirmed by the following sets of experiments.

2. Protein-containing fractions were collected after gel filtration, and the protein denatured in 8M urea. This would release any coenzyme that was not covalently bound to the enzyme into the surrounding solution. The protein was precipitated, centrifuged and the pellet removed. The supernatant was then analysed for free NAD using the Nisselbaum-Green recycling assay which is sensitive to >0.1nmol NAD. No coenzyme could be detected.

3. "Dummy" conjugation experiments were also carried out. Here the experimental conditions were identical to a typical conjugation experiment, apart from the omission of the coupling reagent - again, no coenzyme could be detected, either using the Nisselbaum-Green recycling assay or by analysing 100µl of each protein-containing sample using TLC. The presence of ≥0.5nmol of nucleotide material can be detected on PEI-cellulose TLC plates under ultra-violet light (Randerath and Randerath, 1967).

After the above experiments were completed it was assumed that the only coenzyme present in protein fractions was enzyme-bound via a covalent linkage. However, after gel filtration of native dehydrogenases, small quantities of coenzyme were evident. The native and modified enzymes were therefore further purified after gel filtration by washing with activated charcoal (*Section 3.8.1*).

After purification, each enzyme-coenzyme preparation was ready to be characterised and was analysed to determine both the extent of conjugation and the size of any intrinsic activity present.

3.7 Determination of the success of the coupling reaction - the calculation of the amount of coenzyme conjugated to each enzyme molecule

In order to determine the extent of conjugation between coenzyme and enzyme, the molar ratio of bound coenzyme to enzyme subunit was determined for a purified enzyme-coenzyme preparation. This was achieved using two different methods: one method served to determine the molar ratio of nucleotide to enzyme protein, while the other determined the molar ratio of reducible coenzyme to enzyme protein.

3.7.1 Determination of ratio of coenzyme molecules to enzyme subunit molecules by exploitation of the ultra-violet absorption properties of an enzyme-coenzyme complex.

Because an enzyme-coenzyme complex contains nucleotide coenzyme material (λ_{max} 260-270nm) and protein material (λ_{max} 280-290nm) the individual concentrations of each can be determined simply by taking two different absorbance readings in the ultra-violet region of the spectrum. Immediately after gel filtration the absorbance of each fraction was recorded at 266nm and 290nm and, by using the equation system first applied by Mansson *et al* (1978), the concentration ratio of coenzyme to enzyme could be calculated. The procedure involves the formation and solvation of two simultaneous equations as shown *Figure 3.14*:

$$a[E] + x[NAD] = A_{266}$$

$$b[E] + y[NAD] = A_{290}$$

Enzyme	Molar absorption coefficient	
	a	*b*
LDH	a = 1.33 $mg^{-1}mlcm^{-1}$	b = 1.20$mg^{-1}mlcm^{-1}$
GltDH	a = 1.23 $mg^{-1}mlcm^{-1}$	b = 1.13 $mg^{-1}mlcm^{-1}$
YADH	a = 1.22 $mg^{-1}mlcm^{-1}$	b = 1.15$mg^{-1}mlcm^{-1}$

Coenzyme	Molar absorption coefficient	
	x	*y*
NAD	18,000$cm^{-1}M^{-1}$	1600$cm^{-1}M^{-1}$
N^6CM-NAD	19,500$cm^{-1}M^{-1}$	2000$cm^{-1}M^{-1}$
N^6AHCM-NAD	20,000$cm^{-1}M^{-1}$	1800$cm^{-1}M^{-1}$

Figure 3.14 *The equation system used to estimate the individual concentration of coenzyme and enzyme in an enzyme coenzyme complex* where *a* and *b* represent the molar absorption coefficient of each enzyme used, and *x* and *y* represent the molar absorption coefficient of each coenzyme used. The molar absorption coefficients were calculated from the absorbance of solutions of known concentration. Purified samples of both enzyme and coenzyme were taken and the absorption of their solutions were read at 266nm and 290 nm. The Beer-Lambert law was applied to calculate E, the molar extincton coefficient.[A=E.c.l, where A=absorbance, E= molar absorption coefficient, c= concentration of solution (M), and l = length of light path (= 1cm)]

The figures are based on the molar absorption coefficents at 266 and 290nm for each coenzyme and enzyme used. The coefficients were calculated by reading the absorbance of samples of known concentrations at those wavelengths (*Figure 3.14*).

This spectrophotometric method has been validated by Mansson *et al* (1978) by measuring coenzyme concentration using both ^{14}C-labelled coenzymes and phosphate concentration determination of NAD, and by measuring protein concentration with the Lowry method. This method is a convenient way with which to measure the concentrations of coenzyme and enzyme in an enzyme-coenzyme conjugate. However, because the biological activity of the bound coenzyme is of paramount importance to the ability of an enzyme-coenzyme complex to act as a self sufficient catalytic unit an

additional method was used to determine whether the bound coenzyme was actually *reducible*.

3.7.2 Determination of the molar ratio of reducible coenzyme to enzyme subunit

The amount of covalently bound coenzyme available for reduction by a second enzyme was determined to give an indication as to what proportion of the immobilised coenzyme molecules were active. Although such a measurement would not be the same as a direct measurement of the coenzyme available for reduction with the same enzyme to which it was bound, it would provide an indication of the accessibility of the bound coenzyme and provide for an interesting comparison. The amount of protein available was determined using the Coomassie method, whilst the amount of bound coenzyme available for reduction by a second enzyme was determined using the Nisselbaum-Green recycling assay (*Figure 3.6*) with alcohol dehydrogenase and/or a similar recycling assay using lactate dehydrogenase.

Once the proportion of reducible bound coenzyme had been established, the specific activity of the modified enzyme and the endogenous catalytic activity of the enzyme-coenzyme complex was determined.

3.8 Activity determinations

After purification, each enzyme-coenzyme complex was assayed for intrinsic activity and specific activity: The intrinsic activity was measured by using a coenzyme-recycling assay, otherwise the activity would be limited by one catalytic cycle for each reducible enzyme-bound coenzyme. Assays for intrinsic activity should detect whether a bound coenzyme is available for catalysis, either with the very same enzyme molecule to which it is bound (**intra**molecular reaction), or with neighbouring enzyme molecules in solution (**inter**molecular reaction). The specific activity was also determined using a coenzyme-recycling assay in order to provide a consistent approach for comparison

with the intrinsic activity results. Specific activities were measured in order to estimate whether the coupling process had seriously decreased the efficiency of enzyme catalysis. Any serious inactivation of the enzyme would probably decrease the chances of obtaining a good intrinsic activity with its coenzyme complex.

Two types of coenzyme-recycling assay were used: one employed a DCPIP/PES redox couple and the other employed an MTT/redox couple (*Figure 3.6*). Whenever the same enzyme preparation was assayed for activity using both recycling assays, the two assays showed good agreement in terms of (μmoles substrate) converted / min / mg protein. The results from both assays were, therefore, converted into the same units (μmol/min/mg) making it possible to directly compare the activities of different enzyme-coenzyme complexes that had been assayed using the different recycling assays.

During initial use of the DCPIP/PES recycling assay, a problem was encountered: upon assay of a native dehydrogenase *with no covalently bound coenzyme*, a significant decrease in absorbance at 610nm was observed. This absorption decrease must have been due to the oxidation of a reduced species by the redox dye, dichlorophenolindophenol (see *Figure 3.6b*) which consequently resulted in a change of its absorption properties at 610nm. The decrease in absorbance was too large and erratic to simply be taken into account in an assay for the intrinsic activity of a enzyme-coenzyme conjugate. In an attempt to identify the reduced species and to eliminate the problem some interesting results were obtained.

3.8.1 Modification of the DCPIP recycling assay

The theory behind the DCPIP assay is explained in *section 3.2.2.2*. The decrease in absorbance, due to oxidation of DCPIP, upon addition of native enzyme but without addition of coenzyme is a phenomenon that has been observed before (Slater *et al*, 1964; Bernofsky and Schwann, 1973). Several explanations have ensued, including proposals that a substance present in the tissue sample interferes with the assay, that

there are protein sulfhydryl groups present which have a redox activity low enough to be able to reduce an NAD molecule (Bernofsky and Schwann, 1973), or that there are coenzyme impurities remaining in a supposedly purified sample. Because companies selling enzymes admit to the presence of coenzyme in their samples, the latter theory was tested first. The decrease in absorbance might be due to the presence of free NAD, tightly bound to the enzyme by non-covalent forces.

After dialysis and gel filtration of native lactate dehydrogenase, an absorbance decrease of 0.03 A_{610}/min/mg was still evident in the DCPIP/PES recycling assay. In order to totally remove all non-covalently bound coenzyme a charcoal washing procedure was employed (Velick *et al*, 1953). Each enzyme solution was washed with activated charcoal which strongly adsorbs any non-covalently bound NAD.

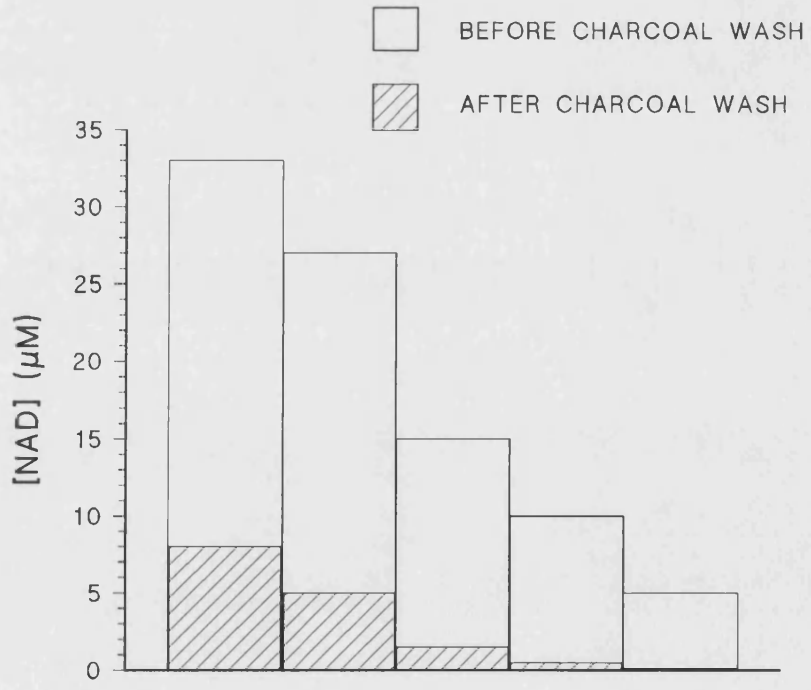


***Figure 3.15:** The adsorption of NAD by charcoal.* 1mg of active charcoal was added to 1ml of NAD (concentration at zero time = 70μM, 60μM, 30μM, 20μM or 10μM) in H_2O at room temperature, and mixed periodically for 30 min, before NAD concentration was measured at 266nm.

As illustrated in *Figure 3.15,* activated charcoal was very effective at adsorbing NAD in free solution, removing up to 25μmol NAD (gm charcoal)$^{-1}$. Because activated charcoal is also known to adsorb protein, the NAD-washing protocol was applied to a protein solution to determine how much protein was lost. Charcoal-washing of lactate dehydrogenase or alcohol dehydrogenase solutions with a charcoal:protein ratio of 1:1 (w/w) in all cases resulted in a loss of 5-10% of protein material, independent of whether each enzyme had been covalently modified with coenzyme or not. The loss of this small amount of protein material during the washing of an enzyme-coenzyme sample was deemed to be acceptable.

Native dehydrogenases that had been washed with activated charcoal showed an undetectable decrease in absorbance when analysed for intrinsic activity with the DCPIP/PES recycling assay (*Figure 3.16*) - the reduced species was no longer present in solution.

A similar stability of the blank rate occurred upon the addition of phosphodiesterase (PDE) to a native dehydrogenase solution prior to the DCPIP/PES assay (*Figure 3.16*), although the effect was somewhat reduced, with 30% of the absorbance decrease remaining. PDE is an enzyme which catalyses the hydrolysis of phosphate esters. There are two such esters present in the structure of NAD and PDE breaks it down into AMP and NMN, components having no biological activity in this system. Thus NAD was effectively removed

These results add weight to the theory that the presence of NAD associated with native dehydrogenases through non-covalent forces leads to an apparent intrinsic activity in the DCPIP recycling assay. Pre-washing of dehydrogenase solutions with active charcoal before assaying is a convenient and effective way to make measurements of actual intrinsic activities easier to follow, the only drawback being a small loss of protein.

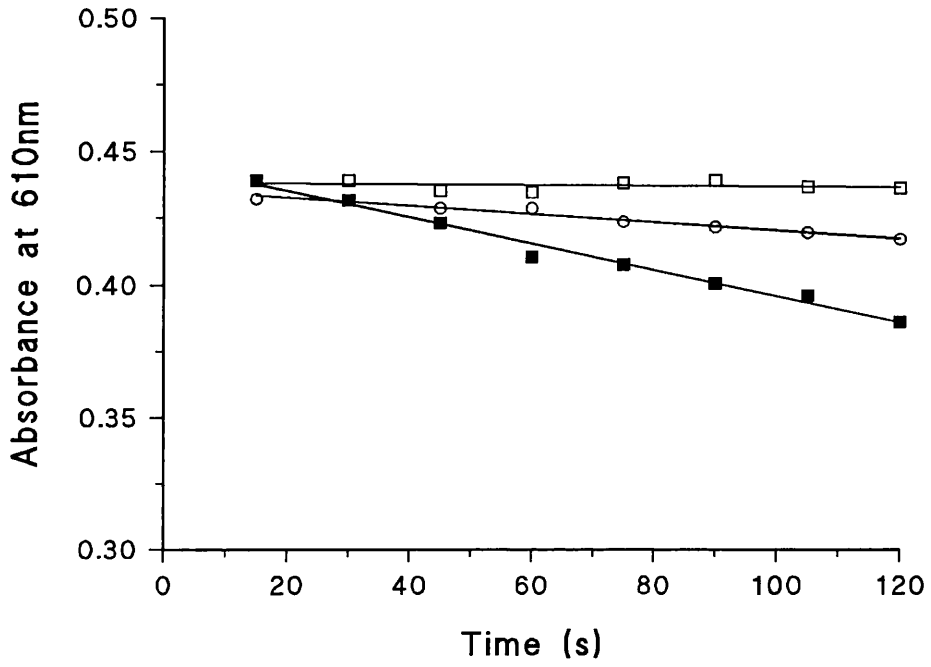


Figure 3.16: *The effect of charcoal washing and phosphodiesterase (PDE) addition on the instability of the blank rate in the DCPIP recycling assay.* ■ = blank rate after gel filtration; □ = rate after charcoal-washing; O = rate after PDE addition. Assay solution: 0.75ml 100mM potassium phosphate buffer (pH 7.5), 2μmol PES, 50nmol DCPIP, 50μmol lactate and 100μl LDH (1mg ml^{-1}). *Charcoal washing:* 0.1 mg active charcoal was added to 1ml of enzyme solution at room temperature, and mixed periodically for 30mins before assaying. *Phosphodiesterase (PDE) addition:* 10μl *Crotalus durissus terrificus* PDE (1mg ml^{-1}) was added to 0.5 ml of enzyme solution, mixed and left at room temperature for 60mins before assaying.

PART TWO: Properties of enzyme-coenzyme conjugates

The first part of this chapter dealt with the approach used to prepare and analyse enzyme-coenzyme conjugates synthesised via amine and carboxyl functional groups. The results that were generated from this approach will be presented in this section. The results are categorised depending on the constituent enzyme of the enzyme-coenzyme complex. Relevant properties of each enzyme used will also be discussed here, whereas the properties of each coenzyme and coupling agent used were discussed in part one of this chapter.

Certain aspects of each dehydrogenase that may have affected the formation and properties of their coenzyme conjugates are now considered.

3.9 General properties of NAD-dependent dehydrogenases

The three-dimensional structure of a coenzyme-dependent enzyme often includes the presence of a deep cleft, formed between the interface of two major domains. This cleft serves as a coenzyme "docking bay" (Anderson *et al*, 1979) and is often present in NAD-dependent dehydrogenases. Amongst the dehydrogenases that possess the classic double-domain structure (*Figure 3.17*) are lactate dehydrogenase (Adams *et al*, 1970), malate dehydrogenase (Hill *et al*, 1972), glyceraldehyde-3-phosphate dehydrogenase (Buehner *et al*, 1974), alcohol dehydrogenase (Eklund *et al*, 1976) and glutamate dehydrogenase (Rice *et al*, 1987). Another structural motif that is common to these enzymes is a region of alternating parallel β-sheets and α-helices. At the centre of this motif is often found the NAD-binding loop, a flexible stretch of peptide which, upon substrate binding, creates a more favourable environment for substrate catalysis (Hill *et al*, 1972; Baker *et al*, 1992a). The general function of the two major domains is now clear: one organises molecular assembly and provides residues for the

binding of non-coenzymic substrate, while the other carries the NAD-binding capability (Baker *et al*, 1987).

It is not surprising, then, that most dehydrogenases appear to have the same mode of NAD binding, even though the residues involved may differ (Moras *et al*, 1975). The strategy of all NAD-dependent dehydrogenases is to orient the C-4 position of the nicotinamide ring of the coenzyme towards the reactive centre of the substrate on the enzyme surface (Zubay, 1988). The coenzyme is bound in an open structure (Baker *et al*, 1992a), the adenine ring being sandwiched between hydrophobic residues, whilst the ribose and phosphate and sometimes the nicotinamide moieties are involved in hydrogen bond interactions with residues of the enzyme.

Because of the similar mode of NAD-binding, the same approach could be used in this study to prepare a number of different dehydrogenase-coenzyme conjugates. However, certain properties that were unique to each enzyme would undoubtedly affect the formation of the individual enzyme-coenyme conjugates. For example, the sensitivity of the enzyme to modification or the availability of certain residues for reaction with the coupling reagent may differ between dehydrogenases. Each dehydrogenase will now, therefore, be discussed independently.

3.10 Lactate dehydrogenase: properties relevant to its conjugation with coenzymes

Lactate dehydrogenase (LDH) is one of the most thoroughly investigated of the NAD-dependent dehydrogenase enzymes. It has many potential commercial applications (*Section 1.3*). Along with alcohol dehydrogenase, it has proved to be amenable to the formation of conjugates with NAD or NAD derivatives (Venn *et al*, 1977; Warth *et al*, 1989; Schafer *et al*, 1986; Yomo *et al*, 1992). Consequently, LDH was chosen as a

model dehydrogenase in this study and two of the LDH-coenzyme conjugates formed were incorporated into enzyme electrode systems (*Chapter 5*).

LDH is an NAD-dependent enzyme and it catalyses the reversible reaction of pyruvate to lactate, a reaction which is found at the end of the glycolytic pathway.

$$\text{LACTATE} + \text{NAD}^+ \overset{\text{LDH}}{\Leftrightarrow} \text{PYRUVATE} + \text{NADH} + \text{H}^+$$

The equilibrium of the above reaction lies very much to the left, with an equilibrium constant, K_{eq}, of around 3×10^{-5}M at pH 7.0, in phosphate buffer, at 25°C (Hakala *et al*, 1956). Because the ultimate interest lay in the reverse reaction, the pH of the intrinsic activity assays was raised slightly, to pH 7.5-8.0, in order to help shift the reaction equilibrium towards lactate oxidation. The lactate oxidation reaction was important because of the eventual enzyme electrode applications of the LDH-coenzyme conjugates where lactate would be the substrate of analytical interest. In addition, the coenzyme is more successfully regenerated from its reduced state (NADH) to its oxidised state (NAD) by the electrode used, and so the lactate oxidation reaction could be more easily monitored than the pyruvate reduction reaction in the enzyme electrode.

3.10.1 Structure

LDH exists as a tetramer of MW 140,000, consisting of subunits of MW 35,000 (see *Figure 3.17*). Each of these subunits is globular, apart from an elongated peptide stretch at the N-terminus. Two major structural forms of the subunit exist: the M chain, usually found in anaerobic tissues and the H chain, usually found in aerobic tissues. The chains differ in amino acid composition and they also possess different physical, kinetic and immunological properties (Everse and Kaplan, 1974). Nevertheless, the M and H chains can readily combine with each other in any

combination to form a tetramer, and consequently five isoenzymes of LDH are known: H_4, MH_3, M_2H_2, M_3H and M_4. The isoenzyme chosen for use in these studies was H_4 LDH from pig; the reasons for its choice are explained below. The subunits of LDH have been shown to act independently of the each other and to bind NAD at the same rate (Holbrook *et al*, 1975). Each subunit was treated as a separate catalytic unit in this study.

Each LDH molecule can bind one molecule of NAD per subunit, each subunit consisting of a catalytic domain and a nucleotide binding domain (*Section 3.9)*. In pig H_4 LDH, the latter domain consists of around 150 amino acids out of a total of 333 residues (Eventoff *et al*, 1975). LDH shows an ordered substrate binding mechanism: NAD must bind before lactate can and, after catalysis, pyruvate leaves before the coenzyme (see *Figure 1.5*) (Holbrook *et al*, 1975). Upon transition from a binary complex (coenzyme bound to enzyme) to a ternary complex (coenzyme and substrate bound to enzyme) a pronounced confirmational change occurs in each LDH subunit (Holbrook *et al*, 1975). This structural movement includes the closing of a flexible peptide loop over the active centre to provide a favourable environment for catalysis (Grau *et al*, 1981).

A basic scheme for the catalytic mechanism of LDH is shown in *Figure 3.18*. As with all other NAD-dependent dehydrogenases, the pyridine ring of NAD is the active nucleus of the coenzyme, while the remainder of the molecule is responsible for enzyme binding and recognition. Several studies with LDH have confirmed the importance of the ribose, phosphate and, to a lesser extent, the adenine moieties in coenzyme binding (Everse and Kaplan, 1974). X-ray crystallographic studies have been carried out at low resolution on various LDHs and have confirmed the role of these moieties (Adams *et al* 1970, Eventoff *et al,* 1975, Grau *et al,* 1981). In the case of pig H_4 LDH, X-ray diffraction data was obtained at 0.27nm resolution (Grau *et al*, 1981). *Figure 3.19* shows the bonds that form between the pig H_4 LDH and NAD

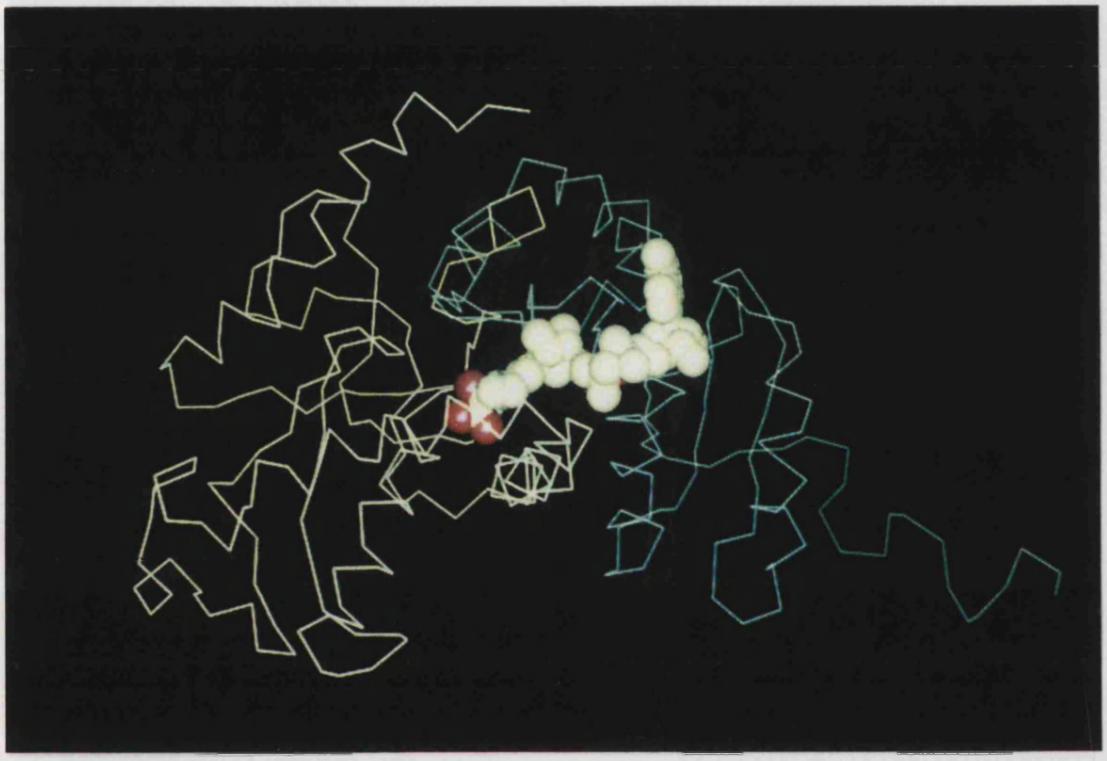


Figure 3.17: *One subunit of LDH showing two domains (green and yellow) separated by the coenzyme-binding cleft. A molecule of NAD is shown bound in the catalytic position.*

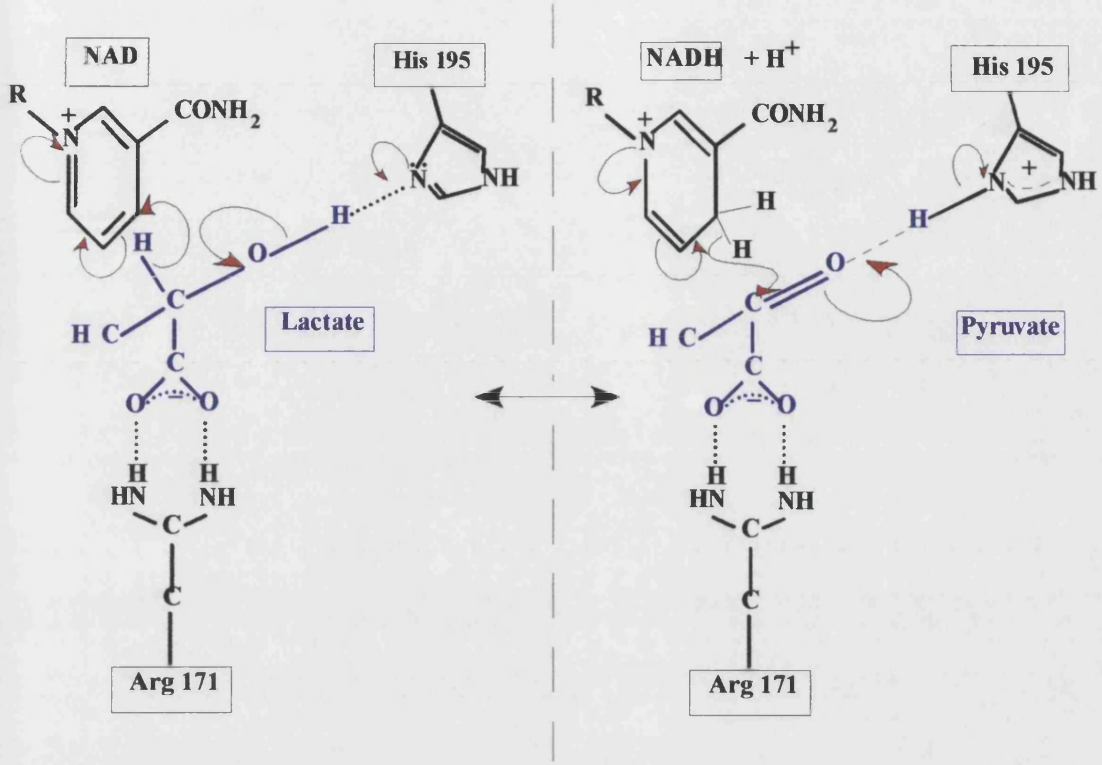


Figure 3.18. *The catalytic mechanism at the active site of lactate dehydrogenase.* The nicotinamide moiety of NAD accepts a hydride ion from lactate in the oxidation reaction, and donates a hydride ion in the reduction of pyruvate. Substrate is shown in blue, and hydrogen bonding is denoted by dotted lines.

during coenzyme binding, and illustrates the relative availability of the C-8 and N^6 positions of the adenine ring for modification by solvated reagents. Indeed, Grau *et al* (1981) observed that in pig H_4 the amine group of NAD protruded noticably further into the solvent when bound to LDH than it did in the dogfish M_4 isozyme. The data of Grau *et al* was used to prepare *Figure 3.3*, which clearly illustrates the solvent accessibility of the adenine N^6 group of NAD when it is bound to pig H_4 LDH.

Having established that the N^6 amine of NAD appears to be a suitable position for attachment of one end of a molecular bridge between LDH and the coenzyme, we shall now consider the opposite end of the bridge and the most suitable positions for its anchorage to the surface of the LDH molecule.

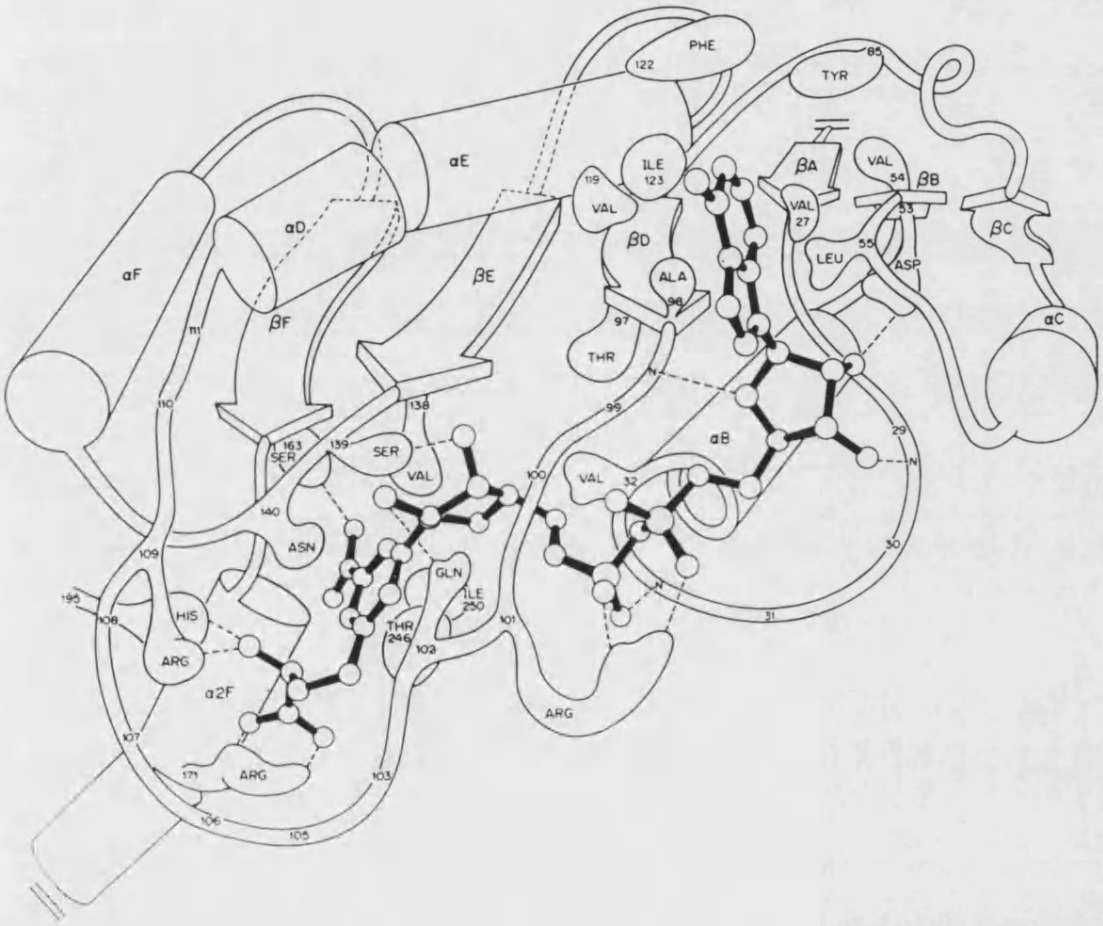


Figure 3.19 *Diagrammatic representation of a molecule of NAD bound at the catalytic site of pig heart LDH.* Hydrogen bonds are denoted as dotted lines.

There are several reactive residues commonly found on the surface of proteins. The ε-amine group of lysyl residues and the side chain carboxyl groups of aspartyl and glutamyl residues are amongst the most widely used in modification and immobilisation studies. They were used in this study as points of attachment for NAD.

3.10.2 ε-Amine groups of lysyl residues

Structural studies show that the main source of solvent accessible amine groups present on the protein surface are most commonly the ε-amine groups of lysyl residues (Creighton, 1993). As discussed in *Section 3.4*, glutaraldehyde was used to attach each amine-containing coenzyme to LDH via the enzyme's lysine amine groups, and carbodiimide was used to attach the carboxyl containing coenzyme, N^6CM-NAD to LDH, also via its surface amine groups.

LDH has been immobilised using ε-amine groups as anchorage points for spacer groups in previous studies. For example, chicken H_4 LDH was immobilised onto alkylamine-derivatised glass beads using both glutaraldehyde coupling and carbodiimide coupling (Stolzenbach and Kaplan, 1978). The immobilised enzyme was stable, immobilisation actually improving its stability, and over 50% of its lactate activity remained.

3.10.2.1 Solvent accessibility

The total lysine content of pig H_4 LDH has been reported by Klitz *et al* (1977) in their primary structure determination of the enzyme. There were 24 lysyl residues per enzyme subunit out of a total of 333 residues. Because the location of polar and hydrophobic side chains in LDH has been reported to be typical of that in most other proteins (Holbrook *et al*, 1975) it was assumed that many of these lysyl residues were present on the protein surface where they were available for interaction with reactive ligands present in free solution. In an X-ray crystallographic structure determination of

dogfish M_4 Apo-LDH, which is very similar in primary structure to pig H_4 LDH (Klitz *et al*, 1977), the relevant solvent accessibility of each residue was calculated (Abad-Zapatero *et al*, 1987). The surface of each amino acid residue, X, was normalised with respect to the same residue (X) as found in the tripeptide Gly-X-Gly. If the enzyme residue had a side chain accessible surface area which was greater than 20% of its available area in the extended Gly-X-Gly tripeptide, it was labelled accessible, and if the value was greater than 5%, it was labelled partially accessible. 18 out of 29 lysyl residues were accessible, and another 6 partially accessible, leaving only 17% of lysyl residues inaccessible to the solvent. The terminal amino group of pig H_4 LDH is acetylated in nature, and so it will not be subject to modification by either glutaraldehyde or carbodiimide reagent.

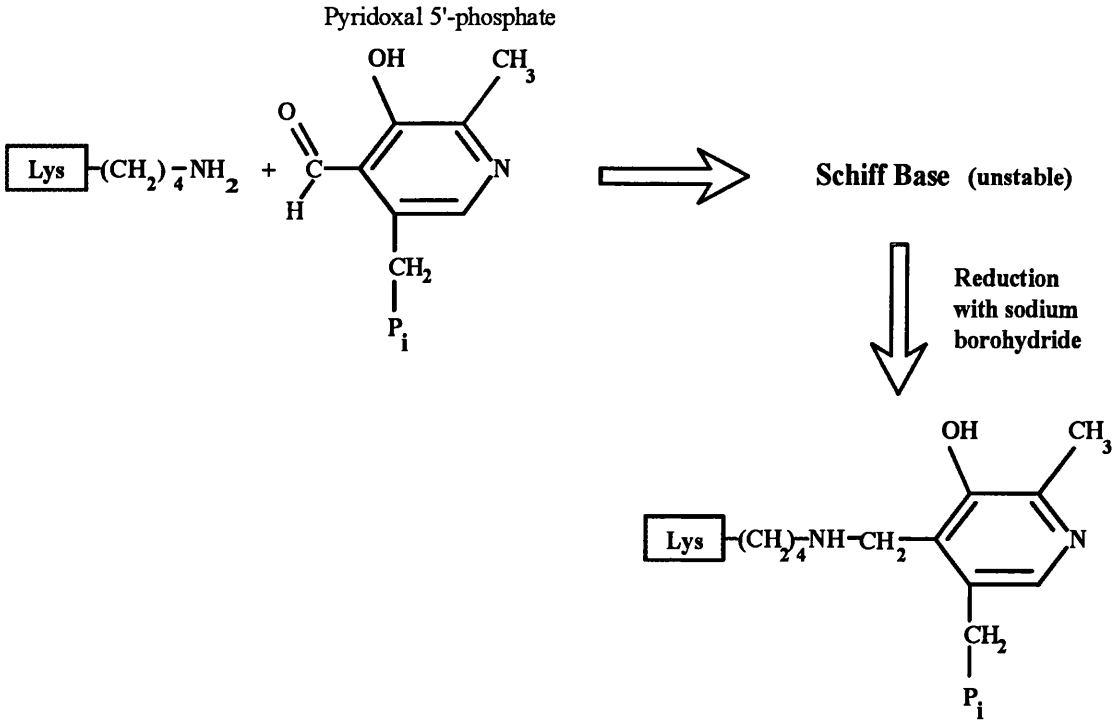
3.10.2.2 Sensitivity

Certain lysyl residues of LDH are involved in interactions with other residues, and so their modification may affect the functioning of the enzyme. Around five lysyl residues per subunit are thought to form ion pairs with negatively charged residues, such as glutamate or aspartate, and another six are thought to be involved in hydrogen bond interactions (Holbrook *et al*, 1975). Lys 58 of LDH is thought to be involved in hydrogen bonding with one of the hydroxyl groups of the adenine ribose of NAD. There is also thought to be a lysine generally present at the 250 position which is involved in interaction with the carboxyamide group of the nicotinamide ring of NAD. However Lys 250 was not recognised in X-ray crystallography studies of pig H_4 LDH (Grau *et al*, 1981). If any of the above residues are accessible to solvent, their reaction with modification reagent may affect the activity of LDH. Indeed, as we shall now discuss, there does appear to be a lysyl residue that is sensitive to modification in both pig H_4 and M_4 LDH isoenzymes.

The modification of lysyl residues with pyridoxal 5'-phosphate is known to cause a reduction of enzymic activity in many dehydrogenases, including lactate dehydrogenase

(Chen and Engel, 1975). The effect of this modification upon the activity of LDH is of particular interest to us because pyridoxal 5'-phosphate is similar to glutaraldehyde in that it reacts with primary amine-containing protein residues to form Schiff bases (see diagram below). Upon modification, up to 66% inactivation of LDH pyruvate activity was observed. Activity was totally restored after dialysis but inhibition was made permanent by reduction with sodium borohydride, indicating that the inactivation was related to Schiff base formation. Although several lysyl residues were modified, only one was responsible for the loss of activity. One interesting observation was that the incorporation of pyridoxal 5'-phosphate could be increased from 1.3 to 5.8 molecules per LDH subunit if the modified protein was first purified before the addition of a second aliquot of pyridoxal 5'-phosphate. However, further modification was accompanied by a further deterioration in activity to 9% of the original enzyme activity, and so this technique was not employed in this study to increase the incorporation of glutaraldehyde. Another important observation was that when 3mM NAD was added to the reaction mixture, the inhibition effect was reduced by 40%. The inhibitory effect of pyridoxal 5'-phosphate may go some way to explaining the 70% inactivation of LDH caused by modification with an NAD-glutaraldehyde adduct as described in *Section 1.6.1* (Venn *et al*, 1977).

Chen and Engel (1975) suggested that modification of a specific lysine by pyridoxal 5'-phosphate may sterically hinder the catalytic action of LDH. This theory was supported by the work of Pfleiderer *et al* (1968) who found that 14 of the 24 ε-amino groups of pig H_4 LDH could be acetylated without loss of activity. Chen and Engel suggested that it is possible that the structure of pyridoxal 5'-phosphate is such as to direct the reagent towards certain lysyl residues and the inhibitory effect that was observed may be a property that is unique to pyridoxal 5'-phosphate and molecules of similar structure. However, carbodiimide-mediated modification of pig H_4 LDH lysyl residues (with NAD derivatives) has also been carried out, and a 35-40% inhibition of activity was observed (Warth *et al*, 1989).



3.10.2.3 Location of lysyl residues relative to the coenzyme binding site

If the immobilised NAD molecule is to be catalytically active with the very same enzyme subunit to which it is bound, it is imperative that solvent-accessible lysyl residues are present in the vicinity of the coenzyme binding site so that the immobilised coenzyme molecule can interact with the active centre. In order to discover whether there are lysyl residues in sufficient proximity to the coenzyme binding site of pig H_4 LDH, the data of Grau *et al* (1981) was used to design a diagramatic representation of all the lysyl residues of Pig H_4 LDH that were located in close proximity to the N^6 group of an NAD molecule bound at the active centre (*Figure 3.20*). This exercise was also performed to give some idea of length of the spacer that would be required to provide a flexible link between the ε-amine of the lysyl residue and the N^6 amine group of NAD. *Figure 3.20* shows there are four such "proximal" lysyl residues and they are present at the edge of the coenzyme-binding cleft, at the interface between the two major domains. The suitability of each proximal lysyl residue as a coenzyme anchorage site will now be discussed.

Lysine 243: the ε-amine group of Lys 243 is 1.73nm away from the amine group of an NAD molecule bound at the active site. If N^6AHCM-NAD was coupled to Lys 243 via glutaraldehyde it would have a total spacer length of at least 2.1nm and the NAD moiety would probably be within comfortable reach of the coenzyme binding site. However, although the ε-amine of Lys 243 protrudes outwards, away from any interfering neighbouring residues, the molecular spacer between LDH and the NAD molecule would have to be extremely flexible in order to allow the coenzyme to interact with the active site (*Figure 3.20*).

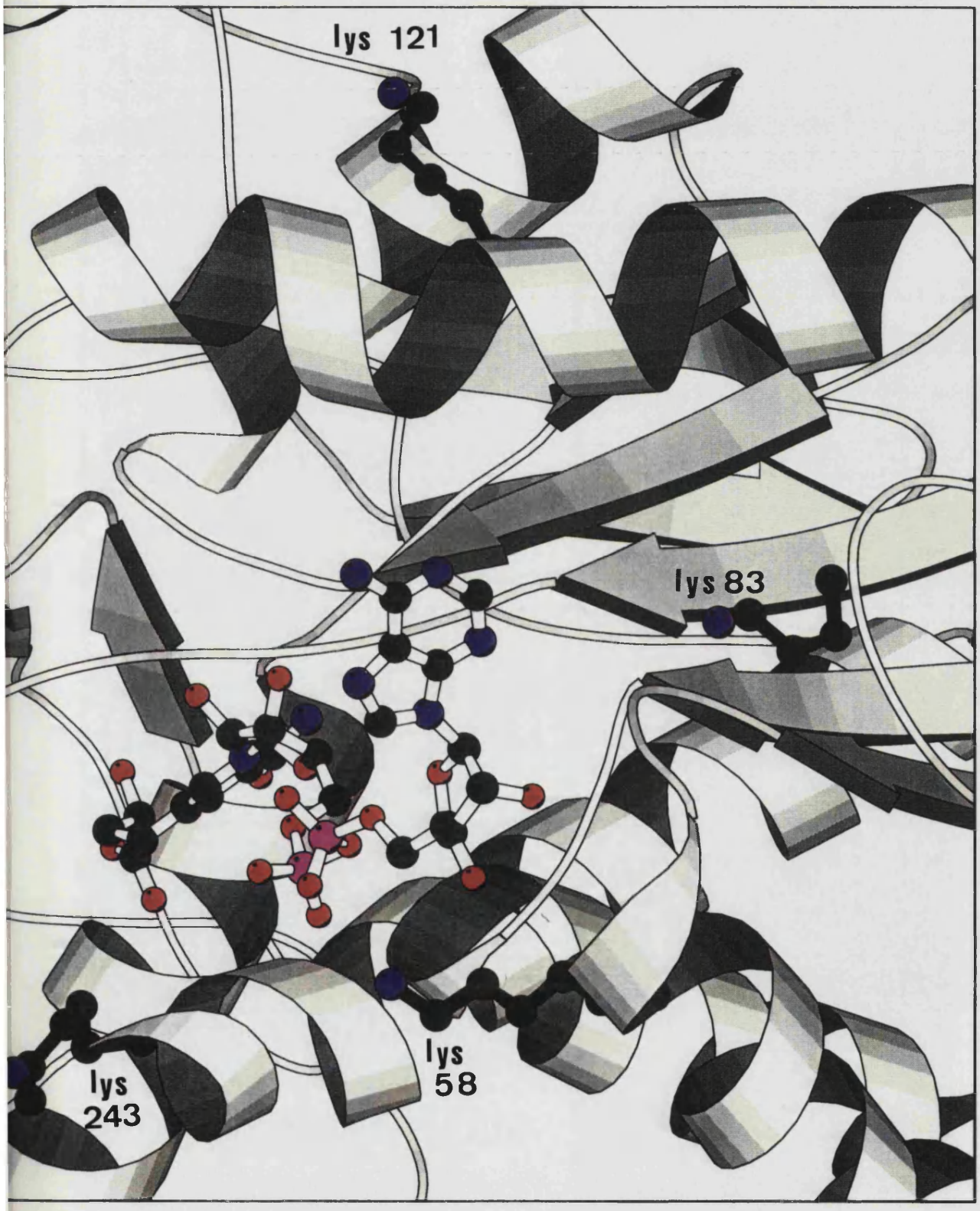
Lysine 58: this residue is thought to interact with the hydroxyl groups of the adenine ribose of NAD (Holbrook *et al*, 1975). Although its ε-amine group is only 1.1nm away from the amine group of NAD, it seems to be the most inaccessible ε-amine of the four proximal lysyl residues. This residue may also be protected from modification in the conditions of excess coenzyme that prevail during conjugation experiments.

Lysine 121: the ε-amine group of Lys 121 is 1.3nm away from the amine group of bound NAD and it protrudes into the solvent. As such, it may function as a good anchorage site, either for N^6AHCM-NAD or NAD coupled via glutaraldehyde or N^6CM-NAD coupled via carbodiimide.

Lysine 83: this is another good candidate; its ε-amine group is only 1.2nm away from the N^6 position of the adenine ring of the bound NAD, and it clearly protrudes into the surrounding solvent.

If the mechanism of intrinsic activity of an LDH-NAD complex is intramolecular, then it is hard to see how N^6CM-NAD coupled directly, via carbodiimide, to any of the above lysyl residues will interact with the catalytic centre. It must be stressed that

Figure 3.20: *Representation of the ε-amine groups of four "proximal" lysyl residues in relation to the adenine moiety of an NAD molecule bound at the active centre of pig H_4LDH.* NAD and residues are shown as ball and stick models.
Key for atoms: blue = nitrogen, red = oxygen, grey = carbon, pink = phosphate (hydrogens not shown).
Key for surrounding protein: coiled ribbons = α-helix, arrows = parallel β-sheet.
The picture was prepared using the data of Grau *et al* 1981, on a Molscript™ package. The bound NAD molecule is actually a molecule of *S*-lac-NAD which was designed to simulate the ternary complex structure formed between LDH, NAD and lactate.



this is a theoretical exercise, is highly speculative and was only performed for pig heart LDH, for which appropriate data is available. This exercise was carried out to see if it would be possible to predict the success of each LDH-coenzyme conjugate on the basis of its spacer length. The accuracy of this prediction relies on a complete intramolecular reaction between NAD and LDH. Nevertheless, the three-dimensional structure of pig H_4 LDH does show that an intramolecular reaction, with possible anchimeric effects* is theoretically feasible.

3.10.3 Carboxyl-containing residues

LDH has been immobilised onto derivatised glass beads via its surface acidic residues using a carbodiimide reagent (Stolzenbach and Kaplan, 1978); the final preparation was stable and only a small decrease in activity resulted. Perhaps the best examples of the modification of LDH with small ligands via carbodiimide coupling are actual previous coenzyme conjugation studies. No serious loss in activity has yet been reported, the specific activity of a native enzyme after modification remaining between 65-90% of its original activity (Warth *et al*, 1989; Schafer *et al*, 1986).

3.10.3.1 Solvent accessibility

According to the amino acid sequence analysis of pig H_4 LDH carried out by Klitz *et al* (1977) there are 69 carboxyl-containing amino acids, comprising 36 aspartyl and 33 glutamyl residues. Many of these are present on the protein surface, where they are readily accessible (Holbrook *et al*, 1975). As with lysyl residues, the solvent accessibility of each glutamyl and aspartyl residue of dogfish M_4 LDH has been calculated (Abad-Zapatero *et al*, 1987). There were only 12 glutamyl residues in dogfish M_4 LDH, five of which were accessible, another one of which was partially accessible. There were 22 aspartyl residues, eight of which were accessible and another

* Effect of "close concentration" of coenzyme covalently bound to enzyme upon enzyme activity at a particular coenzyme concentration. For further explanation refer to *Section 1.6.3*

eight partially accessible. In summary, out of all the carboxyl-containing residues, 65% showed a degree of accessibility. If it is assumed that the same degree of accessibility exists for each residue in pig H_4 LDH, then there will be 44 accessible carboxyl-containing residues compared to 22 accessible lysyl residues. It will be interesting to see whether more coenzyme molecules were incorporated when carboxyl-containing residues or lysyl residues were used to anchor the coenzyme to LDH.

3.10.3.2 Sensitivity

Although no reports of inactivation of LDH caused by modification of a specific carboxyl residue could be found, the side chains of certain aspartyl and glutamyl residues have been reported to be involved in ionic and hydrogen bond interactions with coenzyme, substrate, or other enzyme residues. Thus their modification may contribute towards a certain degree of enzymic inactivation (Holbrook *et al*, 1975). For example, the carboxylate group of Asp 168 is involved in hydrogen bonding with the essential catalytic His 195 and has been reported in pig H_4 LDH (Grau *et al*, 1981), and in other forms of LDH (Holbrook *et al*, 1975). Asp 53 and Asp 30 are also thought to be involved in coenzyme binding interactions in LDH. It must be stressed that some "sensitive" active centre residues will be protected by an excess of coenzyme, the bound coenzyme molecule acting as a physical barrier to the action of modificaton reagents. Conditions of excess coenzyme exist during the conjugation experiments.

3.10.3.3 Location of aspartyl and glutamyl residues relative to the coenzyme binding site

The data of Grau *et al* (1981) was used to prepare a representation of any acidic residues in close proximity to the active site of pig heart LDH (*Figure 3.21*). This exercise was performed to give some idea of the length of spacer required to provide a flexible link between the carboxyl carbon atom of proximal acidic residues and the N^6 atom of NAD when bound at the active site.

Asp 53: Although the carboxyl group of Asp 53 is only 0.8nm away from the N^6 atom of the adenine ring of NAD, it becomes buried upon coenzyme binding and is involved with hydrogen bond interactions with the adenine ribose moiety of NAD (Grau *et al*, 1981). It will probably not be available for covalent modification in the presence of coenzyme and modification of this residue may have undesirable effects upon the coenzyme affinity of LDH.

Asp 57: The carboxyl group of this residue lies 1.2nm away from the N^6 position of NAD. It protrudes into the solvent and may be a suitable anchorage point for N^6AHCM-NAD, the spacer arm of which is 1.4nm in length.

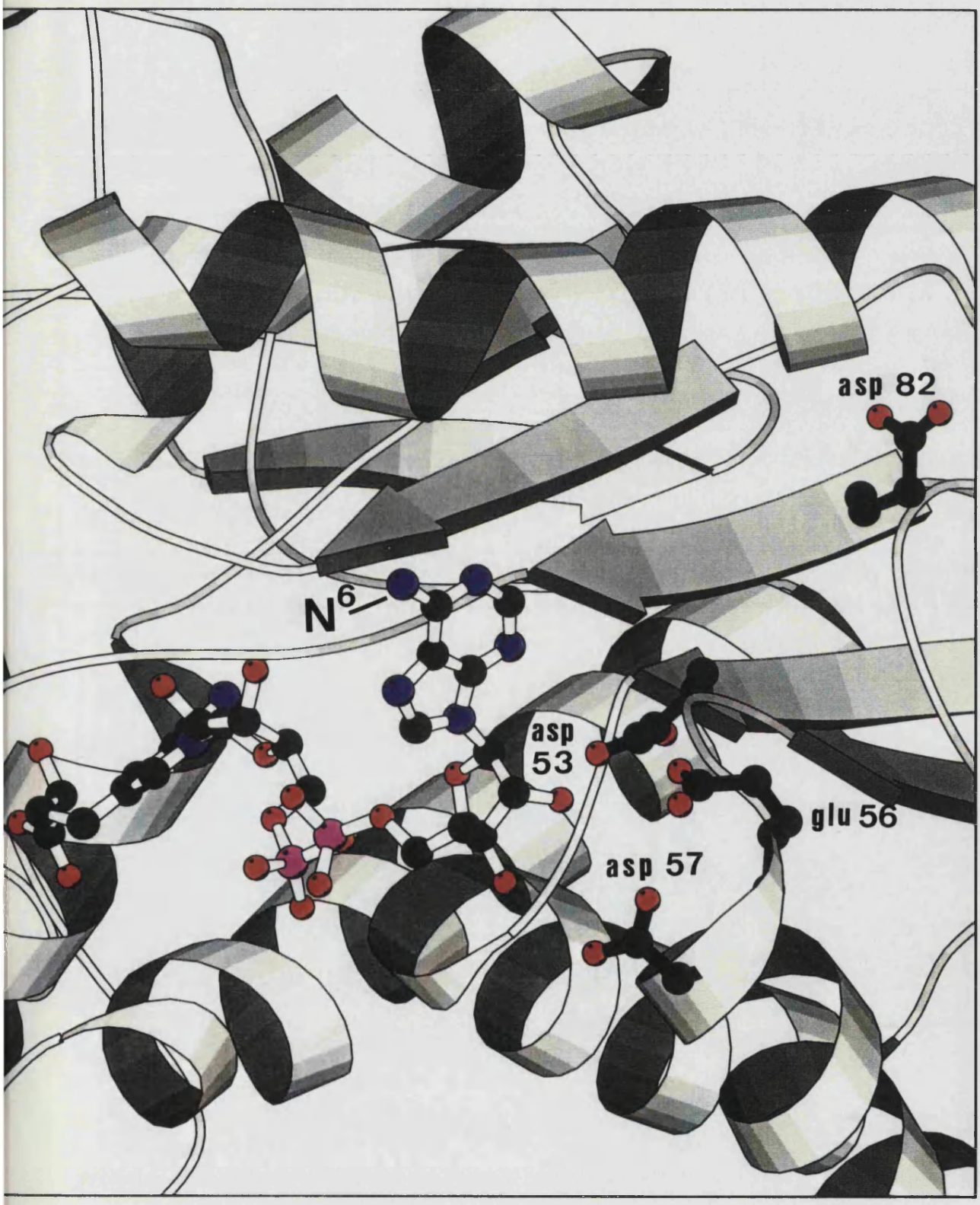
Asp 82: The carboxyl group of this residue is 1.4nm away from the N^6 atom of NAD and it may also be a suitable anchorage point for N^6AHCM-NAD.

Glu 56: The proximal glutamyl residue is only 1.0nm away from the adenine ring of the active site-bound coenzyme and may also be a suitable anchorage position for N^6AHCM-NAD, although it is not clear whether this residue protrudes into the surrounding solvent.

Because there are no appropriate acidic residues close enough to allow a covalently bound NAD molecule (coupled via carbodiimide) to interact with the active centre, it could be argued that LDH-NAD conjugates coupled using carbodiimide will not have intramolecular activity in the absence of exogenously added coenzyme.

The properties of LDH have been discussed in detail because it is used as a model dehydrogenase enzyme in this study. We shall now briefly consider aspects of the other enzymes used: glutamate dehydrogenase and alcohol dehydrogenase.

Figure 3.21: *Representation of the ε-amine groups of four "proximal" acidic residues in relation to the adenine moiety of an NAD molecule bound at the active centre of pig H_4LDH.* NAD, glutamyl and aspartyl residues are shown as ball and stick models.
Key for atoms: blue = nitrogen, red = oxygen, grey = carbon, pink = phosphate (hydrogens not shown).
Key for surrounding protein: coiled ribbons = α-helix, arrows = parallel β-sheet.
The picture was prepared using the data of Grau *et al* 1981, on a Molscript™ package. The bound NAD molecule is actually a molecule of *S*-lac-NAD which was designed to simulate the ternary complex structure formed between LDH, NAD and lactate.



3.11 Glutamate dehydrogenase: properties relevant to coenzyme conjugate formation

If the approach used in this chapter is to be applicable to a wide range of dehydrogenases, then it must work with those enzymes of a more complex nature. Glutamate dehydrogenase (GDH) was partly chosen because of its many complex characteristics.

GDH is an NAD(P)-dependent enzyme which catalyses the reversible reaction of α-ketoglutarate to glutamate.

$$\text{glutamate} + NAD^+ + H_2O \overset{\text{GDH}}{\Leftrightarrow} \alpha\text{-ketoglutarate} + NH_4^+ + NADH + H^+$$

NADP-specific GDHs are usually involved in ammonia assimilation, whilst NAD-specific GDHs are involved in glutamate catabolism. The equilibrium of the above reaction lies very much to the left, with an equilibrium constant, K_{eq}, of $1x10^{-14}$M in Tris buffer at pH 8.0 and 25°C. The equilibrium of the reaction is strongly dependent upon ionic strength (Smith *et al*, 1975). As with LDH, we are interested in the NAD reduction reaction because of the method of coenzyme regeneration and our interest in glutamate as a substrate (*Section 1.3*). Ox liver was chosen as the source of GDH in our studies because this form of the enzyme is amongst the most thoroughly investigated of GDHs; it is readily available and relatively inexpensive.

3.11.1 Structure

In its smallest catalytically active form, liver GDH exists as a hexamer of six identical subunits, the molecular weight of each ox liver GDH subunit being around 56,000 and consisting of around 500 residues (Bell and Bell, 1984; Ozturk *et al*, 1992). So far attempts to crystallise ox liver GDH in a form suitable for X-ray analysis have failed. X-ray crystallographic studies have, however, been carried out on another NAD-

dependent GDH from *Clostridium symbosium.* Although this is a bacterial GDH, its primary structure is very conserved on comparison with those of vertebrate GDHs (Baker *et al*, 1987). X-ray diffraction studies have been performed at 0.60nm resolution (Rice *et al*, 1987) and more recently at 0.19nm resolution (Baker *et al*, 1992a). The three dimensional structure was typical of NAD-dependent dehydrogenases in that it possessed two globular domains with an intervening cleft. The NAD molecule bound in an open conformation as it did in other dehydrogenases (Baker *et al*, 1992b). Earlier studies showed that the adenine moiety of NAD could be replaced with other bases without loss of function (Smith *et al,* 1975).

3.11.2 Allosteric behaviour

In contrast to LDH, the subunits of GDH demonstrate complex allosteric behaviour. There are thought to be six catalytic and regulatory sites present in each ox liver GDH subunit. These comprise a catalytic NAD binding site, a regulatory NAD binding site, two ADP regulatory sites and two GTP regulatory sites (Ozturk *et al*, 1992). The enzyme is inhibited by GTP and high concentrations of NADH but is activated by ADP. NAD binds with less affinity at the regulatory site in comparison to the catalytic site, although the opposite is the case for NADH. It is not surprising, then, that NAD-binding in GDH is a complex process with non Michaelis-Menten kinetics (Bayley and O'Neill, 1972).

3.11.3 Polymerisation

Another unusual property of GDH is that its hexamers have a tendency to polymerise with an increasing concentration of enzyme; this is probably due to complementary areas of electrostatic attraction on the surface of each hexamer (Olson and Anfinsen, 1952). Enzymic activity is independent of the degree of polymerisation and polymerised GDH subunits are fully active (Josephs *et al*, 1973). However, the availability of some residues for modification will no doubt be reduced, the higher the concentration of enzyme. For this reason, GDH concentration was kept below 2 mg

ml^{-1} during modification reactions in an attempt to maintain the availability of surface residues. Polymerisation of the hexamers also has a light scattering effect, resulting in a misleading increase in the spectrophotometric absorption properties of GDH. For example, it has been observed that for a 0.5 mg ml^{-1} solution of ox liver GDH, 20% of the extinction at 280nm was due to light scattering caused by polymerisation of the hexamers (Josephs *et al*, 1973). This was therefore taken into account in the measurements of GDH concentration here in this study. During glutaraldehyde cross-linking studies on GDH (Josephs *et al*, 1973), it was observed that at higher concentrations (>1mg ml^{-1}) an insoluble gel tended to form when a 50-100 excess of glutaraldehyde was used. The formation of such a gel in our study was avoided by keeping the GDH concentration below 2mg ml^{-1} during modification studies.

3.11.4 Kinetics

GDH subunits are not kinetically equivalent with respect to substrate activity (Syed and Engel, 1984). However molar ratios of coenzyme:enzyme were expressed in terms of subunit concentration for ease of comparison with LDH conjugates. The kinetics of NAD-dependent GDHs are difficult to measure because of their complex allosteric behaviour and most kinetic studies have been carried out on NADP-dependent GDHs. The mechanism was generally thought to be a partial random order mechanism, in which substrate and coenzyme could bind to the enzyme in any order (Smith *et al*, 1975), although more recent reports suggest that, in the presence of ADP, glutamate binds before the coenzyme; this is, again, very unusual for a dehydrogenase enzyme (Syed *et al*, 1991).

The effects of chemical modification upon different GDH residues are now considered. Amino acid comparisons of different GDH enzymes have shown that their primary structures are similar to each other, but distinct from those of other dehydrogenases. GDHs have an especially high arginine : lysine ratio (Moon *et al*, 1972).

3.11.5 ε-amine groups of lysyl residues

Amino acid analysis of ox liver GDH revealed 33 lysyl residues (Moon *et al*, 1972). Assuming that they are of the same order of accessibility as the lysyl residues of dogfish M_4 LDH (*section 3.11.2*) then there would be plenty of these residues available for interaction with solvated reagents, despite the high arginine : lysine ratio of GDH. Lysine modification studies with ox liver GDH showed that in the presence of a 100-fold excess of pyridoxal 5'-phosphate with respect to subunit concentration, the glutamate activity of the enzyme decreased to 10% of the original activity (Syed and Engel, 1984). Although 4-5 lysyl residues were modified, only one modified residue was thought to be responsible for inactivation: Lys 126*. Lys 126 is essential for activity towards glutamate and is uniquely reactive in all GDHs studied because it has an abnormallly low pK_a (7.7-8.0 at 30°C) (Smith *et al*, 1975). Acetylation of Lys 126 leads to an 80% loss in activity. Other important lysyl residues of ox liver GDH include Lys 143, Lys 425 and Lys 428 which are all present at regulatory sites (Dombrowski *et al*, 1992). Modification of Lys 126 was completely prevented in the presence of NADH, GTP and α-ketoglutarate, although no other substrate combinations were investigated. A separate investigation by Piszkiewicz *et al* (1971) observed that NAD alone did not protect against loss of activity in the presence of pyridoxal 5'-phosphate, as was the case for LDH. However, Josephs *et al* (1973) found that they were able to cross link polymers of GDH in a 50-100 excess of glutaraldehyde with respect to subunit concentration, with no substrates present with only a 20% loss in activity, although some allosteric properties were affected. This again suggests that modification of lysyl residues with pyridoxal 5'-phosphate and glutaraldehyde does not affect enzyme activity in quite the same way.

*According to the numbering of Smith *et al* (1975)

3.11.6 Carboxyl-containing residues

There are 61 carboxyl containing residues in ox liver GDH, comprising 29 aspartyl and 32 glutamyl residues (Moon and Smith, 1972). As with LDH , there are probably more accessible carboxyl groups than amine groups (Creighton, 1993). Although there is some evidence that carboxyl residues are involved in side chain interactions with residues from other subunits (Smith *et al*, 1975), no reports of inhibition being caused by the specific modification of a glutamate or aspartyl residue in GDH could be found.

3.12 Alcohol dehydrogenase: properties relevant to coenzyme conjugate formation

Alcohol dehydrogenase (ADH) was chosen for use in these studies because of the interest in ethanol as an analyte (*Section 1.3*) and because coenzyme conjugation studies have never been carried out on the yeast enzyme. Yeast alcohol dehyrogenase (YADH) is distinct from mammalian forms of ADH, usually purified from liver (LADH), in that it has a narrower substrate specificity and is 30 times more efficient at catalysing the oxidation of ethanol, as shown in the reaction below (Branden *et al*, 1975). In addition, YADH is a tetramer whereas LADH is a dimer.

$$\text{ACETALDEHYDE} + \text{NADH} + H^+ \xrightleftharpoons{\textbf{ADH}} \text{ETHANOL} + NAD^+$$

The equation constant, K_{eq}, for the above reaction is around 8×10^{-12}M, in phosphate buffer, pH7.0, 20°C (Backlin, 1958). As with LDH and GDH the concerned was with the NAD reduction reaction because of the interest in ethanol as an analyte and the potential method of coenzyme regeneration in an enzyme electrode.

3.12.1 Structure

The molecular weight of each YADH subunit is 36-37,000, and the native enzyme exists as a tetramer of MW 140-150,000 (Branden *et al,* 1975). There is thought to be one coenzyme binding site per subunit, although several studies have estimated the value to be slightly less (Branden *et al,* 1975). The lower estimates are thought to be caused by a degree of negative cooperativity between subunits. Although the three-dimensional structure of liver alcohol dehydrogenase (LADH) has been studied extensively (Eklund *et al,* 1981; Eklund *et al,* 1976; Cedergen-Zeppezauer *et al,* 1983) there was no interpretable X-ray crystallographic data for YADH available until recently. Ramaswamy *et al* (1994) have obtained data at 0.32nm resolution but have not yet published their findings. However, over half of the primary sequence has been determined (Jornvall, 1973) enabling a comparison with corresponding peptide segments of LADH. Such comparisons show that the NAD-binding mode of YADH is similar to that of other dehydrogenases. Differences between certain residues in the substrate binding cleft give YADH a smaller substrate pocket, which reflects its narrower substrate specificity. Unlike LDH and GDH, YADH is a zinc-containing enzyme; the metal ion is linked to the ligands Cys 46, Cys 174, the imidazole of His 67 and either a water, ethanol or acetaldehyde molecule, depending on the state of catalysis.

3.12.2 Reaction mechanism

The reaction mechanism for LADH is shown in *Figure 3.22* and the reaction mechanism of YADH is thought to be similar.

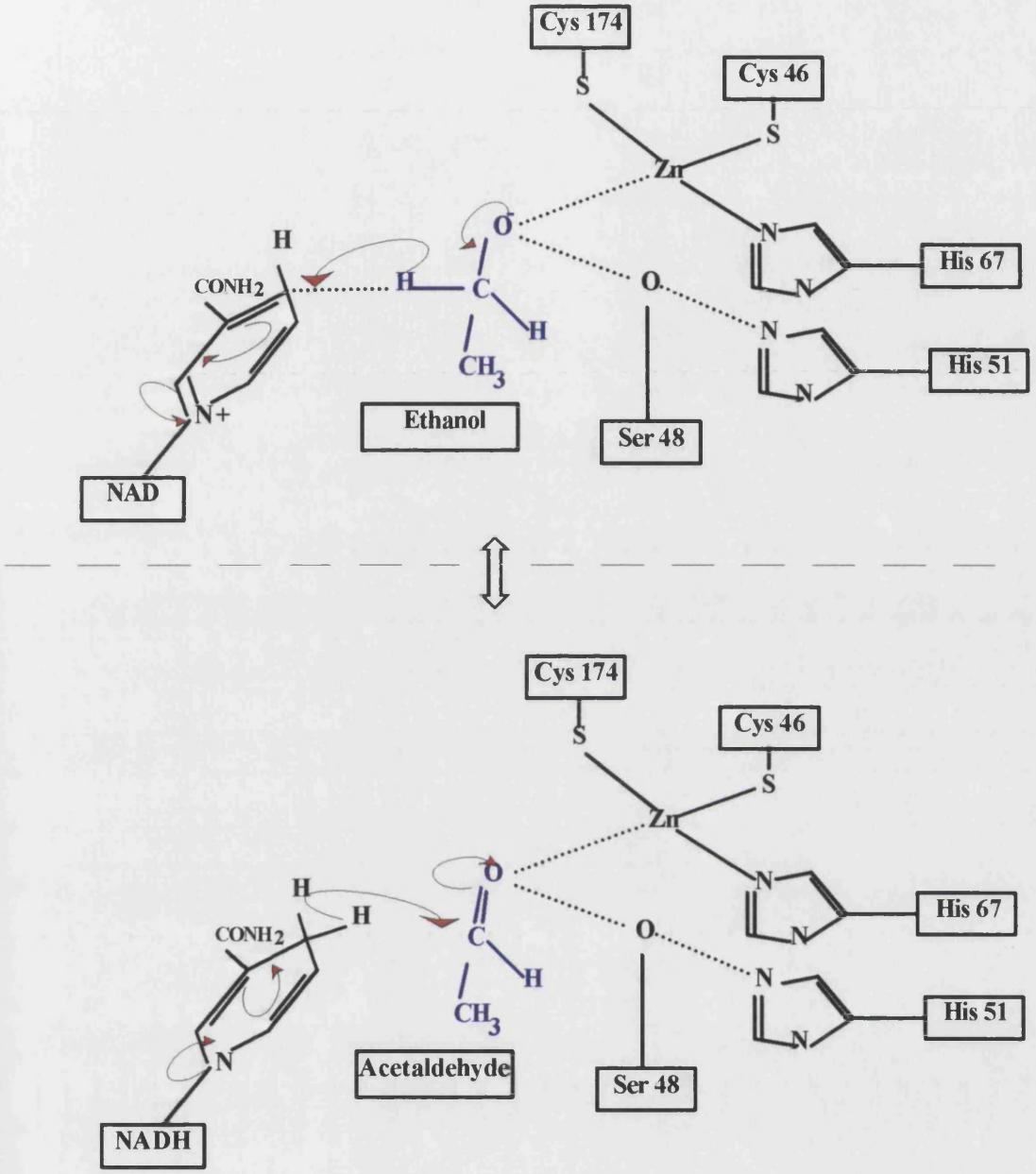


Figure 3.22: *The catalytic mechanism of liver alcohol dehydrogenase.* The nicotinamide moiety of NAD accepts a hydride ion from ethanol in the oxidation reaction and donates a hydride ion to acetaldehyde in the reduction reaction. Hydrogen bonds are shown as dotted lines and the substrate is coloured in blue.

3.12.3 Kinetics

A partial random order mechanism for ethanol oxidation (NAD or ethanol accepted first) with a compulsory order for product dissociation was reported (Dickson and Monger, 1973), although a compulsory ordered mechanism has been suggested by Plapp *et al* (1973) between pH 7 and pH 9.

Because there is no three-dimensional structure and no complete amino acid sequence available for YADH we cannot say how many glutamyl, aspartyl or lysyl residues are available for modification. It is probably safe to assume, however, that several of each residue will be present on the surface of the enzyme, where they are accessible to solvated reagents. We shall now consider the "sensitivity" of certain residues in YADH.

3.12.4 ε-amine groups of lysyl residues

Lys 228 of YADH is thought to be involved in hydrogen bonding with the O-3' atom of the adenine ribose of NAD. Chemical modification of this residue in LADH with picolinamide increased the dissociation rate of the enzyme-coenzyme complex (Plapp *et al*, 1973) and, because the dissociation of NAD from the enzyme is a rate limiting step, the catalytic turnover of LADH was increased by tenfold. Pyridoxal 5'-phosphate modification has been reported for LADH, but not YADH. The results for LADH modification were very similar to those results obtained for LDH and GDH: after pyridoxal 5'-phosphate modification the residual activity was 20% (McKinlee-McKee and Morris, 1972). The inactivation was related to Schiff base formation and again there was thought to be an essential lysyl residue in the enzyme. Up to 11 lysyl residues were modified by pyridoxal 5'-phosphate, showing that several lysyl residues in LADH are solvent-accessible. LADH was completely protected from inhibition in the presence of saturating concentrations of NAD. Glutaraldehyde has been used to stabilise a YADH preparation that had been pre-immobilised onto porous glass (Ooshima *et al*, 1981). Although there was no siginificant decrease in activity caused by an excess of glutaraldehyde, enzymic activity had already been reduced by 82% after the enzyme had been immobilised onto glass (acrylamide monomers had probably modified cysteine residues of YADH).

3.12.5 Carboxyl-containing groups

Carboxyl groups near the active site zinc are important for catalysis in YADH (Ganzhorn and Plapp, 1988). Asp 49 forms a hydrogen bond with the imidazole group of His 67, and Glu 68 is located behind the metal ion, opposite the binding site. These acidic residues are thought to neutralise the positive charge of the metal ion and modification of these residues may cause a reduction in the activity of the enzyme. In LADH, Asp 223 hydrogen bonds to the O-2' atom of adenine ribose of NAD; it is present in many other dehydrogenases and is probably present in YADH (Eklund *et al*, 1981; Plapp *et al*, 1973).

Properties of enzyme-coenzyme conjugates synthesised

Each dehydrogenase-coenzyme complex was purified using gel filtration chromatography to remove excess coenzyme that had not conjugated to the enzyme (*Section 3.6.1*). The appropriate protein fractions were concentrated, if necessary to around 1 mg ml^{-1} against solid polyethyleneglycol and then washed with activated charcoal to remove any traces of non-covalently bound coenzyme still present. The modified enzyme was then characterised. The amount of coenzyme bound to each enzyme subunit was determined in two ways - either with respect to the total nucleotide concentration (called the **total** molar ratio) or with respect to the *reducible* coenzyme concentration (called the **active** molar ratio) bound to each subunit.

Experimental error

The synthesis approach described in part one was carried out once for each conjugate synthesised. In general, the results showed close agreement upon duplication although, where a large discrepancy arose (more than 20%), the experiment was again repeated. The synthesis of ADH-coenzyme conjugates using glutaraldehyde was only carried out once because it was found that glutaraldehyde had a strong inhibitory effect on the ADH (*Section 3.15.1.2*).

3.13 Properties of lactate dehydrogenase-coenzyme conjugates

The properties of the LDH-coenzyme conjugates were investigated in detail because LDH was used as models for other enzyme-coenzyme conjugates.

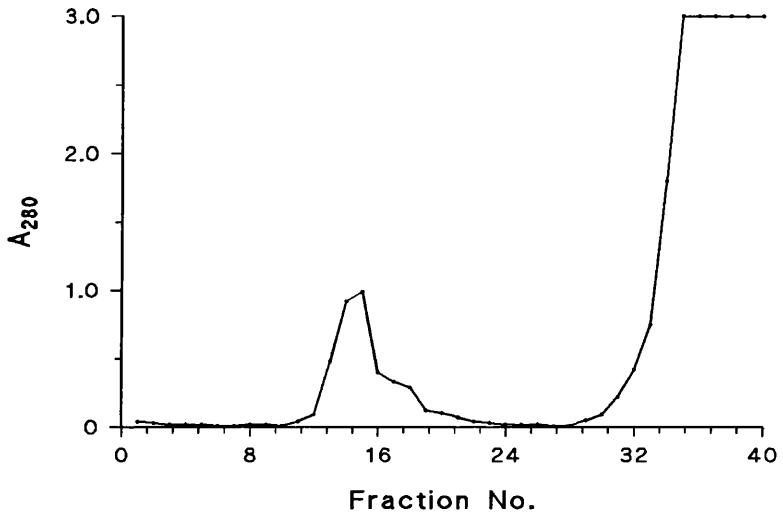


Figure 3.23: *Separation of LDH-N^{6}AHCM-NAD conjugate from uncoupled coenzyme by gel filtration chromatography*

A Sephacryl S-100 column (80 x 1.5cm) was used to separate a glutaraldehyde coupled LDH-N^{6}AHCM-NAD complex from uncoupled N^{6}AHCM-NAD. The column was run at 4°C with a flow rate of 0.2ml min^{-1}. Fractions were collected in 2ml volumes in a dropwise manner using an automatic fraction collector. The eluted fractions were taken immediately for analysis to determine their absorbance at 280nm in a spectrophotometer.

A gel filtration elution profile for LDH-N^{6}AHCM-NAD is shown in *Figure 3.23*. It is also typical of other LDH-coenzyme elution profiles, and as with GDH-coenzyme conjugates, two minor protein peaks were observed, although the second peak was less obvious with the LDH conjugates. Protein fractions were taken from only the first peak for further analysis because of the reasons described in *Section 3.6* which also provides further evidence of a complete separation of free coenzyme from the protein fractions.

3.13.1 Conjugates coupled using glutaraldehyde

3.13.1.1 Coenzyme incorporation

Values for the "total" and "active" molar ratio of NAD or N^6AHCM-NAD to pig heart LDH subunit are presented in *Table 3.4*. Only 0.5 molecules of NAD were incorporated per subunit compared to 1.8 molecules of N^6AHCM-NAD. Similar values were obtained by Venn *et al* (1977) and they suggested that the difference in incorporation was due to the presence of the long hexamethyl spacer arm on the NAD derivative, rendering it more sterically available for interaction with enzyme residues. The lower incorporation of NAD was probably also due to the lower reactivity of its amine group towards glutaraldehyde meaning that less coenzyme-glutaraldehyde adducts were available for reaction with surface LDH residues. The bound NAD derivative was also slightly more available for intermolecular reaction with a second enzyme, ADH, with 67% of covalently bound N^6AHCM-NAD molecules being reduced compared to only 40% of bound NAD molecules. The extra accessibility of the derivative may be indicative of the steric advantage conveyed by the "hexyl" arm, enabling the NAD moiety of N^6AHCM-NAD to protrude further into the solution where it is more accessible for interaction with neighbouring ADH molecules. Despite the advantages gained in the use of N^6AHCM-NAD, the results confirm that NAD itself can be conjugated to an enzyme using glutaraldehyde, thus avoiding the laborious preparation involved in the synthesis of its N^6-modified coenzyme derivatives.

3.13.1.2 Specific activity

Modification of LDH with glutaraldehyde resulted in a decrease in the lactate activity of the enzyme (*Table 3.4*). The activity was reduced to 12% of the activity of native LDH after modification with an excess of NAD-glutaraldehyde adduct, and to 25% after modification with an excess of N^6AHCM-NAD-glutaraldehyde adduct.

Table 3.4
Properties of lactate dehydrogenase-cofactor complexes synthesised using glutaraldehyde coupling

Enzyme	Molar ratio cofactor / LDH subunit			Activity (Umg^{-1})[c]	
	Total[a]	Active[a]	%[b]	Intrinsic[d]	Specific
LDH	0.0	0.0	0	0.00	18.9
LDH-NAD	0.5	0.2	40	0.09	2.3
LDH-N^6AHCM-NAD	1.8	1.2	67	0.12	4.7

[a]"Total" molar ratio determined using A_{266} and A_{290} values. "Active" molar ratio determined using MTT/PES coenzyme-recycling assay with ADH and using Coomassie method to determine protein concentration.
[b]("active" molar ratio of coenzyme:enzyme subunit/ "total" molar ratio of coenzyme:enzyme subunit) x 100.
[c] Umg^{-1} = μmole min^{-1} (mg enzyme)$^{-1}$ Both intrinsic and specific activities were determined in a recycling assay with a DCPIP/PES redox couple. 0-0.1ml of enzyme sample (~1mg/ml) were added.
d Intrinsic activity is defined as the acivity of the enzyme(-coenzyme) in the absence of exogenously added coenzyme but in the presence of other substrate to saturating concentration. The specific activity is determined in the presence of 3mM NAD.

3.13.1.3 Intrinsic activity

The intrinsic activity of each LDH-coenzyme conjugate is shown in *Table 3.4*. The values are of the same order of magnitude as those of Gacesa and Venn (1979) who observed an intrinsic activity of 0.06 Umg^{-1} for the LDH-N^6AHCM-NAD conjugate, and 0.04 Umg^{-1} for the LDH-NAD conjugate*. As expected, the charcoal-washed native enzyme showed no intrinsic activity.

When covalently bound, NAD was a more active coenzyme than N^6AHCM-NAD in terms of substrate activity with LDH. Despite there being three times as many N^6AHCM-NAD molecules bound per subunit, the intrinsic activity of its LDH-conjugates was only 0.12 Umg^{-1} compared to a value of 0.09 Umg^{-1} for the LDH-NAD conjugate. The difference in substrate activity between NAD and its "hexyl"-containing derivative are similar when each coenzyme is assayed in its free state (*Table 3.3*), N^6AHCM-NAD being 12% less active as a substrate for LDH in comparison to NAD. The intrinsic activity of each conjugate was only a fraction (2-4%) of the

*Values converted into Umg^{-1} for ease of comparison, assuming a DCPIP extinction coefficient of 21,000/M/cm (Armstrong, 1964)

specific activity of the modified enzyme. Similar results were reported by Gacesa and Venn (1979).

Kinetic studies were carried out in order to determine whether the intrinsic activity of the LDH-coenzyme conjugates was generated through an inter- or intramolecular reaction.

3.13.1.4 Kinetic studies on the intrinsic activity of LDH-N^{6}AHCM-NAD: an inter- or intra- molecular mechanism?

Figure 1.10 represents the two principal types of interaction that are possible between LDH and coenzyme in a LDH-coenzyme conjugate - an *inter*molecular reaction and an *intra*molecular reaction. Both mechanisms of interaction may contribute towards the overall intrinsic activity of an enzyme-coenzyme conjugate. In order to find out whether one mechanism predominates over the other, a simple logarithmic plot of velocity versus enzyme concentration was used (Mansson *et al*, 1978). The theory behind this plot will now be explained for a LDH-NAD conjugate, although it would also apply to the other enzyme-coenzyme conjugates discussed in this study.

If each LDH-NAD molecule behaves as a self-sufficient catalytic unit requiring only non-coenzymic substrate for catalysis (intramolecular mechanism), then it follows that the rate of the reaction will be proportional to the concentration of individual catalytic units, all other factors (including non-coenzymic substrate) being constant. It will therefore be a first order reaction and a rate equation can be written as follows (Crockford and Knight, 1964).

$$v_1 = k_1 [\text{LDH-NAD}]$$

or logarithmically,

$$\log v_1 = \log k_1 + \log [\text{LDH-NAD}]$$

A plot of log v_1 versus log [LDH-NAD] should give a straight line with a gradient of 1. However if intrinsic activity is generated by an intermolecular reaction, where an LDH molecule interacts with NAD bound to neighbouring LDH molecule, then the rate of the reaction will be proportional to the product of the concentrations of the two enzyme molecules. The reaction will be a second order reaction and a rate equation can be written as follows,

$$v_2 = k_2[\text{LDH-NAD}]^2$$

or logarithmically,

$$\log v_2 = \log k_2 + 2 \log [\text{LDH-NAD}]$$

In this case a plot of log v_2 versus log [LDH-NAD] will give a straight line with a slope of 2. The latter case should also be true for a system which contains native LDH and a corresponding concentration of NAD added exogenously.

Mansson *et al* (1978) were able to confirm the validity of this technique and they observed an intramolecular mechanism for intrinsic activity generated by an LADH-N^6AHCM-NAD conjugate. They were able to confirm the mechanism by immobilising the LADH-coenzyme conjugate onto Sepharose 4B, thus reducing drastically the probability of an interaction between neighbouring LADH molecules - the intrinsic activity was hardly affected, thus confirming the intramolecular mechanism.

Figure 3.23a shows a plot of log velocity versus log enzyme concentration for two systems:

A. LDH-N^6AHCM-NAD, with 1.8 "active" molecules covalently bound per subunit.

B. Native LDH with 2.0 molecules of exogenously added N^6AHCM-NAD per LDH subunit.

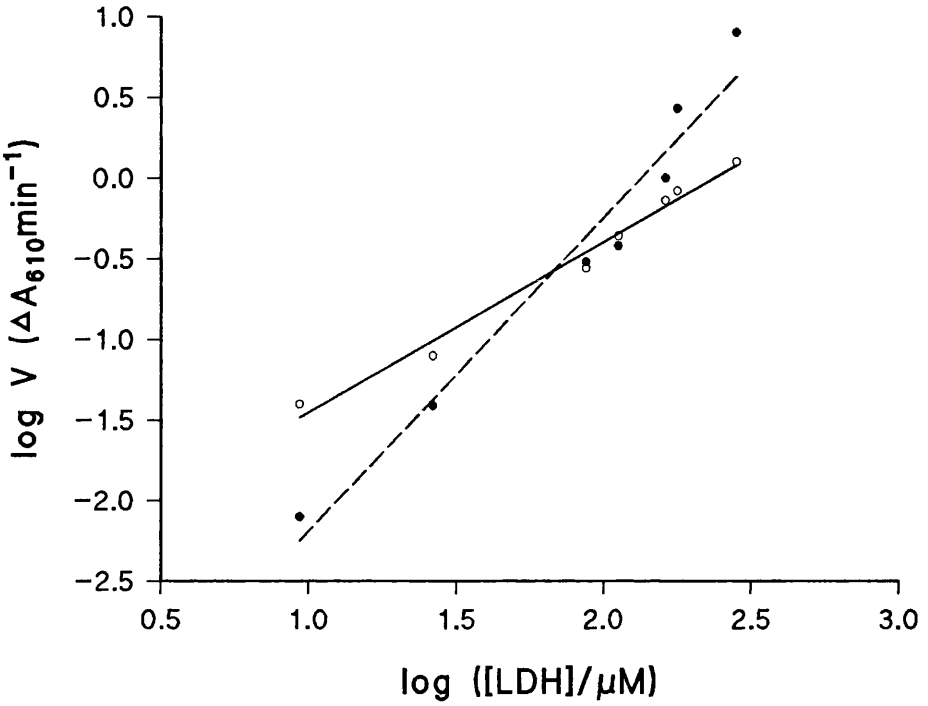


Figure 3.23. *Logarithmic plot of reaction velocity versus enzyme concentration* for: i. LDH-N^{6}AHCM-NAD complex with 1.8 molecules of coenzyme bound per subunit (O), ii. native LDH and free N^{6}AHCM-NAD with 2:1 ratio of coenzyme to subunit (•). The enzyme concentration is given as subunit concentration in the assay mixture. Reaction velocity was determined at pH7.5 using a DCPIP/PES recycling assay. Various concentrations of enzyme sample were added to: 0.75ml 100mM phosphate buffer, 50μl 1mM DCPIP; 50μl 40mM PES; 50μl 1M lactate; 10μl of various concentrations of NAD were also added to the native LDH assays

As expected, the slope of the linear plot for the native enzyme in the presence of free coenzyme is very close to 2 (*1.9*) because reactivity is generated through an intermolecular mechanism. The slope for system A was closer to 1 (*1.1*). It would seem that the main mechanism of intrinsic activity for the LDH-N^{6}AHCM-NAD conjugate is intramolecular, because the reaction is very close to being a first order reaction. It may be worth pointing out that at an enzyme concentration of 63μM, both free enzyme and enzyme-coenzyme conjugate had the same activity with a coenzyme ratio of around 2:1 whether coenzyme was covalently bound or not. In other words, the enzyme-coenzyme system was more efficient below an enzyme subunit concentration of 63μM even though the specific activity of the modified enzyme was less than half of that of the native enzyme.

This experiment illustrates that comparisons between enzyme-coenzyme conjugate activity and the activity of an unmodified enzyme with a corresponding concentration off free coenzyme cannot be directly made because the kinetic mechanisms differ. However, from an economical standpoint the two systems can be compared at a defined enzyme concentration to see which is the more efficient.

3.13.1.5 Stability of the intrinsic activity present in a LDH-N^{6}AHCM-NAD conjugate

It was important to establish that the intrinsic activity of the LDH-coenzyme conjugates was sufficiently stable to justify their incorporation into an enzyme electrode system. Before the stability of the LDH-N^{6}AHCM-NAD conjugate activity was determined, the stability of its component parts was measured. The stability of N^{6}AHCM-NAD in free solution at various temperatures is given in *Section 3.2.2.4.* The activity remained above 80% after 50 days at room temperature. *Figure 3.23b* shows the stability of native LDH at 4°C and at room temperature.

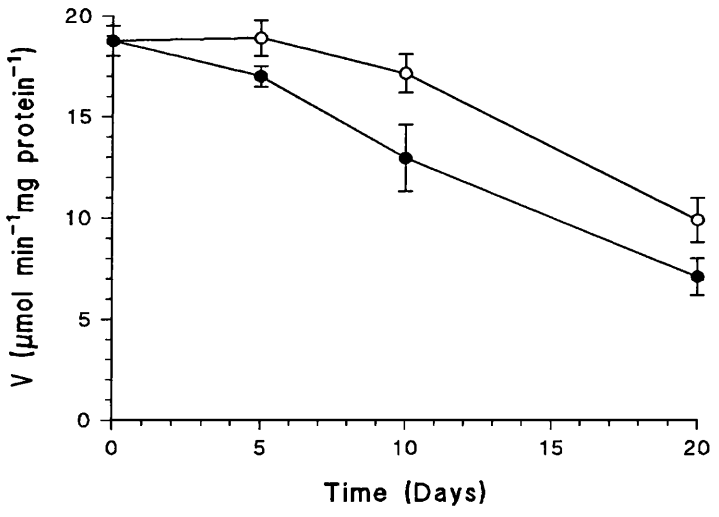


***Figure 3.23b:** Stability of pig H_4 LDH at 4°C (o) and at room temperature (•).* LDH was stored at 1mgml^{-1} in 100mM phosphate buffer, pH7.5. *Assay mixture*: 20µl of 20µg/ml LDH sample were added to 2.83ml 100mM phosphate buffer, pH 7.5; 0.1ml 500mM lactate; 50µl 100mM NAD. The reaction velocity was followed at 340nm in a spectrophotometer.

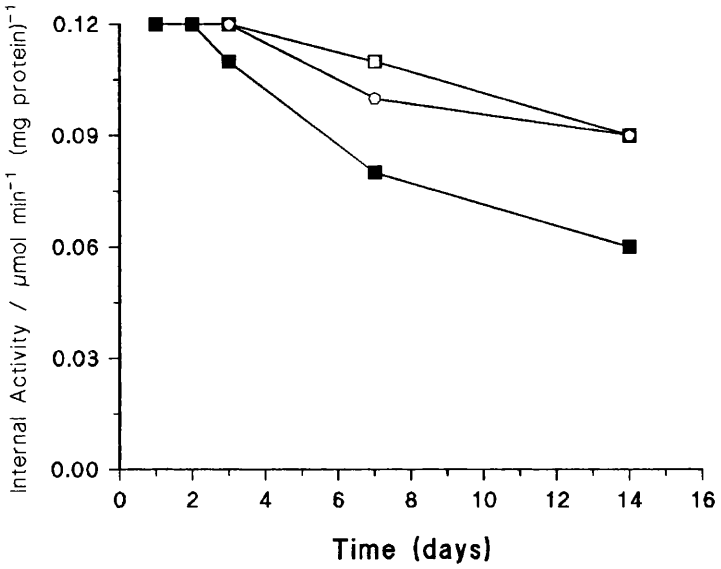


Figure 3.23c: *The stability of the intrinsic (internal) activity found in a LDH-N⁶AHCM-NAD conjugate at room temperature (■), at 4°C(□) and at -20°C (○).*
Reaction velocity was determined at pH7.5 using a DCPIP/PES redox couple. 100μl of 1mgml^{-1} enzyme-coenzyme sample were added to: 0.75ml 100mM phosphate buffer; 50μl 1mM DCPIP; 50μl 40mM PES; 50μl 1M lactate.

The native enzyme was sufficiently stable when stored in phosphate buffer for ten days at 4°C, with residual specific activity remaining above 80%. The stability of the LDH-N⁶AHCM-NAD conjugate is shown in *Figure 3.23c*.Considering the stabilities of its constituent parts, the formation of an enzyme-coenzyme conjugate does not seem to have detrimental effects upon the stability of the enzyme or the coenzyme. Residual intrinsic activity remains above 75% after 14 days when stored at 4°C or -20°C in phosphate buffer. The stability was deemed sufficient for use of the conjugate in enzyme electrode studies *(Chapter 5)*.

3.13.2 Carbodiimide Coupling

Carbodiimide coupling involves the covalent fixation of coenzyme via either amine- or carboxyl-containing residues. We may therefore expect to see differences between

conjugates formed related to the type of residue that anchors the coenzyme to the enzyme.

3.13.2.1 Coenzyme incorporation

The values for molar ratio of coenzyme to LDH subunit are presented in *Table 3.5*.

Table 3.5
Properties of lactate dehydrogenase-cofactor complexes synthesised using carbodiimide coupling

Enzyme	Molar ratio cofactor / LDH subunit			Activity (U/mg)[c]	
	Total[a]	Active	%[b]	Intrinsic	Specific
LDH	0.0	0.0	--	0.00	18.9
LDH-NAD	0.3	0.3	100	0.01	15.0
LDH-N^6CM-NAD	1.8	0.3	17	0.01	14.1
LDH-N^6AHCM-NAD	1.0	0.2	20	0.01	7.8

[a] "Total" molar ratio determined using A_{266} and A_{290} values. "Active" molar ratio determined using MTT/PES coenzyme-recycling assay with ADH and using Coomassie method to determine protein concentration

[b] ("active" molar ratio of coenzyme:enzyme subunit/ "total" molar ratio of coenzyme:enzyme subunit) x 100.

[c] Umg^{-1} = $\mu mole\ min^{-1}$ $(mg\ enzyme)^{-1}$ Both intrinsic and specific activities were determined in a recycling assay with a DCPIP/PES redox couple. 0-0.1ml of enzyme sample (~1mg/ml) were added.

The covalently bound NAD derivatives were poor substrates for recycling by a second enzyme, in this case ADH. It would seem that the direct coupling of the derivatives to LDH results in an enzyme-coenzyme bridge that is too short to allow the NAD molecule to interact with the active centre of the second enzyme (ADH). The glutaraldehyde-coupled conjugates would therefore be expected to have a higher proportion of reducible coenzyme molecules, and this was indeed the case. NAD appears to be successfully coupled using carbodiimide and *N*-hydroxysuccinimide as was observed by Larsson and Mosbach (1971) although a water-insoluble carbodiimide, dicyclohexylcarbodiimide, was used by them. The N^6 amine group of NAD reacted with a carbodiimide activated cation at the same order of magnitude as it did with glutaraldehyde with 0.3 molecules incorporated per subunit using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride compared to 0.5 molecules

incorporated using glutaraldehyde. Carbodiimide coupling is, therefore, another method by which unmodified NAD can be incorporated onto the surface of an enzyme molecule. This has not been reported before.

Comparing the two NAD derivatives, 1.8 molecules of N^6CM-NAD were incorporated per LDH subunit compared to 1.0 molecule of N^6AHCM-NAD. We can therefore conclude that although the acidic residues have been reported to have a greater solvent accessibility (*Section 3.10.3.1*), lysyl residues are no less amenable with respect to the anchoring of NAD derivatives to LDH via carbodiimide coupling.

3.13.2.2 Specific activity of modified LDH

15-50% of the original lactate activity of LDH was lost during carbodiimide coupling experiments (*Table 3.5*). Hence, with the LDH-coenzyme conjugates, the use of carbodiimide was more desirable, with respect to maintaining the original activity of the enzyme, than the use of glutaraldehyde which showed ~70% reduction in activity.

3.13.2.3 Intrinsic activity

The intrinsic activity of each conjugate was well below the expected values for native LDH with a corresponding concentration of coenzyme added exogenously. Considering that Mansson *et al* (1978) found that a carbodiimide-coupled LADH-N^6AHCM-NAD conjugate had an activity of 4.2μmol min^{-1} (mg protein)$^{-1}$ and that this was 15% of the specific activity of the native enzyme, the result for the LDH-N^6AHCM-NAD conjugate at 0.01μmol min^{-1}(mg protein)$^{-1}$ was disappointing and represented less than 0.5% of the specific activity of the modified enzyme. It appears that the "zero length" spacer provided by carbodiimide coupling is either too short to enable the NAD molecule to interact with the active site of the molecule it is bound to, or it is too short to interact with neighbouring enzyme molecules. The low intrinsic activity of the carbodiimide-coupled N^6CM-NAD complex was predicted in our studies of lysyl residues that were proximal to the active centre (*Section 3.10.2.3*);

there were no lysyl residues that were close enough to allow an anchored N^6CM-NAD molecule to interact with coenzyme binding site. The nearest accessible lysine was 1.1nm away (Lys 58). The small amount of intrinsic activity may have been generated through an intermolecular mechanism. The only conjugate with a spacer arm long enough to reach the active centre was LDH-N^6AHCM-NAD, with a spacer arm of 1.4nm. However, this conjugate did not possess a higher intrinsic activity and it may have been that this length was still not long enough to allow a flexible link. It is more likely, however, that the coenzymes did not become anchored at the proximal lysyl residues.

Even though the conditions of glutaraldehyde coupling caused a 70% decrease in LDH activity, the intrinsic activity of each of the final conjugates was higher than the intrinsic activity of those conjugates synthesised using carbodiimide. If the higher intrinsic activity was due to the longer bridge that was provided by the glutaraldehyde between the enzyme and coenzyme then this bridge may also allow a better interaction between the covalently bound coenzyme and the electrode surface. For this reason glutaraldehyde-coupled LDH-NAD and LDH-N^6AHCM-NAD were incorporated into an enzyme electrode system (*Chapter 5*). After these studies GDH and ADH were conjugated to NAD and its derivatives using the same coupling methods.

3.14 Properties of glutamate dehydrogenase-coenzyme conjugates

Section 3.6.1 shows a typical gel filtration elution profile for a GDH-coenzyme conjugate.

3.14.1 Glutaraldehyde-coupled conjugates

3.14.1.1 Coenzyme incorporation

The values for the molar ratio of glutaraldehyde-coupled coenzyme to GDH subunit are shown in *Table 3.6*. The extent of conjugation was relatively large with around five molecules of unmodified NAD incorporated per subunit of GDH. Before comparison with LDH it must be noted that the molecular weight of the ox liver GDH subunit is 56,000 compared to 35,000 for pig H_4 LDH. However, even when this is taken into account, six times as many NAD molecules were incorporated into GDH, via glutaraldehye coupling, than in LDH. This may reflect a difference in the accessibility of surface lysyl residues in LDH and GDH. Incorporation of N^6AHCM-NAD into the latter enzyme was even more extensive with 6.6 molecules of coenzyme incorporated per subunit. This was twice as many molecules as were incorporated into LDH, again taking into account the molecular weight difference. The rates of reduction of the bound coenzymes were excellent, with 90% of both coenzymes being available for catalytic interaction with a second enzyme in an intermolecular reaction with LDH or ADH. This suggests that the bound coenzymes were more solvent accessible or were subject to less steric hindrance by surrounding residues in GDH than in LDH. The accessibility of a bound coenzyme was not obviously related to the length of the spacer arm involved.

Table 3.6
Properties of glutamate dehydrogenase-cofactor complexes synthesised using glutaraldehyde coupling

Enzyme	Molar ratio cofactor / GDH subunit			Activity (U/mg)[c]	
	Total	Active	%[b]	Intrinsic	Specific
GDH	0.0	0.0	--	0.000	0.15
GDH-NAD	5.0	4.7	94	0.012	0.06
GDH-N^{6}AHCM-NAD	6.6	5.7	86	0.018	0.16

a "Total molar ratio determined using A_{266} and A_{290} values. "Active" molar ratio determined using MTT/PES coenzyme recycling assay with ADH and using Coomassie method to determine protein concentration.
[b] % of active molar ratio of coenzyme:enzyme subunit/ total molar ratio of coenzyme:enzyme subunit.
[c]Both intrinsic and specific activities were determined in a recycling assay with a DCPIP/PES redox couple and a MTT/PES redox couple, both assays being in good agreement (S.D. =2.4%).

3.14.1.2 Specific activity

Glutaraldehyde had no strong inactivation effects upon beef liver GDH. Indeed coupling with the NAD analogue resulted in no loss of activity whatsoever. As was the case with LDH, the inactivation effect of glutaraldehyde was slightly greater when it was attached to NAD than when it was attached to N^{6}AHCM-NAD.

3.14.1.3 Intrinsic activity

The intrinsic activities of GDH-NAD and GDH-N^{6}AHCM-NAD were 20% and 11% of the specific activity of modified enzyme, respectively, and greater than the corresponding activities of glutaraldehyde-coupled LDH-coenzyme conjugates. Evidence for an intramolecular mechanism is apparent for these conjugates because their intrnsic activity is noticeably higher than the activity generated by native LDH in the presence of a corresponding concentration of coenzyme, added exogenously. This may be a result of an apparent "close concentration" of coenzyme around the LDH active site. The more efficient catalytic unit is again the N^{6}AHCM-NAD conjugate, its intrinsic activity being 50% greater than that of the GDH-NAD conjugate. NAD was not a more active substrate for GDH than its analogue, when it was covalently bound to GDH, the opposite being the case for LDH conjugates. Even though, on average, 0.76 NAD molecules were bound to the GDH

molecule for every 1.0 N^6AHCM-NAD molecule, the intrinsic activity generated by the GDH-NAD conjugate was only 66% of the activity generated by the GDH-N^6AHCM-NAD. This was probably because of the shorter bridge that exists between NAD and the enzyme; thus more bound N^6AHCM-NAD molecules were able to reach the GDH active sites.

Because the GDH-NAD conjugate possessed substantial intrinsic activity, the activity of the conjugates in the absence of exogenous coenzyme was compared to the activity of unmodified GDH with a corresponding concentration of coenzyme, exogenously added. The glutaraldehyde-coupled GDH-NAD preparation was assayed at a concentration of 0.1mg/ml using the MTT/PES recycling assay (Methods and Materials). The GDH-NAD conjugate was over five times more efficient at 0.1mg/ml than the same concentration of unmodified GDH assayed with the same molar ratio of coenzyme per subunit: the activity of the latter system was 0.002 U/mg compared to an intrinsic activity of 0.012 U/mg for the enzyme-coenzyme conjugate. However, if an intramolecular mechanism is assumed for the GDH-coenzyme conjugates, such comparisons are of limited value as the efficiency of the systems will vary according to the protein concentration used.

3.14.2 Carbodiimide-coupled conjugates

3.14.2.1 Coenzyme incorporation

As can be seen in *Table 3.7*, good incorporation of coenzyme derivatives onto the GDH surface was also achieved using carbodiimide coupling, with between 5 and 7 molecules of each NAD derivative being incorporated. However, it would seem that unmodified NAD is better coupled using glutaraldehyde, both with LDH and GDH, with only 1.6 molecules being incorporated with carbodiimide compared to 5.0 molecules with glutaraldehyde. NAD incorporation via carbodiimide coupling was the same per 1000 MW both in GDH and in LDH. Again, there does not seem to be a

significant difference between coupling via acidic residues and lysyl residues on the enzyme surface. It was also apparent that each bound coenzyme was less available for reduction with a second enzyme when coupled directly using carbodiimide. That is, 25-34% of each directly bound coenzyme was available for reduction with a second enzyme, compared to an availability of more than 80% when coupled via glutaraldehyde.

Table 3.7
Properties of glutamate dehydrogenase-cofactor complexes synthesised using carbodiimide coupling

Enzyme	Molar ratio cofactor / GDH subunit			Activity (U/mg)[c]	
	Total[a]	Active	%[b]	Intrinsic	Specific
GDH	0.0	0.1	--	0.000	0.22
GDH-NAD	1.6	0.4	25	0.000	0.19
GDH-N^6CM-NAD	5.9	2.0	34	0.020	0.09
GDH-N^6AHCM-NAD	6.8	2.0	29	0.021	0.18

[a]"Total" molar ratio determined using A_{266} and A_{290} values. "Active" molar ratio determined using MTT/PES coenzyme-recycling assay with ADH and using Coomassie method to determine protein concentration (Materials and Methods)
[b]("active" molar ratio of coenzyme:enzyme subunit/ "total" molar ratio of coenzyme:enzyme subunit) x 100.
Both intrinsic and specific activities were determined in a recycling assay with a DCPIP/PES redox couple and an MTT/PES redox couple, the two assays in close agreement (S.D. 2.4%). 0-0.1ml of enzyme sample (~1mg/ml) were added.

3.14.2.2 Specific activity

The specific activities displayed in *Table 3.7* show that carbodiimide modification did not seriously affect the activity of GDH, the largest reduction in specific activity being 55% for the GDH-N^6CM-NAD conjugate. There may be a lysyl residue in GDH which is sensitive to carbodiimide coupling; this may possibly be Lys 126 as discussed in *Section 3.11.5.* However, it must be noted that this sensitive residue was not modified by glutaraldehyde under the conditions used because there was no loss of activity.

3.14.2.3 Intrinsic activity

The GDH-NAD conjugate did not possess any intrinsic activity. The direct link that is provided between GDH and NAD by carbodiimide coupling was therefore too short for interaction with the active site of the enzyme to which it was bound. There does not appear to be an accessible acidic residue that is close enough to the active centre (intramolecular), or that protrudes far enough into the surrounding solution to generate activity (intermolecular). However, the intrinsic activity generated by each of the N^6CM-NAD and N^6AHCM-NAD derivative conjugates was quite acceptable with respect to the specific activity of the modified enzymes, being 22% and 11%, respectively. The intrinsic activity for each conjugate was 4-5 times greater than that expected from an intermolecular mechanism at an enzyme concentration of 0.1 mg/ml. Again, N^6AHCM-NAD was the best overall catalytic unit in terms of units per mg of protein. Glutaraldehyde and carbodiimide-coupled conjugates were comparable in terms of intrinsic activity.

3.15 Properties of alcohol dehydrogenase-coenzyme conjugates

A gel filtration elution profile for ADH-N^6AHCM-NAD is shown in *Figure 3.24*. It is typical of other ADH-coenzyme elution profiles, and as with GDH-coenzyme conjugates, two minor protein peaks were observed. *Section 3.6.1* provides evidence of a complete separation of free coenzyme from the protein fractions.

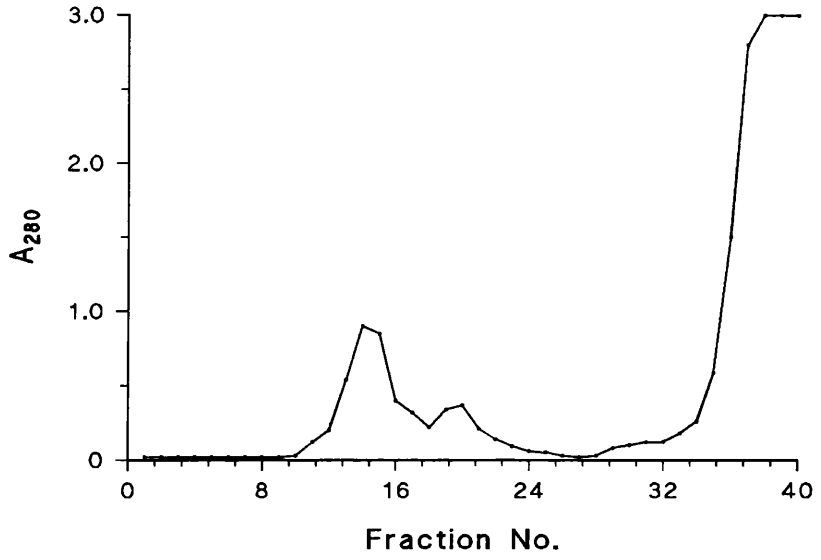


Figure 3.24: *Separation of ADH-N^{6}AHCM-NAD conjugate from uncoupled coenzyme by gel filtration chromatography*

A Sephacryl S-100 column (80 x 1.5cm) was used to separate a terephthalaldehyde coupled ADH-N^{6}AHCM-NAD complex from uncoupled N^{6}AHCM-NAD. The column was run at 4°C with a flow rate of 0.2ml min^{-1}. Fractions of 2ml were collected in a dropwise manner using an automatic fraction collector. These fractions were taken immediately for analysis to determine their absorbance at 280nm (A_{280}) in a spectrophotometer.

3.15.1 Glutaraldehyde-coupled conjugates

3.15.1.1 Coenzyme incorporation

As can be seen in *Table 3.8*, 1.2 NAD molecules were incorporated compared to 0.5 molecules in glutaraldehyde-coupled LDH-coenzyme conjugates. However, more NAD molecules were incorporated onto the surface of GDH (5.0 moles per mole subunit). A similar situation was seen for the bound derivative, N^{6}AHCM-NAD, where 3.5 molecules were incorporated per subunit compared to 1.8 molecules with LDH and 6.6 molecules with GDH. Surprisingly, the accessibility of the covalently-bound NAD for interaction with a second enzyme, LDH, in a DCPIP/PES recycling assay was actually higher than the accessibility of the covalently bound N^{6}AHCM-NAD: 41% of bound NAD molecules were available for reduction by a second enzyme (LDH) compared to only 17% of bound N^{6}AHCM-NAD molecules. These results may

reflect a difference in the structure of the active sites of LDH and ADH, the second enzymes.*

Table 3.8
Properties of alcohol dehydrogenase-cofactor complexes synthesised using glutaraldehyde coupling

Enzyme	Molar ratio cofactor / ADH subunit			Activity (U/mg)[c]	
	Total[a]	Active	%[b]	Intrinsic	Specific
ADH	0.0	0.1	0	0.00	120
ADH-NAD	1.2	0.5	41	0.00	0.40
ADH-N^{6}AHCM-NAD	3.5	0.6	17	0.00	0.00

[a]"Total" molar ratio determined using A_{266} and A_{290} values. "Active" molar ratio determined using DCPIP/PES coenzyme-recycling assay with LDH and using Coomassie method to determine protein concentration

[b]("active" molar ratio of coenzyme:enzyme subunit/ "total" molar ratio of coenzyme:enzyme subunit) x 100.

[c]Both intrinsic and specific activities were determined in a recycling assay with an MTT/PES redox couple. 0-0.1ml of enzyme sample (~1mg/ml) were added.

3.15.1.2 Specific and intrinsic activity

A strong inhibitory effect was observed after glutaraldehyde modification of YADH. Because there was almost total inhibition of enzyme activity it was not surprising that no intrinsic activity could be detected. In order to investigate this inhibitory effect, further experiments were carried out which eventually led to the successful employment of an alternative bialdehyde reagent.

3.15.1.3 The effect of various aldehydes on the specific activity of YADH

In order to confirm that the inhibition of YADH in the presence of excess glutaraldehyde was actually due to the glutaraldehyde, the enzyme was mixed in increasing amounts of this cross-linking reagent. The ethanol activity of the treated YADH was then measured in the MTT/PES chemical recycling assay. As *Figure 3.25* shows, inhibition became apparent at higher concentrations of glutaraldehyde.

* The second enzyme experiments are to show that the bound coenzyme is available for reduction in intermolecular enzyme reactions. ADH was used as a second enzyme in studies of the LDH-coenzyme conjugates, whereas LDH was used as a second enzyme in studies of ADH-coenzyme conjugates.

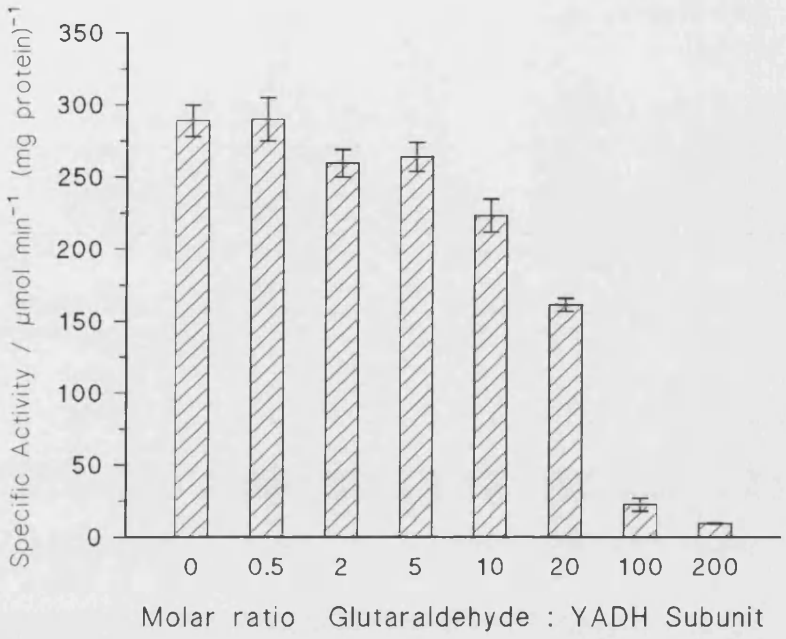


Figure 3.25: *Effect of excess glutaraldehyde on the specific activity of YADH.* 20µl of glutaraldehyde solution of varying strength was added to a 1mgml^{-1} buffered YADH solution and mixed. After 2 hours the enzyme was assayed in a MTT/PES recycling assay. To 1.95ml (100mM TEA/100mM nicotinamide/ 500mM ethanol) buffer, pH7.8 were added 0.1ml 100mM NAD, 50µl 12mM MTT and 0.8ml 3mM PES. The molar ratio of glutaraldehyde to YADH subunit was calculated knowing that the strength of the stock solution was 25%.

Because of the complexity of the chemistry of glutaraldehyde (*section 3.4.1*) the loss of activity could be due to a number of reasons. Glutaraldehyde is not absolutely specific for lysyl residues; it has also been known to react with thiol groups, imidazole groups and hydroxyl groups (Ji, 1980). In order to investigate whether inactivation was due to the reactive aldehyde group, the effect of excess aromatic aldehyde, benzaldehyde (*Figure 3.26*), upon YADH activity was examined.

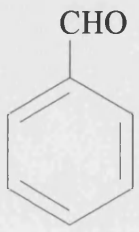


Figure 3.26: *The structure of benzaldehyde*

Benzaldehyde has previously been used in substrate studies with YADH (Bowen *et al*, 1986). Benzaldehyde was not a substrate, but inhibited the enzyme by 22% when in a 400-fold molar excess of the YADH subunits in phosphate buffer, at 22°C and at pH 7.0 for 1 hour. This amount of inhibition would be acceptable if, for example, an aromatic dialdehyde was used as a homobifunctional coupling reagent. Thus, the effect of an aromatic dialdehyde upon YADH activity was also examined under our conditions. This experiment was complicated because the aromatic dialdehydes are only slightly soluble in water and so an organic solvent was required that had no effect on the catalytic activity of YADH. Two solvents were investigated: formamide and 1,3 dioxan . As can be seen in *Figure 3.27*, dioxan had a serious detrimental effect upon the activity of YADH, and so only formamide was used in further experiments involving YADH.

The effect of various excesses of benzaldehyde upon the activity of YADH is shown in *Figure 3.28*. Inhibition only became apparent when the benzaldehyde was present in 150-fold excess over the YADH subunit. The loss of activity was around 20% when the benzaldehyde was present in 180-fold excess over the subunit concentration.

This led us to investigate the possibility of using an aromatic dialdehyde as a bifunctional coupling reagent in the place of glutaraldehyde. Terephthalaldehyde was the molecule of choice and its structure is shown in *Figure 3.29*.

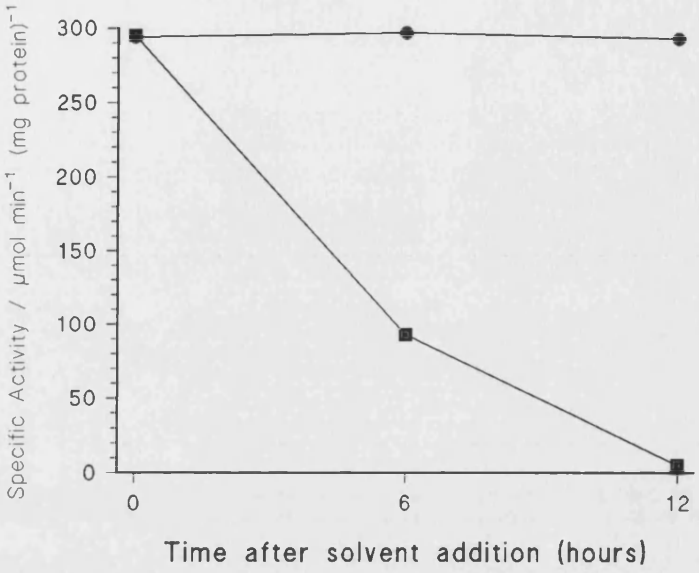


Figure 3.27 *The effect of organic solvents, formamide and dioxan, on the activity of YADH.*

● = formamide; ■ = dioxan. A 0.8ml solution of YADH (2mgml^{-1}) in 100mM phosphate buffer, pH 7.5 was made up and left at 4°C. 0.2ml of 100% formamide or dioxan was added. 0.1ml of the solution was taken for assay at intermittent periods using a MTT/PES recycling assay for YADH activity. To 1.95ml (100mM TEA/ 100mM nicotinamide/ 500mM ethanol) buffer, pH7.8, were added 0.1ml 100mM NAD, 50µl 12mM MTT, 0.8ml 3mM PES.

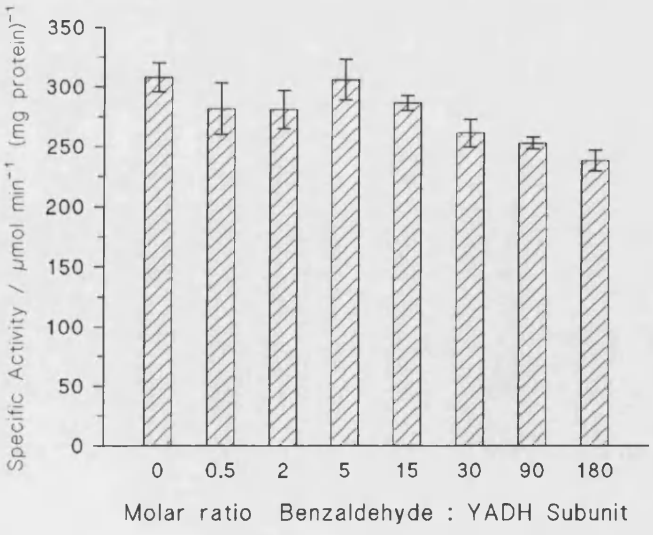


Figure 3.28: *Effect of excess benzaldehyde on the specific activity of YADH.* 20µl of benzaldehyde solution (50% formamide) were added to 1ml of a 1mgml^{-1} YADH solution (10% formamide) and mixed. After 2 hours the enzyme was assayed in a MTT/PES recycling assay: to 1.95ml (100mM TEA/ 100mM nicotinamide/ 500mM ethanol) buffer, pH7.8) were added 0.1ml 100mM NAD, 50µl 12mM MTT, 0.8ml 3mM PES.

Figure 3.29: *The structure of terephthalaldehyde*

The structure of terephthalaldehyde is more defined than glutaraldehyde because it does not have polymeric forms in solution. Therefore, any conjugates that it forms will be more clearly defined in structure than those formed with glutaraldehyde (Ji, 1980). Terephthalaldehyde would also act as a bridge of around 0.6 nm in length between the enzyme and coenzyme. Interestingly, the dialdehyde did not seriously inhibit YADH, even in a large excess over the subunit concentration, as shown in *Figure 3.30*. This was somewhat surprising, because although the coupling of terephthalaldehyde is defined, it is very hydrophobic and aromatic in nature.

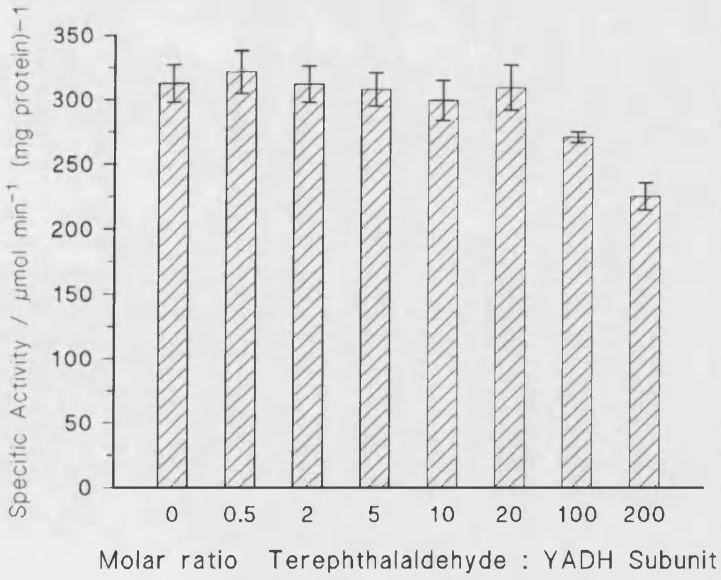


Figure 3.30: *Effect of excess terephthalaldehyde on the specific activity of YADH.*

20µl of terephthalaldehyde solution (50% formamide) was added to a 1mg/ml YADH solution in 10% formamide and mixed. After 2 hours the enzyme was assayed using a MTT/PES recycling assay: to 1.95ml (100mM TEA/ 100mM nicotinamide/ 500mM ethanol) buffer, pH7.8, were added 0.1ml 100mM NAD, 50µl 12mM MTT, 0.8ml 3mM PES.

Terephthalaldehyde was not a substrate for YADH as are some aromatic aldehydes (Bowen *et al*, 1986; Long *et al*, 1989). The reagent was used in the same way as glutaraldehyde, except it was first dissolved in 10% formamide before addition to the reaction mixture.

3.15.2 Terephthalaldehyde-coupled ADH-coenzyme conjugates

3.15.2.1 Coenzyme incorporation

It was exciting to find that more coenzyme molecules were incorporated using terephthalaldehyde than using glutaraldehyde. That is, 3.2 molecules of NAD were bound, on average, compared to only 1.2 when glutaraldehyde was used. With the derivative, 5.1 molecules of N^6AHCM-NAD bound to the enzyme compared to only 3.5 with glutaraldehyde coupling. The relative electron deficiency of the carbonyl atoms of terephthalaldehyde causes it to be less reactive with primary amines but it would appear that the aromatic dialdehyde gives rise to conjugates with extra stability, enabling more coenzyme molecules to remain anchored to lysyl residues. The percentage reduction of each coupled coenzyme was of the same order of magnitude as the glutaraldehyde-coupled coenzymes: 25% of the bound coenzyme molecules were available for reduction with a second enzyme (LDH) compared to the 17-41% seen with glutaraldehyde. This may be a reflection of the shorter spacer length of terephthalaldehyde compared to glutaraldehyde.

3.15.2.2 Specific activity

As shown in *Table 3.9*, there was no inhibition of YADH activity after terephthalaldehyde-mediated coupling. The enzyme appeared to be soluble throughout the coupling experiment, despite the presence of the water-insoluble reagent, terephthalaldehyde. No precipitate was evident, although it was possible that any insoluble protein was lost during purification. Protein insolubility would not show up

as a reduction in specific activity of the enzyme because the assays were carried out after purification.

Table 3.9
Properties of alcohol dehydrogenase-cofactor complexes synthesised using terephthalaldehyde coupling

Enzyme	Molar ratio cofactor / ADH subunit			Activity (U/mg)[c]	
	Total[a]	Active	%[b]	Intrinsic	Specific
ADH	0.0	0.1	--	0.01	115
ADH-NAD	3.2	0.8	25	0.29	124
ADH-N^{6}AHCM-NAD	5.1	1.3	25	0.65	127

[a]"Total" molar ratio determined using A_{266} and A_{290} values. "Active" molar ratio determined using DCPIP/PES coenzyme-recycling assay with LDH and using Coomassie method to determine protein concentration
[b]("active" molar ratio of coenzyme:enzyme subunit/ "total" molar ratio of coenzyme:enzyme subunit) x 100.
[c]Both intrinsic and specific activities were determined in a recycling assay with an MTT/PES redox couple. 0-0.1ml of enzyme sample (~1mg/ml) were added.

3.15.2.3 Intrinsic activity

Considering the high specific activities of the modified enzymes, the intrinsic activities were quite disappointing. The intrinsic activity of the ADH-NAD and ADH-N^{6}AHCM-NAD conjugates represented only 0.2% and 0.65% of their specific activities, respectively.

Terephthalaldehyde would seem to be an alternative coupling reagent to glutaraldehyde, with the added advantage that it does not affect the specific activity of YADH. The final structure of enzyme-coenzyme conjugates coupled using terephthalaldehyde is also more defined, but it must also be noted that the link between enzyme and coenzyme would be more rigid.

3.15.3 Carbodiimide-coupled conjugates

3.15.3.1 Coenzyme incorporation

Table 3.10
Properties of alcohol dehydrogenase-cofactor complexes synthesised using carbodiimide coupling

Enzyme	Molar ratio cofactor / ADH subunit			Activity (U/mg)[c]	
	Total[a]	Active	%[b]	Intrinsic	Specific
ADH	0.0	0.1	--	0.00	140
ADH-NAD	0.4	0.5	100	0.10	6.0
ADH-N^6CM-NAD	1.5	1.0	75	0.16	7.4
ADH-N^6AHCM-NAD	4.5	0.7	15	0.01	9.2

[a] "Total" molar ratio determined using A_{266} and A_{290} values. "Active" molar ratio determined using DCPIP/PES coenzyme-recycling assay with LDH and using Coomassie method to determine protein concentration

[b] ("active" molar ratio of coenzyme:enzyme subunit/ "total" molar ratio of coenzyme:enzyme subunit) x 100.

[c] Both intrinsic and specific activities were determined in a recycling assay with an MTT/PES redox couple. 0-0.1ml of enzyme sample (~1mg/ml) were added.

Incorporation of unmodified NAD was poor with, on average, less than half of the YADH subunits having an NAD molecule attached (*Table 3.10*). This also was the case with the LDH-NAD conjugate couple formed using carbodiimide. Incorporation of N^6AHCM-NAD was good at 4.5 moles incorporated per mole of subunit. However, N^6CM-NAD coupled only to the extent of 1.5 moles per mole subunit. This suggests that there are more accessible carboxyl groups than accessible lysyl residues in YADH. Surprisingly, the accessibility of each bound coenzyme to recycling with a second enzyme (LDH) seems to increase with a *decreasing* length of spacer arm.

3.15.3.2 Specific activity

YADH is sensitive to the carbodiimide coupling conditions and shows a significant decrease in specific activity when treated in this way. Native YADH was subjected to the same conditions as occurred in the conjugation except that 1-(3-

dimethylaminopropyl)-3-ethyl-carbodiimide.HCl and *N*-hydroxysuccinimide were omitted. Thus, the inhibition of activity seen in the modified enzymes must be due to the modification of the enzyme by the coupling reagent.

3.15.3.3 Intrinsic activity

The intrinsic activity of the ADH-N^{6}AHCM-NAD conjugate was only 0.1% of its specific activity. The intrinsic activity was higher (3%) for the ADH-N^{6}CM-NAD conjugate suggesting that modified lysyl residues were in a more suitable position for coenzyme anchorage than were the carboxyl residues of YADH. The intrinsic activity of the ADH-NAD conjugate was 1.6% of its specific activity. Another surprising property of the carbodiimide-coupled YADH-coenzyme conjugates was that the intrinsic activity was lowest in the conjugates that possessed the highest spacer length between the enzyme and coenzyme. One explanation may be that if a long spacer arm is bound in very close proximity to the active site, it may not be able to fold or curl up so that it can insert the NAD moiety into the active site. This explanation assumes an intramolecular reaction.

CHAPTER 4

Enzyme-coenzyme conjugates coupled via a disulphide bridge

Introduction

A novel method for coupling NAD to an NAD-dependent dehydrogenase is via a disulphide bridge. Such a linkage could be formed by the oxidative dimerisation of two sulphydryl groups, one of which is attached to the protein and the other attached to the coenzyme (*Figure 4.1*).

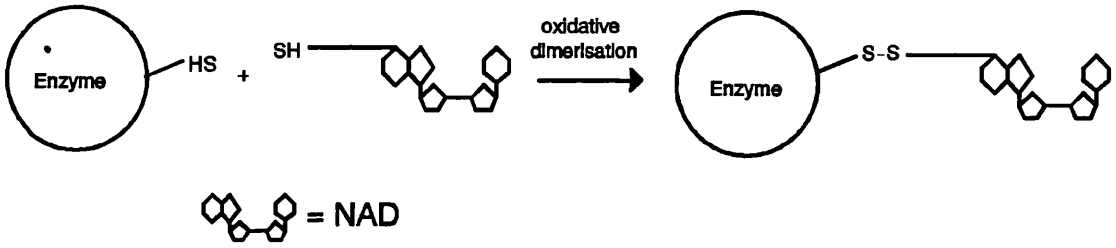


Figure 4.1: *The "disulphide" method of enzyme-coenzyme conjugate formation*

In order to couple NAD to a dehydrogenase in this way, attempts were made to synthesise a free sulphydryl-containing NAD derivative that would react specifically with free sulphydryl groups present on the surface of a protein. Protein sulphydryl groups may be inherently present on the surface of the native enzyme where they are found in cystyl residues. The sulphydryl group of cysteine is, in general, the most reactive functional group present on a protein surface (Liu, 1977), readily undergoing oxidative dimerisation with another sulphydryl group to form a disulphide bridge. Some dehydrogenase enzymes, however, may not possess thiol groups that are accessible to the surrounding solvent, but such groups can be introduced onto the protein surface using a thiolating reagent.

A coupling reaction involving thiol dimerisation would be more specific than the carbodiimide and glutaraldehyde coupling reactions described in *Chapter 3*, for which certain side reactions are known. The results presented in *Chapter 3* also suggested that a successful approach to enzyme-coenzyme conjugate formation will be limited, depending on the sort of dehydrogenase used. For example, although glutaraldehyde could be used to couple NAD to LDH and GDH, it could not be used to couple NAD to YADH because of the serious inhibitory effect that it caused. The same could be said of the approach described in this chapter: a "disulphide" coupling approach will not be suitable for use with dehydrogenases that are sensitive to thiol reagents, for example YADH and LADH, because of the covalent modification of thiol groups that is involved. Nevertheless, there were several reasons why the formation of a disulphide-containing spacer arm covalently linking an enzyme to its coenzyme was of interest:-

1. Enzyme electrode studies using enzyme-coenzyme conjugates formed using glutaraldehyde suggested that the link formed between the enzyme and coenzyme might be unstable under the conditions of electrode operation (*Chapter 5*), and it would be interesting to find out whether a different sort of molecular link would be more stable in the environment of an enzyme electrode system. The redox value, E_o, for SH/SS* systems averages between -0.2V and -0.3V at pH 7.0 (Liu, 1977). Consequently, a disulphide bridge linkage formed between an enzyme and its coenzyme would be stabilised under the oxidising conditions of an enzyme electrode that are required to regenerate NAD from NADH (a potential of +0.15V is applied to the enzyme electrode).

2. Although rotation about a disulphide bond is restricted, such a connection formed between an enzyme and coenzyme may allow the spacer arm to be more flexible than

* SH = free sulphydryl group; SS = disulphide bridge

either the peptide bond or Schiff base connections that were used in carbodiimide-mediated conjugations and in those conjugations made via glutaraldehyde.

3. A disulphide bond linkage is a reversible one; it can easily be broken by reduction with a suitable reagent, such as dithiothreitol, thus providing an excellent method with which to confirm that the intrinsic activity of an enzyme-coenzyme conjugate is due to the presence of covalently bound coenzyme.

To date, only one study is known that deals with the binding of a coenzyme to an enzyme via a disulphide bridge linkage (Persson *et al*, 1991; *Section 1.6.3*). A single cystyl residue was incorporated into glucose dehydrogenase using site-directed mutagenesis, followed by the coupling of a thiolated NAD adduct, synthesised from N^6AHCM-NAD and *N*-succinimidyl-3-(2-pyridyldithiopropionate), to the cysteine sulphydryl group (see *Figure 1.6i*). This approach was quite complex, requiring the use of genetic engineering techniques. The native glucose dehydrogenase that was used did not, unlike most dehydrogenases, contain any cystyl residues, and so it was not subject to inhibition by thiol-containing molecules. Consequently, the technique could not easily be applied to most dehydrogenases, which inherently contain cystyl residues in their structure.

Figure 4.2 outlines the approach used in the synthesis of protein-coenzyme conjugates coupled via a disulphide linkage in our study. Several steps were common to the approach used in the production of enzyme-coenzyme conjugates linked via amine and carboxyl groups *(Chapter 3)*.

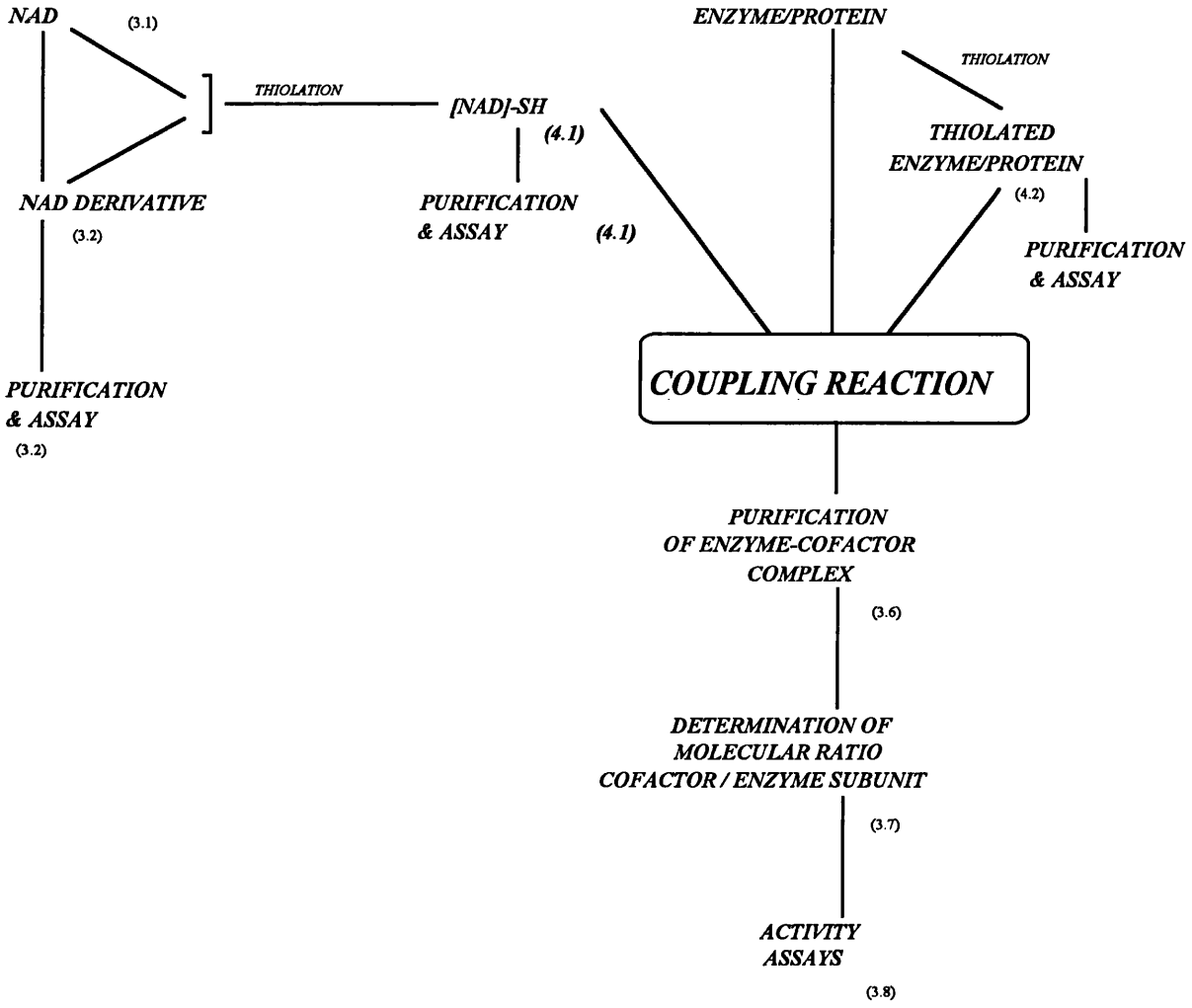


Figure 4.2: *An overview of the approach used in the synthesis and characterisation of protein-coenzyme conjugates coupled via a disulphide bridge.* The numbers in brackets relate to relevant sections of *Chapters 3 & 4*..

The approach described here differs from that used in the previous chapter, mainly because both coenzyme and enzyme are modified in order to introduce thiol groups before conjugation. The first part of this chapter deals with the approach used to introduce thiol groups into the structure of NAD and onto protein surfaces. Bovine serum albumin (BSA) was chosen as a model protein: experiments were carried out to see if NAD could be covalently coupled onto the typical globular protein surface of BSA using the "disulphide method" before further attempts were made to form an enzyme-coenzyme conjugate using LDH as the consitituent protein. If BSA could be

conjugated to NAD, as well as serving as a protein model, it would be of interest as a control in the enzyme electrode applications because it would not have catalytic activity. The covalent coupling of a coenzyme molecule to a macromolecule such as BSA also provides a number of ways in which the coenzyme can be immobilised, for example, by entrapment behind a dialysis membrane or by adsorption. The water-soluble macromolecular conjugate could then be used as an immobilised coenzyme unit (*Figure 4.3*).

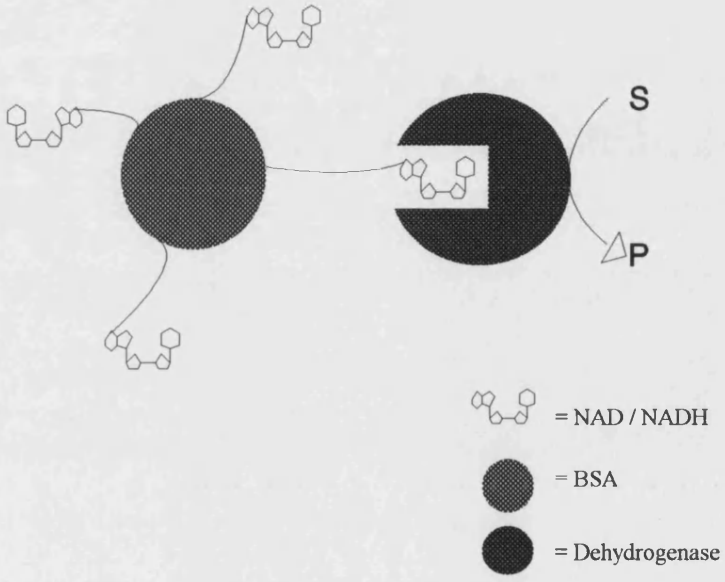


Figure 4.3: *Use of BSA-NAD conjugate as immobilised NAD unit.*

As in the previous chapter, this approach involved the preassembly of the constituent parts (in this case thiolated enzyme and thiolated coenzyme) before they were brought together in a coupling reaction (*Figure 4.1*). The advantages of the "preassembly approach" are described in *section 3.5*.

The second part of this chapter describes the properties of the protein-coenzyme conjugates that were synthesised.

PART ONE: The approach used to synthesise dehydrogenase-coenzyme conjugates linked via a disulphide bridge.

4.1 The synthesis of a thiolated NAD derivative

In order to synthesise a protein-NAD conjugate linked via a disulphide bridge, a thiolated NAD derivative was required (*Figure 4.1*). The amine group of the adenine ring of NAD was chosen as the point of derivatisation because modification at this position does not lead to a serious loss in the substrate activity of NAD and also because this position does not become buried during the interaction of the coenzyme with the enzyme binding site. (*section 3.1*). The oxidative dimerisation of 6-mercaptopurine-NAD (*Figure 4.4*) with protein sulphydryl groups was considered for its simplicity but it was not attempted because of the unreactive nature of the aromatic sulphydryl group of 6-mercaptopurine-NAD, caused by the electron delocalisation effects of the aromatic ring. The proximity of the bulky adenine ring to the sulphydryl group in 6-mercaptopurine-NAD might also have sterically hindered the approach of a protein-bound thiol ligand during coupling reactions. Moreover, an enzyme-coenzyme conjugate formed by oxidative dimerisation of 6-mercaptopurine-NAD and a surface cystyl residue of a dehydrogenase would undoubtedly possess a spacer arm that would be too short to allow a flexible interaction between the bound coenzyme and the active site.

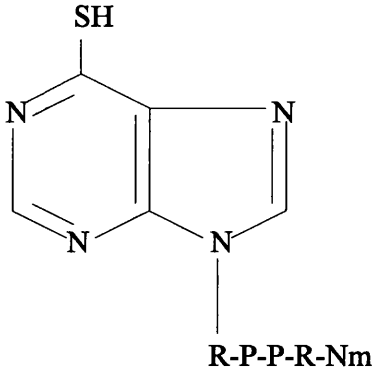


Figure 4.4: *The structure of 6-mercaptopurine-NAD*

Attempts were made to introduce an aliphatic sulphydryl group into the structures of NAD and the two NAD derivatives previously synthesised, N^6AHCM-NAD and N^6CM-NAD (*Chapter 3*). Each of these NAD derivatives contains a functional group that is both more reactive and more accessible to modification reagents than the amine group of NAD, and they are therefore easier to derivatise. If the two NAD derivatives could be thiolated, they would result in the formation of enzyme-coenzyme conjugates with longer spacer arms than would be found in those conjugates formed using a similarly thiolated NAD. The criteria used to synthesise a thiol-containing NAD derivative will now be discussed taking, in turn, derivatisation of the primary amine-containing coenzymes, NAD and N^6AHCM-NAD, and the carboxyl containing coenzyme, N^6CM-NAD.

4.1.1 Thiolation of NAD and N^6AHCM-NAD

The structures of NAD and N^6AHCM-NAD are shown in *Figure 1.3*. The primary amine group of N^6AHCM-NAD is a stronger nucleophile than the aromatic amine of NAD, because of the electron delocalisation effects caused by the aromatic character of the purine ring. Although the pK_a of a primary aliphatic amine is 10.5, the pK_a of the aromatic amine of aniline (*Figure 4.5*) is 4.6 (Finar, 1967).

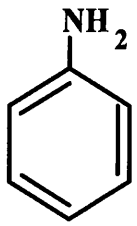


***Figure 4.5:** The structure of aniline*

The pK_a of the amine group of NAD is difficult to measure because it is actually less basic than either of the endocyclic nitrogen atoms (Abrams and Kallen, 1976). The presence of endocyclic nitrogen atoms in an aromatic ring causes it to be relatively electron deficient, thus accentuating the delocalisation effect and making the amine of

adenine even less reactive (Streitweiser and Heathcock, 1985). Despite these unfavourable characteristics, attempts were made to modify the amine position of NAD because it showed a certain reactivity with both glutaraldehyde and carbodiimide-activated carboxyl groups (*Chapter 3*). In addition, if a simple one-step thiolation of NAD could be achieved it would prove interesting, for example, as a simple coenzyme immobilisation technique or in the reversible formation of NAD dimers for use in enzyme studies.

A heterobifunctional reagent was employed to introduce a free sulphydryl group into the structures of NAD and N^6AHCM-NAD. The prerequisites of such a reagent included:

1. An amine-reactive group for attachment to the primary amine of the coenzyme.
2. A terminal sulphydryl group for oxidative dimerisation with a protein thiol.
3. In the case of NAD, an intermolecular spacer between the amine-reactive and sulphydryl groups to act as a spacer arm in the final enzyme-coenzyme conjugate (*Figure 4.1*).

Traut's reagent, 2-iminothiolane, described below, was chosen for this role because, not only does it possess the properties described above, but it is also simple to use, relatively inexpensive and has been successfully employed in the past to introduce thiol groups onto protein surfaces (Jue *et al*, 1978).

4.1.1.1 Traut's reagent (2-Iminothiolane)

Traut's reagent, 2-iminothiolane (*Figure 4.6*), has most often been used as a protein modification reagent.

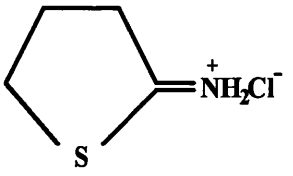


Figure 4.6: The structure of Traut's reagent, 2-iminothiolane

It reacts with primary amines to introduce thiol groups, as shown below.

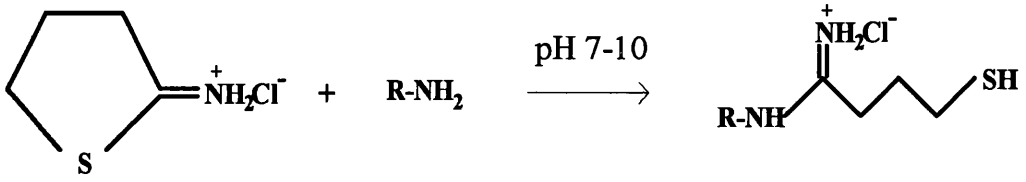


Figure 4.7: The reaction of Traut's reagent with primary amines

Originally, the structure of Traut's reagent was reported to be a straight chain compound, 4-mercaptobutyrimidate, although in 1978, Jue *et al* reported its true ring structure. The reagent contains a substituted imidoester which reacts with amines to form an amidine group. It is water soluble and reaction with amines is optimal between pH 7 and pH 10, so it may be used under the mild conditions that are suitable for maintaining the structure and function of dehydrogenases. Upon oxidation, amines that have been modified with Traut's reagent react with proteins to form linkages that are reversibly cleaved by mild reduction; this suggests the absence of N-alkylimidate linkages caused by side reactions, a property typical of other imidates (Jue *et al*, 1978). The reagent undergoes slow hydrolysis in aqueous solution, but this is dependent on temperature: the presence of free thiol groups is minimal at 7°C but at 25°C the thiol groups become slowly exposed (Jue *et al*, 1978). The reagent has a spectrophotometric absorption maximum at 248nm, the molar extinction coefficient being $8840cm^{-1}M^{-1}$, a property that is lost upon its reaction with amines. The reaction between Traut's reagent and primary amines can be easily followed by monitoring the decrease in absorbance at 248nm in a spectrophotometer.

Traut's reagent introduces a thiol group that is 0.8nm away from the point of derivatisation of the modified molecule. It has been successfully used to introduce up

to 43 thiol groups onto the protein surfaces of the 30S ribosome of *Escherichia coli* (Jue *et al*, 1978) and has been widely used in studies of the topography of ribosomal proteins (Gulle *et al*, 1988) and in the modification of liposomes (Lasch *et al*, 1987).

Before the reagent was used in the present study it was analysed to confirm some of its characteristics. The percentage of free sulphydryl groups in a solution of Traut's reagent made up in 50mM triethanolamine.HCl buffer, pH 7.0 was determined at 24°C using Ellman's reagent (see below). 5% of the reagent's molecules possessed a free sulphydryl group. However, as expected, this figure was dependent upon the temperature, being reduced to only 2% at 4°C. In addition, at 4°C the concentration of free sulphydryl groups remained stable, but at 24°C a slow increase of 0.5% hr^{-1} free sulphydryl groups was observed.

Next, the reaction of Traut's reagent with glycine was monitored to confirm that the reaction of the reagent with a primary amine results in a stoichiometric production of free sulphydryl groups (*Figure 4.7*). The glycine was added in a 13-fold molar excess of Traut's reagent and left to react for 24 hours at 4°C (*Figure 4.8*). The release of thiol groups was monitored using Ellman's reagent (see below). As can be seen the reaction was in fact 85% complete after only one hour.

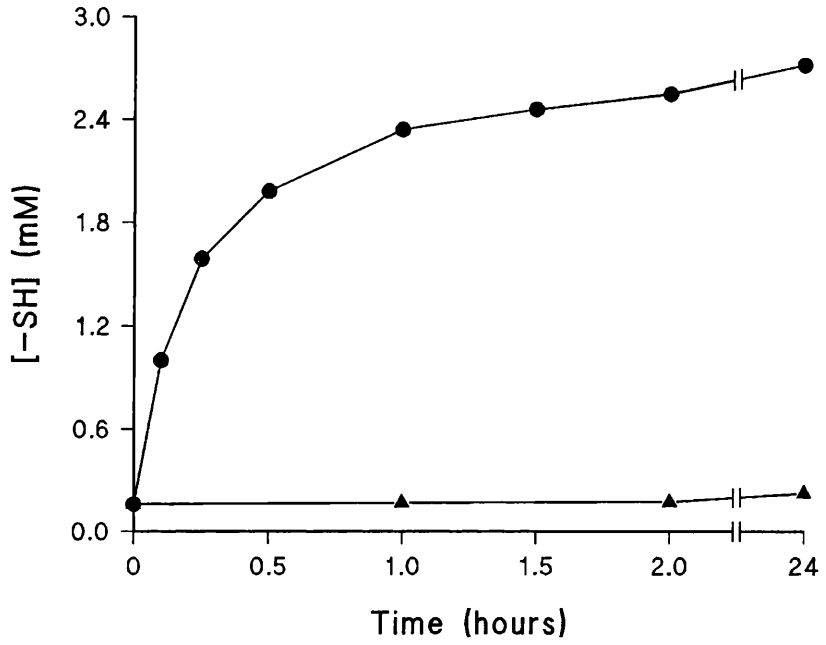
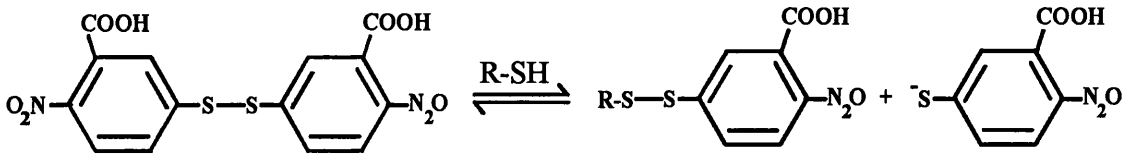


Figure 4.8: *The reaction of Traut's reagent with glycine:* • = glycine added (40μmol ml^{-1}; ▲= glycine absent. The reaction was carried out at 4°C in 2ml of TEA, 50mM, pH 8.0. Traut's reagent was added to 3μmol ml^{-1} and the reaction was monitored by following the release of free sulphydryl groups upon the reaction of Traut's reagent with primary amine groups of glycine using Ellman's reagent as follows: A 0.2ml sample of the reaction mixture was added to 1.6ml of phosphate buffer, 0.1M, pH8.0 and 0.2ml of Ellman's reagent (4$mgml^{-1}$) in a cuvette and mixed thoroughly. After 15mins the absorbance of the solution at 412nm was recorded and the concentration of sulphydryl groups calculated from a calibration curve.

4.1.1.2 Ellman's Reagent

Ellman's reagent is one of the most popular reagents for the determination of thiol groups. The reaction of 5,5-dithiobis-(2-nitrobenzoic) acid with a free thiol group results in the release of 2-nitro-5-thiobenzoic acid, which has a characteristic yellow colour, and a molar extinction coefficient of 13,600 $cm^{-1}M^{-1}$ at its absorption maximum of 412 nm (Lundblad and Noyes, 1978). The structure and mechanism of reaction of Ellman's reagent is shown in *Figure 4.9*.



5,5'-dithio-bis-(2-nitrobenzoic acid)
[Ellman's reagent]

nitrothiobenzoate
(yellow)

Figure 4.9: *Structure and mechanism of Ellman's reagent*

The reaction can be carried out at neutral or mildly alkaline pH. A calibration curve was made using cysteine solutions of various concentration to give a plot of the absorbance at 412nm versus the concentration of free sulphydryl groups in the assay.

4.1.2 Thiolation of N^6CM-NAD

Attempts were also made to modify N^6CM-NAD, a carboxyl containing NAD derivative. Heterobifunctional reagents possessing both a carboxyl-reactive function and a thiol group are not commonly used. 2-aminoethanethiol, cysteamine, was chosen to introduce a thiol group by carbodiimide-mediated reaction of its amine function with the carboxyl group of N^6CM-NAD. The low pH required to promote the carbodiimide-mediated reaction should also help to maintain the thiols in a reduced form, the pK_a of an -SH group being 9.2 (Glazer, 1977). The spacer arm obtained from such a modification would be more polar in character than the spacer arms that would be obtained for the amine-containing coenzymes modified with Traut's reagent and would therefore provide an interesting comparison. 2-Aminoethanethiol would contribute 0.5nm in length to a spacer arm formed between the enzyme and coenzyme. The functional groups of 2-aminoethanethiol should also show normal reactivity, *i.e.* amine and thiol functions should not influence each other's reactivity: a sulphydryl group is less electronegative than a hydroxyl group whose presence does not affect the function of the amine group in ethanolamine, the alcohol equivalent of 2-aminoethanethiol (Sidgewick, 1966). In fact, 2-aminoethanethiol has been used in genetic studies as a cross-linker between two oligonucleotides: O6-phenyl-2'-deoxyinosine was used to activate the C-6 atom of the adenine ring of the nucleotide,

enabling a reaction between the C-6 atom and the amine group of 2-aminoethanethiol. A cross-link was then formed between two modified nucleotides by oxidative dimerisation of their thiol groups. However, this technique required the use of complex organic synthesis for the O6-phenyl-2'-deoxyinosine activating agent (Ferentz and Gregory, 1991).

A fundamental step that was used in both the purification and assay of newly synthesised thiolated NAD derivatives will now be discussed, that is, covalent chromatography using an activated thiol-agarose column.

4.1.3 Preparation and use of activated thiol-agarose: the assay of compounds containing a thiol group using covalent chromatography

A convenient and very specific way with which to assay newly synthesised thiol-containing compounds is to use a thiol-specific chromatography column which will separate the thiol compound from compounds not possessing thiol groups. The procedure of Dean *et al* (1986) was used to synthesise a thiol agarose gel as summarised in *Figure 4.10*. The hydroxyl groups of Sepharose 4B, the support matrix, are subjected to epoxide-activation with epichlorohydrin in order to form a thiosulphate. The latter group was then reduced with dithiothreitol (DTT) to produce free thiol groups. If the thiol-agarose beads are used in the free thiol form, oxidising conditions are required to enhance the binding of the thiolated sample by oxidative dimerisation. This, however, also causes cross-linking between molecules of the thiolated sample that are being assayed and can seriously interfere with the sample binding. The thiol groups of the gel were therefore activated with dipyridyldisulphide (DPDS) (*Figure 4.10*) making possible the spontaneous and reversible reaction of the free thiol groups of the sample with the gel under mild conditions. This activation also reduces the liability of thiol-agarose to oxidation by atmospheric oxygen, a reaction which is enhanced under alkaline conditions. The binding of a thiolated ligand onto

activated thiol agarose can be easily monitored in a spectrophotometer because pyridine-2-thione, a product of the reaction between DPDS-activated thiol-agarose and free thiol groups (*Figure 4.10*), has an absorption maximum at 343nm, and an extinction coefficient of $8080M^{-1}cm^{-1}$.Finally, the product, pyridine-2-thione also has the sulphur moiety as a thione which is unreactive in this system and thus there is no tendency for the reaction to be reversible.

The capacity of the synthesised gel was examined at pH 8.0 and was found to have 0.9 μmol PDS per ml swollen gel; therefore, the thiol group binding capacity must also be 0.9μmol per ml swollen gel. This figure was calculated from the absorbance of eluted fractions at 343nm after the addition of a solution of DTT (3mM). The efficiency of the gel at high capacity was then examined using a cysteine solution. Here a cysteine sample (4.1μmol) was added to 5ml of swollen DPDS-activated thiol agarose gel with a total capacity of 4.5μmol). Each fraction was assayed for free thiol groups using Ellman's reagent to detect any cysteine that did not bind to the column.

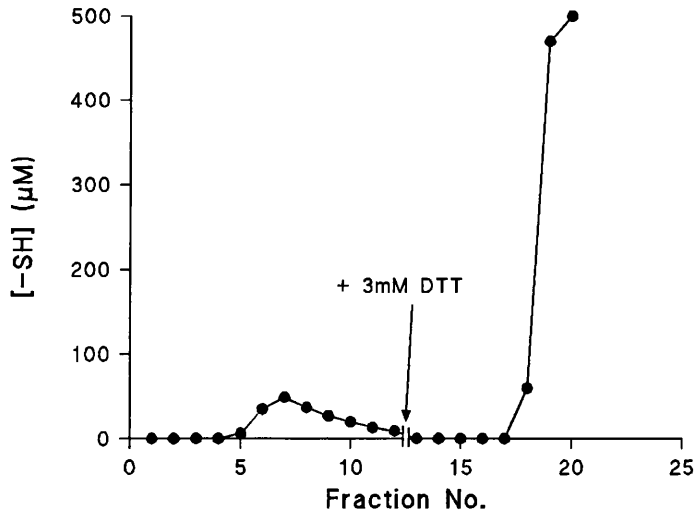
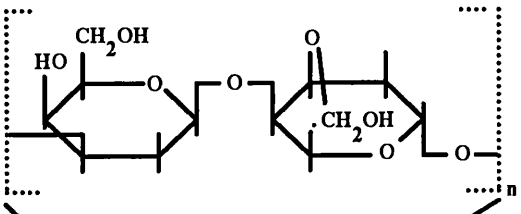


Figure 4.11: *The efficiency of the activated thiol agarose column:* 0.5ml of a $10mgml^{-1}$ cysteine solution (4.1μmol) was added to a gel of capacity 4.5μmol. In other words the column was run at near maximum capacity. Bed volume = 5ml, flow rate = $10ml\ hr^{-1}$, fraction volume = 0.6ml, running buffer = 50mM phosphate, pH8.0. Fractions were collected in a dropwise manner using an automated fraction collector and the thiol content of each fraction was determined using Ellman's reagent as follows: A 0.2ml sample of the reaction mixture was added to 1.6ml of phosphate buffer, 0.1M, pH8.0 and 0.2ml of Ellman's reagent ($4mgml^{-1}$) in a cuvette and mixed thoroughly. After 15mins the absorbance of the solution at 412nm was recorded and the concentration of sulphydryl groups calculated from a calibration curve.



Repeating agarose units

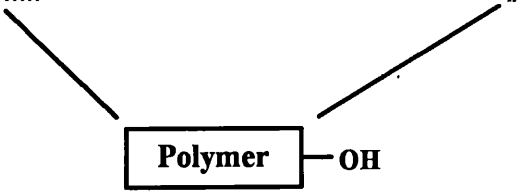
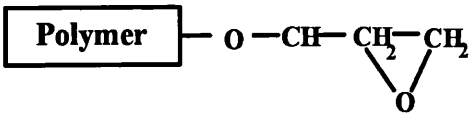
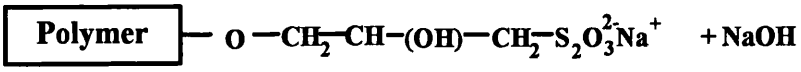


Figure 4.10: The synthesis and activation of thiol agarose starting from agarose

Epoxide activation using epichlorohydrin (1-choro-2,3-epoxypropane)

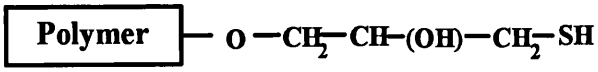


Soidum thiosulphate ($Na_2^+S_2O_3^{2-}$) + H_2O



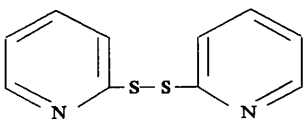
Dithiothreitol

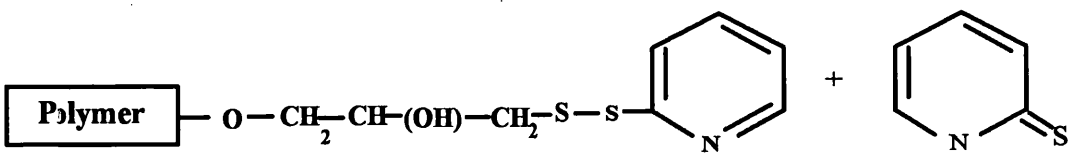
[$HS\,CH_2\,CH(OH)\,CH_2SH$]



Thiol agarose

Dipyridyl disulphide





DPDS-activated thiol agarose

Pyridine-2-thione

It was calculated that 6% of the cysteine did not bind, giving an efficiency of 94% at a high loading capacity (90%) (*Figure 4.11*). The presence of cystine in the cysteine solution may have contributed towards the small inefficiency of the column. Cysteine was used to test the efficiency of the thiol-agarose because it is analagous to the thiol-containing protein samples that were being investigated.

Enough activated thiol-agarose was made so that it could be freshly used each time a column was required. The gel was not regenerated and was only used once.

4.1.4 Modification of NAD with Traut's reagent

A number of different attempts were made to modify NAD using Traut's reagent. The reaction was first carried out at 24°C and at pH8.0 with a reaction volume of 1ml, and with various excesses of Traut's reagent. The reaction was monitored in a Radiometer pH-stat to ensure that there were no changes in pH, and the thiolation of NAD was followed by monitoring the increase in free thiol groups due to the reaction of Traut's reagent with the primary amine of NAD.

According to this analysis only 0.85% of the NAD molecules were modified when the Traut's reagent was present in a 10-fold molar excess over NAD concentration (*Figure 4.12*). The rate of the reaction was only slightly above the rate of hydrolysis of Traut's reagent alone in solution. A series of experiments was then carried out in an attempt to improve the reaction:

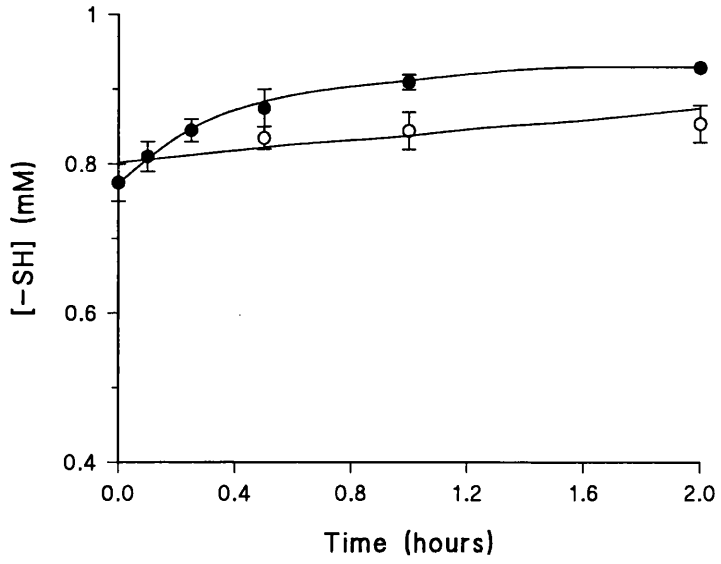


Figure 4.12: *The reaction of Traut's reagent with NAD:* ● = NAD added (6μmol/ml); O = NAD absent. Error bars represent range of error for five separate experiments. The reaction was carried out at 24°C in 1ml of TEA, 50mM, pH 8.0. Traut's reagent was added to 60μmol ml^{-1} and the reaction was monitored by following the release of free sulphydryl groups upon the reaction of Traut's reagent with primary amine groups of NAD using Ellman's reagent as follows: A 0.2ml sample of the reaction mixture was added to 1.6ml of phosphate buffer, 0.1M, pH8.0 and 0.2ml of Ellman's reagent ($4mgml^{-1}$) in a cuvette and mixed thoroughly. After 15mins the absorbance of the solution at 412nm was recorded and the concentration of sulphydryl groups calculated from a calibration curve.

1. The excess of Traut's reagent in the reaction mixture was increased from 10-fold to 20-fold, 100-fold and 500-fold molar excess over NAD concentration in an attempt to improve NAD modification by mass action.

2. The pH of the reaction was varied in an attempt to improve the nucleophilicity of the amine group: the reaction was attempted at pH7.0, pH8.5 and pH10.

3. The volume of the reaction was decreased to 100μl and carried out with a 10-fold excess of Traut's reagent over NAD concentration at pH8.0.

4. The reaction was carried out at 4°C and left for up to 96 hours in attempt to slow the natural hydrolysis of Traut's reagent so that more of it was available to react with NAD for a longer period of time.

Despite the attempts described above the thiolation of more than 0.85% of the NAD molecules could not be achieved.

The small fraction of putatative thiolated NAD was isolated from excess Traut's reagent by purifying the nucleotide material by precipitation in ethanol, followed by centrifugation and thorough washing of the nucleotide pellet. (Traut's reagent is soluble in chilled ethanol). This process was repeated several times in order to remove all traces of unreacted Traut's reagent. At this stage the molar ratio of thiol groups to NAD was determined to be 0.6%. The sample was then passed down an activated thiol-agarose column (*Figure 4.13*).

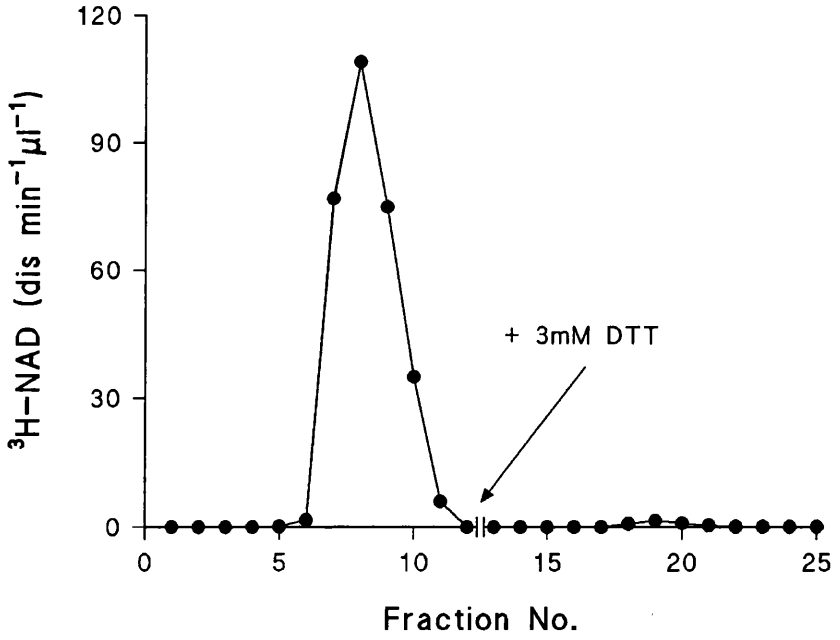


Figure 4.13: *The binding of thiolated ^{3}H-NAD to an activated thiol agarose column:*
0.5ml of a solution containing 40µmol NAD (1µmol of putatative thiolated NAD) was added to a gel of capacity 4.5µmol. Bed volume = 5ml, flow rate = 10ml hr^{-1}, fraction volume = 0.5ml, running buffer = phosphate, pH 8.0, 50mM. The NAD sample added to the column contained 400,000 cpm/mg. Fractions were collected in a dropwise manner using an automated fraction collector and the radiactive counts present in each sample measured as follows: 200µl of each fraction was mixed with 2ml Optiphase "Safe" scintillant in a scintillation vial and ^{3}H-NAD counts measured in a LKB scintillation counter for 15 minutes in triplicate. The ^{14}C and ^{3}H"windows" were open during counting and counting efficiency was calculated as 60% for ^{3}H and 98% for ^{14}C by calculating the effciency of the machine when counting a dated standard sample of ^{14}C or ^{3}H-labelled material. The counts per minute were adjusted to give the disentegrations per minute (dis/min).

Only a tiny fraction (<0.5%) of the ^{3}H-NAD was eluted upon addition of DTT and so the 0.85% conversion of NAD observed might have been due to residual thiol from the Traut's reagent.

To confirm that the N^{6} amine of NAD was unreactive with Traut's reagent, the reaction was also attempted using ^{3}H-adenosine under the various reaction conditions listed above. No thiolation of ^{3}H-adenosine occured. It was therefore concluded that an adenine amine group was unreactive with Traut's reagent.

The poor reactivity of NAD with Traut's reagent was disappointing considering that the amine of NAD showed reactivity with both the glutaraldehyde and carbodiimide-activated carboxyl groups described in the previous chapter.

4.1.5 Modification of N^{6}AHCM-NAD with Traut's reagent

The amine of N^{6}AHCM-NAD appeared to be reactive with a 20-fold molar excess of Traut's reagent at pH 8.0 and at 24°C. Attempts were made to improve the reactivity of the amine group by increasing the pH from pH 8.0 to pH 9.0 but it was surprisingly slightly less reactive at the higher pH (*Figure 4.14*). It was calculated that 33% of N^{6}AHCM-NAD reacted at pH 8.0 and 26% at pH 9.0.

Traut's reagent was removed from the nucleotide preparation by ethanol extraction. A control was run to ensure complete removal of unreacted Traut's reagent from the nucleotide material as follows: A solution containing NAD and Traut's reagent was made up, the volume and concentrations corresponding to the N^{6}AHCM-NAD reaction mixture. Glycine was then added in a 4-fold molar excess of Traut's reagent, and left for 90mins to form the sulphydryl groups. The NAD was precipitated in chilled ethanol, centrifuged at 30,000g and -5°C, and the pellet washed with ethanol. The

washing procedure was repeated until no free thiol groups could be detected by Ellman's reagent.

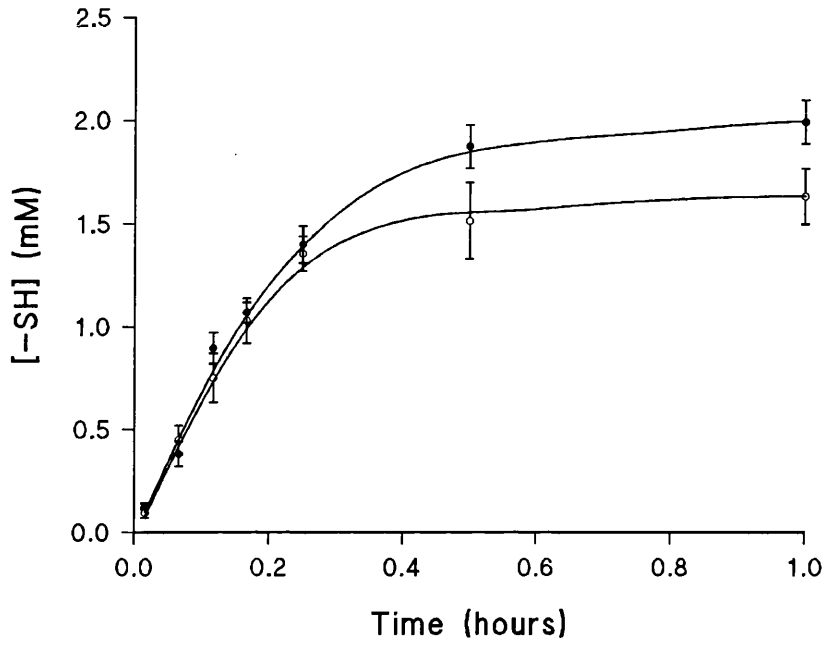


Figure 4.14: *The reaction of Traut's reagent with N^6AHCM-NAD at pH8.0 (•) and pH 9.0 (O)* The reaction was carried out at 4°C in 1ml of 50mM TEA, pH 8.0 containing 6μmol N^6AHCM-NAD. Traut's reagent was added to 120μmol ml^{-1} and the reaction was monitored by following the release of free sulphydryl groups upon the reaction of Traut's reagent with primary amine groups of N^6AHCM-NAD using Ellman's reagent as follows: A 0.2ml sample of the reaction mixture was added to 1.6ml of phosphate buffer, 0.1M, pH8.0 and 0.2ml of Ellman's reagent ($4mgml^{-1}$) in a cuvette and mixed thoroughly. After 15mins the absorbance of the solution at 412nm was recorded and the concentration of sulphydryl groups calculated from a calibration curve.

After purification, the molar ratio of sulphydryl groups to N^6AHCM-NAD was measured using Ellman's reagent. Assuming the molar extinction coefficient of N^6AHCM-NAD to be 21,000 $cm^{-1}M^{-1}$, the ratio was 0.37.

In order to confirm accurately the amount of thiolated coenzyme that had been prepared, ^{3}H-N^6AHCM-NAD was synthesised starting from ^{3}H-NAD, as described in *section 3.2.1.* The tritium atom was attached to the C-2 of the adenine ring and so remained unaffected during the preparation. The final preparation contained

radioactive counts of 20,800 dpm per mg of derivative. ^{3}H-N^{6}AHCM-NAD was modified with Traut's reagent as described above and a sample added to a DPDS-activated thiol-agarose column (*Figure 4.15*).

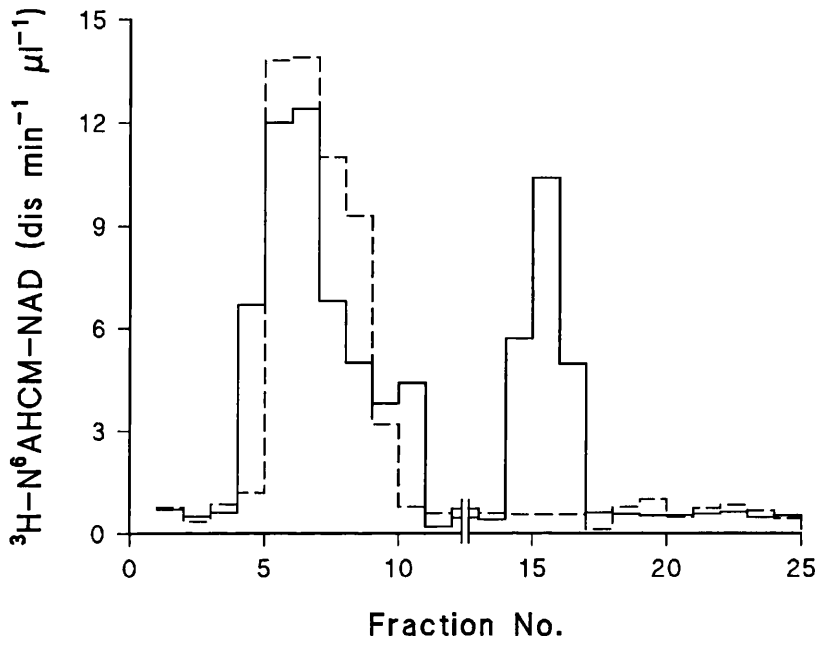


Figure 4.15 *The binding of thiolated ^{3}H-N^{6}AHCM-NAD to DPDS-activated thiol agarose:*

— = binding of unthiolated ^{3}H-N^{6}AHCM-NAD. A 0.5ml sample of thiolated derivative was added to the column (6 mg ml^{-1};20,800 dpm mg^{-1}) of capacity 4.5µmol. Axis break represents addition of a solution of 3mM DTT. Bed volume = 5ml, flow rate = 10ml hr^{-1}, fraction volume = 0.5ml, running buffer = 50mM phosphate, pH 8.0, column run at 25°C. Fractions were collected in a dropwise manner using an automated fraction collector and the radioactive counts present in each sample measured as follows: 200µl of each fraction were mixed with 2ml Optiphase "Safe" scintillant in a scintillation vial and ^{3}H-NAD counts measured in a LKB scintillation counter for 15 minutes in triplicate. The ^{14}-C and ^{3}-H "windows" were open during counting and counting efficiency was calculated as 60% for ^{3}H and 98% for ^{14}C by calculating the efficiency of the machine when counting a dated standard sample of ^{14}C- or ^{3}H-labelled material. The counts per minute were than adjusted to give the disentegrations per minute (dis/min).

28% of ^{3}H-N^{6}AHCM-NAD bound to the column, and was eluted with DTT. This figure compares well to the ratio of sulphydryl groups to coenzyme molecules determined above (37%) and the percentage of derivative that reacted with Traut's reagent according to the concentration of free thiol groups released (33%). The efficiency of the column (94%) may account for the binding being slightly lower than would be expected if 37% of the coenzyme molecules were thiolated.

The substrate activity contained in each fraction eluted from the DPDS-activated thiol-agarose column was measured using the Nisselbaum-Green recycling assay with alcohol dehydrogenase (*Figure 4.16*), bearing in mind that N^{6}AHCM-NAD had 58% of the substrate activity of NAD in this assay (*Table 3.3*).

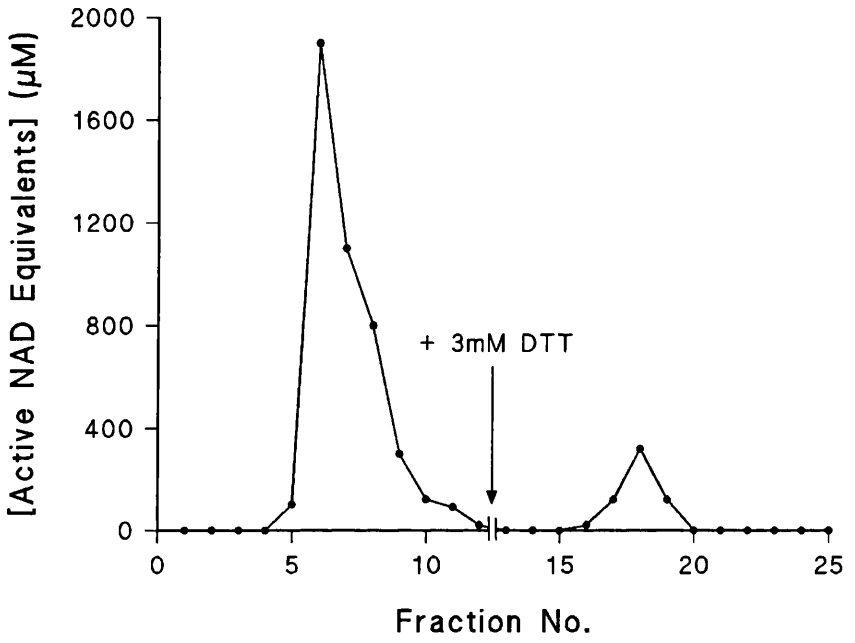


Figure 4.16: *The binding of thiolated N^{6}AHCM-NAD to a DPDS-activated thiol agarose column measured in terms of its substrate activity:*

A 0.5ml sample of thiolated derivative containing 4mg N^{6}AHCM-NAD was added to the column of capacity 4.5μmol. Bed volume = 5ml, flow rate = 10ml hr^{-1}, fraction volume = 0.5ml, running buffer = 50mM phosphate, pH 8.0, column run at 24°C. Fractions were collected in a dropwise manner using an automated fraction collector, and analysed for coenzyme activity using the Nisselbaum-Green recycling assay for NAD as follows: The cycling experiment was carried out at pH 7.4 using a MTT/PES redox couple with yeast alcohol dehydrogenase. To 1.95ml 100mM TEA/ 100mM nicotinamide/ 500mM ethanol buffer, was added 100μl of diluted coenzyme sample. 50μl of 12mM MTT, 800μl of 3mM PES and 100μl of yeast alcohol dehydrogenase (30μgml^{-1}) were also added. The reaction was monitored by following the change in absorbance at 556nm over 10mins.

If the substrate activity of N^{6}AHCM-NAD was unaffected after modification with Traut's reagent, then the portion of NAD that bound to the column might be expected to contain 28% of the activity of the total nucleotide preparation applied to the column (because this was the percentage of ^{3}H-N^{6}AHCM-NAD that bound to the column). However, the portion of nucleotide that bound to the column contained only 11.5% of the total substrate activity of the nucleotide material. This low activity, which was 53%

of the expected activity, could either be due to the presence of NAD, a more active substrate for alcohol dehydrogenase than N^6AHCM-NAD, in the N^6AHCM-NAD preparation or it could indicate a difference in activity between N^6AHCM-NAD and its thiolated derivative. In order to determine which was the case, the thiolated NAD derivative was precipitated in ethanol, centrifuged, dissolved in phosphate buffer and freeze-dried. The substrate activity was 55% of the normal activiy of N^6AHCM-NAD, here determined with ADH in an MTT/PES recycling assay. The molar ratio of sulphydryl groups to N^6AHCM-NAD could not be determined accurately at this stage because traces of DTT were still present in the preparation, and there was not enough thiolated derivative left to re-precipitate in ethanol to remove residual DTT.

Further evidence for the derivatisation of N^6AHCM-NAD with Traut's reagent was provided using thin layer chromatography.

Thin layer chromatography (TLC) of thiolated N^6AHCM-NAD

After Traut's reagent was removed from a HS-N^6AHCM-NAD preparation (see above), the nucleotide material was analysed using TLC.

Considering the net charge of each derivative, the R_f values follow the pattern that might be expected from past TLC results of N^6-derivatives of NAD (Lindberg *et al*, 1973). Other factors may have also influenced the final R_f values, such as molecular size and shape, and charge distribution. At the pH of the solvent (pH 5.0) N^6AHCM-NAD will possess a net charge of 0, but the thiol-modified derivative will have a single net negative charge because the protonated amine is no longer present and the Traut's molecule does not introduce an overall charge.

Table 4.1
Thin layer chromatography of a thiolated N^6AHCM-NAD derivative

	R_f values in chromatography systems	
Coenzyme	0.1M LiCl	0.5M LiCl
N^6AHCM-NAD	>0.90[a]	>0.90
HS-N^6AHCM-NAD	0.67±0.05	0.70±0.03

[a] R_f values above 0.90 could not be recorded accurately due to spot diffusion
TLC was performed on PEI-cellulose coated plates at room temperature. 10µl of each coenzyme (1 mgml) was spotted onto a plastic sheet pre-coated with PEI-cellulose using a 10µl capillary tube, 1cm from the labelled bottom of the sheet. The sheet was placed upright in a glass tank containing lithium chloride solution to a height of 0.5cm. After the solvent front had reached 2-4cm from the top of the sheet, the sheet was removed from the tank and dried using a stream of cool air. The sheet was then analysed for nucleotide spots under ultra-violet light.

4.1.6 Thiolation of N^6CM-NAD by carbodiimide-mediated addition of 2-aminoethanethiol

The conditions for the reaction of N^6CM-NAD with 2-aminoethanethiol were based on those described by Lindberg *et al* (1973) for the carbodiimide-mediated modification of N^6CM-NAD with diaminohexane (*Materials and Methods*). 2-Aminoethanethiol was added in excess of N^6CM-NAD in 1.5ml water, and the reaction carried out at pH4.5. The pH of the reaction was kept constant by the addition of hydrochloric acid in a Radiometer pH-stat. The reaction was judged to be complete after 50 minutes, when there was no further change in pH. The reaction was left for a further 60 minutes at room temperature before the thiolated derivative was isolated from unreacted 2-aminoethanethiol by ethanol precipitation under which conditions the thiol reagent is soluble. When no traces of thiol groups could be detected in a control sample containing NAD and 2-aminoethanethiol at corresponding concentrations, the molar ratio of sulphydryl groups to N^6CM-NAD was found to be 0.51 by using Ellman's reagent. Here the assumption is that N^6CM-NAD has an extinction coefficient of 19,000 $M^{-1}cm^{-1}$ at 266nm.

The thiolated derivative was then purified and separated from unreacted N^6CM-NAD and traces of carbodiimide by using a DPDS-activated thiol agarose column. In order to determine accurately the amount of thiolated N^6CM-NAD, ^{3}H-N^6CM-NAD was synthesised starting from ^{3}H-NAD (*section 3.2.1*) and a product containing 35,000dpm per mg of final derivative was obtained. A solution containing 2.0mg of the latter was added to the column (*Figure 4.17*), of which 42% bound. The sample applied to the column had a ratio of thiol groups to coenzyme of 0.51 and it was therefore concluded that most of the modified coenzyme molecules contained one sulphydryl group each. If, for example, most of the modified coenzyme preparation had passed straight through the PDS-thiol agarose column, then it would have been concluded that a minority of the coenzyme molecules contained most of the thiol groups.

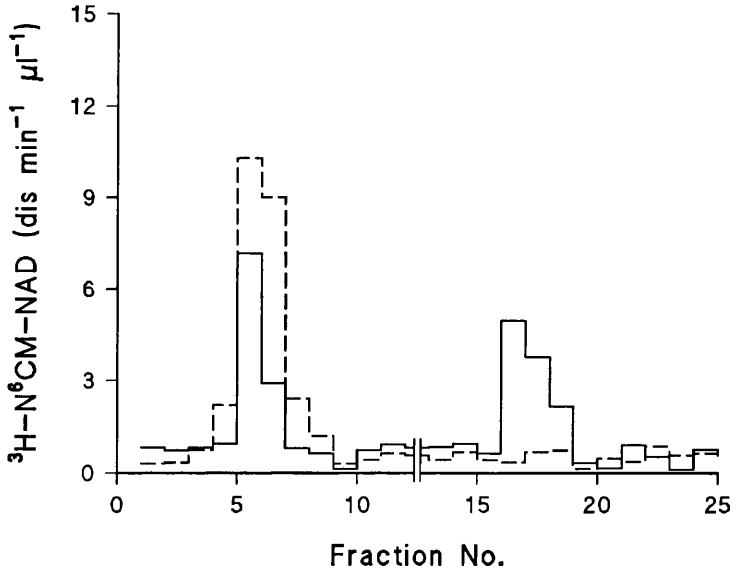


***Figure 4.17** The binding of thiolated ^{3}H-N^6CM-NAD to activated thiol agarose:*
— = binding of unthiolated ^{3}H-N^6CM-NAD. A 0.5ml sample of thiolated derivative was added to the column (3 mg ml^{-1};35,000 dpm mg^{-1}) of capacity 4.5µmol. Axis break represents addition of a solution of 3mM DTT. Bed volume = 5ml, flow rate = 10ml hr^{-1}, fraction volume = 0.5ml, running buffer = 50mM phosphate, pH 8.0, column run at 24°C. Fractions were collected in a dropwise manner using an automated fraction collector and the radioactive counts present in each sample measured as follows: 200µl of each fraction were mixed with 2ml Optiphase "Safe" scintillant in a scintillation vial and ^{3}H-NAD counts measured in a LKB scintillation counter for 15 minutes in triplicate. The ^{14}C and ^{3}H "windows" were open during counting and counting efficiency was calculated as 60% for ^{3}H and 98% for ^{14}C by calculating the efficiency of the machine when counting a dated standard sample of ^{14}C- or ^{3}H-labelled material. The counts per minute were than adjusted to give the disentegrations per minute (dis/min).

The thiolated N^6CM-NAD derivative was isolated from DTT by repeated precipitation in ethanol; it was then centrifuged and then freeze dried in phosphate buffer. The ratio of sulphydryl groups to N^6CM-NAD in this final preparation was 0.91, confirming the presence of unmodified N^6CM-NAD before purification using the PDS-thiol agarose column. The substrate activity of the pure HS-N^6CM-NAD was also determined, using the Nisselbaum-Green recycling assay, to be 23% of the activity of NAD.

Further evidence for derivatisation of N^6CM-NAD was provided using thin layer chromatography.

Thin layer chromatography of thiolated N^6CM-NAD

After separation of the thiolated nucleotide preparation from all traces of 2-aminoethanethiol, the nucleotide material was subjected to analysis by TLC.

Table 4.2
Thin layer chromatography of a thiolated N^6AHCM-NAD derivative

	R_f values in chromatography systems	
Coenzyme	0.1M LiCl	0.5M LiCl
N^6CM-NAD	0.18±0.01	0.82±0.03
HS-N^6CM-NAD	0.51±0.05	0.15±0.04

TLC was performed on PEI-cellulose coated plates at room temperature. 10µl of each coenzyme (1 mgml) was spotted onto a plastic sheet pre-coated with PEI-cellulose using a 10µl capillary tube, 1cm from the labelled bottom of the sheet. The sheet was placed upright in a glass tank containing lithium chloride solution to a height of 0.5cm. After the solvent front had reached 2-4cm from the top of the sheet, the sheet was removed from the tank and dried using a stream of cool air. The sheet was then analysed for nucleotide spots under ultra-violet light.

Considering the net charge of each derivative, the R_f values were as expected from past TLC of nucleotides (Lindberg *et al*, 1973). At the pH of the solvent (pH5.0) N^6CM-NAD will have a net charge of -2, but the SH modified derivative will have

only a single negative charge (-1) because the acidic carboxyl group is no longer present and the 2-aminoethanethiol molecule does not introduce charge overall.

A comparison of the properties of each of the thiolated NAD derivatives is shown in *Table 4.3*. NAD was not used in conjugation experiments with proteins because of its poor reactivity with Traut's reagent.

Table 4.3
Summary of characteristics of thiolated NAD derivatives

Coenzyme	Conversion[a] (%)	% Bound to activated thiol agarose column	Substrate activity with ADH (% of NAD)
HS-NAD[b]	0.60[c]	< 0.5	--
HS-N^{6}CM-NAD[d]	51	42	23
HS-N^{6}AHCM-NAD[b]	37	28	47

a Percentage conversion from original unthiolated coenzyme. This figure was calculated by determining the ratio of sulphydryl groups to coenzyme molecules, after purification, with Ellman's reagent.

b Thiolated using Traut's reagent

c Could not be confirmed

d Thiolated using carbodiimide and 2-aminoethanethiol

4.2 The preparation of proteins for oxidative dimerisation with a thiolated coenzyme adduct

4.2.1 Considerations for the modification of proteins by a thiolated coenzyme adduct

If an enzyme-coenzyme conjugate formed via a disulphide linkage is to have intrinsic activity it is important that the constituent enzyme is not seriously inhibited by covalent modification of its surface thiol groups. Relevant experiments were carried out, as will be described, but first the number of thiols inherently available in native BSA, our protein model, and in native LDH will be considered along with a brief consideration of the relative properties of dehydrogenases in general.

4.2.1.1 Bovine serum albumin (BSA)

The molecular weight of BSA is 66,000. It has been widely reported that there is only one accessible thiol group in BSA, Cys 34 (Shaw *et al*, 1984) despite there being 26 cysteine groups in the primary sequence of BSA reported by Dayhoff (1976). Most of these cysteine groups are presumed to be buried in the interior of the protein structure or involved in disulphide bond interactions. Only one case is known in which NAD has been covalently attached to the surface of a BSA molecule (Wahl and Chang, 1987). Carbodiimide coupling was employed to create a peptide bond between the carboxyl surface residues of BSA and the NAD derivative N^6AHCM-NAD. An active preparation of immobilised NAD was obtained, although the conjugate was not well characterised.

Introduction of extra thiol groups onto the surface of BSA

There are 61 lysyl residues in BSA (Dayhoff, 1976), of which at least 30 are available for modification by a bifunctional reagent (Pierce Chemical Company, 1990), and so the introduction of thiol groups onto the protein surface by Traut's reagent should prove feasible.

4.2.1.2 LDH

The pig heart (H_4) isoenzyme of LDH was used and its general properties are described in *Section 3.10*. Pig H_4 LDH contains 5 cystyl residues. In the dogfish muscle isoenzyme there are 7 cystyl residues of which none were reported to be either accessible or partially accessible in a study carried out by Abad-Zapatero *et al* (1987). The function of the cystyl residues of LDH is not well understood (Klitz *et al*, 1977). The pig H_4 isoenzyme of LDH is unique among LDHs in that the essential thiol, Cys 165, reacts with maleimide while the enzyme is in its native state, whereas in other

cases it must be denatured first (Holbrook *et al*, 1975). However, protection from modification by the presence of coenzyme was reported. Indeed, modification of pig H_4 LDH by para-mercuribenzoate was completely prevented in the presence of coenzyme (Holbrook *et al*, 1975).

The essential cysteine, Cys 165, is 1.0nm from the substrate binding site and is accessible only from the active centre. Any molecule modifying this thiol will be situated between Asp 168 and His 195, preventing movement of His 195 towards the active centre and therefore preventing the formation of a hydrogen bond from the imidazole of His 195 to lactate or pyruvate, which is essential for substrate stabilisation during catalysis. However, upon coenzyme binding the coenzyme binding loop is lowered, introducing a molecular barrier, so that Cys 165 will be inaccessible to an approaching molecule. The peptide around the essential cysteine of LDH was sequenced (Holbrook and Stinson, 1970) and was found to be homologous to sequences found in YADH, LADH and G-3-PDH for which a similar scenario might be expected.

Introduction of extra thiol groups onto the surface of LDH

As reported in *Chapter 3*, 24 lysines are present in pig H_4 LDH isoenzyme. 83% of these residues showed some degree of accessibility in the dogfish muscle isoenzyme, and so the introduction of thiol groups by Traut's reagent should prove feasible.

4.2.1.3 Other dehydrogenases

ADH contains 8-9 cysteine groups per subunit and is seriously inhibited by thiol reagents or thiol oxidation (Branden *et al*, 1975). Consequently, an approach involving the binding of a thiolated NAD adduct would not be suitable for LADH or YADH. The strong inhibitiory effect is due to modification of the two essential cystyl residues that bind to the catalytic zinc atom. No protection from inhibition is afforded by coenzyme. Conversely, any inhibitory effects observed in GDH due to thiol

modification are prevented by the presence of coenzyme (Smith *et al*, 1975). Whether the inhibition is due to a catalytic role of one or more cystyl residues is dubious but it may, instead, be due to protein unfolding caused by modification of cystiene thiol groups. Maleimide modification does not result in inhibition, even in the absence of coenzyme, thus illustrating that the extent of inhibition is largely dependent on the thiol reagent used (Smith *et al*, 1975). In bovine liver GDH, the enzyme used in our study, there are no reactive cystyl residues present i.e. none are accessible to solvated reagents. This is probably due to steric hindrance or hydrophobic protection caused by the surrounding enzyme structure (Rasched and Bayne, 1982). Bovine liver GDH contains 33 lysyl residues (see *section 3.11.5*), and so there should be several accessible amine groups that are available to react with Traut's reagent on the surface of GDH.

Malate dehydrogenase (MDH) was also used in our thiol modification studies. The overall function and NAD-binding mechanism of MDH is similar in many ways to LDH (Roderick and Banaszak, 1986). The enzyme from rat mitochondria was used, and this is also similar to pig H_4 LDH which contains 7 cystyl and 24 lysyl residues, in that it contains 8 cystyl residues and 26 lysyl residues. The thiol modification of, and the NAD conjugates formed by MDH and LDH in this study, may therefore be expected to be similar.

In summary, previous studies suggest that the introduction of thiol-containing reagents to LDH, GDH and perhaps MDH should be feasible, without a serious loss in activity due to thiol modification.

In order to investigate whether or not covalent thiol modification inhibits native dehydrogenases, and to what extent, various dehydrogenases were modified with a thiol reagent in the following study.

4.2.2 The effect of dipyridyldisulphide (DPDS) upon the activity of various dehydrogenases

Dipyridyldisulphide (DPDS) is very reactive with free thiol groups and so can be used in cysteine modification studies. Upon reaction with cysteine, DPDS introduces a bulky thiopyridine group (*Figure 4.18*) and so any steric hindrance or detrimental hydrophobic effects that a bifunctional reagent might cause should become apparent upon modification with DPDS.

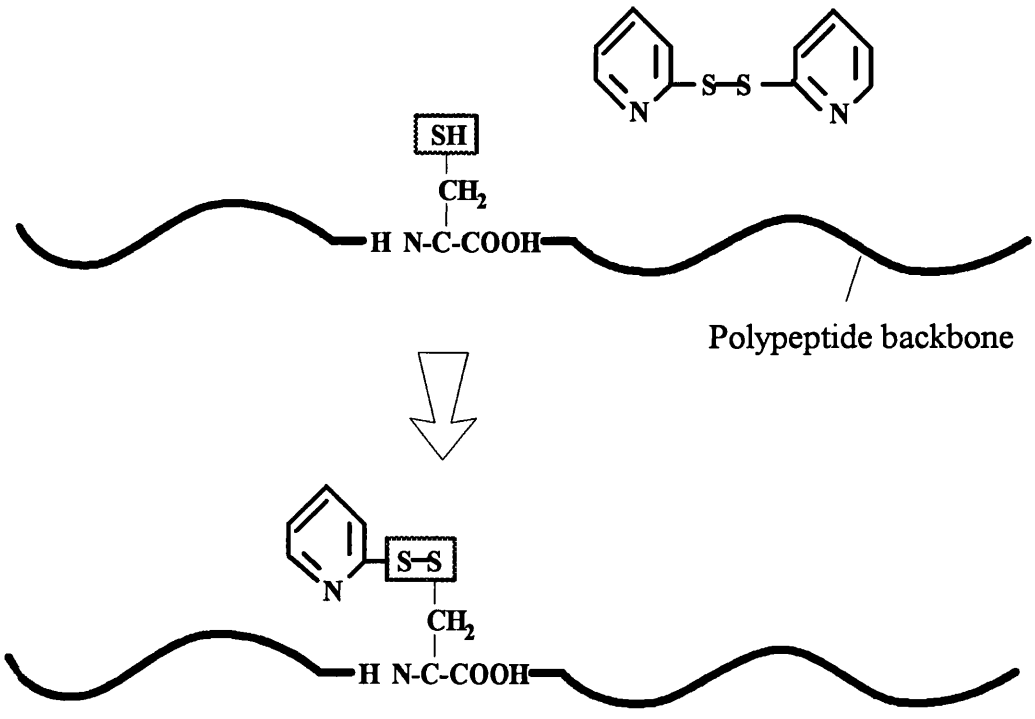


Figure 4.18: *Modification and activation of cystyl residues with DPDS*

In order to solubulise DPDS, a small amount of acetone is required in an aqueous solution. Because acetone is known to react with amine groups (Lundblad and Noyes, 1984) a corresponding amount of acetone was added to a control enzyme sample to ensure that any inhibition was not caused by reaction with acetone. No inhibitory

effects were observed upon any of the dehydrogenases tested. It must also be noted that the coenzyme was not added in any of the DPDS-modification experiments, and so no protection of sensitive active site residues was afforded by its presence.

The effect of various concentrations of DPDS upon the activity of LDH, GDH, YADH, LADH, and MDH was investigated. Various volumes (0-200µl) of a 1mgml^{-1} solution of DPDS in 50% (v/v) acetone:water were added to a 1ml solution containing 140µgml^{-1} of dehydrogenase, mixed and left at room temperature for 60mins before extensive dialysis. Each enzyme was then assayed for specific activity using a standard assay at 340nm, to detect the reduction or increase in the concentration of NADH.

As was expected thiol modification of ADH resulted in strong inhibitory effects, especially at larger excesses of DPDS (*Figures 4.19 & 4.20*). This was probably due to the modification of cystyl residues bound to the catalytic zinc.

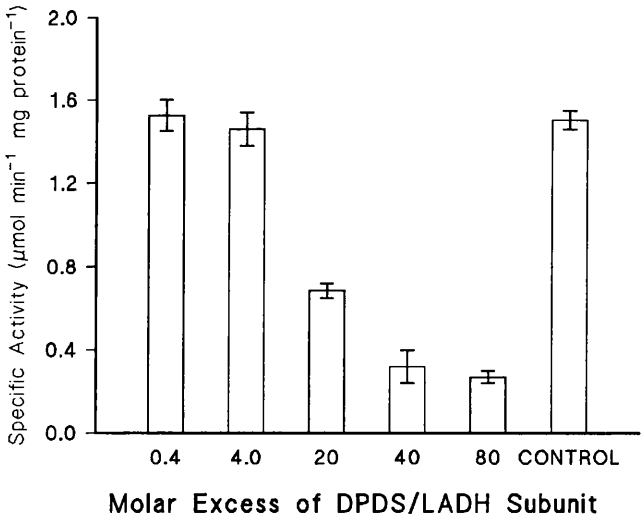


***Figure 4.29**: The effect of excess DPDS on the specific activity of LADH.* The specific activity of the enzyme was measured as follows: To 2.5 ml of 75mM glycine-sodium pyrophosphate buffer, pH 9.0 were added 0.1ml of 2.2M semicarbazide; 0.1ml of 95% ethanol (v/v); 0.2ml of 28mM NAD; 0.1ml of 0.3M glutathione, and finally 20µl of enzyme sample. The solution was mixed and the rate of the reaction followed by monitoring the increase in absorbance at 340nm.

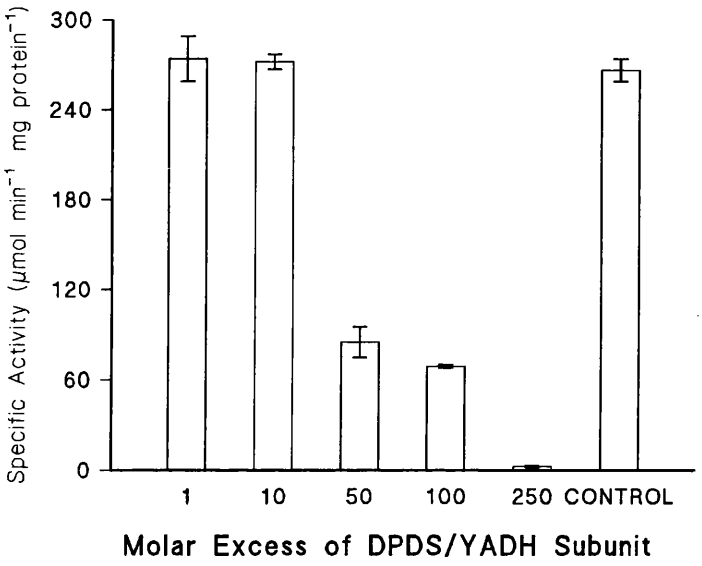


***Figure 4.20**: The effect of excess DPDS on the specific activity of YADH.* The specific activity of the enzyme was measured as follows: To 2.5 ml of 75mM glycine-sodium pyrophosphate buffer, pH 9.0 were added 0.1ml of 2.2M semicarbazide; 0.1ml of 95% ethanol (v/v); 0.2ml of 28mM NAD; 0.1ml of 0.3M glutathione, and finally 20μl of enzyme sample. The solution was mixed and the rate of the reaction followed by monitoring the increase in absorbance at 340nm.

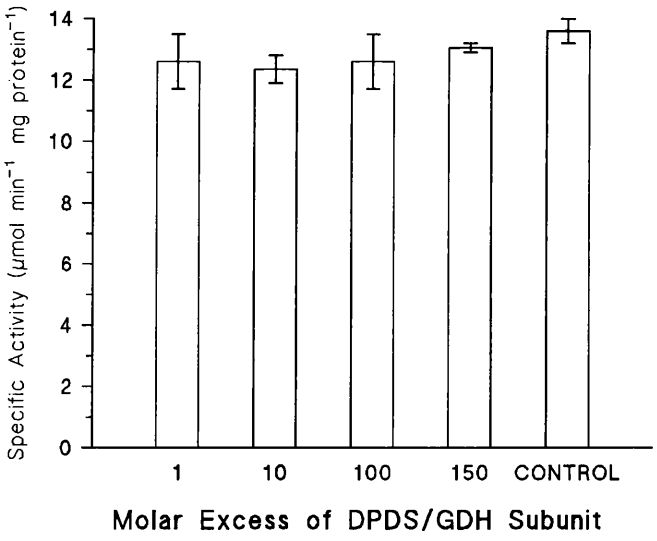


***Figure 4.21**: The effect of excess DPDS on the specific activity of GDH.* The specific activity of the enzyme was measured as follows: To 2.5 ml of Imidazole buffer, 0.1M, pH 7.9 were added 0.2ml of 0.2M oxoglutarate, 0.05ml of 12.8M ammonium acetate, 0.03ml of 12mM NADH, 0.1ml of 26mM EDTA, 0.05ml of 0.1M ADP, and finally 0.02ml of enzyme sample. The solution was mixed and the rate of the reaction followed by monitoring the decrease in absorbance at 340nm.

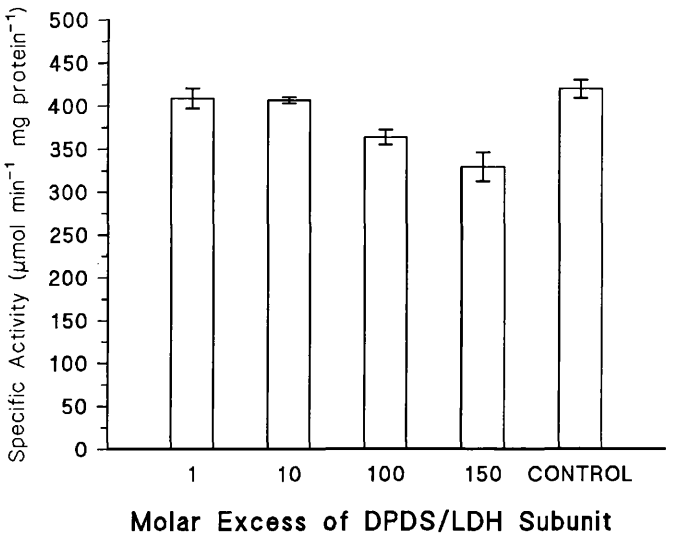


***Figure 4.22**: The effect of excess DPDS on the specific activity of LDH.* The specific activity of the enzyme was then measured as follows: To 2.83 ml of sodium pyrophosphate buffer, 0.1M, pH 7.0 were added 0.1ml of 23mM sodium pyruvate, 0.05ml of 12mM NADH, and 0.02ml of enzyme sample. The solution was mixed and the rate of the reaction followed by monitoring the decrease in absorbance at 340nm.

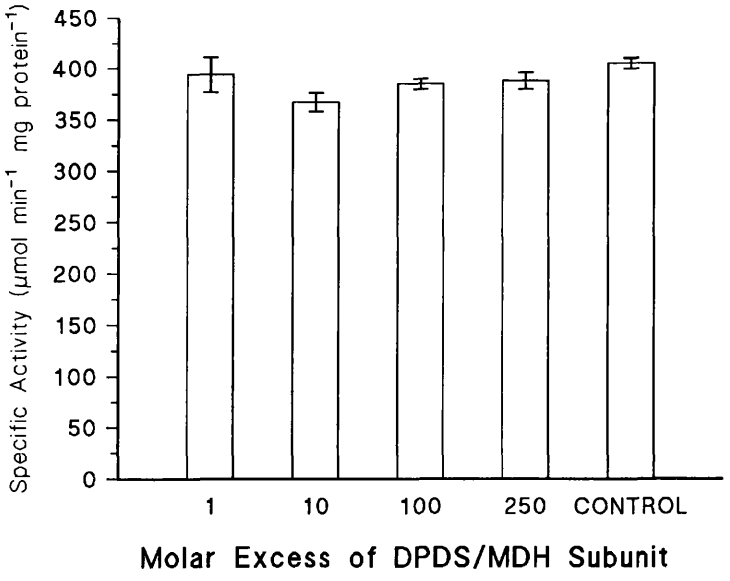


***Figure 4.23**: The effect of excess DPDS on the specific activity of MDH.* The specific activity of the enzyme was measured as follows: To 2.83 ml of 0.1M phosphate buffer, pH 7.5 were added 0.1ml of 15mM oxaloacetate; 0.05ml of 12mM NADH and 0.02ml of enzyme sample. The solution was mixed and the rate of the reaction followed by monitoring the increase in absorbance at 340nm.

However, the results also showed that LDH, GDH and MDH were not seriously inhibited, especially when bearing in mind that any inhibition might be reduced in the presence of coenzyme which acts as a physical barrier to "sensitive" active site residues. Another way, perhaps, in which to reduce any inhibitory effects and also to dramatically improve the incorporation of NAD-SH adducts onto protein surfaces is to introduce thiol groups by modifying surface lysyl residues using Traut's reagent. The presence of extra thiol groups may prevent inhibition by decreasing the molar excess of thiol adduct to sensitive thiol groups.

4.2.3 Modification of proteins with Traut's reagent in order to introduce free sulphydryl groups

Traut's reagent, described in *Section 4.1.1*, was used to modify the surface amine groups of proteins in order to introduce free sulphydryl groups. The protocol of Jue *et al* (1978) was employed, which has been used extensively to modify the proteins of the 30S ribosome of *Escherichia coli*.

4.2.3.1 BSA modification

The reaction of Traut's reagent with BSA was monitored at 248nm to confirm that a reaction occurs between Traut's reagent and protein residues (the intact Traut's molecule has an absorption maximum at 248nm which is lost upon its reaction with amine groups).

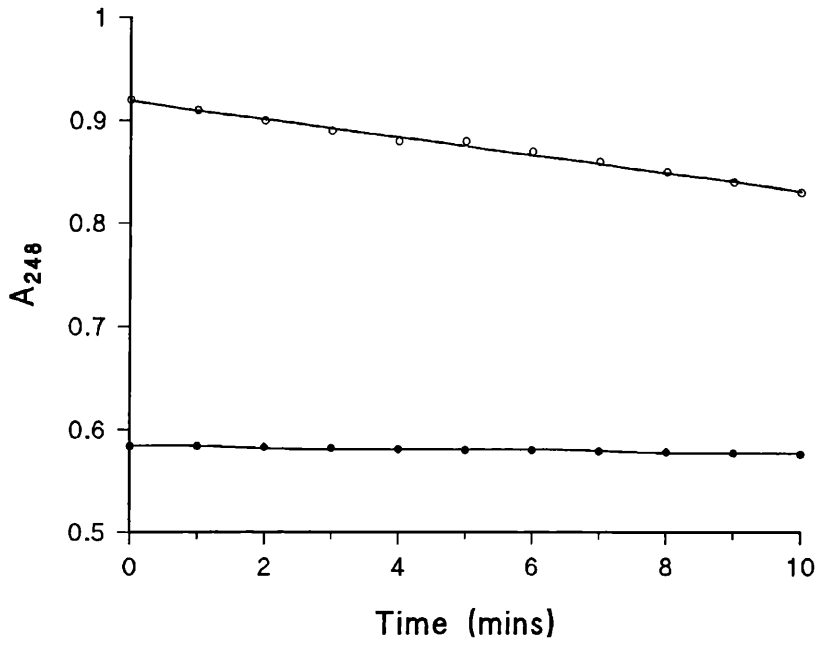


Figure 4.24: *The reaction between Traut's reagent and bovine serum albumin (BSA):*

O = BSA added; ● = BSA absent. To 1ml of 2mgml^{-1} BSA in 50mM TEA buffer, pH8.0, 2mg of Traut's reagent was added. The reaction was monitored for 10mins at room temperature by following the decrease in absrobance at 248nm due to the reaction of Traut's reagent with a primary amine group and the conversion rate calculated knowing that the extinction coefficient of Traut's reagent was 8840cm^{-1}M^{-1} at 248nm.

The reaction rate, calculated from *Figure 4.24*, with a 17:1 molar excess of Traut's reagent over the surface lysine concentration, was 0.6μM/min with respect to the modification of amine groups, assuming that 32 out of 64 lysyl residues are accessible (see below). However, when the same conditions of excess are applied using glycine, the rate is 7.0μM/min. The slower rate of the BSA reaction is probably due to steric hindrance of the approach of Traut's reagent caused by surrounding protein residues. After BSA had been modified it was isolated from unreacted Traut's reagent using a Sephacryl S-100 gel filtration column. As can be seen in *Figure 4.25*, a baseline separation was effected.

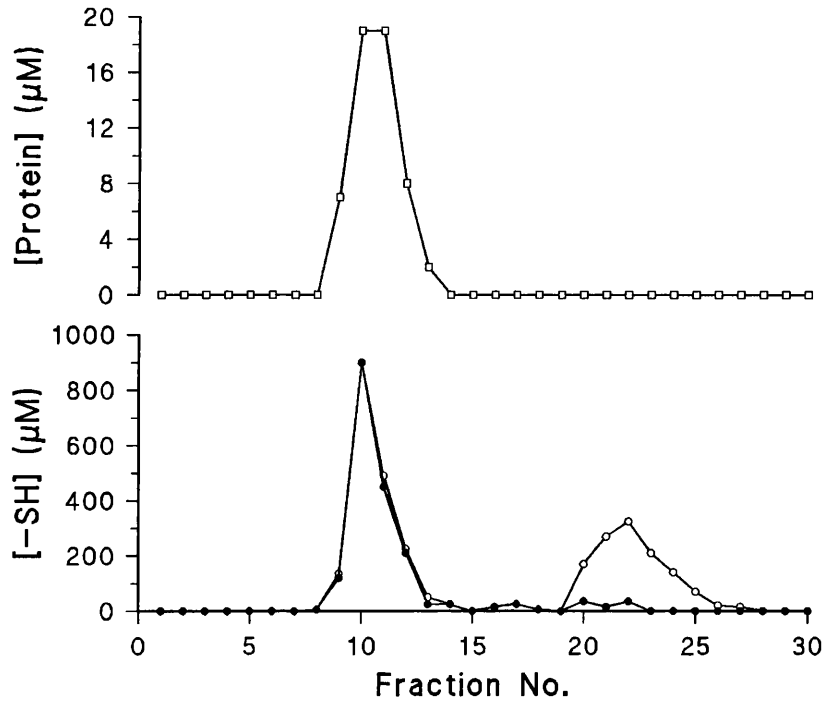


Figure 4.25: *The elution profile of a typical separation of a thiolated BSA preparation from unreacted Traut's reagent by gel filtration chromatography:* □,• = glycine absent; O.= 5μmol glycine added to each fraction.
A Sephacryl S-100 column (60 x 1.5cm) was run at 4°C with a flow rate, of $0.2mlmin^{-1}$. Fractions were collected under gravity in 2.2ml volumes in a dropwise manner using an automated fraction collector. The eluted fractions were taken for analysis to determine the following properties:-
Protein concentration: measured using the Coomassie method. 50μl of each fraction were mixed with 2ml of a 50:50 mixture of Saline:Coomassie Brilliant Blue G and left for 5minutes. The absorbance of the solution was then read at 620nm and the concentration calculated from a standard curve made from BSA solutions of known concentration.
Sulphydryl concentration: A 0.2ml sample of the reaction mixture was added to 1.6ml of 0.1M phosphate buffer, pH8.0 and 0.2ml of Ellman's reagent ($4mgml^{-1}$) in a cuvette and mixed thoroughly. After 15mins the absorbance of the solution at 412nm was recorded and the concentration of sulphydryl groups calculated from a calibration curve.

Evidence for the modification of BSA by Traut's reagent was provided by adding glycine to each fraction eluted from the gel filtration column, effecting a reaction between the primary amine group of glycine and the excess, unreacted Traut's reagent resulting in the formation of sulphydryl groups. By monitoring each fraction for any increase in free sulphydryl group concentration after this reaction, the presence of any unreacted Traut's reagent in the protein-containing fractions was detected. As can be seen in *Figure 4.25*, there was no increase in sulphydryl concentration after the addition of glycine in the protein-containing fractions (first peak) - the Traut's reagent was present in a reacted state because the sulphydryl groups had been formed.

However, in the second peak there was an increase in the concentration of sulphydryl groups resulting from reaction of unreacted Traut's reagent with excess glycine.

The protein-containing fractions were pooled and concentrated to $1mgml^{-1}$ against solid polyethyleneglycol. By measuring the protein concentration using Coomassie reagent and the sulphydryl concentration using Ellman's reagent, a preparation of modified BSA (BSA-[SH]), with 31 sulphydryl groups incorporated per mol of BSA was obtained. Further evidence for the incorporation of free thiol groups onto the surface of BSA was provided by:-

1. Passing the modified protein down an activated thiol agarose column (Section 4.1.3). From *Figure 4.26* it was calculated that 165 nmoles of pyridine-2-thione were displaced from the DPDS-thiol agarose column per mg of thiolated BSA molecule, whereas 27nmol were displaced per mg of native BSA molecule.

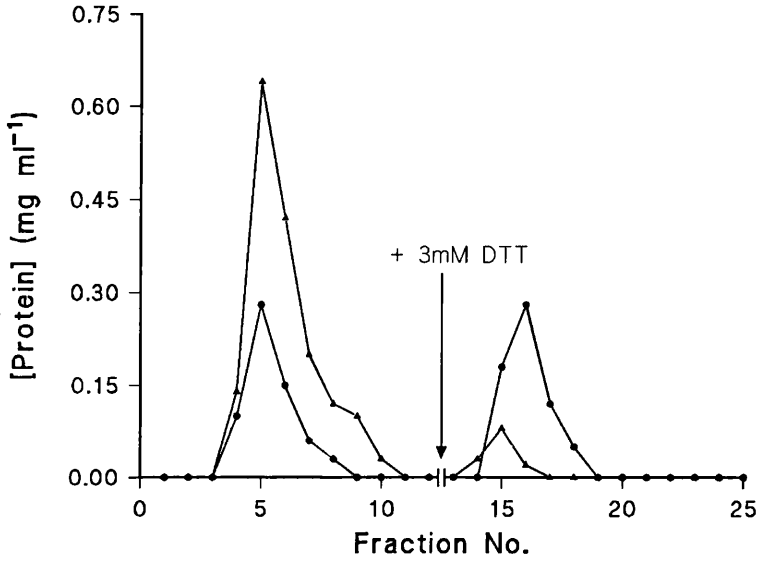


Figure 4.26: *The binding of thiolated BSA to an activated thiol agarose column.*
• = thiolated BSA,▲ = native BSA. 0.5ml of BSA solution (1.75mgml^{-1}) was applied to the column. Bed volume = 5ml, flow rate = 10ml hr^{-1}, fraction volume = 0.5ml, running buffer = phosphate, pH 8.0, 50mM, column run at 24°C. Fractions were collected in a dropwise manner using an automated fraction collector and the protein concentration of each fraction measured using the Coomassie method: 0.05ml of each fraction were mixed with 2ml of a 50:50 mixture of saline:Coomassie Brilliant Blue G and left for 5minutes. The absorbance of the solution was then read at 620nm and the concentration calculated from a standard curve made from BSA solutions of known concentration.

The reaction of all of the 31 sulphydryl groups introduced onto the surface of BSA with activated thiol ligands on the modified agarose column may not have been feasible due to steric limitations imposed by the dimensions of the thiol agarose beads (*Figure 4.27*).

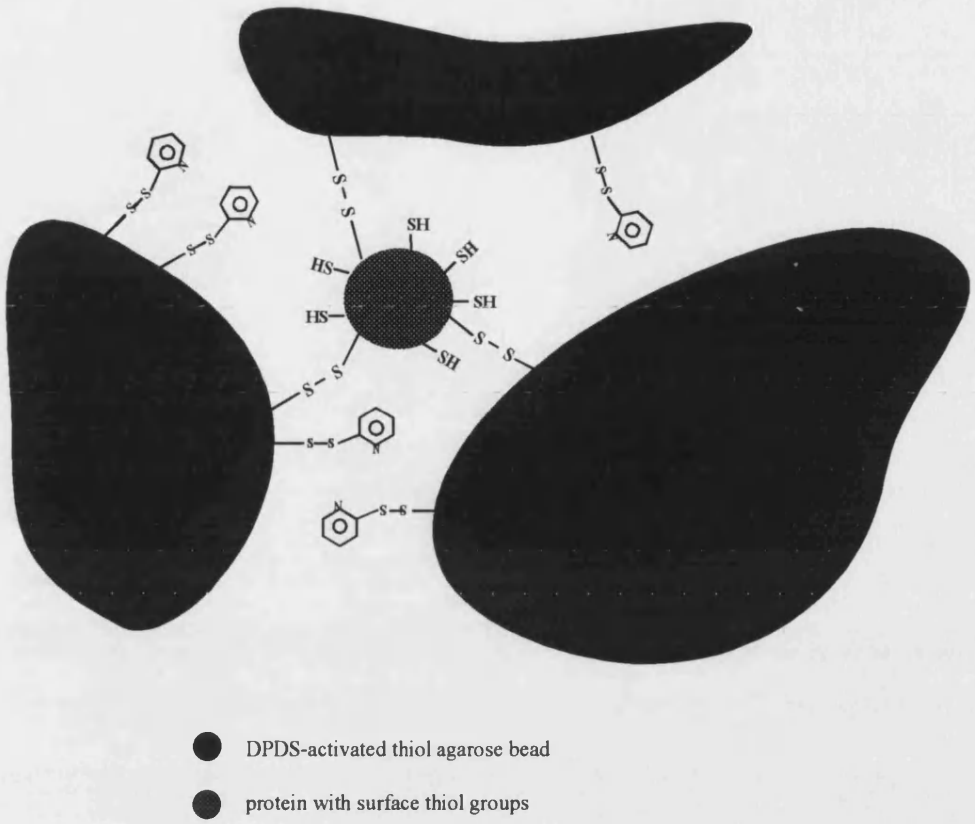


Figure 4.27: *Possible steric limitations of protein-thiol binding to a DPDS-activated thiol-agarose column.* After a few of the protein thiol groups have formed disulphide bridges with the agarose beads, the protein may be held in position so that its remaining thiol groups cannot interact with the DPDS-activated thiol groups of the agarose beads.

The bound BSA was eluted with DTT and the protein-containing fractions pooled. The DTT was removed by gel filtration and the protein material concentrated and analysed as described above. There were, on average, 31 sulphydryl groups per BSA molecule indicating that, because the number of thiol groups did not increase after fresh reduction with DTT, the conditions used for protein purification and analysis were not conducive to the oxidation of free thiol groups.

2. Reaction of thiolated BSA with DPDS. This was attempted for two reasons: firstly, to qualitatively confirm the incorporation of sulphydryl groups into BSA by monitoring the increase in absorbance at 343nm due to the release of pyridine-2-thione, and secondly, to activate the protein for conjugation with thiolated coenzyme adducts in

the same way that thiol agarose was activated to react with thiolated NAD adducts (*Figure 4.28*).

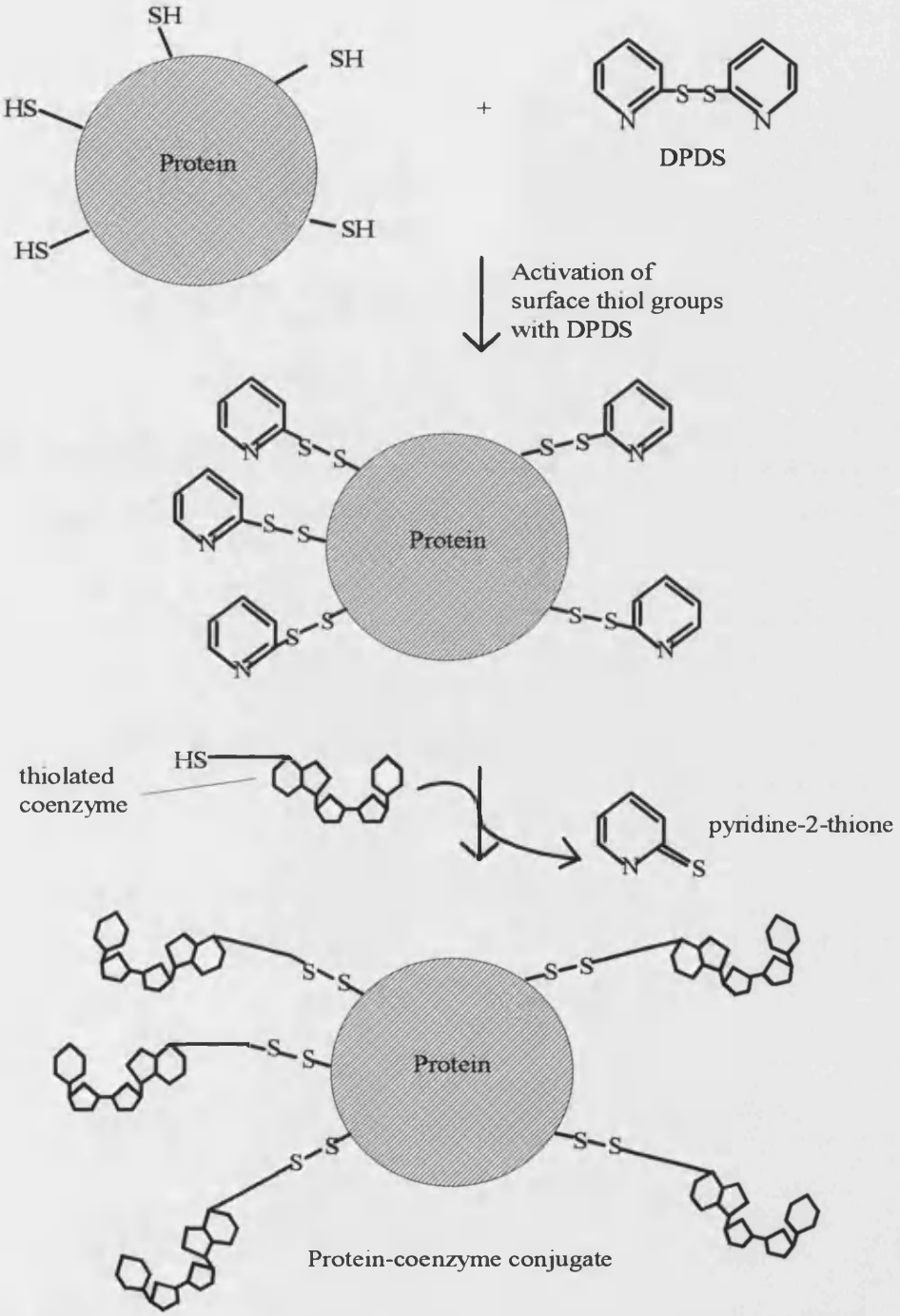


***Figure 4.28**: Scheme for activation of protein surfaces for thiol dimerisation with thiol-containing coenzyme adducts - the "disulphide" method of protein-coenzyme conjugate formation*

Activation of protein thiol groups with DPDS would reduce the possibility of cross-linking between thiolated coenzyme derivatives and intramolecular cross-linking

between BSA thiol groups caused by the oxidising conditions that would, otherwise, be required. Activation would also make conjugation under mild conditions possible, without the introduction of strong oxidising conditions detrimental to proteins and enzymes. It was calculated that 28 moles of free sulphydryl groups were present per mole of thiolated BSA molecule compared to 31 moles detected by Ellman's reagent and only 1.0 mole of thiol groups was present per mole of freshly reduced native BSA.compared to 1.8 moles detected by Ellman's reagent.

The effect of various excess of Traut's reagent upon the incorporation of free sulphydryl groups into BSA was investigated (*Table 4.4*). As can be seen, up to 32 lysyl residues were available for modification with Traut's reagent when it was present at high excess.

Table 4.4

The effect of varying excess of Traut's reagent during modification of BSA upon the incorporation of free sulphydryl groups

Molar excess 2-iminothiolane/ BSA	Moles of [-SH] detected per mole of BSA (maximum possible = 64)*
0	1.8
10	7.6
25	12
50	25
100	31
200	32
500	32

2mg of BSA was added to one of the following volumes of a solution of $2mgml^{-1}$ Traut's reagent in 50mM triethanolamine, pH 8.0 in a reaction vial. The solution was made up to 1ml with triethanolamine buffer: 0.00ml, 0.02ml, 0.05ml, 0.10ml, 0.20ml, 0.40ml, and 1.0ml, giving respective molar excesses of Traut's reagent over BSA molecule of 0, 10, 25, 50, 100, 200 and 500. The reaction was left at room temperature for 4 hours before gel filtration with Sephacryl S-100 at $4^{o}C$ and further analysis.

* or 65 including N-terminal amine group

It was decided that conditions of 100-fold molar excess of Traut's reagent per 66,000 molecular weight were adequate for subsequent protein-modification experiments.

A model for protein modification by Traut's reagent had now been established and so dehydrogenase enzymes were modified with Traut's reagent in a similar manner.

4.2.3.2. Modification of lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH), malate dehydrogenase (MDH).

A 100-fold molar excess of Traut's reagent over LDH and MDH subunit (MW 35,000) and GDH subunit (MW 56,000) was used in the modification reactions. Each enzyme thiolation reaction was treated and purified as described for the BSA model.

1. LDH

The activity of LDH after modification with Traut's reagent was analysed using a DCPIP/PES recycling assay (*Figure 3.6*) and it was 85 % of the specific activity of the native enzyme. This small reduction in activity was comparable to the 18% inhibition caused by DPDS-modification of thiol groups (*Figure 4.22*) and so may have been caused by a residue of free thiol groups present in the Traut's reagent. However, because only 2% of the Traut's reagent molecules had formed thiol groups at 4°C (*section 4.1.1*), the inhibition of LDH may also have been due to lysine modification by the Traut's reagent.

After gel filtration, the thiolated LDH was analysed for thiol group concentration with Ellman's reagent and protein concentration was determined using Coomassie reagent. On average, 7 thiol groups were intoduced onto the surface of each LDH subunit. The introduction of thiol groups was confirmed by passing the thiolated LDH solution down an activated thiol agarose column (*Figure 4.29*).

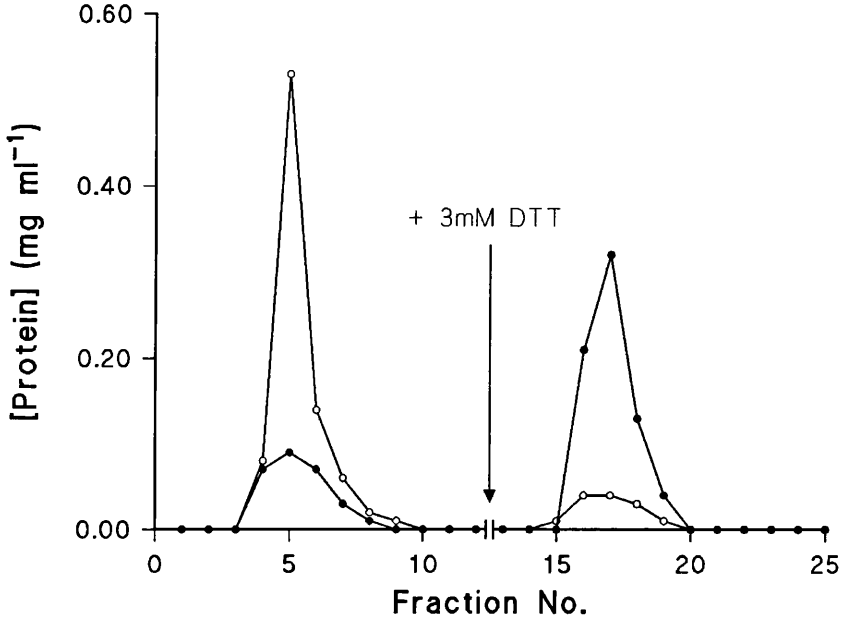


Figure 4.29: *The binding of thiolated LDH to an activated thiol agarose column.*
• = thiolated LDH, O = native LDH. 0.5ml of LDH solution (1.10mgml^{-1}) was applied to the column. Bed volume = 5ml, flow rate = 10ml hr^{-1}, fraction volume = 0.5ml, running buffer = 50mM phosphate, pH 8.0, column run at 24°C. Fractions were collected in a dropwise manner using an automated fraction collector and the protein concentration of each fraction measured using the Coomassie method: 50µl of each fraction were mixed with 2ml of a 50:50 mixture of saline: Coomassie Brilliant Blue G and left for 5minutes. The absorbance of the solution was then read at 620nm and the concentration calculated from a standard curve made from LDH solutions of known concentration.

The thiolation of LDH resulted in improved binding to the thiol agarose column: only 10% of a sample of native LDH, that had been freshly reduced with DTT (and excess DTT removed by gel filtration), bound to the column compared to 77% of the thiolated LDH sample, indicating the presence of extra thiol groups.

GDH and MDH were modified with Traut's reagent and analysed as described for LDH; the results are summarised in *Table 4.5*, along with the LDH modification results.

Table 4.5

Characterisation of dehydrogenases modified using Traut's reagent

Enzyme	Molar ratio [SH]/subunit[a]	% Binding to activated thiol-agarose column	Activity[b] (% of native enzyme)
LDH	0.84	10	100
LDH-[SH]	7.6	77	85
GDH	2.2	32	100
GDH-[SH]	9.2	35	9
MDH	0.32	2.1	100
MDH-[SH]	7.2	49	71

[a] *Protein concentration*: measured using the Coomassie method. 50µl of each fraction were mixed with 2ml of a 50:50 mixture of Saline:Coomassie Brilliant Blue G and left for 5minutes. The absorbance of the solution was then read at 620nm and the concentration calculated from a standard curve made from enzyme solutions of known concentration.
Sulphydryl concentration: A 0.2ml sample of the reaction mixture was added to 1.6ml of 0.1M phosphate buffer, pH8.0 and 0.2ml of Ellman's reagent (4mgml^{-1}) in a cuvette and mixed thoroughly. After 15mins the absorbance of the solution at 412nm was recorded and the concentration of sulphydryl groups calculated from a calibration curve prepared using cysteine standards.
b Activity of LDH and MDH determined using a DCPIP/PES recycling assay and activity of GDH determined using an MTT/PES recycling assay in presence of 3mM NAD and saturating concentrations of respective substrates.

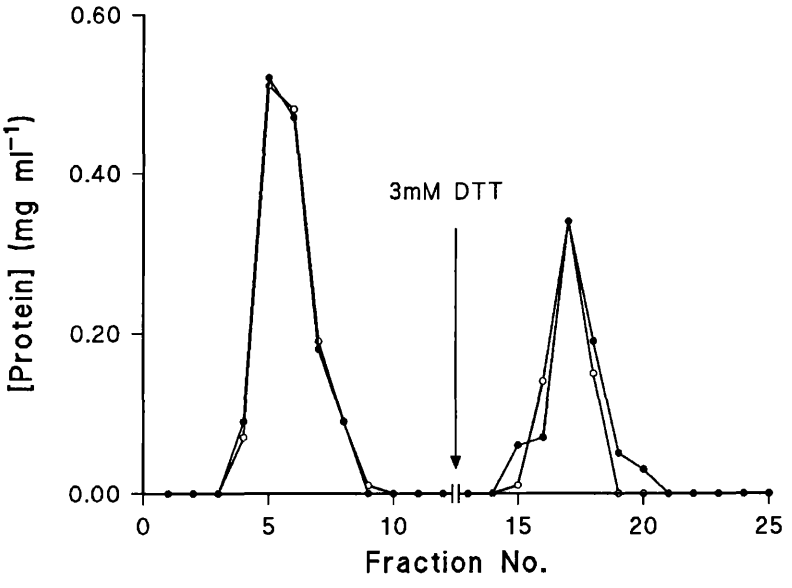


Figure 4.30: *The binding of thiolated GDH to an activated thiol agarose column.*
• = thiolated GDH, O = native GDH. 0.5ml of GDH solution (1.50mgml^{-1}) was applied to the column. Bed volume = 5ml, flow rate = 10ml hr^{-1}, fraction volume = 0.5ml, running buffer = 50mM phosphate, pH 8.0, column run at 24°C. Fractions were collected in a dropwise manner using an automated fraction collector and the protein concentration of each fraction measured using the Coomassie method: 50µl of each fraction were mixed with 2ml of a 50:50 mixture of saline:Coomassie Brilliant Blue G and left for 5minutes. The absorbance of the solution was then read at 620nm and the concentration calculated from a standard curve made from GDH solutions of known concentration.

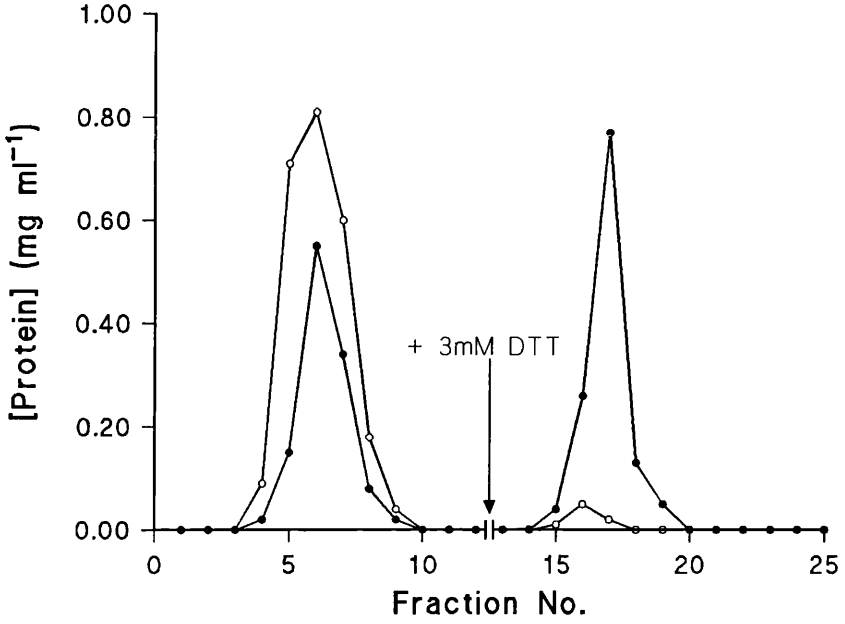


Figure 4.31: *The binding of thiolated MDH to an activated thiol agarose column.*
• = thiolated MDH, O = native MDH. 0.5ml of MDH solution (1.70mgml^{-1}) was applied to the column. Bed volume = 5ml, flow rate = 10ml hr^{-1}, fraction volume = 0.5ml, running buffer = , 50mM phosphate, pH 8.0, column run at 24°C. Fractions were collected in a dropwise manner using an automated fraction collector and the protein concentration of each fraction measured using the Coomassie method: 50µl of each fraction were mixed with 2ml of a 50:50 mixture of saline:Coomassie Brilliant Blue G and left for 5minutes. The absorbance of the solution was then read at 620nm and the concentration calculated from a standard curve made from MDH solutions of known concentration.

As can be seen, a similar incorporation of thiol groups was achieved for each dehydrogenase subunit, 7.2 moles being incorporated onto the surface per mole of MDH subunit; the incorporation of thiols onto the surface of GDH was slightly higher at 9.2 because of the presence of more accessible lysyl residues. The binding of native GDH to the thiol-agarose column was surprising because of reports of the absence of any accessible cystyl residues on the surface of GDH (Rasched and Bayne, 1982) (*Figure 4.30*). The binding was hardly improved upon by thiolation. The topography of the GDH molecule may have been such as to allow protruding DPDS-activated thiol groups of the agarose beads to react with cystyl residues that are buried within the enzyme structure. The improved binding of MDH caused by thiolation was comparable to that of thiolated LDH, with binding being improved by 47% after thiolation (*Figure*

4.31). The inhibitory effect of Traut's reagent modification upon MDH was also similar to the inhibition of LDH, indicating further similarities between LDH and MDH. The strong inhibition of GDH, 91% of the activity being lost, was probably due to modification of the sensitive Lys 126 residue by Traut's reagent (*Section 3.11.5*).

BSA and LDH were now ready for conjugation with the previously prepared thiolated coenzyme adducts (*Section 4.1*)

PART TWO: Conjugation experiments

BSA, LDH, thiolated BSA and thiolated LDH were activated with DPDS as described in *Section 4.2.3*. To a purified, DPDS-activated protein solution, thiolated coenzyme derivative was added in a 15-fold molar excess over BSA molecule or LDH subunit. The reaction was followed by observing the change in absorbance of the solution at 343nm due to the release of pyridine-2-thione. When the reaction was complete, the putatative protein-coenzyme conjugate was subjected to gel filtration to remove all traces of free thiolated coenzyme. It was then concentrated, if necessary, using polyethyleneglycol, washed with activated charcoal and stored at 4°C for no longer than 12 hours before further analysis (for details see *Materials and Methods*).

The "active" and "total" molar ratio of coenzyme to enzyme was determined as described in the legend to *Table 4.6*.

4.3 BSA-coenzyme conjugates formed by reaction of HS-N⁶CM-NAD with activated thiol groups on the protein surface

The results from the analysis of a BSA-N⁶CM-NAD conjugate preparation are shown in *Table 4.6*. Native BSA did not react with the thiolated N⁶CM-NAD derivative, probably because the reaction conditions were not suitable for oxidative dimerisation between the one solvent-accessible cysteine of BSA and the sulphydryl group of the coenzyme. Activation of the single cysteine thiol group with DPDS promoted the binding of 0.3 thiolated coenzyme molecules per BSA molecule, although none of these bound coenzyme molecules could be detected in a second enzyme recycling assay with alcohol dehydrogenase (see "active" ratio).

Table 4.6

The properties of BSA-N⁶CM-NAD conjugates

	Molar ratio of coenzyme:protein	
Conjugated protein	Total[a]	Active[b]
BSA	0.0	0.0
Activated BSA[c]	0.3	0.0
Activated BSA[d]-[SH]	4.2	4.5

a "Total" molar ratio of bound nucleotide to protein was determined by reading the absorbance of the conjugate solution at 266nm and at 290nm and calculating, with known extinction coefficients for BSA and N⁶CM-NAD at both of these wavelengths, the separate concentrations of BSA and N⁶CM-NAD by the solvation of simultaneous equations (*section 3.7*).

b "Active" molar ratio of bound coenzyme to BSA molecule was determined by assaying for coenzyme in a recycling assay with ADH.The cycling experiment was carried out at pH 7.4 using a MTT/PES redox couple with yeast alcohol dehydrogenase. To 1.95ml (100mM TEA/ 100mM nicotinamide/ 500mM ethanol buffer was added 100µl coenzyme present at 0.1µM in the assay solution. 50µl of 12mM MTT, 800µl of 3mM PES and 100µl of yeast alcohol dehydrogenase ($30\mu g ml^{-1}$) were also added. The protein concentration was determined in a Coomassie assay: 0.1ml of sample was added to 1.9ml of a 50:50 mixture of saline: Coomassie reagent and left for 5mins before reading absorbance at 620nm.

c A native BSA solution was subjected to a 50-fold excess of DPDS and purified before conjugation with HS-N⁶CM-NAD.

d BSA was first treated with a 100-fold excess of Traut's reagent and purified before activation with a 50-fold excess of DPDS, followed by conjugation with HS-N⁶CM-NAD.

The thiolated BSA molecule contained 30 activated thiol groups per molecule and so it was not surprising that its conjugate with N^6CM-NAD contained the highest number of covalently bound coenzyme molecules per BSA molecule, with 4.2 N^6CM-NAD moles detected per mole of BSA. The bound coenzyme molecules demonstrated a complete interaction with the active site of alcohol dehydrogenase in the second enzyme recycling assay, 4.5 active molecules of thiolated N^6CM-NAD derivative being detected per molecule of BSA. This suggested that the chemical nature of the spacer arm did not interfere with the interaction between BSA-bound N^6CM-NAD and the active site of ADH, and it was of a suitable length to allow flexibility into and out of the active site of the second enzyme.

Although only 14% (4.2 out of 30) of the activated thiol sites were modified with coenzyme, the conjugation could probably be improved upon by increasing the excess of thiolated coenzyme.

Further evidence for the presence of covalently bound coenzyme on the surface of BSA was provided by the following experiment:-

Binding of thiolated ^{3}H-N^6CM-NAD to DPDS-thiolated BSA.

The radioactive coenzyme, ^{3}H-N^6CM-NAD, was conjugated to DPDS-thiolated BSA as described previously. After purification by gel filtration chromatography, the protein was concentrated using polyethyleneglycol, washed with activated charcoal to a final concentration of 0.80 $mgml^{-1}$, precipitated and the acid-insoluble radioactive counts measured. As *Figure 4.32* shows, a significant amount of radioactivity was detected in the precipitated conjugate.

The amount of radioactivity detected, 704 dpm $(mg\ protein)^{-1}$, represented only 2.1 moles of coenzyme per mole of BSA compared to estimates of 4.2-4.5 using other methods, but this was calculated using the protein concentration estimated before

precipitation with trichloroacetic acid. A significant amount of conjugate material was probably lost during the process of precipitation and filtration. The experiment serves to qualitatively support the covalent fixation of coenzyme to BSA via a disulphide bridge linkage.

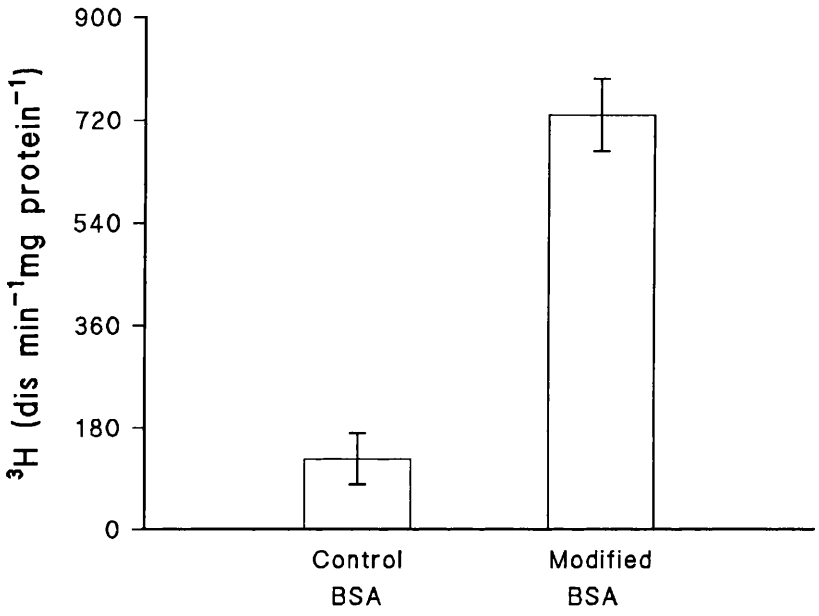


Figure 4.32: *Radioactive counts present in a BSA-coenzyme conjugate synthesised using thiolated ^{3}H-N^{6}CM-NAD:* The conjugate was concentrated using polyethyleneglycol to a concentration of 0.80 mgml^{-1}, and precipitated by adding trichloroacetic acid to 20% and leaving to stand at 4°C overnight. The precipitate was collected under suction on a GFC Millipore filter, and the radioactive counts collected by washing the filter paper with 3ml optiphase in a scintillation vial. The counts were then measured in a LKB scintillation counter for 15 minutes in triplicate. The ^{14}C and ^{3}H "windows" were open during counting and counting efficiency was calculated as 60% for ^{3}H and 98% for ^{14}C by calculating the efficiency of the machine when counting a dated standard sample of ^{14}C or ^{3}H-labelled material. The counts per minute were than adjusted to give the disentegrations per minute (dis/min)

4.4 BSA-coenzyme conjugates formed using HS-N^{6}AHCM-NAD

Because the proposed mechanism of the reaction of activated BSA with HS-N^{6}CM-NAD and HS-N^{6}AHCM-NAD was the same, *i.e.* reaction of an aliphatic thiol group attached to the coenzyme with DPDS-activated thiol groups attached to BSA, it was

expected that similar amounts of the two thiolated NAD derivatives would bind to BSA, and this was indeed the case (*Table 4.7*). 0.2 molecules of thiolated N^6AHCM-NAD bound to native BSA that had been activated with DPDS, compared to 0.3 molecules of HS-N^6CM-NAD, and 3.3 molecules bound to thiolated BSA that had been similarly activated, compared to 4.2 molecules of HS-N^6CM-NAD. However, there was a marked difference in the accessibility of the bound coenzyme derivatives to alcohol dehydrogenase in the second enzyme recycling assay, with only 0.8 out of 3.3 N^6AHCM-NAD molecules being active. This could have been caused either by the spacer arm being too long or by the chemical nature of the spacer arm interfering with the interaction between the coenzyme with the second enzyme.

Table 4.7
Properties of BSA-N^6AHCM-NAD conjugates

	Molar ratio of coenzyme:protein	
Conjugated protein	Total[a]	Active[b]
BSA	0.0	0.0
Activated BSA[c]	0.2	0.0
Activated BSA-[SH][d]	3.3	0.8

[a] "Total" molar ratio of bound nucleotide to protein was determined by reading the absorbance of the conjugate solution at 266nm and at 290nm and calculating, with known extinction coefficients for BSA and N^6AHCM-NAD at both of these wavelengths, the separate concentrations of BSA and N^6AHCM-NAD by the solvation of simultaneous equations (*section 3.7*).

[b] "Active" molar ratio of bound coenzyme to BSA molecule was determined by assaying for coenzyme in a recycling assay with ADH.The cycling experiment was carried out at pH 7.4 using a MTT/PES redox couple with yeast alcohol dehydrogenase. To 1.95ml (100mM TEA/ 100mM nicotinamide/ 500mM ethanol) buffer was added 100µl coenzyme present at 0.1µM in the assay solution. 50µl of 12mM MTT, 800µl of 3mM PES and 100µl of yeast alcohol dehydrogenase ($30\mu g ml^{-1}$) were also added. (Materials and Methods) The protein concentration was determined in a Coomassie assay: 0.1ml of sample was added to 1.9ml of a 50:50 mixture of saline:Coomassie reagent and left for 5mins before reading absorbance at 620nm.

[c] A native BSA solution was subjected to a 50-fold excess of DPDS and purified before conjugation with HS-N^6AHCM-NAD.

[d] BSA was first treated with a 100-fold excess of Traut's reagent and purified before activation with a 50-fold excess of DPDS, followed by conjugation with HS-N^6AHCM-NAD.

After the promising results of the BSA conjugates formed using thiolated N^6CM-NAD, attempts were now made to form LDH-coenzyme conjugates and to discover whether such conjugates possessed intrinsic activity

4.5 LDH conjugates formed using HS-N^6CM-NAD

The results from the analysis of an LDH-N^6CM-NAD preparation are shown in *Table 4.8*.

Table 4.8

Properties of LDH-N^6CM-NAD conjugates

	Molar ratio of coenzyme:protein		Activity (U/mg)[e]	
Conjugated enzyme	Total[a]	Active[b]	Intrinsic	Specific
LDH	0.0	0.0	0.00	17.5
Activated LDH[c]	0.1	0.0	0.00	3.9
Activated LDH-[SH][d]	2.3	1.7	0.27	5.1

a "Total" molar ratio of bound nucleotide to protein was determined by reading the absorbance of the conjugate solution at 266nm and at 290nm and calculating, with known extinction coefficients for LDH and N^6CM-NAD at both of these wavelengths, the separate concentrations of LDH and N^6CM-NAD by the solvation of simultaneous equations (*section 3.7*).

b "Active" molar ratio of bound coenzyme to LDH molecule was determined by assaying for coenzyme in a recycling assay with ADH.The cycling experiment was carried out at pH 7.4 using a MTT/PES redox couple with yeast alcohol dehydrogenase. To 1.95ml (100mM TEA/ 100mM nicotinamide/ 500mM ethanol) buffer was added 100µl coenzyme present at 0.1µM in the assay solution. 50µl of 12mM MTT, 800µl of 3mM PES and 100µl of yeast alcohol dehydrogenase ($30\mu gml^{-1}$) were also added. The protein concentration was determined in a Coomassie assay: 0.1ml of sample was added to 1.9ml of a 50:50 mixture of saline:Coomassie reagent and left for 5mins before reading absorbance at 620nm.

c A native LDH solution was subjected to a 50-fold excess of DPDS and purified before conjugation with HS-N^6CM-NAD.

d LDH was first treated with a 100-fold excess of Traut's reagent and purified before activation with a 50-fold excess of DPDS, followed by conjugation with HS-N^6CM-NAD.

e Both intrinsic and specific activities were determined in a recycling assay with a DCPIP/PES redox couple.

Native LDH did not conjugate with the thiolated N^6CM-NAD derivative, probably because the oxidising conditions required for oxidative dimerisation were not used. The small loss in specific activity (7.1% because activity of the untreated native

enzyme = 18.9 Umg^{-1}) was probably due to the modification of one or more cystyl residues by the thiolated coenzyme. LDH activated with DPDS showed only a small degree of conjugation with, on average, only a small fraction of the subunit molecules (~10%) containing a bound coenzyme molecule. The 5 cysteines of pig H_4 LDH were largely inaccessible to both DPDS and, therefore, thiolated coenzyme. The most successful enzyme preparation was the thiolated preparation, activated with DPDS, as was the case with the BSA conjugates. 2.3 moles of N^6CM-NAD were bound per mole of LDH subunit, showing 73% of their normal substrate activity in a second enzyme recycling assay with alcohol dehydrogenase.

Activity

The intrinsic and specific activity of each LDH-coenzyme conjugate was also determined using a DCPIP/PES coenzyme recycling assay (*Section 3.8*). A further loss of activity of each of the activated and thiolated enzyme preparations was apparent upon conjugation with the thiolated coenzyme. The specific activity of the thiolated LDH was slightly higher, at 27%, than that of the unthiolated DPDS-activated LDH, which had only 20% of the activity of native LDH. This suggests that thiolation affords some protection against inhibition by reaction with thiolated coenzyme by reducing the possibility of reaction of an activated native cystyl residue with coenzyme.

Intrinsic activity was only generated by the coenzyme-conjugate formed by the thiol-modified enzyme. It was 5% of the specific activity of the conjugate or 1.3% of the specific activity of native LDH.

The following experiment was carried out to confirm that the intrinsic activity generated by the LDH-N^6CM-NAD conjugate was due to the presence of coenzyme covalently bound to the surface of LDH via a disulphide bridge linkage:-

Treatment of the enzyme-coenzyme conjugate with DTT

1μmol DTT was added to 1ml of a LDH-N^6CM-NAD conjugate solution (0.4 mg ml^{-1}) which was then agitated with an overhead stirrer at room temperature for 30mins. After gel filtration with Sephacryl S-100 the conjugate was again assayed for intrinsic and specific activity. The intrinsic activity of the conjugate had totally disappeared, confirming that the previous intrinsic activity was caused by the presence of coenzyme that was covalently bound to the surface of LDH via a disulphide bridge and was interacting with the active site of the same LDH molecule that it was bound or with neighbouring LDH molecules. However, the specific activity of the molecule after DTT treatment was reduced from 5.1 Umg^{-1} to 4.2 Umg^{-1}. It was hoped that if the original inhibition was due to covalently bound coenzyme the activity might have been restored upon reduction of the disulphide linkages by DTT.

4.6 LDH conjugates formed using HS-N^6AHCM-NAD

The results from the analysis of LDH-coenzyme conjugates formed using thiolated N^6AHCM-NAD derivative are shown in *Table 4.9*.

Native LDH activated with DPDS again showed only a small degree of conjugation, with, on average, up to 10% of the subunit molecules containing a bound coenzyme. This figure was the same for both thiolated N^6CM-NAD and N^6AHCM-NAD. The thiolation of an enzyme undoubtedly improves the chances of conjugate formation with a thiolated coenzyme, with 2.7 moles of coenzyme binding per mole of LDH subunit with only a 15-fold excess of coenzyme. Again, this result compares well with the N^6CM-NAD result. However, the bound N^6AHCM-NAD molecules showed only 33% of their normal substrate activity with ADH in the second enzyme recycling assay, whereas bound N^6CM-NAD molecules possessed 73% of their normal activity. A similar result was obtained from assay of the BSA conjugates and it suggested that the spacer arm formed between thiolated N^6CM-NAD and the protein surface was more

suitable for interaction of the coenzyme with a second enzyme than the spacer arm formed by similar conjugation of a thiolated N^6AHCM-NAD molecule. This suggestion was supported by the results for the intrinsic activity.

Table 4.9

Properties of LDH-N^6AHCM-NAD conjugates

	Molar ratio of coenzyme:protein		Activity[e]	
Conjugated enzyme	Total[a]	Active[b]	Intrinsic	Specific
LDH	0.0	0.0	0.00	16.9
Activated LDH[c]	0.1	0.0	0.00	3.5
Activated LDH-[SH][d]	2.7	0.9	0.05	8.6

[a] "Total" molar ratio of bound nucleotide to protein was determined by reading the absorbance of the conjugate solution at 266nm and at 290nm and calculating, with known extinction coefficients for LDH and N^6AHCM-NAD at both of these wavelengths, the separate concentrations of LDH and N^6AHCM-NAD by the solvation of simultaneous equations (*Section 3.7*).

[b] "Active" molar ratio of bound coenzyme to LDH molecule was determined by assaying for coenzyme in a recycling assay with ADH.The cycling experiment was carried out at pH 7.4 using a MTT/PES redox couple with yeast alcohol dehydrogenase. To 1.95ml 100mM TEA/ 100mM nicotinamide/ 500mM ethanol buffer was added 100µl coenzyme present at 0.1µM in the assay solution. 50µl of 12mM MTT, 800µl of 3mM PES and 100µl of yeast alcohol dehydrogenase ($30\mu g ml^{-1}$) were also added. The protein concentration was determined in a Coomassie assay: 0.1ml of sample was added to 1.9ml of a 50:50 mixture of saline: Coomassie reagent and left for 5mins before reading absorbance at 620nm.

[c] A native LDH solution was subjected to a 50-fold excess of DPDS and purified before conjugation with HS-N^6AHCM-NAD.

[d] LDH was first treated with a 100-fold excess of Traut's reagent and purified before activation with a 50-fold excess of DPDS, followed by conjugation with HS-N^6AHCM-NAD.

[e] Both intrinsic and specific activities were determined in a recycling assay with a DCPIP/PES redox couple.

Activity

The intrinsic and specific activity of each LDH-coenzyme conjugate was also determined using a coenzyme recycling assay with dichlorophenolindophenol indicator (*Section 3.8*). The activity of each of the DPDS-thiolated enzyme preparations was further reduced by conjugation with the thiolated coenzyme. The specific activity of the thiolated LDH was higher, at 45%, than that of the unthiolated LDH that was activated with DPDS, which possessed only 18% of the activity of native LDH.

Intrinsic activity was, again, only generated by the conjugate formed using the thiolated enzyme. It was only 0.6% of he specific activity of the conjugate, and 0.2% of the specific activity of native LDH. The intrinsic activity of the LDH-N^6AHCM-NAD conjugate did not compare well with that of the LDH-N^6CM-NAD conjugate which possessed an intrinsic activity that was ten-fold greater.

CHAPTER 5

The incorporation of LDH-coenzyme conjugates into a reagentless enzyme electrode for lactate

Introduction

This chapter is included to demonstrate that an enzyme-coenzyme conjugate preparation is able to act as the fully contained catalytic constituent of a reagentless enzyme electrode for lactate. A full consideration of recent developments in enzyme electrode technology, relating to the use of NAD-dependent dehydrogenases, was made before attempting to construct a lactate biosensor using preparations containing NAD covalently bound to LDH. The reasons for interest in an amperometric biosensor for lactate are given in the *General Introduction.*

The LDH-NAD and LDH-N^6AHCM-NAD conjugates that contained a glutaraldehyde cross-linker were used in the preparation of enzyme electrodes because they each included a methylene-containing spacer arm which was most likely to allow the enzyme-bound coenzyme the greatest freedom of movement between active sites of the LDH molecules and the electrode surface (*Figure 1.5*). Each of the LDH-coenzyme conjugates formed using carbodiimide-mediated coupling possessed a shorter spacer arm. In addition, the LDH-coenzyme conjugates that were synthesised using glutaraldehyde coupling possessed a ten-fold higher intrinsic activity than those formed using carbodiimide coupling. They were therefore likely to generate a larger electrode response when immobilised on to the surface of an enzyme electrode in the presence of lactate.

The length of the molecular link between the adenine N^6 position of NAD and the polypeptide backbone of the LDH molecule was at least 1.5nm for the LDH-NAD

conjugate and at least 3.1nm for the LDH-N^6AHCM-NAD conjugate. The longer spacer arm of LDH-N^6AHCM-NAD would be expected to provide a more flexible link allowing the enzyme-bound coenzyme a greater freedom of movement and greater accessibility into the surrounding environment. This was supported by the results reported in *Chapter 3* which showed that the LDH-coenzyme conjugate containing the extra hexamethylene spacer contained coenzyme that was 27% more accessible to the active sites of ADH molecules in free solution.

The approach used to construct the lactate biosensor using LDH-NAD and LDH-N^6AHCM-NAD conjugates will be discussed in *Part One* of this chapter followed by a description of the properties of the final enzyme electrode in *Part Two*.

Part One

5.1 A consideration of recent developments in the technology used to construct enzyme electrodes incoporating NAD-dependent enzymes

Figure 5.1 displays the layout of a generalised enzyme electrode constructed using an NAD-dependent enzyme. Several decisions must be taken before constructing such a device: namely, how the enzyme is to be immobilised, how the coenzyme is to be retained and regenerated, and which type of electrode material is to be used. These issues will now be discussed, in turn.

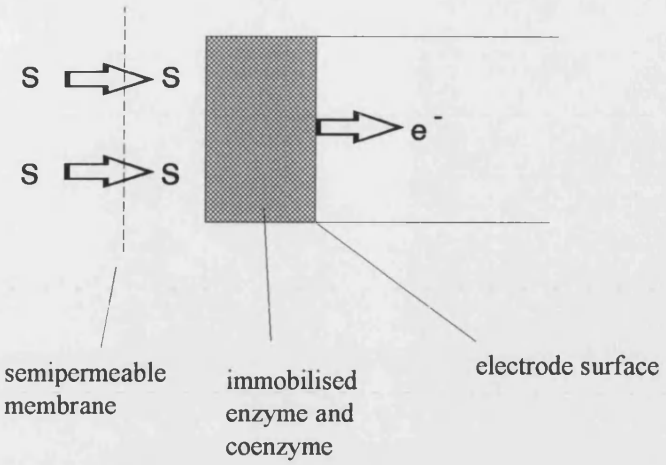


Figure 5.1: *Generalised layout of an enzyme electrode based on an NAD-dependent enzyme*. The enzyme and coenzyme must be immobilised in the same phase, usually in the intimate vicinity of the electrode surface, to enable coenzyme to be regenerated and the reaction between enzyme and substrate to be detected.

5.1.1 Coenzyme regeneration

Electrocatalytic coenzyme regeneration is becoming the preferred method of regeneration in the construction of NAD-dependent enzyme electrodes, and the reasons why this method of coenzyme regeneration was chosen were discussed in *Section 1.5*. The substrates of NAD-dependent dehydrogenases that are of analytical interest - for example, ethanol, lactate, glutamate, glucose and glycerol - are all oxidised during catalysis by their corresponding dehydrogenases and electrons are passed to the coenzyme NAD which is consequently reduced to form NADH. Thus, the coenzyme must be regenerated by oxidation, and only very rarely by reduction. This is indeed fortunate because the electrocatalytic oxidation of NADH is, by far, an easier reaction to achieve, although the corresponding reduction is also possible (Aizawa *et al*, 1976b). The formal potential of the NADH/NAD couple is -520mV vs Ag/AgCl* (Schumann and Schmidt, 1992; Dominguez *et al*, 1993a), reflecting that the oxidising potential of NAD is actually quite low. However, this figure was determined

* This overpotential was actually reported versus a standard calomel electrode and recalculated for consistency for reference against a Ag/AgCl electrode in this chapter using standard tables given in the literature (Bard and Faulkner, 1980)

enzymatically and the practical electrocatalytic oxidation of NADH actually requires an overpotential of +700mV vs Ag/AgCl in order to proceed (Kulys *et al*, 1991). Unfortunately, when such a high overpotential is used the oxidation of NADH proceeds via the irreversible formation of radicals which facilitate the formation of NAD dimers and other side products (Elving *et al*, 1976). The application of large overpotentials also generally leads to increased electrode fouling and a high level of background interference from other redox compounds. Despite these difficlties, Bonnefoy and his co-workers were able to demonstrate that the electrocatalytic regeneration of NAD from NADH was greater than 99.99% pure with respect to its enzymic activity (Bonnefoy *et al*, 1988). In addition, a reagentless enzyme for lactate was constructed by Blaedel and his co-workers who were able to slightly reduce the overpotential required to oxidise NADH to 450mV vs Ag/AgCl by using a glassy carbon electrode in place of a platinum electrode (Blaedel and Engstrom, 1980). In this study, LDH was immobilised alongside NAD by entrapment with an acetylated dialysis membrane. However, although the lactate electrode was quite sensitive, detecting down to 3μM lactate, it demonstrated a largely non-linear dependence and the response time was quite sluggish (at least 7mins). Moreover, the overpotential used in this study was still too high for lactate detection in a 'dirty' solution containing redox substrates other than NADH (Heller, 1992). In fact, the optimum range of overpotential for an amperometric biosensor is beween -150 and 50mV vs Ag/AgCl (Dominguez *et al*, 1993a). Fortunately, there are now ways in which the minimum overpotential required to oxidise NADH can been reduced, as will now be described.

Redox mediators

A redox compound capable of oxidising NADH may itself be electrocatalytically oxidised at a lower overpotential than NADH. Such a compound can act as a redox mediator between the coenzyme and the electrode surface. Suitable mediators can also be immobilised in the same phase as the coenzyme, for example by covalent fixation, to keep the enzyme electrode in a 'reagentless' state. The use of mediators has

significantly reduced the overpotential required to regenerate NAD in recent studies. For example, the covalent fixation of quinones to the electrode surface reduced the required overpotential to between 50 and 200mV vs Ag/AgCl, but the reported low stability of this system has restricted its application (Cenas *et al*, 1985; Kulys *et al*, 1991). A similar decrease in overpotential was obtained upon the use of phenazines and phenoxines as mediators, but again the resultant enzyme electrodes were unstable (Schumann and Schmidt, 1992). More succesful mediators have been found: electrodes made from the conducting organic salt N-methylphenazinium tetracyanoquinodimethanide (NMP $TCNQ^{-}$) suffered no deterioration in response to NADH after several days (Albery and Bartlett, 1984; Kulys *et al*, 1991). Ferricyanide or ferricinium ions deposited onto electrode surfaces also act as stable mediators (Cass *et al*, 1984; Yon Hin and Lowe, 1987). Such electrodes can oxidise NADH at an optimum overpotential of between 0 and 200mV vs Ag/AgCl. More recently, phenazine methosulphate acted as a succesful mediator when it was immobilised by adsorption onto a graphite electrode (Miyawaki and Yano, 1993) but other reports suggest that phenazine methosulphate may be harmful to enzyme preparations (Huck *et al*, 1984). Also recently, tetrachloro-*p*-benzoquinone was found to be a particularly stable quinone and its recent adsorption onto the surface of a graphite electrode also resulted in a stable electrode preparation (Schumann and Schmidt, 1992).

The use of redox catalysts for the electrochemical oxidation of NADH has significantly decreased the overpotential required to oxidise NADH. However, the long term stability of these electrodes is bound to be limited when the mediator is immobilised by adsorption because of leaching effects. The covalent linking of mediators to the electrode surface implies the functionalisation of the electrode. Unfortunately, certain useful electrode materials, such as platinum, are not very efficient in such functionalisation processes. Platinum electrodes are often preferred because of the reduction in electrode fouling and the ease with which they can be developed by machining (Schumann and Schmidt, 1992). Functionalisation of platinum electrodes

can now be easily achieved by the growing up of a conductive polypyrrole matrix onto the electrode surface. By covalently binding redox mediators to the surface of this matrix, very stable electrodes have been prepared that catalyse the oxidation of NADH at an overpotential between 150 and 250mV vs Ag/AgCl (Schumann and Schmidt, 1992).

Carbon paste electrodes

Recently, the value of carbon paste as an electrode material in the construction of dehydrogenase-based biosensors has been assessed (Persson *et al*, 1993). The use of carbon paste results in low background currents and the organic environment that predominates is not detrimental to the biocomponent. Chemical modification of carbon paste electrodes is possible and results in electrodes with increased stability at alkaline pH, and so the electrodes that are formed are suitable for use with the alkaline-stable dehydrogenase enzymes. An ADH-based biosensor for alcohol was made by chemical modification of a carbon paste electrode with a polymer containing a covalently-bound phenothiazine dye. The overpotential applied was 100mV vs Ag/AgCl (Persson *et al*, 1993). Another promising carbon paste electrode for ethanol detection was constructed by the entrapment of ADH and NAD in a polyethyleneimine matrix (Dominguez *et al*, 1993b).

Because of its favourable characteristics, especially with respect to dehydrogenase-based biosensors, carbon paste was chosen as the electrode material in the construction of enzyme electrodes described here. Moreover, an activated carbon material was available from the collaborating body, Cambridge Life Sciences, that did not require chemical modification to achieve NADH oxidation at a low overpotential. This activated carbon paste will now be described.

In 1988, Bennetto used a commercial electrode source as a glucose sensor following the simple adsorption of glucose oxidase to the electrode material (Bennetto, 1988).

The electrode material consisted of a platinum-treated carbon mixture which was mixed with a teflon binder material to form a paste, before being compressed onto a carbon substrate. An electrode prepared using this activated carbon possesses the following properties which are conveyed by the material used.

1. The electrode functions as a solid platinum electrode, presumably due to the activation treatment with colloidal platinum; the electrode is consequently prone to less fouling, and, at the same time, is less expensive to produce.

2. The electrode operates at a lower operating potential than a platinum electrode; it is able to reduce NADH at an overpotential of +150mV vs Ag/AgCl.

3. The electrode is expected to have a longer lifetime compared to electrodes operated at high overpotentials, due to less interference and fouling. The stability of an enzyme electrode constructed using this electrode material is determined by the catalytic component, only.

4. Because the material is predominantly made of carbon, high enzyme loading by adsorption is possible.

5. The simplicity of the electrode construction overcomes major manufacturing problems.

The ability of the activated carbon paste electrode to regenerate NAD at a reduced overpotential was confirmed before experiments were carried out using the enzyme-coenzyme conjugates.

Oxidation of reduced coenzyme at an overpotential of +150mV vs Ag/AgCl using an activated carbon paste electrode

A description of the electrode set-up is given in *section 5.3*. The response of the activated carbon paste electrode to 0.0, 1.0, 2.0, 4.0 and 5.0 mM solutions of NADPH was examined, and the results shown in *Figure 5.2*. Although NADPH was used in place of NADH, the two coenzymes have a very similar oxidising potential (Elving *et al*, 1976).

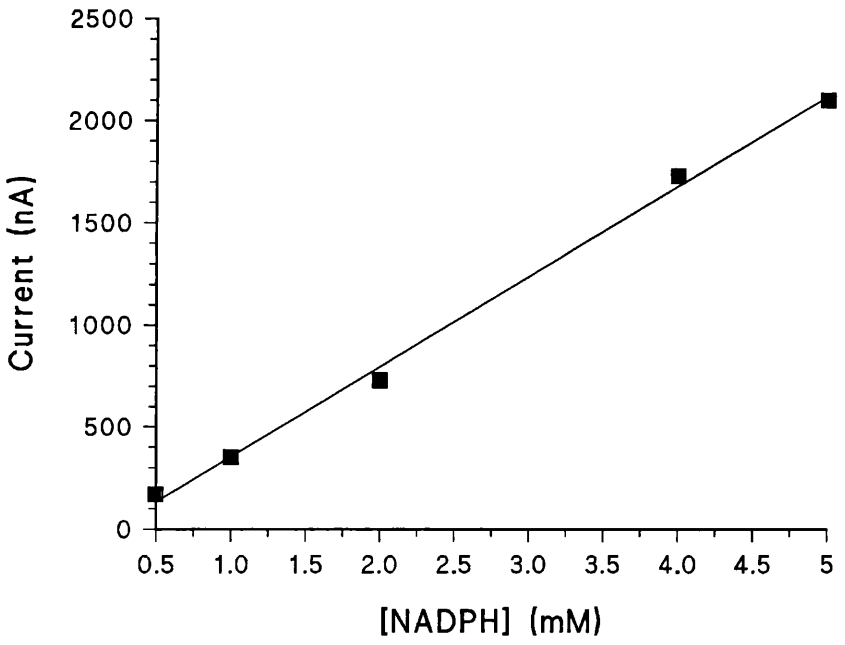


Figure 5.2: *The response of an activated carbon paste electrode to NADPH at an applied overpotential of +150mV vs Ag/AgCl.*

The response shown has been corrected with respect to a working electrode containing no activated carbon at its surface. *Electrode preparation:* activated carbon particles (25mg) were mixed with 0.25ml phosphate buffer, 100mM, pH 7.5 and spun down in a microfuge (5 mins). A commercial teflon binder material (0.04ml) was added to the pellet and mixed to form a paste. The paste was compressed onto the surface of a carbon support material ('Toray' paper) using a glass slide and left to dry for 15mins. A disc (0.2cm in diameter) was cut out and inserted into the linings of a working electrode The cell halves were assembled (reference = Ag/AgCl) and a potential of +150mV was applied to the cell, which was then left to attain a steady current before NADPH samples in PESK buffer were added to the sample cell, and the response recorded.

The electrode clearly responded well to NADPH with a change in current of 400nA/mM NADPH due to the oxidation of NADPH at a low overpotential of +150mV.

5.1.2 Coenzyme immobilisation

The problem of coenzyme retention is solved, in our case, upon immobilisation of the enzyme because the coenzyme molecules are covalently bound to the enzyme molecules. Because the coenzyme is strongly bound to enzyme, any leaching will be due to the method of enzyme immobilisation. An enzyme electrode has been prepared using this sort of coenzyme immobilisation technique only once before (Torstensson and Johansson, 1980). An ADH-N^6AHCM-NAD conjugate preparation, synthesised by carbodiimide-activation of accessible surface acidic residues of ADH (Mansson *et al*, 1978) was adsorbed onto the surface of a glassy carbon electrode to form a biosensor for ethanol. However, a large overpotential was required to regenerate the coenzyme (550mV vs Ag/AgCl) and the enzyme-coenzyme conjugate was only intrinsically active for one catalytic cycle, reportedly due to decomposition of NADH at the surface of the glassy carbon electrode. Despite these problems it was pointed out that a more stable electrode could be attained by using a modified electrode surface and an electrode material that allows the oxidation of NADH at a reduced overpotential. The idea of a self-contained enzyme-coenzyme conjugate acting as the biocatalytic component of an NAD-dependent enzyme-electrode was still a good one: the problems that arose were electrochemical in nature.

The question still remains of how to immobilise the enzyme-coenzyme conjugate in our study.

5.1.3 Enzyme immobilisation

Immobilisation of enzyme, in our case, is partly required to fix the enzyme in the close vicinity of the electrode surface so that each enzyme-bound coenzyme molecule can move 'back and forth' from the active sites of the LDH molecules to the electrode surface for regeneration (*Figure 1.5*). :

The possibility of covalent immobilisation (*Section 1.2*) inhibiting the enzyme is particularly relevant to the immobilisation of enzyme-coenzyme conjugates: the enzyme has already been covalently modified and, in most cases, its specific activity reduced as a consequence. Because of this, and because there are less surface residues on the enzyme available for covalent modification, this method of immobilisation was not chosen. The cross-linking method (entrapment) leads to a high density of immobilised enzyme activity but complicates the construction of the enzyme electrode (*Section 1.2*). This method has, however, been used in the preparation of dehydrogenase-based biosensors (*Section 1.4*) (Blaedel and Jenkins, 1976; Kulys, 1991). The adsorption method was chosen for the immobilisation of the LDH-coenzyme conjugates in this study because:

1. It allowed a high enzyme loading. The adsorption method would allow a high density of intrinsic enzyme activity in the closest proximity to the electrode surface

2. It maintained the simplistic theme of the activated carbon paste electrode: the carbon electrode surface also acted as the adsorption support.

3. It was the most non-invasive of the immobilisation methods in that it was least likely interfere in the 'back-and-forth' movement of the enzyme-bound coenzyme to and from the electrode surface.

Any desorption that might have occurred was minimised by maintaining constant pH, temperature, solvent conditions and ionic concentration throughout the electrode experiments. It was reasoned that desorption would not significantly affect the electrode experiments, which lasted up to a few hours at a time.

In order to confirm that the desorption effect would not affect the electrode studies, an experiment was carried out to investigate the stability of an enzyme preparation adsorbed onto the activated carbon material.

5.2 The stability of LDH immobilised by adsorption onto an activated carbon electrode surface.

The criteria for successful adsorption of the enzyme was that a significant amount of active enzyme must be adsorbed to the carbon paste and its activity must remain stable for at least several hours. In order to examine the success of adsorption, native LDH was adsorbed to the activated carbon particles and an activated carbon paste prepared. The LDH activity present on the paste was measured intermittently over a period of several days. The method of Bennetto (1988) was modified to adsorb LDH onto activated carbon particles as follows:

Preparation of immobilised LDH

Native LDH was mixed with activated carbon particles in a ratio of 10:1 (v/w) phosphate buffer. The suspension was left for one hour before the activated carbon was spun dow, the supernatant discarded and the pellet containing the adsorbed LDH formed into a paste by the addition of a teflon binder material. The paste was spread onto a layer of a conductive carbon paper or support and left to dry. The immobilised enzyme preparation was then stored in PESK buffer; one preparation was stored at

room temperature and another at 4°C. The potential enzyme electrode material was analysed for LDH activity intermittently, over a period of 28 days.

Assay of immobilised LDH for activity

A sample disc was cut out of the electrode material, using a punch of 0.5cm in diameter, and assayed for LDH activity (legend *Figure 5.4*). After assay, the immobilised enzyme disc was dried in a warm oven and weighed. The LDH specific activity present per mg of electrode material was then calculated.

The stability of the immobilised LDH preparations at 4°C and at room temperature are shown in *Figure 5.4.*

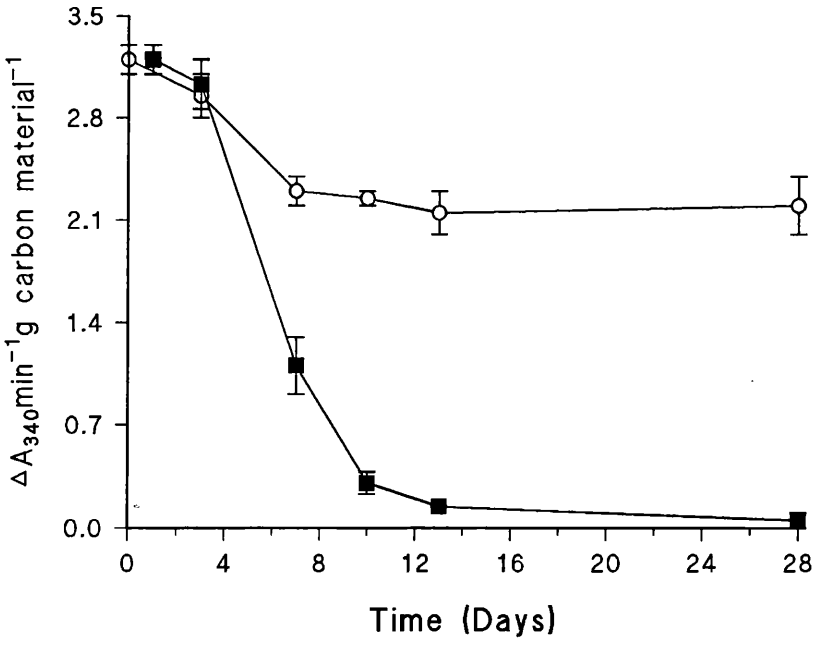


Figure 5.4 *The stability of LDH activity present on activated carbon pastes prepared by adsorption of LDH.* ○ = storage at 4°C; ■ = storage at room temperature. A sample disc was cut out of the bioelectrode material (see text) using a punch of 0.5cm in diameter, and added to a cuvette containing: 2.6ml 100mM phosphate buffer, pH 7.5; 0.1ml 25mM pyruvate; 0.05ml 12mM NADH. The solution was mixed with an overhead stirrer and the absorbance of the solution at 340nm was measured at intermittent periods to monitor the decrease in NADH concentration. After assay, the immobilised enzyme disc was dried in a warm oven and weighed. The LDH specific activity present per mg of electrode material was then calculated.

As can be seen, it was very important to store the prepared carbon pastes at 4°C because the stability at room temperature deteriorated rapidly after 3 days. However, on the third day, the LDH activity present on the pastes had only dropped slightly indicating that any desorption effects occurring within a few hours use of the pastes would not seriously convey instability into an enzyme electrode prepared from them.

In summary, each LDH-coenzyme preparation was immobilised onto the surface of an activated carbon paste electrode by adsorption. An overpotential of 150mV vs Ag/AgCl was applied to the electrode which was sufficient to oxidise any LDH-bound NADH that might be formed upon the addition of lactate solution.

5.3 Construction of an enzyme electrode

A simplified representation of the enzyme electrode is shown in *Figure 5.1*, whilst a diagram of the apparatus used is shown in *Figure 5.5*.

Each LDH-coenzyme conjugate solution was concentrated against solid polyethyleneglycol before immobilisation onto the surface of adsorbtive activated carbon particles as described above. The final concentrations of each LDH-coenzyme conjugate used are shown in *Table 5.1*. Once the carbon paste biosurface had been prepared, discs of 0.2cm diameter were cut out of it using a punch and inserted into the lining of a working electrode (*Figure 5.6*).

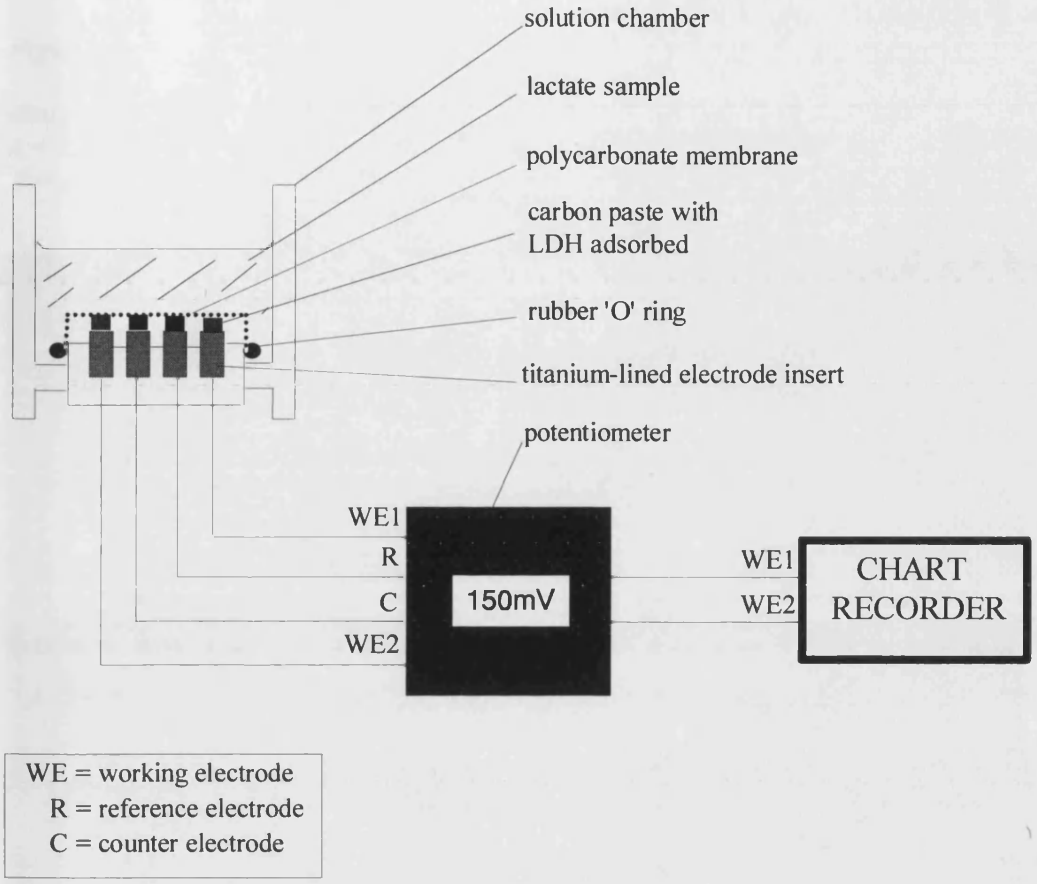


Figure 5.5: *Diagram of the apparatus used to study a reagentless enzyme electrode for lactate incorporating LDH-coenzyme conjugates.* Purpose of the counter electrode is to account for any potential difference occurring between the reference and working electrodes.

As *Figure 5.5* shows there were two working electrodes. This allowed the response of two different enzyme electrodes to the same substrate to be studied at the same time; an electrode containing an LDH-coenzyme conjugate could be run alongside one containing native LDH and no coenzyme.

Figure 5.6: *Photographical representation of the construction of an enzyme electrode.*
A *= titanium electrode lining;* ***B*** *= platinised carbon surface (with adsorbed enzyme);*
C *= polycarbonate membrane;* ***D*** *= "O" ring*

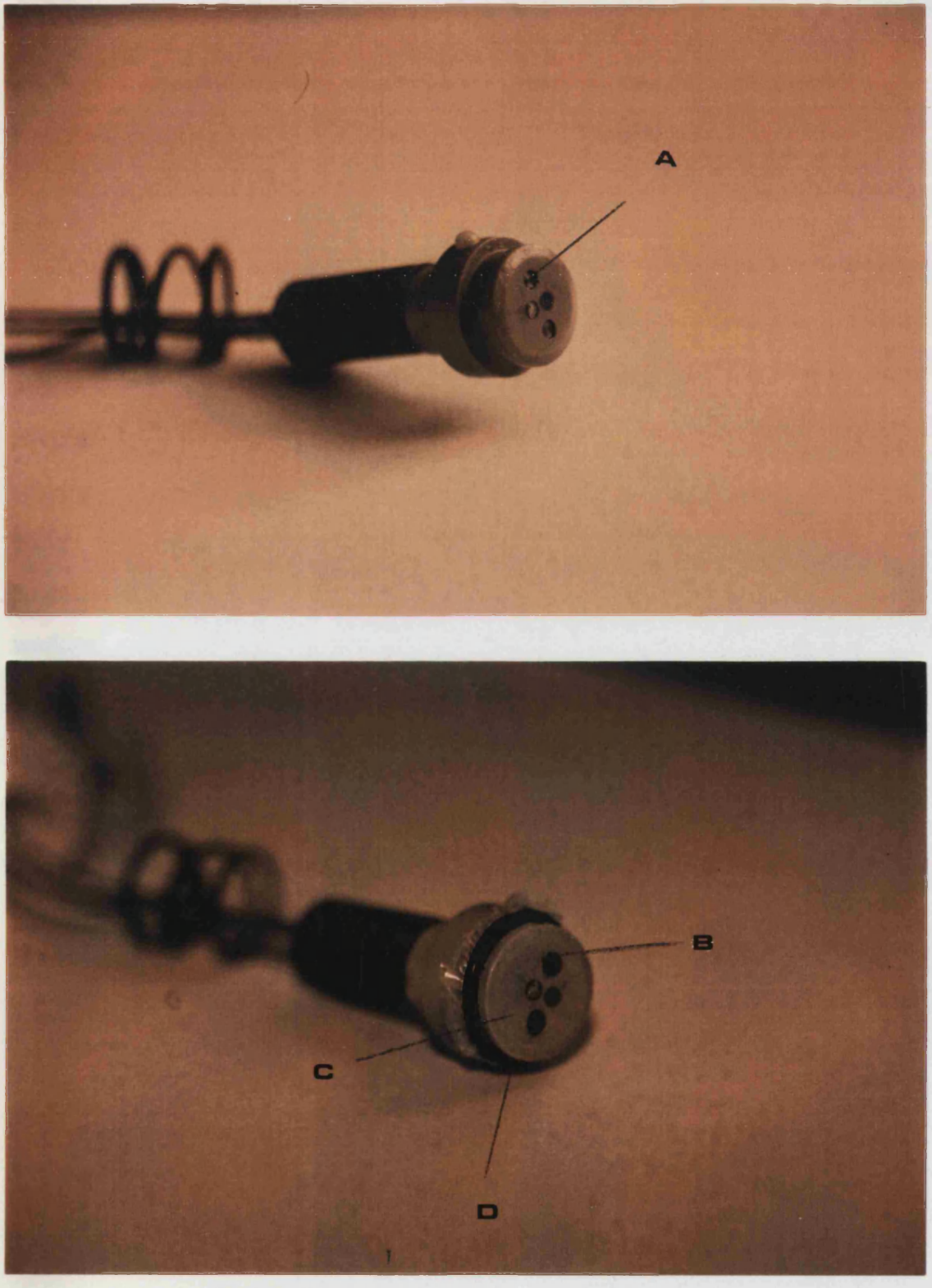


Table 5.1
Properties of enzyme-coenzyme conjugates before and after use in electrode studies

Enzyme	Activity (U/mg) Intrinsic	Specific	Protein concentration (mg/ml)
LDH	0.00 **0.00**	18.9 **18.0**	1.1
LDH-NAD	0.09 **0.07**	2.3 **2.0**	1.0
LDH-N^6AHCM-NAD	0.12 **0.10**	4.7 **3.9**	2.0

Figures in bold denote activities of conjugates after a 14-day electrode study period

The electrodes were covered with a polycarbonate membrane (pore size 50nm) which was held in place with a rubber 'O' ring (*Figure 5.6*). This membrane provided a physical barrier against interfering proteins that might be present in the sample solution. The cell halves were assembled and the solution chamber filled with PESK buffer, pH 7.4. An overpotential of +150mV was applied to the cell using a potentiometer and the cell was then left until a steady current was reached in both working electrodes (30mins). The current flowing through each electrode was recorded on a twinned-pen chart recorder. A sample solution containing lactate was pipetted into the solution chamber and any change in the current of each working electrode was measured by the chart recorder. The response time was generally between 1 and 2 minutes. The cell was washed with PESK buffer and each electrode allowed to reach a steady current against a blank buffer solution before the addition of the next lactate sample (5 minutes).

Part Two

5.4 Properties of reagentless NAD-dependent enzyme electrodes constructed using LDH-coenzyme conjugates

5.4.1 Response to lactate

Native LDH was used to prepare a control enzyme electrode which did not possess immobilised coenzyme. The response of this control electrode was examined alongside the LDH-coenzyme conjugate electrode for each lactate measurement.

5.4.1.1 Response of enzyme electrodes prepared using an LDH-NAD conjugate

The modified-enzyme-containing carbon paste electrode was constructed and tested immediately after preparation. An electrode was constructed several times, but no discernible response to lactate concentrations of 0.5mM, 2mM, 10mM, 100mM or 500mM was obtained.

5.4.1.2 Response of enzyme electrodes prepared using an LDH-N^6AHCM-NAD conjugate

The modified-enzyme-containing carbon paste electrode was constructed and tested immediately after preparation. The average responses of the electrodes prepared to concentrations of lactate ranging from 0.5 to 10mM are shown in *Figure 5.7*.

The corrected or 'real' response, was calculated by subtraction of the response obtained from the electrode prepared using native LDH from the response obtained from the electrode prepared using the LDH-N^6AHCM-NAD conjugate. A clear difference in the two responses was evident, the difference in response being 12nA/mM lactate greater for the coenzyme-containing electrode. The response was linear for the concentration range measured, 0.5-10mM, and the detection limit was between 0.5 and 1mM.

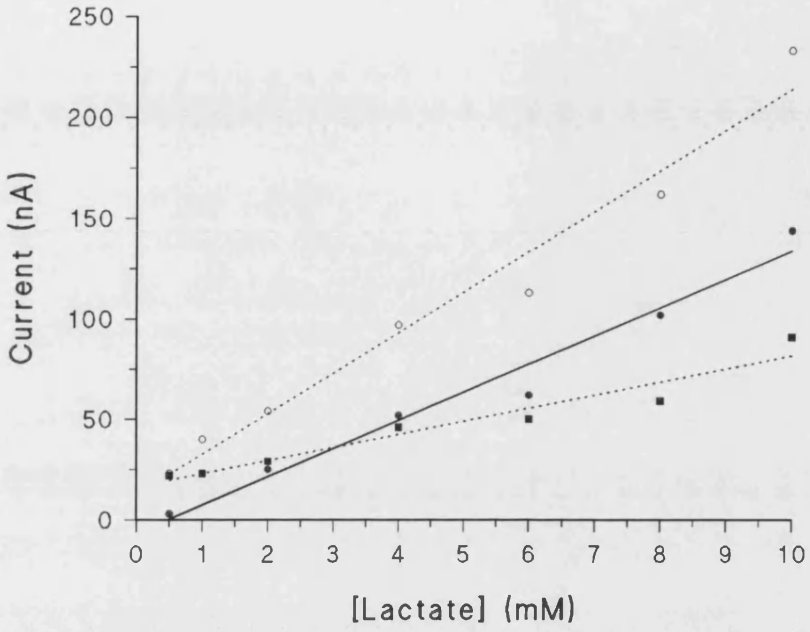


Figure 5.7: *The response of a reagentless enzyme electrode for lactate* ○ = uncorrected response; ● = corrected response; ■ = control response. *Electrode preparation:* activated carbon particles (25mg) were mixed with 0.25ml LDH or LDH-N^{6}AHCM-NAD in 100mM phosphate buffer, pH 7.5 and spun down in a microfuge (5 mins). A commercial teflon binder material (0.04ml) was added to the pellet and mixed to form a paste. The paste was compressed onto the surface of a carbon support material ('Toray' paper) using a glass slide and left to dry for 15mins. A disc (0.2cm in diameter) was cut out and inserted into the linings of a working electrode The cell halves were assembled (reference = Ag/AgCl) and a potential of +150mV was applied to the cell, which was then left to attain a steady current, before lactate samples in PESK buffer were added to the sample cell, and the control and normal response recorded on a twinned-pen chart recorder.

The response to 100mM lactate was also measured using an electrode prepared in the same way as the LDH-N^{6}AHCM-NAD electrode, but using an unplatinised carbon preparation as the electrode base. A clear difference in response can be seen (*Figure 5.8*); the response of the latter was lower at 5nA/mM lactate. In addition, the response time of the electrode was slower (3mins) and less linear when the lactate concentration was varied.

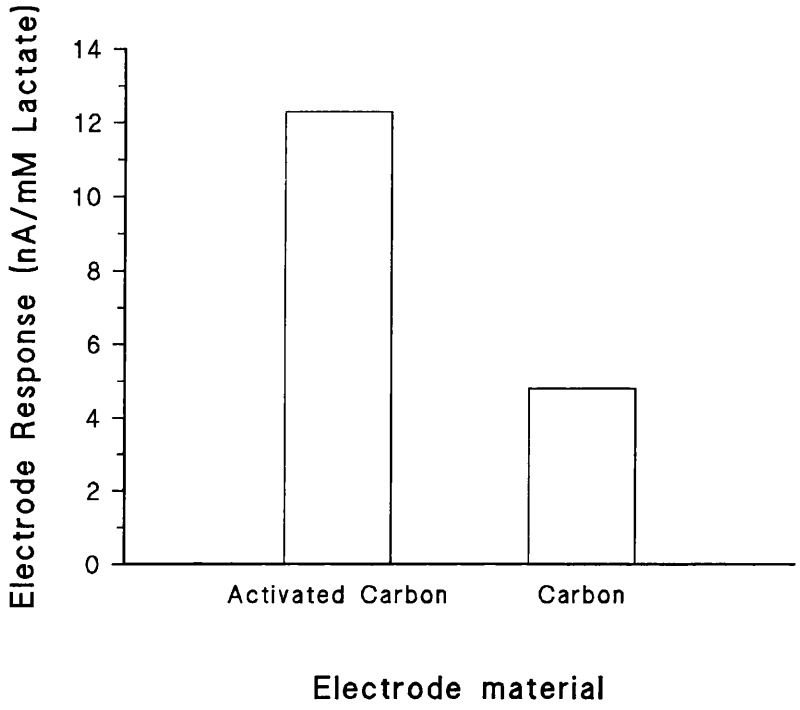


Figure 5.8: *Comparison of responses of reagentless enzyme electrodes for lactate prepared using different electrode materials.* *Electrode preparation:* activated carbon particles (25mg) were mixed with 0.25ml LDH or LDH-N^{6}AHCM-NAD in 100mM phosphate buffer, pH 7.5 and spun down in a microfuge (5 mins). A commercial teflon binder material (0.04ml) was added to the pellet and mixed to form a paste. The paste was compressed onto the surface of a carbon support material ('Toray' paper) using a glass slide and left to dry for 15mins. A disc (0.2cm in diameter) was cut out and inserted into the linings of a working electrode The cell halves were assembled (reference = Ag/AgCl) and a potential of +150mV was applied to the cell, which was then left to attain a steady current, before lactate samples in PESK buffer (*Materials and Methods*) were added to the sample cell, and the control and normal response recorded on a twinned-pen chart recorder.

5.4.2 Reproducibility of the lactate response

The reproducibility of the lactate response generated by the same enzyme electrode could not be measured because of an instability effect (see below). However, *Table 5.2* shows the reproducibility of the response to 10mM lactate constructed by using three different fresh preparations of LDH-N^{6}AHCM-NAD adsorbed to activated carbon.

It is clear that there was a definite increase in response to 10mM lactate due to the covalent immobilisation of NAD onto molecules of LDH.

Table 5.2

Reproducibility of lactate response

Preparation	Control response	Uncorrected response	Corrected response
A	7.9	20.5	12.6
B	6.8	20.7	13.9
C	9.0	19.2	10.2
Mean	7.9	20.1	12.2
S.D.	1.1	0.82	1.8

Control response = response from electrode prepared with native LDH
Uncorrected response = response from electrode prepared with LDH-N^{6}AHCM-NAD
Corrected response = Uncorrected response - control response
Electrode preparation: activated carbon particles (25mg) were mixed with 0.25ml LDH or LDH-N^{6}AHCM-NAD in 100mM phosphate buffer, pH 7.5 and spun down in a microfuge (5 mins). A commercial teflon binder material (0.04ml) was added to the pellet and mixed to form a paste. The paste was compressed onto the surface of a carbon support material ('Toray' paper) using a glass slide and left to dry for 15mins. A disc (0.2cm in diameter) was cut out and inserted into the linings of a working electrode The cell halves were assembled (reference = Ag/AgCl) and a potential of +150mV was applied to the cell, which was then left to attain a steady current, before lactate samples in PESK buffer (*Materials and Methods*) were added to the sample cell, and the control and normal response recorded on a twinned-pen chart recorder.

5.4.3 Electrode stability

After the electrode material prepared using the LDH-N^{6}AHCM-NAD conjugate was subjected to 6 days storage in PESK buffer at 4^{o}C a cell was again constructed and the response of the electrode to lactate concentrations between 0.5 and 10mM measured. The response was again rapid and linear, but had decreased in size to 7.2nA, 60% of the original response. Desorption of enzyme heavily contributed towards this instability (*Figure 5.2*). However, the half-life of the electrode material was dramatically reduced when the enzyme-coenzyme conjugate paste was incorporated into a polarised electrode, with an overpotential of +150mV.

The responsive enzyme electrode prepared using LDH-N^{6}AHCM-NAD adsorbed onto activated carbon particles demonstrated a pronounced lack of stability when used for extensive periods. The instability did not depend on whether activated carbon or untreated carbon was used as the electrode base.

For example, the enzyme electrode that initially gave rise to the response shown in *Figure 5.7* was also tested after 3 hours of polarisation. A comparison of the corrected responses, before and after polarisation is given in *Figure 5.9.* As can be seen, the response to 10mM lactate was very much reduced, in fact, to only 28% of the original response, indicating a half-life of 1.5 hours.

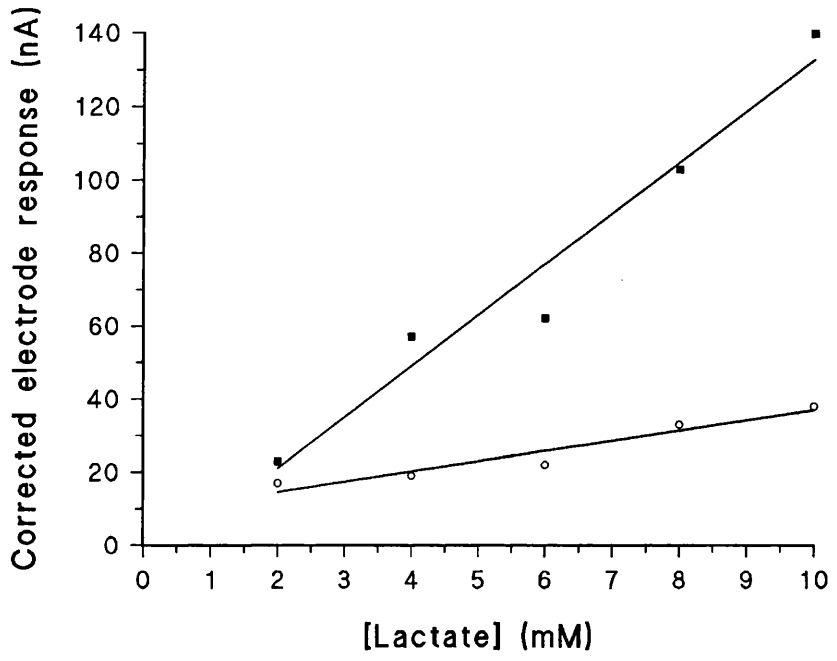


Figure 5.9: *Comparison of enzyme electrode response to lactate after 3 hours of polarisation at an overpotential of 150mV vs Ag/AgCl.* ■ = before polarisation; O = after polarisation. *Electrode preparation:* activated carbon particles (25mg) were mixed with 0.25ml LDH or LDH-N^6AHCM-NAD in 100mM phosphate buffer, pH 7.5 and spun down in a microfuge (5 mins). A commercial teflon binder material (0.04ml) was added to the pellet and mixed to form a paste. The paste was compressed onto the surface of a carbon support material ('Toray' paper) using a glass slide and left to dry for 15mins. A disc (0.2cm in diameter) was cut out and inserted into the linings of a working electrode The cell halves were assembled (reference = Ag/AgCl) and a potential of +150mV was applied to the cell, which was then left to attain a steady current, before lactate samples in PESK buffer (*Materials and Methods*) were added to the sample cell, and the control and normal response recorded on a twinned-pen chart recorder.

The response of a fresh electrode preparation to 10mM lactate was measured repeatedly at ten minute intervals. As *Figure 5.10* shows a half-life of 1.5 hours was confirmed.

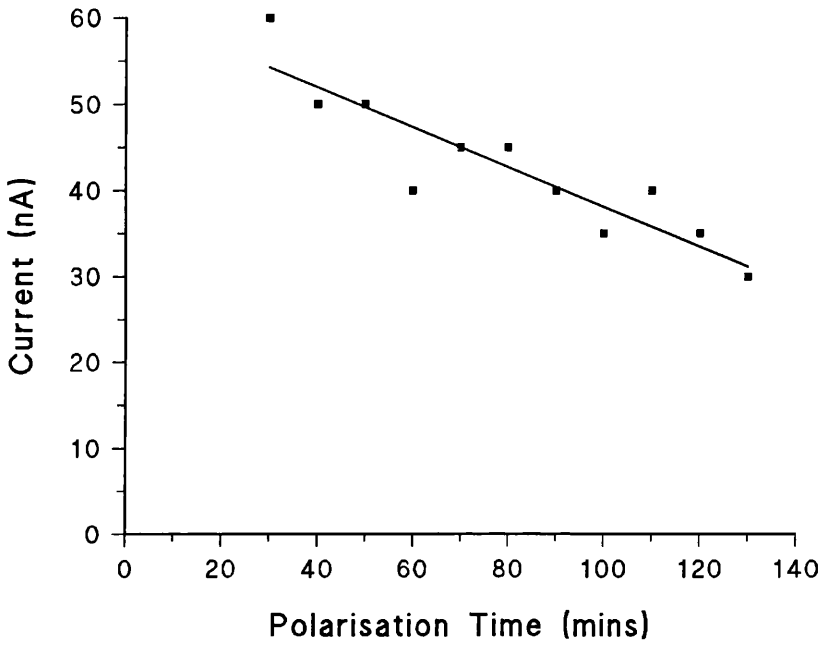


***Figure 5.10**: Instability of enzyme electrode response to repeated applications of 10mM lactate.* *Electrode preparation:* activated carbon particles 25mg were mixed with 0.25ml LDH or LDH-N^6AHCM-NAD in 100mM phosphate buffer, pH 7.5 and spun down in a microfuge (5 mins). A commercial teflon binder material (0.04ml) was added to the pellett and mixed to form a paste. The paste was compressed onto the surface of a carbon support material ('Toray' paper) using a glass slide and left to dry for 15mins. A disc (0.2cm in diameter) was cut out and inserted into the linings of a working electrode. The cell halves were assembled (reference = Ag/AgCl) and a potential of +150mV was applied to the cell, which was then left to attain a steady current, before lactate samples in PESK buffer (*Materials and Methods*) were added to the sample cell, and the control and normal response recorded on a twinned-pen chart recorder.

Further investigations were then carried out into the cause of this stability. An LDH-N^6AHCM-NAD activated carbon paste disc was inserted into the lining of each working electrode; both electrodes were polarised with an overpotential of +150mV. The response of each electrode to 5mM lactate was measured; one electrode was then disconnected from the potentiometer, whilst the other remained polarised, and each electrode left for 5 hours. No electrode response to 5mM lactate could be obtained from the polarised electrode after 5 hours, whereas the response from the unpolarised electrode remained the same (*Table 5.3*). Upon addition of a solution of 3mM NAD in PESK buffer, a steady response of 110nA was obtained in the polarised electrode, indicating that the immobilised enzyme was still fully active, because the current

obtained from the fresh electrode in the presence of 3mM NAD was 120nA *(Table 5.3)*.

Table 5.3
The effect of an applied overpotential of +150mV upon the intrinsic and specific activities of immobilised LDH-N^{6}AHCM-NAD

Polarisation time (hrs)	Current change (nA)	
	+ 5mM lactate	+ 5mM lactate + 3mM NAD
0.5	50 (57)[a]	120 (109)
5.0	2 (49)	110 (103)

[a] Figures in brackets represent response of a similar working electrode that was left unpolarised overnight.
Responses shown for LDH-N^{6}AHCM-NAD activated carbon paste electrode after polarisation time shown.*Electrode preparation:* activated carbon particles (25mg) were mixed with 0.25ml LDH or LDH-N^{6}AHCM-NAD in 100mM phosphate buffer, pH 7.5 and spun down in a microfuge (5 mins). A commercial teflon binder material (0.04ml) was added to the pellet and mixed to form a paste. The paste was compressed onto the surface of a carbon support material ('Toray' paper) using a glass slide and left to dry for 15mins. A disc (0.2cm in diameter) was cut out and inserted into the linings of a working electrode. The cell halves were assembled (reference = Ag/AgCl) and a potential of +150mV was applied to the cell, which was then left to attain a steady current, before lactate samples in PESK buffer (*Materials and Methods*) were added to the sample cell, and the control and normal response recorded on a twinned-pen chart recorder.

As *Table 5.3* shows the instability of the electrode was only increased when an overpotential was applied to the cell. The specific activity of the enzyme moiety of the LDH-N^{6}AHCM-NAD conjugate was unaffected by the applied overpotential, indicating that the loss of intrinsic activity of the enzyme-coenzyme conjugate was due to a loss in functioning coenzyme.

CHAPTER 6

Conclusions

The adenine-modification of NAD can be used to introduce functional groups that can improve the coupling chemistry of the coenzyme with respect to its conjugation to proteins. To this end, N^6CM-NAD and N^6AHCM-NAD were synthesised and the properties of the final derivatives were in good agreement with those found in previous similar syntheses (Lindberg *et al*, 1973). The derivatives showed good substrate and recycling activity with both ADH and LDH, both activities ranging between 52 and 88% of the corresponding activities of unmodified NAD. The K_M of LDH for each of the free coenzyme derivatives was three times larger than the corresponding K_M for NAD, but past enzyme-coenzyme conjugate studies have shown that the behaviour of an enzyme towards a coenzyme derivative can change dramatically upon the covalent linkage of the derivative to the enzyme surface (Nakamura *et al*, 1986). Hence, although the affinity of LDH for NAD in free solution was slightly reduced upon modification of the coenzyme, the effect of the covalent binding of the NAD derivatives in the vicinity of the active site of the enzyme may improve the substrate activity of each of the derivatives.

Table 6.1

The effects of various coupling techniques upon the specific activity of the enzyme

Enzyme	Coupling method[b]			
	Glutaraldehyde	Carbodiimide	Terephthalaldehyde	"disulphide"[c]
LDH	12-22[a]	41-79	--	30-49
ADH	0.0-0.3	4-6	100	--
GDH	40-100	41-86	--	--

[a] in units of μmoles/min/mg protein
[b] used to couple NAD, N^6AHCM-NAD (and N^6CM-NAD)
[c] Involves the modification of lysyl residues with Traut's reagent, followed by DPDS modification, followed by modification with thiolated coenzyme molecules

Upon modification of a dehydrogenase using glutaraldehyde or carbodiimide coupling techniques, a reduction in the specific activity of the enzyme generally occurred, but

this was usually not serious (*Table 6.1*). A notable exception was the glutaraldehyde modification of yeast ADH, with an almost total loss of enzyme activity occurring after modification. It was subsequently found that the aromatic dialdehyde, terephthalaldehyde, may provide a good alternative to glutaraldehyde as a coupling reagent in cases where glutaraldehyde is not suitable.

There was no detectable loss in specific activity of the yeast ADH after modification with terephthalaldehyde. Previous dehydrogenase modification studies using pyridoxal-5-phosphate go some way to explaining the loss in activity of the dehydrogenases after modification with glutaraldehyde as the mechanism of reaction of pyridoxal-5-phosphate with lysyl residues is thought to be the same as that of glutaraldehyde. However, glutaraldehyde also reacts with other amino acid residues such as cysteine, histidine and tyrosine (Habeeb and Hiramoto, 1968). Similarly, carbodiimide compounds are also known to react with residues other than glutamate or aspartate and so the loss in specific activity cannot be totally put down to modification of sensitive acidic residues. Chemical modification using glutaraldehyde and carbodiimide is random in nature and, although they are established coupling reagents, the reason for any loss of specific activity cannot be clearly defined. In this respect the "disulphide" coupling approach taken in *Chapter 4* is preferable: it is a more specific coupling technique.

In order to establish, firstly, the number of coenzyme molecules incorporated onto an enzyme surface and, secondly, the intrinsic activity of an enzyme-coenzyme conjugate, the isolation of the conjugate from all traces of free coenzyme must first be confirmed. During the preparation of each enzyme-coenzyme conjugate a "baseline" separation of the protein material from free coenzyme on a Sephacryl S-100 gel filtration column was sufficient to ensure that there were no traces of free coenzyme that were detectable by the sensitive coenzyme-recycling assay of Nisselbaum and Green (1969). In addition, the conjugates were washed with activated charcoal which removed any non-covalently bound coenzyme that was firmly bound to the enzyme by electrostatic, hydrophobic or other forces. It was established that any coenzyme that was detected in any of the assays used was covalently bound.

From the analysis of the total number of bound coenzyme molecules per enzyme subunit, it could be inferred that, proportionately, more GDH and ADH residues were accessible for coupling than LDH residues. The difference in average coenzyme incorporation of the GDH subunit could not be accounted for by its larger molecular weight (56,000) compared to LDH (35,000) or ADH (36,000). The table below shows the average number of coenzyme molecules incorporated per 1000 molecular weight of subunit for the glutaraldehyde and carbodiimide coupling of N^6AHCM-NAD to GDH, ADH and LDH. It must be noted that past studies with malate dehydrogenase have shown the chemical modification of enzymes with coenzymes to be a random process, with not all enzyme subunits having coenzyme incorporated to an equal extent (Eguchi *et al*, 1986). The "total" molar ratio of coenzyme to enzyme subunit can only be taken as an average value. The coupling of N^6AHCM-NAD was indicative of the proportion of incorporation of the other coenzymes used, NAD and N^6CM-NAD.

Table 6.2

Incorporation of N^6AHCM-NAD into various dehydrogenases using glutaraldehyde or carbodiimide-mediated coupling techniques

Enzyme	Coupling method	
	Glutaraldehyde	Carbodiimide
LDH	0.05	0.03
ADH	0.10	0.13
GDH	0.12	0.12

Values represent the number of N^6AHCM-NAD molecules incorporated per 1000 MW of subunit.They were estimated by measuring the 266/290nm absorbance ratio of an enzyme-coenzyme conjugate solution

As can be seen, despite the hexameric contacts formed between surfaces of the glutamate dehydrogenase subunits compared to the tetrameric contacts of the LDH subunit surfaces, proportionately more lysine and acidic residues were available for coupling on a GDH subunit surface. Glutaraldehyde and carbodiimide coupling methods did not differ in their success with respect to coenzyme incorporation. In general, more N^6AHCM-NAD molecules were incorporated than either NAD or N^6CM-NAD molecules using glutaraldehyde, terephthalaldehyde, carbodiimide and

the "disulphide" coupling techniques. The reactive amine function of this derivative enabled the coenzyme to be used in all of these couplings. The long "hexyl" spacer arm of N^6AHCM-NAD is 1.1nm longer than the spacer arm of N^6CM-NAD and 1.4nm longer than NAD, which does not possess a spacer arm. The longer spacer arm of N^6AHCM-NAD could have enabled the terminal amine function of the coenzyme to act as an effective probe, making it more accessible to enzyme residues in folds and clefts on the globular surface of the enzyme molecule. The amine of N^6AHCM-NAD was also more reactive than the amine function of NAD, which is subject to the electron delocalisation and steric hindrance effects caused by the proximity of the purine ring. The coupling experiments performed using N^6AHCM-NAD served to further establish the usefulness of this coenzyme as an activated NAD derivative.

Although carbodiimide-mediated reaction is promoted by an excess of nucleophilic amine there was no obvious difference in the incorporation of N^6CM-NAD or N^6AHCM-NAD (*Table 6.3*). In the use of N^6CM-NAD in carbodiimide-mediated coupling experiments, the coenzyme was itself activated and in excess of the nucleophilic amine of the enzyme subunits, this did not seem to influence the success of carbodiimide-mediated amide bond formation.

Table 6.3
Difference in incorporation of carboxyl- and amine-containing NAD derivatives onto enzyme subunit surfaces using carbodiimide coupling

Enzyme	Coenzyme	
	N^6CM-NAD	N^6AHCM-NAD
LDH	1.8	1.0
GDH	5.9	6.8
ADH	1.5	4.5

Considering the reported accessibility of lysyl, aspartyl and glutamyl residues of LDH it is not known why only a small fraction of them was available for coupling with coenzymes. Over half of the 24 lysyl residues of pig heart LDH are completely exposed. However, despite a large excess of coupling reagent and coenzyme, no more than two of these residues reacted with glutaraldehyde or carbodiimide to incorporate

coenzyme onto the enzyme surface. Incorporation of coenzyme was slightly greater using terephthalaldehyde; the small aromatic molecule may have reached lysyl residues that the polymeric aliphatic glutaraldehyde molecule could not, but still no more than three lysyl residues were modified. The apparent low accessibility of surface residues was also found in the case of the GDH and ADH coenzyme-modification studies; far more lysyl and acidic residues were expected to be accessible to modification than were actually modified. Low coenzyme incorporation has also been observed in past enzyme-coenzyme conjugate studies, with often less than one coenzyme being incorporated per subunit. One reason for this may be the hindrance of the approach of coupling reagent or coenzyme derivative by surrounding enzyme residues. This hindrance may be steric or may be due to hydrophobic or charge repulsion effects. For example, the approach of the hydrophobic spacer arm of N^6AHCM-NAD towards a carbodiimide-activated acidic residue may be repelled by the surrounding hydrophilic surface residues. Even though a lysyl or acidic residue may be exposed, the reactivity of the residue may be reduced by the conditions of the microenvironment created by surrounding enzyme residues. This has indeed been known to happen in previous protein modification studies (Lundblad and Noyes, 1984) and this may be another reason which explains the low incorporation of coenzyme molecules. It can also be envisaged that, when one or two bulky coenzyme molecules have been covalently bound to a subunit they form physical or chemical barriers to the approach of further coenzyme derivatives towards the appropriate subunit residues. Whatever the reason for the low coenzyme incorporation, the formation of a successful enzyme-coenzyme conjugate with high intrinsic activity is not necessarily related to the number of incorporated coenzyme molecules. All coenzymes were incorporated to some extent using the coupling methods employed in this study and this led to the chance that some molecules would bind near the active site where they would be available for interaction with the coenzyme binding site, and would therefore form enzyme-coenzyme conjugates with high intrinsic activity.

An "active" molar ratio of covalently-bound coenzyme to enzyme subunit was determined to indicate the activity of the bound coenzymes with neighbouring enzyme molecules, in other words the intermolecular substrate activity of the bound coenzymes. On the whole, a substantial proportion of bound coenzyme molecules were

intermolecularly active, proving that coenzyme molecules bound to one enzyme can act as immobilised coenzymes for other enzyme reactions. Initially, it was thought that the success of the intermolecular activity of a bound coenzyme would be related to the length of the spacer arm separating it from the surface of the enzyme anchor, the longer spacer arm allowing the coenzyme to protrude further into the surrounding solution where it would be more available for interaction with the active sites of neighbouring enzyme molecules. However, the intermolecular activity of a bound coenzyme did not seem to be dependent on any one factor. For instance, the long spacer arm of N^6AHCM-NAD did not seem to make the NAD moiety any more accessible for interaction with a neighbouring enzyme molecule than NAD or N^6CM-NAD. The same could be said for the spacer arm of N^6CM-NAD compared to the non-existent spacer arm of NAD. The lower substrate activity of each of the NAD derivatives was taken into account during the calculation of intermolecular substrate activity and so this could not explain why the longer spacer arms did not provide a better interaction between the bound NAD moiety and surrounding enzyme molecules. If we look at the availability of carbodiimide-coupled LDH-coenzyme conjugates we can see that 100% of the 0.3 NAD molecules bound, on average, per subunit were available for reaction with ADH. However, only 20% of the 1.8 N^6AHCM-NAD molecules bound per subunit and only 17% of the 1.0 N^6CM-NAD molecules were available for reaction with a second enzyme. The NAD molecules were likely to bind to the most accessible activated residue on the LDH surface whereas the spacer arms of the NAD derivatives made them more accessible to other more buried activated residues. The availability of covalently bound N^6AHCM-NAD, for example, to a neighbouring enzyme molecule could be explained if the residue anchoring the bound NAD molecule was the only position that was accessible to neighbouring enzyme molecules, and only 20% of the N^6AHCM-NAD molecules were bound at this position. The intermolecular activity of the bound coenzyme molecules may be interesting as the enzyme-coenzyme conjugates may be used in this way as coupled regeneration units, for example, in enzyme reactors. It also shows that the bound coenzyme molecules may be available to interact with an electrode surface when the enzyme-coenzyme conjugates are immobilised in the close vicinity of the potential gradient of an electrode. However, it must be noted that, with respect to enzyme electrodes there was also a requirement for an intramolecular activity of the

conjugates. The covalently bound coenzymes that can interact with an electrode surface must also be able to interact with the active site of a neighbouring enzyme-coenzyme molecule or the active site of the enzyme to which it is bound to, in order for coenzyme regeneration and the generation of a physicochemical signal (reduced coenzyme) to occur. However, intramolecular activity would probably be more successful because the enzyme molecules were not free to diffuse and interact when immobilised at the electrode surface and thus an intermolecular reaction would be restricted.

By using the kinetic method first applied by Mansson *et al* (1978) it was possible to determine a predominantly intramolecular mechanism for the intrinsic activity of the glutaraldehyde-coupled LDH-N^{6}AHCM-NAD conjugate of Gacesa and Venn (1979). Thus, the method of Gacesa and Venn used to couple coenzyme to LDH was firmly established and suggestions made by Eguchi *et al* (1986) that conjugate formation in this particular case was dubious, were refuted. The glutaraldehyde-coupled LDH-N^{6}AHCM-NAD conjugate adds to the increasing list of other enzyme-coenzyme conjugates that have an intramolecular mechanism for their intrinsic activity: ADH-N^{6}AHCM-NAD coupled via carbodiimide (Mansson *et al*, 1978), GlcDH-PEG-NAD (Nakamura *et al*, 1986) and GlcDH-SPDP-N^{6}AHCM-NAD (Persson *et al*, 1991). The presence of an intramolecular mechanism proved for the LDH-N^{6}AHCM-NAD conjugate that some of the bound molecules were able to interact with the active site. The intermolecular mechanism was probably less dominant for two reasons: firstly, the conjugated coenzyme was covalently bound to the enzyme and was therefore in a permanent close contact with the active site of the enzyme molecule. It therefore has more chance of reacting than coenzymes that are covalently attached to neighbouring enzyme molecules which are freely diffusing through solution. Secondly, the bound coenzyme probably interferes with the reaction between the active site of an enzyme and coenzyme molecules attached to surrounding enzyme molecules.

Because the mechanism of intramolecular and intermolecular activity is different, a direct comparison could not be made between the activity of an enzyme-coenzyme conjugate and the activity of an unmodified enzyme with a corresponding concentration ratio of free coenzyme to enzyme subunit. The chances of an enzyme-

coenzyme molecule with an intramolecular mechanism reacting with substrate is independent of the enzyme concentration in solution, but an unmodified enzyme molecule with a corresponding ratio of free coenzyme in solution has more chances of interacting with coenzyme with a higher enzyme concentration. In other words, the two systems cannot be compared because one is dependent upon the enzyme concentration, while the other is not. However, we can compare activity at a defined concentration of enzyme to give us a comparison of the efficiency of the two systems. For example, as was seen in *Figure 3.24,* at an enzyme subunit concentration of less than 63μM (2mg/ml), the LDH-N^6AHCM-NAD conjugate was more efficient in terms of μmoles lactate converted / min / mg protein, despite the modified enzyme having only 12% of the specific activity of the unmodified enzyme. Above this concentration the unmodified enzyme in the presence of free coenzyme became more efficient. Despite the efficiency of the LDH-N^6AHCM-NAD conjugate, below a certain enzyme concentration the intrinsic activity of the conjugate was only 2.5% of the activity of the conjugate in the presence of exogenously added coenzyme. In order to realise fully the potential of enzyme-coenzyme conjugates it was hoped that the intrinsic activity of the conjugates would have been comparable to the specific activity of the conjugate. The enzyme-coenzyme conjugate with the highest proportion of intrinsic activity to specific activity was the GDH-NAD conjugate formed using glutaraldehyde, with the intrinsic activity of this conjugate representing 20% of its specific activity. The activity of this conjugate was also compared to the activity of native GDH in the presence of a corresponding ratio of free coenzyme, both enzymes being present in a recycling assay at 0.2mg/ml. The conjugate was ten times more active at the defined concentration than the native enzyme with an equal ratio of coenzyme (exogenously added) per enzyme subunit.

Despite the greater efficiency of the enzyme-coenzyme conjugates at a lower enzyme concentration, the fact remains that a full interaction of covalently bound coenzyme with the enzyme active site does not seem to have been realised in any of the conjugates synthesised in this study. In order to understand why this was the case we shall now consider the intrinsic activity of the LDH-coenzyme conjugates in relation to their spacer lengths. The proximity of suitable lysyl residues to the coenzyme binding site of pig heart LDH was discussed in *Section 3.10.* The ε-amine groups of the most

suitable residues were 1.3nm (Lys 121) and 1.2nm (Lys 83) away from the N^6 amine of an NAD molecule catalytically bound at the active site. This distance was thought to reflect the approximate length of a suitable spacer arm. Indeed, the minimum limit for the optimum length of a spacer arm was set at 1.2nm by Warth *et al* (1989) using pig heart LDH. The length of the spacer arm of N^6AHCM-NAD coupled using glutaraldehyde was at least 2.1nm but the intrinsic activity of the conjugate was only 0.12 U/mg - only 2.5% of the specific activity of the conjugate. The spacer arm, in this case, may have been too long and not flexible enough too allow the NAD moiety to interact with the active site. It seems more likely, however, that most of the 1.8 N^6AHCM-NAD molecules bound per subunit did not bind at one of the desired lysines. Chemical modification of enzymes to introduce coenzymes, especially using glutaraldehyde which polymerises readily in solution, is a very random approach and it may well be that a more defined approach is needed in order to fully realise the potential of the enzyme-coenzyme conjugate. The spacer length of the glutaraldehyde-coupled LDH-NAD molecule was at least 1.2nm because an intrinsic activity was obtained at 0.09U/mg, 4% of the specific activity of the modified enzyme. This proves that the glutaraldehyde polymerises to a certain extent as a single glutaraldehyde molecule would only introduce to an LDH-NAD conjugate a spacer arm of 0.7nm in length and would not allow the bound coenzyme to interact with the active site. If we look at carbodiimide-coupled conjugate formed between activated coenzyme carboxyl groups and lysyl residues of LDH, it can be seen that the intrinsic activity was less than 0.2% of the specific activity of the modified enzyme. This reflects that the 0.3nm spacer of LDH-N^6CM-NAD is too short to allow the lysine-bound coenzyme to interact with the active site of LDH. The small intrinsic activity observed was probably due to an intermolecular reaction mechanism. In *Section 3.10* it was also found that the carboxyl carbon atom of each of 3 accessible acidic residues were within 1.0-1.4nm of the adenine moiety of an NAD molecule catalytically bound at the active site. As expected, the LDH-NAD conjugate coupled via carbodiimide which did not have a spacer arm had an intrinsic activity that was less than 0.2% of the specific activity of the modified enzyme, but so did the LDH-N^6AHCM-NAD conjugate, which had a spacer arm of 1.4 nm. It would seem that the coenzyme did not bind to any of the suitable acidic residues described.

Attempts at forming enzyme-coenzyme conjugates via a disulphide bond were made to observe the effect of a higher incorporation of coenzyme, and to see if the specific reaction of cystyl residues with thiol-containing coenzyme derivatives would lead to the formation of enzyme-coenzyme conjugates with intrinsic activity. In an effort to form a thiolated NAD derivative the inertness of the N^6amine group of NAD was demonstrated: all attempts to react NAD with the iminium-activated carbon of 2-iminothiolane failed despite attempts to vary pH, temperature, excess of 2-iminothiolane and reaction volume. Attempts were also made to modify NAD using the *N*-hydroxysuccinimide-activated carbon of *N*-hydroxysuccinimidyl-*S*-acetylthio-acetate. Again the coenzyme failed to react, probably due to the steric hindrance or poor nucleophilicity of the amine group. It seems a defined reaction of the amine is difficult to achieve, but Dimroth rearrangement of N-1 modified NAD is now possible under mild reaction conditions and this may be an alternative way in which the unmodified coenzyme can be thiolated (Buckmann, 1987).

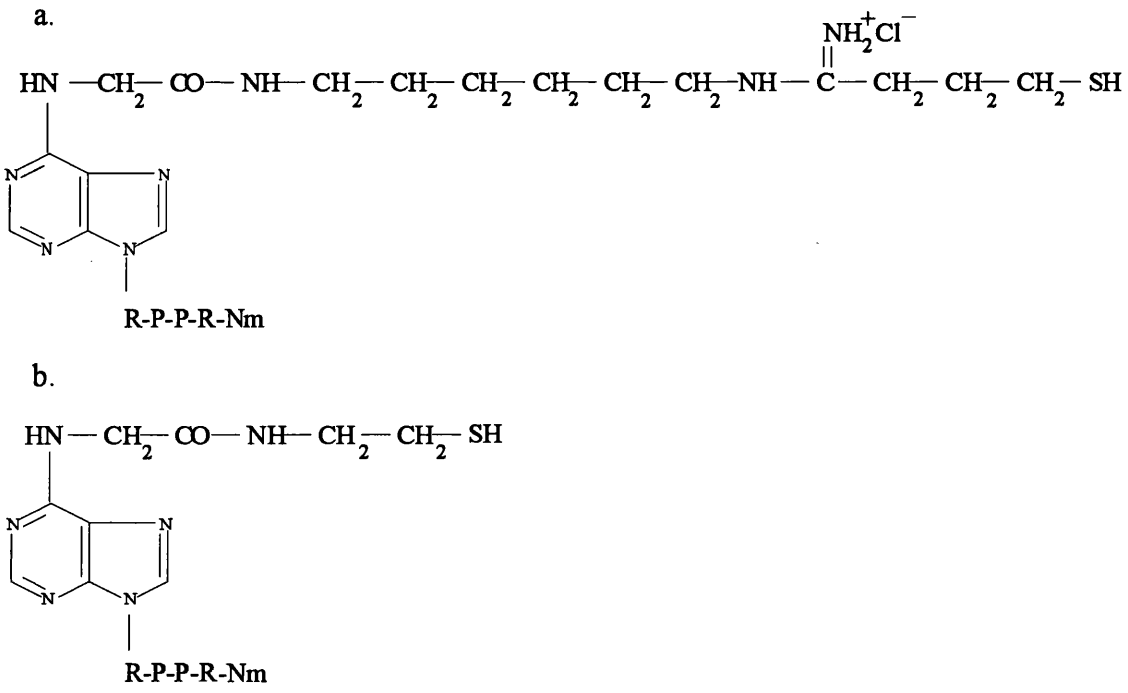


Figure 6.1: *The structures of thiol-containing NAD derivatives, (a.) N^6thiobutyrimidate-[(aminohexyl)carbamoylmethyl]-NAD and (b.) N^6(2-thioethane)-1-carboxamido-methyl-NAD*

The aliphatic amine group of N^6[(aminohexyl)carbomylmethyl]-NAD was more reactive with 2-iminothiolane than the amine of NAD. 37% of the N^6AHCM-NAD

molecules were thiolated, although only limited attempts were made to optimise the reaction conditions. The proposed structure of the new derivative is shown in *Figure 6.1.* The thiolated coenzyme has a total spacer length of 2.2nm consisting of a hexyl and propyl stretch with an intervening amidine bond and a terminal thiol group. A different modification was carried out to thiolate the carboxyl-function of N^6CM-NAD: an excess of 2-aminoethanethiol was added in the presence of a small amount of carbodiimide to promote the formation of an amide bond between the coenzyme carboxyl function and the amine function of 2-aminoethanethiol. The extent of thiolation was 51% and the structure of the thiolated coenzyme is shown in *Figure 6.1*; the total spacer length is 0.9nm.

The substrate activity of each of the thiolated coenzyme derivatives was determined at saturating concentrations in a recycling assay with ADH. Upon thiolation, the substrate activity of N^6AHCM-NAD was reduced from 51% of the substrate activity of unmodified NAD to 31%. Similarly, the thiolated N^6CM-NAD had 23% of the substrate activity of NAD compared to a value of 77% for N^6CM-NAD alone. These thiol-conataining NAD derivatives have a better substrate activity with ADH than the SPDP-N^6AHCM-NAD dervative described by Persson *et al* (1991) did with glucose dehydrogenase, but glucose dehydrogenase is known to be sensitive to changes at the adenine position of NAD (Nakamura *et al*, 1986). The introduction of a thiolated methylene chain at the amine and carboxyl functions leads to a further decrease in substrate activity compared to the original derivatives. We can conclude that the extension of the spacer arm weakens the efficiency of the enzyme-coenzyme interaction perhaps by interfering unfavourably with the enzyme residues surrounding the coenzyme binding site of ADH. However, the thiolated coenzymes have significant substrate activity and they may be useful for the specific and reversible anchorage of coenzymes to thiol residues in coenzyme immobilisation applications. In our study it was possible that the covalent anchorage of the coenzymes to the enzyme molecule might improve the interaction and increase the substrate activity of each coenzyme derivative.

In order to form successful enzyme-coenzyme conjugates using the thiolated coenzymes it was essential that the dehydrogenases used were not seriously inhibited upon modification with thiol-containing adducts. In order to investigate this, the activity of each of several dehydrogenases was compared before and after modification with dipyridyldisulphide (DPDS). None of the native cystyl residues of LDH were completely accessible for reaction with DPDS and this may explain the small loss of activity of LDH upon treatment with DPDS despite reports that LDH was inhibited in the presence of maleimide. This study supports past observations that inhibition by thiol modification in dehydrogenases depends, to a large extent, on which thiolation reagent is being used (Smith *et al*, 1975). Because cystyl residues of the pig heart LDH were not accessible to any significant extent, the thiolation of lysyl residues was carried out in order to enable the increased incorporation of thiolated coenzyme. The potential of this method was demonstrated using BSA. Out of 64 lysyl residues, 31 had detectable thiol groups after BSA modification, reflecting the availability of lysyl residues on the protein surface to small molecules like 2-iminothiolane. Although the same proportion (48%) of modified lysyl residues was not recorded for LDH, 7.6 lysines were modified out of 24 (31%). This was higher than the coenzyme incorporation achieved using glutaraldehyde and carbodiimide reagents, demonstrating that the potential of the lysyl residues as anchorage sites had not been fully realised. The incorporation of a large number of thiol groups was confirmed when DPDS was used to activate the thiol groups for coupling with thiolated coenzyme molecules under mild conditions. It was consequently concluded that the thiol groups were in a largely accessible state before modification with DPDS and had not become involved in interaction with side chain residues of the enzyme. However, when the DPDS-activated thiol groups were added to thiolated coenzyme molecules, only a small proportion of the DPDS-thiol groups reacted: on average, 3.3 HS-N^6AHCM-NAD molecules and 4.2 HS-N^6CM-NAD molecules were incorporated onto the surface of BSA, representing modification of 12 and 18% of the DPDS activated thiol groups, respectively. The coenzyme incorporation was slightly improved in the case of LDH, with 2.7 HS-N^6AHCM-NAD and 2.3 HS-N^6CM-NAD molecules being incorporated per LDH subunit, representing modification of 38% and 32% of the available DPDS-thiol residues. The low incorporation can be partly explained by the fact that only a 15-fold excess of thiolated coenzyme to enzyme subunit was used; however it is still not clear

why more residues did not react. Modification of the introduced thiol groups with DPDS would have led to the structure shown below.

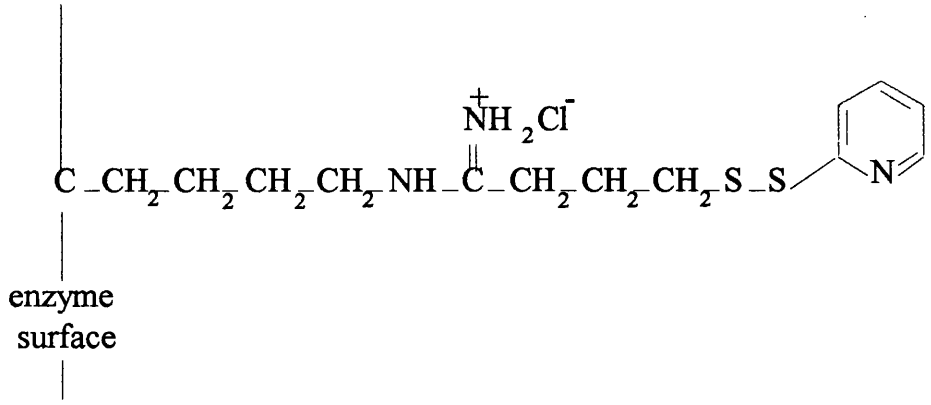


Figure 6.2: *Structure of a PDS-thiolated lysine residue.* Original lysine shown in blue

The DPDS-activated thiol seems very accessible to the surrounding solvent. It may be that the long spacer arm "flops about" too much and eventually finds a place on the enzyme surface where it forms hydrophilic, hydrophobic or electrostatic interactions with side chain residues of the enzyme surface which means that the pyridyl-2-disulphide (PDS) group does not protrude into solution. Although the availability of the PDS-thiol groups was confirmed on reduction with DTT, it could be envisaged that the thiolated coenzyme molecule was not as accessible as DTT to the same areas of the enzyme surface. This explanation is a speculative one but the evidence showed that the reactivity of the PDS-thiol and coenzyme thiol groups were not to blame- the problem was one of accessibility. It was also possible that the bound coenzyme molecules hindered the approach of other coenzyme molecules towards activated thiol groups on the enzyme surface. Attempts were made to activate the thiolated coenzyme with DPDS in an attempt to react it with free thiol group on the enzyme surface, but the extra loss of coenzyme during the purification step of the coenzyme that was required proved too costly: not enough PDS-thiol coenzyme could be obtained in a pure form.

Despite the incorporation of coenzyme being lower than expected using the "disulphide" coupling technique, the incorporation of coenzyme onto the surface of LDH was higher than with glutaraldehyde and carbodiimide coupling methods, and

was also slightly more defined in that any loss in specific activity of the conjugate could be attributed to thiol modification. An intermolecular substrate activity of the coenzymes coupled to BSA and LDH was evident. 74-100% of the covalently bound N^6CM-NAD molecules, but only 24-33% of the N^6AHCM-NAD molecules, were accessible to a second enzyme, emphasising that the intermolecular activity of an enzyme-coupled coenzyme does not depend on spacer length.

The intrinsic activity of the "disulphide" coupled LDH-N^6CM-NAD conjugate was 5% of the specific activity of the modified enzyme, but the intrinsic activity of the LDH-N^6AHCM-NAD conjugate was only 0.6% of its specific activity. The total length of the spacer arm of the LDH-N^6CM-NAD conjugate was 1.9nm, measured from the ε-amine of the lysine anchor to the N^6amine of the NAD molecule. This was greater than the minimum spacer length of 1.2nm determined by the distance of the nearest accessible lysyl residue to the active site. An intramolecular mechanism was probable for the intrinsic activity of the LDH-N^6CM-NAD conjugate because the percentage of specific activity seemed too high for an intermolecular mechanism as the coenzyme was present at a concentration of 30 times less than the K_M of LDH for free NAD. The spacer length of the LDH-N^6AHCM-NAD conjugate was 3.2nm, and may have been too long to allow the NAD moiety to interact with the coenzyme binding site.

Although thiol modification has shown how many thiols can be introduced onto the surface of LDH without a serious loss in enzyme activity, a way of coupling coenzyme to a significant proportion of these thiols was not found. The chances of one of the thiolated lysines that are proximal to the active site being modified with thiolated coenzyme remained low.

Although there is a relationship between the length of the spacer arm and the intrinsic activity of the conjugate, spacer arms that are sufficient in length do not seem to allow a full interaction with the enzyme active site. Because the chemical modification of enzymes is undefined and rather random in nature there is no easy way of knowing whether this was because the coenzymes were unable to bind near enough to the active site. After these studies it appeared that the chemical modification technique to covalently bind coenzymes to enzymes was not sufficient to fully realise the potential

of enzyme-coenzyme conjugates. In order for enzyme-coenzyme conjugates to be fully exploited for use in enzyme electrodes, the intrinsic activity should approach the specific activity of the enzyme-coenzyme conjugate in order to generate a maximum physicochemical signal. Only two of the past chemical modification studies have reported a high intrinsic activity (Mansson *et al*, 1978 and Schafer *et al*, 1986); the intrinsic activity was around 40% of the specific activity of the modified enzymes involved. One of the studies involved the carbodiimide coupling of N^6AHCM-NAD to LADH, but our study has shown that success of this approach cannot be applied to LDH, GDH or ADH, and cannot, therefore, be seen as generally applicable. In addition, an attempt by Kato *et al* (1987) to apply the carbodiimide method to FDH, resulted in a conjugate with an intrinsic activity representing only 10% of its specific activity.

The randomness of the chemical modification approach has been demonstrated in this study, particularly by the availability of bound coenzyme molecules to neighbouring enzymes. In most cases, the loss of specific activity after modification could not be defined because the modification reagents involved were not absolutely selective. Only one ordered and defined approach has so far been studied to form an enzyme-coenzyme conjugate - that of Persson *et al* (1991) who exploited the fact that native glucose dehydrogenase contains no cysteine residues. A single cysteine residue was introduced at an accessible position in the vicinity of the active site by site-directed mutagenesis and exactly one thiolated coenzyme molecule was attached per glucose dehydrogenase subunit. It was probably no coincidence that the resulting enzyme-coenzyme conjugate had the highest percentage of intrinsic activity compared to specific activity of any conjugate studied so far at 85%. Despite the extra work involved in this approach, which required the use of genetic engineering techniques, it may be worthwhile in the long run. As was pointed out, although only glucose dehydrogenase and glucose-6-phosphate dehydrogenase contain no cysteines in their native structure, the cysteines of other dehydrogenases may be replaced by other residues using site-directed mutagenesis. In addition, results from this study here show that the cysteines of LDH, MDH and GDH are almost totally inaccessible to modification with DPDS and so a cysteine introduced at an accessible position, proximal to the active site, would be almost totally exclusively modified by DPDS, to

react with a thiolated coenzyme under mild conditions. The advantages of this more defined approach are several fold. The exact location of the incorporated coenzyme is known and conclusions can be drawn about surrounding residues; the exact spacer length of the conjugate is known in relation to its proximity to the active site and by introducing different spacer arms the optimum length of a spacer arm can be established. The specific activity of the enzyme is more likely to be maintained because the enzyme will only be modified at one insensitive position.

Electrode studies

The glutaraldehyde-coupled LDH-NAD and LDH-N^{6}AHCM-NAD conjugates were chosen over carbodiimide-coupled conjugates for incorporation into enzyme electrodes because the glutaraldehyde spacer arm gave the best chance of the enzyme-bound coenzyme reaching the electrode surface, as was indicated by assaying the LDH-N^{6}AHCM-NAD conjugates for intermolecular activity: 67% of the 1.8 N^{6}AHCM-NAD molecules bound per LDH subunit were accessible to ADH in a coenzyme recycling assay compared to only 20% of 1.0 N^{6}AHCM-NAD molecules that were available in a carbodiimide-coupled conjugate. The glutaraldehyde-coupled conjugates were also chosen because they possessed a higher intrinsic activity, at 0.12U/mg for the LDH-N^{6}AHCM-NAD conjugate and 0.09U/mg for the LDH-NAD conjugate. The intrinsic activity of each carbodiimide-coupled conjugate was only 0.01U/mg. The higher intrinsic activity of the glutaraldehyde-coupled conjugates meant that they would have a greater chance of generating a higher physicochemical signal upon immobilisation onto the surface of an electrode, and therefore a larger electrode response would be obtained. It was also known that the LDH-N^{6}AHCM-NAD conjugate had an intramolecular mechanism; if the mechanism had been intermolecular, intrinsic activity may not have been present upon immobilisation of the enzyme by adsorption to the electrode surface which makes diffusion of enzyme molecules impossible. The loss of adsorbed enzyme was less than 60% over 28 days at 4^{o}C and the loss of enzyme activity observed over the first few hours was not enough to affect the electrode experiments.

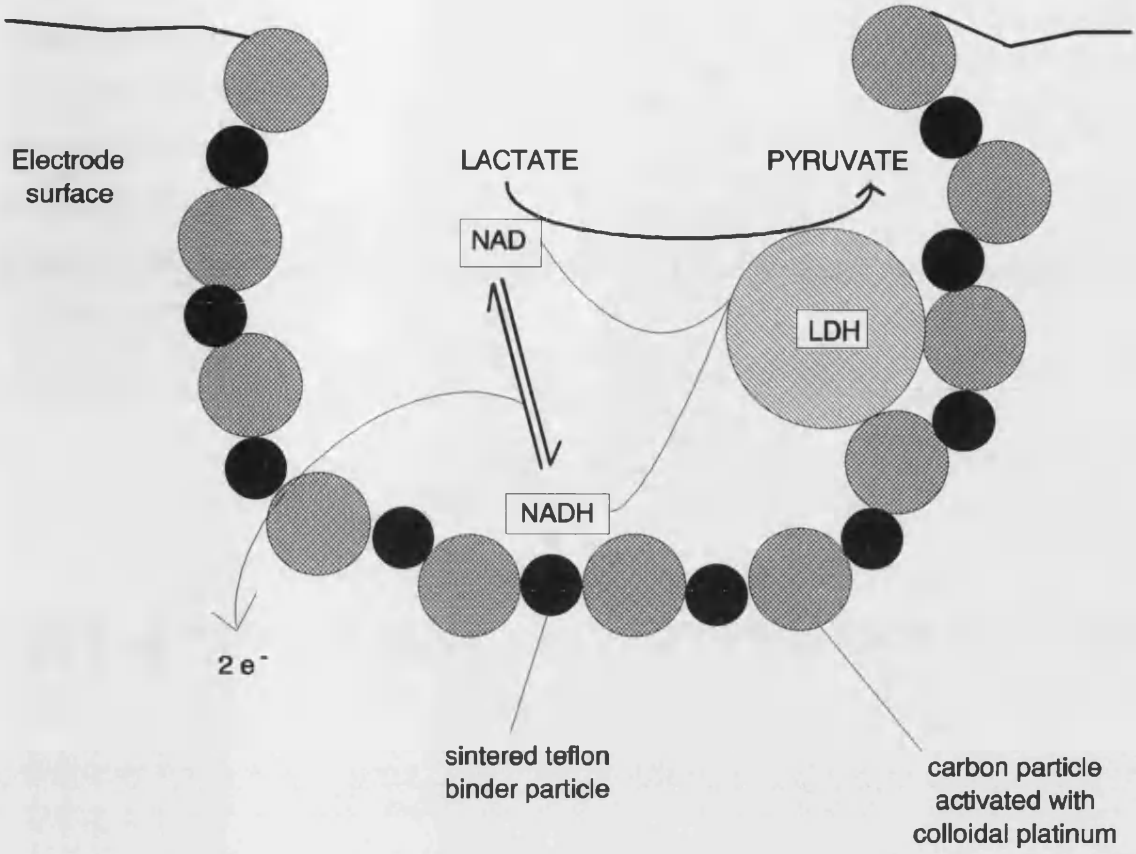


Figure 6.3: *A schematic of the theoretical mechanism behind the lactate response of an enzyme electrode constructed using an LDH-coenzyme conjugate*

A simplified representation of the mechanism of the enzyme electrode is shown in *Figure 6.3*. The LDH-coenzyme conjugate adsorbed at the surface of the electrode forms a ternary complex between covalently bound coenzyme, lactate and enzyme. Lactate is oxidised to pyruvate and the covalently bound coenzyme is reduced. Pyruvate is released into the surrounding solvent and some of the enzyme-coenzyme molecules are positioned so that the spacer arm allows the reduced NADH moiety to swing out of the active centre of the enzyme and into the vicinity of the potential gradient at the electrode surface. Here, NAD is regenerated by the electrocatalytic oxidation of NADH. In this way a steady current, or finite electrode response, is eventually obtained in the presence of lactate.

There was no electrode response at all from an enzyme electrode constructed using an LDH-NAD conjugate preparation but an electrode response of 12nA/mM lactate was obtained from an enzyme electrode constructed using an LDH-N^6AHCM-NAD

preparation. From this we can infer that the spacer arm of the glutaraldehyde cross-linker was not long enough to allow NAD to reach the surface of the electrode but the presence of an extra (aminohexyl)carbamoylmethyl spacer arm allowed the coenzyme to interact (*Figure 6.4*). It seems unlikely that the intrinsic activity of the LDH-NAD conjugate was generated by an intermolecular mechanism, and so this cannot be an explanation for the lack of electrode response.

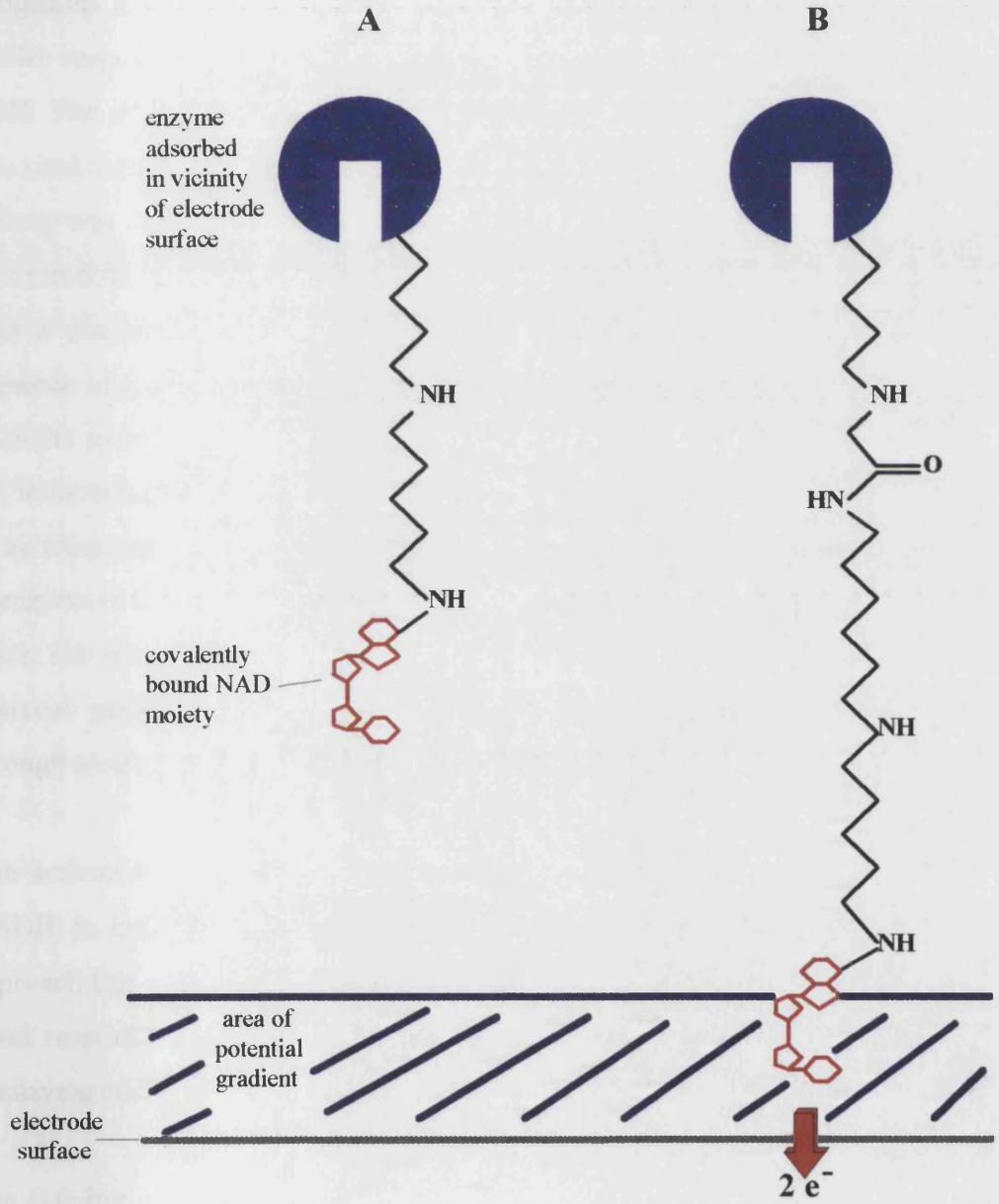


Figure 6.4: *Schematic showing how the longer spacer arm of LDH-N⁶AHCM-NAD conjugate (B) might allow the bound coenzyme to be regenerated by the electrode whereas the spacer arm of the LDH-NAD conjugate (A) might not.*

Interestingly, in the presence of exogenous NAD the electrode response of 12nA/mM lactate was only increased to 24nA/mM. This was surprising because the specific activity of the LDH-N^6AHCM-NAD conjugate free in solution was over 40 times greater than the intrinsic activity. The enzyme-coenzyme conjugate immobilised at the electrode surface had reached 50% of its potential activity without the presence of exogenous coenzyme. It is not clear why there was so little specific activity but a similar response was obtained for a control enzyme electrode prepared using native LDH. The intrinsic activity was better represented when electrocatalytic regeneration was used than chemical regeneration in a coenzyme-recycling assay, where the intrinsic activity was only 4% of the specific activity and therefore, the electrode would seem to be a more favourable and efficient method of regeneration of enzyme-bound coenzyme than a chemical recycling assay. The small size of the electrode response in the presence of free coenzyme could be due to a small amount of adsorbed enzyme and attempts must be made to find out how much enzyme was adsorbed and to improve the immobilisation. It must also be noted that most of the free NAD would diffuse away from the area of potential gradient at the electrode surface whereas the bound coenzyme is fixed in its vicinity. It could also be that the exogenous coenzyme cannot reach the enzyme for catalysis; its access could be slowed down by the activated charcoal paste, although past studies have shown that the free NAD can diffuse through a carbon paste (Dominguez *et al*, 1993a)

The activated carbon paste electrode had a potential response of 400nA/mM free NADH in bulk solution. The size of the enzyme electrode response will have to approach this order of magnitude for commercial application (Turner *et al*, 1987). The good response time of 1-2 mins was probably due to the proximity of the enzyme-coenzyme conjugate to the area of potential gradient at the electrode surface.

The stability of the electrode was poor. This was not due to the electrode materials used or the stability of the conjugate alone. The functional electrode had a half-life of less than 1.5 hours which is not long enough for commercial applications. Wallace and Coughlin (1978) found that NADH can be decomposed at a graphite electrode surface

and Torstensson and Johansson (1980) showed that the reduced coenzyme was degenerated at an activated glassy carbon electrode surface. In our case, experiments demonstrated that the instability was not due to a denaturing of the enzyme which remained stable for 12 hours in a polarised electrode. The stability of the intrinsic activity only decreased rapidly when an overpotential was applied to the cell; the intrinsic activity remained unaltered if the electrode was left unpolarised between lactate measurements. The instability seemed to be due to a loss in functioning coenzyme upon polarisation of the electrode. This may be because the covalently-bound coenzyme becomes adsorbed onto the carbon electrode surface after a while but this does not happen without the application of an overpotential. Another explanation was that the link between coenzyme and enzyme could also be affected by the polarisation of the cell due to an unstable coupling chemistry in the presence of an overpotential of +150mV. The NAD moiety, itself, may also have been degenerated in some way. In the only other study of an enzyme-coenzyme conjugate in an enzyme electrode, carbodiimide-coupled ADH-N^6AHCM-NAD was adsorbed onto an activated glassy carbon electrode and, although instability due to loss of coenzyme substrate activity was observed, no connection was made between stability and electrode polarisation (Torstensson and Johansson, 1980). It may be worth constructing enzyme electrodes using conjugates with different coupling chemistries or using different enzyme immobilisation techniques.

Despite the instability problems it was demonstrated that an enzyme-coenzyme conjugate was able to act as a fully contained catalytic constituent of a reagentless enzyme electrode. The biosensor had a rapid response time, unlike devices constructed using membrane entrapment or macromolecular support techniques to immobilise coenzymes.

Even though measures were taken in this study to ensure that there were accessible residues near active centre of LDH the chance that they would be modified using the chemical modification approach was low because few coenzyme molecules became incorporated. Consequently, the intrinsic activity of the enzyme-coenzyme conjugates

was generally disappointing. In order to improve the performance of reagentless enzyme electrodes constructed using enzyme-coenzyme conjugates the cause of instability observed must be more clearly defined and the response of the electrode in the presence and absence of exogenous coenzyme must be improved. Several goals were reached during the course of this study:

a. Established chemical coupling techniques were used to form enzyme-coenzyme conjugates using several dehydrogenases. It was found that the chemical modification approach was too random in nature to be successful for a wide range of NAD-dependent dehydrogenases.
b. Novel NAD derivatives were synthesised containing a very useful terminal thiol group attached to a spacer arm on the adenine moiety of NAD.
c. New coupling techniques were established which may prove useful for future applications. Terephthalaldehyde was used as an able replacement for glutaraldedehyde in a case where the effect of glutaraldehyde modification upon the activity of the enzyme was too costly and a "disulphide" coupling technique was developed which may be used in the future to exploit the reactivity and specificity of cysteine residues.
d. The potential of reagentless enzyme electrodes constructed using enzyme-coenzyme conjugates was demonstrated.

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