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THE APPLICATION OF IMMOBILIZED NAD^+ IN AN ENZYME ELECTRODE AND IN MODEL ENZYME REACTORS

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SUMMARY

1 Coenzymically active dextran-bound NAD^+ was incorporated into an enzyme electrode with glutamate dehydrogenase and lactate dehydrogenase

2 The enzyme electrode was used to determine glutamate concentrations in the range 1 10^{-4}–1 10^{-3} M, within which the millivoltage deflection was linearly proportional to the logarithm of the glutamate concentration

3 The enzyme electrode was also used to measure pyruvate concentrations in the range 2 10^{-5}–8 10^{-4} M

4 The dextran-bound NAD^+ was incorporated in model enzyme reactors containing galactose dehydrogenase and alanine dehydrogenase, or lactate dehydrogenase and alanine dehydrogenase, both of which produced alanine

5 In these reactors ultrafilters retained the dextran-bound NAD^+ in the reaction chamber while allowing the products of the reaction to pass through

6 In one enzyme reactor a constant rate of alanine production was maintained over a period of 6 5 h during which time the bound coenzyme was recycled over 90 times

INTRODUCTION

The application of enzyme electrodes in monitoring and determining metabolite concentrations has been reviewed [1] Enzyme electrodes have an advantage over conventional methods of enzymic analysis in that they can retain their enzymes in an active form for continuous or serial measurements which do not perturb the condition of the media to be analysed Until now their application has been restricted to relatively simple reactions which are predominantly hydrolytic [2–5] As a result most enzyme electrodes contain a single enzyme The introduction of a second enzyme has on occasion improved the performance of an electrode as shown by the inclusion of catalase in an L-amino acid electrode to remove H_2O_2 and regenerate O_2 [6] The versatility of enzyme electrodes could be greatly increased if enzymes which require cofactors were utilized [7] However, as also has been pointed out, the cost of adding non-rate-limiting amounts of free coenzyme to the samples to be assayed would make their use uneconomical

Clearly then the availability of immobilized cofactors which can be included in

the electrode and retain their cofactor activity will greatly extend the range of enzymes which can be used in enzyme electrodes It may also allow such enzyme electrodes to be used for monitoring the concentration of a metabolite in physiological fluids in vivo where it would not be possible to add a free coenzyme

Part of this paper demonstrates the application of one such immobilized cofactor (dextran-bound NAD^+) in an enzyme electrode

The availability of immobilized cofactors which can be enzymically regenerated allows a similar extension in the scope of enzyme reactors As with enzyme electrodes most enzyme reactors hitherto studied are based on simple hydrolytic reactions [8, 9] The potential of other enzymes like various dehydrogenases, which utilize coenzymes in amounts stoichiometric to the substrate converted, has been limited by the cost of including the free coenzyme in the reaction mixture and the separation problems involved in recovering the coenzyme or purifying the product These disadvantages have been overcome in the model enzyme reactors described in this report In these model systems substrates were pumped into a chamber containing dextran-bound NAD^+ and two NAD^+-linked dehydrogenases, while the products of the reaction and any unconverted substrates were removed at the same rate by ultrafiltration The soluble coenzyme derivative was retained within the reaction vessel by the ultrafiltration membrane together with the enzymes that effect its recycling

MATERIALS

All enzymes were purchased from Boehringer (Tutzing, W Germany), with the exceptions of glutamate–pyruvate transaminase (EC 2 6 1 2) and beef heart lactate dehydrogenase (EC 1 1 1 27) which were obtained from Sigma Chemical Co (St Louis, Mo , U S A), as were ADP, sodium pyruvate and L-lactic acid The dialysis membranes were manufactured by the Union Carbide Corp (Chicago, Ill , U S A) They were treated before use by being boiled for 1 h in 1 mM EDTA (sodium salt) (pH 7 0) and 1 h in distilled water

Dextran-bound NAD^+ was prepared by the linkage of NAD^+-N^6-(*N*-(6-aminohexyl)acetamide) through its terminal amino group to cyanogen bromide-activated dextran T40 (Pharmacia, Uppsala, Sweden) as described elsewhere [10] The coupling step was in principle similar to that carried out in the synthesis of Sepharose-bound NAD^+ [11] The sample of dextran-bound NAD^+ used throughout this work contained 30 μmoles of nucleotide per g dry wt of dextran

METHODS

Preparation of reagents for enzyme electrode assays

The NH_4^+-sensitive glass electrode (Beckman 39137 cation-sensitive electrode) used in these assays responds to protons [4] Therefore all solutions used in determinations with this electrode were adjusted to pH 8 0 with Tris base at 25 °C Since the electrode can also detect monovalent ions like Na^+ and K^+, all reagents used with the electrode were converted to their respective Tris salts Sodium pyruvate was first converted to the free acid by passage through a column of Dowex 50 × (400–200 mesh) (H^+ form) and then adjusted with Tris base to pH 8 0 ADP (sodium salt) was directly converted to the Tris salt by passage through Dowex 50 × (400–200 mesh) in the Tris form followed by adjustment to pH 8 0 as above

Preparation of enzyme electrode (lactate dehydrogenase/glutamate dehydrogenase electrode)

The enzyme electrode was prepared by enclosing soluble glutamate dehydrogenase (EC 1 4 1 3) and rabbit muscle lactate dehydrogenase, and dextran-bound NAD^+, in a piece of dialysis membrane stretched around the bulb of the NH_4^+-sensitive glass electrode Prior to formation of the lactate dehydrogenase/glutamate dehydrogenase electrode the two enzymes, glutamate dehydrogenase (360 units), and lactate dehydrogenase (410 units), were combined and dialysed against 50 mM Tris–HCl buffer (pH 8 0) containing 10 μM Tris–EDTA and 100 μM Tris–ADP to remove NH_4^+ The enzyme solution contained within the dialysis bag was then concentrated against sucrose to give a volume of 150–200 μl (compared to 350 μl before dialysis) Dextran-bound NAD^+ (25 mg dry wt) was dissolved in with the enzymes and the resulting viscous solution was placed on a 5-cm square of dialysis membrane in contact with the tip of the electrode After formation of the lactate dehydrogenase/glutamate dehydrogenase electrode according to an earlier procedure [5], about three quarters of the enzyme solution remained entrapped by the membrane

Equilibration and storage of the lactate dehydrogenase/glutamate dehydrogenase electrode

Immediately after its preparation and each time before use the lactate dehydrogenase/glutamate dehydrogenase electrode was equilibrated in 50 mM Tris–HCl buffer (pH 8 0) made 10 μM in Tris–EDTA and 100 μM in Tris–ADP When not in use the enzyme electrode was kept at 4 °C in this same equilibration solution

Determinations of glutamate and pyruvate using the lactate dehydrogenase/glutamate dehydrogenase electrode

Glutamate and pyruvate concentrations were routinely determined in 50 ml of equilibration solution at 25 °C Glutamate was determined in the presence of 2 mM pyruvate, pyruvate was measured in the presence of 10 mM glutamate The millivoltage deflections caused by the serial addition of aliquots of 1 M glutamate or 0 2 M pyruvate to the stirred solutions were measured on a Radiometer ion meter (PHM 53) connected to a recorder A standard fiber junction saturated calomel electrode was used as a reference electrode

As a control the enzymes of the lactate dehydrogenase/glutamate dehydrogenase electrode were inactivated by soaking the electrode in 8 M urea at 25 °C for 40 min The electrode was then rinsed in distilled water and equilibrated in 50 mM Tris–HCl buffer (pH 8 0) to remove urea before being retested

Enzyme reactors

Enzyme reactor experiments were carried out in a Model 8MC ultrafiltration apparatus (Amicon Corp , Lexington, Mass , U S A) fitted with a PM 10 ultrafiltration membrane The enzyme pairs together with Dextran-bound NAD^+ were present in the small chamber of the apparatus in 2 ml of reaction mixture which contained the buffered substrates Up to 100 ml more of the same reaction mixture were placed in the reservoir chamber The ultrafiltration was done at 40–50 lb/inch^2 with the apparatus setting on "push liquid" such that the volume in the small chamber remained constant at 2 ml throughout the experiment The filtrate was collected in 5-ml fractions for analysis

Composition of the enzyme reactor solutions

The reaction mixture used in the enzyme reactor containing β-galactose dehydrogenase (EC 1 1 1 48) and L-alanine dehydrogenase (EC 1 4 1 1) consisted of 50 mM sodium pyrophosphate buffer made 10 mM in galactose, 2 mM in sodium pyruvate, 200 mM in NH_4Cl and 0 1 mM in EDTA The mixture was adjusted to pH 8 8 with NaOH

The reaction mixture used in the other enzyme reactor containing beef-heart lactate dehydrogenase and alanine dehydrogenase consisted of 100 mM Tris base made 100 mM in L-lactic acid, 200 mM in NH_4Cl and 0 01 mM in EDTA The pH of this mixture was adjusted to pH 8 5

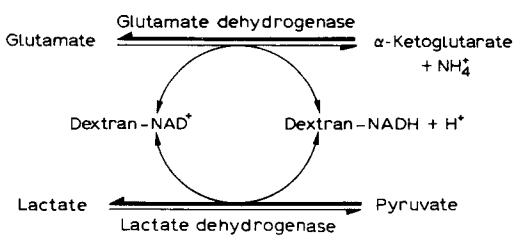
Measurement of pyruvate and alanine concentrations

Pyruvate and alanine were determined spectrophotometrically using methods described by Lowry and Passonneau [12] Rabbit-muscle lactate dehydrogenase was used for the assay of pyruvate Alanine was measured in a coupled assay by using glutamate–pyruvate transaminase in the presence of α-ketoglutarate to convert alanine to pyruvate, which was in turn assayed by using rabbit muscle lactate dehydrogenase Alanine was determined in the presence of pyruvate by correcting for the contribution made to the reaction by the latter substance

RESULTS AND DISCUSSION

Determination of glutamate concentrations using the lactate dehydrogenase/glutamate dehydrogenase electrode

The lactate dehydrogenase/glutamate dehydrogenase electrode was used to determine glutamate concentrations in the range 10^{-4} M–10^{-3} M as described in Methods The presence of glutamate in the assay medium containing pyruvate generated NH_4^+ according to the following scheme



Scheme 1 Generation of NH_4^+ as a reflection of glutamate concentration

The generation of NH_4^+ in the vicinity of the NH_4^+-sensitive electrode caused a millivoltage deflection which was shown to be directly proportional to the logarithm of the glutamate concentration in the range 10^{-4}–10^{-3} M (Fig 1) Over a series of assays a slight deviation from the linear semilogarithmic standard curve was observed at the lowest glutamate concentration tested (1 10^{-4} M) Also shown in Fig 1 are the millivoltage deflections produced at similar concentrations by the lactate dehydrogenase/glutamate dehydrogenase electrode after it had been inactivated in 8 M urea This control was done to compensate for any change in the composition of the assay medium caused by the addition of glutamate per se

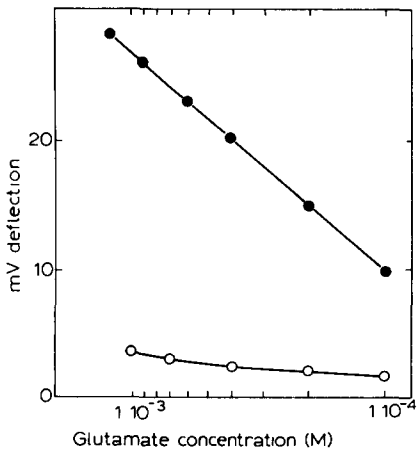


Fig 1 Estimation of glutamate using the lactate dehydrogenase/glutamate dehydrogenase electrode Glutamate was assayed with the lactate dehydrogenase/glutamate dehydrogenase electrode as described in Methods A representative curve (●), made by plotting millivoltage deflection against *p*[glutamate], is shown here and compared to a curve (○) obtained after denaturation of the lactate dehydrogenase/glutamate dehydrogenase electrode in 8 M urea

Determination of pyruvate concentrations using the lactate dehydrogenase/glutamate dehydrogenase electrode

The addition of excess glutamate (10 mM) to the lactate dehydrogenase/glutamate dehydrogenase electrode in the absence of pyruvate caused a millivoltage deflection This can be explained by the glutamate dehydrogenase reaction taking place until all available dextran-bound NAD^+ is reduced Under these conditions the lactate dehydrogenase/glutamate dehydrogenase electrode was used to determine pyruvate concentrations in the range 10^{-5}–10^{-4} M (Fig 2) as described in Methods

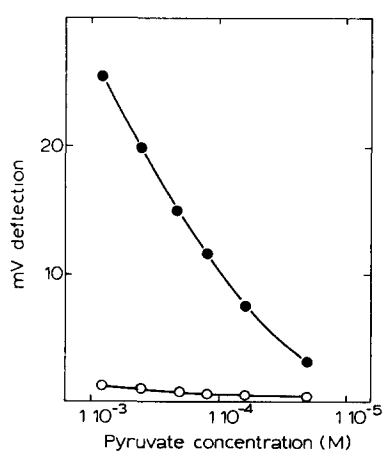


Fig 2 Estimation of pyruvate using the lactate dehydrogenase/glutamate dehydrogenase electrode Pyruvate was assayed with the lactate dehydrogenase/glutamate dehydrogenase electrode as described in Methods A representative calibration curve (●), made by plotting millivoltage deflection against *p*[pyruvate] is shown here and compared to a curve (○) obtained after denaturation of the lactate dehydrogenase/glutamate dehydrogenase electrode in 8 M urea

The relationship between millivoltage deflection and the logarithm of the pyruvate concentration was not linear, unlike the corresponding relationship involving glutamate (Fig 1) Also shown in Fig 2 are the control additions of pyruvate to the lactate dehydrogenase/glutamate dehydrogenase electrode after its inactivation by 8 M urea These controls, and similar ones involving glutamate (Fig 1), demonstrate that the deflections registered by the lactate dehydrogenase/glutamate dehydrogenase electrode are a result of enzymatic activity and are not due to the addition of NH_4^+ or other cations along with the sample

Response time of the lactate dehydrogenase/glutamate dehydrogenase electrode

After each addition of glutamate or pyruvate to the electrode assay solution a steady state of NH_4^+ production was attained within 3–4 min

Stability of the lactate dehydrogenase/glutamate dehydrogenase electrode stored at 4 °C

The response of the lactate dehydrogenase/glutamate dehydrogenase electrode decreased over a period of 15 days (Table I) However, at any stage it was possible to recalibrate the electrode and determine glutamate concentrations as before Considering the nature of the enzymes used the lactate dehydrogenase/glutamate dehydrogenase electrode was remarkably stable Moreover it might be possible to further stabilize this enzyme electrode by storing it in glycerol or other protecting agents, or by immobilizing the enzymes

TABLE I

STABILITY OF THE LACTATE DEHYDROGENASE/GLUTAMATE DEHYDROGENASE ELECTRODE STORED AT 4 °C

The stability of the lactate dehydrogenase/glutamate dehydrogenase electrode on storage at 4 °C was tested by assaying two glutamate concentrations over a period of 2 weeks The assay and the storage of the enzyme electrode were done as described in Methods The control values were obtained after denaturation of the lactate dehydrogenase/glutamate dehydrogenase electrode in 8 M urea

Glutamate (M)	mV response				
	1st day	4th day	8th day	15th day	Control
$2\ 10^{-3}$	30 0	19 2	16 7	11 4	3 7
$2\ 10^{-4}$	14 7	9 0	6 9	4 2	0 9

Application of the lactate dehydrogenase/glutamate dehydrogenase electrode

The lactate dehydrogenase/glutamate dehydrogenase electrode has potential use in the assay of α-ketoglutarate and L-lactate by the reverse of the reactions already considered, in which the electrode would respond to the uptake of NH_4^+, during the formation of glutamate However, lactate concentrations of the order of 10^{-2}–10^{-1} M would undoubtedly be necessary for the efficient working of the electrode in this direction, which would seriously restrict its utility

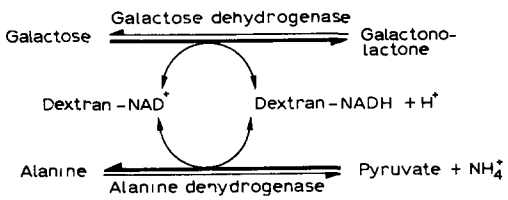
The application of the lactate dehydrogenase/glutamate dehydrogenase electrode to the measurement of glutamate and pyruvate has been demonstrated in this report Whereas in conventional assays for glutamate and pyruvate the enzymes and

coenzyme are used once and then discarded, the lactate dehydrogenase/glutamate dehydrogenase electrode advances a method for the determination of these compounds in which both enzymes and coenzyme can be conserved and reutilized

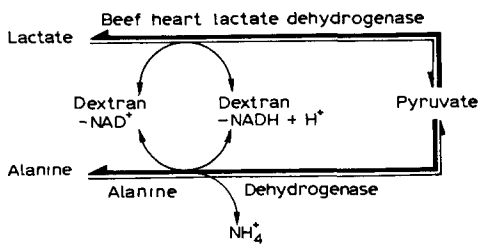
The use of this enzyme electrode to measure glutamate and pyruvate in physiological fluids would, however, be affected by the presence of cations like Na^+ and K^+ which are detected by the cation-sensitive electrode ($K^+ > NH_4^+ > Na^+$ [4]) This difficulty might be overcome by referencing against an identical lactate dehydrogenase/glutamate dehydrogenase electrode in which the enzymes have been denatured, by using a glass pH electrode [5], or by improvements in the design of the electrode itself Finally by modification of such parameters as enzyme concentration, coenzyme concentration and enzyme to coenzyme ratios, it might be possible to increase the observed slopes from 15 to 20 mV/decade (Figs 1 and 2) to nearer the Nernstian limit of 59 mV/decade

Design of the model enzyme reactors

Both of the model enzyme reactors studied were set up to produce alanine through the reaction of L-alanine dehydrogenase However, the two systems used different enzymes for the reduction of dextran-bound NAD^+ as shown below (Scheme 2)



Lactate dehydrogenase/alanine dehydrogenase reactor



Scheme 2 Model enzyme reactors

In the galactose dehydrogenase/alanine dehydrogenase reactor β-galactose dehydrogenase reduces dextran-bound NAD^+ Although this enzyme has a low activity (5 units/mg protein at 25 °C) it is one of the few commercially available NAD^+-linked dehydrogenases which catalyse a reaction in which the equilibrium lies in favour of NADH formation The formation of NADH is further promoted by the spontaneous hydrolysis of galactonolactone at alkaline pH values [13]

In the lactate dehydrogenase/alanine dehydrogenase reactor, where beef heart

lactate dehydrogenase is used to generate dextran-bound NADH, the equilibrium position in this direction is unfavourable However, this reaction is promoted by the high lactate concentration used (100 mM) and by the consumption of the products, pyruvate and dextran-bound NADH, by the second enzyme, alanine dehydrogenase

Performance of the model enzyme reactors

The concentration of alanine in the effluent from the galactose dehydrogenase/alanine dehydrogenase reactor was 0 45 mM (Fig 3) Since the pyruvate concentration entering the reactor was 1 5 mM this represented 30% conversion The rate of production of alanine was constant over a period of 6 5 h, during which time the volume passed through the reactor was 30 times that contained in the reaction chamber

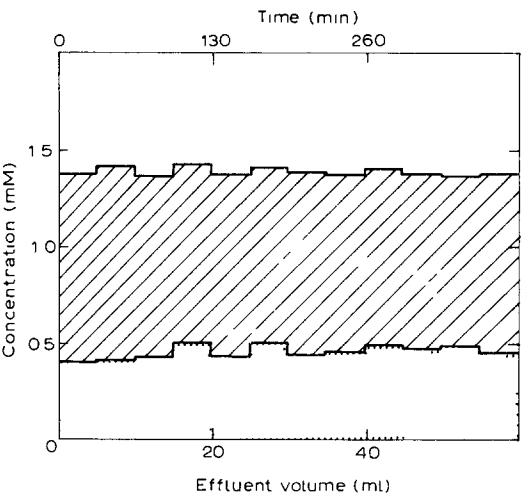


Fig 3 Continuous conversion of pyruvate to alanine in an enzyme reactor containing dextran-bound NAD^+ The galactose dehydrogenase/alanine dehydrogenase reactor, containing galactose dehydrogenase (2 units), alanine dehydrogenase (3 units) and dextran-bound NAD^+ (10 mg dry wt) in the reaction chamber, was run as described in Methods The pressure on the apparatus was maintained at 50 lb/inch^2 to give a flow rate of 9 2 ml/h The stippled area represents the alanine concentration in the effluent from the reactor The cross-hatched area above represents the concentration of unreacted pyruvate in the same effluent

The lactate dehydrogenase/alanine dehydrogenase reactor containing beef heart lactate dehydrogenase (85 units), alanine dehydrogenase (24 units) and dextran-bound NAD^+ (12 mg dry wt) was maintained at a pressure of 40 lb/inch^2 to give a flow rate of 11 5 ml/h The concentration of alanine in the effluent from this reactor was initially 1 0 mM However, after 5 5 h of operation the concentration had decreased to 0 3 mM This decrease in activity cannot be explained by an accumulation of pyruvate in the reaction chamber inhibiting lactate dehydrogenase since throughout the experiment the pyruvate concentration was below 1 10^{-5} M

Recycling rate of dextran-bound NAD^+

The dextran-bound NAD^+ was recycled in the galactose dehydrogenase/alanine dehydrogenase reactor at a rate of 14 times/h This rate is calculated on the

assumption that all the immobilized NAD^+ is sterically available to the enzymes However, the percentage of the bound NAD^+ which can be enzymically reduced even in the presence of excess enzyme is less than 100% [10, 11] Therefore the rate at which the coenzymically active NAD^+ can be recycled is probably greater than the figure quoted

In the lactate dehydrogenase/alanine dehydrogenase reactor the maximum rate of recycling of the immobilized NAD^+ was 33 times/h Again the same qualification applies to this figure as it does to that obtained from the galactose dehydrogenase/alanine dehydrogenase reactor

Conclusions on the use of dextran-bound NAD^+ in enzyme reactors

Water-soluble derivatives of NAD^+ such as dextran-bound NAD^+ have obvious advantages over less-soluble derivatives like Sepharose-bound NAD^+ [11] when used in enzyme reactors, since they are sterically more available At the same time their size is compatible with methods used to retain enzymes inside reactors enclosed by a membrane

Another water-soluble derivative of NAD^+ (polyethyleneimine-succinyl-NAD^+) has been previously described and applied in a two-enzyme recycling system [14] This derivative, however, has an acyl linkage to NAD^+ which is labile above pH 7 In contrast, the dextran-bound NAD^+ used throughout this work, has a far more stable alkyl linkage which makes it more suitable for use in enzyme reactors under continuous flow-through conditions Furthermore the dextran-bound NAD^+ does not contain extra charged groups which might interact with the enzymes used in the reactor

The two enzyme reactors described here are presented only as models In the galactose dehydrogenase/alanine dehydrogenase reactor the yield of alanine might approach 100% if the parameters of enzyme concentration, dextran-bound NAD^+ concentration and flow rate were suitably adjusted

The lactate dehydrogenase/alanine dehydrogenase reactor is of interest as a "multi-step enzyme system" in which the product of the first reaction is the substrate for the second enzyme Such systems have been studied in which the enzymes were bound to particulate matrices such as Sepharose [15] It may also serve as a model for an enzyme therapy system in which enzymes and coenzymes immobilized together could function as a self-contained unit

ACKNOWLEDGEMENTS

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