Potentiometric Determination of NAD(P)H Coenzymes Using a Nitrite-Selective Membrane Electrode and a Nitrate Reductase Enzyme and the Application to Glucose Assay in Human Serum

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NAD(P)H coenzyme concentrations were determined with the combination of a nitrite-selective membrane electrode and a nitrate reductase enzyme. The method was based on the detection of nitrite formed by the enzymatic reaction of NAD(P)H with nitrate catalyzed by nitrate reductase. The lower detection limits for both these coenzymes were around 100 μM . This system was applied to detect glucose by combining an additional glucose dehydrogenase enzyme. Serum glucose levels could be determined through the detection of nitrite.

Keywords Enzymatic method, nitrite-selective membrane electrode, nitrate reductase, NAD(P)H determination, serum glucose assay, coupled enzyme reactions

The application of ion-selective electrodes coupled with chemical reactions has recently attracted much interest, because various substances including non-ionic species can easily be detected with the inherent advantages of ion-selective electrodes, *i.e.*, high selectivity, sensitivity and freedom from optical interference.¹⁻⁷ For this purpose, 1-fluoro-2,4-dinitrobenzene¹⁻⁴ and Girard's reagent^{3,5-7} are widely used. The former reagent can release fluoride by the reaction with amino acids, amines, hydrazines or phenols, so that a fluoride-selective electrode can be used for the determination of these compounds. The latter reagent can convert aldehydes or glucose, for example, to ionic forms which can be measured with appropriate ion-selective electrodes.

Enzyme reactions are also applied for converting biologically important substances including amino acids, phosphatidylcholine and adenosine-5'-triphosphate to suitable ionic forms.^{3,8-10} For example, phosphatidylcholine concentrations have been determined by detecting choline released by the enzymatic reaction of the phosphatidylcholine with phospholipase D, using a choline-sensitive electrode.⁸

We are particularly interested in extending the use of various ion-selective electrodes coupled with enzymatic reactions, because this approach will lead to the detection of specific substances especially with high selectivity under mild conditions. In the present study, a nitrite-selective membrane electrode and a nitrate reductase enzyme were applied to determine the concentrations of NAD(P)H coenzymes. The method is

based on the following enzyme-catalyzed reaction of the coenzymes with nitrate.

Thus, the concentration of NAD(P)H can be determined through the detection of NO_2^- . This approach is very useful because many enzyme reactions can be coupled with the formation of the coenzymes.^{11,12} For example, glucose can be determined when the following enzyme reaction (2) is further coupled to the reaction (1).

In this study, we noted the determination of NAD(P)H coenzymes and glucose using the combination of a nitrite-selective electrode and the above enzymatic reactions.

Experimental

Reagents

The sources of the reagents were as follows: nitrite ionophore I (aquo-cyano-cobyrinic acid-heptakis(2-phenylethyl ester)) and bis(1-butylpentyl)decane-1,10-diyl diglutarate (ETH 469) from Fluka (Buchs, Switzerland); poly(vinyl chloride) (PVC) (degree of polymerization, 1020) from Nacalai Tesque (Kyoto,

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Japan); nitrate reductase (from *Aspergillus* sp.) and glucose dehydrogenase (NADP⁺ dependent, from *Cryptococcus uniguttulatus*) from Sigma (St. Louis, MO, USA); and NADPH, NADH and NADP⁺ from Oriental Yeast (Tokyo, Japan). All other chemicals used were of analytical-reagent grade.

Electrode system

The nitrite-selective electrode was constructed similarly to that reported previously.^{13,14} The components of the sensor membrane were 2 mg of nitrite ionophore I, 60 μ l of ETH 469 and 30 mg of PVC. The materials were dissolved in tetrahydrofuran (about 1 ml) and poured into a flat Petri dish (16 mm diameter), then the solvent was evaporated at room temperature. The resulting membrane was excised and attached to a PVC tube (4 mm o.d., 3 mm i.d.) with tetrahydrofuran adhesive. In the present study, a small-sized dish was used to make the sensor membrane instead of an ordinary-sized (28 mm diameter) one⁸⁻¹⁰, because the response characteristics of the electrode were remarkably improved. The PVC tube was filled with an internal solution comprising 10 mM NaNO₂ and 10 mM NaCl, and the sensor membrane was conditioned overnight. The electrochemical cell arrangement was

Ag, AgCl/internal solution/sensor membrane/sample solution/1 M NH₄NO₃ (salt bridge)/10 mM KCl/Ag, AgCl.

The electromotive force (emf) between the silver/silver chloride electrodes was measured using a voltmeter with high input impedance produced by a field-effect transistor operational amplifier (LF356; National Semiconductor, Sunnyvale, CA, USA; input resistance >10¹² Ω) and was recorded. The detection limit was defined as the intersection of the extrapolated linear regions of the calibration graph.¹⁵ All measurements were performed at a constant temperature of 25°C.

Determination of NAD(P)H

Standard aqueous solutions of NAD(P)H coenzymes in the range of 1×10^{-6} – 1×10^{-2} M were prepared. To 100 μ l of the standard solution, 50 μ l of an assay solution containing 0.1 U nitrate reductase, 150 mM NaNO₃ and 30 mM NaH₂PO₄/Na₂HPO₄ (pH 7.4) was added, and the mixture was incubated at 25°C for 10 min. Subsequently, 50 μ l of 500 mM NaH₂PO₄/Na₂HPO₄ (pH 5.0) was introduced to adjust the pH of the solution to around 5, and the nitrite formed was measured using the nitrite-selective electrode. It should be emphasized that the present electrode system, including the reference electrode¹⁶, is compact, and therefore, assay solution volumes as small as 200 μ l can be used. Between measurements, the nitrite electrode was soaked in distilled water for 20 – 30 s, rinsed several times with distilled water and wiped. The electrode was stored in 10 mM NaNO₂ and 10 mM NaCl when not in use.

Determination of glucose

To 100 μ l of the standard aqueous solution containing

1×10^{-6} – 1×10^{-2} M glucose, 50 μ l of an assay solution containing 0.1 U nitrate reductase, 150 mM NaNO₃, 0.5 U glucose dehydrogenase, 0.5 mM NADP⁺ and 30 mM NaH₂PO₄/Na₂HPO₄ (pH 7.4) was added, the mixture was incubated at 25°C for 10 min, and 50 μ l of 500 mM NaH₂PO₄/Na₂HPO₄ (pH 5.0) was introduced. Then, the nitrite formed was determined by the electrode as described above. The determination of glucose in human serum was performed after the serum (20 μ l) was initially diluted with distilled water to 100 μ l. The glucose level in serum was calculated by subtracting the blank level of NO₂⁻ which was produced in the absence of glucose dehydrogenase. The glucose concentration in serum was also determined by a colorimetric method using a reagent kit available from Iatron Laboratories (Iatro-Chrom GLU-LQ; Tokyo, Japan).

Results and Discussion

Response characteristics of the electrode

The potentiometric response of the nitrite-selective electrode was first examined in buffer solutions consisting of 100 mM NaH₂PO₄/Na₂HPO₄ (pH 7.4 and pH 5). The calibration graphs were obtained by measuring known amounts of sodium nitrite added to the buffers and plotting the concentrations against the corresponding emf values obtained. As shown in Fig. 1, the electrode responded to nitrite more sensitively at acidic pH 5 than at pH 7.4. The slope of the electrode response and the lower limit of detection at pH 7.4 were –46 mV per concentration decade and 300 μ M, respectively, while those at pH 5 were –57 mV per concentration decade and 8 μ M, respectively. The low sensitivity of the electrode at higher pH is shown to be due to large

Fig. 1 Response to nitrite at pH 7.4 (line a) and pH 5 (line b).

Fig. 2 Response to NADPH in an assay solution (200 μ l) containing 0.1 U nitrate reductase, 50 mM NaNO_3 and 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.4). At the time indicated by the arrow, 1 μ l of 100 mM NADPH was added (corresponding to 0.5 mM NADPH when diluted in the assay solution).

interference from hydroxide ion.¹⁷ The nitrite electrode is reported to be free from interference from various anions including phosphate, nitrate and chloride, but the hydroxide ion is an exception.^{13,14}

In order to confirm directly whether the concentration of the coenzyme can be determined through the detection of nitrite, we monitored the time response of the enzyme reaction of NADPH and nitrate reductase. A typical chart of the response to NADPH is shown in Fig. 2. When 1 μ l of 100 mM NADPH aqueous solution (final concentration: 0.5 mM) was added at the time indicated by the first arrow to an assay solution (200 μ l) containing 0.1 U nitrate reductase, 50 mM NaNO_3 and 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.4), a rapid response was observed and the final potential level reached corresponded to 0.5 mM nitrite. The same amount of NADPH was added again at the times indicated by the second and third arrows, and these potential levels also corresponded to 1.0 mM and 1.5 mM nitrite, respectively. This result demonstrated clearly that the present system is suitable for determining the concentration of coenzyme; however, one problem that remained was the low sensitivity of the electrode at pH 7.4. We performed measurements at pH 5, at which electrode response was improved remarkably as shown in Fig. 1. At this pH, however, the enzyme did not work. Thus, the assay sample was acidified after the enzyme reaction at pH 7.4. According to the procedure described in the experimental section, we constructed calibration graphs of the NADPH and NADH coenzymes. As shown in Fig. 3, NADPH showed a near-Nernstian response (line a) with a slope of -57 mV per concentration decade except for the higher concentration range of 5 to 10 mM, while NADH showed a much worse response (line b). The lower limits of detection for both these coenzymes were around 100 μ M. The deviation from the linear

Fig. 3 Calibration graphs for NADPH (line a) and NADH (lines b and c). In line c, the enzymatic reaction with NADH proceeded completely.

response at high concentration of NADPH would be induced by the product inhibition due to the high accumulation of NADP^+ in the reaction system, and this will be discussed further in the next section. The lower sensitivity to NADH than to NADPH indicated the lower reactivity to NADH on the nitrate reductase of *Aspergillus* sp. used in the present study. When the reaction time was prolonged from 10 min to 60 min and the enzyme unit increased from 0.1 U to 0.5 U, the calibration graph of NADH (line c) was almost superimposed on that of NADPH (line a), except that the linear response was further prolonged even at high concentration (10 mM) of NADH over the case of NADPH.

Determination of glucose using coupled enzyme reactions

Then, we determined glucose concentrations, using the coupled enzyme reactions (1) and (2) shown in the introduction. In the reaction of glucose with NADP^+ catalyzed by glucose dehydrogenase, NADP^+ is converted to NADPH which is, in turn, the substrate of nitrate reductase. Thus, NADP^+ will be regenerated and available for the glucose dehydrogenase again. This indicates that the amount of NADP^+ initially added can be suppressed. To determine the optimum concentration of NADP^+ in this system, we examined the concentration dependence of the coenzyme on potential response (Fig. 4). It was found that glucose was efficiently converted to nitrite even at the low concentration of NADP^+ (0.2 – 1 mM showing the concentration contained in 50 μ l assay solution defined in the experimental section). This demonstrated that the enzyme recycling occurred effectively as discussed above. In contrast, when NADP^+ concentrations increased up to 10 mM, the conversion did not effec-

Fig. 4 Effect of NADP^+ concentration on potential response. Glucose concentrations were 1 mM (line a) and 10 mM (line b); these were contained in initial standard solution (100 μL), while the NADP^+ concentrations shown in the abscissa were those contained in assay solution (50 μL). See the experimental section for the definition of standard and assay solutions.

Fig. 5 Calibration graph for glucose.

tively proceed, suggesting that the enzyme activity of nitrate reductase was affected significantly in the presence of a large amount of NADP^+ . This was consistent with a decrease in potential change at high concentration of NADPH observed in Fig. 3 (line a), where a large amount of NADP^+ was formed in the reaction mixture, leading to so-called product inhibition. Thus, we added 0.5 mM NADP^+ to make the calibration graph for glucose. As shown in Fig. 5, the calibration graph for glucose was similar to that for NADPH , and glucose could be determined down to 100 μM .

We determined serum glucose levels using this method, because the nitrite electrode suffered no serious interference from Cl^- , HCO_3^- or other anions pre-

Table 1 Glucose concentrations in serum samples determined by potentiometry and by colorimetry

Serum sample	Potentiometry		Colorimetry	
	Glucose ^a /mM	RSD, %	Glucose ^a /mM	RSD, %
1	4.4	2.6	4.2	2.9
2	5.0	2.0	5.0	3.2
3	5.2	2.3	5.0	3.4

a. Average of three experiments.

sent in biological fluids.^{13,14} The normal range of glucose was reported to be 3.3 – 5.6 mM.¹⁸ Table 1 summarizes the results in three different donors determined by the present method, along with those obtained by colorimetry. The glucose levels estimated by the present method showed similar values to those obtained by colorimetry.

These results demonstrate that the combination of the nitrite electrode and enzyme reactions involving nitrate reductase is useful for the determination of coenzyme and glucose concentrations. Since there are many enzymatic reactions coupled with NADP^+ (or NAD^+)^{11,12}, the present system can be applied for the determination of various biologically important substances.

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