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Photon Counting Determination of Ultratrace Levels of Nicotineamide Adenine Dinucleotide, Reduced Form (NADH) by Use of Immobilized Luciferase

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A photon counting method based on the detection of bioluminescence was developed for the determination of ultratrace levels of β-nicotineamide adenine dinucleotide, reduced form (NADH). A commercial bacterial luciferase (*Vibrio fischeri*) which contains both flavin mononucleotide (FMN) reductase and luciferase was immobilized by the diazocoupling method onto arylamine glass beads. The immobilized luciferase beads were packed in a tube and placed in front of the photomultiplier tube of a photon counter. A flow injection system with a packed-bed reactor was used for the determination of NADH. The immobilized enzyme system is superior to soluble luciferase in that it is reusable and much more stable. In the present method, the lower limit of detection of NADH was 130 fmol.

Ethanol and L-lactic acid determinations in serum were carried out by using immobilized alcohol dehydrogenase and lactic dehydrogenase, respectively. NADH produced in each enzyme reaction was assayed by using the immobilized luciferase column. The serum background emission is negligibly small. The methods are simple and suitable for routine use.

Keywords——immobilized bacterial luciferase; photon counting method; NADH ultratrace assay; flow injection analysis

The extreme sensitivity and specificity of light-producing bacterial luciferase have been utilized for analytical purposes in studies on biological fluids.[1,2] The purified bioluminescent system from marine bacteria, consisting of an nicotineamide adenine dinucleotide, reduced form (NADH): flavin mononucleotide (FMN) oxidoreductase and a luciferase (EC 1.6.99.3 and 1.6.99.1), emits blue light in the presence of FMN, NADH, a long chain aliphatic aldehyde and molecular oxygen as a result of the following reactions:[3]

$$NADH + H^+ + FMN \xrightarrow{\text{oxidoreductase}} NAD^+ + FMNH_2$$

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + h\nu\ (490\,\text{nm}).$$

Recently, DeLuca *et al.*[4] reported an automated flow system assay of various metabolites. In their paper, the above two enzymes, which were chromatographically purified from *Beneckea harveyi*, were coimmobilized on CNBr-activated Sepharose and packed into small flow cells. By using a photometer as the detection system, they reduced the lower limit of detection of NADH to the picomol level. It was pointed out, however, that the most serious problem affecting the analytical performance of the Sepharose-immobilized enzymes was bacterial contamination. This was minimized by filling the packedbeds of immobilized enzyme with a solution containing the antibiotic gentamicin and storing them at 0—4 °C.

Kurkijärvi *et al.*[5] also described a successful immobilization of *Beneckea harveyi* luciferase on CNBr-activated Sepharose and used a continuous flow column for NADH

monitoring. To determine the lifetime of the immobilized luciferase, they carried out 80—100 measurements/d for a week. After 4 d the light emission began to decrease. They concluded that the gel was packed tightly in the process of continuous assays, leading to disruption of the gel matrix, which was confirmed with a microscope. Moreover, they pointed out that the lifetime of the enzyme column seemed to be more dependent on the mechanical strength of the support than on inactivation or leakage of the enzymes, so that the lifetime can be lengthened by immobilizing the enzyme on more rigid matrixes.

We describe here a successful immobilization[6,7] of commercial *Vibrio fischeri* luciferase on arylamine glass and its application to the determination of ultratrace levels of NADH. Because arylamine glass is rigid, unlike Sepharose, and does not absorb light around 490 nm, it is suitable as a support for a packed-bed reactor generating light. As the commercial extract of marine bacteria contains both oxidoreductase and luciferase,[8] we immobilized it on glass without further purification.

In the present flow injection analysis (FIA),[9,10] a photon counter was used for the detection of light emission. The photon counter has several advantages, that is, only a few photons of light emission can be detected, a sample signal can be electrically separated from noise and the detection limit can be lowered. The packed-bed reactor of immobilized luciferase was placed in front of the photomultiplier tube of a photon counter. The injection of a liquid sample containing NADH into a moving, continuous carrier stream produced remarkable gains in sensitivity and detection limit, and we were able to lower the limit of detection of NADH to the femtomol level. Even without the purification of commercial bacterial luciferase, the detection limit for NADH was lowered about 50-fold compared with that of DeLuca.[4]

We have also considered an immobilized system for assaying alcohol and lactic acid in serum. In this assay, the NADH produced by alcohol dehydrogenase and L-lactate dehydrogenase (EC 1.1.1.1 and 1.1.1.27) is determined by the use of bacterial luciferase in a coupled assay. The light produced is directly proportional to the concentration of the alcohol or lactic acid present in serum.

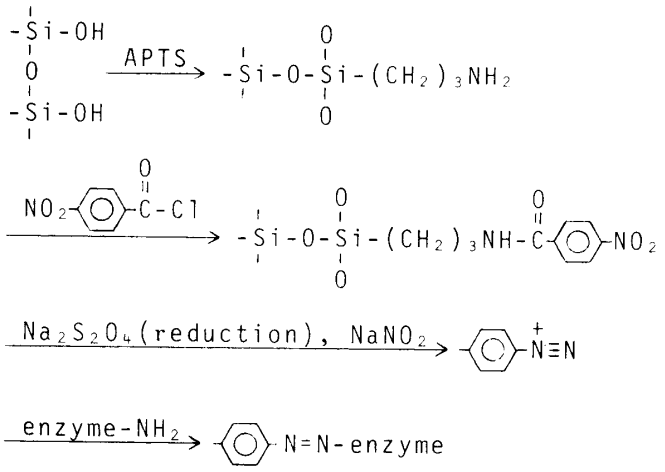
Experimental

Reagents——Controlled-pore glass (CPG-500, mean pore diameter 547 Å, surface area 44.5 m^2/g) was a product of Electro Nucleonics, Inc. NADH and β-nicotinamide adenine dinucleotide (NAD) were purchased from Sigma Chemical Company (Grade III). FMN, decanal and dithiothreitol (DTT) were obtained from Nakarai Chemicals. Bacterial luciferase (Type V), consisting of the luciferase and the NADH : FMN oxidoreductase from *Vibrio fischeri*, was purchased from Sigma Chemical Company. Alcohol dehydrogenase (ADH) (alcohol : NAD oxidoreductase; EC 1.1.1.1) from yeast was obtained from Boehringer Mannheim. Catalase (hydrogen-peroxide : hydrogen-peroxide oxidoreductase; EC 1.11.1.6), diaphorase (reduced-NAD : lipoamide oxidoreductase; EC 1.6.4.3) and L-lactic dehydrogenase (LDH) (L-lactate : NAD oxidoreductase; EC 1.1.1.27) (Type II) from rabbit muscle were obtained from Sigma Chemical Company.

All other reagents were of analytical grade.

Immobilization of Bacterial Luciferase——The arylamine glass was prepared from the alkylamine porous glass beads by reaction with *p*-nitrobenzoyl chloride, followed by reaction with sodium dithionite. The diazotization procedure was carried out in an ice bath by addition of 2 N HCl and solid $NaNO_2$ to the arylamine glass. Diazotized glass beads were added to the enzyme which had previously been dialyzed against 500 ml of deaerated 50 mM sodium bicarbonate solution (pH 8.0) to remove DTT (stabilizer), since it inhibits the immobilization.[8] The mixture was shaken gently at 4 °C for 20 h. The procedure of immobilization is shown in Chart 1. The beads were then washed with cold water, 1 M NaCl and finally with 0.1 M phosphate buffer (pH 7.0). After the washings, the beads were suspended in 0.1 M phosphate buffer containing 0.5 mM DTT, 1 mM ethylenediaminetetraacetic acid (EDTA) and 10% glycerol, which are necessary components for maintaining the enzyme activity during storage, and stored at 4 °C in the dark.

Immobilization of Other Enzymes——Immobilizations of catalase, diaphorase, ADH and LDH were carried out by coupling the amino groups of the enzyme with the aldehyde groups that were introduced on the alkylamine glass beads with glutaraldehyde.[11] Coupling was achieved under the following reaction conditions: 0.1 M phosphate buffer



APTS: γ-aminopropyl triethoxysilane

Chart 1

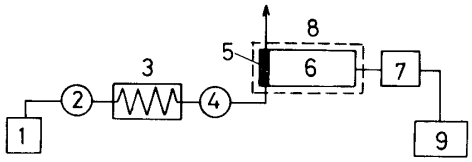


Fig. 1. Schematic Diagram of the Flow System for NADH-Monitoring

1, substrate solution (FMN, decanal, DTT); 2, pump (0.45 ml/min) (Kyowa Seimitsu Co., Ltd.: KHD-16); 3, water bath; 4, injecter; 5, column (immobilized enzyme) (1.5 mm × 3.5 cm glass tube); 6, photomultiplier (H.V. −670 V); 7, photon counter; 8, light-shielded box; 9, recorder (Shimadzu: R-11M).

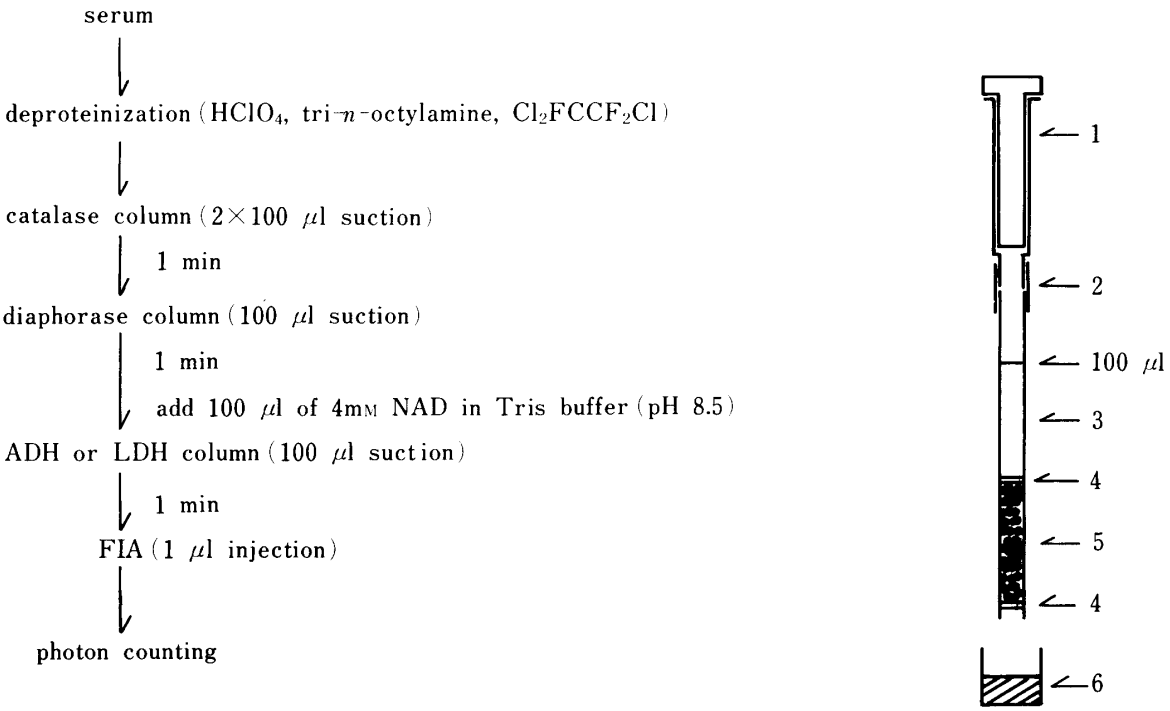


Fig. 2. Determination Procedure with the Immobilized Enzyme by a Simplified Method

1, syringe for suction and ejection of serum; 2, nylon tube; 3, disposable micropipette of 100 μl (Drummond Scientific Co.); 4, glass wool; 5, immobilized enzyme; 6, serum.

(pH 7.0) at 4 °C for 20 h. Immobilized enzyme was stored in the above buffer solution.

Substrate Solution——A 0.1 M phosphate buffer was filtered through a 0.22 μ Millipore membrane to remove dust and microorganisms, and was then used to prepare a substrate solution containing 40.2 μM FMN, 10 μM DTT and 0.001% decanal, which were freshly prepared each day.

Standard Solution of NADH——A stock solution of about 0.1 mM NADH in phosphate buffer was standardized daily from the absorbance reading on the spectrophotometer. It was diluted as required in the above buffer. Very dilute solutions of NADH are unstable. Hence dilutions were done shortly before use.

Instrumentation——Light emission was measured with a photon counter (Hamamatsu TV Co., Ltd.: C-1230) equipped with a photomultiplier tube (Hamamatsu TV: R-585). Spectrophotometric measurements were made with a Shimadzu UV-350 machine.

Construction of Enzyme Reactor——A schematic representation of the whole measuring system is shown in Fig. 1. The substrate solution was kept in a light-shielded vessel in an ice bath (1 in Fig. 1). Before reaching the column, the substrate solution was passed through a coil in a water bath (3 in Fig. 1) to increase the temperature to 20 °C. The NADH samples were injected through an injecter (4 in Fig. 1) by using a micro syringe. The samples contained 130 fmol (100 pg) to 1.3 nmol (1 μg) of NADH.

A glass tube (1.5 mm × 3.5 cm) (5 in Fig. 1) packed with immobilized enzyme beads was fixed just in front of a photomultiplier tube (6 in Fig. 1).

Determination Procedure——A serum added to the substrate was deproteinized with perchloric acid and extracted with tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane. Contaminating hydrogen peroxide and NADH in the sample solution were removed by passing the solution through tubes containing immobilized catalase and diaphorase as shown in Fig. 2. A syringe was used to move the sample solution. NAD in Tris buffer was added to the eluent and the main enzymatic reaction for immobilized ADH or LDH was done by a similar method. The sample solution containing NADH produced with immobilized enzyme was injected into the FIA system and the intensity of bioluminescence was recorded. The substrate concentration was determined by means of a calibration curve for NADH standards obtained under the same determination procedure.

Results and Discussion

Properties of Immobilized Luciferase

NADH concentration injected into the FIA system was determined on the immobilized luciferase column by monitoring the light emission. This procedure was rapid and sensitive. We made a direct comparison of NADH-stimulated light emission from the immobilized luciferase *vs.* soluble luciferase. There was scarcely any difference in levels of light emission between the immobilized and soluble enzymes at low concentration of NADH (100 pg—1 μg). In the determination processes, the immobilized luciferase column produced stable light emission, but it was observed that the light intensity was decreasing in the case of soluble luciferase after several hours.

Recently it was found that addition of 10% glycerol greatly increases the stability of the immobilized enzyme beads during storage, and this could be done routinely without any effect on the enzymatic activity. Therefore, the beads were stored at 4 °C in 0.1 M phosphate buffer (pH 7.0) containing 0.5 mM DTT, 1 mM EDTA and 10% glycerol. There was no loss of coupled activity during more than six months of storage under these conditions.

The immobilized luciferase column was stable at room temperature during use, but slowly lost its activity depending upon the system and conditions of the assay.

Factors Influencing the Assay

Factors influencing the assay with the glass tube packed with immobilized luciferase were tested by using the substrate solution and by injection of solution containing a constant level of NADH to FIA system.

Flow Rate——Light intensity levels at three different flow rates (0.36, 0.45 and 0.54 ml/min) were examined. The flow rate of 0.45 ml/min was selected for subsequent experiments because it gave maximum bioluminescence intensity.

pH and Buffer Molarity——The effect of pH on the bioluminescence reaction is shown in Fig. 3A. The pH–activity curve was quite restricted and the optimum pH in phosphate buffer was 7.0. As for the effect of phosphate buffer molarity on the bioluminescence reaction, the

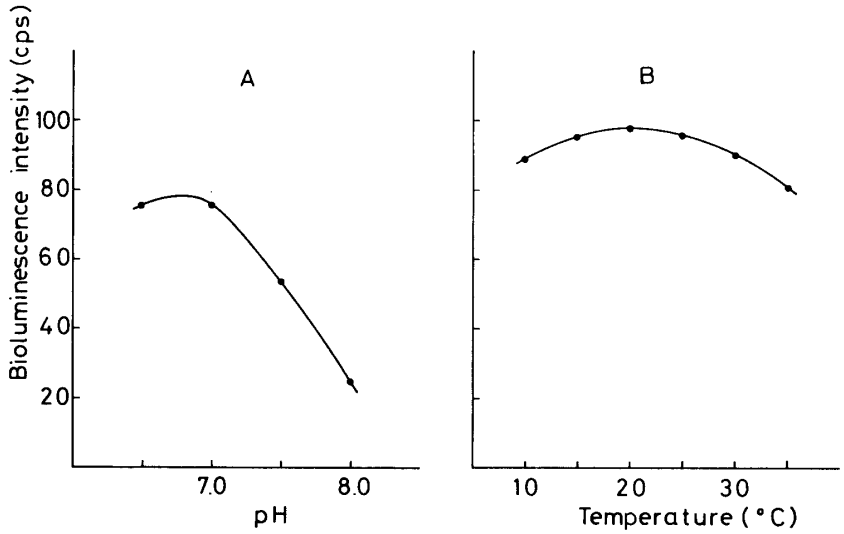


Fig. 3. Effects of pH and Temperature on the Immobilized Luciferase Column

A 1 μl aliquot of 1.3 pmol/μl NADH was injected into the FIA system. Other conditions were the same as in Fig. 1. Counts per second for a 1 μl injection differ from those in other figures because a different packed-bed reactor was used.

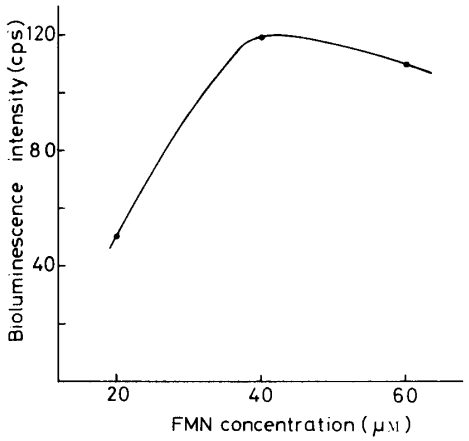


Fig. 4. Effect of FMN Concentration on the Immobilized Luciferase Column

A 1 μl aliquot of 1.3 pmol/μl NADH was injected. The substrate solution contains 10 μM DTT and 0.001% decanal in 0.1 M phosphate buffer (pH 7.0). Counts per second for a 1 μl injection differ from those in other figures because a different packed-bed reactor was used.

optimum value was 0.1 M and it was interesting to note the considerable loss of bioluminescence at higher and lower molarities. Similar effects were observed when the ionic strength was changed with sodium chloride. The molarity–activity relationship exhibited an even narrower optimum. The effect of ionic strength on the luciferase system has been reported previously.[3]

Temperature——The effect of temperature on the assay is shown in Fig. 3B. The optimum temperature for the reaction was 20 °C, and this was adopted in the standard assay procedure. The bioluminescence reaction was comparatively temperature-independent in the range of 10—30 °C. However, if another coupled enzyme system was required to follow the production of NADH, a constant temperature was necessary.

FMN Concentration——The effect of FMN concentration on the bioluminescence reaction is shown in Fig. 4. FMN is known to play a vital role in bacterial luminescence.

However, with excess FMN the reaction was light-sensitive, with erroneous emission in the absence of immobilized luciferase. The optimum FMN concentration was 40.2 μM.

Aldehyde Concentration——A long-chain aliphatic aldehyde greatly stimulates the bioluminescence reaction but, as with FMN, its role is not completely defined. Hastings *et al.*[12] investigated the efficiency of various aldehydes as a function of their chain length. In the

present investigation it was found that decanal gave the best effect. Decanal has a very low solubility and is labile in water, and so it was found more convenient to dispense it as a saturated solution in methanol (200 ppm). Since only small amounts are necessary to catalyze the reaction (0.001% in whole substrate solution), the methanol was not inhibitory.

Dithiothreitol Concentration——Thiol compounds have been commonly used in the bacterial luciferase assay systems.[13] DTT greatly stimulates light intensity. When mercaptoethanol rather than DTT was used, the light intensity was greatly diminished. However, higher amounts of DTT produced erroneous emission in the absence of NADH. The optimum concentration of DTT for the bioluminescence reaction was 10 μM.

Background Light Emission in Photon Counting of FIA

The detectability of FIA depends on the relation between the signal and the background caused by the presence of contaminating traces of dehydrogenase and electronic noise. The latter can be reduced by discriminator. One of the most serious factors limiting detection of small amounts of either substrates or enzymes is the presence of contaminating traces of dehydrogenases, particularly an apparent aldehyde dehydrogenase activity, which is always present. Some of the commercial enzymes used also contained significant amounts of various dehydrogenases. DeLuca *et al.*[14] immobilized both luciferase and oxidoreductase on solid glass supports attached to a glass rod. These immobilized enzymes were used for detecting low concentrations of enzymes that produce NAD(P)H; specifically, glucose-6-phosphate dehydrogenase and malate dehydrogenase. In the measurement they pointed out that background light emission, which was observed in the absence of any added NAD(P)H, was the limiting factor in preparing a more sensitive detection system and they suggested that the emission might be reduced to an insignificant intensity by the purification of these dehydrogenases with NAD- and NADP-affinity columns. Moreover, in a typical assay, light production was initiated by immersing the immobilized enzyme rod into 0.5 ml of a mixture containing all the necessary components at optimal concentrations except for a limiting amount of the enzyme or substrate to be assayed. Light output was integrated for longer than 60 s with a photometer in the stationary state.

In the present FIA system which was equipped with the immobilized luciferase column in front of a photomultiplier tube, a few microliters of serum sample which had been diluted with the enzyme reagent and the buffer was injected directly into the carrier stream. The bioluminescence produced on the immobilized luciferase column was determined by the integrating photon technique (gate time: 1 s). As a result, the contaminating traces of aldehyde dehydrogenase were highly diluted, minimizing the erroneous reaction during the time of column elution. Even in the case of direct injection of serum without pretreatment, background light emission was negligibly small. Such a system permits the use of the photon counter as an extremely sensitive detector.

Measurements of NADH

Control experiments were performed with NADH in 0.1 M phosphate buffer (pH 7.0). When 1 μl of sample containing 130 fmol (100 pg/μl)—1.3 nmol (1.0 μg/μl) of NADH was injected, a good proportionality was found between the amount of NADH and light emission. Typical light emission peaks from the immobilized luciferase reactor are shown in Fig. 5, for 1 μl of 1.0—5.0 ng/μl NADH injected. The reproducibility of the column system in NADH determination was good (coefficient of variation: 3.0% in 5 measurements). Moreover, the sensitivity for NADH is comparable with that of the soluble luciferase method.

About 30 measurements could be carried out within one hour by injecting samples one after another.

Stability of Immobilized Luciferase

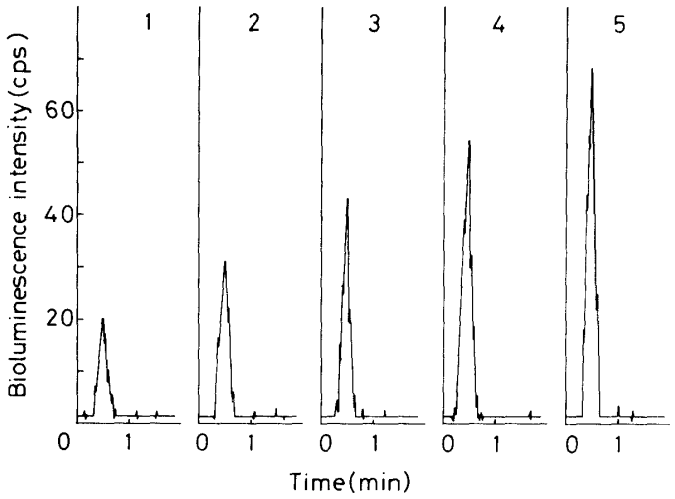


Fig. 5. Bioluminescence Detection of NADH by Flow Injection Analysis with an Immobilized Luciferase Column

The 1 μl injection of NADH contained the following amounts. 1, 1.3 pmol/μl (1.0 ng); 2, 2.6 pmol/μl (2.0 ng); 3, 3.9 pmol/μl (3.0 ng); 4, 5.2 pmol/μl (4.0 ng); 5, 6.5 pmol/μl (5.0 ng). Counts per second for a 1 μl injection differ from those in other figures because a different packed-bed reactor was used.

To determine the lifetime and operational stability of the same luciferase column, we made 50—70 measurements/d during two weeks. After each daily use the column was washed with 0.1 M phosphate buffer (pH 7.0) containing DTT, and stored overnight in a refrigerator. Prior to use, the column was equilibrated with the substrate solution. During the first 7—10 d there was no change in the peak light intensity. Thereafter, the peak light intensity began to decrease, and fell to about one-twentieth of the initial value after two weeks. Obviously, the lifetime of luciferase was lengthened by immobilizing the enzyme on glass beads, as compared with that of dissolved luciferase. About 700 NADH measurements were done with an immobilized enzyme reactor without any change in sensitivity or accuracy. As compared to the soluble reagent (sufficient for 50 NADH measurements and stable for 1 d), at least 50 times more analyses could be carried out with the immobilized bacterial luciferase during a period of several weeks.

Substrate Assays with the Immobilized Luciferase Reactor

We next applied the above described FIA system to the assay of other substrates by coupling a suitable immobilized enzyme column with the bioluminescence enzyme reactor.

Ethanol——Ethanol can produce NADH through the reaction of ADH according to the following equation:

$$\text{ethanol} + \text{NAD}^+ \overset{\text{ADH}}{\rightleftharpoons} \text{acetaldehyde} + \text{NADH} + \text{H}^+$$

ADH catalyzes the reaction in the direction of NADH production at pH 8.5. The pH value of 7.0 of the phosphate buffer for the immobilized luciferase system inhibits NADH production and results in low sensitivity in the detection of ethanol. Accordingly, ADH was immobilized on glass beads and utilized to produce NADH by the suction system (immobilized ADH was packed in a disposable pipette), as described in Experimental.

Serum to which ethanol had been added was successively reacted with the immobilized catalase and diaphorase for the removal of interfering factors by suction and ejection with a syringe. Then 100 μl of 0.4 mM NAD in Tris-buffer (pH 8.5) was added to the eluate from the immobilized diaphorase column, and the solution was reacted with immobilized ADH again.

TABLE I. Analytical Characteristics of the Packed-Bed Reactor

Analyte	Linear range (pmol)	Recovery and precision		
		Level (pmol)	Mean recovery (5 measurements) (pmol)	C.V. (%)
NADH	1.3×10^{-1}—1.3×10^{3}	1.3	1.2	3.0
Ethanol	75.9—3.8×10^{3}	75.9	70.0	8.1
Lactic acid	76.0—3.8×10^{3}	76.0	72.0	7.5

After one minute, NADH produced was injected into the continuous-flow system containing the immobilized luciferase column. NADH was determined from a calibration curve produced from the light intensities of NADH standards obtained by the same determination procedure, and ethanol concentration was calculated mathematically. The detection limit of ethanol was 75.9 pmol (Table I).

L-Lactic Acid——L-Lactic acid can produce NADH through the reaction of LDH according to the following equation:

$$\text{L-lactic acid} + \text{NAD}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{acetaldehyde} + \text{NADH} + \text{H}^+$$

Immobilized LDH was applied to a continuous-flow system with immobilized luciferase as well as ADH. The detection limit for L-lactic acid was similar to that for ethanol (Table I).

The technique described here may be used when only small amounts of NADH or FMN are to be analyzed, especially when they cannot be detected by spectrophotometric or fluorometric means. In addition the system may be used for measuring compounds which are conjugate in their action with NADH. For example micro quantities of ethanol can be determined by measuring the reduction of NAD in the presence of ADH. The cost of the enzymes and chemicals is very small. It is probable that bacterial luciferase offers many other potential applications for the assay of metabolites in very small amounts of samples.

Kurkijärvi *et al.*[5] immobilized bacterial bioluminescence enzymes on CNBr-activated Sepharose. However, Sepharose is not suitable for the FIA system because it is a soft gel and is crushed by the flow of substrate solution. Rigid glass beads are very suitable for use in the immobilized luciferase reactor in the FIA system.

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