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Review

Immobilized Bacterial Luciferase and Its Applications

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ABSTRACT

This review discusses the properties of the bioluminescent bacterial system as well as the methods for immobilization of bacterial luciferases and for their co-immobilization with other enzymes. The analytical systems using immobilized bacterial luciferases and their applications in analytical biochemistry and biotechnology have been described.

Index Entries: Bacterial luciferase; immobilization, of bacterial luciferase; bioluminescent microassay.

INTRODUCTION

The development of high-sensitivity enzymatic assays for various physiologically active substances has been a subject of much scientific research (*1*). These methods have found practical applications in medical diagnosis, biotechnology, and environmental protection. The most promising is, probably, the luminescent assay, since its sensitivity is close to that of the radioisotopic assay, but the former is nontoxic (*2*). Bioluminescent systems help to combine the advantages of enzymatic and luminescent methods. Among a host of bioluminescent systems known, the bioluminescent bacterial system is probably the most practical, because, first, the range of enzymes and metabolites detectable by this system is fairly wide and, second, the bacterial enzymes are less costly and easily available compared to nonbacterial enzymes (*3*).

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Immobilization of enzymes lowers the cost of assays because of the manifold applications and higher stability of immobilized enzymes.

The development of high-sensitivity bioluminescent assays is closely related to some biotechnological problems, such as the preparation of luciferases from microbiological raw material, the development of methods for immobilization of luciferases, and their co-immobilization with other enzymes, as well as the optimization of storage and application of biocatalysts. Of particular interest for bioluminescent microassays are the co-immobilized multienzymatic systems, which notably widen the range of detectable substances. This review covers some biotechnological problems related to immobilization and stabilization of bacterial luciferases and multienzymatic bioluminescent systems. Their applications in bioluminescent microassay are discussed.

SOME PROPERTIES OF THE BIOLUMINESCENT BACTERIAL SYSTEM

Luminous microorganisms are widely found in ocean waters (*4*). The most studied are the luminous bacteria of the following species: *Beneckea harveyi, Photobacterium fisheri, Photobacterium phosphoreum,* and *Photobacterium leiognathi.* Luciferases from bacteria of various species have similar properties and differ somewhat in substrate specificity, stability to proteases, thermal and pH stabilities, and catalytic activities. The highest catalytic activity was observed for bacterial luciferase from *B. harveyi* (*5*).

Bacterial luciferase catalyzes the reaction:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + h\nu \quad (1)$$

where $FMNH_2$ and FMN are the reduced and oxidized forms of flavinmononucleotide, and RCHO and RCOOH are the long-chain aldehyde and relevant acid. The aldehydes, active in Reaction (*1*), have a chain length of C_8 to C_{16}, depending on the strain. The $FMNH_2$ in the presence of oxygen is unstable and is rapidly oxidized, even in the absence of luciferase. The rates of nonenzymatic and enzymatic oxidations of $FMNH_2$ are close, although the nonenzymatic oxidation is not followed by bioluminescence. For this reason, in order to carry out a reaction (*1*), FMN is reduced directly in the reaction mixture chemically or photochemically (*4*). The bacterial cell has a special enzyme, NADH:FMN–oxidoreductase, catalyzing the enzymatic reduction of FMN by Reaction (*2*):

$$FMN + NADH + H^+ \rightarrow FMNH_2 + NAD^+ \quad (2)$$

These enzymes were shown to be nonconductive (*6*). The problem of a possible complexing of luciferase with oxidoreductase, as well as of the mode of their interaction, remains open (*6*).

The bacterial luciferase is a heterodimer consisting of nonidentical α-and β-subunits, with the molecular masses 42 and 37 kdaltons, respectively (*6*). The active site is located on the α-subunit. The role of the β-subunit has not been fully elicited. It is supposedly important as a factor in protecting against the attack of proteases and temperature (*6*).

The NADH- and NADPH-specific oxidoreductases reducing FMN are discriminated. There exists an oxidoreductase specific for both cofactors. The portion of one or the other reductase in the bacterial cell varies depending on the strain. The mode of action of bacterial luciferase is rather complicated (*6*). The reaction runs via a number of intermediate steps, including the enzyme complexing with oxygen and long-chain aldehydes, as well as the formation of an emitter that admittedly has the structure of 4-α-peroxiflavin linked with luciferase. The energy for the emitter formation is supposed to be produced during the aldehyde oxidation to a corresponding acid (*6*). There is no common view in the literature concerning the nature of the emitter in the reactions catalyzed by bacterial luciferases. The phenomenon is widely used for scientific and practical purposes (*7–10*), primarily in analytical biochemistry. Since the bioluminescent bacterial system is NAD(P)H-dependent (Reactions 1 and 2), it is used to detect any component of various multienzymatic systems consuming or producing NAD(P)H. On the basis of bacterial luciferase and NAD(P)H:FMN–oxidoreductase, the commercial analytical reagents were created to detect NAD(P)H. Such reagents are produced by Boehringer and LKB-Wallak. In the USSR, the analogous soluble preparations have been elaborated at Moscow University and Krasnoiarsk Institute of Biophysics. The LKB-Wallak reagent is used to detect NADH at 0.1 n*M*–1 μ*M* (*11*). The efficiency of multienzymatic systems can be notably increased by their immobilization (*12*). The co-immobilization of luciferase and oxidoreductase essentially increases the sensitivity of the NAD(P)H assay (*13–15*).

METHODS FOR IMMOBILIZATION OF THE BIOLUMINESCENT BACTERIAL SYSTEM

First researches on immobilization of bacterial luciferases had been published by the 1970s, when Erlanger obtained the insoluble preparations of *P. fischeri* and *P. leiognathi* luciferases linked with azide of polyacrylic acid (*16*). The enzyme coupling did not affect their specific activity, emission spectrum, pH-optimum of activity, and bioluminescence quantum yield, but strongly influenced thermostability. For instance, the immobilized *P. fischeri* luciferase was 10 times as stable as soluble enzyme. Both immobilized enzymes were stable in suspension at room temperature (23°C) for many hours. Operational stability of insoluble lu-

ciferases was also fairly high: a 20-fold use of the same preparation did not attenuate the luminescence. This carrier binds about 60% protein. Immobilization supposedly runs mostly via lysine ϵ-amino groups (*16*). Yablonsky and De Luca co-immobilized luciferase and oxidoreductase from *P. fischeri* on glass rods (*17*) using the method of diazotization to activate the carrier (*18*). Immobilization notably inactivated enzymes, but the quantum yield of bioluminescence and the pH optimum of activity remained unchanged. The enzyme stability markedly increased. One rod was used more than 100 times and stored for 2 wk at 4°C in the presence of 1 m*M* dithiothreitol without loss in activity (*17*). Immobilization of the bienzymatic system of luminescent bacteria on glass was also described elsewhere (*19*).

The same method was applied later to obtain more complex co-immobilized multienzymatic systems (*15,20,21*). Bacterial luciferase and oxidoreductase were co-immobilized with glucose-6-phosphate, lactate, and malate dehydrogenases to quantify their substrates. The flow-injection method for NADH and lactate assay in blood serum was created on the basis of commercial luciferase and oxidoreductase co-immobilized on alkylamino glass beads (*22*). As carrier, an insoluble, high-polymer collagen was also used. The surface carboxyl groups of collagen were pretransformed to acyl–azide groups (*23*) to carry out the co-immobilization of luciferase and oxidoreductase via amino groups by the azide method (Table 1). The authors reported a high mechanical resistance of the enzymatic preparation obtained. The pH optimum of their activity was shifted from 6.8, corresponding to soluble bacterial luciferase, to 6.4, attributable to the pH optimum of oxidoreductase. This fact allows us to suggest that there occurs a change in the limiting step from luciferase to oxidoreductase (Reaction 2) (*24*).

The bienzymatic bacterial system was entrapped into a gel of bovine serum albumin crosslinked with glutaric aldehyde (*25*). The preparations were used for creatine kinase assay and showed an exclusive operational and thermal stability, which afforded up to 200 assays/d for 2 mo, with no loss in sensitivity to creatine kinase. Preparations with high catalytic activity were obtained by the entrapment of the luciferase system into starch gel (*19*). Immobilization of the bioluminescent bacterial system on nylon, preactivated with 1,6-diaminohexane, retained 50–70% of the enzyme activity. The nylon helix, containing immobilized enzymes, was used to detect NADH (1–2500 nmol) by the flow-injection method. The enzymatic preparations afforded up to 50 assays/d for 2 mo, with no loss in sensitivity to NADH. Over 500–700 samples can be analyzed with only a few milligrams of thus immobilized enzymes (*26*).

The most applicable immobilization methods for the bioluminescent bacterial enzymatic system and for the co-immobilized bioluminescent multienzymatic systems proved to be immobilization on polysaccharide carriers, mainly Sepharose preactivated by cyanogen bromide (*27,28*).

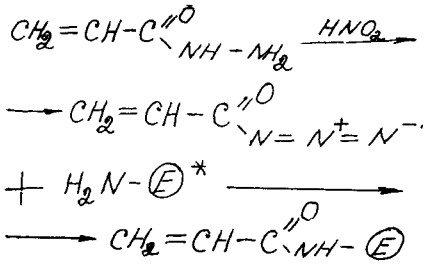
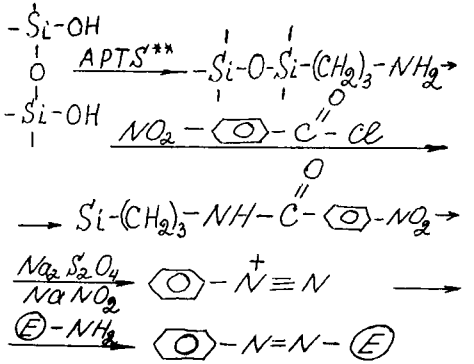
These carriers have a large specific surface and a large number of active functional groups, which makes it possible to obtain the immobilized enzymes with a high specific activity and to use dilute suspensions of immobilized enzymes in analysis (*13*).

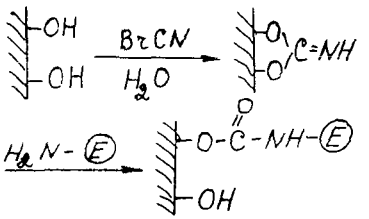
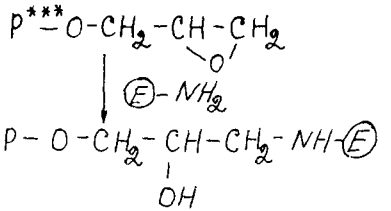
Sepharose and agarose have a high mechanical resistance and are well preserved in the lyophilized state. With immobilization on BrCN–Sepharose, the carrier binds up to 50% of luciferase activity and up to 90% of oxidoreductase activity (*27*). The activity of the bienzymatic system often increases 3–5 times after immobilization (*13,29,30*). This can usually be explained by the flavinmononucleotide reduction by oxidoreductase in close proximity to luciferase, which has a far higher local concentration compared to soluble systems. Analogous effects were observed for other coupled multienzymatic systems immobilized on Sepharose (*31*). Immobilization of the luminescent bienzymatic system on BrCN–Sepharose essentially stabilizes it. The suspension of immobilized enzymes can be stored for a few weeks at 4°C in 1 m*M* dithiothreitol with no loss in activity. Bacterial luciferase and oxidoreductase, immobilized on BrCN–Sepharose, are stable to lyophilization and rapid freezing in liquid nitrogen. They also retain 70–100% activity for up to a few months of storage (*28*). The pH optimum of the BrCN–Sepharose-immobilized bienzymatic system lies within 6.5–7.0 and can be easily shifted by coupling positively or negatively charged ligands to the carrier. The possibility of varying the pH optimum of immobilized enzymes is quite useful for elaboration of active preparations of the co-immobilized multienzymatic systems (*28*).

A disadvantage of the immobilization methods, using BrCN-activated carrier, is the high toxicity of the activator. In one of our researches (*29*) we used epoxyagarose to immobilize the extracts from *B. harveyi*. The preparations were active and stable for a fortnight in suspension (4°C) and for 3 mo in the lyophilized state. The sensitivity of the assays using these preparations was 10 times as low as with the enzymes co-immobilized on BrCN–Sepharose. Immobilization on epoxyagarose needs a long-term incubation of enzyme solutions at a temperature of over 20°C, which inactivates luciferase. For this reason, this method is not always feasible to obtain the high-activity preparations of the co-immobilized multienzymatic systems. At present, BrCN–Sepharose and BrCN–agarose are the best carriers for the immobilized bioluminescent systems.

One of the factors limiting the analytical application of bacterial cells is that it is difficult to produce bacteria with constant physiological properties. It is because of this that immobilized luminescent cells were used for the oxygen assay (*32,33*). *Beneckea harveyi* cells entrapped in calcium alginate gel show a high light emission (Table 2) and possess catalase activity. The last fact permits their application to assay both oxygen and hydrogen peroxide. Living *B. harveyi* cells, immobilized in calcium alginate gel, retain a stable light emission for 1 d. Table 2 lists the gel carriers used for luminescent bacterial cell immobilization.

TABLE 1
Immobilization Methods for Bacterial Luciferases and NAD(P)H:FMN-Oxidoreductases

Carrier	Enzymatic preparation	Immobilization method	Stability	Refs.
Polyacrylhydrazide	*P. fischeri* and *P. liognathi* luciferases partially purified		100% Activity remains at 23°C longer than 15 h 15 d at 4°C	*16*
Porous arylamino glass	Purified luciferase and NAD(P)H:FMN–oxidoreductase from *P. fischeri*		100 assays per day, 15 d at 4°C	*15,17,20*
	Partially purified luciferase and NAD(P)H:FMN–oxidoreductase from *P. fischeri*		100% Activity remains at 4°C for 6 mo in a 10% glycerol, 0.5 m*M* DTT, 700 assays with one preparation of immobilized enzymes	*22*
Insoluble high-polymer collagen	Partially purified luciferase and NAD(P)H:FMN–oxidoreductase from *P. fischeri*	Surface-accessible carboxyl groups of collagen were transferred into acyl–azide groups. Further modification is analogous to method 1	70% Activity remains at 4°C for a fortnight	*24*

Gel of bovine serum albumin and glutaraldehyde	Partially purified luciferase and NADH:FMN–oxidoreductase from *P. fischeri*	Solution of enzyme and BSA is dispersed on cellophane membrane; glutaraldehyde is then added to form gel	A 100% activity remains at 4°C for 8 wk; 200 assays/d, with one membrane	25
Nylon, activated by 1,6-diaminohexane	Purified luciferase and NADH:FMN–oxidoreductase from *P. fischeri*	Enzyme was attached to carrier by glutaraldehyde	A 100% activity remains at 4°C for 2 mo; 50 assays/d with one membrane	25
Agarose, activated by BrCN	*B. harveyi* extracts. Partially purified luciferase and NADH:FMN–oxidoreductase from *B. harveyi*		90% Activity remains at 4°C for a fortnight	13
Sepharose, activated by BrCN	Purified luciferase and NADH:FMN–oxidoreductase from *B. harveyi*		90% Activity remains at 4°C for 5 d	27
Epoxyagarose	*B. harveyi* extracts		90% Activity remains at 4°C for a fortnight	29

*APTS-γ-aminopropyltriethoxysilane.
**E-enzyme.
***P-carrier.

TABLE 2
Properties of Immobilized Cells *B. harveyi* in H_2O_2 Assay[a]

Immobilization method	Bioluminescence activity, arbitrary units	Catalase activity
Photoinduced polymers		
ENT = 104 (600 Å)	—	−
ENT = 101 (400 Å)	1510	+
Calcium alginate[b]	1100	+
Agar	0	+
Cellulose triacetate	0	+
Polyacrylate	0	−
Carrageenan	0	+
Pectic acid	0	+

[a]From ref. (*33*).
[b]A mixture of cell suspension (0.5 mL) and sodium alginate (2 g) was slowly injected into $CaCl_2$ solution. Diameters of resultant gels were 1.5–2.0 mm

APPLICATION OF IMMOBILIZED LUMINESCENT BACTERIAL ENZYMES FOR SCIENTIFIC PURPOSES

Immobilization of bacterial luciferase was first employed as a novel approach to study the mode of enzyme action (*16*). Many applications of immobilized bacterial luciferases showed that the enzymes are capable of turnover without special regeneration, except at the step of FMN reduction.

To study the subunit interactions of bacterial luciferases (*34*), to elucidate the mechanism of coupling bacterial luciferase with oxidoreductase (*35*) and to model various cell processes (*36*), the enzymes immobilized on BrCN–Sepharose were used.

Separately immobilized α- and β-subunits of bacterial luciferase did not possess any catalytic activity. The activity was restored only upon addition of the contrary subunit, i.e., bacterial luciferase was shown to exhibit catalytic activity only in the case of recombination of α-β-subunits (*34*). Immobilization of bacterial luciferase on BrCN–Sepharose does not alter the catalytic constant of the enzyme. Since, with this immobilization method, mainly protein amino groups are modified (*37*), one can conclude that either the amino group is not part of the active site of bacterial luciferases or the amino group of the active site is strongly screened. The K_m of $FMNH_2$ increases 10^3 and 10^2 times for immobilized bacterial luciferase, compared to the native one, on binding via α- and β-subunits, respectively. So, the amino groups of both subunits participate in $FMNH_2$ binding to the enzyme, the role of the α-subunit being more important. The immobilization does not affect the interaction of long-chain aldehydes with bacterial luciferase.

Tu and Hastings (*35*) studied the interaction of bacterial luciferase immobilized on BrCN–Sepharose with soluble oxidoreductase. The immobilized bacterial luciferase was shown to be an affinity sorbent for NADH:FMN–oxidoreductase from the same strain. The functional efficiency of the thus co-immobilized bienzymatic system increased several times compared to soluble enzymes. These results confirm the existence of a specific interaction between the two major enzymes of the bacterial bioluminescent system.

De Luca and Kricka (*36*) carried out co-immobilization of the 11-enzyme system converting glucose to ethanol with the aid of a bienzymatic system of luminescent bacteria. The sensitivity of the bioluminescent assay of ethanol proved, in this case, sufficient to explore the glucose metabolism in the conditions close to those in intact cells. The authors consider that similar systems were convenient models to study the membrane-bound enzymes and their functioning in proximity to membrane surface (*36*).

The reaction kinetics with immobilized and co-immobilized luciferase and oxidoreductase has not been studied in detail. It was noted, however, that the maximum light intensity is achieved for immobilized luciferases 10 times as fast as for soluble enzymes (*38*).

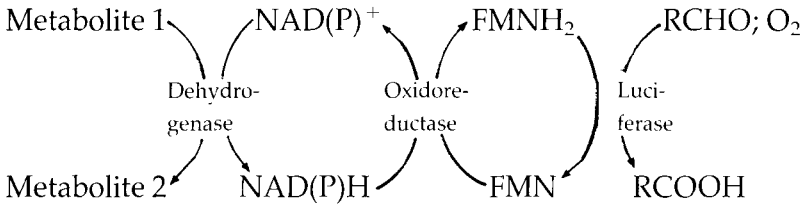
IMMOBILIZED ENZYMES IN LUMINESCENT MICROASSAY

The immobilized enzymes of the bacterial bioluminescent system are fairly promising for analytical biochemistry (Table 3) primarily for assaying NAD(P)H, and $NAD(P)^+$NAD(P)H-dependent enzymes and their

TABLE 3
Assay of Metabolites and Enzymes By Use of The Immobilized Bioluminescent Bacterial System

Substance	Concentrations assayed, *M*	Refs.
NADH	10^{-12}–10^{-7}	*13,14,27,41*
NADPH	10^{-10}–10^{-6}	*27*
NAD^+	10^{-10}–10^{-7}	*14,20,30*
Glucose-6-phosphate	10^{-12}–10^{-10}	*58*
Glucose	2.10^{-11}	*58*
FMN	5.10^{-12}	*58*
Ethanol	2.10^{-6}	*20*
Testosterone	10^{-10}–10^{-8}	*38,59*
Androsterone	10^{-10}–10^{-8}	*38,59*
Hexokinase	2.10^{-11}	*58*
Creatine kinase	1–8U/L	*24*
Sodium formate	10^{-12}–10^{-7}	*14*
Formate dehydrogenase	10^{-12}–10^{-7}	*14*

substrates. Scheme 1 shows the reactions for detection of enzymes and metabolites using a three-enzyme system:



(Scheme 1)

Assay of Pyridine Nucleotides and Metabolites

The bioluminescent assay of NADH (or NADPH) is based on the bienzymatic system—bacterial luciferase and oxidoreductase—in the conditions for which the light intensity is proportional to the concentration of reduced pyridinenucleotide (*39,40*). In recent years, the composition optimized reagents have been elaborated for bioluminescent detection of NADH (*11,41*). The authors (*11*) payed principal attention to the choice of optimum concentration for reductase and luciferase. Two factors were regarded: the light intensity and the duration of the maximum light intensity. The latter factor is very important for the assay of activities of NADH-dependent enzymes and their substrates. The duration of maximum light intensity was shown to depend on oxidoreductase concentration, whereas the light intensity dependent on luciferase concentration. The method with the use of internal calibration permits the detection of 0.1 n*M*–1 μ*M* $NADH_2$ and 1–100 μ*M* ethanol and 0.01–0.3 U/L alcohol dehydrogenase (*11*). High-sensitivity methods to assay NADH and FMN have been developed on the basis of immobilized crude bacterial extracts (*13*). The activity of the immobilized samples increased 2–5-fold compared to that of the soluble extracts. The preparations thus obtained had no background associated with NADH and FMN and permitted the detection of NADH from 0.1 n*M* to 1 μ*M* and FMN from 0.1 n*M* to 0.1 μ*M*. This indicates the presence about 0.1 pmol of cofactor in a sample. The advantages of using crude extracts in obtaining bioluminescent reagents are primarily their low cost, simple production, and high enzymatic activity of the sample (*13*). Partially purified luciferase and oxidoreductase co-immobilized on BrCN–agarose sensitized the NADH assay 100-fold (*14*).

Bioluminescent assays of NAD^+ have been developed by coupling various dehydrogenase reactions with a bienzymatic bacterial reaction (*14,20,30,42*) (Scheme 1). The sensitivities of methods with the use of lactate, malate, alcohol, and formate dehydrogenases were 10^{-10} and $10^{-12}M$ for soluble and immobilized systems, respectively. There are patents on the application of partially purified luciferase and NADH: FMN–oxidoreductase from *P. fischeri* and *P. phosphoreum* immobilized on BrCN–Sepharose to assay NADH, NAD^+, and alcohol dehydrogenase

(*43–45*). In practice, the detection limit for nucleotides depends not on the sensitivity of the bioluminescent assay proper, but mainly on the amount of nucleotide admixtures in the reagents and enzymes in use. For this reason, many authors suggest that the enzyme and substrate preparations should contain a minimum amount of admixtures or they should be additionally purified.

The bioluminescent detection of NADH and NADPH is a basis of the assays for a multitude of various metabolites of clinical importance (*46–48*). Feasibility of multienzymatic systems in which the metabolite under assay generates NADH or NADPH was reported by De Luca et al. (*30*). Various metabolites—D-glucose, 6-phosphogluconate, L-lactate, L-malate, L-alanine, and L-glutamate—were assayed by using a specific dehydrogenase (or hexokinase and dehydrogenase) and bacterial luciferase with oxidoreductase co-immobilized on BrCN–Sepharose. The bioluminescent detection was performed for a wide range of metabolite contents (0.01–100 nmol). This rapid, well-reproducible assay needed minimum amounts of enzymes since the immobilized biocatalysts could be used many times. Co-immobilized enzymes proved to be 1000–2000 times more active in coupled reactions than the relevant soluble enzymes and tens of times more active than separately immobilized ones. In general, the longer the sequence of reactions catalyzed by co-immobilized enzymes, the higher the efficiency of co-immobilization (*49,50*).

Assay of NAD^+-Dependent Dehydrogenases

Ismailov et al. (*51*) studied the activity of dehydrogenases in human blood serum using a coupled system with soluble and immobilized enzymes from *P. fischeri*. The activity of dehydrogenases in normal human blood can be assayed with 10 μL of a 1000–fold diluted serum for an assay time of about 1 min. The assays have been developed for the isozymes of lactate, malate, and isocytrate dehydrogenases at clinically important concentrations.

Assay of Bile Acids

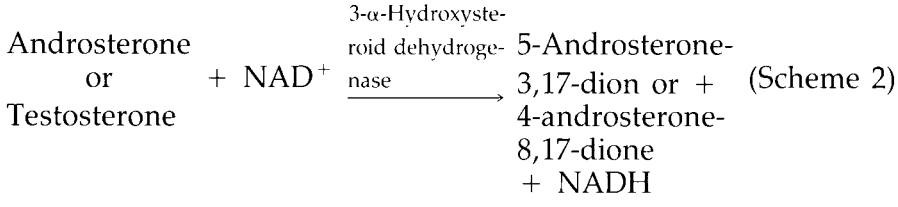
An increased concentration of bile acids in blood serum is caused by liver diseases (*52–54*). Because of the complexity and long duration of chromatographic and radioimmunological assays, the test has not been employed thus far for medical diagnosis. The enzymatic spectrophotometric assay is not very sensitive. The bioluminescent assay with the use of 3-α-hydroxysteroid dehydrogenase and bacterial luciferase proved to be most convenient and sensitive for clinical applications (*55*). One assay needed 20 μL serum. The serum enzymes are preinactivated with trichloroacetic acid. After addition of NAD^+ and luciferase, the luminescence is initiated by introduction of 3-α-hydroxysteroid dehydrogenase. The initial rate of light intensity increase is compared with the standard, a mixture of equimolar amounts of cholate, deoxycholate, and henode-

oxycholate. The linear range for cholate is observed within 0.3–300 μ*M*. The assay correlates well with the spectrophotometric assay, but is 10 times as sensitive. Primary bile acids (*56*) and 12-α-hydroxy acids (*57*) were assayed using bacterial luciferase and 12-α-hydroxysteroid dehydrogenase co-immobilized on BrCN–Sepharose. The calibration plots were obtained using cholyltaurine, which also served as an internal calibration standard in assaying serum samples. Linearity was observed within 1–200 μ*M* bile acids, regardless of their nature. Androsterone, cortisone, 12-α-ketocholic acids, and other metabolites (lactate, pyruvate, acetoacetate, β-hydroxybutyrate, or malate) did not hinder the determination. The assay correlates well with the chromatographic assay of bile acids, but is more sensitive by a few factors.

Kricka et al. (*58*) successfully used the above co-immobilized enzymes in a flow column reactor and obtained a linear dependence of bioluminescence intensity on the content of cholic acids within 8–325 pmol.

Assay of Hormones

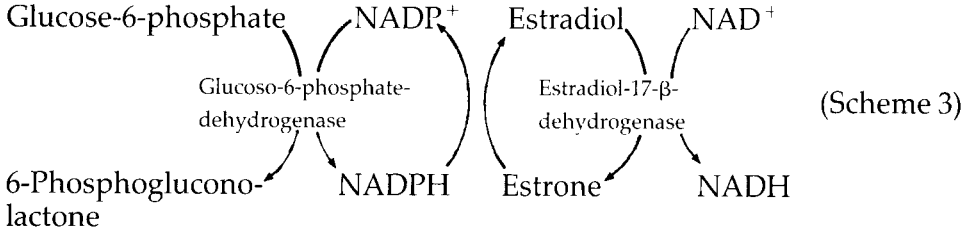
Hormones are assayed by using specific hydroxysteroid dehydrogenases co-immobilized with bacterial luciferase. The assay is based on the reaction:



(Scheme 2)

The assay sensitivity was 0.8 pmol of androsterone and testosterone (*38*).

A high-sensitivity assay for other hormones—estrone and estradiol—was reported elsewhere (*59,60*). The assay consists of two steps: the transhydrogenase reaction, generating NADH in the system (transhydrogenase activity of estradiol-17-β-dehydrogenase is used), and the bioluminescent reaction of NADH determination. Below is the transhydrogenase reaction:



(Scheme 3)

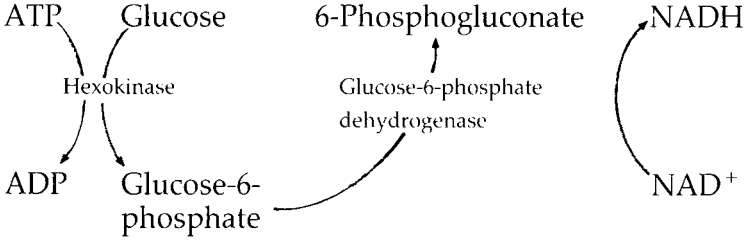
With a large excess of estradiol-17-β-dehydrogenase in respect to the steroid assayed, the rate of NADH formation is a linear function of the

overall concentration of estrone and estradiol. Since NADH is continuously regenerated, its formation rate is constant for a few hours. After incubation of the reaction mixture, NADH formed for 15, 30, or 60 min is assayed by the bioluminescent method. Because of the high specificity and activity of estradiol–transhydrogenase, the bioluminescent assay permits the determination of 0.1–50 pg estrogen, whereas the radioimmunoassay has a detection limit of 5 μg.

Assay of Glucose and Its Derivatives

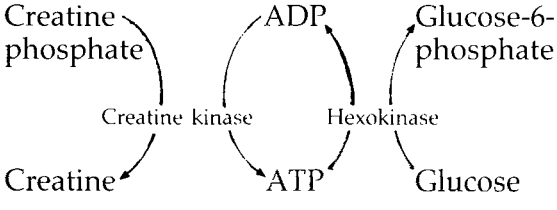
Glucose-6-phosphate and 6-phosphogluconate within 1–100 pmol can be quantified by fluorometry or chromatography. Both methods need expensive equipment. In addition, the fluorometry is complicated by the necessity of using cyclic enzymatic reactions for the high-sensitivity assay.

The bioluminescent assay of these compounds is based on the following sequence of reactions:



(Scheme 4)

Following this, the NADH is assayed by means of a bienzymatic bacterial system. Kricka et al. (*58*) developed the assays for glucose, glucose-6-phosphate, and 6-phosphogluconate using multienzymatic systems co-immobilized with a bioluminescent bacterial system on BrCN–Sepharose. The sensitivities of assays were 10 pmol–1.5 nmol for glucose, 10 pmol–100 nmol for glucose-6-phosphate, and 10 pmol–100 nmol for 6-phosphogluconate. Blum et al. (25) immobilized the bienzymatic system of *P. fischeri* in the gel of bovine serum albumin to assay creatine kinase activity (Scheme 5).



(Scheme 5)

Glucose-6-phosphate was assayed according to Scheme 4. The detection range for creatine kinase by this method was 0.5–10 U Sigma/mL, which corresponded to the clinically important concentration range for the enzyme in the blood serum.

BIOLUMINESCENT REACTORS BASED ON IMMOBILIZED BACTERIAL LUCIFERASES

One of the main advantages of immobilized enzymes—their manyfold applicability—is most efficiently used in various bioluminescent sensors: membranes, rods, and column reactors. Guilbault et al. (*25*) incorporated a commercial bienzymatic bacterial system into the gel of bovine serum albumin. The samples obtained were used 100 times and more, the assay error being not more than 15%.

Immobilized bacterial luciferases were also used in a flow column reactor for the bioluminescent assay (*9,25,58,61,62*). In this case, the cuvet for measuring the luminescence was replaced by a small, 0.5-mL column filled with a carrier with immobilized enzymes. A solution of substrates was pumped through a column. The assay sample was introduced through a special device. The signal was followed by a recorder. One assay took not more than 1.5 min. The column did not need washing before measurements. The operational stability of the column with immobilized enzymes was 80–100 assays/d for 1 wk. This method was used to assay 10–10^6 pmol NADH (*61*) and 5–1000 pmol glucose-6-phosphate or bile acids (*58*). The method with the column reactor correlates well with the chromatographic methods.

APPLICATION OF IMMOBILIZED CELLS OF LUMINESCENT BACTERIA

Beneckea harveyi cells, entrapped in alginate gel, were used to determine low concentrations of hydrogen peroxide (*33*). In this case, the substance measured was oxygen forming from H_2O_2 under the action of catalase containing in bacterial cells. The linear range of measured H_2O_2 concentrations depended on the amount of immobilized cells taken for an assay. For 0.5, 0.8, and 1.0 g of immobilized cells the range of assayed concentrations was 0.6–6.0, 0.5–5.0, and 0.4–4.0 μM H_2O_2. A continuous assay halved the activity of immobilized cells for 20 h.

An oxygen sensor containing intact *P. fischeri* cells, placed between two semipermeable polypropylene membranes, helped to measure oxygen in gaseous and liquid phases at the concentration of 0.4 μM and higher. A continuous work of the sensor at 20°C for 8 h decreased not more than 10% of cell activity (*63*). These oxygen sensors can help to monitor the photosynthesis and to assay herbicides inhibiting photosynthesis (*64*), as well as any substances influencing oxygen concentration in the medium.

A promising approach is the use of luminescent bacterial cells to assay toxins, narcotics, and poisons inhibiting the luminescence in vivo. Kratasjuk et al. (*65*) reviewed in detail the methods for assaying toxins with the use of bacterial bioluminescence. Lyophilized cells of *P. phospho-*

reum were used to assay microquantities (0.9–25 μg/mL) of mycotoxins in foodstuffs (*66*). The dark mutants of luminescent bacteria, because of their capability to recover the luminescence in the presence of various mutagens, appeared to be sensitive test systems in assaying nanogram quantities of various mutagens, carcinogens, and inhibitors of DNA synthesis (*67*).

CONCLUSION

Literature analysis shows wide prospects for application of bioluminescent bacterial systems in clinical biochemistry, environmental control, researches, and biotechnology. Most publications of the 1980s are devoted to the preparation and application of co-immobilized multienzymatic systems in bioluminescent microassay. Application of these systems lowers the cost, increases reproducibility, and provides an opportunity for automation and standardization of the assay. A principal biotechnological problem for the near future is, presumably, the foundation of the industrial production of reagents (kits) and equipment for bioluminescent assay. This will help to implement widely the practice of the above methods, which exceed many bioanalytical procedures in sensitivity simplicity, and diversity of applications.

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