Chemiluminescent Enzyme Immunoassay
A Review

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Highly sensitive chemiluminescent enzyme immunoassays developed in our laboratory are reviewed. Oxidases, peroxidase and glucose oxidase, generating \( \text{H}_2\text{O}_2 \) can be assayed by isoluminol/microperoxidase or peroxynitrate/fluorescent dye. \( \beta \)-D-Galactosidase is also assayed by chemiluminescence reaction based on the coupled enzyme reaction using lactose/glucose oxidase. \( \beta \)-D-Galactosidase can be measured by using o-nitrophenyl \( \beta \)-D-galactoside as substrate and chemiluminescence reaction of NADH after coupled enzyme reaction of galactose dehydrogenase. Sucrose and lucigenin are used as substrate and chemiluminescent reagent for the assay of invertase. Glucose-6-phosphate is determined by using glucose 6-phosphate as substrate and chemiluminescent assay of NADH. Alkaline phosphatase is assayed by using NADP\(^+\), alcohol dehydrogenase and chemiluminescence reaction of NADH. These methods can be applied successfully to the chemiluminescent enzyme immunoassays for various hormones and drugs in biological fluids.

Keywords
Chemiluminescent enzyme immunoassay, chemiluminescence, enzyme, luminol, isoluminol, lucigenin, bis[2,4,6-trichlorophenyl]oxalate, nicotinamide adenine dinucleotide, 17a-hydroxyprogesterone, thyroxin, mass screening, congenital hypothyroidism, congenital adrenal hyperplasia

1 Introduction
Since its introduction by Yalow and Berson 30 years ago, radioimmunoassay (RIA) has made it possible to measure many hormones and drugs at extremely low concentrations, thus opening a new avenue of sensitive assay. In spite of its success, it involves the use of an isotopically tagged antigen or antibody which may pose problems associated with waste disposal, short half-life and radiolysis of the labeled marker. To avoid these drawbacks while retaining the specificity of an immunoassay, various alternatives to RIA\(^1\) are developed, such as enzyme immunoassay (EIA), fluorescent immunoassay, particle immunoassay, spin immunoassay, chemiluminescent immunoassay and bioluminescent immunoassay.

Among these methods, the enzyme immunoassay methods are the most useful technique, since they are sensitive as RIA. The laboratory equipment required is relatively inexpensive, readily available, and the reagents are reasonably priced and have a long shelf life.

Recently, chemi- and bioluminescence (CL, BL) have had an impact on biochemical analysis, on medicine and on clinical diagnosis.\(^2\) CL is the phenomenon observed when the excited product of an exoergic chemical process reverts to its ground state with emission of light. A large number of molecules are capable of exhibiting CL, but only a few yield intense CL. The best known CL reactions are those of luminol, lucigenin and active oxalates. Many investigators have
explored the use of CL or BL in order to increase the sensitivity of the immunoassay. These CL and BL immunoassays have been classified into four types, as follows: (1) immunoassay using CL labels (e.g. isoluminol derivatives or acridinium esters), (2) EIA monitored by means of a CL reaction (e.g., luminol/ peroxidase/H₂O₂), (3) EIA monitored by means of a BL reaction (e.g., luciferase/luciferin), and (4) immunoassay using cofactors as labels and monitored by means of a BL reaction.

In this review, the study on CL EIAs developed in our laboratory has been described.

2 Chemiluminescence Reaction

2.1 Luminol and isoluminol

Luminol has been one of the most studied CL molecules (Fig. 1), although its detailed reaction mechanism in aqueous solution is still not clear in spite of very extensive research. Luminol emits light in the presence of alkaline hydrogen peroxide and a catalyst. Various oxidants are capable of initiating the reaction and a large number of compounds catalyze or influence the reaction of luminol. These compounds range from simple transition metal ions to macromolecules, such as peroxidase enzymes. The reaction in aqueous solution is very pH dependent, and only peroxidase enzymes catalyze luminol/H₂O₂ system to produce light efficiently at near neutral pH range from 7 to 8.

Various isoluminol derivatives were synthesized because the emission intensity of luminol was diminished substantially by alkylation and particularly acylation of the 5-amino-substituent of luminol. Its 6-amino-isomer, isoluminol, actually increases in efficiency on alkylation and finds use in derivative form. Aminobutylethyl isoluminol (ABEI) and aminohexylethyl isoluminol (AHEI) have been used in CL immunoassay.

We have developed CL EIAs using horseradish peroxidase (HRP) as labels for cortisol and dehydroepiandrosterone.

2.2 Bis(2,4,6-trichlorophenyl)oxalate (TCPO)/fluorescent dye

The peroxyoxalate-type CL reaction reported by Rauhut is the most efficient nonenzymatic CL reaction known, having a quantum yield as high as 25%. The reaction mechanism using TCPO/H₂O₂/fluorescent dye is illustrated in Fig. 2. Hydrogen peroxide and oxalic ester (TCPO) react, forming intermediates capable of transferring as much as 105 kcal/mol of energy to the fluorescent acceptor. 1,2-Dioxetiethanedione is the postulated key intermediate in this reaction. The fluorescent acceptor is excited and then emits its characteristic light.

Sherman et al. showed that perylene exhibits the strongest CL response among various fluorescent aromatic hydrocarbons. We have reexamined various water-soluble fluorescent dyes because it should be possible to adapt the TCPO CL method to the EIA system. The highest value of S/N ratio was obtained by using 8-anilinonaphthalene-1-sulfonic acid (ANS). Flourescin showed also a good response with this reaction. The CL assay for H₂O₂ using TCPO/ANS is illustrated in Fig. 3. The detection limit of this method is 0.1 pmol/assay tube, corresponding to 10⁻⁹ M of H₂O₂. The detection limit is lower than those obtained by colorimetric and fluorometric methods, and a CL method using isoluminol-microperoxidase, as shown in Table 1.

Fig. 1 Chemiluminescence reactions of luminol and isoluminol.

Fig. 2 Chemiluminescence reaction of bis(2,4,6-trichlorophenyl)oxalate with H₂O₂ and fluorescent dye.
2.3 Lucigenin and N-methylacridinium ester

Lucigenin (N,N'-dimethyl-9,9'-bisacridinium dinitrate) is one of the classical organic CL reagents, its CL being produced by addition of either H₂O₂ or organic reducing compounds to an alkaline solution. Nieman et al. reported the CL assays of biologically important reducing substances, such as glucose, glucuronic acid, hyaluronic acid, ascorbic acid and creatinine. We have also examined CL reaction of lucigenin with various organic compounds, and found that the compounds having α-hydroxycarbonyl groups gave intensive CL. Nieman et al. suggested that the CL reaction of lucigenin with reducing sugars is based on the formation of 1,2-enediol tautomer which is oxidized by lucigenin in a subsequent reaction step. Reducing sugars, such as glucose, galactose, fructose, produce light, while methyl glycosides, sucrose and sorbitol give no CL because they have no α-hydroxycarbonyl group.

In contrast to luminol compounds, the CL reaction is well established. As shown in Fig. 4, the emitting species is N-methylacridone. Lucigenin can not be used as the CL label in immunoassay because it has no coupling group. Recently, a CL acridinium ester has been synthesized and used widely for the CL labeling of antibodies.

2.4 NADH

Co-factors, NADH and NADPH, are important substances in biology. Almost every substance of biological interest can be measured with use of these co-factors in the presence of dehydrogenases. Many papers have been published with analytical procedures using these co-factors. The sensitivities of spectrophotometric and fluorophotometric methods are 10⁻³ and 10⁻⁸ mol/l, respectively. Though the bioluminescence methods are very highly sensitive, the reactant, bacterial luciferase, is too expensive and unstable to use in routine assays. Therefore, we have developed a highly sensitive method for the CL assay of NADH and NADPH based on the CL reaction using an electron mediator and isoluminol-microperoxidase.

The principle of CL reaction of NADH is illustrated in Fig. 5. NADH or NADPH reduces molecular oxygen in the reaction solution to superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂) in the presence of electron mediator. Williams and Seits used Methylene Blue as an electron mediator, but its reaction rate was slow compared with phenazinium methylsulfate. However, it was not so stable, especially under light. A more stable electron mediator, 1-methoxy-5-methylphenazinium methylsulfate (1-MPMS) has been used in this CL reaction. Generated O₂⁻ and H₂O₂ react with isoluminol in the presence of microperoxidase mPOD to emit light.

As shown in Fig. 6, the working curves of NADH and NADPH are linear in the range from 1 pmol/assay (20 µmol/l) and the detection limit is 2 pmol/assay. The sensitivity of this method is lower than that of a bioluminescent method using bacterial luciferase/FMN oxidoreductase, but higher than those of UV and fluorescent method.

In order to increase the sensitivity of CL assay of NADH, the enzyme cycling reaction of NAD⁺/NADH is used. The principle is illustrated in Fig. 7. Ethanol/alcohol dehydrogenase and oxaloacetate/malate dehydrogenase are used as the substrate and co-enzyme, respectively. After the enzyme cycling reaction, the malate generated in the reaction mixture is converted to oxaloacetate and NADH by adding excess NAD⁺ and malate dehydrogenase. This step is an equilibrium reaction. Therefore, glutamate and glu-
The detection limit is 0.03 pmol/assay, corresponding to 0.05 µmol/l. Detection limits of NADH obtained by various methods are summarized in Table 2. This enhanced CL assay is more sensitive than the CL assay mentioned above and almost comparable to the bioluminescent method.

3 Chemiluminescent Assay of Enzymes

Measurement of enzyme activity via reactions that produce light has a number of advantages: chemiluminescent assays are very sensitive and very rapid; typically measurements are completed within 1 min. Therefore, in order to increase the sensitivity of enzyme immunoassay, conventional enzymes, peroxidase (POD), glucose oxidase (GOD), invertase (INV), β-α-galactosidase (BGase), glucose-6-phosphate dehydrogenase (G6PDH) and alkaline phosphatase (ALP), have been determined by the CL reactions mentioned above. The principles of CL assay for enzymes are illustrated in Table 3.

3.1 Peroxidase

Peroxidase (POD) can be measured sensitively by the CL reaction of luminol/H₂O₂. The luminol...
3.2 Glucose oxidase

Glucose oxidase (GOD) is an enzyme which produces $\text{H}_2\text{O}_2$ during a preincubation period that is appropriately selected in order to increase the sensitivity of the system. GOD oxidizes glucose to yield $\text{H}_2\text{O}_2$, which can be measured by CL reaction of TCPO/ANS. The detection limit of this assay is $6 \mu\text{U}/\text{assay}$, which corresponds to $320 \text{ amol}/\text{assay}$. When measured by a Picolite Model 6500 Luminometer, the detection limit is 30 amol/assay.

3.3 $\beta$-d-Galactosidase

$\beta$-d-Galactosidase (BGase) can be assayed by three CL methods, as illustrated in Table 3. BGase is an enzyme which catalyzes the hydrolysis of lactose to $\alpha$-d-glucose and $\beta$-d-galactose. Hydrogen peroxide generated from the coupled enzyme (GOD) reaction is measured by the CL reaction using isoluminol/microperoxidase or peryoxoxalate/fluorescent dye. Typical standard curves of BGase ranged from $6.3 \times 10^{-3}$ to $6.3 \times 10^{-3} \mu\text{U}/\text{assay}$, corresponding to 7.4X10$^{-6}$ mol.

3.4 Invertase

The principle of CL assay of invertase (INV) is illustrated in Table 3. INV ($\alpha$-d-fructofuranosidase) is an enzyme which catalyzes the hydrolysis of sucrose to $\alpha$-d-glucose and $\beta$-d-fructose. Sucrose is not a reducing sugar but both $\alpha$-d-glucose and $\beta$-d-fructose are reducing sugars. Therefore, intensive light is produced by adding lucigenin solution into the hydrolyzed sucrose solution. A typical standard curve ranges from $1.25 \times 10^2$ to $1 \times 10^4 \mu\text{U}/\text{assay}$. The detection limit is $125 \mu\text{U}/\text{assay}$, corresponding to $7.4 \times 10^{-16} \text{ mol}$.

3.5 Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PDH) is frequently used for various homogeneous EIAs. Its activity is generally assayed by either UV or fluorometric methods. The principle of CL assay of G6PDH is shown in Table 3. NADH generated from the enzymatic reaction of G6PDH is assayed by the CL reaction of NADH reported by Ishikawa et al. The detection limits of NADH obtained by various methods are listed in Table 3. INV (a-D-fructofuranosidase) is an enzyme which catalyzes the hydrolysis of sucrose to $\alpha$-d-glucose and $\beta$-d-fructose. Sucrose is not a reducing sugar but both $\alpha$-d-glucose and $\beta$-d-fructose are reducing sugars. Therefore, intensive light is produced by adding lucigenin solution into the hydrolyzed sucrose solution. A typical standard curve ranges from $1.25 \times 10^2$ to $1 \times 10^4 \mu\text{U}/\text{assay}$. The detection limit is $125 \mu\text{U}/\text{assay}$, corresponding to $7.4 \times 10^{-16} \text{ mol}$.

3.6 Alkaline phosphatase

Alkaline phosphatase (ALP) is also widely used in EIAs. It catalyzes the hydrolysis of phosphate monoesters in alkaline medium. Several substrates have been proposed for spectrophotometric or fluorophotometric assay of ALP. A CL assay of ALP based on the coupled enzyme reaction is developed. Three methods have been devised. Their principles are shown in Table 3: Method I using glucose-1-phosphate as substrate and GOD as coupled enzyme, Method II using galactose-1-phosphate as substrate and $\beta$-galactose dehydrogenase, and Method III using NADP$^+$ as substrate and alcohol dehydrogenase as coupled en-
enzyme. In Method I, the H$_2$O$_2$ generated is determined by the CL method using isoluminol/microperoxidase. The CL assay for NADH is used in Method II and III. The ALP detection limits obtained by these CL method and other methods are compared in Table 5. The Method III using NADP$^+$ is more sensitive than the other two methods. The detection limit is 0.25 pg/ml, corresponding to $2 \times 10^{-18}$ mol/assay. This value is lower than that of the colorimetric method using p-nitrophenyl phosphate but one order larger than that of

### Table 3 Chemiluminescent assay of various enzymes used in EIA

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Chemiluminescence reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase (POD)</td>
<td>Luminol$+\text{H}_2\text{O}_2 \xrightarrow{\text{POD}} \text{pH 7-8} \rightarrow$ 5-Aminothalate$+\text{N}_2+h\nu$</td>
</tr>
<tr>
<td>Glucose oxidase (GOD)</td>
<td>$\alpha$-Glucose$+\text{O}_2 \xrightarrow{\text{GOD}} \alpha$-Glutonic acid$+\text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O}_2+\text{TCPO} \xrightarrow{\text{O}} \text{C}-\text{C} +$ 2,2,4,6-Trichlorophenol</td>
</tr>
<tr>
<td></td>
<td>$\text{O}-\text{O}$ $\text{C}-\text{C} + \text{ANS} \rightarrow$ 2CO$_2+\text{ANS}^*$ $\downarrow$ $h\nu$</td>
</tr>
<tr>
<td>$\beta$-d-Galactosidase (BGase)</td>
<td>1) Lactose$+\text{H}_2\text{O} \xrightarrow{\text{BGase}} \alpha$-d-Glucose$+\beta$-d-Galactose</td>
</tr>
<tr>
<td></td>
<td>$\alpha$-d-Glucose$\Leftrightarrow\beta$-d-Glucose</td>
</tr>
<tr>
<td></td>
<td>$\beta$-d-Glucose$+\text{O}_2 \xrightarrow{\text{GOD}} \alpha$-Glutonic acid$+\text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td></td>
<td>or $\text{H}_2\text{O}_2+\text{TCPO}+\text{ANS} \rightarrow h\nu$</td>
</tr>
<tr>
<td></td>
<td>2) $\alpha$-Nitrophenyl-$\beta$-d-galactoside$+\text{H}_2\text{O} \xrightarrow{\text{BGase}} \alpha$-Nitrophenol$+\beta$-Galactose</td>
</tr>
<tr>
<td>Invertase (INV)</td>
<td>Sucrose$+\text{H}_2\text{O} \xrightarrow{\text{INV}} \alpha$-Glucose$+\alpha$-Fructose</td>
</tr>
<tr>
<td></td>
<td>Lucigenin+Reducing sugar$\rightarrow N$-Methylacridone$+h\nu$</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase (G6PDH)</td>
<td>G6P$\xrightarrow{\text{G6PDH}}\text{NAD}(P)^+ \rightarrow \text{1-MPMS}$$\xrightarrow{\text{O}<em>2}$ $\text{O}</em>{2}^-+\text{H}_2\text{O}_2$ $\downarrow$ $h\nu$</td>
</tr>
<tr>
<td></td>
<td>G6P$\xrightarrow{\text{6PG}}\text{NAD}(P)^+ \rightarrow \text{1-MAP}$$\xrightarrow{\text{O}<em>2}$ $\text{O}</em>{2}^-+\text{H}_2\text{O}_2$ $\downarrow$ $h\nu$</td>
</tr>
<tr>
<td>Alkaline phosphatase (ALP)</td>
<td>1) $\alpha$-Glucose-1-phosphate$+\text{H}_2\text{O} \xrightarrow{\text{ALP}} \alpha$-Glucose$+\text{Pi}$</td>
</tr>
<tr>
<td></td>
<td>$\alpha$-Glucose$+\text{O}_2 \xrightarrow{\text{GOD}} \alpha$-Gluconic acid$+\text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O}_2+\text{1L}+\text{mPOD} \rightarrow h\nu$</td>
</tr>
<tr>
<td></td>
<td>2) $\alpha$-Galactose-1-phosphate$+\text{H}_2\text{O} \xrightarrow{\text{GalDH}} \alpha$-Galactose$+\text{Pi}$</td>
</tr>
<tr>
<td></td>
<td>$\alpha$-Galactose$+\text{NAD}^+ \rightarrow $ $\alpha$-Galacto-1,5-lactone$+\text{NADH}$</td>
</tr>
<tr>
<td></td>
<td>$\text{NADH}+\text{1-MPMS}^+\text{1L}+\text{mPOD} \rightarrow h\nu$</td>
</tr>
<tr>
<td></td>
<td>3) $\text{NADP}^+\text{H}_2\text{O} \xrightarrow{\text{ALP}}\text{NAD}^++\text{Pi}$</td>
</tr>
<tr>
<td></td>
<td>$\text{NAD}^+\text{C}_2\text{H}_2\text{OH} \xrightarrow{\text{ADH}} \text{NADH}+\text{CH}_3\text{OH}$</td>
</tr>
<tr>
<td></td>
<td>$\text{NaDH}+\text{1-MPMS}^+\text{1L}+\text{mPOD} \rightarrow h\nu$</td>
</tr>
</tbody>
</table>

G6P, glucose 6-phosphate; 6PG, glucose-6-lactone 6-phosphate; ADH, alcohol dehydrogenase.
the enzyme amplification method using NADP+/alcoholdehydrogenase/diaphorase. The sensitivity of CL assay may be enhanced by a coupling enzyme cycling method.

4 Chemiluminescent Enzyme Immunoassay

Many investigations concerning with CL EIA have been carried out to improve the sensitivity of the assay system. We have developed various CL EIAs, which are summarized in Table 6. A representative of the system used in CL EIAs is illustrated in Fig. 8. The double antibody solid phase method is used to separate the bound and free fractions after and immune reaction. After separation, the enzyme activity is assayed by the CL reaction. Several typical CL EIAs developed in our laboratory are described in this chapter.

4-1 Thyroxin

Congenital hypothyroidism is one of the more common preventable cause of mental retardation. At present the mass screening test of neonates for congenital hypothyroidism has been carried out with both or either of RIA for thyrotropic hormone (TSH) and/or thyroxin (T4) in dried blood samples spotted on filter paper cards. Recently, needs for non-isotopic methods for a screening test exists in many screening centers which have no laboratory of radioisotopes. In Japan, we have succeeded in starting routine screening with a fluorescent EIA for TSH using BGase, and improved it to the ELISA method using HRP as a label enzyme. It is necessary to perform a parallel assay of TSH and T4 in the same sample. Although several T4-EIAs were reported, none of them could be used in the mass screening test because of their low sensitivity. Therefore, we have developed a highly sensitive CL EIA for T4 which can be used for screening of congenital hypothyroidism.

Table 4 Comparison of BGase detection limits obtained by various assay methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Detection limit (mol/assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectrophotometry</td>
<td>5×10⁻¹⁰</td>
</tr>
<tr>
<td>(o-NPGal)</td>
<td></td>
</tr>
<tr>
<td>Fluorophotometry</td>
<td>2×10⁻¹⁴</td>
</tr>
<tr>
<td>(4-MU-Gal)</td>
<td></td>
</tr>
<tr>
<td>Chemiluminescence</td>
<td></td>
</tr>
<tr>
<td>(Lactose/GOD/IL/mPOD)</td>
<td>5×10⁻¹⁵</td>
</tr>
<tr>
<td>(Lactose/GOD/TCPO/ANS)</td>
<td>2×10⁻¹⁶</td>
</tr>
<tr>
<td>(o-NPGal/GADH/NADH-CL)</td>
<td>1×10⁻²⁰</td>
</tr>
<tr>
<td>Bioluminescence</td>
<td></td>
</tr>
<tr>
<td>(o-NPGal/GADH/NADH-BL)</td>
<td>2×10⁻²²⁸</td>
</tr>
</tbody>
</table>

Table 5 Comparison of alkaline phosphatase detection limits obtained by various assay methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Substrate</th>
<th>Detection limit/pg</th>
<th>RSD, % (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemiluminescent</td>
<td>NADP⁺</td>
<td>0.25</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Glucose-1-phosphate</td>
<td>2.5</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>Galactose-1-phosphate</td>
<td>2.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Spectrophotometric</td>
<td>p-Nitrophenol phosphate</td>
<td>10.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Enzyme-amplification</td>
<td>INT—Violet</td>
<td>0.3⁵</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1⁵</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 6 Chemiluminescent enzyme immunoassays developed in our laboratory

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Chemiluminescent assay</th>
<th>Analyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>POD</td>
<td>Luminol/H₂O₂</td>
<td>Steroids</td>
</tr>
<tr>
<td>GOD</td>
<td>Glucose/Luminol/Fe(CN)₃⁻</td>
<td>Insulin, 17-OHP</td>
</tr>
<tr>
<td></td>
<td>Glucose/TCPO/ANS</td>
<td>T₄, 17-OHP</td>
</tr>
<tr>
<td>β-GAL</td>
<td>Lactose/GOD/TCPO/ANS</td>
<td>Drug, α-FP, TSH</td>
</tr>
<tr>
<td></td>
<td>o-Nitrophenyl-β-D-galactoside/GalDH/NAD/1-MPMS/IL/mPOD</td>
<td>17-OHP</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate/NAD/1IL/mPOD</td>
<td>17-OHP</td>
</tr>
<tr>
<td>INV</td>
<td>Sucrose/Lucigenin/CH₃OH⁺</td>
<td>17-OHP, T₄</td>
</tr>
</tbody>
</table>

POD, peroxidase; GOD, glucose oxidase; β-GAL, galactosidase; G6PDH, glucose-6-phosphate dehydrogenase; INV, invertase; TCPO, bis(2,4,6-trichlorophenyl)oxalate; ANS, 8-anilinonaphthalene-1-sulfonic acid; GalDH, galactose dehydrogenase; 1-MPMS, 1-methoxyphenazinium methylsulfate; mPOD, microperoxidase.
In EIAs of haptens, the sensitivity is markedly influenced by the combination of antibody and enzyme-labelled hapten antigen: "homologous" or "heterologous" combination in which the same or different derivative (site or bridge) are used. Therefore, various combinations of four antisera against T4 and four T4-GOD conjugates were examined to obtain the highest sensitivity. Given the displacement results shown in Fig. 9, the T4-GOD conjugate prepared by glutaraldehyde treatment seems to be the most effective label for every antisera prepared in this study. The bridge heterologous system using anti-T4-hemiglutarate-BSA and T4-GOD conjugate prepared with glutaraldehyde was most sensitive, because it was selected. We have established a CL EIA using GOD and peroxyoxalate CL reaction of TCPO/ANS system. The procedure and a typical standard curve are shown in Fig. 10. The detection limit of T4 is 0.25 µg/dl, equivalent to 5 pg (6.43 fmol)/assay. The precision of this method is adequate and is similar to that of other EIAs or RIAs for T4 in dried blood samples.

Using this CL EIA and Autopack Neonate-T4 RIA kit, we assayed 50 samples of dried blood from neonates. The linear correlation coefficient between the T4 values as determined by those two methods was 0.91, the slope 0.454, and the y-intercept 0.343. The proposed method is applicable to large-scale preliminary screening of neonates for congenital hypothyroidism.

4-2 17α-Hydroxyprogesterone

Congenital adrenal hyperplasia (CAH), a disorder of adrenal steroidogenesis, is known to be caused mostly by HLA-linked deficiency of 21-hydroxylase which is necessary for cortisol synthesis. This 21-hydroxylase defect results in excessive ACTH secretion with over-production and accumulation of cortisol precursors, particularly 17α-hydroxyprogesterone (17-OHP). In newborns, measurement of 17-OHP using RIA ro EIA is frequently used for the diagnosis, management and mass-screening of CAH. Fluorescence EIAs and ELISAs using HRP as label enzyme were developed and a CL EIA using GOD was also established with the peroxyoxalate CL reaction of bis(2,4,6-trichlorophenyl)oxalate–fluorescent dye system.

Recently, CL EIAs for 17-OHP using G6PDH and

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Fig. 8 General system of chemiluminescent EIA.

Fig. 9 Dose-response curves for thyroxin in homologous and heterologous systems.

**Step 1** Dried blood filter paper disc (3 mm, i.d.) 1
Anti T₄-g-BSA serum (1×10⁻⁵) 200 µl
T₄-GOD (GA) (2×10⁻⁴) 100 µl
DASP bead 1

- incubate for 3 h at 37°C
- washed with saline (2 ml×3)

**Step 2** 0.5 M Glucose solution (pH 5.0)300 µl

- incubate overnight at 4°C

**Step 3** Chemiluminescent assay by TCPO–ANS

Fig. 10 Procedure and standard curve of chemiluminescence EIA for T₄.
BGase based\(^3\) on the CL reaction of NADH mentioned above were developed.

As mentioned above, the "homologous" and "heterologous" systems are examined. Various combinations of anti-17-OHP antisera and 17-OHP-enzyme conjugates were examined to obtain the highest sensitivity. The 50% displacement values and detection limits of 17-OHP obtained from working curves of various combinations are listed in Table 7. When thioether derivatives (4CMT and 4CET) were used for preparing immunogens and enzyme-labelled conjugates, the bridge heterologous system\(\text{III}\) using steroid-enzyme conjugate obtained from 17-OHP having a shorter bridge than that used for preparing antibody gave higher sensitivity, whereas the system using a longer bridge\(\text{II}\) was not effective. The sensitivity of the homologous systems\(\text{I, IV, V}\) was also lower. This result is similar to that obtained by Hosoda et al.\(^4\) This phenomenon can be explained in terms of the steric interaction between antibody and labelled enzyme. The bridge heterologous system can increase the sensitivity by using a shorter or longer bridge in enzyme labelling than is used in the homologous case, because the steric hindrance between antibody and labelled enzyme decreases the affinity of antibody for the antigenic determinant in the labeled antigen. The sensitivities of site heterologous systems\(\text{VI, VII, VIII}\) were lower than that of the bridge heterologous system\(\text{II}\). From these results, it was concluded that the bridge heterologous system using anti-17-OHP antiserum against 17-OHP-4CET-BSA and 17-OHP-4CMT-enzyme conjugate was the most sensitive combination for 17-OHP EIA.

From the above results, CL EIAs using G6PDH and BGase as the label enzyme were developed with the bridge heterologous combination system between anti-17-OHP antiserum and 17-OHP-enzyme conjugates. Activities of enzyme conjugates bound on double antibody solid phase are assayed by the CL methods described in sections 3.3 and 3.5. The procedure and typical standard curve of CL EIA for 17-OHP using BGase as the label enzyme are shown in Fig. 11. The measurable range was from 0.05 to 100 pg/assay and the detection limit was 0.05 pg/assay, corresponding to 0.1 amol.

Table 7 Comparison of various EIA systems: homologous and heterologous

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Enzyme conjugate</th>
<th>System</th>
<th>50% Displacement/pg</th>
<th>Detection limit/pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I) 4-CMT</td>
<td>4-CMT</td>
<td>Homolo.</td>
<td>7.3</td>
<td>1.0</td>
</tr>
<tr>
<td>(II) 4-CMT</td>
<td>4-CET</td>
<td>Bridge heterolo.</td>
<td>19.4</td>
<td>2.5</td>
</tr>
<tr>
<td>(III) 4-CET</td>
<td>4-CMT</td>
<td>Bridge heterolo.</td>
<td>2.6</td>
<td>0.25</td>
</tr>
<tr>
<td>(IV) 4-CET</td>
<td>4-CET</td>
<td>Homolo.</td>
<td>6.4</td>
<td>1.0</td>
</tr>
<tr>
<td>(V) 3-CMO</td>
<td>3-CMO</td>
<td>Homolo.</td>
<td>15.8</td>
<td>1.0</td>
</tr>
<tr>
<td>(VI) 11-HS</td>
<td>3-CMO</td>
<td>Site heterolo.</td>
<td>482.0</td>
<td>25.0</td>
</tr>
<tr>
<td>(VII) 4-CET</td>
<td>3-CMO</td>
<td>Site heterolo.</td>
<td>12.4</td>
<td>2.5</td>
</tr>
<tr>
<td>(VIII) 7-BSA</td>
<td>3-CMO</td>
<td>Site heterolo.</td>
<td>6.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

4-CMT, 4-carboxymethylthio-17-OHP; 4-CET, 4-carboxyethylthio-17-OHP; 3-CMO, 17-OHP-3-(O-carboxymethyl)-oxime; 11-HS, 11-a-hemisuccinoxy-17-OHP; 7-BSA, derivative at C7 of 17-OHP.

![Fig. 11 Procedure and standard curve of chemiluminescence EIA using BGase as the label enzyme.](image-url)
The relative standard deviation at each 17-OHP level were in the range from 1.0 to 11.7%. The standard curve obtained by the CL EIA using G6PDH was similar to that of the CL EIA using BGase; the measurable range from 0.1 to 100 pg/assay. The relative standard deviation were from 6.0 to 8.2%.

The 17-OHP values of neonatal dried blood samples spotted on filter paper (3 mm i.d.) were assayed by the CL method using G6PDH and the ELISA using HRP. The correlation between 17-OHP values obtained by methods was satisfactory; $Y(\text{CL EIA})=0.98X(\text{ELISA})+1.25$, $r=0.93$, $n=29$.

5 Conclusion

Use of CL reactions can enhance the sensitivity of enzyme assay. Various CL assays of enzymes based on luminol, lucigenin, TCPO/ANS and NADH CL reactions are developed. CL reagents used in the assays are inexpensive. These methods can be applied successfully to the CL EIAs for various hormones and drugs in biological fluids. The CL assays described here might be applicable to other various EIAs using H$_2$O$_2$ or NADH-generating enzyme system. Moreover, the sensitivity might be improved by using the enzyme cycling method.

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References


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