ANALYTICAL BIOCHEMISTRY 149, 309–315 (1985)

Bioluminescent Enzyme Immunoassay for Estriol. Use of Reversibly Inactivated Bacterial Luciferase as Label[1]

FREDRICK S. YEIN,[2] CHARLES K. MARSCHKE, PHILIP C. DEMING, THOMAS F. HOLZMAN, AND PAUL S. SATOH

Upjohn Diagnostics, Kalamazoo, Michigan 49001

Received February 8, 1985

A bioluminescent enzyme immunoassay using estriol labeled with reversibly inactivated bacterial luciferase is described. An estriol derivative bearing an alkylthiolsulfonate is linked to the cysteinyl thiols of luciferase by formation of mixed disulfide linkages; thus, luciferase becomes inactive. After immunoassay, the inactive luciferase of the label bound to the immunoprecipitate is reactivated by incubation with dithiothreitol and the luciferase activity then is quantitated by a 20-s reaction performed with an automated luminometer (LKB 1251). Under the defined conditions, the labels are stable for at least 14 days as tested at 4°C. A standard curve with a wide linear range from 50 to 6000 pg is demonstrated. This unique technology discussed here, therefore, offers exciting possibilities as a sensitive and rapid enzyme immunoassay for estriol.

KEY WORDS: luminescence; reversibly inhibited luciferase; enzyme immunoassay; estriol.

Radioimmunoassay (RIA)[3] is a very powerful tool for various analyses of bioanalytes, and is now widely used as an alternative method for analytical chromatography (1). Recently, extensive research has enabled the development of several nonisotopic immunoassay methods which avoid many of the drawbacks in RIA, i.e., short half-life, disposal problems, and the health hazard of radioisotopes (2). One of the most promising nonisotopic approaches is enzyme immunoassay (EIA). However, due to limitations in either the signal amplification of the enzyme or the detecting systems by spectrophotometry for the enzyme-generated products (3), EIA has been most effective for measurement of analyte concentrations in the range milligrams per liter to micrograms per liter, while RIA effectively measures the analyte in concentrations of nanograms per milliliter. Most enzyme-labeled ligands used in EIA are very stable and are not hazardous as compared to the isotopic labels.

Analytical EIA methods based on bioluminescence are about 100 times more sensitive than spectrophotometric EIA, and thus potentially have a sufficiently low detection limit to compete with RIA (4,5). Many bioluminescent reactions involve the use of luciferase-catalyzed oxidation of a substrate to an electronically excited intermediate which emits light on decay. The chemical reaction catalyzed by bacterial luciferase is

$$RCHO + O_2 + FMNH_2 \rightarrow FMN + RCOOH + H_2O + \text{light}.$$

Luciferase from *Vibrio harveyi* containing a total of 16 cysteinyl residues has demonstrated the existence of a highly reactive cysteinyl sulfhydryl located at the active center. Disulfide modification of this cysteinyl residue re-

[1] U. S. and foreign patents pending.

[2] To whom correspondence should be addressed.

[3] Abbreviations used: RIA, radioimmunoassay; EIA, enzyme immunoassay; THF, tetrahydrofuran; estriol-6-CMO, estriol-6-(*O*)-carboxylmethyl-oxime; 5-MTSA, 5-methanethiosulfonylamine; EMTS, 5-(estriol-6-(*O*)-carboxyamidemethyl-oxime)-pentylmethanesulfonothioate; DTT, dithiothreitol; RCHO, decanal; IgG, immunoglobulin G; BSA, bovine serum albumin; PBS, phosphate-buffered saline; EL, estriol–luciferase conjugate.

0003-2697/85 $3.00

sults in loss of enzymatic activity which can be recovered by treating the modified luciferase with a reducing agent, i.e., 2-mercaptoethanol (6,7). On the basis of these observations, we report here the development of a bioluminescent enzyme immunoassay for estriol using the reversibly inactivated luciferase as reporter enzyme. In principle, the derivatized estriol bearing an alkylthiolsulfonate is specifically attached to the reactive sulfhydryl of the luciferase by a disulfide linkage. This modification converts the active luciferase (luciferase–SH) to the inactive form (luciferase–S–S–estriol). The conjugates of the inactivated luciferase/estriol are used as labels for a competitive immunoassay. After separating the bound from the free label, the enzyme activity of luciferase in the immunoprecipitate is measured after reduction of the disulfide linkage by dithiothreitol.

This report details much of the experimental evidence which establishes the validity of the described concept and demonstrates a bioluminescent EIA for estriol with sensitivity comparable to RIA.

MATERIALS AND METHODS

Materials. Estriol-6-CMO was purchased from Steraloids, Inc. 5-Methanethiolsulfonylamine and goat antiserum to rabbit IgG were obtained from Upjohn Diagnostics. Rabbit anti-estriol antibody was purchased from Arnel Company, New York. *N,N'*-Carbonyldiimidazole was obtained from Pierce Chemical Company. Polysorbate 80 was purchased from ICN Pharmaceutical, Inc., Cleveland, Ohio. Oxidoreductase (*P. fischeri*) was obtained from Boehringer-Mannheim. The solvents were of analytical grade from Burdick and Jackson Laboratory, Inc. Other reagents used in this research were of analytical grade.

Equipment. Bioluminescence was measured with an automated Luminometer 1251 (LKB-Wallac) with three autodispensers and an online printer. The NMR spectrometric analysis was performed using a CFT-20 NMR spectrometer (Varian Instrument Co.). The uv–vis spectrophotometric analysis was performed on a Hitachi 100-80A spectrophotometer.

Derivatization of estriol-6-(O)-carboxylmethyl oxime. Estriol-6-CMO was derivatized with 5-methanethiosulfonylamine (5-MTSA) HBr to provide an active thiol group for the coupling of estriol via a disulfide linkage onto the sulfhydryl group(s) on the luciferase. Five milligrams (12 μmol) of E-6-CMO was dissolved in 0.5 ml of dry THF in a Reacti-Vial (Pierce). Then 2.5 mg (15 μmol) of carbonyldiimidazole was added to the solution and dissolved by vortexing. The reaction was continued for 20 min at 25°C, after which 3.7 mg (14 μmol) of 5-MTSA was added. This mixture was carefully titrated to pH 7.5–8.0 with 0.1 N NaOH. During the titration, the mixture became clear. Complete reaction was ensured by incubation at 25°C for 2 h and then overnight at 4°C. The whole solution was chromatographed on thin-layer plate (Silica GF) using chloroform/methanol (7:3, v/v) as the solvent system. The desired product, 5-(estriol-6-(*O*)-carboxyamidemethyl-oxime)-pentylmethanesulfonothioate (EMTS), with R_f = 0.75, was eluted with the mixture of chloroform/methanol (1.0/1.0, v/v), dried under nitrogen, and redissolved in methanol and stored at −20°C. The structure of EMTS was confirmed by spectrophotometry and NMR. The concentration was determined spectrophotometrically using a molar extinction coefficient of 11,000 at 262 nm.

Purification of luciferase. Bacterial luciferase was purified from *V. harveyi* (8). The activity of the luciferase was determined by the standard $FMNH_2$-injection method (9). The reaction was initiated by rapid injection of 0.1 ml of catalytically reduced $FMNH_2$ into 1.02 ml of reaction mixture consisting of 0.01 ml of enzyme, 0.01 ml of 0.05% decanal in 1% isopropanol, and 1.0 ml of 0.02 M phosphate buffer with 0.2% BSA, pH 7.0. The peak light intensity was measured by a luminometer (LKB 1250). The luciferase concentration was determined on the basis of 79,000 molecular

weight and an extinction coefficient of 0.1% solution = 0.94 absorbance at 280 nm.

Preparation of luciferase-labeled estriol. The estriol–luciferase conjugate (EL) was prepared as follows. Luciferase in 0.1 M phosphate buffer with 0.9% NaCl (PBS) was mixed with equal volume of pure EMTS in methanol (prepared as above) diluted to micromolar concentration in PBS with 0.1% polysorbate 80. The mixture was incubated for 15–30 min at 25°C, chromatographed on Bio-Gel P6, and then diluted into PBS with 0.2% BSA (PBS–BSA). The EL was stored at 4°C until use.

Assay for luciferase activity in labels and immunoassay. The oxidoreductase-coupled assay (9) for luciferase activity measurement was modified. The principle of this assay is summarized as

$$NADH + H^+ + FMN \rightarrow FMNH_2 + NAD^+.$$

The $FMNH_2$ reduced *in situ* by the oxidoreductase-catalyzed reaction is used for the reaction catalyzed by luciferase as shown in the previous equation. The assay consisted of 0.2 ml of sample, 0.5 ml of FMN in assay buffer (0.02 M phosphate buffer with 0.2% BSA and 7.1 μM FMN), 0.05 ml of oxidoreductase (10 U/ml in 0.05 M phosphate buffer with 20% glycerin and 5 mM dithiothreitol), and 0.1 ml of substrate mixture (0.12 mM decanal and 15 mM NADH in PBS with 0.05% BSA). The reaction was initiated and monitored automatically by the 1251 Luminometer, a microprocessor-controlled, programmable luminometer capable of measuring the rate, detecting the peak, and integrating the total signals simultaneously. For this assay, 0.1 ml of the substrate mixture was injected (by the injection pump) into the reaction mixture which was continuously stirred by a stirrer in the instrument. The peak light intensity (mV) was recorded and the total light output was integrated (total counts) for the first 20 s after the start of the reaction.

Luciferase-linked immunoassay for estriol. One-tenth milliliter ml of sample or estriol standard solution (range from 50 to 6000 pg), 0.1 ml of EL (1000–1500 mV), and 0.1 ml of rabbit anti-estriol antibody solution (1/32000 dilution in PBS–BSA with 1% normal rabbit serum) were incubated for 1 h at 25°C. Then 0.1 ml of the second antibody solution (goat anti-rabbit IgG; 1/25 dilution in PBS–BSA with 6.25% PEG 3350) was added and incubated for an additional 30 min. At the end of the incubation, 2.0 ml of PBS was added to each tube and centrifuged for 15 min at 3000*g*, 4°C. The supernatants were decanted and the pellet in each tube was resuspended into 0.2 ml of 50 mM dithiothreitol in PBS and incubated for 3.0 h at 25°C to reactivate the luciferase of the label in the immunoprecipitate. Then the luciferase activity was measured by the oxidoreductase-coupled assay as described above.

RESULTS

The derivatization of estriol-6-CMO (bearing a reactive sulfhydryl group), and the subsequent attachment of the synthesized derivatives (EMTS) to luciferase through the disulfide linkage are summarized in Fig. 1. The EMTS moved as a single uv-positive component on TLC, and gave a uv spectrum with the maximum absorption peak at 262 nm and a minor peak at 308 nm. In the proton-NMR analysis, two singlets were observed: $\delta(CDCl_3)$ 0.76 (18-CH_3) and δ3.37 (–SO_2–CH_3), as well as a multiplet δ1.9–1.25 (–NH–CH_2–CH_2–

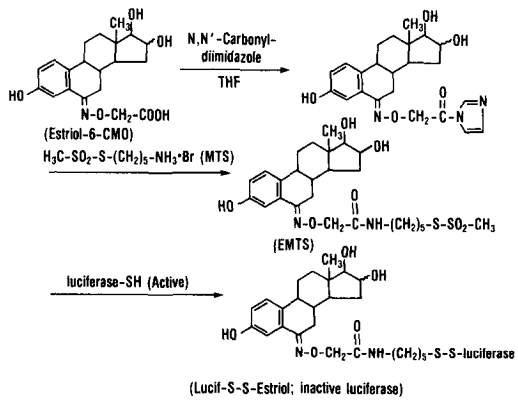

FIG. 1. Synthesis scheme for luciferase-labeled estriol.

CH_2–CH_2–CH_2–S–) and a very weak singlet δ4.6 (16-*OH*). While the compound was not very soluble in $CDCl_3$, it was very soluble in CD_3OD. But in this solvent the solvent peak at δ(3.30) obscured any peak due to –SO_2–CH_3, whereas the multiplets at δ(1.9–1.25) and δ(4.6) were quite pronounced due to the higher solubility. The luciferase lost its activity after mixing with EMTS for the synthesis of the luciferase–estriol conjugate by the disulfide linkage. Both EMTS and EL were immunologically active in a radioimmunoassay (10). The EL specifically bound to the anti-estriol antibody. When the EL was prepared by reacting luciferase with twofold molar excess of EMTS, the luciferase activity of the resulting conjugate was 100% recovered only from the EL treated with 50 mM dithiothreitol for 30 min at 25°C, but not from the untreated EL. These observations suggested that the estriol was attached onto the cysteinyl-SH at the active center of the luciferase by the disulfide linkage that can be reduced by dithiothreitol to reactivate the luciferase (luciferaseS–S–estriol) back to the catalytically active form, luciferase–SH. It was also observed that 20 mM dithiothreitol resulted in 70% reactivation after 30 min, while 50 or 100 mM dithiothreitol results in 42% reactivation after 10 min incubation at 25°C.

The luciferase activity was measured by the automatic luminometer (LKB 1251) using the modified assay described under Materials and Methods. A linear relationship between the light intensity and the various amounts of luciferase was obtained. 1770 mV of peak signal was generated by 30 ng of luciferase.

a). Binding

Ab_1 + Lucif-S-S-E ⟶ Ab_1-E-S-S-Lucif

b). Separation (immunoprecipitation)

Ab_2 + Ab_1-E-S-S-Lucif ⟶ Ab_2-Ab_1-E-S-S-Lucif ↓

c). Reactivation (reduction of disulfide linkage)

Ab_2-Ab_1-E-S-S-Lucif + RSH ⟶ Lucif-SH + Ab_2-Ab_1-E-SH

d). Quantitation by oxidoreductase coupled assay for luciferase activity.

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

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + Light$$

FIG. 2. Immunoassay procedures using luciferase-labeled estriol. Lucif-S–S–E (luciferase–S–S–estriol synthesized as in Fig. 1), Ab_1 (rabbit anti-esteriol antiserum), Ab_2 (goat anti-rabbit-IgG antiserum), RSH (dithiothreitol).

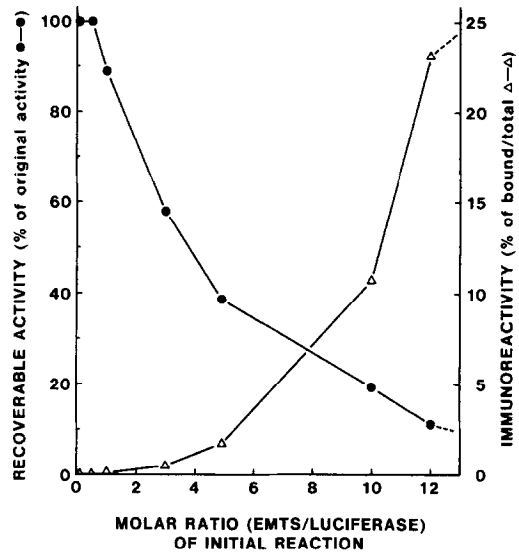

FIG. 3. Immunoreactivity and recoverable luciferase activity of label prepared by different ratios of EMTS/Luciferase.

To produce a label with optimal enzyme activity and immunoreactivity, luciferase was reacted with various amounts of EMTS as described above. The resulting labels with luciferase in the inactive form were reactivated with 50 mM dithiothreitol for 30 min at 25°C. Then the activity of the reactivated luciferase was measured as recoverable activity. The immunoreactivity of the same label was determined using the previously described immunoassay (Fig. 2). As shown in Fig. 3, recoverable enzyme activity decreases drastically as the ratio of EMTS/luciferase is increased in the conjugation reaction. However, the immunoreactivity increases. The optimal ratio is found to be 12:1 (EMTS/luciferase) and results in an EL with 24% binding (bound/total) and 12% recoverable enzyme activities. With a labeling ratio of 20:1, the recoverable activity of the resulting conjugate is so low that the signal

from the label is insufficient to construct a useful standard curve. The labels are not stable in PBS (0.1 M phosphate buffer with 0.9% NaCl, pH 7.0) but very stable in PBS containing either 0.1% BSA (PBS–BSA) or 0.5% gelatin (PBSG) in which it retains full activity over 14 days at 4°C.

Figure 4 shows the time course of reactivation of luciferase in the immunoprecipitate and the titration of the second antibody to optimally precipitate the immune complexes. The activation plateaus at 3 h incubation with 50 mM dithiothreitol at 25°C regardless of the concentrations of the second antibody. The optimal concentration of the second antibody for precipitation is 1/25 dilution of the goat-anti-rabbit IgG.

The principle of the competitive-binding immunoassay is that the EL (luciferase-labeled

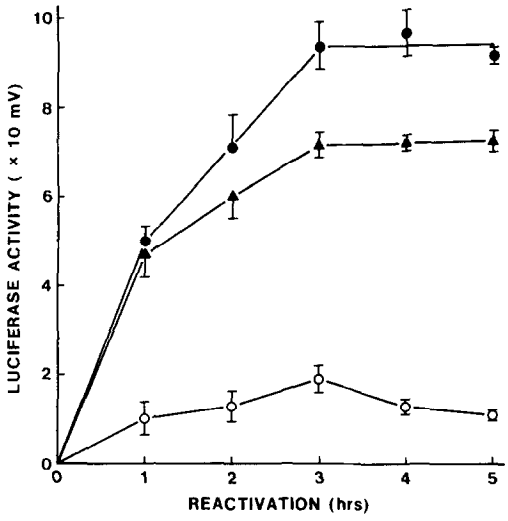

FIG. 4. Reactivation of luciferase-labeled estriol bound to immunoprecipitate. The immune complex of label (prepared by 12:1 of EMTS:luciferase) and antibody was precipitated by the second antibody of different concentration: ● 1:25 dilution; ▲, 1:50 dilution; ○, 1:125 dilution. After a washing, the label in the immunoprecipitate was reactivated with 50 mM dithiothreitol. At different time intervals, the luciferase activity was determined by the "$FMNH_2$ Injection Method" (9). Total label of 483 mV was used for each assay. The values represent avg ± 1 SD from triplicate samples.

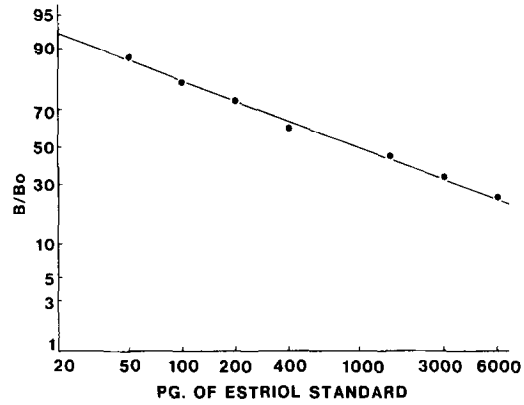

FIG. 5. Standard curve of estriol. The label prepared by 12:1 of EMTS:luciferase was used. Total label of 1200 mV approximately equal to 0.2 pmol of luciferase was added to each tube. The luciferase activity was determined by the oxidoreductase coupled assay. Standard curve: slope = −1.382(logit/log), $r = 0.986$. The abscissa and ordinate are in log and logit function, respectively.

estriol) and the free estriol in the sample compete for a limited number of binding sites on the estriol antibody. The EL bound to the antibody decreases with increasing concentration of free estriol, thus, a decrease of signal generated by the luciferase of the bound EL is observed. Figure 5 shows a typical standard curve for estriol. The linear region of detection for estriol is 50 to 6000 pg as tested. The slope of the curve is −1.382 (logit/log) with 50% displacement at 964 pg and a correlation coefficient of 0.99 for linearity. The labeling procedures and the standard curves are quite reproducible. The compository slope (logit/log) of −1.562 ± 0.140 and 50% displacement at (950 ± 25) pg were obtained from three standard curves using three separate preparations of the EL prepared with a labeling ratio of 12:1 (EMTS:luciferase).

DISCUSSION

One of the products from the luciferase-catalyzed reaction is luminescent light. The luminescence amplification by the luciferase is very great. Under the described assay condi-

tions, the signal amplification power of the luciferase is approximately equal to 4600 mV/pmol. The modification of the reactive sulfhydryl groups of *V. harveyi* luciferase with twofold excess of methyl methanethiolsulfonate led to complete inactivation of the luciferase activity, and treating the modified luciferase with 97 mM 2-mercaptoethanol for 10 min resulted in a 100% recovery of the lost activity (11,12). When the luciferase reacts with twofold excess of EMTS, only 42% of the original luciferase activity is recovered after 10 min reactivation by 100 mM dithiothreitol. The dramatic difference in reactivation apparently is due to the different size and hydrophobicity between the moieties introduced into the highly hydrophobic active center (13). The hydrophobic interaction between the active center and the pentyl-estriol moiety of EMTS is expected to be much stronger than that of $-SCH_3$ of methanethiolsulfonate. The even slower reactivation of the luciferase in the immunoprecipitate (Fig. 4) may have resulted from the steric hindrance of the active site by the relatively large immune complex.

The EL prepared by reacting EMTS with luciferase in a 2:1 or 5:1 ratio has very low immunoactivity whereas the EL with 1:1 labeling shows no detectable binding to the estriol antibody (Fig. 3). Since the essential reactive sulfhydryl resides in a cleft at least 17 Å in length (14), our observations suggest that the spacer in the EL conjugate (approx 14–16 Å in length) is not long enough to allow the estriol to be fully accessible for the antibody binding. The higher labeling ratio of EMTS/luciferase may result in mixed disulfide linkages that may cause irreversible conformational change in the luciferase to a structure with lower quantum yield. Thus, the recovery of light is much lower than desired; however, the drastic increases in the immunoreactivity suggest that estriol may be deposited onto the sulfhydryl groups other than that deep in the active site of the luciferase. The slow reactivation and the low recovery of luciferase activities may be overcome by using (a) thiosulfonate with a longer, less hydrophobic alkyl chain (i.e., polyhydroxyl alkyl) to link the estriol specifically onto the reactive sulfhydryl of the luciferase by a ratio of 1:1, and (b) solid-phase immunoassay (i.e., primary-antibody-coated tubes or beads).

Using the automated luminometer and oxidoreductase-coupled assay for detecting the luciferase activity as described in this report simplifies the assay procedures. The 20-s incubation to produce adequate amplification of the signal increases the throughput efficiency, while other enzyme immunoassays need 30–60 min incubation for their detecting systems to achieve the desired sensitivities. The standard curve obtained by using the luciferase-labeled estriol exhibits sensitivity comparable to that of RIA and covers a wide linear range of estriol concentration (50–6000 pg/tube) useful for measuring the very low concentrations of estriol in the peripheral circulation of nonpregnant women (0.05–0.5 ng/ml) and to detect the variable high concentrations of estriol for normal pregnancies or fetal distress (3.0–35 ng/ml) (15).

The luciferase-labeled estriol immunoassay reported here demonstrates the feasibility of using the reversibly inactivated luciferase to label the antigen for use in the immunoassay and also shows the possibilities of using this bioluminescent immunoassay to substitute for RIA where a high degree of sensitivity is required, i.e., estriol quantitation.

ACKNOWLEDGMENTS

We are grateful to T. O. Baldwin and W. P. Schneider for their invaluable discussions and continued encouragement.

REFERENCES

1. Ebersole, R. C., and Chait, E. M. (1981) *Anal. Chem.* **53**, 682A–692A.
2. Schall, R. F., and Tenoso, H. J. (1981) *Clin. Chem.* **27**, 1157–1164.

3. Wisdom, G. B. (1976) *Clin. Chem.* **22,** 1243–1255.
4. Seitz, W. R. (1981) *Crit. Rev. Anal. Chem.* **13,** 1–58.
5. Wannlund, J., and DeLuca, M. (1982) *Anal. Biochem.* **122,** 385–393.
6. Hasting, J. W., Weber, K., Friedland, J., Eberhard, A., Mitchell, G. W., and Gunsalus, A. (1969) *Biochemistry* **8,** 4681–4689.
7. Nicoli, M. Z., Meighen, E. A., and Hasting, J. W. (1974) *J. Biol. Chem.* **249,** 2385–2392.
8. Holzman, T. F., and Baldwin, T. O. (1981) *Biophys. J.* **33,** 255a.
9. Hasting, J. W., Baldwin, T. O., and Nicoli, M. Z. (1978) in Methods in Enzymology (DeLuca, M. A., ed.), Vol. 57, pp. 135–152, Academic Press, New York.
10. Coat-A-Count I(^{125})-Estriol Kit, Diagnostic Products, Inc., Calif.
11. Welches, W. R., and Baldwin, T. O. (1981) *Biochemistry* **20,** 512–517.
12. Ziegler, M. M., and Baldwin, T. O. (1981) *in* Bioluminescence and Chemiluminescence: Basic Chemistry and Analytical Applications (DeLuca, M. A, and McElroy, W. D., eds.), pp. 155–160, Academic Press, New York.
13. Nicoli, M. Z., and Hasting, J. W. (1974) *J. Biol. Chem.* **249,** 2393–2396.
14. Merritt, M. V., and Baldwin, T. O. (1980) *Arch. Biochem. Biophys.* **202,** 99–506.
15. Tulchinsky, D., and Abraham, G. E. (1971) *J. Clin. Endocrinol.* **33,** 775–782.