0273–2289/85/1103–0063$02.00

# Separation-Free Amperometric Enzyme Immunoassay

T. T. NGO,* J. H. BOVAIRD, AND H. M. LENHOFF

*Department of Developmental and Cell Biology, University of California, Irvine, CA 92717 USA*

Received July 3, 1984; Accepted July 26, 1984

## ABSTRACT

A new technique for conducting a separation-free amperometric enzyme immunoassay is described using DNP-aminocaproic acid as the analyte. The technique is based on the combined use of a recently described separation-free enzyme immunoassay (*19*) and an electrode system that senses $H_2O_2$. Oxidation of glucose to gluconate and $H_2O_2$ by the enzyme reconstituted from DNP-conjugate apoglucose oxidase (DPN-CAGO) and FAD was continuously measured amperometrically. The reconstitution was inhibited by preincubation with anti-DNP antibody before adding FAD. This antibody-induced inhibition of the reconstituting of the holoenzyme was reversed by adding DNP-amino caproic acid to DNP-CAGO before adding the antibody to DNP-CAGO. Based on (a) the antibody-induced inhibition of holoenzyme reconstitution, (b) a specific ligand-induced reversal of the inhibition, and (c) an electrochemical system that measures $H_2O_2$, we developed a separation-free (homogeneous) amperometric enzyme immunoassay.

**Index Entries:** Separation-free immunoassay; immunoassay, separation-free amperometric enzyme; amperometric enzyme immunoassay, separation free; enzyme immunoassay, separation-free amperometric.

## INTRODUCTION

There is a continuing and expanding interest in developing simple, convenient, and sensitive non-isotopic-labeled immunoassays as alternatives for radioisotope-labeled immunoassays. Some of these

alternative labels include bacteriophage, luminescent groups, microspheres, metallic substances, enzyme modulators, and enzymes [*see (1)* for a review]. Among these non-isotopic alternatives, enzymes appear to be the most practical, sensitive, and versatile label because: (a) they can be used as labels for both low and high molecular weight compounds; (b) they are very efficient catalysts, thereby greatly amplifying weak signals; (c) they can be used in separation-free (homogeneous) assays, i.e., those not requiring a separation of unbound ligand-label conjugate from that bound to the antibody; and (d) their activites can be measured by simple optical instruments, such as colorimeters or spectrophotometers (*2–4*). More recently, however, there has been growing interest in using electrochemical probes as detectors in enzyme-labeled immunoassays (*5–18*). Electrochemical detectors offer two practical advantages over optical ones: (a) they are relatively inexpensive and simple instruments, and (b) they are capable of making measurements in highly turbid samples.

We described here a new separation-free amperometric enzyme immunoassay. To illustrate this technique, we have employed DNP-conjugated apoglucose oxide (DNP-CAGO) as the label ligand and DNP-aminocaproic acid as the model analyte. This assay uses our recently developed approach called antibody-induced conformational restriction enzyme immunoassay (AICREIA) (*19*) and an electrode sensitive to hydrogen peroxide (*20*).

## MATERIALS AND METHODS

### Chemicals

2,4-Dinitrofluorobenzene, flavin adenine dinucleotide (FAD), horseradish peroxidase and *N*-2,4-dinitrophenyl-ϵ-aminocaproic acid (DNP-ACA) (Sigma Chemical Co.); 3-dimethyl-aminobenzoic acid (DMAB) and 3-methyl-2-benzothiazolinone hydrazone (MBTH) (Aldrich Chemical Co.,); rabbit anti-DNP serum and highly purified glucose oxidase from *Aspergillus niger* (Miles Labs, Inc.).

*Glucose oxidase labeled with 2,4-dinitrophenyl* (DNP-GO) and *DNP-conjugated apolglucose oxidase* (DNP-CAGO) were prepared by methods described previously (19).

### Amperometric Assay for Glucose Oxidase

The rate at which glucose oxidase catalyzed the formation of $H_2O_2$ from glucose and $O_2$ was monitored amperometrically by using a YSI-Clark 2510 electrode and a YSI model 25 oxidase meter that was supplied with a polarizing voltage of 700 mV. The response was displayed on a Fisher chart recorder. Glucose oxidase was assayed in a 5 mL solution with the electrode immersed in it. The assay solution consisted of 0.1*M*

sodium phosphate buffer pH 6.5 containing 0.15*M* NaCl and 0.3*M* glucose. It was prepared at least 1 d before use to ensure adequate time for the glucose to mutarotate. The reaction was initiated by adding 110μL of a solution containing either DNP-GO or DNP-CAGO and FAD.

## RESULTS

Apoglucose oxidase was covalently linked with DNP to give DNP-CAGO. The glucose oxidase activity was reconstituted from DNP-CAGO and FAD. This enzyme activity was monitored amperometrically by the rate of $H_2O_2$ production, which was proportional to amount of FAD added (Fig. 1).

In developing a separation-free enzyme immunoassay, it is crucial to demonstrate that an antibody to a ligand can modulate the activity of an enzyme conjugated to that ligand, and that an analyte (i.e., free ligand) can nullify the action of that antibody on the activity of the enzyme having a ligand bound to it (i.e., DNP-CAGO).

Figure 2 shows that by preincubating increasing amounts of anti-DNP to solutions containing a fixed concentration of DNP-CAGO, there was decreasing levels of glucose oxidase activity reconstituted after the subsequent addition of excess FAD to the DNP-CAGO. Hence, the modulation effect of the antibody was inhibitory.

When a fixed concentration of DNP-CAGO, a fixed amount of anti-DNP serum, and a fixed but excess concentration of FAD was sequentially added to solutions containing different concentrations of the analyte, i.e., DNP-ACA, the results showed that glucose oxidase activity increased with increasing concentrations of DNP-ACA (Fig. 3). This experiment indicated that DNP-ACA competed successfully with DNP-CAGO for the antibodies to DNP; hence, fewer antibodies to DNP were available to complex with DNP-CAGO. As a result, the uncomplexed DNP-CAGO was able to combine with FAD to form a catalytically active enzyme, which was measured amperometrically by the $H_2O_2$ produced (Fig. 3).

## DISCUSSION

Two factors dictated our choice for using glucose oxidase as the enzyme label in developing a separation-free amperometric enzyme immunoassay. First, one can assay glucose oxidase catalyzed reaction by measuring the rate of $H_2O_2$ production amperometrically (*20*). Second, we recently have used glucose oxidase successfully in developing a colorimetric separation-free enzyme immunoassay (*19*).

The reconstitution of glucose oxidase activity from DNP-CAGO and FAD, as monitored amperometrically, was shown in Fig. 1. The inhibition of the reconstitution of glucose oxidase activity from DNP-CAGO

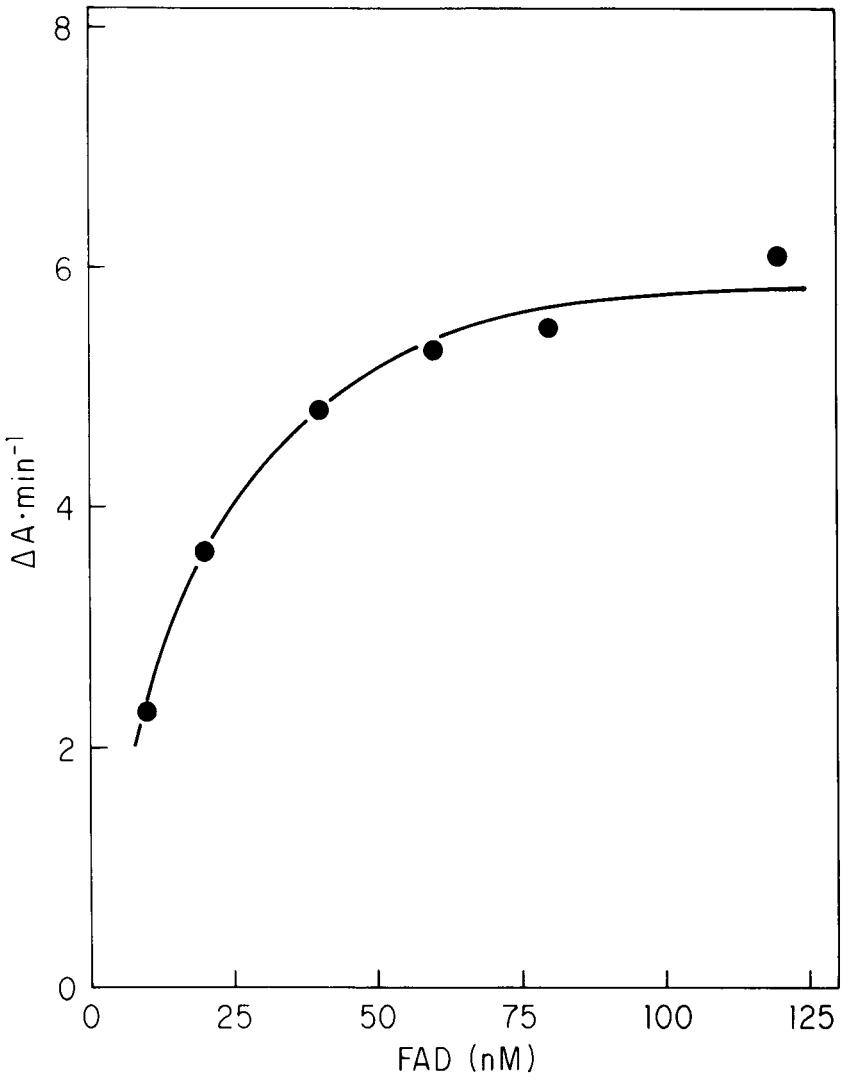


Fig. 1. Amperometric assay for reconstituting glucose oxidase activity from DNP-CAGO and FAD. Solutions (20 μL) containing varying concentration of FAD (50–1500 n*M*) in 0.1*M* sodium phosphate pH 6.5 were added to 100 μL of DNP-CAGO and incubated at 25°C for 15 min. Immediately, 100 μL of each of the above solutions was added to 5 mρ glucose solutions (0.3*M* glucose, 0.15*M* NaCl in 0.1*M* sodium phosphate, pH 6.5) which has been preincubated in a reaction chamber at 25°C, and with the tip of the $H_2O_2$ electrode immersed in the solution.

and FAD by antibody for DNP was also followed amperometrically (Fig. 2). The binding of the antibody to the DNP group of DNP-CAGO presumably restricted the movement of the polypeptide chain of the apoenzyme from assuming a proper conformation for binding FAD and, therefore, for becoming a catalytically active species in the presence of added FAD. Further evidence supporting such a mechanism was pro-

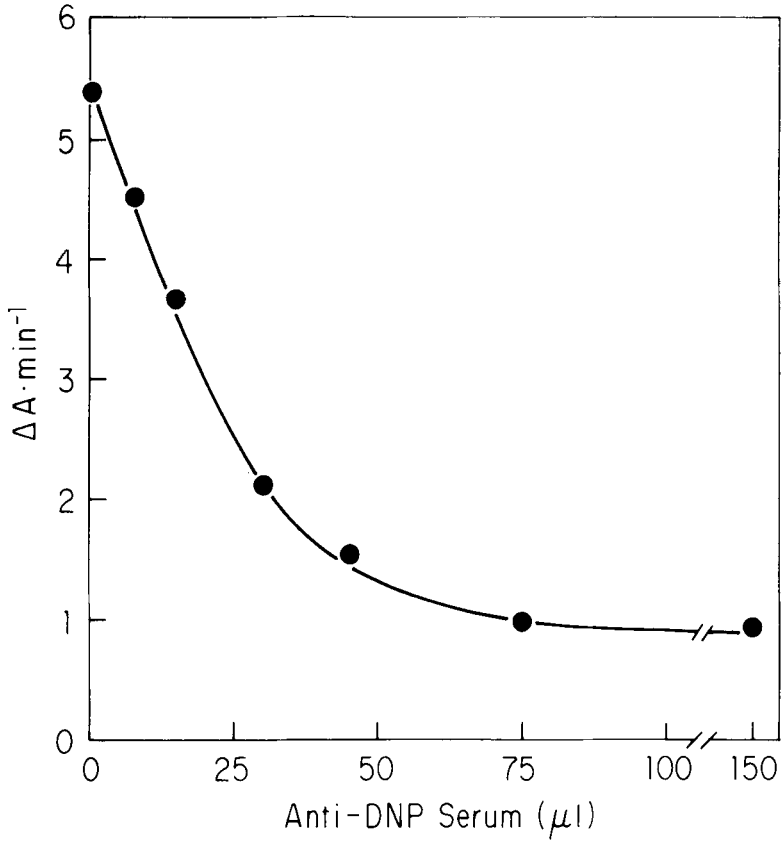


Fig. 2. Amperometric measurement of the inhibition by anti-DNP serum of the reconstitution of hologlucose oxidase from DNP-CAGO and FAD. Varying amounts of anti-DNP serum were added to 60 μL DNP-CAGO. The solutions were adjusted to 310 μL with 0.1*M* sodium phosphte pH 6.5, and incubated at 25°C for 30 min. To these solutions were added 100 μL of 1 μ*M* FAD, and they were further incubated at 25°C for 15 min. Next, 300 μL was removed and assayed for glucose oxidase activity amperometrically (*see* legend to Fig. 1).

vided by the finding that DNP-ACA could counteract the inhibitory action of the antibody for DNP (Fig. 3).

Figure 4 summarizes the overall steps taking place in the separation-free amperometric enzyme immunoassay described here. In the presence of a large quantity of DNP-ACA (the analyte), most of the antibodies (Ab) for DNP would be tied up as Ab:DNP-ACA via reaction (a). As a result, less of the antibody (Ab) would be available to combine with a fixed amount of DNP-CAGO to form the enzymatically inactive complex Ab:DNP-CAGO [reaction (b)]. Therefore, in the presence of more analyte, there would be more of the free, uncomplexed DNP-CAGO capable of forming with FAD the enzymatically active conjugated holoenzyme DNP-CAGO:FAD via reaction (c). Accordingly, there would be an increase in the enzymatic formation of gluconate and of $H_2O_2$ from

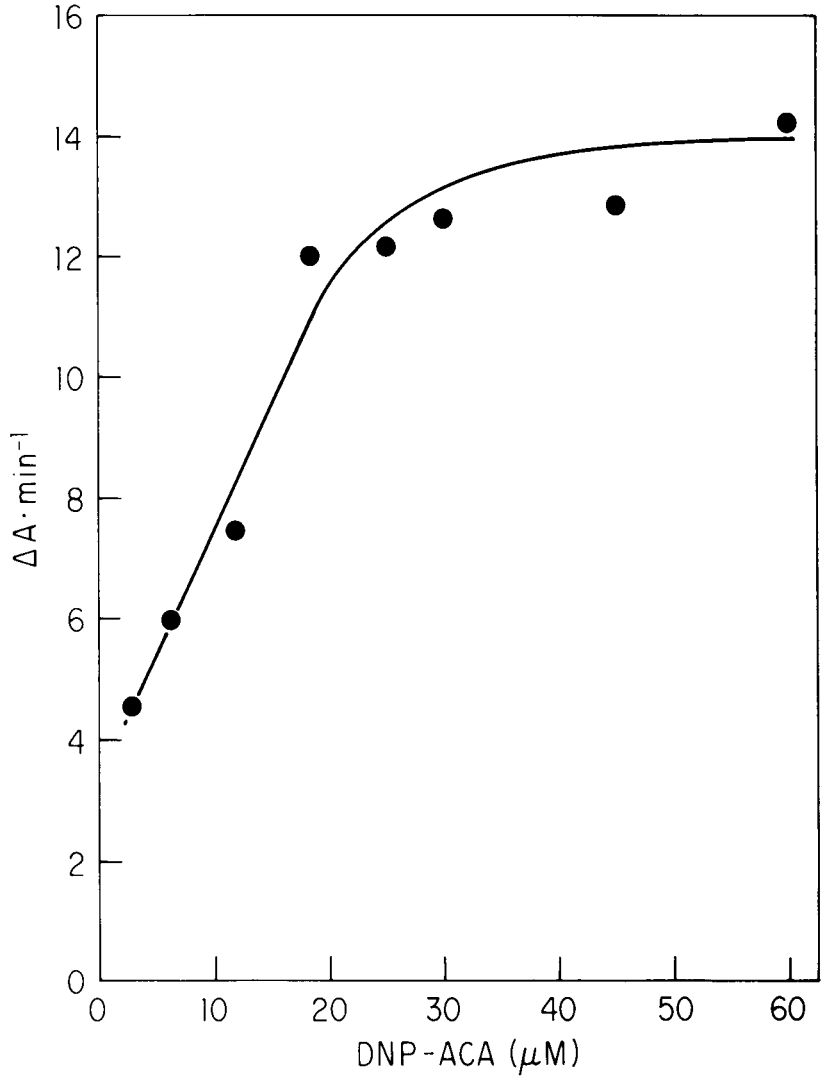


Fig. 3. Standard curve for measuring DNP-ACA by separation-free amperometric enzyme immunoassay. Solutions of 50 μL containing varying amounts of DNP-ACA were added to 60 μL DNP-CAGO solutions. To these solutions were added 40 μL anti-DNP serum, and they were incubated at 25°C for 30 min. Then 60 μL of 1 μ*M* FAD were added to each of these solutions, and they were further incubated at 25°C for 15 min. Next 200 μL was removed from each of the solutions and were assayed for glucose oxidase activity a perometrically (*see* legend to Fig. 1).

glucose and $O_2$. The concentration of $H_2O_2$ can be determined by measuring the amount of current generated upon oxidation of $H_2O_2$ at 700 mV polarizing voltage at the platinum anode of the electrode [reaction (e)]. The electrochemical circuit is completed by the reduction of $O_2$ to $H_2O$ at the silver cathode of the electrode [reaction (f)].

Conversely, if the concentration of the analyte (DNP-ACA) is decreased, reaction (1) would decrease and DNP-CAGO and the anti-DNP

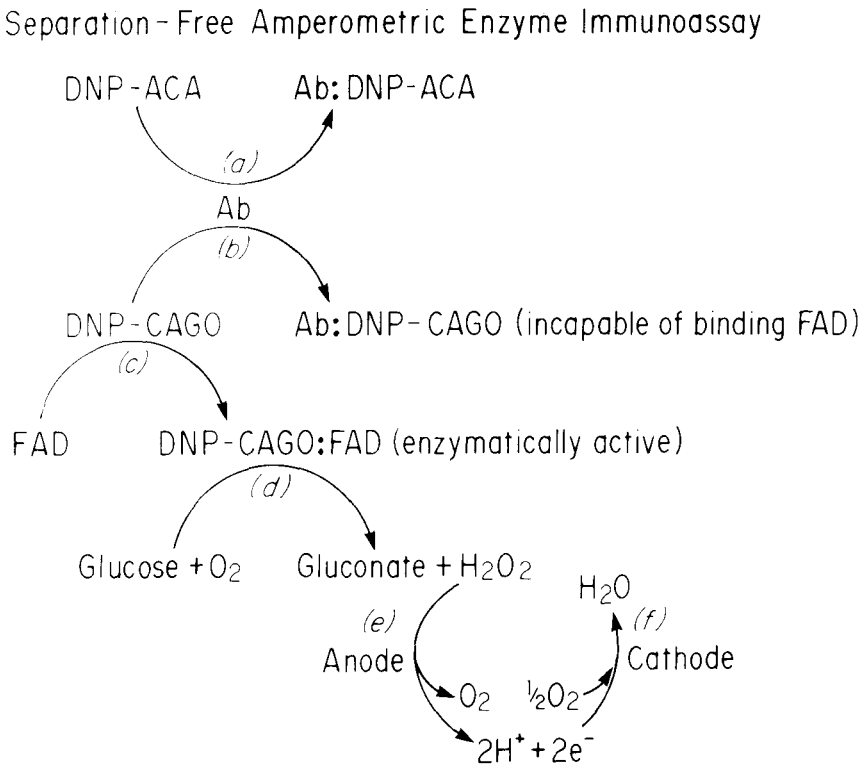


Fig. 4. Overall process of separation-free amperometric (homogeneous) enzyme immunoassay. DNP-ACA, DNP-CAGO, and FAD are defined under Materials and Methods. Ab refers to antibody to DNP.

antibody (Ab) would combine [reaction (b)] leaving less DNP-CAGO to combine with FAD [reaction (c)] to form enzymatically active DNP-CAGO: FAD, and, therefore, less $H_2O_2$.

## REFERENCES

1. Schall, R. F., Jr., and Tenoso, H. J. (1981), *Clin. Chem.* **27,** 1157–1164.
2. Schuurs, A. H. W. M. and van Weemen, B. K. (1977), *Clin. Chim. Acta* **81,** 1–40.
3. Ngo, T. T., and Lenhoff, H. M. (1981), *Appl. Biochem. Biotech. Mol.* **6,** 53–64.
4. Ngo, T. T., and Lenhoff, H. M. (1982), *Mol. Cell. Biochem.* **44,** 3–12.
5. Aizawa, M., Morioka, A., Matsuoha, H., Suzuki, S., Nagamura, Y., Shinohara, R., and Ishiguro, I. (1976), *J. Solid-Phase Biochem.* **1,** 319–328.
6. Mattiasson, B., and Nilsson, H. (1977), *FEBS Lett.* **78,** 251–254.
7. Boitieux, J-L., Desmet, G., and Thomas, D. (1979) *Clin. Chem.* **25,** 318–321.
8. Aizawa, M., Morioka, A., Suzuki, S., and Nagamura, Y. (1979), *Anal. Biochem.* **94,** 22–28.
9. Meyerhoff, M. E., and Rechnitz, G. A. (1979), *Anal. Biochem.* **95,** 483–493.
10. Aizawa, M., Morioka, A., and Suzuki, S. (1980), *Anal. Chim. Acta* **115,** 61–67.
11. Yuan, C-L., Kaun, S. S., and Guilbault, G. G. (1981), *Anal. Chim. Acta* **124,** 169–176.

12. Yaun, C-L., Kaun, S. S., and Guilbault, G. G. (1981), *Anal. Chem.* **53,** 190–193.
13. Boitieux, J. L., Lemay, C., Desmet, G., and Thomas, D. (1981), *Clin. Chim. Acta* **113,** 175–182.
14. Haga, M., Sugawara, S., and Itagaki, H. (1981), *Anal. Biochem.* **118,** 286–293.
15. Gebauer, C. R., and Rechnitz, G. A. (1981), *Anal. Lett.* **14,** 97–109.
16. Alexander, P. W. and Maltra, C. (1982), *Anal. Chem.* **54,** 68–71.
17. Eggers, H. M., Halsall, H. B., and Heineman, W. R. (1982), *Anal. Chem.* **28,** 1848–1851.
18. Pinkerton, T. C., and Lawson, B. L. (1982), *Anal. Chem.* **28,** 1946–1955.
19. Ngo, T. T., and Lenhoff, H. M. (1983), *Biochem. Biophys. Res. Comm.* **114,** 1097–1103.
20. Ngo, T. T., and Lenhoff, H. M. (1980), *Anal. Lett.* **13,** 1157–1165.