# ENZYME MODULATORS AS TOOLS FOR THE DEVELOPMENT OF HOMOGENEOUS ENZYME IMMUNOASSAYS

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## 1. Introduction

Although radioimmunoassay techniques [1–3] have revolutionized both research and clinical methodology, recognition of problems associated with radioactive materials, such as effective waste disposal, potential health hazards, and short half-life of some isoptotes, have prompted searches for non-radioactive markers in immunological assays. Because of the sensitivity, ease of measurement, good stability and longer shelf-life, enzymes have proven to be a good substitute as markers in non-isotopic immunoassays [4–6].

Enzyme immunoassays are of two types, heterogeneous and homogeneous. The heterogeneous ones require a physical separation of the unbound antigen from that bound to the antibody, whereas the homogeneous assay does not. Homogeneous enzyme immunoassays have been developed along the following approaches:

(i) An enzyme is covalently labeled with a ligand (the analyte), and the antibody to the ligand modulates the activity of the enzyme by either inhibiting its activity [7] or activating its activity [8];

(ii) An enzyme substrate is covalently labeled with a ligand so that the binding of the ligand–substrate conjugate by the antibody to the ligand sterically prevents the enzyme from acting on the substrate [9];

(iii) A ligand covalently bound to a prosthetic group and an antibody to that ligand prevents the prosthetic group from combining with an appropriate apo-enzyme. Such prosthetic group modulated enzyme immunoassays have been developed for measurement of haptens and human immunoglobulin G [10].

Here we describe a different approach to homogeneous enzyme immunoassays, one that employs an enzyme modulator (Emmia). An enzyme modulator may be an antibody, inhibitor or receptor to the marker (i.e., indicator) enzyme. The modulator must be covalently linked to a ligand that is similar to the analyte so that the amount of modulator free to regulate the available indicator enzyme is dependent upon the amount of analyte to be determined.

## 2. Principles of Emmia

The principles of Emmia can be illustrated in the following reaction sequences.

(a) *Antibody binding of ligand-substituted modulator*

$$Ab_L + L\text{–}M \rightleftharpoons Ab_L : L\text{–}M$$

when $Ab_L$ is antibody to the ligand, L is the analyte, and L–M is the modulator, M, having the ligand, L, covalently linked to it.

(b) *Competitive antibody binding of analyte (L) and ligand-substituted modulator (L–M)*

$$L + L\text{–}M + Ab_L \rightleftharpoons Ab_L : L + Ab_L : L\text{–}M$$

(c) *Modulation of indicator enzyme*

$$E + L\text{–}M \rightleftharpoons E : L\text{–}M$$

when E is indicator enzyme and E of E:L–M is enzymatically inactive.

(d) *Indicator reaction*

$$\text{Substrates} \xrightarrow{E} \text{Measurable products}$$

In Emmia the amount of analyte, L, is determined by the decrease in indicator enzyme, E, (measured by reaction (d)) caused by its being complexed by a fixed amount of the ligand-substituted modulator, L–M, (reaction (c)). The amount of L–M available to complex E is dependent upon the amount of analyte, L, because both L and L–M compete for a fixed amount of the antibody to L, $Ab_L$ (reaction (b)). Thus, the lower the concentration of L, the more $Ab_L$ will be available to complex with L–M (reaction (a)) so that indicator enzyme E will be less affected as in reaction (c) and will be free to act. On the other hand, more analyte L will tie up more $Ab_L$ (reaction (b)) freeing L–M so that it will react with E (reaction (c)) making less E available to catalyze indicator (reaction (d)). Hence, the enzyme activity measured will be inversely proportional to the amount of analyte present and binding to $Ab_L$.

2.1. *Emmia reactants of model system*

In the system we have developed to demonstrate Emmia, we use the following components: (I) for L, dinitrophenyllysine (DNP-lys); (II) for $Ab_L$, antibody to DNP (AbDNP); (III) for E, peroxidase (POD); and (IV), for M, DNP linked to anti-peroxidase (DNP-AbPOD).

## 3. Materials and methods

Horseradish peroxidase (type VI), purified anti-horseradish peroxidase (produced in rabbits), 2,4-dinitrofluorbenzene and *N*-ε-DNP–L-Lysine · HCl were purchased from Sigma Chem., St Louis, MO. Antiserum to 2,4-dinitrophenol was purchased from Miles Lab., Elkhart, IN. 3-Methyl-2-benzothiazoline hydrazone hydrochloride monohydrate and 3-dimethyl-benzoic acid were purchased from Aldrich Chem., Milwaukee, WI.

The 2,4-dinitrophenylated anti-horseradish peroxidase was prepared as follows: 10 mg antibody to horseradish peroxidase were dissolved in 2 ml of 0.1 M sodium phosphate (pH 7.0). To this antibody solution was added 10 μl 2,4-dinitrofluorbenzene in dioxane (62 mg/ml). The solution was stirred at room temperature for 3 h, then dialyzed 3 times against 2 liters 0.1 M sodium phosphate (pH 7.0) at 4°C for 8 hr each. Using 2,4-dinitrophenyllysine as standards 20. 2,4-dinitrophenyl groups/antibody molecule was determined.

The peroxidase assay was performed according to [11]. The 1 ml assay solution in 0.1 μmol sodium phosphate (pH 7.0) contained 3.5 μmol 3-dimethyl-aminobenzoic acid and 0.075 μmol 3-methyl-2-benzothiazoline hydrazone.

## 4. Results

4.1. *The indicator and modulator reactions*

Antibody to POD labeled with 2,4-dinitrophenyl groups is able to inhibit the peroxidase reaction up to 75% of that inhibited by the unlabeled $Ab_{POD}$. Fig.1 shows the activity of horseradish peroxidase decreased with increasing amounts of DNP-$Ab_{POD}$.

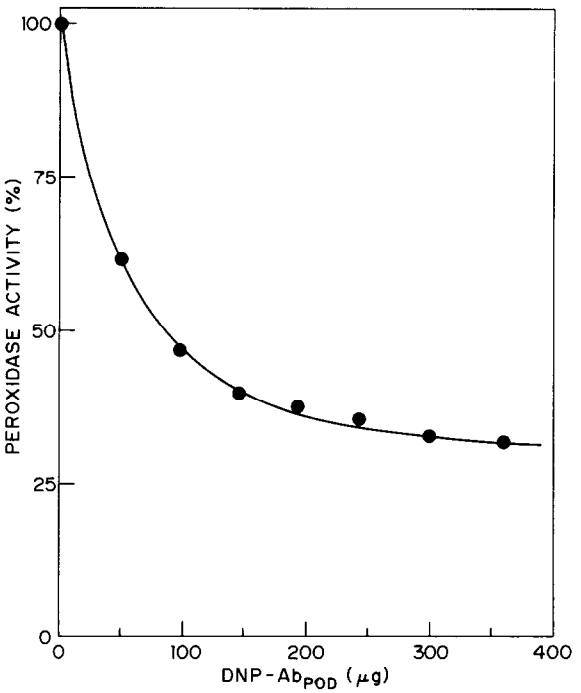



Fig.1. The inhibition of peroxidase activity by DNA-$Ab_{POD}$. Peroxidase (0.1 μg) was incubated at 25° in 0.4 ml 0.1 M sodium phosphate (pH 7.0) containing varying concentrations of DNP-$Ab_{POD}$. After 5 min 0.2 ml of this solution was added to 1 ml peroxidase assay solution and the initial rate was determined. In the absence of the antibody the peroxidase gave $\Delta A$ = 0.57/min.

4.2. *The antibody-binding reaction*

The antibody to the 2,4-dinitrophenyl groups ($Ab_{DNP}$) was able to diminish the inhibitory properties of DNP-$Ab_{POD}$. Fig.2 shows that when the peroxidase was inhibited by the DNP-$Ab_{POD}$ to 35% of its original activity, the addition of increasing amounts of $Ab_{DNP}$ to a solution of the inhibited peroxidase resulted in proportional increases in peroxidase activity to 90% of that originally present.

4.3. *The competitive-binding reaction*

DNP-$Ab_{POD}$ was shown to compete successfully with DNP-Lys for $Ab_{DNP}$ thereby releasing DNP-$Ab_{POD}$ free to bind with and inhibit the POD. Fig.3 showed that when the concentrations of peroxidase, DNP-$Ab_{POD}$ and $Ab_{DNP}$ were kept constant in buffer solutions, the additions of increasing amounts of DNP-Lys resulted in decreasing enzyme acitivity. Thus the concentration of DNP-Lys in an analyte solution can be measured by such a standard curve.

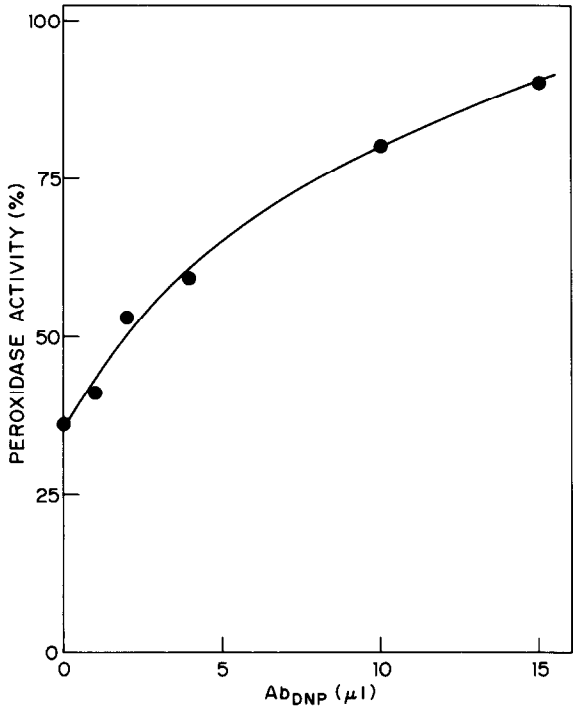



Fig.2. Reversal by $Ab_{DNP}$ on the inhibition of peroxidase caused by DNP-$Ab_{POD}$. DNP-$Ab_{POD}$ (240 μg), varying amounts of $Ab_{DNP}$ and POD (0.1 μg) in 0.3 ml 0.1 M sodium phosphate (pH 7.0) were incubated at 25°C for 5 min. Then 0.2 ml this solution was added to 1 ml peroxidase assay solution to determine the initial rate.

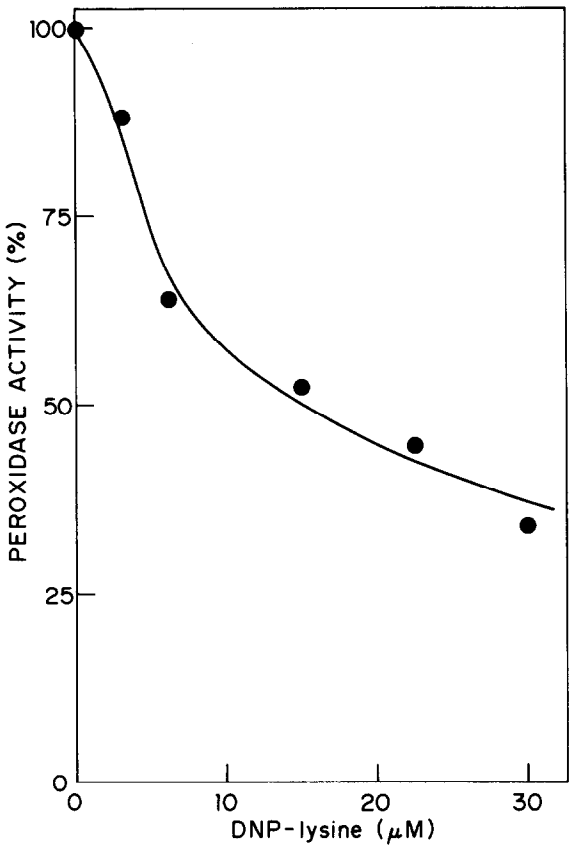


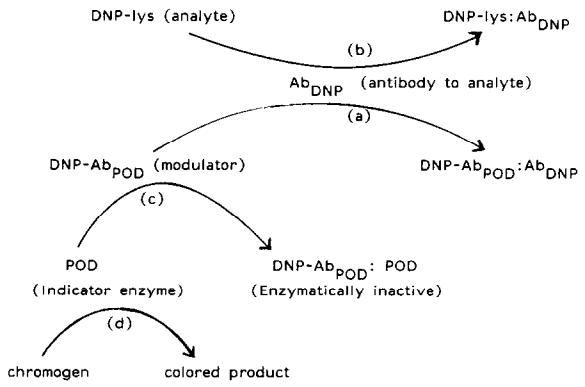

Fig.3. The standard curve for DNP-lysine by Emmia. Solutions of 100 μl containing various amounts of DNP-Lys were added to 300 μl solutions containing 240 μg DNO-$Ab_{POD}$, 20 μl $Ab_{DNP}$ and 0.1 μg peroxidase. The solutions were incubated at 25°C; after 5 min, 200 μl was added to 1 ml peroxidase assay solution to determine the initial rates.

## 5. Discussion

We have described a new approach to homogeneous enzyme immunoassays called enzyme modulator mediated immunoassay (Emmia) and have presented experimental results verifying its feasibility. The overall process of Emmia can be summarized in scheme (1) representing our experimental results. Reactions (a–d) refer to those described in section 2.

It is clear from scheme (1) that in the absence of analyte, reaction (b) would not occur and that the modulator and antibody to the analyte would combine (reaction (a)) leaving most of the indicator enzyme free to act. As the concentration of analyte increases, however, it would compete successfully for

Scheme 1




binding sites on $Ab_{DNP}$ (reaction (b)) leaving more modulator ($DNP\text{-}Ab_{POD}$) free to complex with indicator enzyme (reaction (c)) thereby inactivating it. The overall result is, therefore, as the amount of analyte increases, the amount of chromogen converted by the remaining indicator enzyme (reaction (d)) will decrease. The results presented in fig.1–3 bear out the validity of this approach to homogeneous enzyme immunoassay.

Unlike the substrate-labeled enzyme immunoassay that uses ligand substituted substrate [9], this approach provides an intrinsic amplifying power because any uninhibited enzyme can continuously generate products.

An advantage of Emmia over heterogeneous assays is that it eliminates the cumbersome step of physically separating the unbound and the antibody bound antigens.

The successful development of Emmia requires the availability of an enzyme modulator having a high affinity for the enzyme. There are numerous compounds which qualify. For example, the modulator can be:

(i) An enzyme inhibitor, such as avidin which can inhibit biotin dependent enzymes by high affinity binding [12];

(ii) A transition-state analog, such as coformycin, which inhibits adenosine deaminase with an inhibition constant of $10^{-10}$–$10^{-11}$ M [13];

(iii) An activator, such as an antibody to the wild-type *Escherichia coli* β-galactosidase, which can activate 550-fold the activity of a mutant enzyme known to be inactive by itself [14].

We believe that the concept of Emmia is sufficiently versatile and specific to be a useful addition to the field of homogeneous enzyme immunoassays.

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