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Ultrasensitive Chromogen System for Horseradish Peroxidase Using 3-Methyl-2-benzothiazolinone Hydrazone and *N*-Phenyldiethanolamine

THAT T. NGO

Developmental and Cell Biology, University of California, Irvine, Irvine, California 92717

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ABSTRACT

An ultrasensitive chromogenic system for horseradish peroxidase (HRP) is described. The chromogenic substrate system consists of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and *N*-phenyldiethanolamine (PDEA). The oxidative coupling of MBTH and PDEA using H_2O_2 and HRP, respectively, as the oxidant and catalyst, yields a blue chromophore that most likely is an indamine dye. The chromophore has a rather broad absorption band, with an absorption maximum at 602 nm. In 0.1*M* phosphate, 0.1*M* citrate buffer, pH 7.5, the optimal concentrations of MBTH, PDEA, and H_2O_2 are, respectively, 0.5, 25, and 8 m*M*. Using this assay system, HRP can be determined in lower picomolar levels by either a rate or fixed-time method.

Index Entries: Ultrasensitive chromogen system, for HRP; chromogen system; horseradish peroxidase, ultrasensitive chromogen system for; 3-methyl-2-benzothiazolinone hydrozone, in ultrasensitive chromogen system for HRP; *N*-phenyldiethanolamine, in ultrasensitive chromogen system for HRP.

INTRODUCTION

Enzyme-labeled immunoassay has proven to be an excellent nonisotopic alternative to radioisotope-labeled immunoassay in many areas

of laboratory diagnostics (*1–5*). Among the enzymes used in developing enzyme-labeled immunoassays, such as horseradish peroxidase (HRP), *E. coli* β-galactosidose, fungal glucose oxidase, and intestinal alkaline phosphatose, HRP is still the most widely used. Advantages of using HPR are its high turnover rate, ease and sensitivity of assay, relatively low molecular weight, and ease of conjugation to haptens, antigens, or antibodies. However, several sensitive chromogenic substrates for HRP, such as *o*-phenylene diamine and 2,2-azino-di[3-ethylbenzthiazoline]-6-sulfonic acid were shown to be mutagenic in Ames test (*6*). There is, therefore, a continuing effort to develop highly sensitive, nonmutagenic chromogenic system for HRP assay (*7–14*).

In this paper an ultrasensitive system for HRP, based on oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone and *N*-phenyldiethanolamine, is described.

MATERIALS AND METHODS

Chemicals

Horseradish peroxidase, citric acid, 3-methyl-2-benzothiazolinone hydrazone · HCl, monohydrate (MBTH), and 30% hydrogen peroxide solution were from Sigma Chemical Company. *N*-Phenyldiethanolamine (PDEA), 99%, was from Aldrich Chemical Company. Sodium phosphate was from Fisher Scientific Company.

Reagent Preparations

Phosphate–citrate (PC) buffers: PC (0.02) = 0.02*M* sodium phosphate and 0.02*M* citric acid; PC (0.2) = 0.2*M* sodium phosphate and 0.2*M* citric acid. Citrate was included because Geoghegan et al. (*15*) reported that phosphate–citrate buffer system gave the least background color development.

MBTH solution: 1 mmol (233.7 mg) of MBTH was added to 1000 mL of distilled H_2O and stirred at room temperature for 15 min. The clear solution was then filtered through a 5-μm nylon filter to remove debris. Do not try to dissolve MBTH directly into a buffer, because MBTH is much slower to dissolve in a buffered solution.

PDEA solution: 50 mmol (9.062 g) of granular PDEA was placed in a beaker and was pulverized by using a glass rod. The pulverized PDEA was added to 1000 mL phosphate–citrate buffer. The solution was stirred at room temperature for 30 min and filtered through a 5-μm nylon filter to remove any insoluble material. Either PC (0.02), pH 7.5, or PC (0.2), pH 7.5, can be used as the buffer.

Although PDEA was labeled 99% pure, some granules contained a significant amount of blue color impurity. It was necessary to select those white granules that contained as little a quantity of blue crystals as possi-

ble. Only the white granules were pulverized and used in experiments described in this paper. The effect of the blue impurity was not investigated.

RESULTS

Absorption Spectra

Horseradish peroxidase catalyzed the oxidative coupling of MBTH and PDEA by H_2O_2 to form a deep-blue compound with an absorption peak at 602 nm (Line A, Fig. 1). When 10*N* HCl was added to the blue solution to stop further color development. The absorption spectrum of the acidified solution was only slightly shifted to longer wavelength (606 nm) without changing the absorbance at its new maximum (Line B, Fig. 1).

Influence of Reactant Concentrations on HRP Catalyzed Reaction

The effect of MBTH concentration on the rate of HRP catalyzed reaction, as measured by the rate of blue color formation at 602 μm, is shown in Fig. 2. All rates were measured at constant concentrations of PDEA and H_2O_2. They were kept at 50 and 8 m*M*, respectively. At 0.5 m*M*, MBTH provided 94% of maximal attainable rate, with an apparent Michaelis constant of 52 μ*M*.

Similarly, the rate of HRP-catalyzed color formation increases with increasing PDEA concentrations (Fig. 3), with 94% of the maximal rate obtained at 25 m*M* PDEA. The concentrations of MBTH and H_2O_2 were kept constant at 0.5 and 8 m*M*, respectively. The apparent Michaelis constant for PDEA was 3.1 m*M*.

Increasing the H_2O_2 concentration from 0.1 to 8 m*M* resulted in an increasing reaction rate. However, decreases in the reaction rate were observed when H_2O_2 concentrations were greater than 8 m*M* (Fig. 4). The apparent Michaelis constant for H_2O_2 was 0.1 m*M*.

Effect of pH and Buffer Concentration Rate of Color Formation

Two different concentrations of phosphate–citrate buffer, 0.02 and 0.1*M*, were used in studying the effect of pH on the rate of color formation. In both buffer systems, the optimum pH was approximately 7 (Fig. 5).

When MBTH and PDEA were mixed together, the resulting solution slowly developed blue color. The rate of color development was minimal at pH 7.5. The absorbance increased by 0.036/h in pH 7.5 buffer and by 0.06/h in pH 6.5 buffer (Fig. 6).

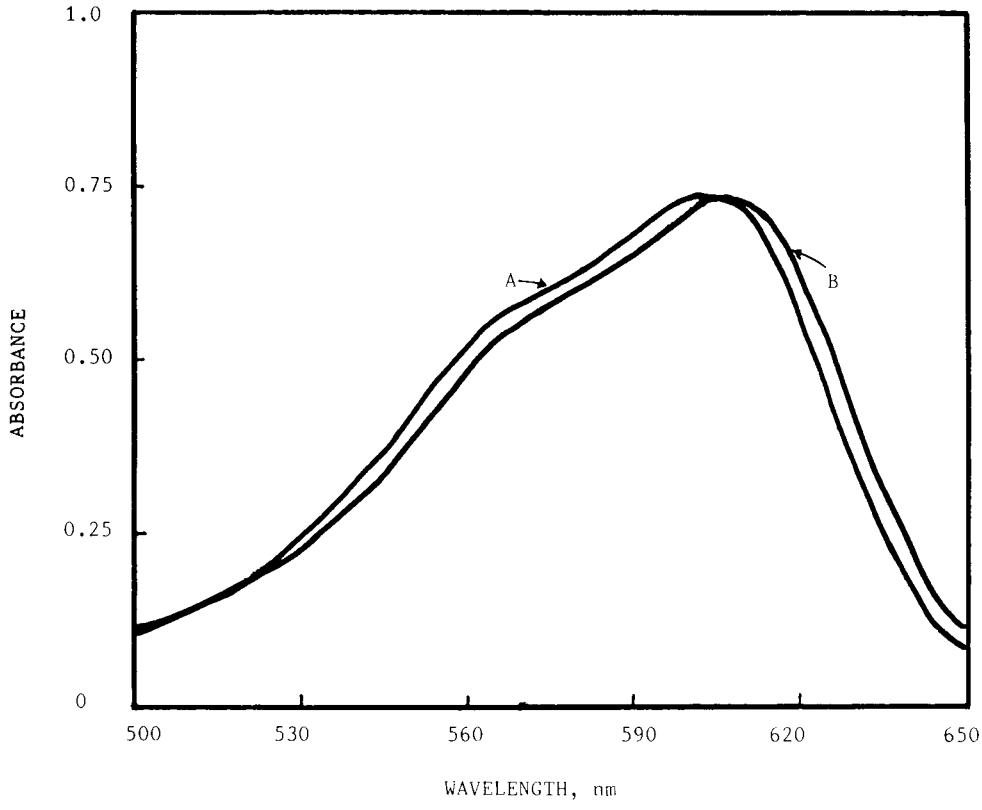


Fig. 1. Absorption spectra of the product of HRP-catalyzed oxidative coupling of MBTH and PDEA. Line A: The solution contained 0.25 m*M* MBTH, 12.5 m*M* PDEA, 4 m*M* H_2O_2, and 2.5 ng of HRP in 0.1*M* phosphate–0.1*M* citrate, pH 7.5. The solution was incubated at 25°C for 15 min, then 1 mL PC (0.02), pH 7.5, was added to the test tube and mixed thoroughly before the absorption spectrum was taken. Line B: The solution was identical to the one used to obtain line A, except that 1 mL PC (0.02), pH 7.5, was replaced by 1 mL of 10*N* HCL to lower the pH of the solution to ≈2.

Horseradish Peroxidase Assay

Horseradish peroxidase at concentration as low as 2 p*M* can be measured by either a rate or a fixed time method (Fig. 7). A linear relationship between the absorbance at 602 nm and the concentration of HRP was observed.

DISCUSSION

A sensitive chromogenic substrate system for HRP based on oxidative coupling of MBTH and *N,N*-dimethyl-*m*-aminobenzoic acid (DMAB) was described by Ngo and Lenhoff (*11*). The sensitivity and versatility of such a system was recently confirmed and improved upon by Geoghegan et al. (*15*). *N,N*-Disubstituted aniline, when reacted with

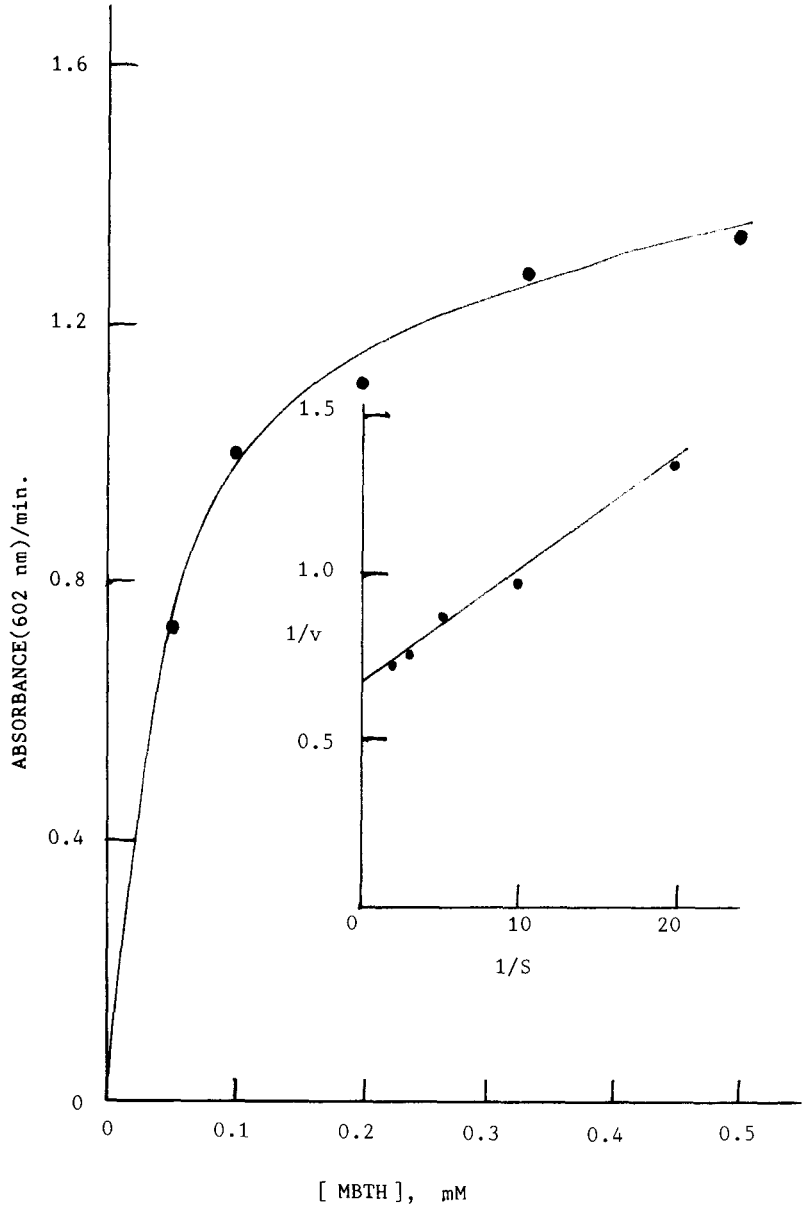


Fig. 2. Dependency of chromophore formation on the MBTH concentration. The reaction solution consisted of 3 mL phosphate–citrate buffer, pH 7.0, containing 150 μmol PDEA, 24 μmol H_2O_2, and MBTH at concentrations indicated in the figure. The reaction was monitored at 602 nm and was initiated by adding 10 μL horseradish peroxidase solution (5 μg/mL).

MBTH in the presence of ferric chloride, gave more intense color than the unsubstituted aniline did. Gochman and Schmitz (*7*) used MBTH and *N,N*-dimethylaniline as chromogens for measuring peroxidase coupled reactions. However, the toxicity, insolubility, and stench of *N,N*-

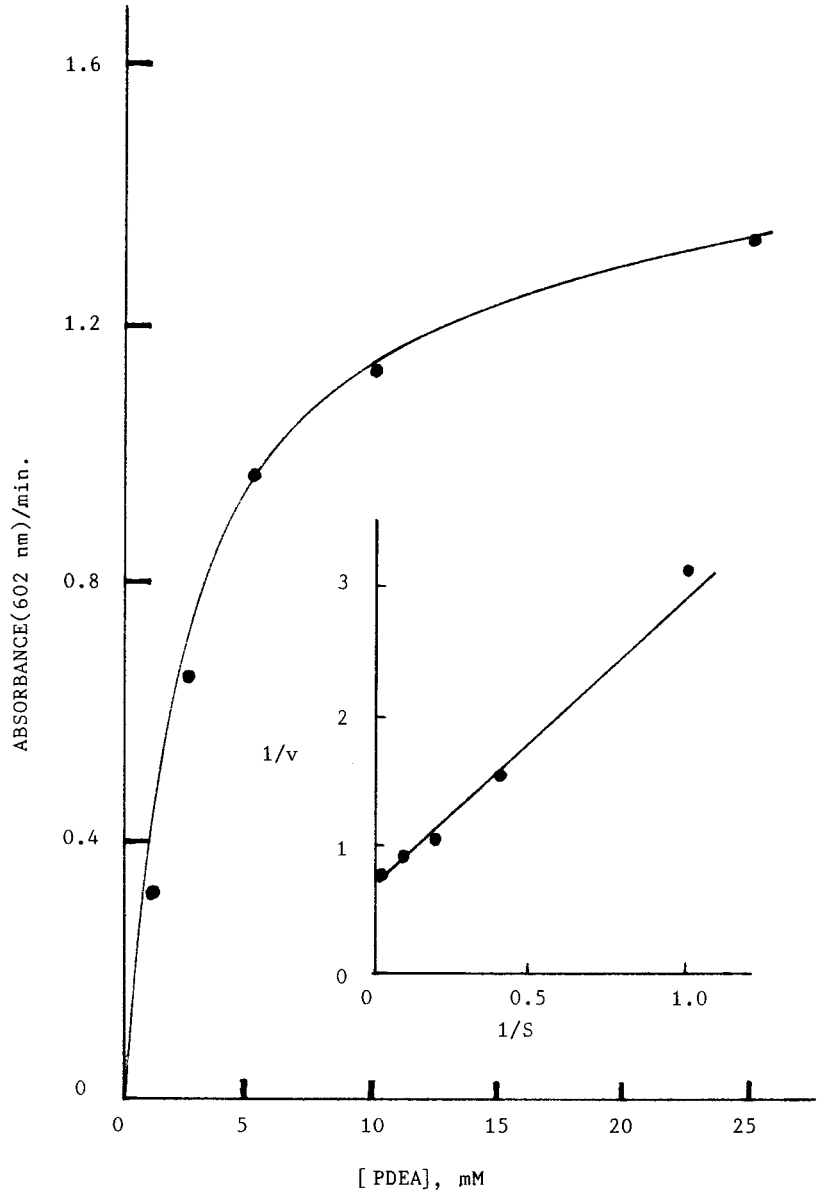


Fig. 3. Dependency of chromophore formation on the PDEA concentration. The reaction solution consisted of 3 mL phosphate–citrate buffer, pH 7.0, containing 1.5 μmol MBTH, 24 μmol H_2O_2, and PDEA at concentrations indicated in the figure. The reaction was monitored at 602 nm and was initiated by adding 10 μL) horseradish peroxidase solution (5 μg/mL).

dimethylaniline are serious disadvantages for its applications. I have chosen *N*-phenyldiethanolamine (PDEA) as a substitute for *N,N*-dimethylaniline because it is more water soluble and less toxic. Furthermore, PDEA has no perceptible smell. The product of HRP-catalyzed oxidative coupling of MBTH and PDEA shows a desirable absorption

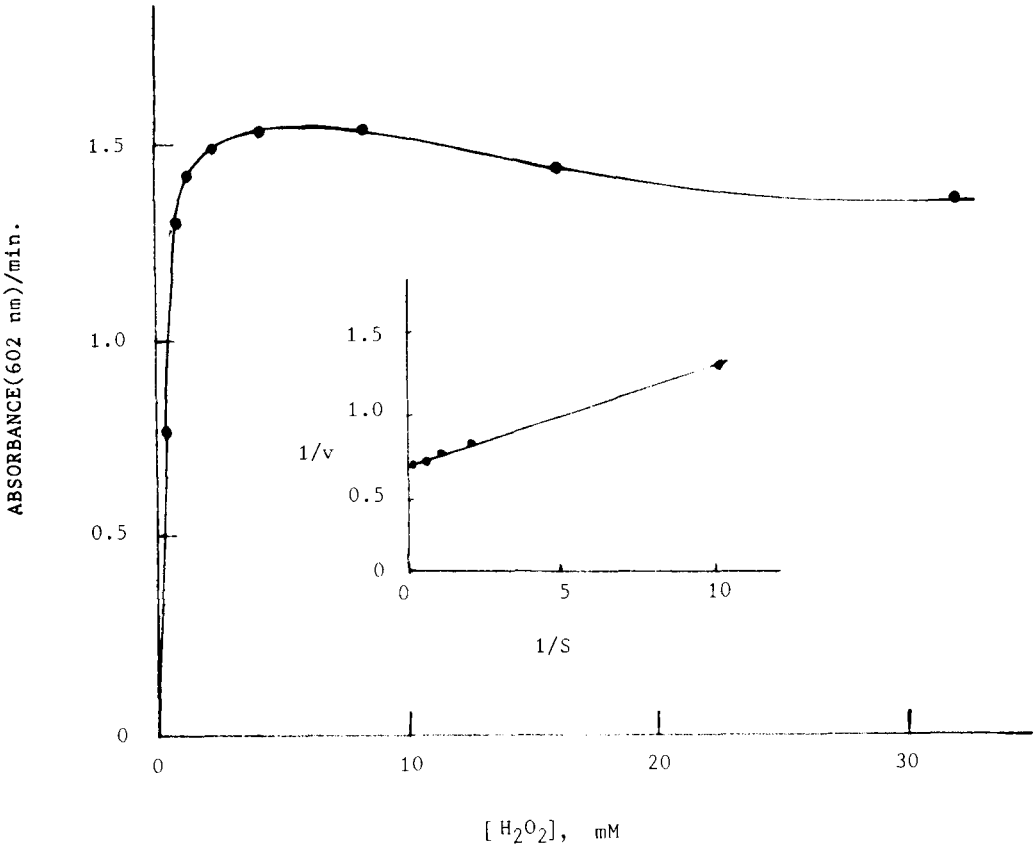


Fig. 4. Effect of hydrogen peroxide concentration on the reaction rate. The reaction was measured at 25°C in 3 mL phosphate–citrate buffer, pH 7.0, containing 1.5 μmol MBTH, 150 μmol PDEA, and H_2O_2 at concentrations as indicated in the figure. The reaction was initiated by adding 10 μL horseradish peroxidase solution (5 μg/mL).

spectrum, with maximal absorption at 602 nm (Fig. 1). This absorption peak is remote from absorption maxima of most interfering substances found in serum or plasma, which absorb light maximally at wavelength shorter than 550 nm.

When strong mineral acid, such as 10*N* HCl, was added to the blue solution to lower its pH in order to stop the enzymatic reaction, the wavelength of maximal absorption was shifted from 602 to 606 nm. The intensity of the blue color decreased only because of the dilution effect. Such a shift of the absorption peak to a longer wavelength when the chromophore solution is acidified is similar to that observed in MBTH and DMAB system (*15*). Since the blue chromophore has not been isolated, its extinction coefficient can only be inferred by reference to the extensive work of Sawicki on the determination of aromatic organic ntirogen coupounds using MBTH (*17*). The blue chromophore would most likely have an extinction coefficient of greater then 50,000.

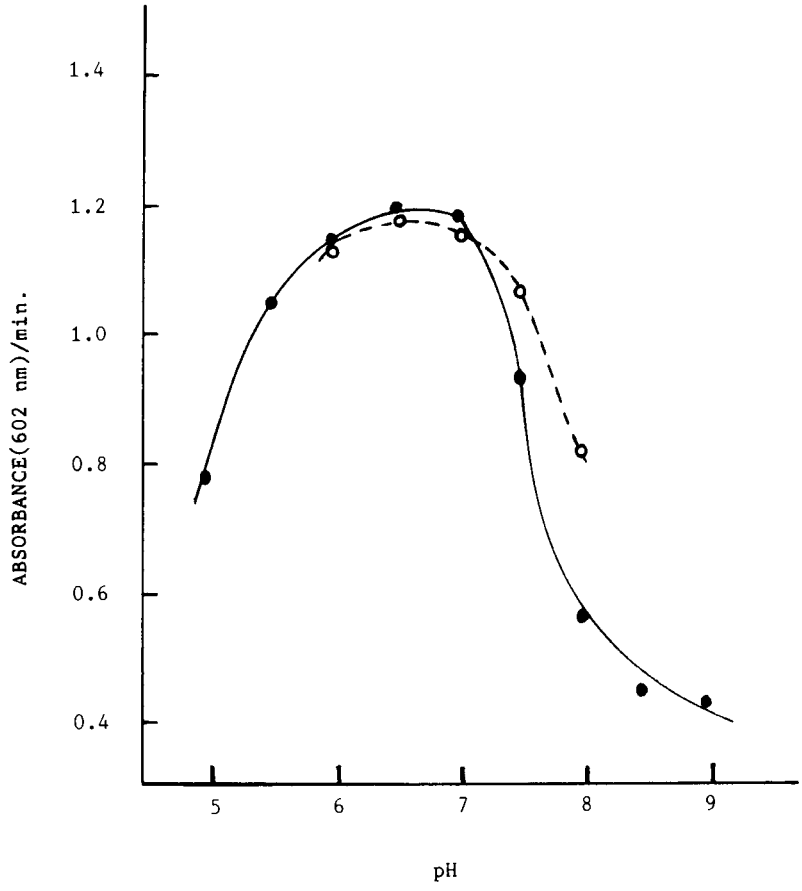


Fig. 5. Effect of pH on the rate of peroxidase-catalyzed chromophase formation. The reaction was measured at 25°C in 3 mL phosphate–citrate buffer, at various pH, and containing 1.5 μmol MBTH, 75 μmol PDEA, and 24 μmol H_2O_2. The reaction was initiated by adding 10 μL horseradish peroxidase solution (5 μg/mL). Filled circles (●) were results obtained in 0.02*M* phosphate–0.02*M* citrate buffer. Open circles (o) were results obtained in 0.1*M* phosphate–0.1*M* citrate buffer.

The chemical nature of the blue chromophore produced from HRP-catalyzed oxidation of MBTH and PDEA has not been elucidated, nor has the mechanism of such HRP-catalyzed reactions been investigated. However, it can be inferred from studies of Hünig (*16*) and Sawicki et al. (*17*) on MBTH reaction that the blue chromophore is likely to be a cationic indamine dye. The reaction mechanism is postulated as follows (Scheme 1).

The optimal concentrations of MBTH, PDEA, and H_2O_2 for the maximal rate of blue color formation were obtained by studying the effect of varying concentrations of one component at fixed and constant concentrations of the other two reagents (Figs. 2–4). The rate increases with increasing concentrations of both MBTH and PDEA. They reached 94%

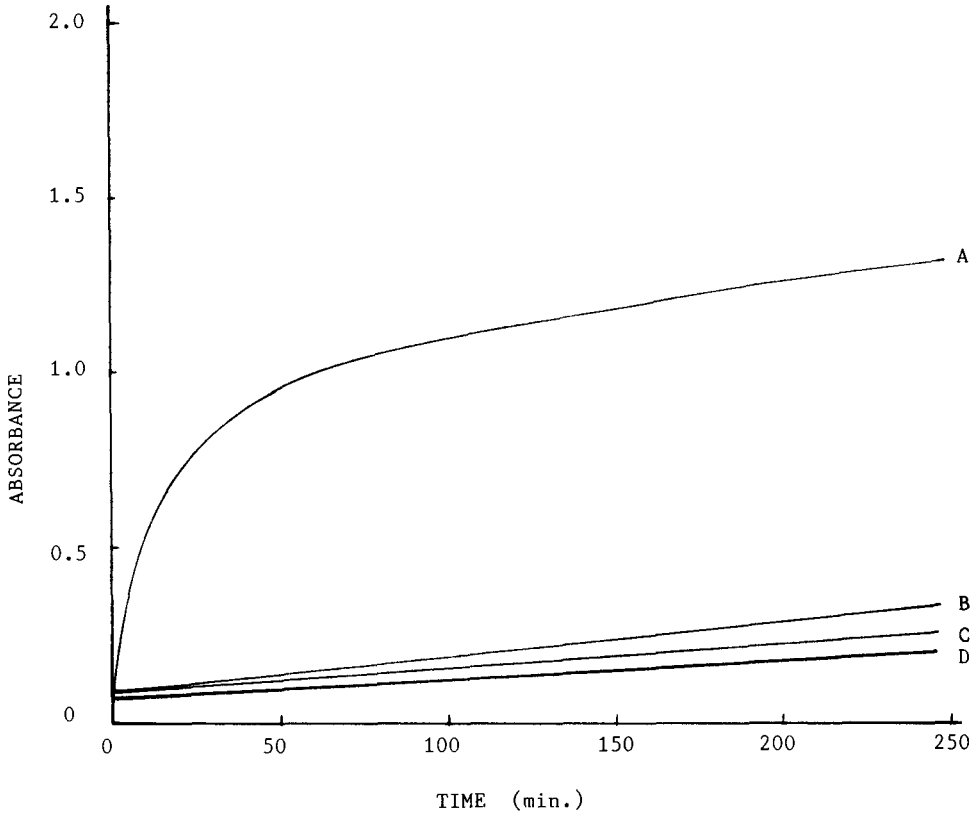


Fig. 6. Time course of HRP-catalyzed blue color formation (Line A) and the stability of solutions of MBTH–PDEA at various pH (Line B = pH 6.5; Line C = pH 7.0; Line D = pH 7.5).

of the maximal rate at 0.5 and 25 m*M*, respectively, whereas with H_2O_2, the rate reaches maximal at 8 m*M* H_2O_2 and falls gradually with further increases in H_2O_2 concentrations. The pH optimum for the rate of blue color formation was 7 under two buffer systems (Fig. 5).

The stability of solutions containing 0.5 m*M* MBTH and 25 m*M* PDEA at pHs 6.5, 7.0, and 7.5 was investigated (Fig. 6). The results showed that minimal background color formation was obtained in pH 7.5 (Line D, Fig. 6). Since the rate of enzymatic reaction at pH 7.5 was approximately 90% of that at pH 7.0, buffers of pH 7.5 were used in all experiments except in the pH-rate profile one.

Two phosphate–citrate buffer systems, high and low concentrations, were used because phosphate at high concentrations rapidly and irreversibly inactivated HRP (*18*).

Using the conditions herein described, MBTH and PDEA are shown to be a very sensitive chromogen system for HRP. Figure 7 showed that picomolar levels of HRP could be quantified by using either a rate or a

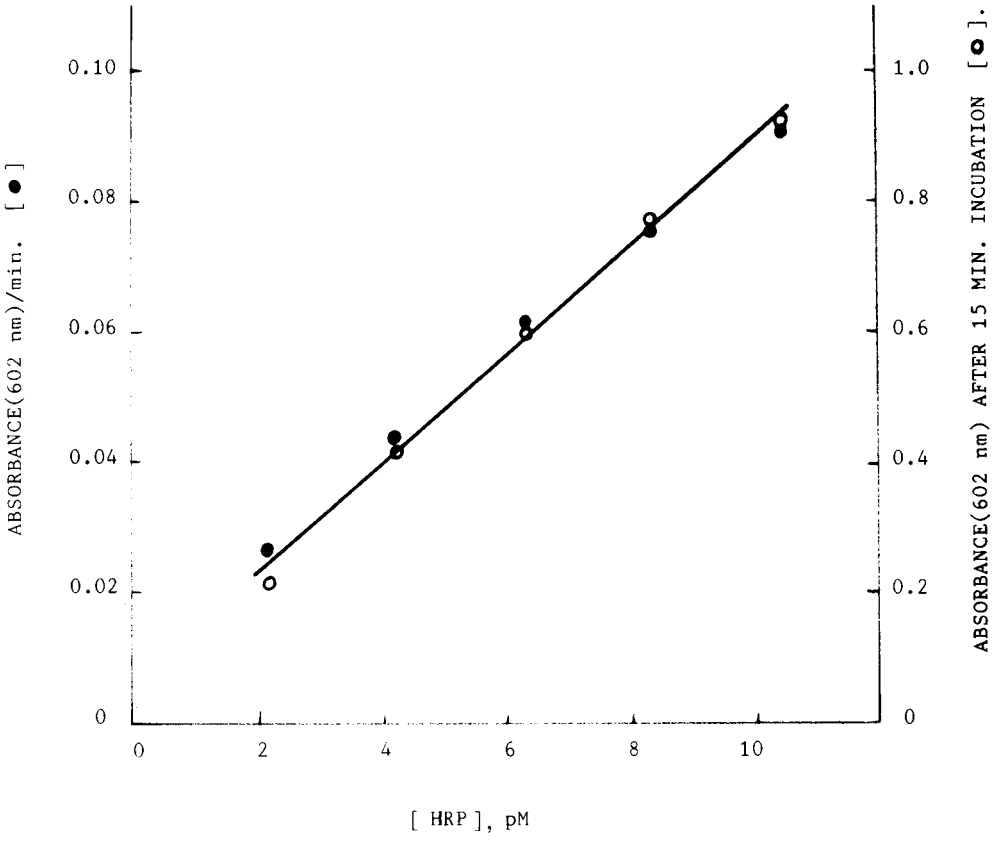
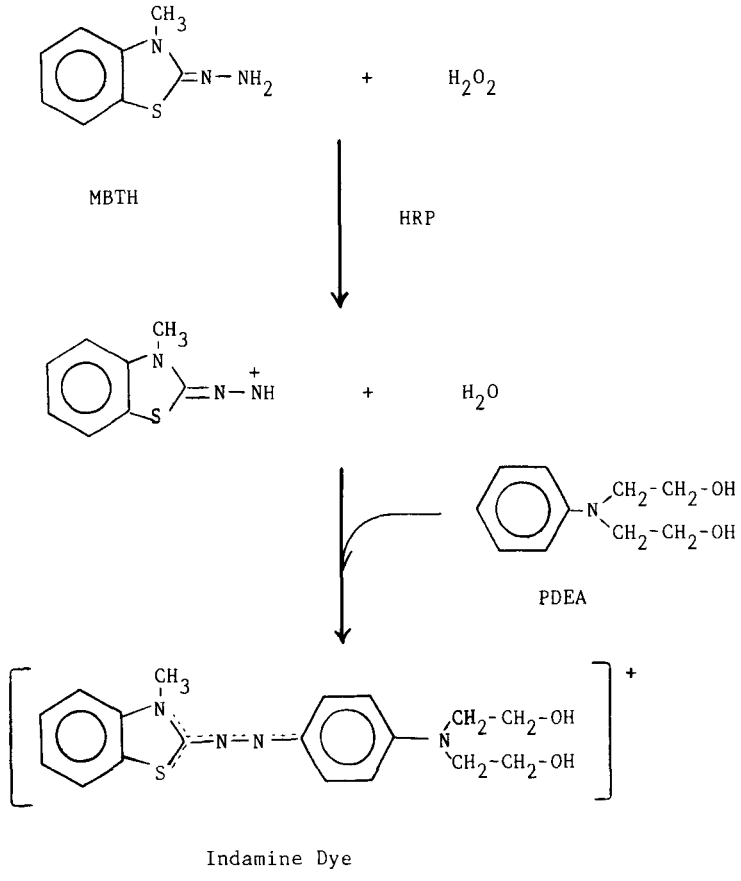


Fig. 7. Calibration plot for HRP determination by a rate (●) and a fixed-time method (o). The reactions were carried out at 25°C in 3 mL of 0.1*M* phosphate and 0.1*M* citrate, pH 7.5, containing 1.5 μmol MBTH, 75 μmol PDEA, and 24 μmol H_2O_2.

fixed time method. The rate of color development in MBTH–PDEA system is at least five times faster than the previously described sensitive method based on MBTH and DMAB (*11*).

Since most analytes measured by using HRP-coupled reactions, such as glucose, uric acid, cholesterol, etc., occur in serum at relatively high concentrations (in millimolar levels), previously developed chromogens have been satisfactory for such applications. For enzyme-labeled immunoassays, however, extremely sensitive chromogen systems would be highly desirable; in fact, in some instances, it is a prerequisite for the successful development of a practical assay. Therefore, it is expected that the most useful application of the method described herein will come from its use in enzyme-labeled immunoassays that use HRP as the label, particularly when a rate assay is used.



SCHEME 1

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